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Perlecan Up-Regulation of FRNK Suppresses Smooth Muscle Cell Proliferation via Inhibition of FAK Signaling

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We previously reported that fully assembled basement membranes are nonpermissive to smooth muscle cell (SMC) replication and that perlecan (PN), a basement membrane heparan sulfate proteoglycan, is a dominant effector of this response. We report here that SMC adhesion to basement membranes, and perlecan in particular, up-regulate the expression of focal adhesion kinase-related nonkinase (FRNK), a SMC-specific endogenous inhibitor of FAK, which subsequently suppresses FAK-mediated, ERK1/2-dependent growth signals. Up-regulation of FRNK by perlecan is actively and continuously regulated. Relative to the matrix proteins studied, the effects are unique to perlecan, because plating of SMCs on several other basement membrane proteins is associated with low levels of FRNK and corresponding high levels of FAK and ERK1/2 phosphorylation and SMC growth. Perlecan supports SMC adhesion, although there is reduced cell spreading compared with fibronectin (FN), laminin (LN), or collagen type IV (IV). Despite the reduction in cell spreading, we report that perlecan-induced up-regulation of FRNK is independent of cell shape changes. Growth inhibition by perlecan was rescued by overexpressing a constitutively active FAK construct, but overexpressing kinase-inactivated mutant FAK or FRNK attenuated fibronectin-stimulated growth. These data indicate that perlecan functions as an endogenously produced inhibitor of SMC growth at least in part through the active regulation of FRNK expression. FRNK, in turn, may control SMC growth by downregulating FAK-dependent signaling events.

INTRODUCTION

Vascular smooth muscle cells (SMCs) demonstrate high rates of replication during embryonic development and are capable of marked increases in replication after injury to the mature vessel wall, a major component of vascular remodeling observed in a variety of vascular fibroproliferative diseases (Clowes *et al*., 1983; Cook *et al*., 1994; Weiser-Evans *et al*., 2000). In the absence of vascular trauma, however, the mature blood vessel remains a highly quiescent tissue, and SMCs are resistant to stimulation by most mitogens, suggesting the existence of active growth-suppressive mechanisms (Lindner *et al*., 1990; Weiser *et al*., 1995). Individual SMCs within the medial layer of mature arteries are surrounded by an extracellular basement membrane matrix consisting predominantly of laminin (LN), collagen type IV (IV), fibronectin (FN), and perlecan (PN) heparan sulfate (Heickendorff, 1989). This basement membrane envelopes the SMCs, providing a barrier between the cell and its local microenvironment, and influences the general biological function of the cell. Previous data indicate that fully assembled basement membranes promote SMC differentiation and are nonpermissive to mitogen-induced SMC growth (Li *et al*., 1994; Weiser *et al*., 1997; Hedin *et al*., 1999). Perlecan is the dominant effector of this growth-suppressive response, and the HS chains of perlecan contribute to SMC growth inhibition (Weiser *et al*., 1997). In contrast, other basement membrane proteins appear to facilitate mitogen-induced SMC replication (Hedin *et al*., 1988; Thyberg and Hultgardh-Nilsson, 1994).

Perlecan is present in a variety of basement membranes, including those surrounding vascular SMCs (Hassell *et al*., 1980; Kleinman *et al*., 1986; Couchman, 1987; Murdoch *et al*.,

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1994). Perlecan is essential for the assembly and maintenance of a functionally complete basement membrane (Arikawa-Hirasawa *et al.*, 1999; Mercedes *et al.*, 1999) and plays a major role in the regulation of a wide variety of cellular processes, including migration, proliferation, adhesion, and regulation of growth factor activities (Iozzo *et al.*, 1994). Homozygous perlecan-null mice die in utero, at least in part because of severe cardiovascular abnormalities. Pertinent to the present study, hyperplasia of SMC-specific α-actin–positive mesenchymal cells was noted (Costell *et al.*, 2002). In addition, a number of studies have clearly demonstrated that perlecan is an important molecule in the control of SMC replication. Available evidence indicates that significant amounts of perlecan are produced by SMCs and that perlecan exists normally within the SMC basement membrane and functions as an endogenous suppressor of SMC replication (Fritze *et al.*, 1985; Weiser *et al.*, 1996, 1997; Bingley *et al.*, 1998; Nugent *et al.*, 2000). However, the intracellular signaling events underlying perlecan-induced SMC growth inhibition are unknown.

Our previous data and studies by others suggest that the assembly of a perlecan-rich SMC basement membrane actively prevents SMCs from replicating in the absence of matrix injury. We showed that growth inhibition by the extracellular basement membrane is driven by perlecan HS compared with chondroitin sulfate–rich proteoglycans and other basement membrane proteins. We therefore sought to determine the mechanism that mediates the effect of perlecan on SMC growth. The proliferation of most nontransformed cells is mediated through the cooperation between extracellular matrix (ECM)–integrin receptor interactions and growth factor signaling pathways (Assoian, 1997; Jones *et al.*, 1997; Howe *et al.*, 1998). Focal adhesion kinase (FAK) integrates integrin and growth factor receptor signaling pathways and transduces such signals to the downstream ERK1/2 pathway (Howe *et al.*, 1998; Renshaw *et al.*, 1999), making FAK important for cell growth (Schaller and Parsons, 1994; Gilmore and Romer, 1996; Sieg *et al.*, 2000). Focal adhesion kinase-related nonkinase (FRNK) is a critical regulator of FAK activity, its expression is highly restricted to vascular SMC lineages, and its in vivo expression patterns are similar to those reported for perlecan and inversely correlated to SMC growth (Taylor *et al.*, 2001). In the present study, we hypothesized that perlecan actively suppresses SMC proliferation via the up-regulation of FRNK. Collectively, we hypothesized that perlecan-induced up-regulation of FRNK mediates the SMC-specific growth-inhibitory effects of perlecan via the active inhibition of FAK-induced, ERK1/2-dependent cell cycle progression.

MATERIALS AND METHODS

Reagents and Antibodies

Human plasma-derived FN, mouse Engelbreth-Holm-Swarm (EHS)-derived, entactin-free LN, human placenta–derived IV, and mouse EHS-derived basement membrane extracts (BM; Matrigel) were from Collaborative Biomedical Products (Bedford, MA). Chondroitin sulfate proteoglycan (CSPG; mixture of versican, neurocan, phosphacan, and aggrecan) was from Chemicon (Temecula, CA). Human arterial endothelium–derived perlecan (PN) was purified as described previously (Whitelock, 2000). Briefly, conditioned media were collected, purified by DEAE chromatography followed by anti-perlecan immunoaffinity chromatography using perlecan domain 1–specific antibodies (specific for the perlecan core protein), and characterized using enzyme-linked immunosorbent assays. Bromodeoxyuridine (BrdU), myelin basic protein (MBP), heparin lyase I and II, chondroitin ABC lyase, HS, and monoclonal human-specific anti-myc (clone 9E10) antibody were from Sigma Chemicals (St. Louis, MO). Monoclonal anti-perlecan antibody was from Zymed Laboratories (South San Francisco, CA). Polyclonal anti–FAK-$^{748-1053}$C, monoclonal anti–FAK-$^{1-423}$N, and human-specific monoclonal anti-IL2R antibodies were from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti–phospho-p42/p44 ERK and anti–total p42/p44 ERK antibodies were from New England Biolabs, Inc. (Beverly, MA). Polyclonal anti–phospho-FAK (Y^{397}) antibody was from Biosource International (Camarillo, CA). Monoclonal anti-paxillin antibody was from Transduction Laboratories (Lexington, KY). Monoclonal anti-BrdU and FITC-conjugated anti-BrdU were from Becton Dickinson (Franklin Lakes, NJ). Rhodamine-labeled phalloidin was from Molecular Probes (Eugene, OR), and Cy3- and FITC-conjugated antibodies to mouse and rabbit IgGs were from Jackson Immunoresearch Laboratories (West Grove, PA). Enhanced chemiluminescence Western blotting detection reagents were from Amersham Life Science, Inc. (Arlington Heights, IL). All tissue culture supplies were from Life Technologies (Gaithersburg, MD). [γ-^{32}P]ATP was from Dupont-New England Nuclear (Boston, MA). The Bradford protein assay kit was from Bio-Rad (Richmond, CA).

Cell Culture and Growth Assays

The aortic media from adult Sprague Dawley rats was aseptically dissected, and SMCs were obtained by explant technique as previously described (Weiser-Evans *et al.*, 2000). The rat thoracic aorta SMC cell line, A10 SMC, was obtained from American Type Culture Collection (Rockville, MD; ATCC CRL 1476) and used for transfection studies. Glass coverslips or bacteriological plastic (Pl) was precoated with 10 μg/ml FN, LN, or IV or 5 μg/ml PN overnight at 4°C and then blocked with 10 mg/ml BSA for 1 h at 37°C. Cell culture flasks were coated with 50 ml/cm^2 Matrigel before cell plating. SMC replication was analyzed by BrdU immunocytochemistry as described previously (Weiser-Evans *et al.*, 2000).

Plasmids and Transfections

A cytomegalovirus (CMV) promoter–based plasmid containing wild-type myc-tagged FRNK was a kind gift from Dr. J.T. Parsons (Department of Microbiology, Health Sciences Center, University of Virginia, Charlottesville, VA). CMV promoter–based plasmids containing wild-type IL2R-tagged, constitutively activated FAK and dominant negative, kinase inactive IL2R-tagged FAK^{Y397F} were kind gifts from Dr. K.M. Yamada (Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health [NIH], Bethesda, MD). A10 SMCs were transfected by use of the Effectene transfection reagent (Qiagen, Valencia, CA). Using this protocol, equal transfection efficiencies of myc-FRNK, IL2R-FAKwt, and IL2R-FAK^{Y397F} were obtained (30–40%). For luciferase assays, a 2482–base pair fragment spanning nucleotides $^{-}$1989 and $^{+}$403 of the FRNK genomic sequence (Nolan *et al.*, 1999) was amplified using RT-PCR, chicken genomic DNA as template, and the following primer sets: 5′: GGATCCAAGCTTCTTCATCAG CCTTATGGC and 3′: GGTACCTTGGAGGAGGGAGCTGCCAATT. The PCR product was subcloned into the *Bam*HI and *Kpn*I sites of the pXP2 luciferase reporter vector (Nordeen, 1988). Proper orientation and sequence were confirmed by sequence analysis. A10 SMCs were plated in complete growth medium the day before transfection. Test DNA (1 μg) plus 0.5 μg of CMV-βGal reporter vector were cotransfected overnight by the Effectene method, and the cells were allowed to recover for 48 h. Cells were then replated on dishes precoated with FN, PN, or Matrigel and lysed 4 h after replating in reporter lysis buffer (Promega, Madison, WI). Luciferase activity for all lysates was quantified in a luminometer using Promega's luciferase assay

system. β-Galactosidase activity of each lysate was measured by standard procedures. Luciferase activity measured for each lysate was normalized to the relative β-galactosidase activity of the sample to give relative light units.

Preparation of Cell Lysates and Immunoblotting

Total cell proteins were isolated from SMC cultures, and equal amounts of protein were subjected to SDS-PAGE (4–12% gradient gels; NOVEX system, Invitrogen, Carlsbad, CA) followed by Western blotting as described previously (Weiser-Evans *et al.*, 2000). Ligand–antibody complexes were visualized using enhanced chemiluminescence detection kits and Hyperfilm x-ray film. Densitometry readings of phospho-FAK or total FRNK signals were obtained and normalized to total FAK signals (performed using the public domain NIH Image program; developed at the U.S. NIH and available at http://rsb.info.nih.gov/nih-image/).

ERK 1/2 Activity Assay

SMCs were growth-arrested in serum-free medium (SFM) for 48 h and then replated on specific matrix proteins in the presence or absence of 10% newborn calf serum for 4 h at 37°C. ERK1 and ERK2 immunocomplexes were obtained from 100 μg of total protein, and ERK 1/2 activity was determined as described previously (Li *et al.*, 1994). ERK 1/2 activity was expressed as picomoles of ^{32}P utilized per minute per milligram protein.

Immunofluorescence Microscopy

SMCs were trypsinized, replated on matrix-coated coverslips, and fixed at 4 h (for phosphorylated FAK and phalloidin) or 24 h (for BrdU) with 4% paraformaldehyde for 20 min. To analyze cell replication, SMCs were replated in the presence of 10 mM BrdU. Cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min and incubated with primary antibodies followed by Cy3- or FITC-conjugated secondary antibodies to visualize antigen–antibody complexes. Rhodamine-conjugated phalloidin was used to detect stress fiber formation, and FITC-conjugated anti-BrdU antibodies were used to detect replicating SMCs. Cells were coverslipped with a DAPI mounting medium (to detect all cells; Vector Laboratories, Burlingame, CA) and were analyzed using fluorescent microscopy.

RESULTS

Perlecan Suppresses Mitogen-stimulated SMC Growth

We previously showed that actively replicating SMCs cultured on fully assembled basement membranes fail to replicate in response to serum stimulation (Weiser *et al.*, 1997). Plating SMCs on PN but not on FN, LN, or IV mimics the morphological and gene expression alterations that occur in SMCs plated on intact basement membranes. To verify that PN is the dominant growth-suppressive component of basement membranes, rat aortic SMCs were plated in the presence of 10% NCS on tissue culture Pl or on individual matrices of FN, LN, IV, or PN and analyzed for their ability to replicate DNA (using BrdU immunocytochemistry). To examine the effects of basement membrane glycosaminoglycan side chains on SMC growth, HS or a CSPG mixture was added to SMCs plated on Pl. Although the CSPG mixture contains large, extracellular CSPGs, it was added to adhered SMCs because these proteoglycans are antiadhesive to SMCs (in contrast to PN, which supports adhesion of SMCs; our unpublished observations). Compared with SMCs plated on Pl, FN, LN, and IV, DNA synthesis in response to serum stimulation effectively ceased in SMCs plated on PN (Figure 1A). In addition, compared with PN, HS or CSPG had little effect on serum-stimulated growth.

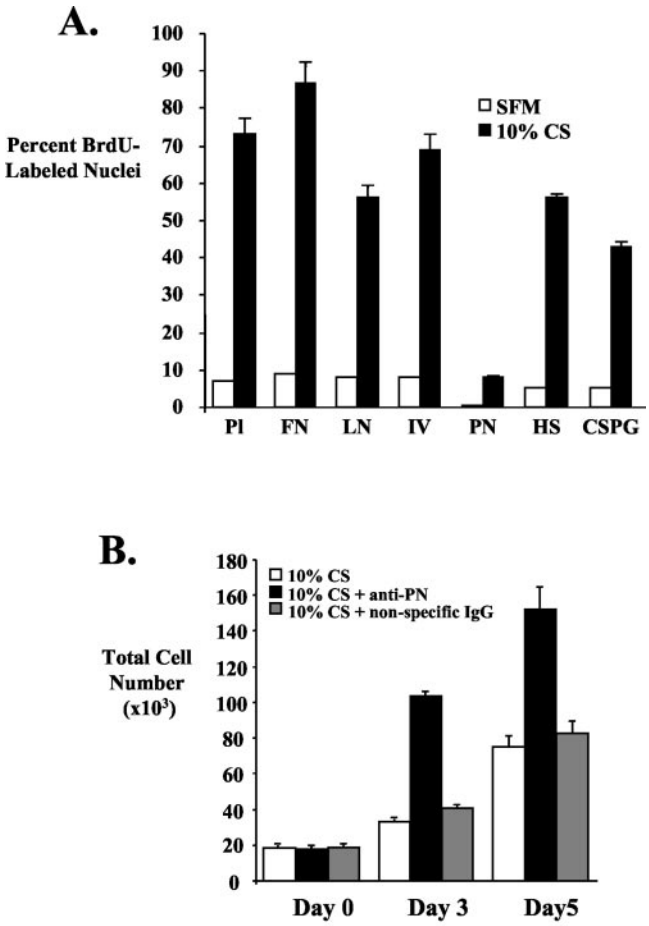

Figure 1. Suppression of serum-induced SMC growth by perlecan. (A) SMCs were plated on Pl, FN, LN, IV, or PN. Some SMCs plated on Pl were treated with 100 μg/ml HS or 10 μg/ml CSPG. SMCs were growth-arrested for 72 h in SFM, then stimulated with 10% CS (black bars) or kept in SFM (white bars) for an additional 24 h in the presence of 10 mM BrdU. SMCs were fixed and immunocytochemically stained for BrdU. The percentage of BrdU-positive SMCs was determined for each condition. A minimum of 500 cells per condition was counted, and the data are presented as the mean ± SEM. (B) SMCs were plated on Pl and allowed to attach overnight. Total cell numbers were determined in triplicate after 3 and 5 d of serum stimulation plus or minus antibodies. White bars, no IgG; black bars, plus 10 μg/ml neutralizing anti-perlecan antibody; gray bars, plus nonspecific IgG. The data are presented as the mean ± SEM. *$p < 0.05$

There was no loss of cell number or viability when SMCs were plated on PN or basement membranes. SMCs replated from growth-inhibitory matrices to growth-promoting matrices, such as FN, regained the ability to replicate in response to serum stimulation (our unpublished results). All experiments, unless otherwise noted, were conducted in the presence of serum to provide SMCs the necessary requirements for maximal growth on individual matrices. Even

under these conditions, perlecan and intact basement membranes retained growth-inhibitory properties.

SMCs cultured on Pl produce abundant amounts of basement membrane materials, including PN, although assembly of a mature SMC basement membrane does not occur and these cells remain in a mitogen-responsive state. To determine whether endogenously produced perlecan attenuates cell growth under normal culture conditions, SMCs were plated on Pl for 5 d in 10% NCS medium in the presence or absence of 10 μg/ml anti-perlecan neutralizing antibody. This antibody neutralizes the SMC growth–inhibitory properties of perlecan (Paka *et al.*, 1999). A nonspecific IgG was used as a negative control. There was a 3.5- to 4-fold increase in cell number in response to serum stimulation in untreated or nonspecific IgG–treated cultures (Figure 1B). In contrast, there was a 7.5-fold increase in cell number in response to serum stimulation in perlecan antibody-treated cultures, suggesting that endogenously produced perlecan attenuates mitogen-induced replication of cultured SMCs.

Intact Basement Membranes and Perlecan Suppress SMC ERK1/2 Activation

Adhesion-dependent growth requires the integration of integrin- and growth factor–mediated signaling events, both of which converge at the level of ERK1/2. We therefore hypothesized that fully assembled basement membranes, and PN in particular, suppress SMC growth by actively inhibiting ERK1/2 signaling events. We first examined ERK1/2 kinase activity after adhesion of SMCs to Pl, to individual matrices of FN, LN, and IV, and to intact basement membranes. Using a kinase assay for total MAPK activity (ERK1 and ERK2), we found that serum-stimulated total MAPK activity was significantly increased in SMCs plated on Pl and on FN, LN, and IV matrices (Figure 2A). In contrast, plating SMCs on basement membranes resulted in suppression of serum-stimulated total MAPK activation.

We next looked at the direct effect of PN on ERK1/2 activation. For these experiments, SMCs were replated on PN or FN matrices in the presence of 10% NCS. Four hours after replating, whole-cell lysates were collected and analyzed by SDS-PAGE for phospho-ERK1/2 and for total ERK1/2. As shown in Figure 2B, SMCs replated on FN showed very high levels of phospho-ERK1/2, whereas SMCs replated on PN showed very low levels of phospho-ERK1/2.

Perlecan Suppresses FAK Activation and Downmodulates Cytoskeletal Organization and Focal Adhesion Complex Formation

Because FAK integrates integrin and growth factor receptor signaling pathways and activates downstream ERK1/2-dependent growth pathways, we examined the effects of basement membranes and PN, compared with Pl and other basement membrane components, on suppressing FAK activation. SMCs were maintained in suspension in medium containing serum as a control for adhesion-mediated FAK activity. Using Western analysis and a total FAK-specific antibody, we found that equal amounts of total FAK protein were expressed by SMCs in all conditions (Figure 3A). Although all matrices supported adhesion of SMCs, tyrosine phosphorylation of FAK (using a Y^{397} phospho-FAK–specific antibody) was suppressed in SMCs plated on PN and intact basement membranes (Figure 3A). Likewise, FAK phosphorylation was minimal in suspended SMCs. Moreover, FAK phosphorylation was markedly reduced in cell lysates isolated from the adult uninjured aortic media, corresponding to very low SMC growth rates in vivo (Figure 3A; in vivo). These data suggest that perlecan contributes to SMC growth suppression at least in part by suppressing FAK-mediated growth signals.

Cell adhesion via integrins is associated with cytoskeletal organization and the formation of focal adhesion complexes, resulting in the generation of intracellular signals involved in cell cycle control. We therefore examined the effects of individual base-

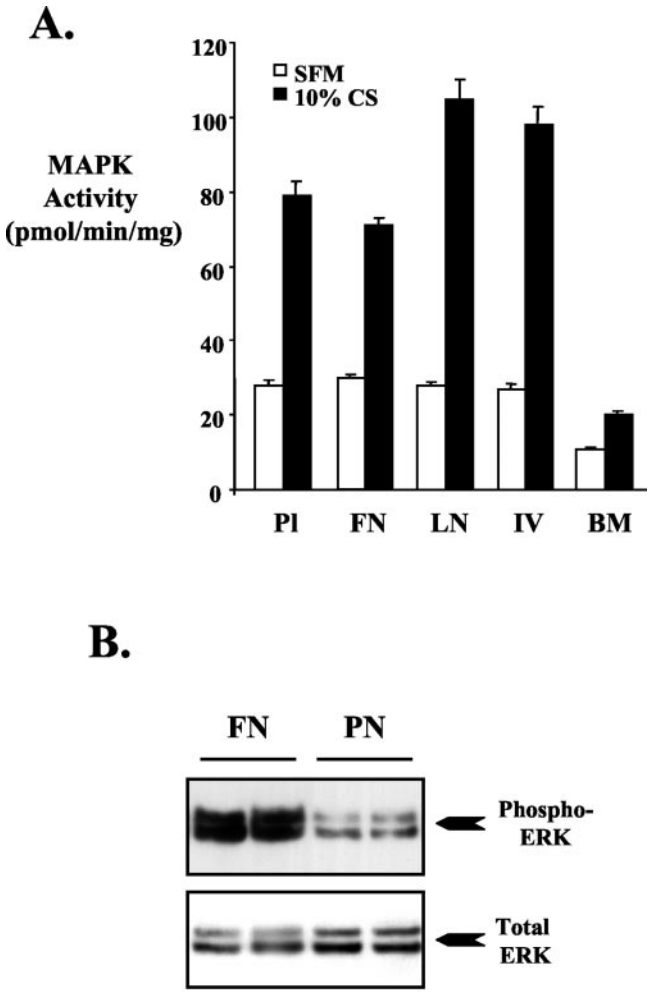

Figure 2. Effects of basement membranes and perlecan on serum-stimulated MAPK activation. (A) SMCs were replated on basement membranes (BM), FN, LN, or IV, or Pl in the presence (black bars) or absence (white bars) of 10% NCS for 4 h. Total MAPK (ERK 1 and 2) activity was measured as described in MATERIALS AND METHODS. The data are shown as picomoles ATP utilized per minute per milligram total protein and are presented as the mean ± SEM of three separate experiments. (B) SMCs were replated on PN or FN matrices in the presence of 10% CS. Whole-cell lysates were collected 4 h after replating, and equal protein concentrations were analyzed by Western analysis for phosphorylated ERK1/2 (top). The filter was stripped and reprobed with a total ERK1/2 antibody (bottom). Shown in the Western blot are two independent experiments for each matrix protein.

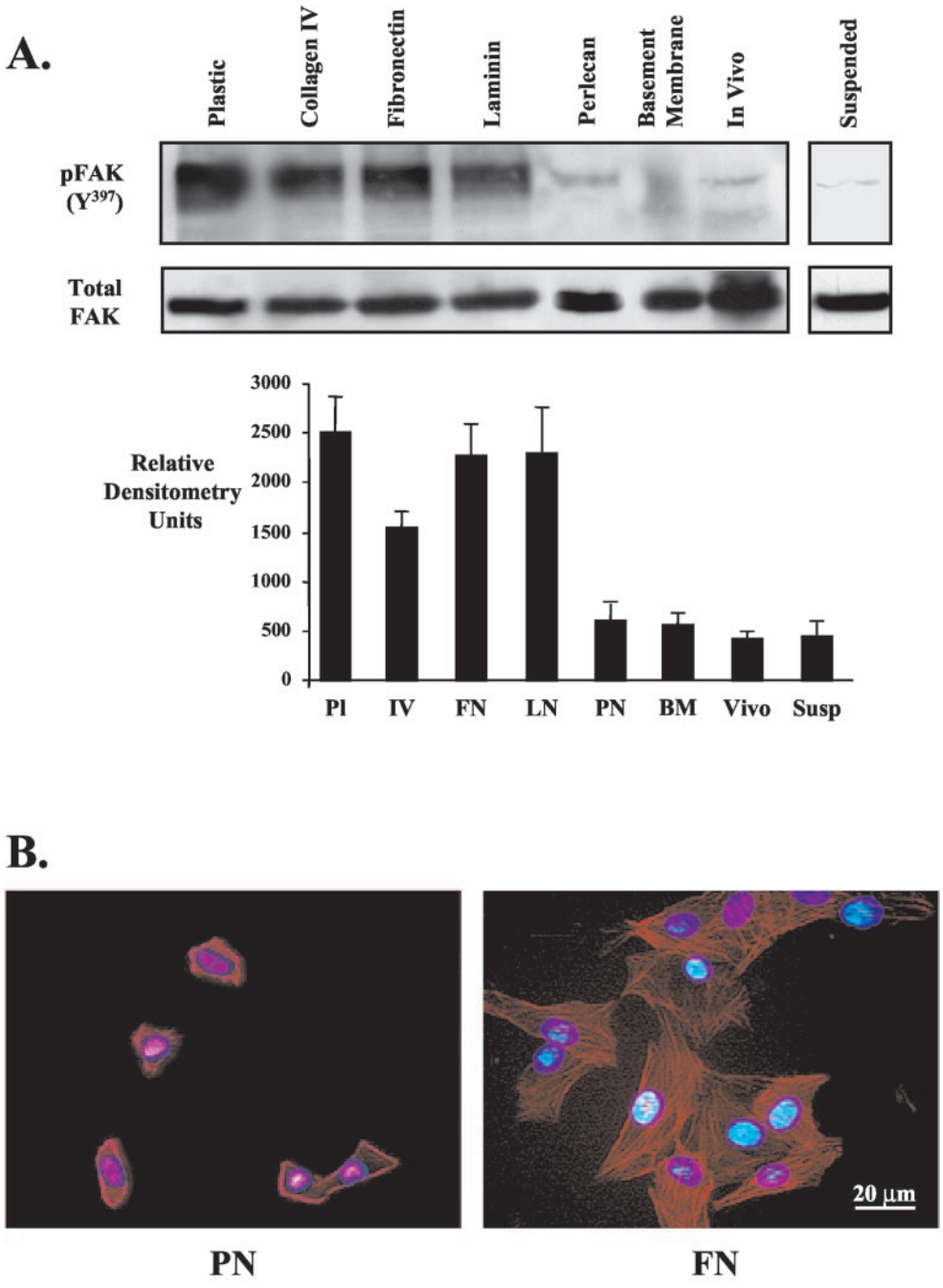

Figure 3. Perlecan suppresses FAK activation and modulates actin microfilament formation. (A) Medial SMCs were isolated from uninjured rat aortas, and total protein was extracted (in vivo). Subcultured SMCs were left in suspension (suspended) in 10% CS or were plated on Pl, IV, FN, LN, PN, or basement membranes (BM) in 10% CS. Cell lysates were prepared after 4 h, and equal protein concentrations were analyzed by Western blotting. Shown is a representative Western blot. Top, FAK phosphorylation levels using an anti–phosphoY^{397}-FAK antibody. Bottom, total FAK protein as well as equal protein loading as determined by anti-FAK immunoblotting. Western blots were scored for relative densitometry, and the data are presented in the graph as the mean ± SEM of at least three independent experiments. (B) SMCs were plated on PN or FN matrices in the presence of 10% CS and allowed to attach for 4 h. SMCs were immunofluorescently stained with rhodamine-labeled phalloidin to detect F-actin stress fiber formation and with DAPI to identify total cells.

ment membrane proteins on SMC spreading and actin microfilament and focal adhesion formation. SMCs were plated on PN, FN, LN, or IV matrices, allowed to attach for 4 h, and were fixed and stained with rhodamine-labeled phalloidin. As shown in Figure 3B, fully developed stress fibers were assembled after adhesion to FN. This pattern of staining was also observed in SMCs plated on LN and IV (our unpublished results). In contrast, after adhesion to PN, cell spreading was reduced and SMCs exhibited poorly assembled stress fibers. Our unpublished results also showed that, although adhesion to FN resulted in the formation of numerous focal adhesions containing paxillin and phosphorylated FAK, adhesion to PN was associated with reduced focal adhesions with little if any phosphorylated FAK present.

Activation of FAK Reverses Perlecan-mediated SMC Growth Suppression

We next determined whether inhibition of FAK signaling was essential for the growth-inhibitory effects of perlecan.

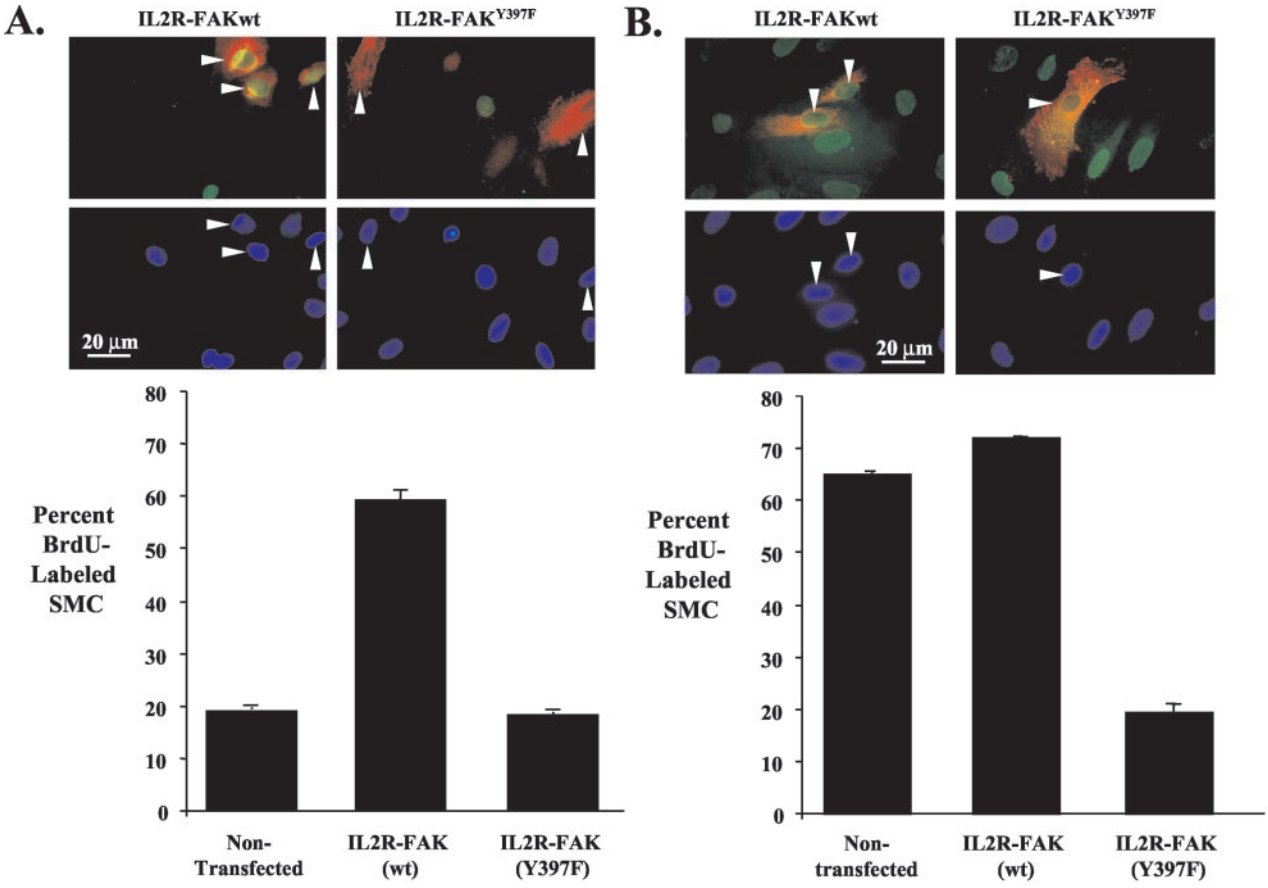

Figure 4. Effects of constitutively active FAK on perlecan-mediated SMC growth suppression. A10 SMCs were transiently transfected with constitutively active, wild-type IL2R-FAK (wt) or with dominant negative, kinase-inactivated IL2R-FAK^{Y397F}, allowed to recover for 24 h, then were replated on coverslips precoated with (A) PN or (B) FN in the presence of 10% CS and 10 mM BrdU. Cells were fixed 24 h later and immunofluorescently stained for BrdU and IL2R. Cells were counterstained with DAPI to identify total SMCs. The percentage of BrdU-positive SMCs was determined independently for nontransfected (IL2R-negative) and transfected (IL2R-positive) SMCs by counting a minimum of 200 SMCs per condition per experiment. The data are presented as the mean ± SEM. *$p < 0.05$. Top, representative immunofluorescence stains. Bottom, the same field, DAPI stain. Arrowheads, IL2R-positive SMCs (red). Green nuclei (yellow with overlay), BrdU-positive SMCs.

For these experiments, we tested whether overexpression of constitutively active FAK could rescue the SMC growth–inhibitory effects of perlecan. A10 SMCs were transiently transfected with plasmids containing IL2R-FAK wild-type fusion protein or IL2R-FAK^{Y397F} kinase dead fusion protein. When expressed, the IL2R-FAK wild-type construct retains constitutive FAK activity, whereas the IL2R-FAK^{Y397F} mutant encodes a dominant negative FAK protein lacking kinase activity (Tamura *et al*., 1999). Transfected SMCs were replated in the presence of 10% NCS and BrdU on coverslips precoated with PN or FN, fixed 24 h later, and immunofluorescently stained for BrdU and for IL2R (human-specific epitope to identify transfected SMCs). IL2R-FAK wild-type transfected SMCs demonstrated high rates of DNA synthesis when plated on PN (comparable to levels observed on FN; Figure 4A), suggesting that overexpression of constitutively active FAK could rescue the SMC growth–inhibitory effects of PN. In contrast, serum-stimulated growth was significantly suppressed when nontransfected SMCs and dominant negative IL2R-FAK^{Y397F} overexpressing SMCs were plated on PN (Figure 4A). In our unpublished results, we also found that SMCs exhibiting high levels of constitutively active ERK1/2 demonstrated significant increases in cell growth when plated on perlecan-rich matrices.

IL2R-FAK wild-type transfected SMCs showed high rates of DNA synthesis when plated on FN (comparable to or slightly higher than nontransfected controls; Figure 4B). In contrast, growth was significantly decreased in dominant negative IL2R-FAK^{Y397F}-overexpressing SMCs. Collectively, these data strongly suggest that suppression of FAK activation is central to perlecan-mediated SMC growth inhibition.

FAK Activity Is Essential for FN-dependent SMC Growth

To verify that FAK is essential for SMC growth, A10 SMCs were transiently transfected with plasmids containing wild-type, myc-tagged FRNK, a dominant negative regulator of FAK activity (Richardson and Parsons, 1996). Transfected SMCs were replated in the presence of 10% NCS and BrdU

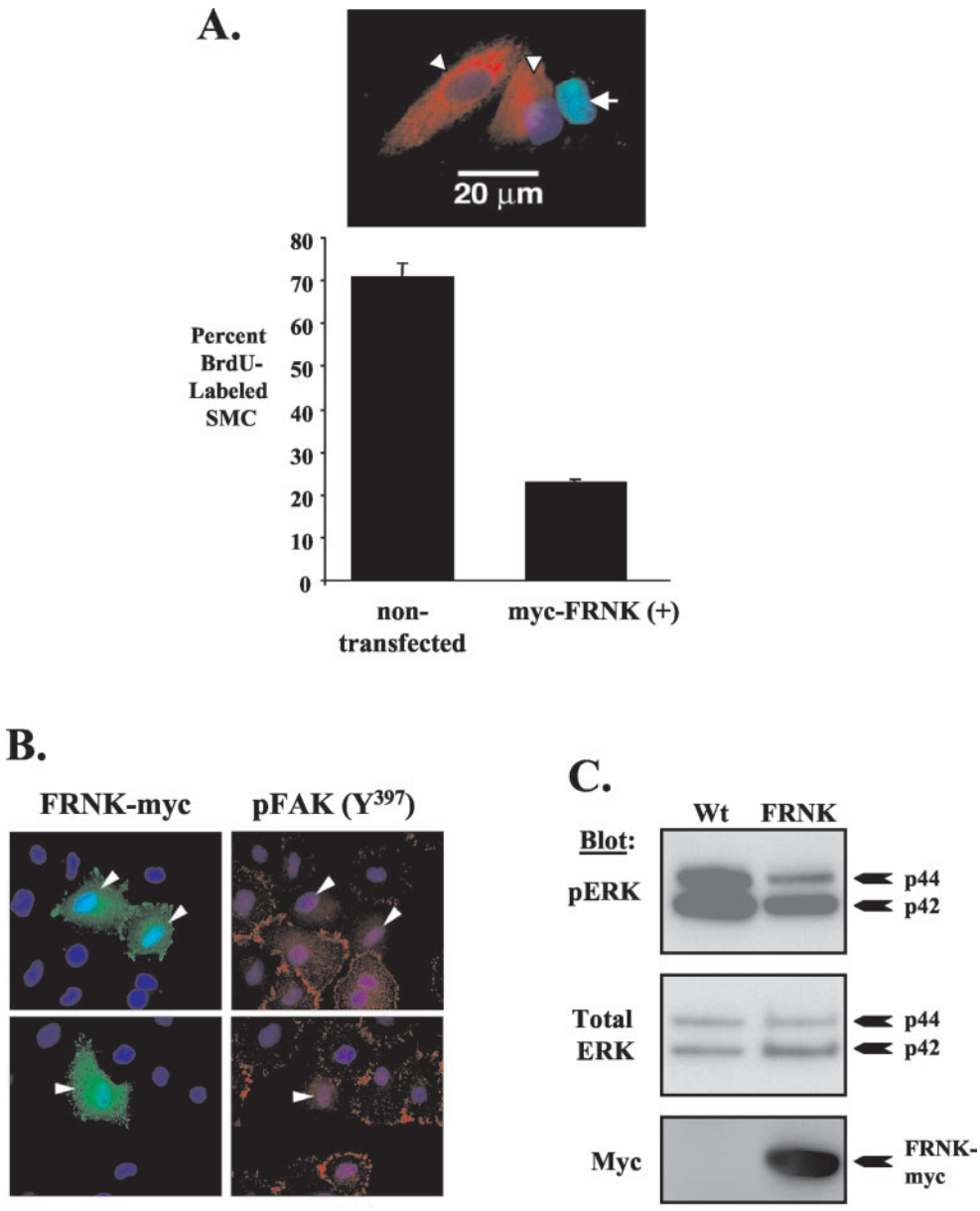

Figure 5. FRNK overexpression suppresses FN-mediated SMC growth and FAK phosphorylation. (A) A10 SMCs were transiently transfected with myc-FRNK, allowed to recover for 24 h, then replated on coverslips precoated with FN in the presence of 10% CS and 10 mM BrdU. Cells were fixed 24 h later and immunofluorescently stained for BrdU and myc. Cells were counterstained with DAPI to identify total cells. The percentage of BrdU-positive SMCs was determined independently for nontransfected (myc-negative) and transfected (myc-positive) SMCs by counting a minimum of 200 myc-negative and/or myc-positive SMCs per experiment. The data are presented as the mean ± SEM. *$p < 0.05$. Top, representative immunofluorescence stain. Arrow indicates BrdU-positive, myc-negative SMCs. Arrowheads indicate a BrdU-negative, myc-positive SMCs. (B) Myc-FRNK–transfected SMCs were replated on FN and fixed 1 h after replating. SMCs were immunofluorescently stained for myc (left) and activated FAK using an anti–phosphoY^{397}-FAK antibody (right). Cells were counterstained with DAPI to identify total cells. Arrowheads indicate myc-positive SMCs. Top and bottom, two independent experiments. The left and right panels of each set are the same field views for myc and phospho-FAK. (C) Cell lysates were prepared from SMCs transfected as described above, and equal protein concentrations were analyzed by Western blotting using anti–phospho-ERK1/2, anti–total ERK1/2, and anti-Myc antibodies. Top, phosphorylated ERK1/2; middle, total ERK1/2 protein and equal protein loading; and bottom, blotting for FRNK-Myc fusion protein to verify transfection. Wt, nontransfected SMCs; FRNK, FRNK-myc–transfected SMCs.

on coverslips precoated with FN, fixed 24 h later, and immunofluorescently stained for BrdU and myc. Nontransfected SMCs exhibited high rates of DNA synthesis when plated on FN. In contrast, SMC growth was significantly decreased in FRNK-overexpressing SMCs (Figure 5A), consistent with a previous report (Taylor *et al.*, 2001).

Parallel cultures were fixed for immunofluorescence analysis of pFAK^{Y397}, or whole-cell lysates were collected for Western analysis of pERK1/2 1 h after replating to determine whether FRNK overexpression alters FAK and ERK1/2 signaling. Nontransfected SMCs were used as controls for Western analysis. As shown in Figure 5B, after 1 h of adhesion, nontransfected SMCs (myc-negative) formed numerous focal adhesions containing phosphorylated FAK. In contrast, although FRNK-overexpressing SMCs (myc-positive) attached and formed focal adhesions on FN, very little, if any, phosphorylated FAK was present. In addition, ERK1/2 activation was significantly suppressed in FRNK-transfected SMCs (Figure 5C). Combined with the IL2R-FAK data (Figure 4), these data confirm that FAK activation is essential for SMC growth. These data also suggest that ERK1/2 acts downstream of FAK in SMCs.

Perlecan–SMC Interactions Suppress FAK Activation through the Up-regulation of FRNK

FAK activity is modulated by FRNK, a 42-kDa SMC-specific protein consisting of the carboxyl-terminal noncatalytic do-

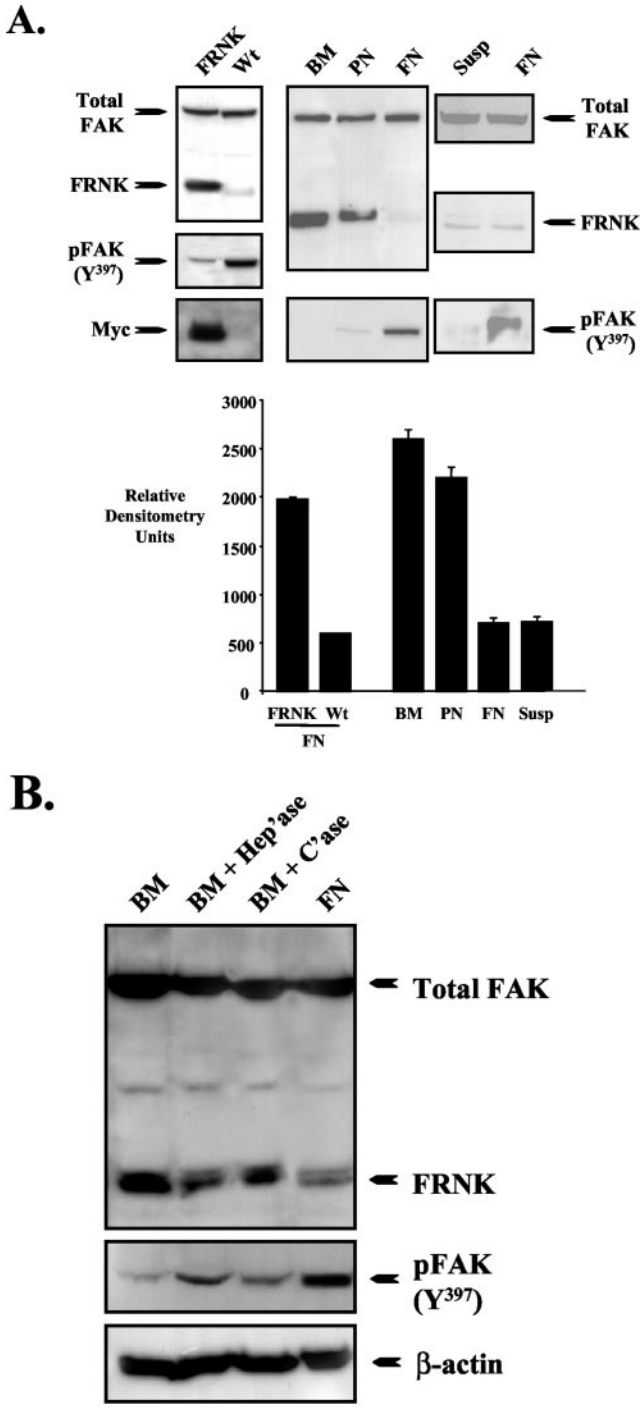

Figure 6. SMC–perlecan interactions up-regulate FRNK expression. (A) SMCs were left in suspension (susp) in 10% CS or were plated on basement membranes (BM), FN, or PN in 10% CS. Cell lysates were prepared after 4 h, and equal protein concentrations were analyzed by Western blotting using anti–total FAK carboxyl terminal or anti–phosphoY^{397}-FAK antibodies. Shown are representative blots. Top, total levels of FAK and FRNK. Bottom, phosphorylated FAK. Left, A10 SMCs were transfected with myc-FRNK and replated on FN as described in Figure 5. Whole-cell lysates were analyzed by Western blotting using anti–total FAK, anti–phosphoY^{397}-FAK, and anti-myc antibodies. Westerns were scored for relative densitometry as described in MATERIALS AND METHODS, and the data are presented in the graph. (B) Basement membranes were digested with 10 U/ml heparin lyase I and II (Hep'ase) or 1 U/ml chondroitin lyase ABC (C'ase) for 4 h at 37°C. SMCs were plated on untreated or treated membranes or on FN in the presence of 10% NCS. Cell lysates were prepared after 4 h, and equal protein concentrations were analyzed by Western blotting using anti–total FAK carboxyl terminal (top) or anti–phosphoY^{397}-FAK (middle) antibodies. β-Actin was used as a control for protein loading (bottom).

main of FAK (Richardson and Parsons, 1996). FRNK is transcribed independently by an alternative promoter within an intron of the FAK gene, 3′ of the FAK kinase domain and 5′ of the focal adhesion targeting domain (Nolan *et al*., 1999). FRNK is expressed selectively in vascular SMCs, and its in vivo expression patterns correlate inversely to high rates of SMC replication during vascular development and after injury to the adult artery (Taylor *et al*., 2001). These in vivo expression patterns are similar to those reported for perlecan (Weiser *et al*., 1996; Weiser-Evans and Stenmark, 1999). We therefore examined whether perlecan–SMC interactions result in the up-regulation of FRNK, which subsequently suppresses FAK activation. SMCs were plated in the presence of 10% NCS on FN, PN, or basement membrane matrices for 4 h, and whole-cell lysates were analyzed for total FAK and total FRNK expression using an anti–FAK carboxyl terminal antibody or for activated FAK using an anti-pFAK^{Y397} antibody. Equal amounts of total FAK protein were expressed by SMCs under all conditions (Figure 6A). Low but detectable levels of FRNK were expressed by SMCs plated on FN (similar to levels in SMCs plated on tissue culture Pl; our unpublished results). In contrast, SMCs plated on basement membranes or on PN matrices exhibited very high levels of FRNK. Correspondingly, whereas SMCs plated on FN exhibited high levels of phosphorylated FAK, FAK phosphorylation was suppressed in SMCs plated on basement membranes or perlecan (Figure 6A). Because perlecan–SMC interactions result in reduced SMC spreading, as a control for cell-shape changes, we also examined the expression of FRNK in suspended SMCs. In contrast to the effects mediated by perlecan, FRNK levels remained low in SMCs maintained in suspension (similar to levels observed in SMCs plated on FN) (Figure 6A), suggesting that perlecan-induced up-regulation of FRNK is independent of cell-shape changes. The endogenous levels of FRNK up-regulated by basement membranes or PN were similar to exogenous, overexpressed levels needed to suppress FAK phosphorylation and growth in SMCs plated on FN (Figure 6A).

We next examined the role of extracellular glycosaminoglycan chains in mediating this effect. In Figure 1, we showed that soluble HS and CSPG were not as effective growth inhibitors as perlecan, suggesting that perlecan core protein–SMC interactions are essential for growth inhibition. To determine whether glycosaminoglycan chains are involved in the up-regulation of FRNK, basement membranes were treated with heparin lyase I/II or chondroitin ABC lyase. SMCs were then plated on untreated or digested membranes in the presence of 10% NCS. In contrast to SMCs plated on untreated or chondroitinase-treated membranes, SMCs plated on heparinase-treated basement membranes exhibited lower levels of FRNK and higher levels of phosphorylated FAK (Figure 6B). However, the de-

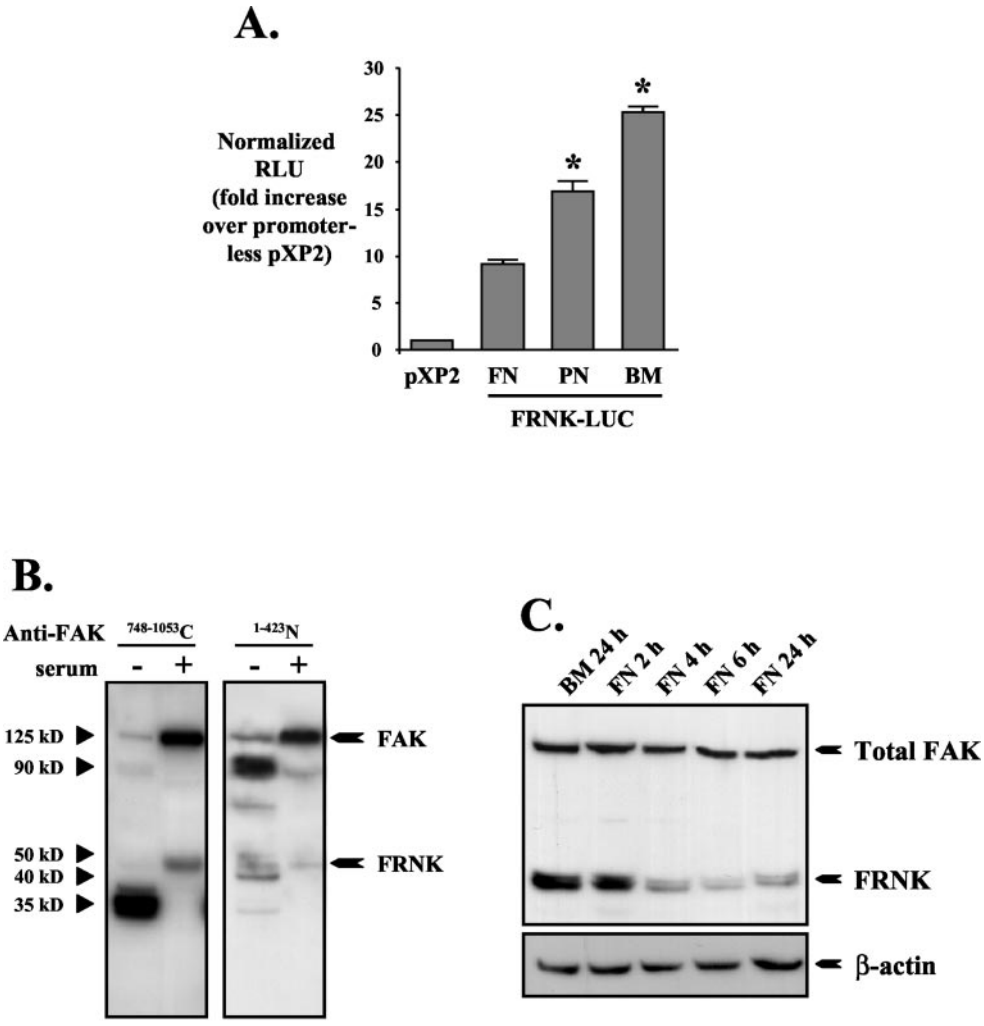

Figure 7. Up-regulation of FRNK by perlecan is actively and continuously regulated. (A) A10 SMCs were transfected with a pXP2-FRNK-LUC construct or a promoterless pXP2-LUC construct, and cell lysates were assayed for luciferase activity as described in MATERIALS AND METHODS (1 μg of test DNA plus 0.5 μg of CMV-βGal per transfection). β-Galactosidase activity of each lysate was measured, and luciferase activity for each lysate was normalized to the relative β-galactosidase activity of that lysate to give relative light units. Data are reported as fold activation $\pm$ SEM over promoterless pXP2 vector (control set to 1) of three separate transfections. *$p < 0.05$. (B) SMCs were growth-arrested in SFM for 72 h, then replated on FN in the presence or absence of 10% NCS. Cell lysates were prepared 1 h later, and equal protein concentrations were analyzed by Western analysis using a polyclonal anti–carboxyl terminal total FAK antibody (residues 748-1053; left). The filter was stripped and reprobed with a monoclonal anti–amino terminal total FAK antibody (residues 1–423; right). Arrowheads indicate approximate molecular weights of FAK and FAK cleavage fragments. (C) SMCs were plated on basement membranes in the presence of 10% NCS for 24 h. Membranes were digested with Dispase, and cells were replated on FN. SMCs were harvested 24 h after plating on basement membranes or at 2, 4, 6, and 24 h after replating. Cell lysates were prepared and analyzed for total FAK and FRNK using an anti–total FAK carboxyl terminal antibody (top). The filters were stripped and reprobed for β-actin as a control for protein loading (bottom).

crease in FRNK (and increase in phosphorylated FAK) was considerably less than that observed in SMCs plated on FN matrices.

To determine whether FRNK protein expression correlates to FRNK transcriptional activity, a 2482-base pair fragment representing the FRNK promoter (Nolan *et al*., 1999) was cloned into the pXP2 luciferase reporter vector (Nordeen, 1988). This construct was tested for the ability to drive luciferase expression after transfection into SMCs. Transfected SMCs were replated in the presence of 10% NCS on dishes precoated with FN, PN, or basement membranes. As shown in Figure 7A, SMCs transfected with the FRNK-LUC construct and replated on FN showed a 10-fold increase in luciferase activity compared with SMCs transfected with a promoterless control construct. This suggests that SMCs exhibit basal FRNK promoter activity, most likely representing SMC-selective expression of FRNK. However, SMCs transfected with the FRNK-LUC construct and replated on PN or basement membranes showed 17- and 25-fold increases in luciferase activity, respectively (Figure 7A), suggesting that SMC interactions with PN or basement membrane matrices can actively increase expression of FRNK above basal SMC levels.

Previous studies suggest that FAK activity may also be regulated by calpain-mediated cleavage of FAK (Carragher *et al*., 1999). To verify that the 42-kDa protein is FRNK and not a cleavage product of FAK, SMCs were growth-arrested under serum-free conditions for 72 h, then replated on FN in SFM or 10% NCS. Western analysis and anti–FAK carboxyl-terminal and amino-terminal antibodies were used to detect total FAK, total FRNK, and FAK cleavage products. Replating SMCs on FN in serum-free conditions induced FAK cleavage, resulting in a significant reduction in native p125FAK and p41FRNK. Using the C-terminal antibody, this reduction in FAK and FRNK was accompanied by an increase in a 35-kDa fragment, consistent with a calpain-sensitive cleavage site in the carboxyl end of FAK (and in FRNK) (Figure 7B) (Carragher *et al*., 1999). Using the N-terminal antibody, a reduction in FAK was detected along with an accompanying increase in predominantly

a 90-kDa fragment but also 50-, 42-, and 40-kDa fragments, consistent with several calpain-sensitive cleavage sites within native FAK (Figure 7B) (Carragher *et al.*, 1999). SMCs replated on FN in serum-rich conditions showed only native p125FAK and p41FRNK.

The above experiments demonstrated that FRNK is up-regulated when SMCs are plated on nonproliferative compared with proliferative substrates. To determine whether loss of perlecan–SMC interactions results in the rapid degradation of FRNK, SMCs were plated on basement membranes for 24 h (to induce high levels of FRNK), gently removed with dispase digestion, and replated on FN matrices. SMCs were harvested at incremental times after replating and analyzed for total FAK and FRNK. As shown in Figure 7C, within 4 h of replating SMCs on FN, FRNK protein levels decreased significantly to levels comparable to those observed in SMCs plated on FN for 24 h. Collectively, these data suggest that SMC interactions with PN result in selective, active, and continuous up-regulation of FRNK rather than in cleavage of FAK.

DISCUSSION

Accumulating evidence suggests that integrin–ECM interactions promote the coclustering of integrin and growth factor receptors, resulting in increased FAK signaling, efficient growth factor signaling to ERK 1/2, and subsequent cell replication (Clark and Brugge, 1995; Aplin and Juliano, 1999; Giancotti and Ruoslahti, 1999; Sieg *et al.*, 2000). However, little is known about how the process of adhesion-mediated, FAK-dependent cell replication is down-regulated. A fully assembled, perlecan-rich SMC basement membrane actively suppresses vascular SMC growth. In the present study, we explored the signaling events underlying basement membrane– and perlecan-mediated SMC growth suppression. We showed that SMC interactions with both intact basement membranes and individual matrices of perlecan result in the active up-regulation of FRNK. Increased levels of FRNK at least in part contribute to SMC growth inhibition via the suppression of FAK- and ERK1/2-dependent growth signals. The effects of perlecan appear to be cell shape independent and unique among the basement membrane components studied, including FN, IV, LN, and extracellular CSPGs. The growth-inhibitory effects of perlecan are overridden by constitutively activating FAK, and by overexpressing FRNK or a kinase-inactivated mutant FAK construct, we verified that FAK signaling plays an integral role in ECM-mediated SMC growth. Taken together, we propose that the deposition of perlecan into the SMC basement membrane acts as an endogenous inhibitor of SMC growth at least in part by increasing FRNK protein levels, which in turn down-modulate FAK-dependent growth signals.

We previously demonstrated that, in the absence of vascular trauma or injury, SMCs in the mature artery remain highly quiescent in large part because of the incorporation of perlecan HS into the basement membrane (Weiser *et al.*, 1996, 1997). Injury-induced removal or proteolysis of perlecan results in increased replicative potential, thus activating SMCs to respond to mitogenic stimuli (Weiser *et al.*, 1997). We proposed that activation of SMCs is mediated through increased ECM–integrin interactions and subsequent integrin-mediated intracellular signaling, and perlecan actively inhibits this process through a previously undefined mechanism. We show here that perlecan–SMC interactions actively up-regulate FRNK expression. Loss of such interactions is associated with rapid degradation of FRNK, further strengthening the hypothesis that active inhibitory and stimulatory mechanisms coordinately regulate SMC growth.

Interestingly, Taylor *et al.* (2001) reported that FRNK is selectively expressed by SMCs. Vascular SMC expression of FRNK is minimal during embryonic development; is up-regulated significantly in the early postnatal vessel, a time point at which SMCs demonstrate significant decreases in replication (Cook *et al.*, 1994); and continues to be expressed at low but detectable levels in the adult vessel. Similar expression patterns are observed after injury to the adult vessel. These in vivo patterns of expression are similar to those reported for perlecan (Weiser *et al.*, 1996; Weiser-Evans and Stenmark, 1999). The present data suggest that promoter sequences within the intronic region of the FAK/FRNK gene representing the FRNK promoter contain perlecan-responsive regulatory elements that are responsive to changes in SMC adhesive events. Although we have yet to describe the specific promoter regions responsible for the up-regulation of FRNK, identification of these regulatory elements will yield important information on the role of active processes regulating SMC quiescence.

The mechanism mediating perlecan-induced up-regulation of FRNK is not known. Perlecan gene knockout studies support the hypothesis that interaction of the perlecan core protein with other basement membrane components contributes to the assembly and structural integrity of a functionally complete basement membrane (Arikawa-Hirasawa *et al.*, 1999; Mercedes *et al.*, 1999). Perlecan is known to interact directly with various ECM proteins, including FN, collagens I, III, IV, and V, and LN (Whitelock *et al.*, 1999). Therefore, one possible mechanism is that perlecan prevents integrins from interacting with basement membrane ligands thus inducing a passive up-regulation of FRNK through the loss of integrin-mediated suppression of FRNK gene transcription. In support of this, Lundmark *et al.* (2001) demonstrated that a combined substrate of perlecan and FN significantly reduces SMC adhesion as well as FAK phosphorylation and SMC growth (our unpublished results) compared with FN alone. However, unpublished data from our laboratory suggest that inhibition of integrin function has no effect on FRNK expression, arguing against a role for integrins in the regulation of FRNK. We are currently vigorously pursuing the potential role of integrins in the control of FRNK regulation.

In addition, although perlecan supports adhesion of SMCs, it also significantly reduces cell spreading compared with other ECM proteins. Cell-shape changes are associated with cell replication, with cell rounding usually inducing growth arrest (Assoian, 1997). Therefore, perlecan-mediated inhibition of cell spreading could result in a passive up-regulation of FRNK and subsequent cell-cycle arrest. However, whereas adhesion of SMCs to perlecan induced increases in FRNK, there were no significant changes in FRNK levels in suspended SMCs, suggesting that up-regulation of FRNK is dependent on perlecan–SMC interactions but not on cell-shape changes. As in the present study, Motamed *et al.* (2002) reported that the SMC antiproliferative effect of SPARC, a matricellular glycoprotein, is independent of changes in cell shape.

Alternatively, all of the cellular processes shown to be regulated by perlecan could be mediated via a cell surface receptor. Our unpublished work, showing that perlecan interacts

directly with the SMC surface, is highly suggestive of the existence of perlecan-binding proteins. Although a perlecan-specific receptor has yet to be identified, it remains possible that these effects occur through a receptor-based mechanism. One report describes a nonintegrin perlecan-specific binding protein present in membrane extracts of a variety of cells (Clement *et al*., 1989), although further identification of this protein or a function were not discussed. In addition, two neural-derived CSPGs inhibit neurite outgrowth, presumably through a receptor-mediated mechanism that actively inhibits cell attachment to growth-promoting ECM and subsequent integrin-induced growth (Condic *et al*., 1999; Li *et al*., 2000). Therefore, it is possible that regulation of FRNK expression by perlecan is direct, through the interaction with a SMC membrane–bound receptor. Clearly, identification of a perlecan binding protein/receptor on the SMC surface will help determine the mechanism of growth arrest induced by perlecan.

In addition, arterial SMCs synthesize perlecan with highly sulfated HS chains, and removal increases the adhesion of SMCs to perlecan (Fritze *et al*., 1985; Whitelock *et al*., 1999). The present data and our previous work (Weiser *et al*., 1997) indicate that at least some of the activity of perlecan resides in its HS side chains, consistent with a large body of evidence implicating heparin-like molecules in the regulation of various SMC functions (Clowes and Karnovsky, 1977; Castellot *et al*., 1981; Campbell *et al*., 1992). However, we showed that addition of soluble HS to cultured SMCs does not elicit the same degree of growth inhibition (Figure 1) or changes in FRNK expression (our unpublished results) as observed with perlecan matrices. Also, although removal of basement membrane HS chains before plating of SMCs resulted in reduced FRNK levels, they were not reduced to the low levels observed in SMCs plated on FN. In addition, the effect of basement membrane perlecan seems to be unique among extracellular proteoglycans, because extracellular chondroitin sulfate and/or basement membrane chondroitin sulfate chains had little effect on SMC growth and FRNK expression. Therefore, heparin/HS, although a strong inhibitor of SMC growth, is much less potent than fully sulfated perlecan, suggesting that the efficacy of perlecan on SMC functions may derive from the coordinate binding of the core protein and the HS side chains to the SMC surface.

The uncontrolled replication of SMCs is a major contributor to the vessel remodeling observed in a variety of vascular pathological conditions. Despite major advances in vascular biology, the mechanisms regulating continual SMC replication remain unknown, thus adding to the largely unsuccessful treatment and poor clinical outcome. Therefore, an understanding of the normal growth-suppressive mechanisms operative in the mature blood vessel could lead to clinical applications targeted to specific endogenous signaling pathways. The results presented here indicate that the interaction of a fully assembled basement membrane or perlecan HS with individual SMCs results in the active up-regulation of FRNK in a cell shape–independent manner. Our results are consistent with an increasing amount of information supporting a role for perlecan in the inhibition of SMC growth and lesion formation. Because the size and complexity of perlecan realistically limits its potential for therapeutic use, studies are ongoing to define the signaling events mediating these effects.

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