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Extracellular Matrix and Nuclear Localization of βig-h3 in Human Bladder Smooth Muscle and Fibroblast Cells

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Abstract The extracellular matrix (ECM) plays an essential role in bladder structure and function. In this study, expression of βig-h3, a recently identified extracellular matrix protein, was investigated in human bladder tissue, and human bladder smooth-muscle (SMC) and fibroblast cells in vitro. SMCs secreted greater than three times the level of this protein compared with fibroblasts. The relative levels of βig-h3 mRNA in the two cell types reflected the protein expression. Immunohistochemical analysis demonstrated protein deposition in the ECM as well as cytoplasmic localization and, unexpectedly, nuclei. Anti-βig-h3 antibodies also stained the matrix surrounding the detrusor SMCs and nuclei of bladder fibroblasts, SMCs, and urothelium in intact bladder tissue. Western blot analyses of medium and matrix fractions obtained from cells in vitro revealed protein of ~70–74 kDa, whereas nuclear extracts contained a 65-kDa reactive protein band. We propose that although this protein is a structural component of bladder ECM, its nuclear localization suggests that it has other regulatory and/or structural functions. J. Cell. Biochem. 79:261–273, 2000.

Key words: extracellular matrix; βig-h3; bladder; localization

The bladder is a dynamic organ whose function is to store and periodically eliminate urine from the body. The filling phase of the micturition cycle is characterized by a gradual increase in volume of urine with little increase in pressure. Once full, the bladder wall experiences a sharp increase in pressure, resulting in contraction of the detrusor SMC that initiate bladder emptying [Shapiro and Lepor, 1995]. Proper bladder function requires precise interactions between the ECM and resident stromal and muscle cells.

The ECM consists of a wide array of secreted proteins, including collagens, laminins, fibronectin, elastin, and proteoglycans [reviewed in Aumailley and Gayraud, 1998]. The ECM components interact with each other, forming a highly organized network that maintains tissue architecture and provides a support structure for resident cellular components. Types I and III collagen comprise the major collagens found in the bladder wall, their primary sources being SMC from the detrusor layer and fibroblasts in the lamina propria [Ewalt et al., 1992; Baskin et al., 1993; Coplen et al., 1994; Deveaud et al., 1998]. Type IV collagen is found as an investment, surrounding individual SMC within the muscularis mucosa and detrusor layer [Deveaud et al., 1998] and a major component of the urothelial basement membrane [Wilson et al., 1996]. Elastin and the elastin-associated microfibrillar proteins, fibrillin-1 and MAGP, are also associated with both compartments of the bladder wall, and are thought to contribute flexibility and elasticity [Koo et al., 1998]. The proper arrangement of these matrix proteins with one another and with the cellular components of the bladder wall all contribute to normal organ function: gradual expansion of the bladder wall on filling and rapid contraction and recoil during micturition [Macarak and Howard, 1997; Chang et al., 1998]. Quantitative changes in bladder ECM

Abbreviations used: ECM, extracellular matrix; PBS, phosphate-buffered saline; PBST, PBS containing 0.1% Tween 20; SMC, smooth-muscle cells; TGF-β1, transforming growth factor-β1.

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composition as well as alterations in the arrangement of matrix proteins can contribute to loss of bladder function and ultimately may lead to renal impairment [Lin and McConnell, 1995; Shapiro and Lepor, 1995; Macarak and Howard, 1997]. Although significant progress has been made in identifying and characterizing the major components of the bladder ECM, comparatively little is known about the "bridging molecules" that interconnect specific components of the ECM with each other and resident cells. These proteins may serve an important function by transferring tension from cells to other matrix components.

The ECM protein, βig-h3, was originally isolated from fetal bovine nuchal ligament by reductive saline extraction (designated MP78 [Gibson et al., 1989]) and was hypothesized to be a microfibrillar protein. More recently, βig-h3 was cloned from TGFβ1-stimulated A549 cells [Skonier et al., 1992]. The nascent protein contains a secretory signal sequence (residues 1–23), four homologous internal domains, and a cell attachment (RGD) site [Skonier et al., 1992, 1994]. The porcine homologue has been purified from a fiber-rich fraction of pig cartilage and designated RGD-CAP [Hashimoto et al., 1997]. The porcine-derived protein binds collagens I, II, and IV. In addition to being closely related to human βig-h3 at the amino acid level, RGD-CAP shows 45% identity with rat osteoblast-specific factor-2 and 22% identity with *Drosophila fasciclin I* [Hashimoto et al., 1997]. Although the physiological function of βig-h3 is not known, it has been suggested that it interconnects different matrix components with each other and resident cells [Gibson et al., 1989, 1997], and consequently, may serve as a bifunctional linker protein.

In bovine tissue, βig-h3 was found associated with collagen fibers in developing nuchal ligament, aorta, lung, and mature cornea [Gibson et al., 1997]. Immunoreactive material was also present in capsule and tubule basement membranes of developing kidney and reticular fibers in fetal spleen. The staining pattern was similar to that observed for type VI collagen [Gibson et al., 1997]. These results indicate that the protein is a widely expressed component of the ECM in several organ systems and may be closely associated with other matrix macromolecules.

In the course of studies directed at elucidating βig-h3 function, we observed that cells obtained from human bladder tissue express relatively high amounts of this protein. In this report, we have investigated βig-h3 production by human bladder fibroblast and SMC and its deposition in tissue.

MATERIALS AND METHODS

Chemicals and Reagents

Biotinylated goat anti-rabbit IgG and avidin D–Texas Red conjugate were purchased from Vector Laboratories (Burlingame, CA). Goat anti-rabbit IgG horseradish peroxidase (HRP) and 4-chloro-1-naphthol were purchased from Sigma (St. Louis, MO). OCT tissue freezing compound was obtained from Sakura Finetek (Torrance, CA). Sodium dodecyl sulfate (SDS)–polyacrylamide gradient minigels were purchased from Novex (San Diego, CA). UDP-[^{14}C]-galactose (300 mCi/mmol) was obtained from NEN (Boston, MA).

Cell Culture

Primary human bladder SMCs and fibroblast cells were isolated as described [Baskin et al., 1993; Coplen et al., 1994] and grown in Dulbecco's Modified Eagle Medium and Ham's F-12 medium (1:1) containing 10% fetal bovine serum and penicillin/streptomycin in an atmosphere of 5% CO_2 in air at 37°C. HeLa (human cervical carcinoma) and K562 (human myelogenous leukemia) cells were grown in RPMI containing 5% fetal bovine serum.

Purification of Nuclei

Cultured SMC or fibroblasts were trypsinized and washed from their flasks with culture media containing 10% fetal calf serum. The cells were pelleted by centrifugation and resuspended in ice-cold sucrose buffer I (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM Mg-acetate, 0.1 mM EDTA, 10 mM Tris (pH 8), 1 mM dithiothreitol [DTT], and 0.1% Triton X-100). Next, cells were lysed with a Dounce homogenizer, and cell breakage was periodically assessed by microscopic examination of the homogenate. When lysis was complete, the homogenate was mixed with sucrose buffer II (2.2 M sucrose, 5 mM Mg-acetate, 0.1 mM EDTA, 10 mM Tris (pH 8) and 1 mM DTT) and layered over a 2 M sucrose cushion. The nuclei were pelleted by centrifugation (30,000*g*, 45 min, at 4°C) and resuspended in glycerol storage buffer

TABLE I. Human Peptide Sequences Used to Develop Antibodies Against βig-h3

Antibody	Peptide sequence	Residue position
1186	IGTNRKYFTNCKQWYQRKIC	55–74
1073[a]	TQLYTDRTEKLRPEMEG-C	118–134
1077[a]	ALPPRERSRLL-C	549–559

[a]A Cys residue was added to the carboxy terminus to facilitate coupling to KLH.

(40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA). For analysis, freshly purified nuclei were spread on glass slides, fixed for 10 min with PBS containing 1.5% formalin, and subsequently incubated with propidium iodide, which specifically stains DNA.

Marker Enzymes

Galactosyltransferase activity (Golgi marker enzyme) was determined using the modified procedure of Rens-Domiano and Roth [1989]. Briefly, reactions contained extract (20–50 μg protein) in 25 mM HEPES (pH 7), 1 mM DTT, 0.5% Triton X-100, 40 mM MnCl, 2 mM ATP, 20 mM *N*-acetylglucosamine, and 2 mM UDP-[^{14}C]-galactose (NEN, specific activity 300 mCi/mmol) in a total volume of 150 μl. The reactions were incubated for 1 h at 37°C and terminated by the addition of 50 μl of 0.2 M EDTA and passed over 0.5 ml of Dowex 1 resin (chloride form). The radiolabeled product [^{14}C]-lactosamine was eluted with 1 ml of water and counted in a liquid scintillation counter.

Cytochrome C reductase activity (ER/microsomal marker enzyme) was assayed as described [Phillips and Langdon, 1962], except that reactions were performed in 25 mM Tris (pH 7.5), 300 mM NaCl, containing 50 μM 2, 6-dichloroindophenol, and 50 μM NADPH. The oxidation of NADPH was monitored at 340 nm ($E_{340} = 6.22\ mM^{-1}\ cm^{-1}$) [Gromer et al., 1998].

Antibody Production and Purification

Three synthetic peptides corresponding to different regions of human βig-h3 (Table I) were synthesized and purified by high-pressure liquid chromatography (BioSynthesis, Lewisville, TX). Each peptide was coupled to maleimide activated-KLH (Pierce, Rockford, IL) at a ratio of 1 mg peptide/mg KLH. Antibodies were raised in rabbits by Cocalico Biologicals (Reamstown, PA) using the following immunization schedule: an initial injection of 250 μg, followed 3 weeks later by three biweekly injections of 100 μg. For affinity purification of antibodies, individual peptides were immobilized on Sulfolink coupling gel (Pierce, Rockford, IL). The IgG fractions were passed over the column, and the column was washed with PBS (~10 column vol) until protein free. Bound antibodies were eluted with 100 mM glycine (pH 2.5), immediately neutralized with Tris base, and adjusted to a concentration of 0.4 mg/ml before storage at −20°C [Harlow and Lane, 1988; Chao et al., 1996].

Enzyme-Linked Immunosorbent Assays

βig-h3 levels in conditioned medium were determined by direct enzyme-linked immunosorbent assay (ELISA) [Kucich et al., 1998]. Briefly, microtiter plates were coated with known amounts of free peptide or conditioned medium diluted in 100 mM Na-carbonate buffer (pH 9.6). The plates were next incubated with Ab1077 (diluted 1:50,000 in PBST) for 1 h at room temperature, washed three times with PBST, and incubated with anti-rabbit-HRP (diluted 1:2,000 in PBST) for 60 min at 37°C. The plates were washed three times with PBST, incubated with peroxidase substrate *O*-phenylethylenediamine (1 mg/ml) in 50 mM citrate buffer (pH 4.6), 0.01% H_2O_2 for 30 min and read at 450 nm. Standard curves, generated by coating the plates with known amounts of immunizing peptide (ALPPRERSRLL-C), were used to quantitate βig-h3 present in media.

Western Blot Analysis

Proteins were resolved on 8%–16% polyacrylamide gradient gels under reducing conditions and transferred to nitrocellulose membranes [Chao et al., 1996]. After transfer, the membranes were blocked in PBST and 5% nonfat dry milk and subsequently incubated with anti-βig-h3 antibodies diluted 1:1,000 (0.4 μg/ml) in PBST, 0.1% bovine serum albumin. Bound antibody was detected with goat anti-rabbit HRP-conjugate, using chloro-naphthol as substrate.

Immunohistochemistry

Human bladder tissue was embedded in OCT freezing compound and stored at −80°C. Five- to 7-μm frozen sections were cut and placed on albumin-coated slides. The tissue was fixed for 5 min in 0.4% formaldehyde/PBS, followed by treatment with two 5-min rinses of

100 mM NH_4Cl/PBS. The tissue was then treated with 6 M guanidine-HCl followed by 100 mM iodoacetamide to unmask epitopes in the extracellular matrix [Gibson et al., 1997] and was subsequently blocked with PBST containing bovine serum albumin (BSA). The tissue was incubated with affinity-purified primary antibody overnight at 9°C (1:20 dilution), washed and incubated with biotinylated anti-rabbit IgG (1:200 dilution), followed by avidin D–Texas Red (1:125 dilution). To assess nuclear localization with antibody Ab 1073, frozen sections of human and mouse bladder tissue were treated directly with the affinity-purified primary antibody (1:20 dilution) in the absence of guanidine-HCl/iodoacetamide treatment.

Cells (2×10^5) were seeded in sterile eight-well Lab Tek chamber slides (Nunc, Naperville, IL) and grown to confluence. The cells were washed twice with PBS, fixed with PBS containing 1.5% formalin for 10 min at 20°C, washed with PBS, and permeabilized with PBS containing 0.1% Triton X-100 and 1% BSA. Next, cells were incubated with primary antibody (diluted 1:400 in PBST, 1% BSA) at 4°C overnight, washed three times with PBST, and subsequently incubated with a goat anti-rabbit IgG-rhodamine conjugate (diluted 1:500 in PBST, 0.1% BSA) for 1 h at 20°C. The slides were washed, coverslipped, and examined with a Zeiss fluorescent microscope equipped with epifluorescence optics. Control slides were treated in an identical manner, but with the addition of 10 μg/ml immunizing peptide to block primary antibody binding.

Northern Blot Analysis

Total cytoplasmic RNA was extracted from bladder cells with guanidinium thiocyanate/phenol-chloroform as described [Chomczynski and Sacchi, 1987]. RNA was size fractionated on 1% agarose-formaldehyde gels and transferred to a Zeta-Probe membrane (Bio Rad, Hercules, CA). The filters were hybridized with a 2.1-kb human βig-h3 cDNA (GenBank Accession Number: M77349) probe (10^6 cpm/ml hybridization mix) labeled with [^{32}P] using a Ready-To-Go DNA labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) to a specific activity of $0.5–1 \times 10^9$ cpm/μg. RNA loading and transfer were evaluated by probing with a 1.4-kb glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe [Kucich et al., 1997]. Equivalent loading and transfer were also verified by quantitative image analysis of ethidium bromide staining of ribosomal RNA, within the blots themselves. The phosphorimages of the filters were digitized (Storm 840, Molecular Dynamics, Sunnyvale, CA), and an identical rectangle was drawn around the probe-specific signal for each lane of the Northern blot. The relative response was quantified by integration of the observed pixel density within each rectangle (Image-Quant V5.1 software; Molecular Dynamics, Sunnyvale, CA) yielding a volume measurement that reflects mRNA content.

RESULTS

Expression of βig-h3 by Cultured Bladder SMC and Fibroblasts

The first series of experiments assessed βig-h3 gene expression in several cell types. Total RNA was extracted from primary human bladder SMC and fibroblasts, as well as HeLa, and K562 cells and analyzed on Northern blots. Both bladder fibroblasts and SMCs expressed a βig-h3 transcript of ~3.4 kb, whereas HeLa and K562 cells did not (Fig. 1). Bladder SMC expressed higher levels of βig-h3 (approximately twofold) than fibroblasts. We have observed comparable levels of expression in primary human lung fibroblasts and SMCs (data not shown), demonstrating that these cell types express βig-h3 RNA in other organ systems, as well.

To assess βig-h3 protein production by bladder wall cells, peptide antibodies were generated against the N- and C-terminal regions of the protein (see Materials and Methods section). SMC and fibroblasts were grown to near confluence [$8 \pm 0.5 \times 10^6$ cells/T75 flask], the medium was changed and, at 24-h intervals, medium samples were collected and the amount of βig-h3 was quantitated by direct ELISA. SMC synthesized approximately three-five times the amount of protein compared with fibroblasts (Fig. 2).

The results presented above (Figs. 1 and 2) demonstrate that bladder cells in vitro expressed βig-h3 that was secreted into the culture medium. To determine protein deposition in the cell-layer/matrix compartment, bladder cells were seeded in slide chambers, fixed and incubated with anti-βig-h3 antibodies. When treated with the C-terminal antibody (Ab

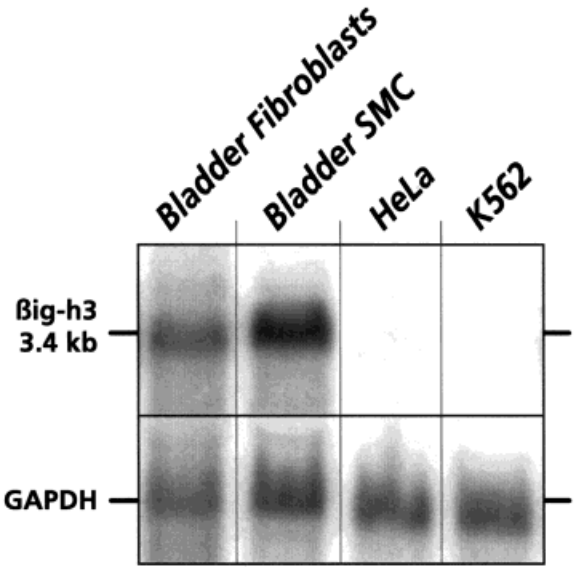


Fig. 1. βig-h3 expression in bladder cells. Total cytoplasmic RNA was extracted from cultured human SMC and fibroblasts and analyzed by Northern hybridization with [^{32}P]-βig-h3 or [^{32}P]-GAPDH probes. RNA was from: bladder fibroblasts, bladder SMC, HeLa, and K562 cells. Note that SMC and fibroblasts express βig-h3 whereas the other cell types do not. Normalization of βig-h3 band densities with that of GAPDH by phosphorimager analysis showed that SMCs expressed approximately twofold higher level of βig-h3 than fibroblasts.

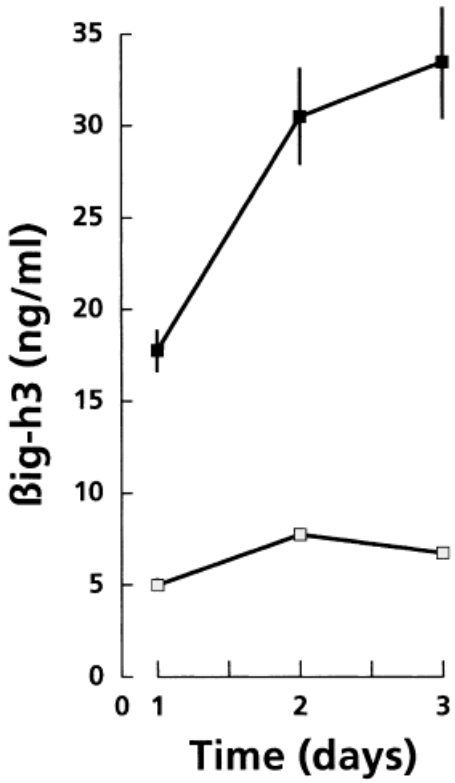


Fig. 2. Protein accumulation in medium. Cells were grown to near confluence, the medium was changed, and at defined time points, conditioned medium samples were collected and assayed for βig-h3 levels by direct enzyme-linked immunosorbent assay and expressed as the mean ± SD. Note that SMC (solid squares) secrete three-five times the amount as fibroblasts (open squares).

1077), fibrous ECM stained brightly (Fig. 3A,C) in cultures of both SMC and fibroblasts. In addition, intracellular cytoplasmic staining was observed surrounding the nucleus, consistent with rough endoplasmic reticulum and Golgi patterns (Fig. 3B). Very fine punctate staining of nuclei was observed with antibody Ab1077 (Fig. 3B,C).

Surprisingly, a different staining pattern was obtained with Ab 1073 (directed at the N-terminal region of the protein). Bladder cells treated with this antibody exhibited prominent nuclear staining, whereas the nucleolar regions were devoid of staining (Fig. 4). This staining was not associated with the outer nuclear membrane as judged by focusing above and below the plane of the cell monolayer. Nuclear staining with Ab1073 had a fine punctate (stippled/spotted) appearance and was more intense than that observed with Ab 1077. Occasionally, very fine staining of the ECM was also observed with Ab 1073. In addition, nuclear staining was also observed when cells were incubated with a third peptide antibody, Ab 1186 (data not shown). To verify antibody specificity, cells were treated with: 1) preimmune serum, 2) primary antibodies that were preincubated with the corresponding immunizing peptide before exposure to cells, or 3) secondary antibody alone. Under these conditions, immunostaining of cells was consistently negative (Figs. 3D and 4C).

Our immunohistochemical studies revealed that βig-h3 produced by bladder cells localized to both intracellular and extracellular locations. To verify the presence of the protein in the extracellular matrix, fibroblasts and SMCs were grown to confluence and the cells were removed from the culture flasks by incubation in PBS, 10 mM EDTA. The remaining matrix was washed, solubilized with 1% SDS, and analyzed on Western blots. βig-h3 (M_r ~70 and 74 kDa) was present in conditioned medium as well as matrix produced by both cell types (Fig. 5). These results confirmed that bladder cells synthesized βig-h3, which was deposited in the ECM.

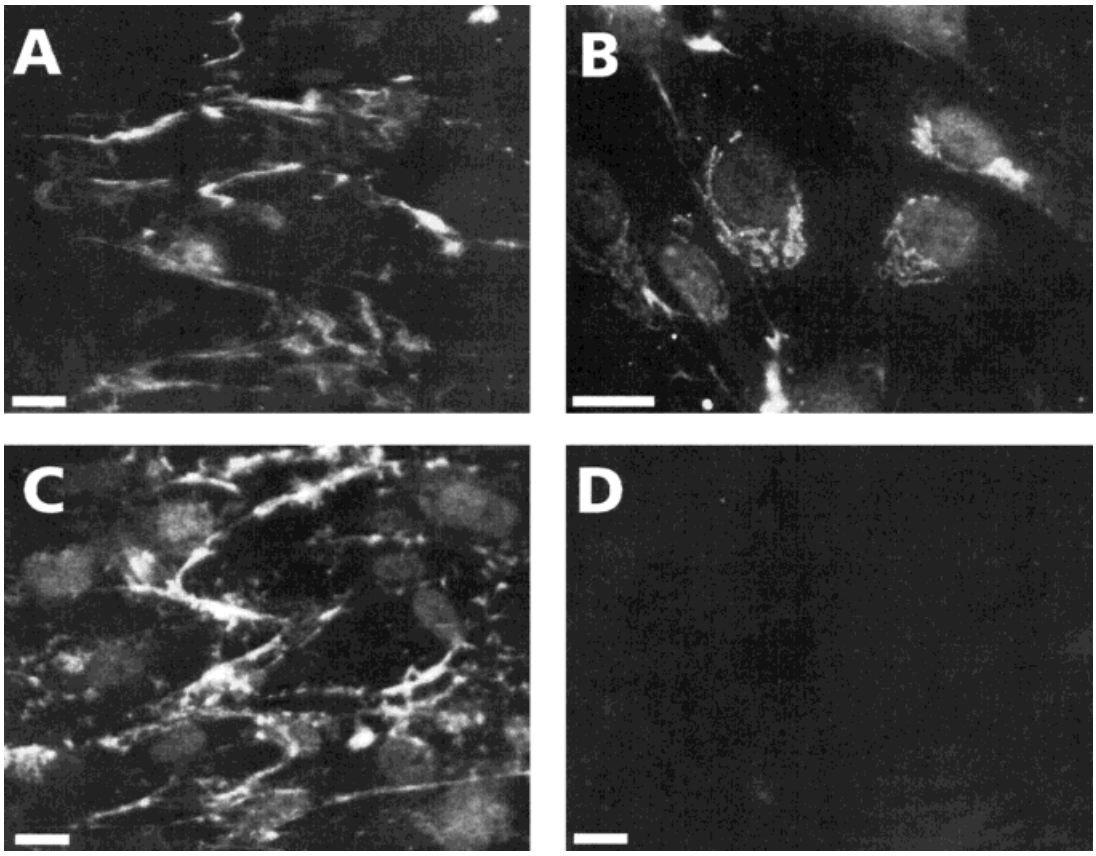


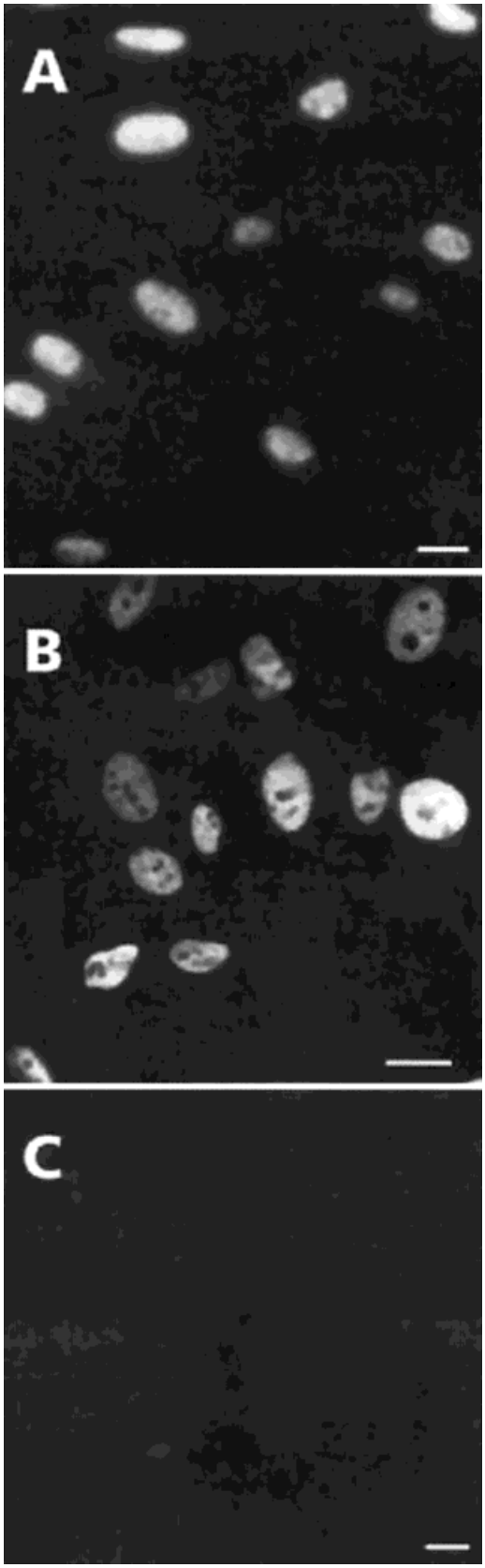
Fig. 3. Immunohistochemical analysis of bladder cells. Cells were grown on slide chambers, washed, fixed, permeabilized, and incubated with βig-h3 Ab 1077. A,B: SMC; C,D: fibroblasts. **A:** Note positive staining of fibrous strands in ECM, and faint cytoplasmic staining of SMC in background. **B:** Intracellular staining of βig-h3 within cytoplasmic organelles (RER and Golgi) in cultured SMC. **C:** Positive staining of extracellular matrix components synthesized and deposited by bladder fibroblasts, as well as fine nuclear and cytoplasmic staining of the fibroblasts in the background. **D:** Fibroblasts incubated with Ab 1077 preincubated with immunizing peptide; molar ratio of Ab:peptide = 1:320. Note immunizing peptide blocks cell staining. Scale bar ≈ 20 μm.

Identification of βig-h3 in Nuclei

Analysis of βig-h3 primary sequence revealed the presence of a signal peptide (residues 1–23), targeting the protein for secretion [Skonier et al., 1992]. Therefore, we were surprised that bladder cell nuclei stained with Ab 1073. To investigate this finding further, nuclei were purified by subcellular fractionation from fibroblasts and SMC. Purified nuclear preparations were examined by phase contrast microscopy (Fig. 6A) and propidium iodide staining (Fig. 6B) and found to be pure at this level of resolution. Furthermore, analysis of marker enzyme activity demonstrated that the nuclear preparations were relatively free of contamination by Golgi and endoplasmic reticulum and comparable in terms of purity to that obtained by others (Table II) [Kihlmark and Hallberg, 1998; Oishi et al., 1996]. Isolated nuclear extracts were analyzed on Western blots, which confirmed the presence of βig-h3 in the nuclear fraction (Fig. 6C). However, the protein present in nuclei had a faster electrophoretic mobility (M_r ~65 kDa) than the secreted form (M_r ~70–74 kDa). These results confirmed the presence of βig-h3 in nuclei whose apparent molecular mass differed from the species present in conditioned medium and associated with matrix.

Immunohistochemical Localization in Bladder Tissue

The results presented above demonstrated synthesis and deposition of βig-h3 by primary human bladder cells, as well as translocation of the protein to the nucleus. To evaluate its an-



atomical distribution in intact human bladder tissue, frozen sections were incubated with βig-h3 antibody Ab 1077. Prominent staining was observed in the detrusor layer of the bladder (Fig. 7A) after epitopes in the matrix were unmasked by treatment of fixed tissue with the chaotropic agent, guanidine–HCl. Staining was primarily associated with the cocoonlike matrix surrounding individual smooth muscle cells that comprise the detrusor muscle bundles. Similar findings were detected in the muscularis mucosa (data not shown). This staining pattern was similar to that seen in human bladder tissue using an antibody against type IV collagen [Devaud et al., 1998]. In addition to this endomysial staining, regions of thickened interstitial matrix that contained blood vessels within the muscle bundles also stained with the βig-h3 antibody. In contrast, the perimysium surrounding smooth-muscle bundles was negative (Fig.7, arrowheads). Although there was some fine fiber staining in the extracellular matrix of the lamina propria compartment (data not shown), the localization was predominantly associated with the smooth-muscle compartment. These data correlate well with both the levels of mRNA and secreted protein from the cultured bladder wall fibroblasts and SMC.

To determine whether Ab 1073 stained nuclei in tissue, as was seen in bladder fibroblasts and SMC in vitro, frozen sections of both human and mouse bladder tissue were initially treated with the chaotrope followed by antibody incubation. No staining was observed, and in some areas, the nucleus appeared to be extracted from the tissue. When frozen tissue sections were stained directly with Ab 1073, in the absence of guanidine treatment, punctate nuclear staining was observed in lamina propria fibroblasts (Fig. 8A), urothelial cells (Fig. 8B), and SMC (Fig. 8C).

Fig. 4. Immunohistochemical analysis of bladder cells. Cells were grown on slide chambers, washed, fixed, permeabilized, and incubated with βig-h3 Ab 1073. **A:** SMC; **B:** fibroblasts; **C:** SMC control, preincubation with Ab 1073 immunizing peptide followed by Ab 1073. Note staining of both SMC (A) and fibroblast (B) nuclei with this antibody. Preincubation of Ab 1073 with immunizing peptide blocks cell staining (C). Bar = 20 μm.

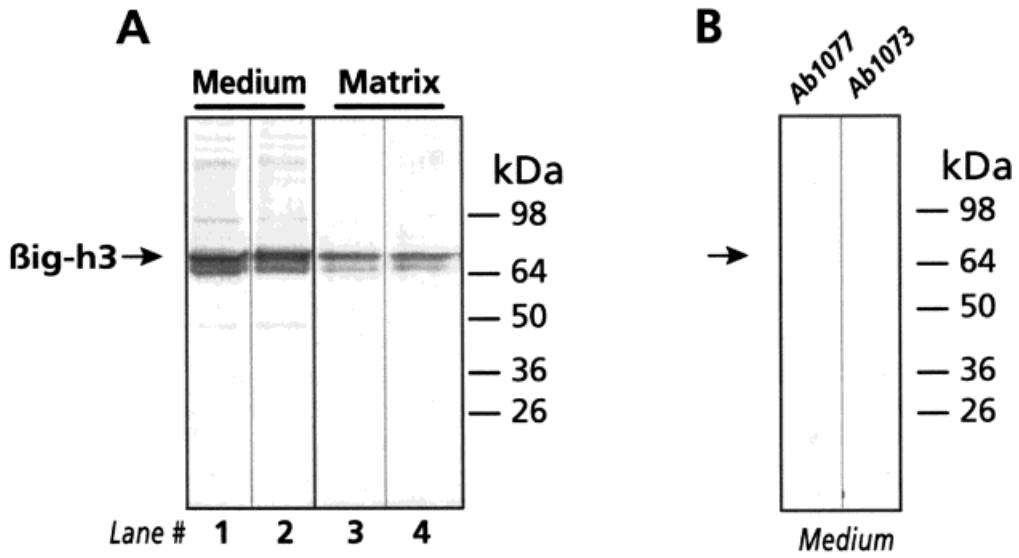


Fig. 5. Analysis of medium and matrix. **A:** Bladder cells were grown to confluence, the culture medium was collected, and the cells were removed from the dishes with PBS, 10 mM EDTA. The remaining matrix was solubilized with 1% sodium dodecyl sulfate and analyzed on Western blots under reducing conditions. Protein was detected using Ab 1077. **Lanes 1** and **2:** Conditioned medium from fibroblasts or SMC, respectively. **Lanes 3** and **4:** Solubilized matrix from fibroblasts or SMC, respectively. Numbers at right indicate relative molecular weight in kilodaltons. Note presence of βig-h3 (arrow on left) in medium and matrix with approximate sizes of 74 and 70 kDa. **B:** Nonconditioned culture medium containing 10% fetal bovine serum was analyzed on Western blots developed with Ab 1073 and Ab 1077, and no immunoreactive proteins are detectable. Numbers on right indicate relative molecular weight in kilodaltons; arrow on left denotes position of βig-h3.

DISCUSSION

In this investigation, we demonstrate that human bladder SMC and fibroblast cells express βig-h3 in vitro. Northern analysis showed approximately two-fold higher levels of βig-h3 RNA from SMC, whereas ELISA analysis demonstrated that SMC expressed approximately three- to five-fold higher levels of the protein compared with fibroblasts. Biochemical and immunohistochemical analyses verified that the expressed protein was secreted into the culture medium and deposited into the ECM produced by both cell types. In bladder tissue, the protein predominantly localized to the detrusor layer, primarily composed of smooth-muscle bundles. These results provide strong evidence that our in vitro findings, using isolated bladder cells, are an accurate reflection of the behavior of these cells in vivo and further suggest that SMC, and possibly fibroblasts, are the major source of βig-h3 in the bladder. The presence of this protein in the detrusor layer surrounding individual SMC suggests that it may play an important role in the active (tension transfer) and passive (structural) properties of bladder tissue. This localization is similar to the cocoonlike investment of type IV collagen [Devaud et al., 1998] seen in detrusor muscle, and also to type VI collagen (our unpublished data). Although colocalization of these matrix proteins suggests, but does not prove, a possible interaction between them, other studies have shown that βig-h3 binds to type IV and type I collagen [Hashimoto et al., 1997]. Such binding of a linker protein could connect the matrix with cell-surface associated proteins, and thereby facilitate tension transfer from muscle bundles to the bladder wall.

An unexpected outcome of our investigation was the different immunostaining patterns observed when intact cells were incubated with antibodies to the N-terminal (Ab 1073) and C-terminal (Ab 1077) regions of the protein. Ab 1077 predominantly stained the matrix, but also exhibited weak nuclear staining, whereas Ab 1073 yielded prominent nuclear staining and little, if any, matrix staining. The presence of βig-h3 was confirmed by Western analysis of purified nuclei, conditioned medium, and solubilized matrix (Figs. 5 and 6). Hence, these results demonstrate that βig-h3 is present in both intracellular and extracellular compartments. Our immunolocalization results also raise some interesting questions about protein

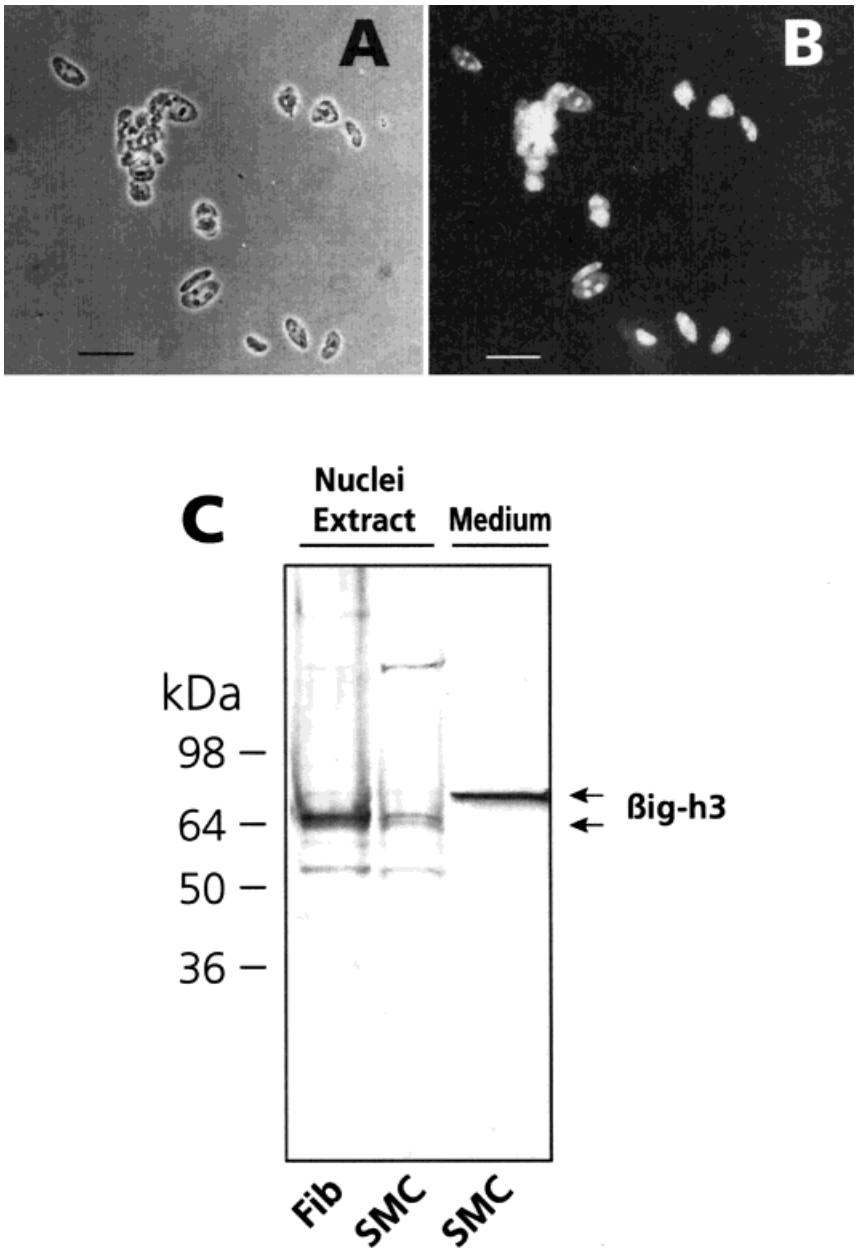


Fig. 6. Purification and analysis of nuclear extracts. SMC were disrupted in a Dounce homogenizer followed by centrifugation through a 2 M sucrose cushion. Nuclei were spread on a glass slide and examined by phase contrast optics (**A**) or fluorescence microscopy of propidium iodode–stained nuclei (**B**) (Scale bar in A and B = 35 μm). Nuclear extracts were prepared from fibroblast and SMC, resolved on 8%–16% sodium dodecyl sulfate gels under reducing conditions, and analyzed on Western blots (**C**). Membranes were incubated with βig-h3 Ab 1077. **Lanes 1** and **2:** Nuclear extracts from fibroblasts and SMCs, respectively. **Lane 3:** Conditioned medium from SMC. Numbers on left indicate relative molecular weight in kilodaltons. Note smaller molecular size of fractions from nuclear extracts compared to medium fraction.

function. Specifically, these data suggest that the epitope recognized by Ab 1073 (residues 118–134) is accessible in nuclei but masked in ECM, whereas the converse is true for Ab 1077 (residues 549–559). This could result from differences in protein conformation, or, alternatively, that βig-h3 interacts with specific protein ligands in nuclei and matrix, whose binding masks or alters other epitopes, making them inaccessible to the antibodies. This is also relevant to the arrangement of βig-h3 in bladder tissue. Staining of frozen bladder tissue

TABLE II. Activity of Marker Enzymes in Bladder SMC[a]

Extract	Galactosyl transferase (μmol/h/mg protein)	Cytochrome-C reductase (μmol/min/mg protein)
Total cell	2.17 ± 0.44	111 ± 14
Nuclei	0.25 ± 0.1	11 ± 5

[a]Galactosyl transferase and cytochrome-C reductase activities were assayed as described in materials and methods.

sections with Ab1077 was initially negative. However, treatment of the tissue with a chaotropic agent followed by iodoacetamide, an alkylating agent that prevents the reformation of disulfide linkages, resulted in exposure of the epitope and positive matrix staining. Therefore, in normal bladder tissue, the C-terminal region of the protein is arranged or interacts with other proteins such that the epitope is masked.

Interestingly, nuclear βig-h3 had a faster electrophoretic mobility than the secreted protein, indicating that its apparent mass is smaller than the soluble and matrix bound forms (Fig. 6C). Several possibilities could account for this: 1) The nuclear protein may undergo different processing steps than the secreted form, 2) The nuclear form may undergo partial proteolytic degradation before nuclear deposition, or 3) The nuclear form may be synthesized from an alternatively spliced message. We are currently investigating these possibilities. However, the fact that the nuclear extracts stained with both the N-terminal and C-terminal region antibodies on Western blots indicates that both of these regions are present in the lower molecular weight form. The difference in molecular size of nuclear βig-h3, as well as the preferential staining of nuclei in intact cells with Ab 1073, suggest that the protein may perform different functions at these sites.

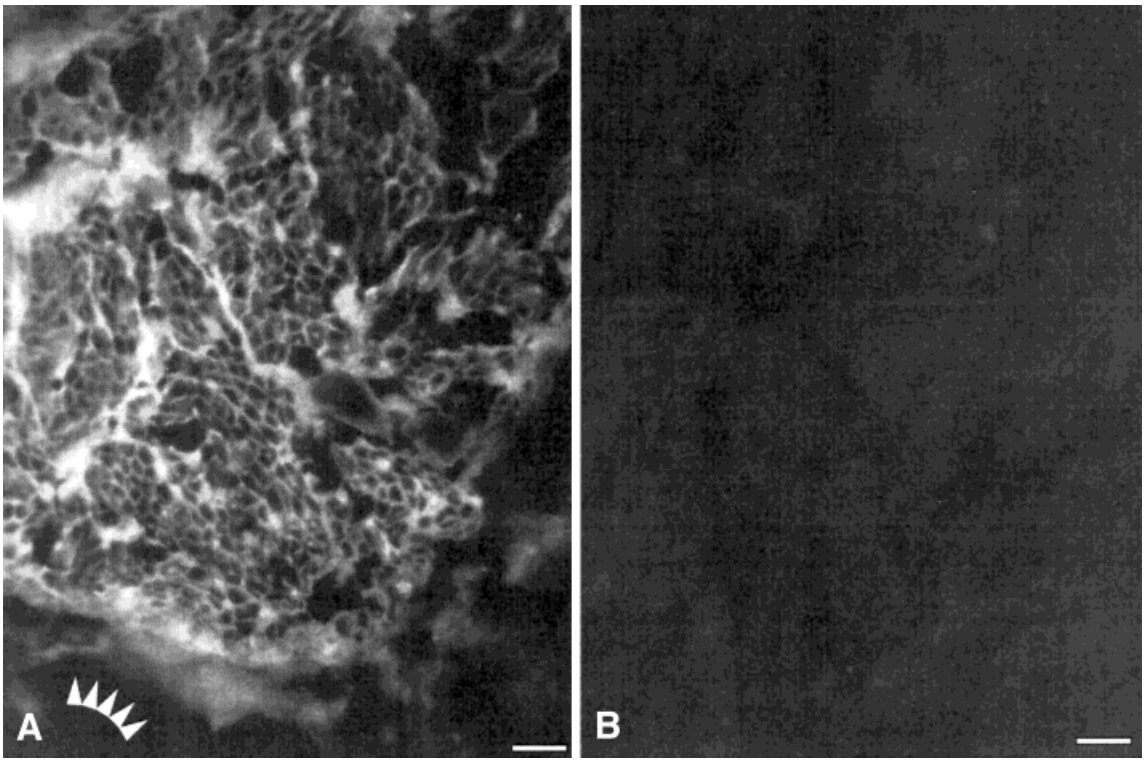


Fig. 7. Immunohistochemical analysis of bladder tissue. Human bladder tissue was embedded in OCT freezing medium, and frozen sections were cut, fixed, and treated with 6 M guanidine HCl /100 mM iodoacetamide to unmask epitopes. Sections were incubated with βig-h3 Ab 1077 (**A**) or preimmune serum (**B**), followed by incubation with biotinylated goat anti-rabbit IgG and avidin D–Texas Red conjugate. (A) Note that βig-h3 is localized to the matrix surrounding individual SMC within a muscle bundle giving a honeycomb pattern, and regions of thickened matrix within the bundle. Perimysial matrix is negative (arrowheads). Scale bar = 20 μm.

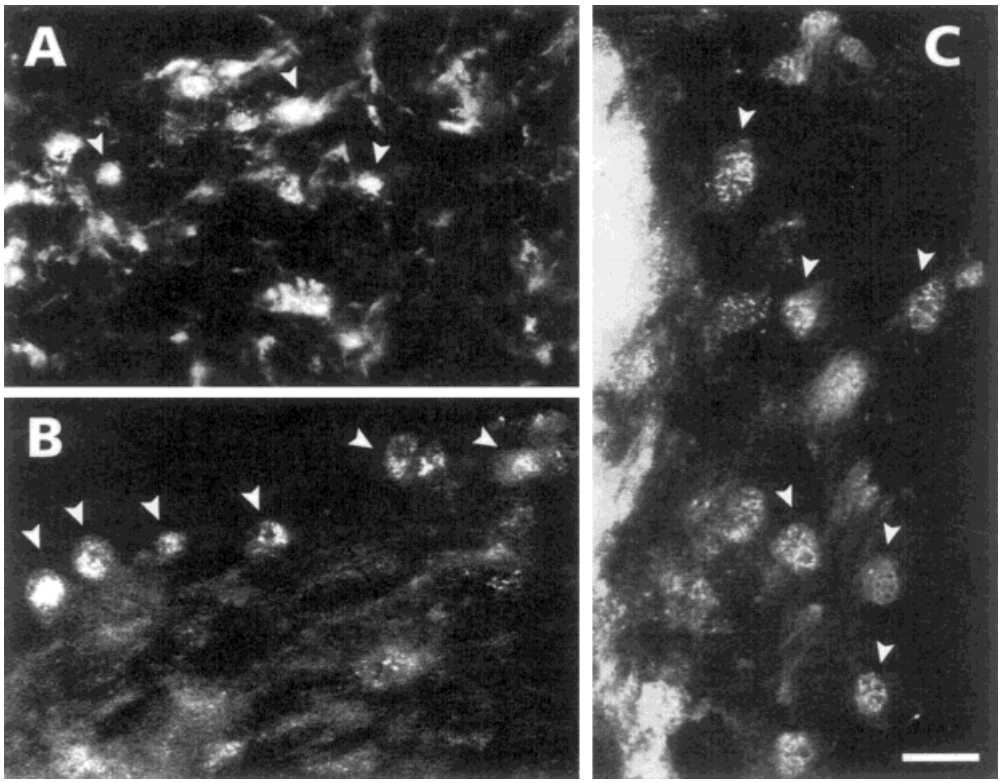


Fig. 8. βig-h3 expression in bladder tissue. Human and mouse bladder tissues were embedded in OCT freezing compound and 5–7-μm frozen sections were cut. Sections were incubated with affinity-purified βig-h3 Ab 1073, followed by incubation with biotinylated goat anti-rabbit IgG and avidin D–Texas Red conjugate. Note the positive punctate staining (arrowheads) within the nucleus of human bladder lamina propria fibroblasts (**A**), urothelial cells (**B**), and mouse detrusor SMC (**C**). Scale bar = 20 μm.

Other proteins that are normally secreted into the extracellular environment have recently been localized to the nuclear compartment of a number of different cell types [Henderson, 1997; Li et al., 1997; Gooden et al., 1999]. SPARC, an acidic and cysteine-rich protein that functions as an inhibitor of cell proliferation and adhesion, is normally localized to the pericellular matrix [Gooden et al., 1999]. However, nuclear matrix staining of this protein was detected in avian embryos, embryonic cells, and in adult bovine aortic endothelium. Nuclear expression was cell-cycle dependent, occurring during interphase. Furthermore, exogenous recombinant SPARC was taken up by the embryonic cells and translocated to the nucleus, indicating that the protein could reenter the cell after secretion and be transported to the nucleus. Lysyl oxidase, an enzyme involved in initiating formation of covalent cross-links in collagen and elastin, has also been localized to the nuclear compartment of both fibroblasts and SMC, in vitro [Li et al., 1997]. Both SPARC and lysyl oxidase have clusters of basic residues that could be potential nuclear translocation signals. However, analysis of βig-h3 amino acid sequences did not identify any nuclear localization signals. Therefore, the mechanism whereby βig-h3 is translocated to the nucleus remains to be defined.

Results from several laboratories indicate that βig-h3 protein may serve important physiological function(s). βig-h3 has been evolutionarily conserved and has been identified in human, bovine, swine, and mouse [Skonier et al., 1992; Skonier et al., 1994; Gibson et al., 1997; Hashimoto et al., 1997]; a closely related protein has also been identified in chickens [Kawamoto et al., 1998]. The chromosomal location of this protein has been conserved in humans and mice [Skonier et al., 1994]. Furthermore, alterations in βig-h3 can have severe consequences, because mutations in this gene

have been directly linked with hereditary corneal dystrophy in humans [Munier et al., 1997]. Genetic analysis has mapped these missense mutations in βig-h3 to positions 124 (conversion of Arg 124 to Cys or His) and 555 (conversion of Arg 555 to Gln or Trp) in individuals with hereditary corneal dystrophy (26). Although the direct role that this protein plays in the cornea is not known, it is hypothesized that mutations at Arg residues 124 and 555 cause the protein to denature, resulting in the formation of "amyloidogenic intermediates" that eventually precipitate [Munier et al., 1997]. More recent immunohistochemical analysis has localized the protein in corneas from patients with granular dystrophy [Klintworth et al., 1998; Streeten et al., 1999]. These results suggest that βig-h3 plays an important role in the cornea and that specific changes in protein primary sequence alter its function. At the present time, it is not known whether these patients also exhibit changes in bladder structure and/or function.

The protein contains an RGD cell attachment/integrin recognition site in its C-terminal region and also binds types I, II, and IV collagen [Skonier et al., 1992; Hashimoto et al., 1997], suggesting that it could function as a linker protein interconnecting specific matrix components with each other and resident cells. For example, surface integrins present on resident cells in the bladder, such as SMC, fibroblasts, or epithelial cells, could bind βig-h3 via the RGD. βig-h3 could, in turn, bind collagen molecules present in bladder ECM. This type of interconnection could provide a supporting structure or tension transfer mechanism between other ECM components and resident bladder cells. Immunohistochemical studies of normal human bladder have shown that a number of integrins are localized to the cellular components of the bladder, including urothelium, fibroblasts, vascular components, nerves, and SMC [Wilson et al., 1996]. The chicken homologue, RGD-CAP, has been shown to interact with an $\alpha_1\beta_1$ integrin in chondrocytes and fibroblasts [Ohno et al., 1999]. Interestingly, βig-h3 has also been shown to enhance attachment and spreading of dermal fibroblasts, suggesting that it functions as an extracellular attachment protein in skin [LeBaron et al., 1995].

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