Fibronectin Matrix Turnover Occurs through a Caveolin-1–dependent Process

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Extracellular matrix remodeling occurs during development, tissue repair, and in a number of pathologies, including fibrotic disorders, hypertension, and atherosclerosis. Extracellular matrix remodeling involves the complex interplay between extracellular matrix synthesis, deposition, and degradation. Factors that control these processes are likely to play key roles in regulating physiological and pathological extracellular matrix remodeling. Our data show that fibronectin polymerization into the extracellular matrix regulates the deposition and stability of other extracellular matrix proteins, including collagen I and thrombospondin-1 (Sottile and Hocking, 2002. *Mol. Biol. Cell* 13, 3546). In the absence of continual fibronectin polymerization, there is a loss of fibronectin matrix fibrils, and increased levels of fibronectin degradation. Fibronectin degradation occurs intracellularly after endocytosis and can be inhibited by chloroquine, an inhibitor of lysosomal degradation, and by caveolae-disrupting agents. Down-regulation of caveolin-1 by RNAi inhibits loss of fibronectin matrix fibrils, fibronectin internalization, and fibronectin degradation; these processes can be restored by reexpression of caveolin-1. These data show that fibronectin matrix turnover occurs through a caveolin-1–dependent process. Caveolin-1 regulation of fibronectin matrix turnover is a novel mechanism regulating extracellular matrix remodeling.

INTRODUCTION

Extracellular matrix (ECM) remodeling is a dynamic, cell-mediated process that occurs during development, tissue repair, and in a variety of pathological events including atherosclerosis, hypertension, and ischemic injury (Clark, 1996; Prescott *et al.*, 1999; Streuli, 1999; Newby and Zaltsman, 2000; Intengan and Schiffrin, 2001). Furthermore, abnormal matrix remodeling is associated with fibrosis, arthritis, reduced angiogenesis, and developmental abnormalities (Liu *et al.*, 1995; Vu *et al.*, 1998; Holmbeck *et al.*, 1999). During tissue repair, the precise deposition of ECM molecules, including collagen I and fibronectin, is required to preserve the structural and functional integrity of tissues (Clark, 1996). Excessive or inappropriate deposition of ECM molecules, as occurs during fibrosis, disrupts normal tissue architecture, leading to impaired organ function (Mutsaers *et al.*, 1997; Zeisberg *et al.*, 2000). The mechanisms that control ECM organization and homeostasis are incompletely understood. We have recently shown that fibronectin matrix polymerization is essential for the organization, as well as the maintenance of ECM architecture (Sottile and Hocking, 2002). Our data show that the cell-dependent process of polymerizing fibronectin into the ECM is required for the

deposition and maintenance of fibrillar fibronectin, collagen-I, and thrombospondin-1 (Sottile and Hocking, 2002). These data are consistent with other studies showing that collagen I and collagen III deposition into the ECM are regulated by fibronectin (McDonald *et al.*, 1982; Velling *et al.*, 2002). Fibronectin has also been implicated in regulating the deposition of tenascin C (Chung and Erickson, 1997), fibulin (Roman and McDonald, 1993; Godyna *et al.*, 1995b; Sasaki *et al.*, 1996), and fibrinogen (Pereira *et al.*, 2002) in the ECM. Hence, fibronectin plays a key role in regulating ECM composition and stability.

ECM remodeling is controlled by a combination of matrix synthesis, deposition, and degradation. Extracellular proteases such as plasmin, plasminogen activators, and matrix metalloproteinases (MMPs) can degrade ECM proteins. ECM turnover is also regulated by endocytosis. ECM proteins such as thrombospondin-1 and vitronectin are known to be internalized by receptor-mediated endocytosis and degraded in the lysosomes (McKeown-Longo *et al.*, 1984; Murphy-Ullrich and Mosher, 1987; Godyna *et al.*, 1995a; Pijuan-Thompson and Gladson, 1997; Memmo and McKeown-Longo, 1998). Recent studies also indicate that collagen I can be endocytosed by the Endo180 receptor (East *et al.*, 2003; Engelholm *et al.*, 2003; Wienke *et al.*, 2003). We previously showed that the loss of ECM fibronectin fibrils could not be inhibited by a variety of protease inhibitors (Sottile and Hocking, 2002), suggesting that turnover of ECM fibronectin may also involve endocytosis and intracellular degradation. Recently published data (Salicioni *et al.*, 2002) also support a role for fibronectin endocytosis in regulating the degradation of soluble fibronectin.

We have developed a model system using fibronectin-null myofibroblasts (FN-null MF) to examine the role of fibronectin polymerization in regulating ECM turnover. The fibronectin-null background has proven to be a valuable tool for determining cell behavior in the complete absence of

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Abbreviations used: BSA, bovine serum albumin; ECM, extracellular matrix; GST, glutathione S-transferase; LPA, lysophosphatidic acid; LRP, low-density lipoprotein receptor-related protein; MFI, mean fluorescence intensity; MMP, matrix metalloproteinase; short hairpin RNA (shRNA); SMC, smooth muscle cell; RAP, receptor-associated protein.

fibronectin and for distinguishing the effects of ECM fibronectin from the effects of soluble fibronectin (Sottile *et al.*, 1998; Hocking *et al.*, 2000; Sottile and Hocking, 2002; Hocking and Chang, 2003). In this article, we have used FN-null MF to determine the mechanism(s) that regulate the turnover of ECM fibronectin. Our data show that turnover of matrix fibronectin involves caveolin-1-mediated endocytosis and intracellular degradation.

MATERIALS AND METHODS

Immunological Reagents and Chemicals

Polyclonal antifibronectin antibody was a generous gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI). Antibodies to LAMP-1, EEA-1, and caveolin-1 were from BD Biosciences (San Jose, CA). Chloroquine, β -cyclodextrin, genistein, and staurosporin were from Sigma (St. Louis, MO).

Proteins

Human fibronectin was purified from Cohn's fractions 1 and 2 (a generous gift from Dr. Ken Ingham, American Red Cross, Bethesda, MD) as previously described (Miekkka *et al.*, 1982). Thrombospondin-1 was purchased from Hematologic Technologies (Essex Junction, VT). Human RAP was purchased from Molecular Innovations (Southfield, MI). A bacterial expression vector containing GST-RAP (Herz *et al.*, 1991) was a kind gift of Dr. Herz (University of Texas Southwestern Medical Center, Dallas, TX). GST-RAP was purified on a glutathione-agarose column as described (Herz *et al.*, 1991). pUR4 was a kind gift of Dr. Hanski (Ozeri *et al.*, 1996) and was provided to us by Dr. Mosher. pUR4 is a 49-mer peptide derived from *Streptococcus pyogenes* adhesin F1, that binds to fibronectin, and inhibits fibronectin matrix polymerization (Tomasini-Johansson *et al.*, 2001). A carboxyl terminal fragment (68mer) of fibronectin's III-11 module (III-11C) was produced with a his tag and expressed in bacteria as described (Morla *et al.*, 1994). Both pUR4 and the control III-11C peptides were purified from bacterial lysates on nickel-agarose columns as described (Tomasini-Johansson *et al.*, 2001). Vitronectin was purified from human serum as described previously (Yatohgo *et al.*, 1988). Recombinant vitronectin was produced in bacteria with (Wojciechowski *et al.*, 2004) or without a GST tag and purified on a heparin-agarose column as described (Wojciechowski *et al.*, 2004). Some recombinant vitronectin was provided by Dr. Hocking (University of Rochester, Rochester, NY).

Cell Culture

We previously described the isolation of fibronectin-null cells from fibronectin-null embryos (Sottile *et al.*, 1998). These cells were adapted to grow in defined media to establish a model system in which all cell- and serum-derived fibronectin was eliminated (Sottile *et al.*, 1998). The integrin repertoire of these cells was previously described (Sottile *et al.*, 1998). We characterized these cells as myofibroblasts (FN-null MF) based on their expression of some smooth muscle cell (SMC) marker proteins (SM α -actin and SM α -calponin) but not others (SM22 and desmin; unpublished data) and on their ability to contract collagen gels (Hocking *et al.*, 2000). Rat and human aortic SMCs were obtained from Cell Applications (San Diego, CA) and maintained in serum containing media (Cell Applications).

Fibronectin Pulse-Chase Experiments

FN-null MF were plated onto glass coverslips precoated with 5 μ g/ml vitronectin or 10 μ g/ml recombinant vitronectin. Cells were grown to 80–90% confluence in defined medium at 37°C and were then incubated ("pulsed") overnight with 20 nM fibronectin. Cells were then either processed for immunofluorescence, or were washed, and then incubated ("chased") with culture medium containing or lacking 20 nM fibronectin. In some experiments, the fibronectin used was conjugated to either Texas Red (Molecular Probes Inc, Eugene, OR) or FITC (Cappel, West Chester, PA) as described (Sottile and Mosher, 1997). The chase medium was supplemented with various inhibitors, as described in the figure legends. Cells were fixed with paraformaldehyde, permeabilized with 0.5% Triton X-100 and then mounted in glycerol gel (Sigma) or in 90% glycerol/10% 0.05 M carbonate buffer, pH 9, with 2 mg/ml ascorbate. After fixing and permeabilizing, cells were incubated with antibodies to caveolin-1, EEA-1, or LAMP-1, followed by Texas Red- or FITC-conjugated secondary antibodies. Cells were examined using an Olympus BX60 microscope (Melville, NY) equipped with epifluorescence. For some experiments, images were obtained with an Olympus scanning confocal microscope.

Protein Colocalization

Confocal z-sections were analyzed using Fluoview software (Olympus). Plots of fluorescence intensity versus position were generated from several separate fields of view. Background fluorescence was determined for each image. Colocalization was defined as those areas where the fluorescent peaks of the

two fluoros coincided and were above background. Peaks lacking one or the other fluor were defined as areas lacking colocalization. One hundred or more peaks were analyzed per field of view. Data represents the average percent overlap $\pm$ SEM of three or more separate images.

Iodination of Proteins and Binding Assays

Fibronectin and thrombospondin were iodinated using the chloramine T method as described (McKeown-Longo and Mosher, 1985). Labeled proteins were separated from unincorporated iodine by gel filtration on Pharmacia (Piscataway, NJ) PD-10 columns (fibronectin) or by using a heparin-Sepharose column (thrombospondin). Iodinated proteins were dialyzed against phosphate-buffered saline (PBS) at room temperature for 3 h. The specific activity of iodinated fibronectin was: $2.5\text{--}5.7 \times 10^{11}$ μ Ci/mol. The specific activity of iodinated thrombospondin was 1.56×10^{12} μ Ci/mol. Binding assays were performed essentially as described (Sottile and Wiley, 1994). Briefly, FN-null MF were seeded into 24-well cluster dishes in Cellgro:Aim V (1:1) and allowed to grow to 80–90% confluence for 2–3 d. The cell culture medium was then replaced with medium containing iodinated fibronectin. Rat SMCs were grown to confluence in serum containing media. Binding assays with SMCs were done in either 5% fibronectin-depleted fetal bovine serum/DMEM or in 0.2% bovine serum albumin (BSA)/DMEM. After an overnight incubation with iodinated fibronectin, cells were either processed as described below or were washed with Cellgro:Aim V and then incubated in culture medium containing or lacking unlabeled fibronectin for 6–24 h. After this incubation period, the cell culture supernatant was collected to determine the amount of released fibronectin. The cell layers were washed and then solubilized in 0.2 N NaOH to determine the amount of cell- and matrix-associated counts. The culture supernatant was precipitated with 13% trichloroacetic acid (TCA) at 4°C for ≥ 1 h. Samples were then centrifuged at 4°C at $12,000 \times g$ for 15 min, and the amount of TCA-soluble and -insoluble counts determined using a gamma counter. The amount of TCA-soluble counts is expressed as a percentage of the total counts present in each well. Binding studies with thrombospondin were done by culturing the cells in media containing 125 I-labeled thrombospondin for 1–8 h in the presence and absence of inhibitors as described in the figure legends. Nonspecific degradation was determined by incubating 125 I-labeled thrombospondin in the absence of cells at 37°C and was subtracted from each data point.

Western Blotting

Cells lysates were prepared as described (Sottile and Hocking, 2002). Equal amounts of proteins were analyzed under reducing conditions by SDS-PAGE, transferred to nitrocellulose paper (Towbin *et al.*, 1979), and probed with antibodies that recognize caveolin-1. To ensure equal protein loading, the blots were also probed with antibodies that recognize vinculin. Blots were imaged and analyzed using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

siRNA

Pairs of oligonucleotides based on the mouse caveolin-1 sequence were designed using the RNA interference (RNAi) OligoRetriever program (<http://katahdin.cshl.org:9331/RNAi>). The short hairpin (sh) was targeted to nucleotides 429–459 of mouse caveolin-1. Oligonucleotides used were: Primer A: GACGTAGATG-GAGTAGACGCGGCTGATGGAAGCTTGCCTCGGCTGCTACTCCGCTACGTCATCTTTT; Primer B: GATCAAAAATGGACGATGACGAGTAGACGCGAGCCGACGCAAGCTTCCATCAGCCGCTCTACTCC-ATCTAGCTCCG. Oligonucleotide pairs were annealed and ligated into the vector pSHAG (Paddison *et al.*, 2002), a generous gift from Dr. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). In this vector, shRNA is under the control of RNA polymerase III promoter, U6. A *HincII*-*PvuII* fragment containing the U6-shRNA cassette was subcloned from pSHAG into pCDNA3.1/Zeo (Invitrogen, Carlsbad, CA) that had been previously digested with *NruI* and *EcoRV* to remove the CMV promoter region. The resulting construct expresses shRNA under the direction of the U6 promoter, and also contains a zeomycin resistance gene. shcav/pCDNA was transfected into FN-null MF using FuGENE (Roche Diagnostics, Indianapolis, IN). Zeocin-resistant cell lines were cloned and expanded. The sequence of mouse and human caveolin-1 in the region that is targeted by the siRNA varies by three bases (two of which are internal). Control cells were transfected with pCDNA containing an shRNA based on the firefly luciferase gene (shfluc). The U6-shfluc cassette was obtained from pSHAG-Ffl (Paddison *et al.*, 2002) (a gift from Dr. Hannon) as described for shcav. Flow cytometry experiments demonstrate that cells expressing caveolin-1 siRNA (shcav cells) and parental FN null MF express very similar amounts of $\alpha 5$, αv , $\beta 1$, and $\beta 3$ integrins (unpublished data).

Adenoviruses

The coding sequence of human caveolin-1 was PCR amplified from a construct containing human caveolin-1 cDNA (a gift from Dr. Mundy, University of Texas Southwestern, Dallas, TX) and inserted into the vector pENTR1A (Invitrogen). A cav-1/pENTR1A clone was recombined with the adenoviral vector pAD/pLDEST (Invitrogen) using Gateway LR Clonase according to the manufacturer's protocol. The resulting cav-1/pAD plasmid was trans-

ected into HEK293 cells and amplified according to the manufacturer's protocol. An adenovirus containing the tetracycline regulatable transactivator was a kind gift of Dr. Schmid (Scripps Research Institute, La Jolla, CA) (Fish *et al.*, 2000) and was used as a negative control virus. Viruses were purified and titered using kits from BD Biosciences.

RESULTS

Fibronectin Is Internalized and Degraded During Fibronectin Matrix Turnover

Turnover of ECM proteins is an important mechanism to remove biologically active proteins from the extracellular environment. We previously reported that the maintenance of ECM fibronectin fibrils requires the continual polymerization of a fibronectin matrix (Sottile and Hocking, 2002). Cells cultured in the absence of fibronectin, or in the presence of fibronectin polymerization inhibitors, showed a dramatic loss of fibronectin matrix fibrils (Sottile and Hocking, 2002). The loss of fibronectin matrix fibrils could not be inhibited with a variety of protease inhibitors (Sottile and Hocking, 2002). Hence, we examined the possibility that fibronectin was internalized and degraded intracellularly. We conducted pulse-chase studies with ^{125}I -fibronectin in cells that were actively polymerizing a fibronectin matrix and in cells where fibronectin polymerization was inhibited. We tested whether ^{125}I -fibronectin was degraded by precipitating the cell culture supernatant with TCA. FN-null MF were incubated with ^{125}I -fibronectin overnight and allowed to establish a fibronectin matrix. The cells were washed and then incubated (chased) in media containing or lacking unlabeled fibronectin. We previously showed that $>85\%$ of the cell-associated fibronectin was incorporated into the ECM at the start of the chase, indicating that the great majority of the fibronectin turnover that occurs during the chase involves matrix fibronectin (Sottile and Hocking, 2002). As shown in Figure 1, there was a time-dependent increase in the amount of degraded (TCA soluble) fibronectin in the cell culture supernatant during the chase. As much as 56% of the total counts in cells that were chased in the absence of fibronectin represent degraded fibronectin. This represents a 1.8–2 $\times$ increase in fragment production compared with cells that were chased in the presence of fibronectin. The ability of unlabeled fibronectin to inhibit ^{125}I -fibronectin degradation was dependent on its ability to become polymerized into the ECM, because cells incubated with fibronectin and the fibronectin polymerization inhibitor, pUR4, degraded fibronectin at levels similar to that of cells incubated in the absence of fibronectin. The control peptide had no effect on fibronectin degradation. These data are consistent with our previously published data showing that fibronectin polymerization inhibitors prevent fibronectin from stabilizing preexisting fibronectin matrix fibrils (Sottile and Hocking, 2002). The increased levels of TCA-soluble counts in cells in which fibronectin polymerization is inhibited suggests that there is increased endocytosis and degradation of fibronectin under conditions where the fibronectin matrix is turned over.

Endocytosis is inhibited by culturing cells at 4°C. Therefore, we asked whether incubating cells at 4°C would prevent the turnover of matrix fibronectin fibrils. Cells were allowed to establish a FITC-fibronectin matrix at 37°C (Figure 2A). These cells were then cultured in the absence of fibronectin at 37 or 4°C. The fibronectin matrix was extensively disrupted in cells chased in the absence of fibronectin at 37°C (Figure 2B). As shown previously (Sottile and Hocking, 2002), cell monolayers were intact, and the cells spread despite disruption of the fibronectin matrix (Figure 2E).

Figure 1. Fibronectin degradation is inhibited by fibronectin polymerization. FN-null MF were incubated overnight with ^{125}I -labeled fibronectin. Cells were washed and then incubated for the indicated time with culture medium containing (+FN) or lacking unlabeled fibronectin (–FN) and containing or lacking pUR4 or the control his-tagged peptide, III-11C for the indicated times. The cell culture supernatant was collected and precipitated with TCA as described in *Materials and Methods*. The amount TCA soluble counts is expressed as a percentage of the total counts present in each well. Error bars represent the range of duplicate determinations.

Incubation of cells at 4°C (Figure 2C) prevented the loss of the preestablished fibronectin matrix. These data are consistent with the idea that endocytosis is an important component regulating the turnover of matrix fibronectin.

Other ECM proteins such as thrombospondin, vitronectin, and heparan sulfate proteoglycans are internalized and de-

Figure 2. Fibronectin matrix turnover does not occur at 4°C. FN-null MF were incubated overnight with 20 nM FITC-conjugated fibronectin. Cells were then either processed for immunofluorescence (Pulse, A) or were washed, and then incubated with culture medium lacking fibronectin (Chase) at 37°C (B) or 4°C (C) for 8 h. Cells were fixed and permeabilized. FITC-fibronectin staining (A–C) and the corresponding phase images (D–F) are shown. Bar, 10 μm .

Figure 3. Fibronectin degradation is inhibited by chloroquine. FN-null MF were incubated overnight with ^{125}I -labeled fibronectin. Cells were washed and then incubated with culture medium lacking fibronectin and lacking (control) or containing 50–200 μM chloroquine (C50, C100, C200) for 10 h. The cell culture supernatant was collected, and precipitated with TCA as described in the legend to Figure 1. The amount of degradation that occurred in the absence of chloroquine was set equal to 100%. Error bars represent the range of duplicate determinations.

graded in the lysosomes (McKeown-Longo *et al.*, 1984; Yanagishita and Hascall, 1984; Murphy-Ullrich and Mosher, 1987; Hausser *et al.*, 1992; Godyna *et al.*, 1995a; Pijuan-Thompson and Gladson, 1997; Memmo and McKeown-Longo, 1998). To determine whether fibronectin degradation occurs in the lysosomes, we tested whether fibronectin degradation could be inhibited by the lysosomotropic agent chloroquine, which increases the pH of lysosomes, and inhibits the activity of lysosomal hydrolases (de Duve *et al.*, 1974). Fibronectin pulse-chase studies were conducted with ^{125}I -fibronectin in the presence and absence of chloroquine. Addition of chloroquine to cells resulted in a dose-dependent inhibition of fibronectin degradation. The highest dose of chloroquine blocked >90% of the TCA-soluble counts (Figure 3), suggesting that the major route of fibronectin degradation is in the lysosomes. To directly test whether extracellular fibronectin is targeted to the lysosomes, we used confocal microscopy to ask whether fibronectin colocalizes with the lysosomal marker protein, LAMP-1. FN-null MF were incubated with Texas Red-fibronectin and allowed to establish a fibronectin matrix. The cells were then incubated in the absence of soluble fibronectin for 24 h. Internalized fibronectin (TR-fibronectin) was readily detected in permeabilized cells that have been treated with chloroquine (Figure 4B). A comparison of panels A and B show that fibronectin (B) and LAMP-1 (A) partially colocalize within cells. Some areas of colocalization can be seen as yellow/orange staining in the merged image in panel C. Quantitative analysis of confocal z-sections showed that >50% of the internalized fibronectin colocalized with Lamp-1. These data provide further supporting evidence that extracellular fibronectin is internalized by cells and targeted to the lysosomes for degradation.

To determine whether fibronectin matrix degradation occurs by a similar mechanism in fibronectin producing cells, we asked whether inhibition of fibronectin polymerization induces the loss of fibronectin matrix fibrils, and up-regulates fibronectin degradation in SMC. Incubation of SMC

Figure 4. Colocalization of fibronectin with the lysosomal marker, LAMP-1. FN-null MF were incubated with Texas Red-conjugated fibronectin (20 nM) overnight. Cells were washed and then incubated for 25 h in cell culture media lacking fibronectin, but containing 50 μM chloroquine. Chloroquine was used to inhibit lysosomal degradation and enhance the levels of intracellular fibronectin. Cells were then fixed, permeabilized, and incubated with an antibody to LAMP-1. Localization of Texas-Red-fibronectin (B) and LAMP-1 (A) are shown. (C) An overlay of the fibronectin and LAMP-1 images shown in A and B. Some areas of colocalization (arrowheads) of fibronectin and LAMP-1 are shown in yellow. It should be noted that yellow will only be seen if the intensity of the red and green fluors are approximately equal. The area marked by the arrow in C was magnified and is shown in the inset. The images are optical sections collected from a confocal z-series scan obtained with an Olympus confocal microscope. Bar, 10 μm .

with pUR4 resulted in a marked reduction in the amount of fibronectin matrix fibrils (Figure 5A, panel B) in comparison with cells before pUR4 treatment (panel A) or cells incubated with a control peptide (panel C). To determine whether fibronectin is degraded by SMC in a manner that depends on fibronectin polymerization, SMC were incubated overnight with ^{125}I -fibronectin. The cells were washed and then incubated in culture medium containing the fibronectin polymerization inhibitor, pUR4, or control peptide. As shown in Figure 5B, addition of pUR4 to SMC resulted in a 1.7–2.3 $\times$ increase in fibronectin degradation in comparison with control cells. In addition, chloroquine inhibited ~80% of fibronectin degradation in SMC (unpublished data). These data indicate that fibronectin matrix turnover in fibronectin-producing cells involves intracellular lysosomal degradation that is regulated by fibronectin polymerization.

Fibronectin Degradation in FN-null MF Is Not Lipoprotein Receptor-related Protein Dependent

Others have shown that fibronectin can be degraded in certain cell types but not in others (Godyna *et al.*, 1995a; Salicioni *et al.*, 2002) by a low density lipoprotein receptor-related protein (LRP)-dependent mechanism. These studies examined the degradation of soluble fibronectin; the fate of ECM fibronectin was not tested. We asked whether turnover of matrix fibronectin in FN-null MF depends on LRP by examining whether the LRP inhibitor, receptor-associated protein (RAP), could inhibit loss or degradation of matrix fibronectin. RAP binds to LRP and competes for binding with LRP ligands, thus preventing their internalization and degradation (Herz *et al.*, 1991; Godyna *et al.*, 1995a; Lazic *et al.*, 2003). As shown in Figure 6A, cells incubated with RAP degraded similar levels of fibronectin as control cells. To demonstrate that functional LRP exists on the surface of FN-null MF, we tested whether RAP could inhibit ^{125}I -thrombospondin degradation, because thrombospondin degradation is LRP dependent (Godyna *et al.*, 1995a; Chen *et al.*, 1996a). Thrombospondin degradation was inhibited by RAP in FN-null MF (Figure 6B). These data demonstrate that

Figure 5. (A) Fibronectin matrix stability in SMCs. Human aortic SMCs (passage 4) were seeded in serum-containing medium and grown to confluence. SMCs were then incubated with 250 nM pUR4 (B) or 250 nM control peptide, III-11C (C) for 24 h. At the time of protein addition, some cells were processed for immunofluorescence (A) to determine the amount of matrix deposited by the cells before pUR4 addition ($t = 0$). Cells were permeabilized and then incubated with a polyclonal antibody to fibronectin, followed by a FITC anti-rabbit antibody. Bar, 20 μm . (B) Fibronectin degradation in SMC. Confluent cultures of rat SMCs were incubated overnight with ^{125}I -fibronectin. Cells were washed and then incubated with culture medium lacking (PBS) or containing 250 nM pUR4 or the control III-11C peptide for the indicated times. The amount of TCA-soluble counts was determined as described in *Materials and Methods* and is expressed as a percentage of the total counts present in each well. Error bars represent the average of duplicate samples, and the error bars the range.

turnover of matrix fibronectin in FN-null MF is not LRP dependent.

Agents That Disrupt Caveolae or Interfere with Caveolae-mediated Endocytosis Block Fibronectin Degradation

Fibronectin internalization and degradation could occur via clathrin- or caveolin-mediated endocytosis. Others have shown that a portion of ECM fibronectin colocalizes with caveolin in lipid rafts (Hocking and Kowalski, 2002). Hence, we tested whether β -cyclodextrin, a cholesterol-binding agent that disrupts caveolae (Smart and Anderson, 2002), can inhibit fibronectin degradation. As shown in Figure 7, the presence of cyclodextrin in the chase media partially inhibited fibronectin degradation in FN-null MF. Others have shown that addition of staurosporin (a general kinase inhibitor) or genistein (a tyrosine kinase inhibitor) can block caveolae-mediated endocytosis (Pelkmans *et al.*, 2002; Pang *et al.*, 2004). To determine whether these kinase inhibitors block fibronectin matrix turnover, fibronectin pulse-chase experiments were done with FN-null MF in the presence and absence of genistein or staurosporin. Addition of either genistein or staurosporin prevented the loss of fibronectin matrix fibrils (Figure 8) and caused a dramatic reduction in fibronectin degradation (Figure 7). Caveolae-mediated endocytosis has also been reported to be blocked by jasplakinolide, an actin filament stabilizing agent (Pelkmans *et al.*, 2002). Our previous data demonstrated that fibronectin turnover was also blocked by jasplakinolide (Sottile and Hocking, 2002).

Others have shown that caveolin-containing vesicles do not contain markers of early endosomes and hence are distinct from the early endosomes that are formed during clathrin-mediated endocytosis (Pelkmans *et al.*, 2001; Nichols, 2002). It should be noted that intracellular fibronectin must be derived from extracellular fibronectin by endocytosis,

because FN-null MF do not synthesize fibronectin (Sottile *et al.*, 1998). Internalized fibronectin did not colocalize with the early endosome marker, EEA1 (Figure 9C). Quantitative analysis of confocal z-sections indicated that <5% of the internalized fibronectin colocalized with EEA-1. Fibronectin also failed to colocalize with the transferrin receptor, another marker of classical early endosomes (unpublished data). These data suggest that endocytosis of fibronectin may involve caveolin-mediated endocytosis.

Down-regulation of Caveolin-1 by RNAi Stabilizes Fibronectin Matrix Fibrils

Our data show that agents that disrupt caveolae or that interfere with caveolae-mediated endocytosis partially prevent fibronectin degradation (Figure 7). These data suggest that fibronectin turnover may be regulated by caveolin-1. This could be due to decreased endocytosis via caveolae and/or to caveolin-1-dependent signaling event that is necessary for fibronectin internalization and degradation. To test whether caveolin-1 is important for fibronectin matrix turnover, we used RNAi to generate a stable FN-null cell line in which caveolin-1 was down-regulated. Caveolin-1 was decreased ~70% in cells stably expressing caveolin-1 siRNA (shcav) in comparison with the parental FN-null MF and with control cell lines expressing luciferase shRNA (shluc; Figure 10A). To determine whether down-regulation of caveolin-1 affects fibronectin matrix stability, we performed fibronectin pulse-chase studies with FITC-fibronectin in FN-null MF and shcav cells. Both the parental FN-null MF and FN-null MF expressing caveolin-1 siRNA polymerized a robust fibronectin matrix (Figure 10B, panels A and G) when cultured in the presence of fibronectin. When soluble fibronectin was removed from the culture media, parental FN-null MF showed a dramatic loss of fibronectin matrix fibrils (Figure 10B, panels B and C). In contrast, there was a

Figure 6. (A) Fibronectin degradation is not inhibited by RAP. FN-null MF were incubated overnight with ^{125}I -labeled fibronectin. Cells were washed and then incubated with culture medium lacking fibronectin and lacking (PBS) or containing 1 μM human RAP (hRAP) or recombinant GST-RAP (rRAP) for the indicated times. The amount of TCA soluble counts was determined as described in *Materials and Methods* and is expressed as a percentage of the total counts present in each well. Similar results were obtained when RAP was added at the time of ^{125}I -labeled fibronectin addition. (B) Thrombospondin degradation is inhibited by RAP. FN-null MF were incubated with ^{125}I -labeled thrombospondin in the presence or absence of 0.1–0.5 μM RAP for the indicated times. The amount of degraded thrombospondin was determined as described in *Materials and Methods*. Error bars represent the range of duplicate determinations.

striking retention of fibronectin fibrils in caveolin siRNA-expressing cells (Figure 10B, panels H and I). These data are the first to show that down-regulating caveolin-1 levels stabilizes fibronectin matrix fibrils.

To quantitate the levels of fibronectin degradation in shcav and control cell lines, we compared the levels of ^{125}I -fibronectin degradation using pulse-chase studies.

Figure 7. Fibronectin degradation is inhibited by agents that disrupt caveolae or that interfere with caveolae-mediated endocytosis. FN-null MF were incubated overnight with ^{125}I -fibronectin. Cells were washed, and then incubated with culture medium lacking fibronectin and lacking (control) or containing 10 mM β -cyclodextrin, 200 μM genistein, 100 μM genistein, 0.1 μM staurosporin, or 20 nM fibronectin. Cells were incubated for 6 h (cyclodextrin) or 24 h (all other treatments). The cell culture supernatant was collected and precipitated with TCA as described in the legend to Figure 1. The amount of fibronectin degraded in control FN null MF was 11.4% of the total counts at 6 h. The amount of degradation that occurred in the absence of inhibitors at each time point was set equal to 100%. Error bars represent the range of duplicate or triplicate determinations.

There was a dramatic 3.7–4-fold reduction in fibronectin degradation in cells expressing caveolin siRNA (shcav) in comparison with the parental FN-null MF (FN-/-) or with a control cell line expressing luciferase siRNA (shluc) (Fig-

Figure 8. Kinase inhibitors prevent the loss of fibronectin matrix fibrils. FN-null MF were incubated overnight with 20 nM FITC-conjugated fibronectin. Cells were then either processed for immunofluorescence (Pulse, A) or were washed and then incubated with culture medium lacking fibronectin and lacking (B, control) or containing 200 μM genistein (C) or 0.1 μM staurosporin (D) for 24 h. Cells were fixed and permeabilized. FITC-fibronectin staining. Bar, 20 μm .

Figure 9. Fibronectin does not colocalize with EEA-1-containing vesicles. FN-null MF were incubated with Texas Red-conjugated fibronectin (20 nM) overnight. Cells were washed and then incubated for 24 h in cell culture media lacking fibronectin, but containing 30 μ M chloroquine. Chloroquine was used to inhibit lysosomal degradation and enhance the levels of intracellular fibronectin. Cells were fixed, permeabilized, and then incubated with antibodies to EEA-1. Localization of Texas-Red-FN (A) and EEA-1 (B) are shown. (C) An overlay of the images in A and B. Little or no colocalization of fibronectin and EEA-1 was seen (C). The boxed area in C was magnified and is shown by the inset. The images are optical sections collected from a confocal z-series scan obtained with an Olympus confocal microscope. Bar, 10 μ m.

ure 11). The effect on fibronectin degradation was specific, because the three cell lines showed no difference in thrombospondin degradation (Figure 12).

The data shown in Figures 10 and 11 indicate that there is a dramatic reduction in fibronectin matrix turnover and degradation in cells expressing caveolin siRNA. Our data also show that the majority of fibronectin degradation occurs in the lysosomes. Hence, decreased fibronectin degradation in caveolin-1 siRNA-expressing cells could result from a reduction in fibronectin endocytosis. To determine whether differences in the levels of intracellular fibronectin can be detected in shcav and control cells, we performed fibronectin pulse-chase studies with TR-labeled fibronectin and examined the levels of intracellular fibronectin at different times during the chase using confocal microscopy. As shown in Figure 13A, intracellular vesicular fibronectin was readily detected in the control shluc cells within 5 h of the start of the chase period. In contrast, little or no intracellular fibronectin staining was seen in the shcav cells at this time point, although fibronectin fibrils were readily detected (Figure 13B). At 10 h, increased levels of vesicular fibronectin staining was seen in the control shluc cells (Figure 13C). Some vesicular fibronectin staining was also detected in the

shcav cells, although this staining was less intense and less extensive than in the control cells (Figure 13D). Thus, intracellular accumulation of fibronectin is delayed in cells expressing caveolin-1 siRNA.

To demonstrate that down-regulation of caveolin-1 in the siRNA-expressing cells (shcav) is responsible for decreased turnover of matrix fibronectin, we reexpressed caveolin-1 in these cells using an adenovirus containing caveolin-1 (Ad-cav-1). As shown in Figure 14A, caveolin-1 levels are increased 14-fold in shcav cells transduced with Ad-cav-1 in comparison with cells transduced with control adenovirus. Increased levels of caveolin-1 persisted for >60 h after transduction (unpublished data). To determine the effect of caveolin-1 overexpression on fibronectin matrix fibril stability and fibronectin degradation in shcav cells, fibronectin pulse-chase experiments were performed using either FITC- or 125 I-labeled fibronectin. Reexpression of caveolin-1 resulted in an increased loss of fibronectin matrix fibrils (Figure 14B, panel C) in comparison with control cells (Figure 14B, panels B and D). In addition, there was a 46% increase in fibronectin degradation in shcav cells treated with Ad-cav-1 in comparison with cells treated with a control adenovirus (Figure 15). Fibronectin degradation in shcav cells was

Figure 10. Expression of caveolin-1 siRNA reduces caveolin-1 levels and stabilizes matrix FN fibrils. (A) Cell lysates were prepared from confluent cultures of FN-null MF or FN-null MF-expressing caveolin-1 siRNA (shcav) or luciferase siRNA (shluc). Equal amounts of total protein were run on an SDS-PAGE gel, transferred to nitrocellulose paper, and then incubated with antibodies to caveolin-1 and vinculin. (B) FN-null MF or FN-null MF-expressing caveolin-1 siRNA (shcav) were incubated with 20 nM Texas Red-FN overnight. Cells were then either processed for immunofluorescence (Pulse, A, D, and G), or were washed and then incubated with culture medium lacking fibronectin (Chase, B, C, E, F, H, and I) for 8 (B, E, and H) or 24 h (C, F, and I). Cells were fixed and permeabilized, then examined with an Olympus microscope). (D-F) Nomarski images of cells in A-C. Bar, 20 μ m.

Figure 11. Expression of caveolin-1 siRNA reduces fibronectin degradation. FN-null MF, or FN-null MF-expressing caveolin-1 siRNA (shcav) or fluc siRNA (shluc) were incubated overnight with 125 I-fibronectin. Cells were washed and then incubated with culture medium lacking fibronectin for the indicated times. The cell culture supernatant was collected and precipitated with TCA as described in *Materials and Methods*. The amount TCA-soluble counts is expressed as a percentage of the total counts present in each well. Error bars represent the range of duplicate determinations.

restored to 86% of the levels of the parental FN null MF upon reexpression of caveolin-1. Addition of unlabeled fibronectin to the chase media reduced fibronectin degradation in both control and Ad-cav-1-treated cells. Taken together, these data indicate that caveolin-1 is a key player in regulating ECM remodeling by a novel pathway that involves endocytosis and intracellular degradation of fibronectin.

DISCUSSION

We and others have shown that fibronectin polymerization is a key regulator of ECM architecture (Sottile and Hocking, 2002), cell proliferation (Clark *et al.*, 1997; Bourdoulous *et al.*, 1998; Sechler and Schwarzbauer, 1998; Sottile *et al.*, 1998, 2000; Christopher *et al.*, 1999), and cell migration (Darribere and Schwarzbauer, 2000; Hocking and Chang, 2003), all critically important components of tissue repair and fibrosis. ECM remodeling can occur through extracellular proteolysis by the action of MMPs, and/or other proteases, or by intracellular proteolysis, after endocytosis. We previously showed that fibronectin matrix polymerization regulates the stability of fibronectin and collagen I matrix fibrils (Sottile and Hocking, 2002). In this article, we show that loss of fibronectin matrix fibrils is accompanied by increased endocytosis and degradation of ECM fibronectin. Further, our data demonstrate that caveolin-1 is an important regulator of fibronectin endocytosis and degradation. Down-regulation of caveolin-1 by siRNA in FN null MF results in stabilization of fibronectin matrix fibrils and a reduction in fibronectin degradation. Reexpression of caveolin-1 in the siRNA-expressing cells promotes increased turnover of fi-

Figure 12. Expression of caveolin-1 siRNA does not effect thrombospondin degradation. FN-null MF, or FN-null MF-expressing caveolin-1 siRNA (shcav) or fluc siRNA (shluc) were grown to confluence and then incubated with 125 I-labeled thrombospondin for 8 h. The amount of degraded thrombospondin was determined by collecting the cell culture supernatant and precipitating with 13% TCA. The amount TCA-soluble counts is expressed as a percentage of the total counts present in each well. Error bars represent the range of duplicate determinations. Similar results were obtained when degradation assays were performed for 2 or 4 h.

bronectin matrix fibrils and increases fibronectin degradation. These data are the first to demonstrate a role for caveolin-1 in regulating fibronectin matrix turnover. Because fibronectin is an important regulator of ECM remodeling (Sottile and Hocking, 2002), these data suggest that caveo-

Figure 13. Cells expressing caveolin-1 siRNA show reduced levels of intracellular fibronectin. FN-null MF-expressing caveolin-1 siRNA (shcav) or fluc siRNA (shluc) were incubated overnight with TR-labeled fibronectin. Cells were washed and then incubated in culture medium lacking fibronectin and containing 30 μ M chloroquine. Chloroquine was used to inhibit lysosomal degradation and enhance the levels of intracellular fibronectin. Cells were fixed at the indicated times. The images are optical sections collected from a confocal z-series scan obtained with an Olympus confocal microscope. Bar, 20 μ m.

Figure 14. (A) Caveolin levels in shcav cells transduced with Ad-Cav-1. FN-null MF-expressing caveolin-1 siRNA were incubated with Ad-cav-1 (cav) or Ad-tet TA (tet) at an MOI of 300 for 19 h. The cells were washed and then incubated in culture medium lacking virus. Cell lysates were prepared at 24 h after virus addition. Equal amounts of total protein were run on an SDS PAGE gel, transferred to nitrocellulose paper, and then incubated with antibodies to caveolin-1 (cav) and vinculin (vinc). (B) Reexpression of caveolin-1 in shcav cells increases the turnover of fibronectin fibrils. shcav cells were incubated with Ad-cav-1 (C) or Ad-tet TA (D) at an MOI of 300. Control cells were not infected with virus (none). Four hours after addition of virus, 20 nM FITC-FN was added, and the cells were incubated for an additional 16 h. Cells were then either processed for immunofluorescence (Pulse, A) or were washed and then incubated with culture medium lacking fibronectin (Chase, B–D) for 24 h. Cells were fixed and permeabilized and then examined with an Olympus microscope. Bar, 20 μ m.

lin-1 plays a key role in modulating ECM composition and stability.

Others have shown that fibronectin can be degraded in peritoneal cells by chymases (Tchougounova *et al.*, 2000). However, this process may be specific for peritoneal cells, because chymase production appears to be restricted to mast cells (Yong, 1997; Krishnaswamy *et al.*, 2001). In addition, chymase inhibitors do not block loss of fibronectin matrix fibrils in FN null MF (unpublished data). LRP is involved in the endocytosis and intracellular degradation of a number of extracellular proteins including thrombospondin-1, thrombospondin-2, and plasminogen activators (Grobmyer *et al.*, 1993; Godyna *et al.*, 1995a; Chen *et al.*, 1996b; Herz and Strickland, 2001). It has previously been reported that soluble fibronectin can be degraded in mouse embryo fibroblasts (MEF) by an LRP-dependent pathway (Salicioni *et al.*, 2002). It should be emphasized that these studies examined the degradation of soluble fibronectin; the turnover of ECM fibronectin was not examined. In addition, the LRP inhibitor, RAP, does not inhibit degradation of matrix fibronectin in FN null MF (Figure 6A) or in SMC (Godyna *et al.*, 1995a). Hence, LRP-mediated fibronectin degradation may also be restricted to a subset of cells. Our data demonstrating turnover and degradation of ECM fibronectin in vitro is consistent with in vivo data indicating that tissue fibronectin is turned over relatively rapidly (Rebres *et al.*, 1995). In addition, evidence suggests that turnover of matrix fibronectin in

Figure 15. Reexpression of caveolin-1 in shcav cells increases fibronectin degradation. FN null MF (FN-/-) or FN null MF-expressing caveolin-1 siRNA (shcav) were incubated without virus (-) or with Ad-cav-1 or Ad-tet TA at an MOI of 300 for 19 h. The virus was removed, and the cells were incubated for 12 h with 125 I-labeled fibronectin. Cells were washed and then incubated with culture medium containing (FN) or lacking (-FN) unlabeled fibronectin for 24 h. The cell culture supernatant was collected, and precipitated with TCA as described in *Materials and Methods*. The amount TCA-soluble counts is expressed as a percentage of the total counts present in each well. Error bars, SEM of triplicate determinations.

the liver may involve receptor-mediated endocytosis (Rontundo *et al.*, 1999).

Our data show that extracellular fibronectin is endocytosed and degraded in both FN-null MF and in SMCs. Differences in the kinetics of degradation in FN-null MF and SMCs are likely due to the presence of cell- and serum-derived fibronectin in SMC. Because soluble fibronectin inhibits matrix fibronectin degradation (Figure 1), reduced fibronectin degradation would be expected to occur in cells that produce fibronectin. Our data show that fibronectin degradation in FN-null MF and in SMC is enhanced by blocking fibronectin polymerization (Figures 1 and 5) and inhibited by blocking lysosomal degradation (Figure 3). Thus, endocytosis and intracellular degradation is an important pathway that regulates fibronectin matrix stability.

Receptor-mediated endocytosis can occur via two major pathways, one involving clathrin-coated vesicles, and the other involving caveolae (Schmid *et al.*, 1998; Pelkmans and Helenius, 2002; Nabi and Le, 2003). Our data indicate that internalized fibronectin does not colocalize with a marker of classical early endosomes (Figure 9) and that agents that disrupt caveolae and/or block caveolae-mediated endocytosis partially inhibit fibronectin degradation (Figure 7). Moreover, down-regulation of caveolin-1 results in stabilization of fibronectin matrix fibrils, a 4 $\times$ reduction in fibronectin degradation, and a reduction in the levels of intracellular fibronectin. These data suggest that fibronectin internalization proceeds by a caveolin-1-dependent process. Interestingly, caveolin-1, caveolin-3, and caveolin 1/3 double knockout mice develop fibrosis in the lungs and/or heart (Drab *et al.*, 2001; Razani *et al.*, 2001; Park *et al.*, 2002). Cells from caveolin-1 null mice are also defective in endocytosis of

albumin (Schubert *et al.*, 2001). These data suggest the interesting possibility that fibrosis in mice lacking functional caveolae may, in part, be due to defects in ECM endocytosis and turnover. Recent reports indicate that the levels of caveolin-1 can be modulated by growth factors (Liu *et al.*, 2002; Peterson *et al.*, 2003). Addition of platelet-derived growth factor (PDGF) to smooth muscle cells resulted in down-regulation of caveolin-1 and caveolae (Peterson *et al.*, 2003). PDGF treatment also results in increased levels of fibronectin matrix fibrils (Sarkissian and Lafyatis, 1998). Hence, regulation of caveolin by growth factors may be a physiological mechanism that controls the rate and/or extent of fibronectin matrix turnover. Most proteins that are degraded in the lysosomes are endocytosed in clathrin-coated vesicles (Cavalli *et al.*, 2001). However, FGF degradation has been shown to occur after caveolae-mediated endocytosis (Gleizes *et al.*, 1996). Our data are the first to demonstrate that fibronectin polymerization controls ECM fibronectin turnover by a novel mechanism involving caveolin-1-dependent endocytosis.

The role of integrins in regulating turnover of matrix fibronectin is unknown. Integrins can be endocytosed by receptor mediated endocytosis and recycled to the cell surface (Bretscher, 1989, 1992; Ng *et al.*, 1999). In addition, integrins are involved in the internalization of other ECM proteins, such as vitronectin (Pijuan-Thompson and Gladson, 1997; Memmo and McKeown-Longo, 1998). In one report, fibronectin was shown to colocalize with integrins in intracellular vesicles in cells overexpressing protein kinase C (PKC) α (Ng *et al.*, 1999). Our flow cytometry data indicate that the cell surface levels of β 1, β 3, and α v integrins are unchanged when fibronectin polymerization is inhibited by removal of soluble fibronectin from the cell culture media or when fibronectin degradation is inhibited by chloroquine (unpublished data). Of the integrins tested, the only integrin whose levels change is the α 5 integrin, which increases after removal of soluble fibronectin from the cell culture media, and decreases upon addition of chloroquine. Hence, conditions that promote fibronectin degradation do result in consistent changes in α 5 integrin levels. Endocytosis of most integrins occurs through a clathrin-mediated process (Raub and Kuentzel, 1989; Van Nhieu *et al.*, 1996). However, a recent report indicates that the α 2 β 1 integrin is endocytosed via caveolae (Upla *et al.*, 2004). It will be interesting to determine whether other integrins can be endocytosed by a similar pathway and whether these integrins regulate fibronectin endocytosis.

ECM fibronectin is comprised of high-molecular-weight multimers that are stabilized by disulfide bonds (Ali and Hynes, 1978; Choi and Hynes, 1979; McKeown-Longo and Mosher, 1984). It is unlikely that these large fibrils are directly endocytosed by cells. A more likely scenario is that matrix fibronectin may be proteolyzed before endocytosis. In addition, we previously showed that multimerization of fibronectin may involve a disulfide isomerase activity (Langenbach and Sottile, 1999). Hence, it is also possible that matrix fibronectin may be dissociated into monomers or dimers by an isomerase activity before endocytosis. Future studies will be directed at understanding the mechanism by which extracellular matrix fibronectin fibrils are internalized by cells before degradation.

ECM molecules play critical roles in regulating tissue repair by providing a scaffold for cell migration and by regulating cell growth and cell contractility. Our data demonstrate that the maintenance and structural organization of ECM fibronectin depends on the continuous polymerization of a fibronectin matrix (Sottile and Hocking, 2002). Further-

more, fibronectin matrix polymerization regulates the organization of other ECM molecules, including collagen I (Sottile and Hocking, 2002; Velling *et al.*, 2002), and enhances cell contractility (Hocking *et al.*, 2000). Thus, factors that regulate fibronectin matrix turnover likely play a critical role in regulating ECM remodeling. A variety of agents have been shown to stimulate or inhibit the ability of cultured cells to polymerize fibronectin into fibrils in the ECM. Transforming growth factor- β (Ignatz and Massague, 1986; Allen-Hoffmann *et al.*, 1988), PDGF (Sarkissian and Lafyatis, 1998), PKC (Sommers and Mosher, 1993), integrin-linked kinase (Wu *et al.*, 1998), and agents that stimulate cell contractility (such as lysophosphatidic acid, LPA; Burridge *et al.*, 1997; Zhang *et al.*, 1997; Zhong *et al.*, 1998) up-regulate fibronectin matrix polymerization. LPA binds to G-protein-coupled receptors and triggers intracellular signaling pathways that lead to activation of Rho-A (Moolenaar *et al.*, 1997). In contrast, H-Ras, Raf-1 (Hughes *et al.*, 1997), cAMP, and agents that inhibit cell contractility and Rho activation (Allen-Hoffmann and Mosher, 1987; Burridge *et al.*, 1997; Zhang *et al.*, 1997; Zhong *et al.*, 1998) inhibit fibronectin polymerization. Hence, several physiological mediators have the potential to alter the rate of fibronectin polymerization and thus, the rate of fibronectin turnover. Our data show that fibronectin matrix turnover is regulated by both fibronectin polymerization and by caveolin-1. In addition, our published data show that fibronectin polymerization is a key regulator of ECM remodeling (Sottile and Hocking, 2002). Hence, these data define a novel pathway for controlling ECM remodeling through the caveolin-1-dependent regulation of fibronectin metabolism. Determining the mechanism(s) by which caveolin-1 regulates fibronectin-dependent ECM remodeling will provide important insights into factors that contribute to the development of fibrosis and into mechanisms that could lead to reversal of excess matrix accumulation during fibrosis.

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