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Crosstalk between integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in renal epithelial cells

Tristin D. Abair^{+,#}, Munirathinam Sundaramoorthy⁺, Dong Chen⁺, Jyrki Heino^{##}, Johanna Ivaska⁺⁺, Billy G. Hudson^{+,&}, Charles R. Sanders[&], Ambra Pozzi^{*,+,#}, and Roy Zent^{*,+,#,\$}

* Department of Medicine, Veterans Affairs Hospital, Nashville, TN, 37232 + Department of Medicine (Division of Nephrology), Vanderbilt University, Nashville, TN 37232 # Department of Cancer Biology, Vanderbilt University, Nashville, TN 37232 \$ Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN 37232 & Department of Biochemistry, Vanderbilt University, Nashville, TN 37232 ## Department of Biochemistry and Food Chemistry, University of Turku, Turku, Finland ++ VTT Technical Research Centre of Finland, Medical Biotechnology, Turku, Finland

Abstract

The collagen binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ have profoundly different functions, yet they are often co-expressed in epithelial cells. When both integrins are expressed in the same cell, it has been suggested that $\alpha 1\beta 1$ negatively regulates integrin $\alpha 2\beta 1$ dependent functions. In this study we utilized murine ureteric bud (UB) epithelial cells, which express no functionally detectable levels of endogenous integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, to determine the mechanism whereby this regulation occurs. We demonstrate that UB cells expressing integrin $\alpha 2\beta 1$, but not $\alpha 1\beta 1$ adhere, migrate and proliferate on collagen I as well as form cellular cords in 3D collagen I gels. Substitution of the transmembrane domain of the integrin $\alpha 2$ subunit with that of $\alpha 1$ results in decreased cell adhesion, migration and cord formation. In contrast, substitution of the integrin $\alpha 2$ cytoplasmic tail with that of $\alpha 1$, decreases cell migration and cord formation, but increases proliferation. When integrin $\alpha 1$ and $\alpha 2$ subunits are co-expressed in UB cells, the $\alpha 1$ subunit negatively regulates integrin $\alpha 2\beta 1$ -dependent cord formation, adhesion and migration and this inhibition requires expression of both $\alpha 1$ and $\alpha 2$ tails. Thus, we provide evidence that the transmembrane and cytoplasmic domains of the $\alpha 2$ integrin subunit, as well as the $\alpha 1$ integrin subunit, regulate integrin $\alpha 2\beta 1$ cell function.

Introduction

Integrins are heterodimeric transmembrane receptors for extracellular matrix components that consist of non-covalently linked α and β subunits [1]. Eighteen α and 8 β subunits are identified in mammals [1] and they consist of a large extracellular domain, a transmembrane domain and a short cytoplasmic tail (with the exception of the $\beta 4$ integrin subunit). The extracellular domain confers ligand specificity and is required for ligand interactions. Following ligand binding, the recruitment of intracellular signaling molecules and activation of downstream signaling pathways is coordinated primarily by the cytoplasmic tail [2]. The role of the transmembrane domain in mediating integrin signaling is poorly defined.

Address correspondence to: Roy Zent, Room C3210 Medical Center North, Vanderbilt University, Nashville, Tennessee 37232, USA. Tel. 615-322-4632, Fax. 615-322-4690, E-mail: roy.zent@vanderbilt.edu.

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The two major collagen-binding receptors, integrins $\alpha1\beta1$ and $\alpha2\beta1$ [1], are structurally similar but exhibit significant differences in affinity for collagens. Integrin $\alpha1\beta1$ displays a higher affinity for collagen IV relative to collagen I [3], while integrin $\alpha2\beta1$ preferentially binds collagen I [4,5]. These integrins activate distinct signaling pathways upon binding to collagens. Integrin $\alpha1$ -null fibroblasts show decreased Shc/Grb2/ERK activation and consequent decreased cell proliferation on collagen matrices [6,7]. Integrin $\alpha1$ -null endothelial cells show increased matrix metalloproteinase production and impaired proliferation [8,9] and integrin $\alpha1$ -null mesangial cells show increased basal levels of activated EGF receptor and GTP-Rac1 with consequent increased reactive oxygen species production and collagen synthesis [10]. Thus, integrin $\alpha1\beta1$ is pro-mitogenic and activates ERK, but negatively regulates metalloproteinase synthesis, Rac activation and collagen production. Integrin $\alpha2\beta1$ activates PI3K [11,12], p38 MAPK [13–16] and ERK [15,16] resulting in increased cell migration and proliferation [14,15] as well as collagen and metalloproteinase expression [16–18]. Moreover, activation of integrin $\alpha2\beta1$ by either collagen I or collagen IV leads to increased reactive oxygen species production [19] as well as RhoA activation [20] and deletion of the integrin $\alpha2$ subunit results in increased endothelial cell proliferation and angiogenesis in vivo [21–23]. Thus although both integrin $\alpha1\beta1$ and $\alpha2\beta1$ can regulate cell migration and proliferation they have differential effects on metalloproteinase expression.

Tubule formation in vitro requires complex interactions between cells and the extracellular matrix. Madin-Darby canine kidney (MDCK) cells require integrin $\alpha2\beta1$ for tubulogenesis in collagen I gels, whereas the role of integrin $\alpha1\beta1$ is unknown [24]. Mouse inner medullary collecting duct (IMCD) cells form tubules in collagen I gels in an integrin $\alpha2\beta1$ - and $\alpha1\beta1$ -dependent manner [25]. Ureteric bud cells (UB) derived from the ureteric bud at E11.5, cannot undergo tubulogenesis in collagen I gels as they express no functionally detectable levels of integrins $\alpha1\beta1$ or $\alpha2\beta1$ [25]. However these cells are able to form tubules in collagen I/Matrigel gels, thus making them an excellent system to determine the unique role(s) and possible cross-talk between integrins $\alpha1\beta1$ and $\alpha2\beta1$ in tubulogenesis.

In this study we demonstrate that UB cells expressing integrin $\alpha2\beta1$, but not $\alpha1\beta1$, can form cellular cords but not tubules with lumens in collagen I gels. Utilizing chimeras of these two integrin subunits, we show that replacing the $\alpha2$ transmembrane or cytoplasmic domains with those of $\alpha1$ resulted in altered cell function and co-expression of both integrin $\alpha1$ and $\alpha2$ subunits by UB cells resulted in decreased $\alpha2\beta1$ -dependent cord formation, adhesion and migration. Thus, both the transmembrane and cytoplasmic domains confer specificity for normal integrin $\alpha2\beta1$ -mediated cell function and integrin $\alpha1\beta1$ regulates $\alpha2\beta1$ -dependent cell behavior.

Methods

Generation of integrin constructs

The human full length integrin $\alpha2$ and the chimeric construct $\alpha2\alpha2\alpha1$ ($\alpha2$ extracellular, $\alpha2$ transmembrane and $\alpha1$ cytoplasmic tail) subcloned into the pAWneo2 eukaryotic expression vector were generated as described previously [16,18]. The $\alpha2\alpha1\alpha2$ ($\alpha2$ extracellular, $\alpha1$ transmembrane and $\alpha2$ cytoplasmic tail) chimeric integrin subunit was prepared by introducing two silent point mutations, which introduced new restriction enzyme recognition sites in the $\alpha2$ cDNA (Bcl I at nucleotide 3400 and HindIII at nucleotide 3511). This was performed utilizing the Altered sites II in vitro mutagenesis system (Promega). The $\alpha1$ transmembrane sequence was obtained by annealing two synthetic 113 basepair oligos corresponding to the human $\alpha1$ transmembrane domain flanked with Bcl I and Hind III sites. The synthetic fragment was digested with these enzymes and ligated to the cleaved $\alpha2$ cDNA.

The LZRS-GFP vector, modified from the original LZRS vector [26] to allow bicistronic expression of the protein of interest and green fluorescent protein (GFP), was a generous gift from Dr. A. Reynolds (Vanderbilt University)[27]. This vector was used to generate stable UB cell populations expressing full-length human integrin $\alpha 1$ subunit. Full length human integrin $\alpha 1$ cDNA in the pLEN vector was a generous gift from Dr. E. Marcantonio (Columbia University, New York, NY). The $\alpha 1$ subunit truncation mutant lacking the integrin tail ($\alpha 1\Delta\text{cyt}$) was generated by PCR, utilizing the mature full length $\alpha 1$ as a template. The sense primer was 5'-CGGTACCACCATGTTCAATGTTGATGTGAAAACTC-3', containing a Kpn I restriction site and an ATG start codon and the anti-sense primer was 5'-TAATGGTACCTCATCACCACAGTGCTAAAATGAGCAG-3'. The PCR product and the full length $\alpha 1$ cDNA was subcloned into the LZRS-GFP vector.

Cell culture and generation of cell populations

The mouse ureteric bud (UB) cell line was obtained from the microdissected UB of a day-11.5 mouse embryo transgenic for simian virus 40 (SV40) large T antigen [28]. These cells were cultured in minimal essential medium (MEM) (Mediatech) supplemented with 10% FBS as described previously [29]. UB cells were transfected with empty vector, full-length $\alpha 2$ or the $\alpha 1/\alpha 2$ chimeras using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Selection was started 48 hours after transfection by adding Geneticin (Invitrogen) at 1 mg/ml to the medium. UB cells that expressed integrin $\alpha 1$ were established as following. The retroviral LZRS vector alone or containing the human $\alpha 1$ cDNA were transfected into the packaging cell line Phoenix 293 using FuGENE 6 transfection reagent (Roche Diagnostics). Virus contained in the supernatant was used to infect UB cells every other day for 2 weeks. Stable cell populations of UB cells expressing equal levels of integrin subunits as specified by the extracellular domains were selected by FACStar Plus cell sorter (BD Biosciences) using antibodies to the extracellular domain of human $\alpha 1$ or $\alpha 2$, respectively (Chemicon International). Polyclonal cell populations were used for experiments.

Three-dimensional cell culture

Tubulogenesis assays of UB cells was performed in 3-D collagen gels composed of rat tail collagen I (Becton Dickinson) and Dulbecco's minimal essential media containing 20 mM HEPES (pH 7.2) as previously described [25]. Briefly, 1.5×10^3 cells were suspended in 100 μl of collagen I gel and plated onto 96 well. After 1 hour at 37°C an equal volume of DMEM medium supplemented 10% FBS was added to the gels. The cells were allowed to grow for 9 days at which time the gels were stained with rhodamine phalloiden as described below. In addition phase-contrast photomicrographs were taken using a Nikon Diaphot TMD inverted microscope and the number of branches found in the branching structures (defined as more than 1 branch) were counted in five random high power fields. Three independent experiments were performed in triplicate.

Cell adhesion

Ninety-six-well plates were coated with different concentrations of collagen I in PBS for 1 hour at 37°C. Plates were then incubated with 1% BSA in PBS for 1 hour to block non-specific adhesion. Cells (1×10^5 in 100 μl serum free medium DMEM containing 0.1% BSA) were added to the wells and incubated for 1 hour at 37°C. In some experiments, cells were pre-incubated with 5 μM LY294002 (a PI3K inhibitor), 50 μM PD98059 (a MEK inhibitor), and 10 μM PD169316 (a p38 inhibitor) (all from Calbiochem) for 30 minutes prior to the assay. These concentrations were utilized as we have verified they are not toxic to UB cells [30] and are within the accepted ranges where these inhibitors are known to be specific and effective. Non-adherent cells were removed by washing the wells with PBS. Cells were then fixed with

4% paraformaldehyde, stained with 1% crystal violet, solubilized in 2% SDS, and the O.D. of the cell lysates were read at 570 nm. Cell adhesion to 1% BSA-coated wells was used as background and the O.D. values were subtracted from those obtained on collagen I. Four independent experiments were performed in triplicate.

Cell migration

Cell migration was assayed in transwells consisting of polyvinylpyrrolidone-free polycarbonate filters with 8- μ m pores (Corning). The bottom of the filters were coated with 2.5 μ g/ml collagen I in PBS overnight at 4°C and subsequently incubated with 1% BSA in PBS for 1 hour at 37°C to inhibit non-specific migration. 1×10^5 cells in 100 μ l serum-free medium containing 0.1% BSA were added to the upper wells and cells were allowed to migrate towards the bottom wells for 4 hours at 37°C. Cells on the top of the filters were removed by wiping and the filters were fixed in 4% formaldehyde in PBS. Migrating cells were stained with 1% crystal violet, and five randomly chosen fields counted at 200x magnification. Four independent experiments were performed. Migration was also performed with or without LY294002, PD98059, and PD169316 at the concentrations indicated above in four independent experiments.

Cell Proliferation

UB cell proliferation was evaluated as described previously [25]. Briefly, UB cells (5×10^3) were embedded in 3D collagen I gels (100 μ l final volume) in 96-well plates as described above and incubated in 100 μ l of media supplemented with 10% FCS. After 48 hours, cells were pulsed for an additional 24 hours with [3 H] thymidine (1 μ Ci/well). The gels were removed from the plates and dialyzed against PBS for 24 hours to remove free [3 H]thymidine. The gels were lysed in 100 μ l 10% SDS and measured with a beta counter. Four independent experiments were performed in triplicate. Proliferation was also performed with or without LY294002, PD98059, and PD169316.

Immunofluorescence

To examine cell morphology, serum-starved UB cells were plated in serum-free medium onto chamber slides coated with 10 μ g/ml collagen I. After 1 h, the cells were fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized in 0.1% Triton X-100 in PBS for 10 min. Cells were subsequently blocked with 3% BSA for 1 h. Some slides were incubated with rhodamine-phalloidin (1:1000; Molecular Probes) and DAPI for 1 h. Other slides were incubated with an anti-phosphotyrosine Ab (PY99) (Santa Cruz Biotechnology) at a 1:250 dilution followed by the appropriate fluorescein isothiocyanate-conjugated secondary antibody (Calbiochem) and DAPI. Chamber slides were mounted with ProLong Antifade kit (Molecular Probes), and cells were imaged on a Zeiss Axiophot microscope and an RT Slider Spot digital camera.

To examine cell morphology of UB cells grown in 3D collagen I gels (1500 cells/gel), the gels were fixed as described above and incubated with rhodamine-phalloidin (1:1000; Molecular Probes) in PBS for 3 h and then washed in PBS for 12 h. All the samples were optically sectioned using a Zeiss LSM510 META confocal laser scanning microscope.

Western blot analysis

Serum starved UB cells were plated in serum free medium on collagen I (20 μ g/ml) for 0, 30, and 60 minutes. Cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail, 1 mM Na_3VO_4 , and 1 mM NaF) for 20 minutes. Lysates were clarified by centrifugation at 12,000 g for 20 minutes at 4°C. Twenty μ g of total cell lysates were run onto a 10% SDS gel and subsequently transferred to nitrocellulose membranes.

Membranes were incubated with anti-phospho ERK (antibody #4376, 1:1000 dilution), anti-phospho p38 MAPK (#9215, 1:1000 dilution), anti-phospho Akt, (antibody #4058, 1:1000 dilution), anti-ERK, (antibody #9102, 1:1000 dilution), anti-p38 MAPK (antibody #9218, 1:1000 dilution), or anti-Akt antibodies (antibody #4685, 1:1000 dilution) (all from Cell Signaling and all used at 1:1000) followed by the appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were identified using enhanced chemiluminescence. Phosphorylated and total kinase bands were quantified by densitometry analysis and the levels of phosphorylated signal was expressed as phosphorylated kinase/total kinase ratio. Values were expressed as the mean $\pm$ SD of three experiments.

Statistical analysis

The Student's t test was used for comparisons between two groups, and analysis of variance using Sigma-Stat software for statistical differences between multiple groups. $p < 0.05$ was considered statistically significant.

Results

Integrin $\alpha 2\beta 1$ but not $\alpha 1\beta 1$ mediates UB cell interactions with collagen I

Although UB cells can form multi-branched tubules in collagen I/matrigel gels [25], they cannot undergo tubulogenesis in pure collagen I gels due to their low expression of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ [25]. Based on strategies developed in previous studies [12–16,25,31], we generated UB cell populations expressing either integrin $\alpha 1$ ($\alpha 1$ -UB) or $\alpha 2$ ($\alpha 2$ -UB) subunits by flow cytometry (data not shown). $\alpha 2$ -UB cells adhered significantly better than parental- or $\alpha 1$ -UB cells on collagen I (Figure 1A). Integrin $\alpha 1\beta 1$ was functional, as $\alpha 1$ -UB cells adhered well to collagen IV, the major integrin $\alpha 1\beta 1$ ligand (data not shown). Consistent with the adhesion results, the few parental- or $\alpha 1$ -UB cells that did adhere to collagen I, did not spread and these cells also did not migrate significantly, while the $\alpha 2$ -UB cells both spread and migrated on this matrix component (Figures 1B, 1C)).

When parental UB cells were placed in collagen I gels they demonstrated virtually no proliferative ability, whereas the $\alpha 1$ - and $\alpha 2$ -UB cells showed a two and three fold increase in proliferation respectively when compared to parental cells (Figure 1D). Tubulogenesis assays in 3D collagen I gels were performed over a 9 day period after which the cells were stained with rhodamine phalloidin and subjected to confocal microscopy. As shown in Figure 1E, parental UB cells remained in a ball with little evidence of growth; the $\alpha 1$ -UB cells proliferated more but still formed balls and the $\alpha 2$ -UB formed cellular cords with poorly defined lumens. Thus, integrin $\alpha 2\beta 1$, but not $\alpha 1\beta 1$, supports UB cell adhesion, migration, proliferation, and cord formation on collagen I.

The transmembrane and cytoplasmic domains of integrins $\alpha 1$ and $\alpha 2$ differentially mediate UB cell function and signaling

The differences in integrin $\alpha 1\beta 1$ - and $\alpha 2\beta 1$ -mediated cellular events on collagen I are related for the most part to their discrepancy in affinity for type I collagen fibrils [4]. Thus, to determine differences in signaling between the integrin $\alpha 1$ and $\alpha 2$ cytoplasmic and transmembrane domains, chimeric integrins were made where the extracellular domain remained as $\alpha 2$ and the transmembrane or cytoplasmic domains were replaced by the corresponding domains of the $\alpha 1$ subunit ($\alpha 2\alpha 1$ and $\alpha 2\alpha 2\alpha 1$, respectively) (Figure 2A). Similar expression of these mutants was verified by flow cytometry utilizing an antibody specific for the human $\alpha 2$ extracellular domain (Figure 2B). The $\alpha 2$ - and $\alpha 2\alpha 2\alpha 1$ -UB cells adhered equally well to collagen I, while the $\alpha 2\alpha 1\alpha 2$ -UB cells adhered 50% less (Figure 2C). Despite their ability to adhere, both $\alpha 2\alpha 2\alpha 1$ - and $\alpha 2\alpha 1\alpha 2$ -UB cells spread poorly on collagen I (Figure 2D) and they both migrated approximately 50% less than the $\alpha 2$ -UB cells (Figure 2E). All three cell lines proliferated

significantly more than parental UB cells in 3-D collagen I gels (Figure 2F) with the $\alpha 2\alpha 2\alpha 1$ -UB cells showing the greatest increase.

When placed in collagen I gels, $\alpha 2\alpha 2\alpha 1$ -UB cells did not form cellular cords (Figure 2B) and $\alpha 2\alpha 1\alpha 2$ -UB cells formed fewer and smaller cords than those formed by $\alpha 2$ -UB cells (Figure 2B). Thus, the $\alpha 2$ transmembrane domain plays a specific role in mediating integrin $\alpha 2\beta 1$ -dependent cell adhesion, migration and cord formation, while the cytoplasmic tail regulates cell migration, proliferation and cord formation.

To examine the role of the integrin cytoplasmic and transmembrane domains in collagen I-mediated signaling, we initially performed staining with an anti-phosphotyrosine antibody on cells plated on collagen I for an hour. As shown in Figure 3A, no cell membrane tyrosine phosphorylation was evident in the UB cells, while staining with strong cell membrane localization - consistent with both small and large focal adhesions - was seen in the $\alpha 2$ -UB cells (Figure 3A). Significantly less staining was evident only in restricted areas on the cell membranes in the $\alpha 2\alpha 2\alpha 1$ -UB, and virtually no membrane staining was present in the $\alpha 2\alpha 1\alpha 2$ -UB cells. To further investigate differences in signaling mediated by these integrins, canonical signaling molecules known to be activated by extracellular matrix, were investigated utilizing replating assay on collagen I [30, 32]. As shown in Figure 3B, compared to UB cells, both $\alpha 2$ - and $\alpha 2\alpha 1\alpha 2$ -UB cells showed increased basal ERK activation, although it was more profound in the $\alpha 2$ -UB cells. No basal ERK activation was evident in $\alpha 2\alpha 2\alpha 1$ -expressing cells, although this kinase became highly activated in a time dependent manner following adhesion to collagen I. A basal level of p38 MAPK phosphorylation which decreased over time following adhesion to collagen was present in UB cells. p38 MAPK activation was greater in $\alpha 2$ - than UB-cells at all time points and $\alpha 2\alpha 1\alpha 2$ -UB cell-induced p38 MAPK phosphorylation was similar in pattern, but less intense than that seen in the $\alpha 2$ -UB cells. In the $\alpha 2\alpha 2\alpha 1$ cells basal p38 MAPK phosphorylation was similar to UB cells; however after adhesion to collagen I its activation was not altered. Expression of both full-length $\alpha 2$ and the $\alpha 2$ chimeras resulted in increased Akt phosphorylation, a downstream effector of PI3K, following adhesion to collagen I. This response was similar in the $\alpha 2$ - and $\alpha 2\alpha 2\alpha 1$ -UB cells, but slightly decreased in the $\alpha 2\alpha 1\alpha 2$ -UB cells 30 minutes after adhesion. Thus signals transduced by $\alpha 2\alpha 1\alpha 2$ -UB cells was similar in pattern, but decreased in intensity than the $\alpha 2$ -UB cells, while the both the pattern and intensity of the $\alpha 2\alpha 2\alpha 1$ -UB cells was distinct from the $\alpha 2$ -UB cells.

Specific kinase inhibitors were used to determine the role of ERK, p38 MAPK and PI3K signaling pathways in UB cell function. Cell adhesion in all the cell populations was unaffected by PD98059 (a MEK inhibitor), PD169316 (a p38 MAPK inhibitor), and LY294002 (a PI3K inhibitor) (data not shown). Although ERK activation was increased in full length $\alpha 2$ - or chimera-expressing cells, this kinase played a minor role in cell migration (Figure 3C). In contrast, inhibiting ERK significantly decreased cell proliferation in all the cell populations with the greatest effect seen in $\alpha 2\alpha 2\alpha 1$ -UB cells (Figure 3D). Inhibiting p38 MAPK significantly decreased cell migration and proliferation, and this effect was independent of the cell population analyzed (Figures 3C and 3D). In contrast, inhibition of PI3K did not alter cell migration (Figure 3C), but it significantly decreased cell proliferation in all the cell populations (Figure 3D). Thus integrin-dependent cell migration of all the UB cells is mediated by p38 MAPK and to a lesser extent by ERK pathways, while cell proliferation is mediated by ERK, p38 MAPK and PI3K pathways.

Integrin $\alpha 1\beta 1$ negatively regulates integrin $\alpha 2\beta 1$ -dependent cell functions

To determine whether integrin $\alpha 1\beta 1$ regulates $\alpha 2\beta 1$ -mediated functions when expressed in the same cells, the full length integrin $\alpha 1$ subunit as well as an integrin $\alpha 1$ subunit lacking the cytoplasmic tail (referred to as $\alpha 1\Delta\text{cyt}$) were transfected into $\alpha 2$ -UB cells in order to generate $\alpha 2+\alpha 1$ -UB and $\alpha 2+\alpha 1\Delta\text{cyt}$ -UB respectively (Figure 4A). Cells expressing comparable levels

of the integrin $\alpha 1$ subunits were obtained by FACS sorting (Figure 4A). Expression of the integrin $\alpha 1$ subunits did not change the expression levels of the $\alpha 2$ subunit (data not shown). $\alpha 2 + \alpha 1$ -UB cells adhered, spread and migrated approximately 50% less than the $\alpha 2$ -UB cells (Figure 4B-D). Proliferation in 3-D collagen I gels was significantly increased in cells expressing both integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Figure 4E). When placed in 3D collagen I gels, $\alpha 2 + \alpha 1$ -UB cells failed to form multicellular cords but rather remained as single cells (Figure 4F). In contrast to the $\alpha 2 + \alpha 1$ -UB cells, the $\alpha 2 + \alpha 1\Delta\text{cyt}$ -UB cells adhered, spread, migrated, proliferated and formed cellular cords indistinguishably from $\alpha 2$ -UB cells (Figure 4F).

When anti-phosphotyrosine staining was performed, no membrane-localized staining was evident in the $\alpha 2 + \alpha 1$ -UB cells (Figure 5A), while the $\alpha 2 + \alpha 1\Delta\text{cyt}$ -UB cells showed similar staining to that observed in the $\alpha 2$ -UB cells. In addition, $\alpha 2 + \alpha 1$ -UB cells also showed significantly less ERK, p38 MAPK and Akt activation than $\alpha 2$ -UB cells following replating on collagen I (Figure 5B). No differences in the activation of these three kinases were observed between $\alpha 2$ - and $\alpha 2 + \alpha 1\Delta\text{cyt}$ -UB cells (Figure 5B). Expression of the integrin $\alpha 1$ subunit alone into UB cells does not alter collagen I dependent activation of any of these pathways (data not shown). This data supports a role for the cytoplasmic domain of the integrin $\alpha 1$ subunit in negatively regulating integrin $\alpha 2\beta 1$ -mediated cell adhesion, migration, cord formation and signaling.

We next determined whether the cytoplasmic tail of the integrin $\alpha 2$ subunit is required for the effect exerted by integrin $\alpha 1$ subunit. To do this, we expressed the full length integrin $\alpha 1$ subunit into both $\alpha 2\alpha 1$ - or $\alpha 2\alpha 1\Delta\text{cyt}$ -UB cells and the activation state of ERK, p38 MAPK and Akt was analyzed following replating. No differences in the levels of activated ERK, p38 MAPK or pAKT were observed in $\alpha 2\alpha 1$ -UB cells either expressing or not expressing the $\alpha 1$ subunit (Figure 5C). On the other hand, the basal levels as well as collagen-induced activation of ERK and p38 MAPK were significantly decreased in $\alpha 2\alpha 1\Delta\text{cyt}$ -UB cells expressing the $\alpha 1$ subunit (Figure 5C). Moreover, expression of the $\alpha 1$ subunit led to increased basal AKT activation in the $\alpha 2\alpha 1\Delta\text{cyt}$ -UB cells (Figure 5C). Thus, the $\alpha 2$ cytoplasmic tail is required for the crosstalk between integrins $\alpha 2\beta 1$ and $\alpha 1\beta 1$.

Discussion

The role of the transmembrane domain of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins, as well as the mechanisms whereby they might regulate each other and/or activate downstream signaling pathways in response to collagen I are unexplored. In this study we expressed integrin $\alpha 1$ and $\alpha 2$ subunits as well as chimeras of these subunits into UB epithelial cells that fail to undergo tubulogenesis in collagen I gels due to low expression of endogenous integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$, to demonstrate that: **1)** expression of the collagen receptors integrin $\alpha 1\beta 1$ or $\alpha 2\beta 1$ is not sufficient for UB cells to undergo tubule formation with lumens in 3-dimensional collagen I gels; **2)** integrin $\alpha 2\beta 1$, but not $\alpha 1\beta 1$, is sufficient for UB cells to undergo cord formation, adhesion, migration, and proliferation on collagen I; **3)** substituting the $\alpha 2$ subunit transmembrane domain with that of $\alpha 1$ leads to decreased cell adhesion, migration and cord formation; while replacing the $\alpha 2$ cytoplasmic tail with that of $\alpha 1$ results in migration and cord formation abnormalities; **4)** relative to $\alpha 2$, the cytoplasmic domain of the integrin $\alpha 1$ subunit promotes cell proliferation; **5)** expressing a full-length $\alpha 1$ integrin subunit in $\alpha 2\beta 1$ -expressing UB cells decreases integrin $\alpha 2\beta 1$ -dependent cord formation, adhesion, and migration but increases proliferation; and **6)** the integrin $\alpha 1\beta$ -mediated effects on integrin $\alpha 2\beta 1$ -dependent signaling require the expression of both α and α subunit cytoplasmic tails. Thus, both the transmembrane and cytoplasmic domains impart specificity to integrin $\alpha 2\beta 1$ signaling and function and integrin $\alpha 1\beta 1$ regulation of $\alpha 2\beta 1$ requires the presence of both the integrin $\alpha 1$ and $\alpha 2$ tails.

Transducing the integrin $\alpha 2$, but not the $\alpha 1$, subunit into UB cells is sufficient to promote adhesion and migration on collagen I. These results are consistent with *in vitro* studies indicating greatly increased affinity of the extracellular domain of integrin $\alpha 2\beta 1$ for collagen I relative to integrin $\alpha 1\beta 1$ [3,4] and complement the finding that antisense mRNA and blocking antibodies directed against integrin $\alpha 2\beta 1$ inhibits MDCK and IMCD tubulogenesis in 3-D collagen gels [25]. Although, the $\alpha 2$ -UB cells form cellular cords, they are shorter, less branched and do not form well defined lumens as found in tubules formed by UB cells grown in collagen I/matrigel gels [25,30]. This result implies that that signals transduced from collagen I by integrin $\alpha 2\beta 1$ are not sufficient to fully compensate for integrin $\alpha 3\beta 1$ and $\alpha 6$ signaling mediated by matrigel when UB cells are grown in collagen I/matrigel gels.

Little information is available on the role of the transmembrane domains of $\alpha\beta$ integrins in modulating integrin function; however destabilizing $\alpha IIb\beta 3$ transmembrane domain heterodimers is sufficient to activate the integrin [33] and specific residues in the transmembrane domain of $\alpha IIb\beta 3$ play critical roles in integrin activation [33–37]. Our study is the first to show that an integrin α transmembrane domain plays a role in regulating $\beta 1$ -dependent cell function and signaling and it suggests there are specific structural determinants within the $\alpha 2$ subunit transmembrane domain required for normal $\alpha 2\beta 1$ integrin function. It is highly unlikely that the $\alpha 1$ transmembrane domain substitution is disrupting the overall structure of the $\alpha\beta$ heterodimer, for the following reasons. Most importantly, the $\alpha 2\alpha 1\alpha\beta 1$ chimeric integrin is expressed on the cell surface. This means that $\alpha 2\alpha 1\alpha 2$ and $\beta 1$ are able to associate by a specific tight noncovalent binding of the $\alpha 2$ β -propeller domain to the $\beta 1$ I-like domain. Without the proper dimer formation in the endoplasmic reticulum, the integrins cannot be transported to the cell surface [38]. Furthermore, integrin $\alpha 2\alpha 1\alpha 2\beta 1$ recognized collagen I, which indicates that the ligand binding $\alpha 2$ I domain is correctly folded. The $\alpha 2$ I domain can either be in the “closed” (low affinity) or in the “open” high affinity conformation [39,40], as intermediate conformations are not probable [41].

Conformational changes of integrins are primarily regulated by “inside-out” signaling, e.g. by protein binding to cytoplasmic domains [42], however $\alpha 2\beta 1$ integrins can also form receptor clusters that increase the avidity of cell adhesion to ligands [43]. Homo- and heteromeric interactions of the transmembrane domains participate in the regulation of cluster formation and may account for the decreased adhesion and signaling found in integrin $\alpha 2\alpha 1\alpha 2\beta 1$. The hetero- and homo-dimerization of integrin α and β transmembrane domains are thought to be regulated by the canonical GxxxG dimerization motifs (21, 23–30). Although there is a high level of structural similarity, the 50% difference in amino acid sequence between the $\alpha 1$ and the $\alpha 2$ transmembrane domains has obvious implications for cell function. Specifically, the sequences of the GxxxG motifs of the $\alpha 1$ (GVIGSIIAG) and $\alpha 2$ (WVILLSAFAG) transmembrane domains are distinct. Although both these amino acid sequences are predicted to form helices, they likely pack differently with $\beta 1$, resulting in alterations in subunit oligomerization and change in function and signaling of the $\alpha 2\alpha 1\alpha 2\beta 1$ chimeric integrin.

When the integrin $\alpha 2$ tail was swapped with the $\alpha 1$ cytoplasmic tail, the $\alpha 2\alpha 1$ -UB cells changed their phenotype and become more spindly, migrated and spread less, were unable to undergo tubulogenesis and formed less and smaller focal adhesions, suggesting functional difference between the integrin $\alpha 2$ and $\alpha 1$ cytoplasmic tails. The altered cell morphology might reflect that integrin $\alpha 1\beta 1$ is primarily expressed in cells of mesenchymal origin, while integrin $\alpha 2\beta 1$ is commonly found in epithelial cells. In support of this hypothesis, poorly differentiated fibroblastoid, spindle-shaped cell mammary carcinoma cells that expressed high levels of the integrin $\alpha 1$, but no detectable $\alpha 2$ subunit, underwent a transition to an epitheloid, polygonal-shaped phenotype when transfected with the full-length integrin $\alpha 2$ subunit [31]. Expression of either the integrin $\alpha 2$ or $\alpha 1$ tail promoted cell proliferation, especially in the $\alpha 2\alpha 1$ -UB cells. Integrin $\alpha 1\beta 1$ stimulates cell proliferation in an ERK-dependent manner [6,7] and the principal

difference in signaling between cells that express integrins with either the $\alpha 1$ or $\alpha 2$ tail is the large increase in ERK signaling in cells expressing the $\alpha 1$ tails (Figures 3 and 5). Furthermore, the MEK inhibitor had a great effect on $\alpha 2\alpha 2\alpha 1$ -UB cell proliferation, suggesting ERK is the predominant pathway whereby the integrin $\alpha 1$ tail induces cell proliferation.

Our data demonstrated that integrin $\alpha 1\beta 1$ negatively regulates integrin $\alpha 2\beta 1$ -mediated cell function and signaling and both the $\alpha 1$ and $\alpha 2$ cytoplasmic tails are required for this crosstalk to occur. Although the mechanisms whereby this regulation happens is not presently known, our finding is consistent with the fact that these two collagen binding receptors differentially activate integrin-dependent signaling pathways and display opposing effects with respect to modulating collagen, metalloproteinase and ROS production [8,10,17,18,44]. In addition, integrin $\alpha 1\beta 1$ is pro-angiogenic, while integrin $\alpha 2\beta 1$ is anti-angiogenic [8,9,21,22]. One possible mechanism whereby the integrins regulate each other is by transdominant suppression, which is well described for a number of integrins [45–47]. If this is the mechanism, our results suggest the transdominant effect occurs due to either direct or indirect interactions between the integrin $\alpha 1$ and $\alpha 2$ subunit tails. Another possibility is that following ligand activation, the integrin $\alpha 1\beta 1$ - and $\alpha 2\beta 1$ -dependent pathways are transduced independently resulting in differences in cell function. This possibility might be especially relevant to activation of ERK in cell proliferation, as integrin signaling is well known to modulate this pathway following activation of growth factor receptors [48,49].

In conclusion, we demonstrate that integrin $\alpha 2\beta 1$ expression is sufficient for UB cells to adhere, migrate, spread, proliferate and form cellular cords in a collagen I matrix. We also show that the transmembrane domain of the $\alpha 2$ subunit has specific structural determinants required for normal $\alpha 2\beta 1$ integrin function and integrin $\alpha 1\beta 1$ modulates $\alpha 2\beta 1$ -mediated signaling and this cross-talk requires expression of both integrin $\alpha 1$ and $\alpha 2$ tails. Thus, the specificity of signaling by integrin $\alpha 2\beta 1$, the principal collagen I receptor is not only determined by the ligand binding domain of the extracellular domain, but also by the transmembrane and cytoplasmic domains of $\alpha 2$ as well as the other principal collagen binding integrin, $\alpha 1\beta 1$.

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Figure 1. Integrin $\alpha 2 \beta 1$ but not $\alpha 1 \beta 1$ mediates UB cell interactions with collagen I

(A) The different UB cell populations were plated in serum free medium on collagen I at the indicated concentrations and cell adhesion was evaluated 1 hour after plating. Values are the mean $\pm$ SD of three experiments performed in triplicates. (B) UB cell populations were allowed to adhere to collagen I for an hour after which they were fixed and stained with rhodamine phalloidin to visualize the cytoskeleton. (C) UB cell populations were plated on transwells coated with 2.5 μ g/ml collagen I and migration was evaluated after 4 hours. Values are the mean $\pm$ SD of three experiments performed in triplicates. (D) The UB cell populations were plated within collagen I gels. After 2 days, cells were treated with 3 H-Thymidine and incubated for a further 2 days. 3 H-Thymidine incorporation was then determined as described in the Methods. Values are the mean $\pm$ SD of three experiments performed in triplicates and are expressed as fold changes over UB cells transfected with vector only. (*) indicates statistically significant differences ($p < 0.05$) between UB cells untransfected or transfected with the integrin $\alpha 1$ or $\alpha 2$ subunits. (E) The UB cell populations were embedded in collagen I gels as described in the Methods. Nine days later, the gels were fixed and stained with rhodamine phalloidin to

visualize the cytoskeleton. The number of branches of 40 cellular structures per experiment was quantified and the values represent the mean $\pm$ SD of three experiments.

Figure 2. The transmembrane and cytoplasmic domains of the integrin $\alpha 1$ and $\alpha 2$ subunits differentially mediate UB cell function

(A) Schematic representation of the chimeric integrin $\alpha 1$ and $\alpha 2$ mutants. (B) Histogram representation of the expression levels of the cell populations shown relative to UB cells transfected with pcDNA3 vector only. (C–E) cell adhesion (C), spreading (D), migration (E), and proliferation (F) were evaluated and expressed as described in Fig. 1. (*) indicates statistically significant differences ($p < 0.05$) between $\alpha 2$ -UB and UB cells expressing the integrin $\alpha 1/\alpha 2$ chimeras. (G) The UB cell populations were plated in collagen I gels and branching was evaluated as described in Fig. 1E. The values are the mean $\pm$ SD of three experiments are shown.

Figure 3. Integrin $\alpha2\beta1$ and the $\alpha1/\alpha2$ chimeras signaling differently in response to collagen I binding

(A) Serum starved UB cell populations were plated on collagen I (10 $\mu\text{g}/\text{ml}$) for 1 hour, after which they were stained with anti-phosphotyrosine antibody (PY99). (B) Serum starved UB cell populations were plated collagen I (20 $\mu\text{g}/\text{ml}$) for the minutes indicated. 20 μg of total cell lysates were then analyzed by western blot for levels of activated and total ERK, p38 MAPK, and Akt. Activated kinase and total kinase bands were quantified by densitometry analysis and expressed as activated kinase/total kinase ratio. Values are the mean $\pm$ SD of 3 experiments and represent fold changes relative to UB cells at time 0. (*) indicates significant differences ($p < 0.05$) relative to UB cells at time 0, while (#) denotes significant differences between

α 2UB-cells and the chimera expressing cells at the same time points. **(C, D)** Cell migration **(C)** and proliferation **(D)** were performed as described in Fig 1 with the exception that cells were either untreated or treated with PD98059 (50 μ M), PD169316 (10 μ M), LY294002 (5 μ M). Values are means $\pm$ SD of three experiments performed in triplicate and proliferation is expressed as fold changes relative to UB cells transfected with vector only. (#) denotes significant differences ($p < 0.05$) between untreated or inhibitor treated cells within the same cell populations.

Figure 4. Integrin $\alpha 1\beta 1$ modulates integrin $\alpha 2\beta 1$ -mediated cell function

(A) Histogram representation of the expression levels of the integrin $\alpha 1$ constructs in $\alpha 2+\alpha 1$ -UB ($\alpha 1$) and $\alpha 2+\alpha 1\Delta\text{cyt}$ -UB ($\alpha 1\Delta\text{cyt}$) cells relative to the $\alpha 2$ -UB cell population (control). (B-D) Cell adhesion (B), spreading (C), migration (D) and proliferation (E) were evaluated and quantified as described in Fig. 1. (*) indicates statistically significant differences between $\alpha 2$ -UB and $\alpha 2+\alpha 1$ -UB cells. (F) The UB cell populations were plated in collagen I gels and branching was evaluated as described in Fig. 1E. The values are the mean $\pm$ SD of three experiments.

Figure 5. Integrin $\alpha 1$ subunit downregulates integrin $\alpha 2\beta 1$ -dependent signaling

(A) Serum starved UB cell populations cells plated on collagen I (10 μ g/ml) for 1 hour, after which they were stained with anti-phosphotyrosine antibody (PY99). (B) Activation of ERK, p38 MAPK, and Akt was evaluated as described in Fig. 3. Values are the mean $\pm$ SD of 3 experiments and represent fold changes relative to $\alpha 2$ -UB cells at time 0. (*) indicates significant differences ($p<0.05$) relative to $\alpha 2$ -UB cells at time 0, while (#) denotes significant differences between $\alpha 2$ UB-cells and $\alpha 2+\alpha 1$ -UB cells at the same time points. (C) Activation of ERK, p38 MAPK, and Akt was evaluated in the UB cell populations indicated as described in Fig. 3. Values are the mean $\pm$ SD of 3 experiments and represent fold changes relative to $\alpha 2\alpha 2\alpha 1$ -UB (B) or $\alpha 2\alpha 1\alpha 2$ -UB (C) cells at time 0. (*) indicates significant differences ($p<0.05$)

relative to $\alpha 2 \alpha 2 \alpha 1$ -UB or $\alpha 2 \alpha 1 \alpha 2$ -UB cells at time 0, while (#) denotes significant differences between $\alpha 2 \alpha 2 \alpha 1$ -UB and $\alpha 2 \alpha 2 \alpha 1 + \alpha 1$ -UB or $\alpha 2 \alpha 1 \alpha 2$ -UB and $\alpha 2 \alpha 1 \alpha 2$ -UB cells at the same time points.