Integrin-Linked Kinase Regulates Integrin Signaling in Human Trabecular Meshwork Cells

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PURPOSE. To determine whether integrin-linked kinase (ILK) controls the organization of the actin cytoskeleton in the trabecular meshwork (TM) by regulating integrin co-signaling.

METHODS. The cell binding domain and the Heparin II (Hep II) domain of fibronectin were used to activate α5β1 and α4β1 integrin signaling, respectively, in differentiated human TM (HTM) cells. The role of ILK was determined using either ILK small interfering RNA (siRNA) to knock out ILK expression or the ILK inhibitors, KP392 and QLI0267. The knockdown of ILK expression was verified by Western blot analysis. The presence of actin stress fibers and focal adhesions was determined by labeling cultures with phalloidin and anti-talin or ILK antibodies, respectively.

RESULTS. Cell spreading in differentiated HTM cells required ILK, since ILK siRNA and the ILK inhibitors significantly reduced cell spreading, actin polymerization, and the localization of talin and ILK in focal adhesions (FAs). Both cell spreading and the localization of talin and ILK to FAs in differentiated HTM cells could be rescued by inducing α4β1 integrin signaling with a recombinant Hep II domain of fibronectin, even though α4β1 integrins were not found in FAs. In the absence of ILK inhibition, the Hep II domain had minimal effect on α5β1 integrin-mediated spreading.

CONCLUSIONS. This study suggests that cooperative α5β1/α4β1 integrin signaling may be regulated by ILK trans-dominantly and that alterations in ILK activity may affect actin cytoskeleton organization and contractility in the TM. (Invest Ophthalmol Vis Sci. 2011;52:1684–1692) DOI:10.1167/iovs.10-6397

Recent studies show that the contractile properties of human trabecular meshwork (HTM) cells regulate intraocular pressure (IOP) by increasing aqueous humor outflow and that cell-matrix signaling events mediated by the Heparin II (Hep II) domain of fibronectin and α4β1 integrins1–3 may be involved. In vitro, the Hep II domain activates an α4β1 integrin signaling pathway that triggers the disruption of the actin cytoskeleton in confluent cultures of both differentiated HTM cells and an immortalized TM-1 cell line, which is consistent with its functional role in vivo.1,2,4 Activation of this α4β1 integrin signaling pathway requires co-signaling with either α1β1 or α2β1 integrins, which are receptors for types I and IV collagens.5 TM-1 cells interacting with a fibronectin substrate, presumably via α5β1 integrin, appeared to be refractive to activation of α4β1 integrins via the Hep II domain, suggesting that integrin cross-talk in HTM cells is regulated.

Most cells express more than one integrin and cross-talk among different integrins as well as between integrins and other receptors impacts contractility and cell spreading.5 Co-signaling between integrins can result in both antagonistic, or trans-dominant inhibition, and cooperative signaling. For instance, α4β1 integrin activation inhibits α5β1 integrin mediated stress fiber and focal adhesion formation in melanoma cells6 and suppresses metalloprotease expression transduced by α5β1 integrin in synovial fibroblasts.7 In Chinese hamster ovary cells, αIIbβ3 integrin engagement down-regulates the activity of α5β1 and α2β1 integrins.8 In contrast, co-signaling between α5β1 and α4β1 integrins in proliferating HTM cells induces the formation of actin stress fibers and cell spreading.9

Integrin signaling has been shown to be regulated by a number of cytoplasmic proteins, including integrin-linked kinase (ILK). ILK is an adaptor protein containing an ankyrin repeat domain, a pleckstrin homology-like motif, and a pseudokinase-like domain.10 ILK interacts with the cytoplasmic domain of β1 and β3 integrins and localizes to focal adhesions.10–12 ILK also interacts with phosphatidylinositol-3,4,5-trisphosphate (PIP3)13–15 and plays a major structural role in integrin mediated adhesion complexes through interactions with a heterotrimeric complex of paxillin, PINCH, and α- or β-parvin referred to as the IPP complex,15,16 as well as kindlin-217–19 and mitogen-inducible gene (Mig)-2.17

Since previous studies have shown that activation of α4β1 integrin signaling in HTM cells can have opposite effects on actin organization and hence contractility,4,9 we wanted to determine whether there were any cytoplasmic signaling molecules that could be regulating α4β1 integrin signaling. Here, we show that ILK is involved in regulating α4β1 integrin signaling in HTM cells plated on the cell binding domain of fibronectin. The present studies show that ILK inhibits α5β1/α4β1 integrin co-signaling in a trans-dominant fashion. This is the first evidence that ILK can regulate cooperative integrin signaling in HTM cells.

METHODS

Cell Culture

The A7–1 (71-yr-old) and N27TM-1 (27-yr-old) HTM cell strains isolated as described previously20,21 were grown until differentiated/quiescent as described previously.22 Cells were used in experiments within three
days after achieving differentiation. The N27TM-1 cell strain was used in the small interfering RNA (siRNA) experiments. All other experiments were done with the A7–1 cell strain. The procurement of human tissue was done with the consent of the donors according to the tenets of the Declaration of Helsinki.

Preparation of Recombinant Proteins

Recombinant Hep II domain (type III 12 to 14 repeats of fibronectin), the cell binding domain (CBD; type III 7 to 10 repeats of fibronectin), and the IIICS domain of fibronectin were made as described previously.9 All spreading assays were performed in the presence of cycloheximide to prevent endogenous synthesis of extracellular matrix proteins. All peptides, inhibitors, and antibodies were prepared as previously described.24

ILK siRNA

Human ILK siRNA (ON-TARGETplus SMARTpool L004,499-00-0005) and control siRNA (ON-TARGETplus Non-targeting Pool D-001810-10-05) were obtained from Dharmacon/Thermo Fisher Scientific (Lafayette, CO). N27TM-1 cells were transfected with 25 or 50 nM siRNA using a cationic lipid-based reagent (Lipofectamine 2000; Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s reverse transfection protocol. Cells were incubated for 48 hours before performing a spreading assay (see below).

Western Blot Analyses

ILK expression levels were determined with Western blot analysis of cell lysates. Cells were lysed with ice cold lysis buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% NP-40, 0.25% deoxycholate, and protease inhibitors). Lysates (10 μg) were resolved on a 10% SDS-PAGE and transferred to a membrane (Immobilon-P; Millipore Corp., Billerica, MA). The membrane was blocked and incubated with primary and secondary antibody as previously described.9 ILK was detected using an ILK polyclonal antibody (Cell Signaling Technologies, Danvers, MA) diluted 1:1000. GAPDH, the loading control, was detected with a rabbit anti-GAPDH antibody (Abcam Inc., Cambridge, MA). Bound antibody was detected with an enhanced chemiluminescence kit (ECL Plus Western blotting detection kit; Amersham Biosciences, Piscataway, NJ) and the area of bands was measured using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/iij/index.html).

Spreading Assay

Serum-starved cells were replated on coverslips coated with 10 μg/mL of the CBD of fibronectin and allowed to spread for 3 hours as previously described.9 All spreading assays were performed in the presence of the CBD of fibronectin and allowed to spread for 3 hours as previously described.9

Immunofluorescence Microscopy

HTM cells were permeabilized, fixed, and blocked as previously described.9 Blocked cells were incubated for 1 hour with mouse anti-vinculin (Sigma Aldrich Co., St. Louis, MO), anti-ILK (Millipore Corp.), anti-talin (Millipore Corp.), anti-paxillin (BD Biosciences, San Jose, CA), or anti-FAK (BD Biosciences) monoclonal antibody. Antibodies were diluted 1:3000 (vinculin) or 1:1000 (ILK, talin, paxillin, and FAK) in 0.1% BSA in PBS. Cells were then incubated simultaneously with Alexa Fluor 546–conjugated goat anti-mouse secondary antibody (4 μg/mL; Invitrogen) and Alexa Fluor 488–conjugated phalloidin (0.67 unit/mL; Invitrogen) in 0.1% BSA/0.1% Tween for 1 hour. For localization of α4β1 integrin, HTM cells were fixed in 100% methanol for 15 minutes at −20°C, blocked as above, and then incubated for 1 hour with mouse anti-α4 integrin antibody (clone HP2/1; Millipore Corp.) at 1:1000 in 0.1% BSA/PBS followed by 1 hour incubation with the Alexa Fluor 546–conjugated goat anti-mouse secondary antibody. Fluorescent images were acquired using an epifluorescence microscope (Axioplan 2; Carl Zeiss, Inc., Thornwood, NY) equipped with a digital camera (Axiocam HRm; Zeiss) together with image acquisition software (Axiovision version 4.5; Zeiss). In cells treated with the ILK inhibitors, cell spreading was determined by counting spread cells containing polymerized actin versus cells lacking polymerized actin or rounded cells from 8 to 12 different fields of view per coverslip (n = 100 cells/treatment). Cell area for the siRNA experiments was measured from four to six different fields of view per coverslip using the image acquisition software (n = 125 cells/treatment).

Cell Adhesion Assay

The cell adhesion assay was performed with the A7–1 cells as previously described.9 Briefly, serum-starved HTM cells were replated into 96 well plates in the presence of 10 μg/mL integrin adhesion blocking antibodies or control mouse IgG (Sigma-Aldrich). All wells were pre-coated with 47 nM of the CBD for 1 hour at 37°C. Bound cells were fixed and stained overnight with 0.5% toluidine blue in 4% paraformaldehyde. Bound dye was then redissolved in 2% SDS and detected at 600 nm using a microplate reader. Rat β1 integrin antibody mAb13 and rat α5 integrin antibody mAb16 were kindly provided by Steve Akiyama (National Institutes of Health, Research Triangle Park, NC). The αv integrin monoclonal antibody M9 was purchased from Millipore Corp.

Fluorescence-Activated Cell-Sorting (FACS) Analysis

FACS analysis of the A7–1 cells was performed as previously described9 on a cytometer (FACScan; BD Biosciences). The α4 monoclonal HP2/1, α5 polyclonal, β1 monoclonal Hb1.1, and α5β1 monoclonal HA5 antibodies were obtained from Millipore Corp.

Data Analysis

All comparisons were made as a percentage of the control or percentage of spread cells. Data are presented as mean ± SEM. Statistical comparisons were done using two-tailed Student’s t-test with P < 0.05 considered significant.

Results

Cell Spreading and Actin Polymerization Are Not Affected by the Hep II Domain in Differentiated HTM Cells Plated on the CBD of Fibronectin

Previous studies have suggested that activation of an α4β1 integrin–signaling pathway by the Hep II domain of fibronectin can have opposing effects on contractility and actin polymerization depending on the matrix substrate, cell cycle stage (quiescent/differentiated versus proliferating), and cross-talk with other integrins engaged on the cell surface.3,4,9 To determine whether there was a cell cycle specific factor that could explain these different responses to the Hep II domain, we performed a spreading assay by replating cultures of either proliferating or differentiated HTM cells that had reached quiescence onto the CBD of fibronectin in the absence or presence of the Hep II domain. The CBD of fibronectin was used to minimize effects from other signaling domains of fibronectin (Fig. 1A). Figures 1B–1G show that the differentiated HTM cells responded differently than proliferating HTM cells in this assay and were refractive to the Hep II domain. In the absence of the Hep II domain, differentiated HTM cells showed a polygonal
as previously shown.9 Thus, there appears to be a cell cycle
transition and cell spreading in proliferating HTM cells (Figs. 1F, 1G)
in contrast, the Hep II domain induced actin stress fiber forma-
tion were responsible for the different responses to the Hep II
proliferating HTM cells where 55.8% of the cells were in
G1/G0 and 17.8% of cells in S phase. This was different from
inhibited cell attachment to the CBD of fibronectin (*
P < 0.0004, **P < 0.0001 compared to IgG control). The αv integrin blocking antibody
had no significant effect. Differentiated A7–1 cells were plated on the
CBD for 1 hour in the absence or presence of integrin blocking
antibodies or control IgG. Bound cells were colorimetrically quantified
data are the mean percentage of IgG ± SEM.

To rule out the possibility that changes in integrin expres-
sion were responsible for the different responses to the Hep II
domain, FACS analyses was done. These studies indicated that
differentiated HTM cells, like proliferating cells,7 expressed the
α4 and α5 integrin subunits as well as β1 integrin subunit
(Fig. 2B). Cell binding studies using antibodies against β1, α5
and αv integrins or a control IgG to block cell attachment were
done to demonstrate that both proliferating and differentiating
cells used the same integrins to attach to the CBD of fibronectin.
Previous studies with proliferating HTM cells showed that
these cells used α5β1 integrins to attach to the CBD of fibronectin.9 This study showed that differentiated HTM cells,
like proliferating HTM cells, also used α5β1 integrin to adhere
to the CBD of fibronectin (Fig. 2A). Together, these data indi-
cate that actin polymerization in differentiated HTM cells was
mediated by α5β1 integrins when plated on the CBD of fibronectin,
and that the Hep II domain did not have any effect even though its α5β1 integrin receptor was expressed.

Polymerization of Actin and Cell Spreading Is
ILK Dependent

To determine whether ILK was involved in mediating the α5β1
integrin signaling pathway seen in differentiated HTM cells,
ILK expression levels were analyzed by Western blot analysis.
As shown in Figure 3A, differentiated HTM cells expressed
morphology that typically contained cortical actin structures
with some stress fibers and numerous focal adhesions contain-
ing vinculin (Figs. 1B, 1D). Addition of the Hep II domain to
these differentiated HTM cultures had no apparent effect on
cell spreading or actin stress fiber formation (Figs. 1C, 1E). In
contrast, the Hep II domain induced actin stress fiber forma-
tion and cell spreading in proliferating HTM cells (Figs. 1F, 1G)
as previously shown.9 Thus, there appears to be a cell cycle
specific factor which is regulating integrin signaling.

FIGURE 1. Effect of Hep II domain on differentiated and proliferating
HTM cells. (A) Schematic of fibronectin showing the cell binding
domain (CBD) and the Hep II domain. (B–E) Cell spreading in differenti-
ated A7–1 HTM cells plated on the CBD occurs in the absence
(B, D) or presence (C, E) of 47.2 nM of the Hep II domain of fibronect.
(F, G) In contrast, cell spreading in proliferating A7–1 HTM cells
requires the addition of the Hep II domain. Cells were labeled with
phalloidin (B, C, F, G) to visualize F-actin or vinculin (D, E) to visualize
focal contacts and adhesions. Bar, 20 μm.

FIGURE 2. HTM cell adhesion via α5β1 integrin and integrin expres-
sion. (A) The α5 and β1 integrin blocking antibodies significantly
inhibited cell attachment to the CBD of fibronectin (*P = 0.0004, **P <
0.0001 compared to IgG control). The αv integrin blocking antibody
had no significant effect. Differentiated A7–1 cells were plated on the
CBD for 1 hour in the absence or presence of integrin blocking
antibodies or control IgG. Bound cells were colorimetrically quantified
using toluidine blue. Data are the mean percentage of IgG ± SEM.

(B) FACS analysis of integrin levels in differentiated A7–1 cells. Cells
were labeled with antibodies against various integrin subunits. Non-
immune IgG controls are shown as black peaks, integrin expression is
shown in gray.
more ILK than proliferating HTM cells. GAPDH levels were similar, indicating that the higher levels of ILK in differentiated HTM cells was not due to loading differences. We then re-plated cells on the CBD of fibronectin in the absence or presence of the ILK inhibitors QLT0267 or KP392, to determine whether ILK was involved in α5β1 integrin-mediated actin polymerization in HTM cells. Addition of 20 μM QLT0267 to the medium inhibited differentiated HTM cell spreading by 70% (P < 0.03; Figs. 3B, 3D) when compared to control cells (Figs. 3B, 3D). Similar results were seen with KP392 (Fig. 3D). Differentiated HTM cells that did spread in the presence of QLT0267 exhibited a decrease in stress fiber formation (Fig. 3B) and showed focal contacts rather than focal adhesions (Fig. 3B). In contrast, proliferating HTM cell spreading and actin polymerization was unaffected by KP392 (Fig. 3C). Thus, differentiated HTM cells, but not proliferating cells use ILK to polymerize actin filaments on the CBD of fibronectin.

To further demonstrate that ILK was involved in promoting actin polymerization in differentiated HTM cells, ILK siRNA was used to knock down ILK expression levels before a spreading assay. As shown in Figure 4A, there was a major knock down of ILK expression with ILK siRNA when compared to lipofectamine only or to the non-specific control siRNA. By densitometry, there was approximately 100, 96.8, and 91.6% ILK knock down with 25, 50, and 100 nM ILK siRNA, respectively, compared to lipofectamine only. Similar results were seen with two other HTM cell lines (data not shown). Figure 4B shows that cell spreading and actin polymerization in differentiated HTM cells treated with 50 nM ILK siRNA for 48 hours was impaired compared to lipofectamine-treated controls. Measurements of cell area further verified this observation and showed a reduction in the area of cells of 38.4 ± 4.0% (P < 0.0001) with 25 and 50 nM ILK siRNA, respectively, compared to lipofectamine-only-treated cells (Fig. 4C). The non-specific control siRNA at 25 nM also had a small inhibitory effect on cell spreading (15.4 ± 4.9%, P < 0.05), but not to the same extent as the ILK siRNA.

**ILK Inhibits α4β1 Integrin Signaling**

To determine whether ILK regulated signaling through α4β1 integrin, cells transfected with ILK siRNA or treated with ILK inhibitors were re-plated in the presence of soluble Hep II domain. As shown in Figure 4B, addition of the Hep II domain re-established the formation of cortical actin and restored cell spreading to 90.5 ± 4.2% and 88.2 ± 3.9% in cells treated with 25 and 50 nM ILK siRNA, respectively, compared to lipofectamine cells treated with the Hep II domain (Fig. 4C). The Hep II domain also induced cell spreading with cortical actin and focal adhesions in the presence of both ILK inhibitors KP392 and QLT0267. As shown in Figure 3D, only 36% and 14% of cells spread when treated with the KP392 and QLT0267 inhibitors.
inhibitors, respectively. However, the addition of soluble Hep II domain increased cell spreading to 73% (\(P < 0.001\)) and 50% (\(P < 0.05\)), respectively, when compared to ILK inhibitors alone (Fig. 3D). The Hep II domain by itself had no effect on cell spreading as 84% of both control and Hep II domain–treated cells spread. This suggests that ILK activity trans-dominantly inhibits signaling from the Hep II domain to induce differentiated HTM-cell spreading on the CBD.

To demonstrate that the Hep II domain used \(\alpha 4\beta 1\) integrin to promote actin polymerization when ILK was inhibited, \(\alpha 4\beta 1\) integrin–blocking antibody was added to differentiated HTM cells plated with 50 nM of ILK siRNA compared to control siRNA or lipofectamine 2000 unless the Hep II domain was present. Cells were plated on the CBD for 5 hours in the absence or presence of the Hep II domain and labeled with phalloidin to visualize F-actin. Bar. 20 \(\mu\)m. (C) Treatments of 25 and 50 nM ILK siRNA significantly decreased cell spreading. Statistically significant (*\(P < 0.05\), **\(P < 0.0001\)). Data are the mean ratio of cell area compared to lipofectamine only control (set to 100%) \(\pm\) SEM for non-Hep II domain–treated cells and compared to lipofectamine + Hep II for cells treated with the Hep II domain. Cell spreading was quantified by measuring the area of the cells in four to six fields of view from a triplicate experiment (\(n = 123\)).

![Figure 4. ILK siRNA knocks down ILK expression. (A) Levels of ILK expression in differentiated N27TM-2 cells treated for 48 hours with ILK siRNA. The blot is representative of the same experiment in three different cell strains. (B) Cell spreading was impaired in HTM cells treated with 50 nM of ILK siRNA compared to control siRNA or lipofectamine 2000 unless the Hep II domain was present. Cells were plated on the CBD for 5 hours in the absence or presence of the Hep II domain and labeled with phalloidin to visualize F-actin. Bar. 20 \(\mu\)m. (C) Treatments of 25 and 50 nM ILK siRNA significantly decreased cell spreading. Statistically significant (*\(P < 0.05\), **\(P < 0.0001\)). Data are the mean ratio of cell area compared to Lipofectamine only control (set to 100%) \(\pm\) SEM for non-Hep II domain–treated cells and compared to lipofectamine + Hep II for cells treated with the Hep II domain. Cell spreading was quantified by measuring the area of the cells in four to six fields of view from a triplicate experiment (\(n = 123\)).](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932971/ on 11/08/2018)
adhesions, unlike ILK and talin (Figs. 8E and 8I, respectively). Instead, α4β1 integrins appeared to be distributed throughout the cytoplasm with some discrete clustering along the cell periphery and in cellular protrusions. In cells treated with the ILK inhibitors KP392 or QLT0267, the clustering of α4β1 integrins along the edges of cell protrusions was reduced (Fig. 8C) and there was a loss of ILK and talin localized to focal adhesions (Figs. 8G and 8K, respectively).

The addition of the Hep II domain to KP392 or QLT0267-treated cells restored the localization of α4β1 integrin to cell protrusions (Fig. 8D) and ILK and talin to focal adhesions (Figs. 8H and 8L, respectively). Similar results to ILK and talin were seen with other focal adhesion proteins, including vinculin, paxillin, and FAK (data not shown). Together, these data suggest that cross-talk between α5β1 and α4β1 integrins recruited talin and ILK to focal adhesions even when ILK activity is inhibited.

**Discussion**

This study demonstrates for the first time that ILK mediates trans-dominant integrin signaling in HTM cells and plays a critical role in regulating cooperative signaling between α5β1 and α4β1 integrins. Inhibition of ILK expression with siRNA or the ILK inhibitors KP392 or QLT0267 allowed activation of a cooperative α5β1/α4β1 integrin signaling pathway that regulated cell spreading, actin polymerization, and focal adhesion formation. When ILK was active, however, cell spreading was dependent solely on α5β1 integrin signaling. This role of ILK appeared to be cell cycle dependent because α5β1/α4β1 integrin cooperative signaling was constitutively active in proliferating HTM cells and was unaffected by the ILK inhibitors.

**Figure 5.** Hep II domain-mediated spreading is dependent on α4β1 integrin. Differentiated A7–1 cells were plated on the CBD in the absence or presence of the ILK inhibitor KP392 (100 μM) and/or 472 nM of the Hep II domain. Whereas inhibition of ILK function with KP392 significantly decreased cell spreading compared to control cells (**P < 0.025, n = 2 experiments), the inhibition with KP392 could be overcome with the addition of the Hep II domain. The recovery of cell spreading by the addition of the Hep II domain could be blocked with a soluble α4 integrin-blocking antibody (25 μg/ml; clone P1H4). Compared to untreated control cells, the percent of cell spreading in HTM cells treated with KP392, the Hep II domain, and P1H4 was significantly different (P < 0.03). Data are the mean percentage of cells spread with F-actin ± SEM.

**Figure 6.** α4β1 integrin ligands induce cell spreading when ILK is inhibited. Differentiated A7–1 cells were plated on the CBD in the absence (A, C, E) or presence of the ILK inhibitor KP392 (100 μM; B, D, F); with or without soluble extracellular domain of VCAM-1 (236 nM; C, D); or the IIICS domain of fibronectin (472 nM; E, F). Cells were labeled with phalloidin to visualize F-actin. Bar, 20 μm.
LY294002. Treatment with 25 μM of the PI3K inhibitor LY294002 (LY) or in combination with KP392 or the ILK inhibitor (****P < 0.001, ****P < 0.0001). (B) Spread differentiation. A7–1 cells were incubated with the Hep II domain in combination with KP392 or LY294002. Treatment with 25 μM of the PI3K inhibitor significantly decreased cell spreading induced by 472 nM of the Hep II domain in the presence of 100 μM of the ILK inhibitor compared to cells treated with both the Hep II domain and the ILK inhibitor (**P < 0.01). Statistically significant from Hep II–treated cells or as indicated by brackets (**P < 0.015, ****P < 0.0007). Data are the mean percentage of cells spread with F-actin ± SEM.

most likely because of low ILK expression in these cells. These observations are consistent with the role of ILK in cell cycle progression and differentiation32–35 and suggest that the level of ILK expression regulates cooperative α5β1/α4β1 integrin signaling.

Activation of this α5β1/α4β1 integrin pathway in differentiated HTM cells was not specific for a particular ligand, and all three α4β1 integrin ligands tested (VCAM-1, IIICS domain, Hep II domain) triggered cell spreading, actin polymerization, and focal adhesion formation when ILK activity was disrupted. In addition, α1β1 integrin–blocking antibodies partially blocked the Hep II-mediated rescue of differentiated cell spreading when ILK activity was disrupted. Only partial blockage was seen with the α4β1 integrin–blocking antibody, most likely because insufficient antibody was used for complete blockage. This α5β1/α4β1 integrin signaling pathway did not involve the localization of α4β1 integrin into focal adhesions. This is not the first time that α4β1 integrin signaling has been observed to occur outside of focal adhesions and has been previously seen in Chinese hamster ovary (CHO) cells.30 This suggests that α4β1 integrin regulates α5β1 integrin signaling and focal adhesion formation through separate membrane sites via an intermediate inside-out signaling pathway. Interestingly, both the Hep II–mediated cell spreading in the presence of the ILK inhibitor and the α5β1 integrin–mediated cell spreading

FIGURE 7. PI3K inhibition leads to differentiated HTM cell rounding. (A) Differentiated A7–1 cells were plated on the CBD in the presence of the ILK inhibitor KP392 or the PI3K inhibitor LY294002 (LY) or in combination. Treatment with LY294002 significantly inhibited cell spreading compared to control cells (25 μM; *P < 0.05; n = 3 experiments). Statistically significant from control cells (*P < 0.05, ****P < 0.001, ****P < 0.0001). (B) Spreading differentiation. A7–1 cells were incubated with the Hep II domain in combination with KP392 or LY294002. Treatment with 25 μM of the PI3K inhibitor significantly decreased cell spreading induced by 472 nM of the Hep II domain in the presence of 100 μM of the ILK inhibitor compared to cells treated with both the Hep II domain and the ILK inhibitor (**P < 0.01). Statistically significant from Hep II–treated cells or as indicated by brackets (**P < 0.015, ****P < 0.0007). Data are the mean percentage of cells spread with F-actin ± SEM.

FIGURE 8. α4β1 integrin, ILK and talin localization. Differentiated A7–1 cells plated on the CBD were labeled with an α4β1 integrin (A–D), ILK (E–H), or talin (I–L) antibody. α4β1 integrins appeared clustered along the edges of the cells treated with Hep II domain (472 nM; B), KP392 and Hep II domain (D), or left untreated (A). Cells treated with KP392 (100 μM; C) did not show any labeling along the edges. Arrows indicate areas of α4 integrin localization. ILK and talin were observed in focal adhesions in differentiated A7–1 cells plated on the CBD in the absence (E, F, I, and J), but not the presence, of the ILK inhibitor QLT0267 (20 μM; G, H, K, and L). Addition of the Hep II domain (472 nM; H, L) restored the localization of ILK and talin in focal adhesions. Bars: (A–D) 10 μm; (E–L) 20 μm.
via the CBD of fibronectin required active PI3K. This suggests that although the α5β1 and α6β1 integrin signaling may use separate pathways, these pathways may merge at, or are upstream of, PI3K.

Exactly how ILK is regulating integrin signaling is unknown. When ILK is inactive, Hep II-mediated signaling through αβ1 integrin appears to “re-activate” β1 integrins through an outside-in signaling pathway which would be in keeping with ILK’s role in outside-in signaling. Recent studies suggest that ILK may trigger an active conformation of β1 integrin58 by promoting the interaction of talin with the cytoplasmic tails of the β1 subunit. Integrin β cytoplasmic tails that are unable to bind talin have been shown to lose their ability to mediate trans-dominant integrin inhibition. In support of this idea, the disruption of ILK activity blocked the recruitment of talin to focal adhesions mediated by αβ1 integrin signaling. Recruitment of talin to focal adhesions could be recovered if the cooperative αβ1/αβ1 integrin pathway was activated with the Hep II domain.

In summary, our results suggest that ILK can trans-dominantly control cooperative integrin signaling events used to regulate the organization of the actin cytoskeleton in differentiated HTM cells. Alterations in ILK activity are likely to affect how TM cells in vivo respond to ECM-mediated signals, thereby altering the functional properties of the TM, such as contractility. For instance, it is well established that the contractile state of the actin network in the TM in vivo can regulate aqueous humor outflow from the eye and IOP. Given the importance of the ECM in regulating the function of the TM, these studies show that it is critical we understand not only the molecular pathways involved in ECM-mediated signaling events but how the cell cycle state may alter these events. Such understanding will enable us to develop more specific treatments for disease.

References


