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Fibronectin binding and mesenchymal morphology

### Binding of soluble fibronectin to integrin α5β1 – link to focal adhesion redistribution and contractile shape

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Focal adhesions are randomly distributed across the ventral surface or along the edge of epithelial cells. In fibroblasts they orient centripetally and concentrate at a few peripheral sites connecting long F-actin stress fibers, causing a typical elongated, contractile morphology. Extensive remodeling of adhesions in fibroblasts also takes part in fibronectin (FN) fibrillogenesis, a process that depends on Rho-mediated contractility and results in the formation of a FN matrix. Our current study implicates that all these fibroblast characteristics are controlled by the ability of integrin α5β1 to bind soluble FN molecules in their compact inactive conformation. The hypervariable region of the ligand-binding I-like domain of α5β1 supports binding of soluble FN. This supports the distribution of centripetally orientated focal adhesions in distinct peripheral sites, Rho activation, and FN fibrillogenesis through a mechanism that does not depend on Syndecan-4. Integrin αvβ3, even when locked in high affinity conformations for the RGD recognition motif shows no appreciable binding of soluble FN and, consequently, fails to support the typical fibroblast focal adhesion distribution, Rho activity, and FN fibrillogenesis in the absence of α5β1. The ability of α5β1 to interact with soluble FN may thus drive the cell-matrix adhesion/cytoskeletal organization required for a contractile, fibroblast-like morphology, perhaps explaining why α5β1, like FN, is essential for development.

### INTRODUCTION

Integrins consist of non-covalently linked α and β subunits. Ligands, including extracellular matrix (ECM) components such as fibronectin (FN), are bound to their extracellular globular head domain while integrins interact via their cytoplasmic tails with a multitude of cytoskeletal adaptor proteins. Thus, integrins create a link between the ECM and the actin cytoskeleton. Ligand-binding can be modulated at the level of integrin clustering (avidity) or by activation of integrins through conformational changes in the extracellular ligand-binding domains (affinity) (Cailenwood, 2004; Takagi and Springer, 2002). For instance, binding to soluble ligands through integrins α5β1, αvβ3, or αIIbβ3 can be enhanced >20 fold by divalent cations or stimulatory antibodies, leading to firm adhesion of cells that show no appreciable adhesion to immobilized ligands in the absence of such stimuli (Danen et al., 1995; Mould et al., 1995; Smith et al., 1994). In turn, integrin-mediated adhesion can modulate various intracellular signaling cascades (Hynes, 2002). Following cell adhesion to the ECM, integrins and associated proteins assemble in cell-matrix adhesions, which organize the actin cytoskeleton. In epithelial cells, “focal adhesions” are randomly distributed across the ventral surface or along the cell border. In mesenchymal cells such as fibroblasts they...
orient centripetally and concentrate in a few peripheral sites connecting long F-actin stress fibers, causing a typical elongated, contractile morphology.

FN is essential for embryonic development and is abundantly present in the ECM associated with wound healing and angiogenesis (Hynes and Zhao, 2000). Mesenchymal cells secrete compact and inactive soluble FN dimers which are assembled into insoluble FN fibrils following their interaction with cell surface receptors of the integrin and syndecan families (Wierzbicka-Patynowski and Schwarzbauer, 2003). FN fibrils are assembled into a matrix that is important for anchorage of cells and guides cell migration during embryonic development and wound healing (Hynes and Zhao, 2000). Integrins α5β1, α6β1, α6β3, αlβ3, αvβ5, αvβ6, and αvβ8 recognize the common integrin-binding motif Arg-Gly-Asp (RGD) that is found in many ECM components, including FN (Danen and Sonnenberg, 2003). Of these integrins, α5β1 is particularly efficient in mediating FN matrix assembly, although others can compensate for its absence to some extent (Wennerberg et al., 1996; Wu et al., 1996; Yang and Hynes, 1996). FN fibrillogenesis requires extensive remodeling of cell-matrix adhesions (Pankov et al., 2000), and contractility of the actin-myosin cytoskeleton, which is stimulated by the small GTPase Rho through activation of myosin-II. Contractility drives FN fibrillogenesis by creating sufficient tension to stretch the compact FN dimers and expose intermolecular FN-binding sites (Zhong et al., 1998).

Integrin-mediated cell adhesion triggers a rapid inactivation of the small GTPase RhoA (Ren et al., 1999), which is important to relieve contractility and allow cell spreading. As cell spreading ends, Rho-mediated cytoskeletal contractility gradually increases, which coincides with maturation of focal adhesions and initiation of FN fibrillogenesis. We previously reported that β1 integrins are dispensable for cell spreading but required to support this second phase (Danen et al., 2002; Danen et al., 2005). In the studies reported here, we have expressed different wild type-, chimeric-, and mutant integrin subunits in cells lacking the \( Itgb1 \), \( Itgb3 \), or \( Itga5 \) integrin genes, or silenced expression of integrin \( \beta1 \) or Syndecan-4 to study how they organize cell-matrix adhesions, cytoskeletal contractility and FN matrix assembly. Our findings point to extensive reciprocal signaling between FN and the actin cytoskeleton selectively through integrin α5β1 that couples binding of soluble FN dimers to a fibroblast-like distribution of focal adhesions, Rho-mediated contractility, and FN fibrillogenesis.

MATERIALS AND METHODS

Cell lines and plasmids

The β1-deficient GE11 cell line was described previously (Danen et al., 2002; Gimond et al., 1999). EA5 cells were derived from murine \( Itga5 \) KO embryonic stem cells (provided by Prof. Richard Hynes, MIT, Boston, MA) were differentiated in DMEM supplemented with 10% fetal calf serum and immortalized with SV40 Large T. Integrin β3-deficient MEFs were isolated from \( Itgb3 \) KO FVB mice (mice were provided by Prof. Richard Hynes, MIT, Boston, MA) and immortalized with the SV40 large T antigen. All cells were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. LZRS bicistronic retroviral expression plasmids encoding human integrin β1 and β3 were described before (Danen et al., 2002). cDNA encoding human α5 (provided by Dr. Erkki Ruoslahti, Cancer Research Center, The Burnham Institute, La Jolla, CA) was cloned into LZRS-neo. To generate a cDNA encoding a chimeric human α5v subunit, a fragment containing the extracellular and transmembrane domain of α5 was digested from LZRS-α5v by PCR amplification in which the accagta sequence immediately upstream of the cytoplasmic tail of αv was changed to tacgta, creating a HindIII site. To generate a cDNA encoding a chimeric human α5vα subunit, a fragment containing the extracellular and transmembrane domain of α5 was digested from LZRS-α5v by PCR amplification of the αv cytoplasmic domain in which the aggagt sequence immediately upstream of the cytoplasmic tail of αv was changed to aagctt, creating a HindIII site. To generate a cDNA encoding a chimeric human β13n subunit, the \( Itgb1 \) sequence downstream of the internal SnaBI site (70 nucleotides upstream of the transmembrane region) was replaced by the corresponding sequence derived from LZRS-αv by PCR amplification in which the ccagta sequence was changed to tacgta, creating a SnaBI site. These cDNAs, as well as cDNAs encoding β3[3N305T], β3[T329C;A347C] and
β3[332C;335C] (provided by Dr. Bing-Hao Luo and Prof. Timothy A Springer, CBR Institute for Biomedical Research, Boston, MA) and cDNA encoding β1[D130A] (provided by Dr. Yoshikazu Takada (University of California Davis Medical Center, Sacramento, CA)) were recloned into LZRS-neo. The β1-3-1 expression plasmid was provided by Dr. Yoshikazu Takada. Retroviral constructs were transfected into ecotropic packaging cells to generate virus-containing culture supernatants. β3 KO MEFs, EA5, and GE11 cells were transfected with cDNA using effectene (Qiagen) or transduction with retroviral supernatants and positive cells were bulk sorted at least twice by FACS for the human integrin expressed.

Antibodies and other materials
Monoclonal antibodies that have been used were anti-human α5 NKL-Sam1 (provided by Dr. Carl Figdor, Nijmegen Centre for Molecular Life Sciences, Nijmegen, the Netherlands), anti-human β1 TS/2/16, clone 18 (Transduction Laboratories), anti-human β3 C17 (provided by Dr. Ellen van der Schoot, Sanquin, Amsterdam, the Netherlands), anti-mouse β3 (clone 2C9.G2, Pharmingen), anti-paxillin (clone 349, BD Transduction Laboratories), anti-RhoA (clone 26C4, Santa Cruz), anti-Syndecan-4 (clone KY/8.2, Pharmingen), anti-vimentin (clone K36, provided by Dr. F. Ramaekers, University of Maastricht, Maastricht, The Netherlands), human integrin α6 (clone 18, Transduction Laboratories), anti-human α5333 C17 (provided by Dr. Ellen van der Schoot, Sanquin, Amsterdam, the Netherlands), anti-human α5333 TS/2/16, clone 18 (Transduction Laboratories), anti-human syndecan-4 (clone KY/8.2, Pharmingen), anti-vimentin (clone K36, provided by Dr. F. Ramaekers, University of Maastricht, Maastricht, The Netherlands), Texas Red-conjugated streptavidin was purchased from Pierce Chemical Co. Human plasma FN and biotinylated-FN were prepared as described previously (Danen et al., 2002). GRGDSP-peptide was generated at the Netherlands Cancer Institute and biotinylated using EZ-link Sulfo-NHS-Biotin (Pierce Chemical Co.).

DOC insolubility assays
Cells were labeled with biotinylated FN as described above and lysed in DOC buffer (1% sodium deoxycholate (DOC), 20 mM Tris-HCl pH 8.5, 2 mM N-ethylmaleimide, 2 mM iodoacetic acid, 2 mM EDTA and 2 mM PMSF). Lysates were washed through a 23 GA needle and DOC-insoluble material was collected by centrifugation at 14,000 rpm for 20 min at 4°C. The pellet was washed once with DOC buffer, resolved in reduced sample buffer and analyzed by SDS-PAGE and Western blotting.

RGD and FN binding assays
Cells were harvested, resuspended in DMEM supplemented with 0.5% BSA and 2 mM MnCl₂, and incubated with 10 μM biotinylated GRGDSP-peptide or 10 μg/ml biotinylated human plasma FN for 1 h at 4°C in the absence or presence of...
increasing concentrations unlabeled FN. The cell pellet was washed in 0.9% NaCl and 2 mM MnCl₂ and subsequently labeled with PE-conjugated human plasma FN for 1h at 4°C. Binding of the RGD-peptide or FN was determined by flow cytometry.

**siRNA transfections**

Cells were plated at 40% confluency and were transfected the following day using DharmaFECT 1 reagent and a final concentration of 100 nM of integrin β1 SMARTpool siRNA (M-040783-00), Syndecan-4 ON-TARGET plus SMARTpool siRNA (L-044221-00), or siCONTROL non-targeting siRNA#2 (D-001210-02) purchased from Dharmacon. After replating, the cells were analyzed at 48 h post-transfection for integrin β1 or Syndecan-4 surface expression, and used for immunofluorescence, RhoA activity- and FN binding-assays.

**RESULTS**

Integrin αβ3 promotes fibroblast-like organization of cell-matrix adhesions, Rho activity, and fibrillogenesis

We have previously shown that β1 integrins promote a fibroblast-like distribution of cell-matrix adhesions and contractile, elongated cell shape that is associated with high levels of RhoA activity, focal adhesion turnover, and FN matrix assembly whereas αvβ3 fails to do so in the absence of β1 (Danen et al., 2002; Danen et al., 2005) (see Figure 1A). There is also evidence that overexpression of αvβ3 can stimulate RhoA activity and cytoskeletal contractility in leukocytes and CHO cells (Butler et al., 2003; Miao et al., 2002). Here, we determined the importance of αvβ3 in these processes using mouse embryonic fibroblasts (MEFs) isolated from Itgb3 KO mice. The levels of GTP-RhoA in β3 null MEFs were similar to those in MEFs isolated from wild type littermates, indicating that RhoA activation does not require αvβ3 (Figure 1B, Supplemental Figure A). Similarly, FN matrix assembly mediated by β3 null MEFs occurred equally efficient as by wild type MEFs (Figure 1C). Ectopic expression of β3 did not further stimulate RhoA activity, whereas increased expression of β1 led to enhanced activation of RhoA (Figure 1B). Increased expression of β1 integrins in β3 null MEFs also led to a more elongated, contractile cytoskeletal organization, whereas the presence or absence of β3 did not affect morphology (Figure 1D). Moreover, suppression of endogenous β1 integrins using Itgb1 specific siRNAs caused a phenotypic switch in wild type MEFs characterized by increased cell spreading, more random distribution of focal adhesions, and formation of cell-cell adhesions (Figure 1E,F). We were unable to completely silence β1 expression in MEFs and the level of suppression reached did not lead to detectable reduction of RhoA-GTP levels (not shown). Nevertheless, the conversion to a more epithelial morphology (strongly resembling that of β1-deficient GE3 cells; Figure 1A) upon β1 silencing was in complete agreement with our previous findings using β1 null cells. Silencing expression of β1 integrins in Itgb3 KO MEFs caused cell rounding but the cells that remained attached resembled GE11 β1 KO cells, growing in islands with extensive cell-

**Figure 1. Integrin β3 is dispensable for RhoA activity and contractility.** (A) Schematic representation of previous results: Itgb1 null cells (GE11) have very low levels of GTP-bound RhoA and FN matrix assembly. Re-expression of β1, but not overexpression of β3 induces highly dynamic cell-matrix adhesions, a mesenchymal cell morphology and restores RhoA activity and FN matrix assembly. Images show GEβ3 and GEβ3 cells stained for paxillin (green) and F-actin (red). (B) Western blot analysis of RhoA activity assay on lysates of Itgb3 KO and WT MEFs overexpressing indicated integrins. Quantification shows relative RhoA activation ± standard deviation (SD) compared to WT MEFs of two independent experiments. (C) Images of assembled FN-biotin on Itgb3 KO and WT MEFs; FN (red) and nucleus (blue). Scale bar, 50 μm. (D) Images of Itgb3 KO and WT MEFs overexpressing indicated integrins stained for paxillin (green), F-actin (red) and nucleus (blue). Arrows indicate long actin fibers. Scale bar, 10 μm. (E) Flow cytometry analysis of integrin β1 surface expression on Itgb3 KO and WT MEFs transfected with integrin β1 or control siRNA. (F) Images of WT and Itgb3 KO MEFs transfected with integrin β1 or control siRNA stained for paxillin (green) and F-actin (red). Two representative examples of integrin β1 siRNA transfected WT MEFs are shown.
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A

B

C

D

E

F

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Figure 2. Integrin α5 is required for efficient RhoA activation and FN fibrillogenesis. (A) Western blot analysis of RhoA activity assay on lysates of EA5 cells expressing indicated integrins. Quantification shows relative RhoA activation ± SD compared to EA5 cells of two independent experiments. (B) Images of EA5 cells expressing indicated constructs stained for paxillin (green), F-actin (red) and nucleus (blue). Scale bar, 10 μm. (C) Western blot analysis of assembled FN-biotin and vimentin (loading control) in DOC insoluble lysates of EA5 cells expressing indicated constructs. The different kDa bands from the protein marker on the Western blot are indicated.

Of the β1 integrins, α5β1 most efficiently supports FN matrix assembly but integrins αβ1, α4β1 and αvβ1 can also mediate cell adhesion to immobilized FN. To determine if α5β1 is the β1 integrin responsible for the typical fibroblast-like characteristics described above, we used differentiated Itga5 KO ES cells (EA5). RhoA-GTP levels were very low in EA5 cells and restoring expression of α5 induced efficient RhoA-GTP loading (Figure 2A, Supplemental Figure B). Expression of an α5vβ chimera, consisting of the α5 extracellular and transmembrane domains and the αv cytoplasmic domain similarly induced RhoA-GTP loading, indicating that α5 specific sequences in the cytoplasmic tail are not required for enhancing RhoA activation (Figure 2A). The very low RhoA-GTP levels in EA5 cells were accompanied by a flat circular cell shape with dispersed focal adhesions. Expression of α5 or α5av induced a reorganization of the cytoskeleton with long F-actin stress fibers connecting peripheral focal adhesions (Figure 2B). Likewise, FN fibrillogenesis was strongly enhanced in the presence of α5 or α5av in EA5 cells (Figure 2C), consistent with previous findings (Sechler et al., 1997; Wu et al., 1993). Together, these results demonstrate that the typical fibroblast-like elongated, contractile morphology that is associated with high levels of Rho-GTP and FN matrix assembly do not require αvβ3 and are primarily stimulated by α5β1.
Figure 3. Ligand-binding to the extracellular domain of β1 is required for RhoA activation and FN fibrillogenesis. (A) Images of GE11 cells expressing indicated β1 subunits stained for paxillin (green), F-actin (red) and nucleus (blue). Scale bar, 10 μm. (B) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated β1 subunits. Quantification shows relative RhoA activation ± SD compared to GEβ1 cells of two independent experiments. (C) Images of assembled FN-biotin on GE11 cells expressing indicated β1 subunits; FN (red) and nucleus (blue). Scale bar, 50 μm. (D) Mean fluorescence analyzed by flow cytometry, demonstrating binding of indicated integrins to different concentrations of soluble FITC-FN.
Figure 4. High affinity mutants of β3 fail to stimulate RhoA activity and FN fibrillogenesis. (A) Images of β1-deficient GE11 cells expressing indicated β3 affinity mutants stained for paxillin (green), F-actin (red) and the nucleus (blue). Scale bar, 10 μm. (B) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated constructs. Quantification shows relative RhoA activation ± SD compared to GEβ1 cells of two independent experiments. (C) Western blot analysis of assembled FN-biotin and vimentin (loading control) in DOC insoluble lysates of GE11 cells expressing indicated constructs.

The extracellular ligand-binding domain of α5β1 specifies fibroblast morphology, RhoA activation, and FN fibrillogenesis

Besides the obvious essential role of the extracellular domain in ligand binding, the integrin transmembrane and cytoplasmic domains regulate ligand affinity and avidity by controlling integrin conformation and clustering (Calderwood, 2004; Li et al., 2003; Luo et al., 2007). The β1 cytoplasmic domain is exchangeable with that of β3 without affecting Rho activation, FN fibrillogenesis or fibroblast morphology when expressed in β1-deficient GE11 cells (Danen et al., 2002). To test if specific amino acid residues in the transmembrane domain of β1 are required for α5β1-mediated support of fibroblast morphology, Rho activity, and FN fibrillogenesis, we generated a chimeric β1e3t+i subunit, consisting of a β1 extracellular domain and β3 transmembrane and cytoplasmic domains. When expressed in β1-deficient GE11 cells (Supplemental Figure C), this chimera promoted focal adhesion distribution and enhanced RhoA activity similar to wild type β1 (Figure 3A,B). Moreover like wild type β1, β1e3+i efficiently supported FN matrix assembly (Figure 3C), arguing against a specific role of the transmembrane and cytoplasmic domains of β1 in these processes. By contrast, expression of a β1[D130A] subunit, containing a mutation in the extracellular I-like domain that abrogates ligand binding (Takada et al., 1992), failed to induce RhoA activation, a mesenchymal morphology, or FN fibrillogenesis (Figure 3, Supplemental Figure C).

These findings indicate that the support of a fibroblast-like, contractile
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morphology associated with Rho activity and FN fibrillogenesis by α5β1 is strictly dependent on ligand binding to its extracellular domain. Other regions of this integrin can be exchanged with corresponding regions of αvβ3 without consequence and surface expression of α5β1 without ligand interaction, which could affect (expression of) other surface receptors, is insufficient to support these processes.

The ability to bind to soluble FN dimers correlates with RhoA activity and FN fibrillogenesis

Many different integrins (including α5β1 and αvβ3) can recognize the RGD motif and mediate cell adhesion to immobilized (stretched) FN. However, we observed that all integrin β subunits that supported a fibroblast-like cytoskeletal organization, Rho activity, and FN fibrillogenesis (β1, β1T329C, and β1[D130A]) efficiently bound to soluble (compact and inactive) FN dimers whereas the other β subunits tested (β3, β1[D130A]) did not (Figure 3D). This suggested that differences in binding to soluble FN between α5β1 and αvβ3 could explain not only the particular efficiency with which α5β1 mediates FN matrix assembly but also the different abilities of these integrins to support a fibroblast-like, contractile cytoskeletal organization and Rho activation.

We used mutants of β3, which, in the context of αIIbβ3, are locked in a low- or high affinity conformation for the RGD sequence in fibrinogen, (Luo et al., 2003; Luo et al., 2004), the integrin recognition sequence that is also present in the central cell-binding domain of FN. Expression of a low affinity β3[T329C; A347C] did not affect the poorly spread GE11 cell morphology (Figure 4A center image; compare with Figure 3A left image). Expression of the high affinity β3[N305T] and β3[V332C; M335C] mutants stimulated cell spreading but distribution of focal adhesions in these cells was similar to those expressing wild type β3 (Figure 4A; compare with Figure 1A right image). In line with these morphological similarities, like wild type αvβ3 the high

![Image](image-url)

Figure 5. Integrin-binding of soluble GRGDSP and FN. (A) Mean fluorescence ± SD demonstrating GRGDSP-biotin binding (10μM) to indicated integrins expressed on GE11 cells analyzed by flow cytometry. (B) Mean fluorescence analyzed by flow cytometry, demonstrating binding of indicated integrins to different concentrations of soluble FITC-FN. Binding to integrin β3 was shown previously in figure 3D. (C) Mean fluorescence demonstrating binding of soluble FN-biotin (10μg/ml) to indicated integrins upon competition with increasing concentrations of unlabeled FN analyzed by flow cytometry.
affinity mutants failed to enhance RhoA-GTP levels or FN fibrillogenesis in the absence of β1 (Figure 4B,C). As expected based on findings in CHO cells (Luo et al., 2003; Luo et al., 2004) the β3[N305T] and β3[V332C; M335C] mutations strongly increased αvβ3-mediated binding of GE11 cells to soluble RGD (Figure 5A). However, even in the presence of the activating divalent cation, manganese, locking αvβ3 in a high affinity state for RGD failed to induce binding to soluble FN (Figure 5B,C). Again, α5β1 efficiently bound soluble FN in these experiments which could be competed with unlabeled FN (Figure 5B,C).

These results demonstrate that while RGD is a common recognition motif, affinity for RGD does not necessarily indicate binding to all RGD-containing ligands. The central cell-binding domain of compact soluble FN dimers appears only available for binding to α5β1 integrins and not to αvβ3. Notably, this indicates that efficient binding to soluble FN dimers might in fact underlie the efficiency with which α5β1 supports a fibroblast-like distribution of focal adhesions, contractile cell shape, and Rho-mediated cytoskeletal contractility that drives FN fibrillogenesis.

Figure 6. High affinity binding to the hypervariable region of the β1 I-like domain controls signaling to FN fibrillogenesis. (A) Mean fluorescence analyzed by flow cytometry, demonstrating binding of integrin β1 and β1-3-1 to different concentrations of soluble FITC-FN. (B) Images of assembled FN-biotin on GE11 cells expressing β1 or β1-3-1; FN (red) and nucleus (blue). Scale bar, 50 μm. (C) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated integrins. Quantification shows relative RhoA activation ± SD compared to GEβ1 cells of two independent experiments. (D) Images of GE11 cells expressing β1 or β1-3-1 stained for paxillin (green) and F-actin (red). Scale bar, 10 μm.
Specificity in the I-like domain controls binding of soluble FN, focal adhesion distribution, Rho-mediated contractility, and FN matrix assembly independent of Syndecan-4

Since ligand-binding is required for α5β1 to support a contractile, fibroblast-like morphology and Rho activity (β1[D130A]; Figure 3) and since its ability to efficiently bind soluble FN dimers appeared to underlie the difference between α5β1 and αvβ3 in this respect (Figure 5), we analyzed a more subtle mutation in the I-like domain that participates in ligand binding. Exchanging the CTSEQNC hypervariable sequence in the I-like domain of β1 with the corresponding region of β3 has been shown to cause a shift in ligand specificity that leads to adhesion to vitronectin, von Willebrand factor and fibrinogen without affecting adhesion to immobilized (stretched) FN (Takagi et al., 1997). We expressed such a β1-3-1 integrin subunit in β1-deficient GE11 cells (Supplemental Figure D). Although adhesion to immobilized FN was similar, cells expressing this chimera displayed strongly reduced binding of soluble FN compared to cells expressing wild type α5β1 (Figure 6A). Importantly, besides causing a marked inhibition of FN matrix assembly, this was accompanied by a strong reduction in RhoA-GTP levels and by a random (epithelial-like) distribution of focal adhesions (Figure 6B,C,D).

Rho-mediated contractility is required for FN-fibrillogenesis (Zhang et al., 1997; Zhong et al., 1998) and RhoA-GTP levels and FN matrix assembly correlated for all integrin constructs tested by us. High RhoA-GTP levels were also associated with soluble FN-binding and fibroblast-like distribution of focal adhesions. We wondered i) if the high levels of RhoA-GTP were up- or downstream of soluble FN binding to α5β1 and the typical fibroblast-like distribution of focal adhesions and ii) if FN fibrillogenesis couples back to Rho activation in a positive feedback loop. To investigate these possibilities, binding of soluble FN, FN fibrillogenesis, and RhoA activity were analyzed in cells expressing α5β1 under conditions where actomyosin contractility was blocked using ROCK or Myosin-II inhibitors. In the presence of these inhibitors, FN-fibrillogenesis was strongly suppressed.
(ROCK) or even completely blocked (myosin-II), but binding of soluble FN and RhoA-GTP levels were not affected (if anything slightly enhanced) (Figure 7). These findings suggest that RhoA activity is downstream of FN-binding and they argue against a positive feedback loop from actomyosin contractility or FN-fibrillogenesis back to the regulation of Rho activity. Furthermore, the fact that the inhibitors ultimately led to disruption of focal adhesions (not shown) combined with the finding that silencing α5β1 in MEFs leads to a more epithelial distribution of focal adhesions (Figure 1F) while in both cases RhoA levels remain high, argues against the possibility that the fibroblast-like cytoskeletal organization acts upstream of RhoA.

It has been reported that the transmembrane proteoglycan Syndecan-4 acts in concert with integrins to mediate FN matrix assembly (Chung and Erickson, 1997) and can modulate the formation of cell-matrix adhesions and the activities of Rho-GTPases, including Rac1 (Bass et al., 2007) and RhoA (Dovas et al., 2006; Saoncella et al., 1999). We investigated if Syndecan-4 played a role in the support of fibroblast-like cytoskeletal organization, Rho activity, or FN fibrillogenesis by α5β1. For this purpose, we silenced Syndecan-4 expression in GEβ3 cells using Syndecan-4 specific siRNAs. A complete knockdown of surface expression of Syndecan-4 was achieved within 48 hrs, whereas Syndecan-4 expression was unaffected in control siRNA transfected GEβ3 cells (Figure 8A). The formation and distribution of cell-matrix adhesions and organization of the actin cytoskeleton remained unaltered in cells without Syndecan-4 (Figure 8B). Similarly, FN matrix assembly and binding of soluble FN by α5β1 was still intact in GEβ1 cells lacking Syndecan-4 (Figure 8C, D). Finally, α5β1-supported RhoA activation was also unaffected by Syndecan-4 silencing (Figure 8E) although for this assay cells had to be expanded to obtain sufficient lysate resulting in incomplete Syndecan-4 downregulation (not shown). Notably, a knockdown of Syndecan-4 expression levels in GEβ3 cells induced a dramatic morphological change, and GEβ3 cells lacking Syndecan-4 were unable to form their typical flattened circular shape but instead formed an irregular cell border containing several cytoskeletal extensions (Figure 8F,G). These experiments indicate that Syndecan-4 can act in concert with αvβ3 to regulate cell-matrix adhesion distribution when α5β1 is absent but Syndecan-4 is not required for cytoskeletal organization, focal adhesion formation or distribution, Rho activity, or FN matrix assembly in the presence of α5β1.

Taken together, our data demonstrate that following the initial drop in RhoA activity during cell adhesion, the ability of α5β1 to bind compact soluble FN dimers drives the typical fibroblast-like distribution of focal adhesions and the accumulation of Rho activity, which, in turn, stimulates FN fibrillogenesis (Figure 9). Interactions of FN with αvβ3 or Syndecan-4 are dispensable for all these processes.

DISCUSSION

FN fibrillogenesis requires integrin-mediated binding of soluble FN dimers and depends on Rho-mediated cytoskeletal contractility to stretch integrin-bound FN molecules exposing cryptic FN-binding sites (Geiger et al., 2001; Mao and Schwarzbauer, 2005; Zhong et al., 1998). The α5β1 integrin mediates FN matrix assembly with particular efficiency although other integrins can substitute for α5β1 to some extent (Wennenber et al., 1996; Wu et al., 1996; Yang and Hynes, 1996). Our current study indicates that α5β1 not only promotes FN matrix assembly, but also stimulates the contractile, fibroblast-like morphology. The ability of α5β1 to efficiently bind compact soluble FN dimers appears to drive the appropriate redistribution of focal adhesions to support this cell shape that is associated with increased RhoA activity, which, in turn, stimulates FN fibrillogenesis. Such dynamic behavior of α5β1 was previously implicated directly in FN-fibrillogenesis (Pankov et al., 2000). Our findings indicate that an analogous process underlies the distribution of focal adhesions leading to the typical contractile cell shape observed in fibroblasts.
Figure 8. Syndecan-4 is not required for α5β1 supported RhoA signaling. (A) Flow cytometry analysis of endogenous Syndecan-4 surface expression on GEβ1 cells transfected with Syndecan-4 or control siRNA. (B) Images of GEβ1 cells transfected with Syndecan-4 or control siRNA stained for paxillin (green), F-actin (red) and the nucleus (blue). (C) Flow cytometry analysis of soluble FITC-FN (10 μg/ml) binding to GEβ1 cells with or without Syndecan-4 knockdown. (D) Images of assembled FN-biotin on GEβ1 transfected with Syndecan-4 or control siRNA; FN (red) and nucleus (blue). Scale bar, 50 μm. (E) Western blot analysis of RhoA activity assay on lysates of Syndecan-4 knockdown and control GEβ1 cells. (F) Flow cytometry analysis of endogenous Syndecan-4 surface expression on GEβ3 cells transfected with Syndecan-4 or control siRNA. (G) Images of GEβ3 cells transfected with Syndecan-4 or control siRNA stained for paxillin (green) and F-actin (red).
Integrins that cannot bind soluble FN stimulate cell spreading and focal contact formation but these adhesions distribute randomly and are connected by short F-actin fibers, leading to an overall non-contractile, flat, epithelial-like cell shape. These findings point to a critical role for α5β1 as an efficient mechano-transducer that couples binding of soluble FN dimers to a cytoskeletal organization that supports the assembly of a FN matrix (Figure 9).

In agreement with our findings, α5β1 has recently been demonstrated to support ROCK-mediated contractility in fibroblasts (Gaggioli et al., 2007; White et al., 2007). However, others have shown that overexpression of αvβ3 can stimulate RhoA activity in leukocytes (Butler et al., 2003) and CHO cells (Miao et al., 2002). Notably, these cells express endogenous β1 integrins making it difficult to compare these studies with our own. In addition, multiple different cell surface receptors can regulate the activity of Rho-GTPases and may contribute to the difference between these studies. Nevertheless, in our previous studies we were unable to observe any stimulation of RhoA activity by αvβ3 in the absence of β1 integrins (Danen et al., 2002; Danen et al., 2005). In our current study we use Itgb3 KO MEFs to demonstrate directly, and to our knowledge for the first time, that αvβ3 is dispensable for RhoA activation. Wild type and β3 null MEFs also display the same fibroblast-like cytoskeletal organization. Moreover, a strong RNAi-mediated reduction in the expression of β1 integrins in MEFs produces a cytoskeletal organization that is very similar to that in β1-deficient cells expressing high levels of αvβ3 (GEβ3). Thus, while expression of β1 induces a morphological switch that resembles an “epithelial-to-mesenchymal transition” in β1 null cells (Danen 2002), silencing β1 in MEFs induces what appears like a “mesenchymal-to-epithelial transition”.

We observe a tight correlation between high RhoA-GTP levels and focal adhesion dynamics, contractile morphology, and FN matrix assembly (Danen 2002, 2005, and this study). Experiments using inhibitors
argue against a positive feedback loop from cytoskeletal organization to RhoA GTP-loading but we do not know at present how the ability of α5β1 to bind soluble FN supports the activity of RhoA. Recently, the guanine nucleotide exchange factors (GEFs) Lsc and LARG were shown to link cell adhesion on FN to RhoA GTP-loading and focal adhesion and stress fiber formation (Dubash et al., 2007). However, the integrins tested in our study that do not bind soluble FN and do not stimulate RhoA activity do in fact efficiently support the formation of focal adhesions and short stress fibers. RhoA-mediated contractility appears to be more important for the distribution of focal adhesions and orientation of stress fibers. If regulation of GEFs and GAPs (GTPase activating proteins) is involved, our experiments using chimeric integrins indicate that obligate interactions of such proteins or their regulators with α5β1-specific residues in the cytoplasmic domains do not underlie α5β1-stimulated RhoA activation. It is possible though that binding of soluble FN to α5β1 induces conformational alterations and/or clustering of the integrin that affects the localization and/or activity of such regulatory proteins.

Syndecan-4 can act in concert with integrins to regulate cytoskeletal organization during cell adhesion to FN through interactions at the HepII domain in FN. Using an siRNA approach we rule out that cross-talk with Syndecan-4 is involved in the fibroblast-like cytoskeletal organization, Rho activity, and FN matrix assembly that is supported by α5β1. Our findings do not seem to support the previously reported role of Syndecan-4 in stimulation of RhoA-mediated processes (Saoncella et al., 1999). However, others have recently shown that during adhesion to FN, Syndecan-4 is required for regulation of Rac1, but not RhoA (Bass et al., 2007). This might also explain why a Syndecan-4 knockdown strongly affected cytoskeletal organization in GEβ3 cells in which Rac1, but not RhoA, is highly active (Danen et al., 2002).

FN serves as a ligand for numerous integrins that bind to the central cell binding domain of FN. Both α5β1 and αvβ3 bind to FN through the RGD sequence in the FN type III9 repeat, whereas only α5β1 also requires an interaction with the synergy site located in the adjacent FN type III9 repeat (Aota et al., 1994; Bowditch et al., 1994; Danen et al., 1995). Although α5β1 and αvβ3 mediate adhesion of GE11 cells to immobilized (stretched) FN with similar efficiency (Danen et al., 2002), only α5β1 efficiently binds compact soluble FN dimers (this report). We find that this marked difference in FN-binding affinity of the two integrins can be explained at least in part by the different hypervariable sequences in the ligand-binding I-like domain of the β subunits. In agreement with Takada and colleagues, we observed that replacing this region of β1 with that of β3 did not affect adhesion to immobilized (stretched) FN. However, we find that this swap in fact leads to a marked reduction in binding soluble FN, implicating the hypervariable sequence in the regulation of FN binding.

It has been demonstrated by others that integrins can be locked in low or high affinity states by disulfide bonds or glycan wedges in the integrin I-like domain. However, our findings demonstrate that binding to FN is an intriguingly complex process: despite the fact that αvβ3 only binds to RGD in FN, when it is locked in a high affinity state for RGD it fails to bind soluble and compact FN dimers. This indicates that the RGD sequence in soluble FN is not exposed for binding to integrins. Conformational changes upon binding of α5β1 (but not αvβ3) to the synergy site in FN might induce the exposure of the RGD sequence. Indeed, the tilt angle of the interdomain between FN type III9 and III10 of FN was shown to determine accessibility to the RGD sequence indicating that integrin-binding to the synergy site may act as a modulator for RGD binding (Altroff et al., 2004).

Of the different FN-binding integrins, only deletion of α5β1 leads to a similar, although less severe phenotype in mice as deletion of FN resulting in mesodermal and/or vascular defects causing embryonic
lethality (Yang et al., 1993). The phenotype of FN[RGE/RGE] mice is identical to that of the α5 knockout (Takahashi et al., 2007) demonstrating the importance of the interaction of α5β1 with the RGD motif in FN. However, FN matrix assembly can still occur in the absence of α5β1 or the FN RGD motif (Takahashi et al., 2007; Yang et al., 1993). In vivo and in vitro, αv integrins can compensate for the absence of α5β1 or RGD, which can involve an interaction of αvβ3 with FN type I repeats (Takahashi et al., 2007; Yang et al., 1999; Yang and Hynes, 1996). On the other hand, differentiated α5-deficient CHO-B2 or ES cells and β1-deficient GD25 and GE11 cells poorly assemble a FN matrix even in the presence of overexpressed αv integrins (Danen et al., 2002; Zhang et al., 1993) and this study). Also, the suppression of FN fibrillogenesis observed in many transformed cells is often associated with loss or inactivation of α5β1 function (Giancotti and Ruoslahti, 1990; Plantefaber and Hynes, 1989). Our findings suggest that even under conditions where compensatory mechanisms involving αv integrins and/or Syndecans support FN matrix assembly in its absence, α5β1 may still be required for the typical contractile fibroblast cell shape. Such a function could explain its crucial role in mesoderm development even when FN-matrices appear intact. The very high efficiency with which α5β1 supports Rho-mediated contractility may also be important during processes such as angiogenesis: activated endothelial cells induce expression of α5β1 and RhoA activity is required for angiogenesis to occur (Hoang et al., 2004; Kim et al., 2000).

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REFERENCES


Fibronectin binding and mesenchymal morphology


Evidence for distinct classes of binding sites for Mn$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$.


Fibronectin binding and mesenchymal morphology


SUPPLEMENTARY FIGURE

**Supplemental Figure.** (A) Flow cytometry of murine integrin β3 expression on Itgb3 KO and WT MEFs. (B) Flow cytometry analysis of human integrin α5 expression on EA5 cells expressing indicated constructs. clg, control immunoglobulin; mAb, monoclonal antibody. (C,D) Flow cytometry of human integrin β1 expression on GE11 cells expressing β1, β1[D130A], β1^*3* or β1-3-1 subunits. MF, mean fluorescence.