Caveolin-1-dependent β1 integrin endocytosis is a critical regulator of fibronectin turnover

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Summary

β1 integrins are major cell surface receptors for fibronectin. Some integrins, including β1 integrins, are known to undergo constitutive endocytosis and recycling. Integrin endocytosis/recycling has been implicated in the regulation of cell migration. However, the mechanisms by which integrin endocytosis/recycling regulates cell migration, and other biological consequences of integrin trafficking are not completely understood. We previously showed that turnover of extracellular matrix (ECM) fibronectin occurs via receptor-mediated endocytosis. Here, we investigate the biological relevance of β1 integrin endocytosis to fibronectin matrix turnover. First, we demonstrate that β1 integrins, including α5β1 play an important role in endocytosis and turnover of matrix fibronectin. Second, we show that caveolin-1 constitutively regulates endocytosis of α5β1 integrins, and that α5β1 integrin endocytosis can occur in the absence of fibronectin and fibronectin matrix. We also show that downregulation of caveolin-1 expression by siRNA results in marked reduction of β1 integrin and fibronectin endocytosis. Hence, caveolin-1-dependent β1 integrin and fibronectin endocytosis plays a critical role in fibronectin matrix turnover, and may contribute to abnormal ECM remodeling that occurs in fibrotic disorders.

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Key words: Integrin, Extracellular matrix, Endocytosis, Caveolae, Fibrosis

Introduction

Degradation and removal of ECM proteins is a cell-mediated process, which is involved in a number of physiological processes, such as development, postnatal tissue remodeling and tissue repair (Clark, 1996; Holmbeck et al., 1999; Vu et al., 1998). Impaired or abnormal degradation and removal of ECM components contributes to the onset and progression of many diseases, including fibrosis, arthritis and cancer (Hotary et al., 2003; Kurban et al., 2006; Liotta and Kohn, 2001; Mutsaers et al., 1997; Poole et al., 2003). Two principal molecular mechanisms are believed to be involved in the turnover of ECM. One is extracellular degradation of ECM proteins by matrix metalloproteases (MMPs), plasmin and other proteases (Marchina and Barlati, 1996; Shapiro, 1998). The other pathway occurs via internalization of ECM proteins and intracellular degradation in lysosomes (Godyna et al., 1995; Memmo and McKeown-Longo, 1998; Murphy-Ullrich and Mosher, 1987; Wienke et al., 2003). These two pathways are not mutually exclusive and probably work collaboratively during ECM turnover. Many ECM proteins form supramolecular complexes (Kuivaniemi et al., 1991; McKeown-Longo and Mosher, 1989; Sasaki et al., 2004; Schwarzbauer and Sechler, 1999; van der Rest and Garrone, 1991). Thus, prior to internalization, extracellular degradation may partially breakdown large multimers or crosslinked molecules. Additionally, some fragments derived from ECM proteins possess functions distinct from the intact proteins (Giannelli et al., 1997; O’Reilly et al., 1997; Sasaki et al., 2000, 2001). Hence, cellular internalization can provide a way to regulate the availability of ECM fragments that may have potent biological effects. However, at present, mechanisms and pathways for degradation and removal of supramolecular ECM components are not completely understood.

Integrin receptors play important roles in mediating cell adhesion, contractility, motility and growth (Aplin et al., 1999; Burridge and Chrzanowska-Wodnicka, 1996; Howe et al., 1998; Hynes, 1992; Hynes, 2002). Ligation of integrins with ECM ligands can activate signaling pathways and influence cytoskeleton organization, which account for many of the biological functions of integrins (Brakebusch and Fassler, 2003; Geiger et al., 2001; Ginsberg et al., 2005). Integrins are known to be constitutively endocytosed and recycled (Bretscher, 1989; Bretscher, 1992; Pellinen and Ivaska, 2006). Disruption of integrin endocytosis and recycling can impair cell spreading and migration (Proux-Gillardeaux et al., 2005; Roberts et al., 2001). Relatively little attention has been given to the fate of the ECM proteins that are ligated to integrins during integrin endocytosis, or to how ECM proteins may modify integrin internalization. In addition, most studies on integrin trafficking do not distinguish between ECM-bound and -unbound forms of the receptors. ECM remodeling has been shown to be critical to many cellular functions, including cell migration and invasion (Engelholm et al., 2003; Hocking and Chang, 2003; Hornebeck et al., 2002; Hotary et al., 2000; Sabeh et al., 2004). Therefore, coupling of ECM remodeling with integrin endocytosis/recycling may be an important feature of integrin trafficking.

We established a fibronectin (FN)-null myofibroblast cell culture model system (Sottile et al., 1998). The turnover of fibronectin matrix can be easily induced in FN-null myofibroblasts when soluble fibronectin is removed from the culture medium. Using this model system, we previously demonstrated that fibronectin matrix turnover

and pathway...
plays an important role in governing ECM turnover (Sottile and Hocking, 2002). We also showed that fibronectin matrix turnover occurs through receptor-mediated endocytosis and is followed by lysosomal degradation (Sottile and Chandler, 2005). In this manuscript, we used this experimental model system to explore the role of integrin endocytosis in fibronectin matrix turnover. We demonstrate that β1 integrins, including α5β1, are fibronectin matrix endocytic receptors. Moreover, we show that caveolin-1 (official protein symbol CAV1) constitutively regulates α5β1 integrin endocytosis, regardless of the presence or absence of fibronectin and fibronectin matrix. We previously demonstrated that downregulation of caveolin-1 by siRNA inhibits fibronectin matrix turnover. Here we show that downregulation of caveolin-1 also impairs β1 integrin endocytosis. These data demonstrate the importance of caveolin-1-dependent integrin endocytosis in regulating fibronectin matrix turnover.

Results

β1 integrins colocalize with internalized fibronectin during long-term fibronectin matrix turnover

Fibronectin matrix turnover occurs through receptor-mediated endocytosis and intracellular degradation in a process that is regulated by caveolin-1 (Sottile and Chandler, 2005). Here we investigate the receptors that are involved in endocytosis of fibronectin and turnover of matrix fibronectin. Both integrins and proteoglycans are known to bind fibronectin, and to participate in endocytosis of various ligands, including ECM proteins (Chen et al., 1996; McKeown-Longo and Panetti, 1993; Memmo and McKeown-Longo, 1998; Murphy-Ullrich and Mosher, 1987; Pijuan-Thompson and Gladson, 1997), and hence, both are candidates for mediating fibronectin turnover.

Some integrins, including β1 integrins, undergo constitutive endocytosis and recycling (Bretscher, 1989; Bretscher, 1992; Caswell and Norman, 2006; Pellinen and Ivaska, 2006; Scezkan and Juliano, 1990). α5β1 and αvβ3 are major fibronectin receptors expressed in FN-null myofibroblasts (Sottile et al., 1998). As a first step in determining whether integrins are involved in regulating fibronectin matrix turnover, we tested whether fibronectin and β1 or β3 integrins are trafficked to the same endocytic compartment. FN-null myofibroblasts were incubated overnight with Texas Red (TR)-conjugated fibronectin to allow them to establish a robust fibronectin matrix. Cells were washed, and then chased with culture medium lacking fibronectin and containing chloroquine, an inhibitor of lysosomal hydrolases (de Duve et al., 1974) that also inhibits fibronectin degradation (Sottile and Chandler, 2005). Using this pulse-chase assay, we previously showed that β1 integrin endocytosis. These data demonstrate the importance of caveolin-1-dependent integrin endocytosis in regulating fibronectin matrix turnover.

Fig. 1. Colocalization of internalized fibronectin with β1 integrins. (A–C) FN-null myofibroblasts were incubated with 10 μg/ml TR-fibronectin overnight. Cells were washed and then incubated for 12 hours in cell culture media lacking fibronectin, but containing 50 μM chloroquine. Cells were stained with an anti-β1 integrin antibody (HMβ1-1). (A) TR-fibronectin; (B) β1 integrin; (C) overlay image. (D–F) FN-null myofibroblasts were incubated with 10 μg/ml TR-fibronectin overnight. Cells were washed, and then incubated with 10 μg/ml 9EG7 at 4°C for 30 minutes. Cells were washed, and then chased for 4 hours with cell culture medium lacking fibronectin, but containing 100 μM chloroquine. Cells were then stained with a FITC-conjugated secondary antibody. (D) TR-fibronectin; (E) 9EG7 (β1 integrin); (F) overlay image. (G–I) Smooth muscle cells were incubated with 10 μg/ml TR-fibronectin and 50 μM chloroquine for 12 hours. Cells were stained using an anti-β1 integrin antibody. (G) TR-fibronectin; (H) β1 integrin (FITC); (I) overlay image. All images are optical sections collected from a confocal microscope. Scale bars: 10 μm.

Fibronectin and β1 integrins colocalize shortly after the initiation of endocytosis

To determine whether fibronectin and β1 integrins are similarly colocalized at early times after the initiation of endocytosis, we developed a short-term fibronectin endocytosis pulse-chase assay. In this assay, fluorescently labeled fibronectin is added to the culture medium and allowed to bind to cells for 1 hour at 4°C (pulse phase). The culture medium containing unbound fibronectin is then replaced with culture medium lacking fibronectin, and the cells incubated at 37°C (chase phase) to allow endocytosis to occur. Intracellular fibronectin was detected by confocal microscopy as soon as 30 minutes following the start of the chase (Fig. 2A). The FN-null myofibroblasts in Fig. 2 were co-incubated with FITC-conjugated 9EG7 during the pulse phase. Internalized TR-fibronectin (Fig. 2A) and FITC-9EG7 (Fig. 2B) were extensively colocalized (Fig. 2C, arrowheads). Quantitative analysis of confocal z-sections showed that 75% of the internalized fibronectin colocalized with 9EG7 (β1 integrins).

α5 integrins also colocalize with internalized fibronectin

Six different α subunits form dimeric complexes with β1 integrins and function as fibronectin receptors (Johansson et al., 1997; Plow et al., 1997). Some of these integrins mediate fibronectin turnover. To determine whether α5β1 integrins colocalize with internalized fibronectin, we used the pulse-chase assay developed in this study. Cells were washed, and then incubated for 12 hours in cell culture media lacking fibronectin, but containing 50 μM chloroquine. Cells were stained with an anti-β1 integrin antibody (HMβ1-1). (A) TR-fibronectin; (B) β1 integrin; (C) overlay image. (D–F) FN-null myofibroblasts were incubated with 10 μg/ml TR-fibronectin overnight. Cells were washed, and then incubated with 10 μg/ml 9EG7 at 4°C for 30 minutes. Cells were washed, and then chased for 4 hours with cell culture medium lacking fibronectin, but containing 100 μM chloroquine. Cells were then stained with a FITC-conjugated secondary antibody. (D) TR-fibronectin; (E) 9EG7 (β1 integrin); (F) overlay image. (G–I) Smooth muscle cells were incubated with 10 μg/ml TR-fibronectin and 50 μM chloroquine for 12 hours. Cells were stained using an anti-β1 integrin antibody. (G) TR-fibronectin; (H) β1 integrin (FITC); (I) overlay image. All images are optical sections collected from a confocal microscope. Scale bars: 10 μm.
fibronectin endocytosis is comparable, a given dose of Alexa Fluor 488 (AF488)-fibronectin (10 μg/ml) was incubated with cells in the presence of increasing concentrations of unlabeled fibronectin during the pulse phase. As shown in Fig. 4B, endocytosis of AF488-fibronectin was maximally (81%) inhibited by 800 μg/ml unlabeled fibronectin. The experiment in Fig. 4A was performed in the absence or presence of excess unlabeled fibronectin (800 μg/ml) during the pulse phase. Nonspecific endocytosis is defined as that occurring in the presence of excess unlabeled fibronectin. The nonspecific component in this assay probably represents the noncompetable fraction of fibronectin binding. The linear increase in nonspecific fibronectin endocytosis parallels the linear increase in noncompetable fibronectin binding that is seen when 125I-labelled fibronectin is added to adherent cells in the presence of excess unlabeled fibronectin (McKeown-Longo and Mosher, 1983). Fibronectin internalization is saturated at fibronectin concentrations above 30-50 μg/ml, as shown by the plateau in the endocytosis curve at high concentrations of added fibronectin (Fig. 4A).

β1 integrins function as fibronectin endocytic receptors
Endocytosed fibronectin co-localizes with β1 and α5 integrins (Figs 1-3), suggesting that these integrins may be involved in fibronectin endocytosis. To determine whether β1 integrins are functionally required for fibronectin endocytosis in FN-null myofibroblasts, we used function blocking anti-integrin antibodies and assessed their effect on fibronectin internalization. Prior to the pulse phase, FN-null myofibroblasts were pre-incubated with β1 antibodies at 4°C, followed by the addition of fluorescently labeled fibronectin. Addition of β1 integrin inhibitory antibodies substantially reduced fibronectin endocytosis (Fig. 5A,B). Flow cytometry was used to quantify the level of internalized fibronectin. There was a 70±3.5% reduction in internalized fibronectin in cells treated with β1 inhibitory antibodies (Fig. 5C). We examined the cell surface levels

Fig. 2. Colocalization of internalized fibronectin and β1 integrins shortly after initiation of endocytosis. FN-null myofibroblasts were incubated with 10 μg/ml TR-fibronectin and 50 μg/ml FITC-conjugated 9E9G7 at 4°C for 1 hour during the pulse. After 30 minutes of chase at 37°C, cells were incubated with 0.2% Trypan Blue for 3 minutes to quench extracellular fluorescence. (A) TR-fibronectin; (B) FITC-conjugated 9E9G7; (C) overlay images. Arrowheads in C indicate colocalized TR-Fibronectin and FITC-9E9G7. These images are optical sections collected from a confocal microscope. Scale bar: 10 μm.

Fig. 3. Colocalization of internalized fibronectin with α5 integrins. Smooth muscle cells were incubated with 10 μg/ml TR-fibronectin and 50 μM chloroquine for 8 hours. Cells were stained using an anti-α5 integrin antibody (AB1928). (A) TR-fibronectin; (B) α5 integrin; (C) overlay image. These images are optical sections collected from a confocal microscope. Scale bar: 10 μm.

Fig. 4. Quantification of fibronectin endocytosis. (A) FN-null myofibroblasts were incubated with 2.5-50 μg/ml AF488-conjugated fibronectin at 4°C during the pulse phase (total endocytosis). Some samples were co-incubated with 800 μg/ml unlabelled fibronectin to determine non-specific endocytosis. Cells were chased in the absence of fibronectin for 2 hours at 37°C, and then processed for flow cytometry to quantify endocytosed fibronectin. Specific endocytosis was determined by subtracting non-specific endocytosis from total endocytosis. The graph shows the best-fit curve of the mean fluorescence intensity (MFI) of internalized AF488-fibronectin. Data represent the mean of duplicate samples and error bars indicate the range. (B) FN-null myofibroblasts were incubated with 10 μg/ml AF488-fibronectin in the presence of increasing concentrations of unlabeled fibronectin (0-800 μg/ml) during the pulse phase. Cells were chased in the absence of fibronectin for 2 hours at 37°C, and then processed for flow cytometry to quantify endocytosed AF488-fibronectin. The amount of internalized AF488-fibronectin is reported as percent control internalization (cells incubated in the absence of unlabeled fibronectin, which was set equal to 100%). Data represent the mean of duplicate samples and error bars indicate the range.
of fibronectin both at the beginning and the end of the chase period using western blot analysis. There was a relatively small reduction (25%) in cell surface fibronectin at the beginning of the chase in cells treated with β1 inhibitory antibodies in comparison with control cells (data not shown). When measured at the end of chase period, there was a 14.5% reduction in cell surface fibronectin in cells treated with β1 inhibitory antibodies. The smaller reduction in cell surface fibronectin at 2 hours of chase (14.5% vs 25%) could reflect the higher rate of endocytosis in the control cells, which leads to a greater reduction of cell surface fibronectin over the course of 2 hours. These data show that the reduction in the levels of endocytosed fibronectin in cells treated with β1 inhibitory antibodies cannot be attributed to the reduced availability of fibronectin to cells. β1 inhibitory antibodies did not have any effect on fibronectin endocytosis (Fig. 5C).

When smooth muscle cells were pretreated with β1 integrin inhibitory antibodies (Fig. 5D), there was also a striking decrease in intracellular accumulation of fibronectin in comparison with control cells (Fig. 5E). Flow cytometric analysis indicated that fibronectin endocytosis was reduced 56.5±4.2% in smooth muscle cells treated with β1 inhibitory antibodies (Fig. 5F). This decrease could not be attributed to decreased fibronectin binding to the cell surface, because fibronectin binding was only decreased 2% in cells treated with β1 inhibitory antibodies. β1 integrins play an important role in endocytosis and turnover of matrix fibronectin

To further establish that the effect of β1 inhibitory antibodies on fibronectin endocytosis was not due to differences in the levels of fibronectin bound to the cell surface or present in the matrix at the beginning of the chase, we performed fibronectin endocytosis assays with FN-null myofibroblasts seeded onto fluorescently labeled pre-assembled fibronectin matrices. Some cells were incubated with β1 inhibitory antibodies for 30 minutes prior to seeding. β1 inhibitory antibodies did not affect cell attachment or spreading (data not shown), probably because of the presence of non-β1 integrins, including β3 integrins (Sottile et al., 1998), and other matrix protein in the pre-assembled matrix, including collagen I and heparan sulfate proteoglycans, which could promote adhesion in a β1-integrin-independent manner. As shown in Fig. 6A,B, fibronectin endocytosis was dramatically reduced in cells treated with β1 inhibitory antibodies. Flow cytometric analysis indicated that there

Fig. 5. Blocking of β1 integrin inhibits fibronectin endocytosis. (A-C) FN-null myofibroblasts were incubated with 25 μg/ml β1 antibodies (Ha2/5) or isotype control antibodies at 4°C for 30 minutes. 10 μg/ml TR-fibronectin (A,B) or AF488-fibronectin (C) was then added to the medium and cells were processed for fibronectin endocytosis pulse-chase assays. After 2 hours of chase, cells were either fixed for imaging assay (A, β1 inhibition; B, isotype control), or processed for flow cytometry to quantify endocytosed fibronectin (C). The numbers over the peaks in C are the MFI of internalized AF 488-fibronectin. (D-F) Smooth muscle cells were seeded in serum-free medium on vitronectin-coated dishes. Cells were allowed to adhere for 3 hours and then processed for integrin-blocking assay as described above for FN-null myofibroblasts. (D) 25 μg/ml β1 inhibitory antibodies (Ha2/5); (E) isotype control; blue: DAPI. (F) Quantification of endocytosed fibronectin in smooth muscle cells by flow cytometry. The numbers over the peaks in F are the MFI of internalized AF 488-fibronectin. Scale bars: 10 μm.

Fig. 6. Blocking of β1 integrins inhibits endocytosis of fibronectin from preassembled matrix. FN-null myofibroblasts were incubated with either 30 μg/ml β1 inhibitory antibody (Ha2/5) or isotype control in suspension at room temperature for 30 minutes prior to seeding on pre-assembled TR-(A,B) or AF488 (C,D) fibronectin matrix. Cells were cultured for 24 hours at 37°C, and were then either fixed for imaging assay (A, β1 inhibition; B, isotype control) or processed for flow cytometry to quantify internalized fibronectin (C,D). The numbers over the peaks in C are the MFI of internalized AF488-fibronectin. Graph in D shows fold change relative to the MFI of endocytosed AF488-fibronectin in cells treated with isotype control IgM, which was set equal to 1 (n=4, mean ± s.d.). Scale bar: 10 μm.
was a significant reduction (58.7±4%) in fibronectin endocytosis in cells treated with β1 inhibitory antibodies (Fig. 6C,D).

To further demonstrate the requirement for β1 integrin in fibronectin endocytosis, we asked whether fibronectin endocytosis occurs in fibroblastic cells lacking β1 integrins (GD25 cells). GD25 cells and GD25 cells re-expressing β1 integrins (GD25 β1) were seeded onto preassembled fibronectin matrix for 24 hours. As shown in Fig. 7A,B, there was a dramatic reduction in fibronectin endocytosis in GD25 cells in comparison to GD25 β1 cells. Flow cytometric analysis indicated that there was a 47.8% reduction in internalized fibronectin in GD25 cells (Fig. 7C). It is likely that the flow cytometry data underestimate the percent inhibition because the cells have some intrinsic background fluorescence [mean fluorescence intensity (MFI)=77] and because of the noncomparable portion of fibronectin endocytosis (Fig. 4B). Turnover of preassembled fibronectin matrix fibrils is also reduced in GD25 cells compared with that in GD25 β1 cells (Fig. 7D-G).

Interestingly, in short-term pulse-chase experiments, GD25 cells endocytosed soluble fibronectin at levels comparable with that in GD25 cells re-expressing β1 integrin (supplementary material Fig. S1A). Fibronectin endocytosis was blocked by RGD peptides (supplementary material Fig. S1B), but not by the control RGE peptides (supplementary material Fig. S1C). These data indicate that an alternative integrin receptor can endocytose soluble fibronectin in the absence of β1 integrins, but that this alternative receptor is inefficient at promoting the internalization of matrix fibronectin.

α5 integrins also function as fibronectin endocytic receptors

To determine which α subunit is involved in fibronectin endocytosis, we used the same function-blocking protocol as described in Fig. 5. Because α5 and αv integrins are expressed in FN-null myofibroblasts in relatively high amounts (Sottile et al., 1998), we first focused on these two α subunits. FN-null myofibroblasts were pre-incubated with α5 or αv function inhibitory antibodies at 4°C, prior to the addition of fluorescently labeled fibronectin during the pulse phase. After 2 hours of chase at 37°C, we found that α5 integrin inhibitory antibodies reduced the intracellular accumulation of fibronectin (Fig. 8A,B). Flow cytometric analysis indicated that there was a 45% reduction in internalized fibronectin in cells treated with 50 μg/ml α5 integrin inhibitory antibody (Fig. 8E). Increasing the concentration of α5 integrin inhibitory antibodies did not cause any further reduction in fibronectin endocytosis (data not shown). This reduction in endocytosed fibronectin could not be totally attributed to a reduction of fibronectin initially bound to the cell surface (19% reduction). By contrast, αv integrin inhibitory antibodies had no effect on fibronectin endocytosis at 100 μg/ml (Fig. 8C-E).

A non-clathrin mechanism mediates fibronectin endocytosis

Using long-term fibronectin matrix turnover pulse-chase assays, we previously showed that fibronectin matrix turnover is inhibited by agents that disrupt caveolae/lipid-raft-mediated endocytosis and can be blocked by downregulation of caveolin-1 (Sottile and Chandler, 2005). To determine whether caveolin-1 also regulates fibronectin endocytosis in short-term pulse-chase assays, we examined the levels of internalized fibronectin in FN-null myofibroblasts expressing caveolin-1 siRNA (shcav) or control siRNA (shluc). As shown in Fig. 9, there is a dramatic reduction in fibronectin (Fig. 9A) and β1 integrin (9EG7, Fig. 9B) endocytosis in cells expressing caveolin-1 siRNA in comparison with control cells. Flow cytometric analysis showed that there was a 46±2% reduction in levels of endocytosed fibronectin in cells expressing caveolin-1 siRNA (Fig. 9G).

To further characterize the intracellular vesicles that contain fibronectin, we asked whether internalized fibronectin is found in lipid-raft-enriched compartments. FN-null myofibroblasts were incubated at 4°C with TR-fibronectin and AF488-conjugated cholera toxin subunit B (CTxB), a caveolae/lipid raft marker, and a short-term endocytosis pulse-chase assay was preformed. TR-fibronectin (Fig. 9H) was found in AF488-CTxB-positive

![Fig. 7. Reduced endocytosis and turnover of pre-assembled fibronectin matrix in β1-integrin-null cells. GD25 and GD25 β1 re-expressing cells were seeded on pre-assembled TR (A,B,D,E,F,G) or AF488 (C) fibronectin matrix and incubated for 24 hours at 37°C (A,B) Cells were incubated with 0.2% Trypan Blue for 3 minutes and then fixed. Intracellular fibronectin vesicles are shown (A, GD25; B, GD25 β1). The insets show outlines of cells loaded with CellTracker Green. Scale bar: 10 μm. (C) Cells were processed for flow cytometry to quantify endocytosed fibronectin. The numbers over the peaks in C are the MFI of internalized AF 488-fibronectin. (D-G) Cells were fixed without Trypan Blue treatment to visualize fibronectin fibrils (D,E, GD25; F,G, GD25 β1; E and G are phase images of corresponding fields). Scale bar: 40 μm.](image-url)
Downregulation of caveolin-1 reduces both fibronectin and β1 integrin endocytosis

β1 integrins play a major role in mediating fibronectin endocytosis (Figs 5-7). Thus, we asked whether β1 integrin endocytosis is also caveolin-1 dependent. We performed a long-term fibronectin matrix turnover pulse-chase assay in cells expressing caveolin-1 siRNA (shcav) and control cells (shluc). The level of intracellular β1 integrins was dramatically reduced in shcav cells compared with control cells (Fig. 11A-F). To ensure that the intracellular β1 integrins detected in Fig. 11D are internalized from the cell surface, we labeled cell surface β1 integrins with 9EG7 antibody. Internalized 9EG7 was extensively colocalized with internalized fibronectin in control cells (shluc, supplementary material Fig. S2F). However, there was a dramatic reduction of 9EG7 intracellular accumulation in cells expressing caveolin-1 siRNA (supplementary material Fig. S2A) in comparison with control cells (supplementary material Fig. S2D).

It is unknown whether endocytosis of β1 integrins is regulated by the same mechanisms in the presence and absence of fibronectin. Cells expressing caveolin-1 siRNA (shcav) were incubated with α5 integrin antibodies at 4°C in the absence of fibronectin. After washing, cells were chased at 37°C, and the levels of internalized α5 integrin were measured. There was a dramatic decrease in endocytosed α5 integrins in cells expressing caveolin-1 siRNA (Fig. 11G) compared with control cells (Fig. 11H). The levels of endocytosed α5 integrin were reduced 53.2±1.7% when caveolin-1 was downregulated (Fig. 11I). The levels of endocytosed β1 integrins were also reduced in cells expressing caveolin-1 siRNA (Fig. 11J) compared with control cells (Fig. 11K).

Re-expression of caveolin-1 increases endocytosis of β1 integrins in cells expressing caveolin-1 siRNA

To confirm that downregulation of caveolin-1 in the caveolin-1 siRNA-expressing cells is responsible for decreased endocytosis of β1 integrins (Fig. 11), we re-expressed caveolin-1 in these cells using an adenovirus containing human caveolin-1 (Ad-cav). We performed long-term fibronectin matrix turnover pulse-chase assays with FN-null myofibroblasts expressing caveolin-1 siRNA (shcav). Re-expression of caveolin-1 in shcav cells resulted in increased endocytosis of β1 integrins and fibronectin (arrows, Fig. 12D,E). Internalized β1 integrins and fibronectin were colocalized in endocytic vesicles (overlay image in Fig. 12F).

To determine whether re-expression of caveolin-1 rescues constitutive endocytosis of α5β1 integrin in these cells, we performed integrin endocytosis assays with cells expressing caveolin-1 siRNA that were cultured in the absence of fibronectin. As shown in Fig. 12J, re-expression of caveolin-1 increased the levels of endocytosed α5 integrin by 243±15% compared with cells transduced with control adenovirus (Ad-tet). The endocytosis of β1 integrins was also increased by re-expression of caveolin-1 in shcav cells (Fig. 12L). By contrast, the control adenovirus (Ad-tet) did not rescue constitutive endocytosis of α5β1 integrin (Fig. 12J) or endocytosis of β1 integrins (Fig. 12M). These data show that caveolin-1 regulates endocytosis of β1 integrins, including α5β1 integrin, regardless of the presence or absence of fibronectin and fibronectin matrix.

Discussion

Fibronectin matrix turnover plays a critical role in governing ECM turnover (Sottile and Hocking, 2002). We previously showed that fibronectin matrix turnover occurs through receptor-mediated

Compartments (Fig. 9I) following 2 hours of chase (Fig. 9J, arrowheads point to yellow staining). Furthermore, internalized fibronectin, β1 integrins and caveolin-1 all extensively colocalized, as shown by the white color in the overlay image of Fig. 9N (arrowheads), as well as by the substantial overlap among the fluorescence intensity plots of the three color staining (Fig. 9O).

To directly address whether fibronectin endocytosis occurs via a clathrin- or non-clathrin-mediated pathway, a dominant-negative Eps15 mutant (DN Eps15) (Benmerah et al., 1999) was transiently expressed in FN-null myofibroblasts. Cells expressing DN Eps15 (Fig. 10A) endocytosed fibronectin from preassembled matrix at levels similar to control cells. By contrast, DN Eps15 effectively inhibited endocytosis of the transferrin receptor (Fig. 10I), which is known to be endocytosed by a clathrin-mediated pathway (Harding et al., 1983). DN Eps15 also failed to inhibit soluble fibronectin endocytosis (Fig. 10E). These data demonstrate that fibronectin endocytosis occurs via a non-clathrin-mediated pathway.

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To directly address whether fibronectin endocytosis occurs via a clathrin- or non-clathrin-mediated pathway, a dominant-negative Eps15 mutant (DN Eps15) (Benmerah et al., 1999) was transiently expressed in FN-null myofibroblasts. Cells expressing DN Eps15 (Fig. 10A) endocytosed fibronectin from preassembled matrix at levels similar to control cells. By contrast, DN Eps15 effectively inhibited endocytosis of the transferrin receptor (Fig. 10I), which is known to be endocytosed by a clathrin-mediated pathway (Harding et al., 1983). DN Eps15 also failed to inhibit soluble fibronectin endocytosis (Fig. 10E). These data demonstrate that fibronectin endocytosis occurs via a non-clathrin-mediated pathway.
endocytosis and intracellular degradation (Sottile and Chandler, 2005). In this manuscript, we demonstrate that β1 integrins play an important role in regulating the endocytosis of fibronectin and the turnover of FN matrix fibrils. We also show that caveolin-1 regulates the endocytosis of fibronectin-binding β1 integrins. Furthermore, caveolin-1 constitutively regulates α5β1 integrin endocytosis (Fig. 11), regardless of the presence or absence of fibronectin and fibronectin matrix. Our study also directly shows that α5β1 integrin endocytosis can occur in the absence of its ECM ligand.

Function-blocking antibodies to β1 integrin inhibit fibronectin endocytosis both in short-term pulse-chase assays, and from preassembled fibronectin matrices (Figs 5, 6), indicating that β1 integrins are functionally important for fibronectin endocytosis. In the short-term assay, most or all of the fibronectin that is present at the start of the chase is cell-associated, protomeric fibronectin. In the long-term pulse-chase experiments, ~85% of the fibronectin is incorporated into the matrix at the start of the chase (Sottile and Hocking, 2002). ECM fibronectin is also the predominant (or sole) form present in pre-established matrices. Importantly, our data show that β1 inhibitory antibodies block the endocytosis of ‘soluble’ and matrix fibronectin. Further, β1-null cells show impaired ability to endocytose matrix fibronectin from pre-established matrices. Interestingly, β1-null cells endocytosed soluble fibronectin at levels similar to cells re-expressing β1 integrin (supplementary material Fig. S1A). These data indicate that other receptors can mediate the endocytosis of soluble fibronectin when β1 integrins are absent, but that this pathway is inefficient when matrix fibronectin is the ligand. Endocytosis of soluble and matrix fibronectin share many features: both are clathrin independent, both are inhibited by downregulation of caveolin-1 (Fig. 9G) (Sottile and Chandler, 2005) and both colocalize with β1 integrins in intracellular vesicles. It is not known why β1 integrins play a more prominent role in endocytosis of ECM fibronectin than soluble fibronectin (Fig. 7). We speculate that a certain degree of extracellular degradation of fibronectin may occur prior to
endocytosis of matrix fibronectin. It has been recently shown that clustering of β1 integrins can induce polarized exocytosis of MT1-MMP to invasive structures, which results in localized ECM degradation (Bravo-Cordero et al., 2007). Thus, it is possible that β1 integrins are involved in two aspects of fibronectin matrix turnover: one involving localized matrix fibronectin degradation, and one involving fibronectin endocytosis.

β1 inhibitory antibodies block fibronectin endocytosis, but do not significantly affect initial fibronectin binding to the cell surface in short-term pulse-chase assays. It is possible that the initial cell surface binding and internalization of fibronectin do not rely on the same receptor(s), as has been shown for other matrix proteins (Panetti and McKeown-Longo, 1993). In addition, there are multiple fibronectin-binding integrins in FN-null myofibroblasts, including αvβ3 (Sottile et al., 1998). Fibronectin can also bind to non-integrin receptors, including proteoglycans (Saunders and Bernfield, 1988; Tumova et al., 2000).

Internalized soluble fibronectin and β1 integrins colocalize at early stages of the endocytosis process (30 minutes, Fig. 2). However, endocytosis of fibronectin from preassembled matrices is a slower process, and sufficient intracellular fibronectin does not accumulate at these early times to allow similar colocalization studies to be done. Therefore, it remains uncertain whether β1 integrins can directly internalize fibronectin from the ECM.

Fig. 10. Dominant-negative Eps15 does not inhibit fibronectin endocytosis. Dominant-negative Eps15 (A,C,E,G,K) or control Eps15 (B,D,F,H,J,L) were transiently expressed in FN-null myofibroblasts. (A-D) Transfected cells were seeded onto preassembled matrices and incubated for 24 hours. Endocytosed fibronectin is shown in A,B. (E-H) Short-term pulse-chase experiments were performed with transfected cells. (E,F) show endocytosed fibronectin following 2 hours of chase. (I-L) The effect of dominant-negative and control Eps15 on transferrin receptor endocytosis is shown. Transfected cells were detected by GFP expression (C,D,G,H,K,L) and manually outlined. 15-20 cells were analyzed for each condition; representative images are shown. Scale bar: 10 μm.

Fig. 11. Integrin endocytosis in caveolin-1-knockdown cells. (A-F) Cells expressing caveolin-1 siRNA (shcav) or control cells (shluc) were incubated with 10 μg/ml TR-fibronectin overnight. Cells were washed, and then incubated for 8 hours in cell culture medium lacking fibronectin, but containing 50 μM chloroquine. Cells were stained with anti-β1 integrin antibody (FITC). Upper panels, shcav; lower panels, shluc. A,D, β1 integrin; B,E, TR-fibronectin; C,F, overlay images. (G-K) Cells expressing caveolin-1 siRNA (shcav, G,J) or control cells (shluc, H,K) were incubated with 50 μg/ml antibodies to α5 integrin (G,H) or β1 integrin (J,K) at 4°C for 45 minutes. Cells were then processed for integrin endocytosis assay. The fluorescence intensity of endocytosed α5 integrin (G,H) was quantified using a MATLAB-based program. (I) Fold change relative to the fluorescence intensity of endocytosed α5 integrin in shluc cells, which was set equal to 1 (mean ± range from two independent experiments). All images are optical sections collected from a confocal microscope. Scale bars: 10 μm.
Not all fibronectin-binding integrins can promote fibronectin endocytosis. Of the integrins tested, only α5β1 integrin was shown to participate in fibronectin endocytosis. Integrin function-blocking assays showed that α5 antibodies were not as effective as β1 antibodies in blocking fibronectin endocytosis (Figs 5 and 8). We speculate that this may be due to the α5 antibodies being less potent inhibitors than β1 antibodies, or to the involvement of multiple α-integrin subunits in fibronectin endocytosis. However, to date, we have been unable to identify other α-integrin subunits that may participate in fibronectin endocytosis.

Integrin endocytosis has been reported to occur by clathrin and caveole/lipid-raft-mediated pathways (Caswell and Norman, 2006; Nishimura and Kaibuchi, 2007; Pellinen and Ivaska, 2006). The existence of different pathways and regulatory mechanisms for integrin endocytosis is probably due to the diverse nature of integrin receptors and their ligands. The clearest evidence for the involvement of caveole/lipid rafts in integrin endocytosis comes from studies of the collagen-binding integrin α2β1 and the leukocyte integrin αLβ2 (Fabbri et al., 2005; Ning et al., 2007; Upla et al., 2004). Some data also show that β1 integrins can be endocytosed via caveole upon induction by glycosphingolipids (Sharma et al., 2005). However, direct evidence that fibronectin-binding integrins can be endocytosed by a caveolin-1-dependent pathway is lacking. The α5β1 integrin is endocytosed and recycled in several cell types (Bretscher, 1992; Pierini et al., 2000). However, the endocytic pathway and regulatory mechanism of α5β1 integrin endocytosis have not been clearly established. In this manuscript, we demonstrate that endocytosis of β1 integrins, including α5β1, is caveolin-1 dependent. In the presence of fibronectin matrix, a large portion of the endocytosed β1 integrins are likely to be ligand (fibronectin) bound, because internalized β1 integrins are extensively colocalized with endocytosed fibronectin, and because endocytosed β1 integrins are in a ligand-bound conformation, as shown by their ability to bind the 9EG7 antibody (Figs 1, 2). We further show that disruption of clathrin-mediated endocytosis does not inhibit fibronectin endocytosis (Fig. 10). In addition, internalized fibronectin is found in lipid raft compartments (Fig. 9H-J), and internalized fibronectin and β1 integrins extensively colocalize with caveolin-1-positive vesicles (Fig. 9K-O). We previously showed that fibronectin matrix turnover is dependent on protein kinases, cholesterol content, and the dynamics of the actin cytoskeleton (Sottile and Chandler, 2005; Sottile and Hocking, 2002). Together, these data strongly suggest that fibronectin endocytosis occurs via a non-clathrin-mediated mechanism, and likely involves both integrins and caveole/lipid rafts.

ECM remodeling is a key regulator of cell migration (Davis and Senger, 2005; Engelholm et al., 2003; Giannelli et al., 1997; Hangai et al., 2002; Hocking and Chang, 2003; Hotary et al., 2000). Our data show that caveolin-1 plays a critical role in fibronectin matrix turnover. Interestingly, caveolin-1 has also been shown to be involved in cell migration (Beardsley et al., 2005; Ge and Pachter, 2004; Grande-Garcia et al., 2007; Navarro et al., 2004). Hence, it is possible that caveolin-1 may regulate cell migration in part, by regulating turnover of matrix fibronectin. Moreover, recent studies from caveolin-1-knockout mice and patients with idiopathic pulmonary fibrosis (IPF) highlight the importance of caveolin-1 in the development of lung fibrosis (Drab et al., 2001; Park et al., 2002; Razani et al., 2001; Wang et al., 2006). These data suggest the interesting possibility that mice lacking caveolin-1 and/or patients with IPF may also have defects in ECM endocytosis and turnover.

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**Fig. 12.** Re-expression of caveolin-1 rescues endocytosis of β1 integrin in caveolin-1 siRNA cells. FN-null myofibroblasts expressing caveolin-1 siRNA (shcav) were transduced with Ad-cav or Ad-tet adenoviruses. (A-I) Cells were incubated with 50 μg/ml TR-fibronectin overnight. Cells were washed, and then incubated for 8 hours in cell culture medium lacking fibronectin, but containing 50 μM chloroquine. Cells were stained with anti-β1 integrin antibody (FITC). Upper panels, shcav without virus transduction; middle panels, Ad-cav; lower panels, Ad-tet. A,D,G: TR-fibronectin; B,E,H: β1 integrin; C,F,I, overlay images. Arrows in D show the internalized fibronectin; C,F,I overlay images. Arrows in D show the internalized β1 integrin (K-β1). At 4°C for 45 minutes. Cells were then processed for integrin endocytosis assay. (J) The fluorescence intensity of endocytosed β1 integrin was quantified using a MATLab-based program. Graph shows the relative fluorescence intensity of endocytosed β1 integrin in shcav cells with or without virus transduction. Intracellular fluorescence intensity of shcav cells without virus transduction was set equal to 1 (mean ± range from two independent experiments). All images are optical sections collected by confocal microscopy. Scale bars: 20 μm.
cell outlines and to quantify the fluorescence intensity within individual cells. Background fluorescence was determined for each image, and subtracted from the fluorescence intensity of each cell. The fluorescence intensity within individual cells was normalized by cell area. Sample sizes were 46-171 cells for each condition. The mean value of two independent experiments (± range) is reported.

Imaging assays
Immunostaining was performed as described (Sottile and Hocking, 2002). Cells were examined using an Olympus microscope equipped with epifluorescence, or with an Olympus scanning confocal microscope. Protein colocalization was quantified from confocal images as described previously (Sottile and Chandler, 2005).

Flow cytometry to quantify endocytosed fibronectin
After pulse-chase assays, cells were incubated with a mixture of 0.02% EDTA, 0.1% trypsin and 200 μg/ml protease K for 3 minutes at 37°C to remove cell surface fibronectin. Cells were detached from culture dishes and washed with PBS/0.01% sodium azide. Cell were then suspended in 0.2% Trypan Blue for 2 minutes to quench any residual cell surface fluorescence (Hed, 1977; Van Amersfoort and Van Strijp, 1994). Cells were washed with PBS/0.01% sodium azide and then suspended in 3.5% paraformaldehyde. The intracellular AF488-fibronectin signal was measured immediately by flow cytometry using a FACSCalibur (Becton Dickinson).

Western blotting
Cells were scraped from dishes in lysis buffer at the end of the pulse or chase period. Western blotting was performed as described (Sottile and Chandler, 2005). Blots were quantified using an Odyssey infrared imaging system (Li-COR Bioscience, Lincoln, NE). Levels of ERK on the blots were used as a loading control. The levels of fibronectin were normalized to the levels of ERK. Control experiments were done to determine whether the full-length fibronectin present at the end of the chase is cell surface fibronectin. For these experiments, intact cells were harvested by EDTA, and then treated with trypsin and proteinase K. Western blot analysis showed that all of the full-length fibronectin was extracellular, because it was completely digested by protease treatment (data not shown).

Transient expression of GFP-Eps15 mutant constructs
Dominant-negative GFP-Eps15 EH21 and control GFP-Eps15 DIIIa2 were generous gifts from Alexandra Benmerah (INSERM, Paris, France). The EH21 mutant disrupts clathrin-mediated endocytosis; the control construct does not affect endocytosis (Benmerah et al., 1999; Benmerah et al., 1998). Transient expression was performed using lipofectamine LTX reagent according to the manufacturer’s instructions. Short-term pulse chase assays were performed on cells 24 hours post transfection. To study fibronectin endocytosis from pre-assembled matrix, cells were harvested 18 hours after transfection, re-seeded on pre-assembled matrix and then cultured for an additional 24 hours. Endocytosis of transferrin receptor was monitored by using antibodies to the transferrin receptor.

Adenoviruses
A plasmid adenovirus was a kind gift from Sandra Schmid (Scrips Research Institute, La Jolla, CA). Preparation and transduction of adenoviruses containing the sequence of either human caveolin-1 (Ad-cav) or tetracycline regulatable transactivator (Ad-tet) were described previously (Sottile and Chandler, 2005). Increased levels of caveolin-1 persisted for >60 hours after viral transduction (data not shown).

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References
the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP.


FIGURE S1