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Heparin II domain of fibronectin mediates contractility through an $\alpha 4\beta 1$ co-signaling pathway

Marie K. Schwinn^a, Jose M. Gonzalez Jr.^a, B'Ann T. Gabelt^b, Nader Sheibani^{b,c}, Paul L. Kaufman^b, and Donna M. Peters^{a,b,*}

^aDepartment of Pathology and Laboratory Medicine, University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI 53706, USA

^bDepartment of Ophthalmology and Visual Sciences, University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI 53706, USA

^cDepartment of Pharmacology, University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI 53706, USA

Abstract

In the trabecular meshwork (TM) of the eye, regulation of tissue contractility by the PPRARI sequence within the Heparin II (HepII) domain of fibronectin is believed to control the movement of aqueous humor and dictate the level of intraocular pressure. This study shows that the HepII domain utilizes activated $\alpha 4\beta 1$ integrin and collagen to mediate a co-signaling pathway that down-regulates contractility in TM cells. siRNA silencing of $\alpha 4\beta 1$ integrin blocked the actin disrupting effects of both PPRARI and the HepII domain. The down-regulation of the actin cytoskeleton and contractility did not involve syndecan-4 or other heparan sulfate proteoglycans (HSPGs) since siRNA silencing of syndecan-4 expression or heparitinase removal of cell surface HSPGs did not prevent the HepII-mediated disruption of the actin cytoskeleton. HepII-mediated disruption of the cytoskeleton depended upon the presence of collagen in the extracellular matrix, and cell binding studies indicated that HepII signaling involved cross-talk between $\alpha 4\beta 1$ and $\alpha 1/\alpha 2\beta 1$ integrins. This is the first time that the PPRARI sequence in the HepII domain has been shown to serve as a physiological $\alpha 4\beta 1$ ligand, suggesting that $\alpha 4\beta 1$ integrin may be a key regulator of tissue contractility.

INTRODUCTION

The actin cytoskeleton is a dynamic structure and modulates tissue function by altering its contractile properties. For example, reorganization of the actin cytoskeleton within the trabecular meshwork (TM) of the eye leads to changes in intraocular pressure. The TM is a specialized tissue located within the anterior segment of the eye that regulates intraocular pressure by mediating the flow of aqueous humor through the anterior segment. A decrease in cell contractility or disruption of an assembled actin network in the TM facilitates aqueous humor outflow and consequently decreases intraocular pressure [1–4]. As with other contractile tissues, contractility in the TM is regulated by the activation of Rho-kinase, protein kinase C,

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*Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, 1300 University Avenue, Madison, WI 53706 USA. Phone: +1 (608) 262-4626. FAX: +1 (608) 265-3301. dmpeter2@wisc.edu.

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or myosin light chain kinase which modulate myosin light chain (MLC) phosphorylation and the subsequent contraction of the TM [5,6]. Inhibition of MLC phosphorylation decreases contractility by disrupting actin polymerization and formation of focal adhesions [7,8]. However, the exact mechanisms by which external stimuli trigger contractile responses in the TM require further study.

Integrins are ubiquitously expressed heterodimeric α/β transmembrane receptors that bind extracellular matrix (ECM) proteins. They establish a direct link between the ECM and the actin cytoskeleton, transmitting signals that regulate adhesion, actin organization, and contractility [9]. Integrins control contractility and the organization of the actin cytoskeleton by modulating Rho GTPases. Of all the integrins, $\alpha4\beta1$ integrin is most recognized for its role in decreasing cell contractility by disrupting focal adhesion formation and actin organization [10–12]. $\alpha4\beta1$ integrin binds a wide range of cell surface and extracellular matrix ligands including vascular cell adhesion molecule-1 (VCAM-1), thrombospondin, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), osteopontin, CD14, and the LDV and REDV sequences in the alternatively spliced V region of fibronectin [13–19]. $\alpha4\beta1$ integrin also binds other regions of fibronectin, including the KLDAPT sequence in the III₅ repeat, the EDGIHEL sequence in the alternatively spliced EDA segment, and the PPRARI/IDAPS sequence in the III₁₄ repeat of the heparin II (HepII) domain [20–22]. The interaction between the PPRARI/IDAPS sequence in the HepII domain and $\alpha4\beta1$ integrin, however, has never been shown to produce any physiological response.

The HepII domain of fibronectin comprises the type III₁₂ through III₁₄ repeats. It contains a high affinity heparin binding domain within the III₁₃ repeat, as well as a lower affinity heparin binding site within the PPRARI sequence of the III₁₄ repeat [23,24]. Although, PPRARI has been reported to serve as a ligand for $\alpha4\beta1$ [22], it is best known as a ligand for syndecan-4, a member of the heparan sulfate proteoglycan (HSPG) family of transmembrane receptors [25]. The interaction between PPRARI and syndecan-4 mediates the formation of focal adhesions and actin stress fibers by triggering the clustering of the syndecan-4 core protein and the subsequent activation of protein kinase C α and RhoA [26,27].

A peptide containing the PPRARI sequence of the HepII domain in fibronectin has recently been shown to down-regulate the organization of the actin cytoskeleton in confluent cultures of TM cells [28] as well as lower intraocular pressure when perfused through cultured human and monkey anterior segments [29]. Presumably, the decrease in intraocular pressure is due to the PPRARI site in the HepII domain activating a signaling pathway that triggers a decrease in contractility. Because both syndecan-4 and $\alpha4\beta1$ integrins have been found in TM cell cultures and in vivo [30,31], the purpose of this study was to identify the signaling pathway utilized by the HepII domain to regulate contractility in TM cells and potentially increase aqueous humor movement in cultured anterior segments.

Using a line of human TM cells (TM-1), we showed that the HepII domain of fibronectin uses a co-signaling pathway involving $\alpha4\beta1$ integrin and collagen to trigger the disruption the actin cytoskeleton and a decrease cellular contractility. These data further suggest that it is the PPRARI sequence within the HepII domain which interacts with an activated $\alpha4\beta1$ integrin. The activation occurs independently of syndecan-4 indicating that PPRARI is an $\alpha4\beta1$ ligand as previously proposed [22]. This study demonstrates, for the first time, that interactions between the HepII domain and $\alpha4\beta1$ integrin co-signaling pathway produce a physiological consequence, especially in the regulation of intraocular pressure.

MATERIALS AND METHODS

Cell culture

The immortalized human TM-1 cell line was maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO), 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (Sigma-Aldrich), 1% amphotericin B (Mediatech, Herndon, VA), and 0.05% gentamicin (Mediatech) [32]. In some experiments, cells were plated on 10 µg/ml plasma fibronectin or type IV collagen (Millipore, Billerica, MA) or plasma fibronectin and treated at confluence with serum free medium in the absence or presence of the recombinant HepII domain (type III 12–14 repeats of fibronectin), the HepII/RK double mutant in which Arg⁹ and Lys²⁵ had been converted to serines, or the PPRARI and IDAPS peptides [29,31,33].

Immunofluorescent microscopy

To detect F-actin, cells were permeabilized, fixed, and labeled with Alexa Fluor® 488-conjugated phalloidin (Invitrogen, Carlsbad, CA) as described [31]. To visualize cell surface heparan sulfates, cells were washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde/PBS overnight at 4°C, and then blocked in 1% bovine serum albumin (BSA)/PBS for 1 h. Cells were incubated for 1 h with the mouse 10E4 antibody against a native heparan sulfate epitope (Dr. Guido David, K.U. Leuven, Belgium) [34] and then labeled for 1 h with rabbit anti-mouse IgM (Zymed Laboratories, Inc., San Francisco, CA) followed by Alexa 546-conjugated goat anti-rabbit secondary antibody (Invitrogen) for 1 h. Fluorescent images were acquired using a Zeiss AxioCam HRm camera (Thornwood, NY) mounted on a Zeiss Axioplan 2 Imaging fluorescence microscope equipped with AxioVision version 3.1 software.

Heparitinase treatment

Confluent monolayers of TM-1 cells were pretreated with 0.01 IU/ml heparitinase III (Sigma-Aldrich) for 2 h. Cells were then incubated with 125 µg/ml HepII in serum-free DMEM for 12 h, and additional 0.01 IU/ml heparitinase III was added every 2 h for the duration of the assay.

siRNA-mediated silencing

siRNA against the human $\alpha 4$ integrin (ON-TARGETplus SMARTpool L-005189-00-0005) was obtained from Dharmacon (Lafayette, CO) or Invitrogen (ITGA4 Stealth RNAi Set). Syndecan-4 siRNA (ON-TARGETplus SMARTpool L-003706-00-0005) and non-targeting control siRNA (ON-TARGETplus siCONTROL Non-targeting Pool D-001810-10-05) were purchased from Dharmacon. At 70% confluence, TM-1 cultures were transfected with 100 or 200 nM siRNA using Lipofectamine™ 2000 (Invitrogen), per manufacturer's protocol. After 48 h, cells were either prepared for FACS analysis or were incubated with 500 µg/ml HepII in serum-free medium for 24 h.

Fluorescence-activated cell-sorting (FACS) analysis

FACS was performed as previously described [31]. Cells were incubated with anti- $\alpha 4$ integrin (P1H4, Millipore), anti-syndecan-4 (8G3, provided by Dr. Guido David), anti- $\beta 1$ integrin (HB1.1, Millipore), or anti- $\beta 7$ integrin (FIB504, BD Biosciences, San Jose, CA) prepared to 10 µg/ml in 1% BSA/Tris-buffered saline (TBS) for 30 min on ice. Cells were then incubated with Alexa 488-conjugated anti-mouse secondary antibody prepared to 5 µg/ml in 1% BSA/TBS for 30 min and analyzed with the FACSCalibur System (BD Biosciences). Purified mouse IgG1 (Millipore) or rat IgG2a (BD Biosciences) were used as negative isotype controls.

Contractility assay

Collagen gels were prepared by mixing rat tail type I collagen (BD Biosciences) with an equal volume of 100 mM HEPES/2X PBS on ice. DMEM was then added until the final concentration of collagen was 1.25 mg/ml. The gels were poured into 6-well plates and allowed to solidify at 37°C for 30 min. 1×10^6 TM-1 cells were seeded on the gels in DMEM. After 24 h, cells were dosed with 0, 100, 250, or 500 µg/ml HepII in serum-free DMEM. Some cells were also treated with 0.5, 1, 2, or 4 mg/ml of the PPRARI, PPAARI, or IDAPS peptides. The gels were detached from the side of the dish with a pipette tip, and the diameter of the gel was measured at 1, 6, 18, and 24 h.

Organ culture

Anterior segments were obtained from male rhesus (*Macaca mulatta*) monkeys at the Wisconsin National Primate Research Center ($n = 3$). The anterior segments were placed in culture within 2 h of death, as previously described [35]. Outflow facility was determined by two-level constant pressure perfusion in the anterior segments of the eyes. One segment from each pair was exchanged with 100 µg/ml of HepII, while the other was exchanged with DMEM.

Light microscopy

The anterior segments were exchanged with 4% paraformaldehyde for 30 min, cut into quadrants, and immersed in 4% paraformaldehyde. Quadrants were then embedded in Epon 812 (Polysciences, Inc., Warrington, PA), as described [36]. Semi-thin sagittal sections were cut and stained with toluidine blue (Polysciences, Inc.). Sections from each quadrant were examined for the presence of TM cells, beams, and the integrity of Schlemm's canal.

Cell Adhesion Assays

96-well plates were coated with 10 µg/ml bovine type IV collagen (Millipore), 1.7 µg/ml of the type III 7–10 repeats of fibronectin, or 250 µg/ml HepII in PBS for 1 h at 37°C. Wells were then blocked with 2% BSA/PBS for 1 h at room temperature and washed with PBS. Cells were pre-incubated with antibodies against $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha V\beta 3$, $\beta 1$ or an IgG control diluted to 25 µg/ml in serum free medium at 37°C for 30 min. Cells were then plated and allowed to attach for 1 h at 37°C. Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, and stained with 0.5% toluidine blue overnight. Bound dye was solubilized with 2% sodium dodecyl sulfate and absorbance at 600 nm was measured using a microplate reader. For assays in which either Mg^{2+} or Ca^{2+} was present, cells were pre-incubated with antibodies in an 1:1 dilution of 2X DMEM and 2X HEPES buffered saline (300 mM NaCl, 50 mM HEPES, pH 7.4) with 4 mM divalent cation. The integrin antibodies $\alpha 1$ (FB12), $\alpha 2$ (P1E6), $\alpha 4$ (P1H4), $\alpha v\beta 3$ (LM609), $\beta 1$ (12G10), and the IgG control were purchased from Millipore. A second $\beta 1$ integrin antibody (TS2/16) was purchased from Thermo Fisher Scientific (Rockford, IL).

RESULTS

Knockdown of syndecan-4 does not block the activity of the HepII domain

It has been reported that the PPRARI sequence of the HepII domain interacts with the glycosaminoglycan chains of syndecan-4 to promote the formation of actin stress fibers and focal adhesions [37]. PPRARI has also been identified as the active site in the HepII domain that triggers a disruption in the actin cytoskeleton in TM cells [29]. To determine if interactions of the HepII domain with syndecan-4 [38] could contribute to a disruption of the actin cytoskeleton, syndecan-4 expression in TM-1 cells was silenced with siRNA prior to treatment with the HepII domain. FACS analysis showed the TM-1 cells used in this study displayed low levels of cell surface syndecan-4 and that these levels were reduced by 84% after transfection with siRNA specific for syndecan-4 (Figure 1A). Immunofluorescent staining for F-actin

showed that these cells, as well as cells treated with vehicle or transfected with non-targeting control siRNA, formed intact monolayers with strong actin stress fibers. When the HepII domain was added to these cultures, the actin networks were disrupted and large separations appeared between the cells, regardless of whether syndecan-4 was silenced. These results indicate that syndecan-4 plays a minimal role in this HepII-mediated pathway (Figure 1B).

HSPGs are not involved in HepII-mediated disruption of actin cytoskeleton

To eliminate the possibility that the HepII domain was interacting with other HSPGs on TM cells, TM-1 cells were pre-treated with heparitinase III to remove the heparan sulfate moieties from the cell surface and then incubated with the HepII domain. Staining with the anti-heparan sulfate antibody 10E4 confirmed that cell surface HSPGs detected in vehicle-treated cultures were removed by this heparitinase treatment (Figure 2). When heparitinase-treated cells were incubated with the HepII domain, the HepII domain still triggered the disruption of the actin cytoskeleton. Both heparitinase-treated and untreated cultures lacked the long actin stress fibers observed in control cultures and instead only contained irregular short actin filaments. In contrast, an abundance of long actin stress fibers were observed in both heparitinase-treated and untreated monolayers of TM-1 cells in the absence of the HepII domain. In addition, treating TM-1 cells with a mutant HepII that was defective in HS binding still triggered the disruption of the actin cytoskeleton (data not shown), suggesting that the high affinity HS binding site in the III₁₃ repeat was not involved. Furthermore, treatment with 60 mM sodium chlorate, which has been shown to block the sulfation of all glycosaminoglycan chains including chondroitin sulfate proteoglycans [39,40], did not inhibit the effect of HepII on the actin cytoskeleton (data not shown).

siRNA-mediated knockdown of $\alpha 4$ integrin blocks the actin disrupting activity of HepII

We next investigated whether $\alpha 4$ integrin contributes to the HepII-mediated disruption of the actin cytoskeleton in TM cells, as the PPRARI sequence of the HepII domain has already been identified as a potential $\alpha 4$ binding site [22]. Expression of the $\alpha 4$ integrin subunit was silenced using $\alpha 4$ -specific siRNA. FACS analysis of TM-1 cells transfected with this siRNA revealed that cell surface expression of $\alpha 4$ was reduced by 89% (Figure 3A). F-actin staining of TM-1 cells showed that silencing the expression of the $\alpha 4$ integrin on the cell surface did not affect assembly of actin stress filaments. The actin staining of cells transfected with $\alpha 4$ siRNA closely resembled that of the TM-1 cells treated with vehicle or transfected with non-targeting control siRNA. In all groups, bundles of actin filaments and intact monolayers were observed (Figure 3B). Silencing the expression of the $\alpha 4$ integrin prevented the HepII-mediated disruption of the TM cell actin cytoskeleton, as assembled actin filaments and intact monolayers were observed even after treatment with the HepII domain. In contrast, when control TM-1 cells were incubated with the HepII domain, assembled actin filaments were not observed and large gaps in the monolayers were visible.

Previous studies showed that PPRARI is the active site in the HepII domain that disrupts the actin cytoskeleton in TM-1 cells while IDAPS has no effect [29]. To further demonstrate that the activity of the HepII domain is $\alpha 4$ integrin dependent, $\alpha 4$ integrin expression was silenced in TM-1 cells with siRNA, and the cells were dosed with synthetic peptides containing integrin binding sequences PPRARI and IDAPS. In both the control and IDAPS treatment groups, all cultures displayed extensive actin filament bundles, regardless of whether expression of the $\alpha 4$ integrin subunit was silenced or not (Figure 4). The PPRARI peptide, however, only disrupted the actin cytoskeleton when the $\alpha 4$ integrin subunit was present in the untransfected cells. In cultures in which the $\alpha 4$ integrin was silenced, the levels of F-actin staining were comparable to controls. These findings suggest that the disruption of the actin cytoskeleton of TM cells mediated by PPRARI involves the $\alpha 4$ integrin subunit.

To investigate the possibility that $\alpha 4\beta 1$ and/or $\alpha 4\beta 7$ integrins were involved [41], FACS analysis was conducted to assess expression levels of $\beta 1$ and $\beta 7$ integrin subunits on the surface of TM cells. We observed abundant expression of $\beta 1$ integrin on the surface of TM-1 cells, while $\beta 7$ integrin expression was minimal (data not shown). Thus, $\alpha 4\beta 1$ integrin is most likely responsible for the HepII domain-mediated disruption of the actin cytoskeleton in TM cells.

Activation of $\alpha 4\beta 1$ enhances binding to HepII

The activation state of $\alpha 4\beta 1$ integrin has been shown to be governed by divalent cations. Mn^{2+} increases the affinity for ligand binding to $\alpha 4\beta 1$ integrins by inducing a conformational change in the $\beta 1$ integrin subunit, while Ca^{2+} has been shown to inhibit this effect [42]. To determine if the activation state of $\alpha 4\beta 1$ affected binding to the HepII domain, TM-1 cells were plated on increasing concentrations of the HepII domain in the absence or presence of Ca^{2+} and Mn^{2+} ions. In the presence of 2 mM Mn^{2+} , TM-1 cell binding to HepII was increased compared to cells that were plated in the absence of cation (Figure 5A). Substitution of the Mn^{2+} ion with Ca^{2+} eliminated this effect, suggesting that the affinity states of $\alpha 4\beta 1$ integrin regulate the interaction with the HepII domain.

To confirm that the activation state of $\alpha 4\beta 1$ was involved in its binding to the HepII domain, cells were plated on 250 $\mu g/ml$ HepII in the absence or presence of the activating anti- $\beta 1$ antibodies, 12G10 and TS2/16. Previous have shown that while 12G10 binding to $\alpha 4\beta 1$ integrin is stimulated by Mn^{2+} and inhibited by Ca^{2+} , TS2/16 binding to the integrin is not influenced by the presence of either divalent cation [43]. Figure 5B shows that the 12G10 antibody decreased binding to HepII, which is consistent with other studies in which 12G10 treatment reduced cell binding to $\alpha 4\beta 1$ ligands [12]. 12G10 also blocked cell attachment in the presence of Mn^{2+} , which enhances binding of 12G10 to the $\beta 1$ integrin,. In contrast, addition of Ca^{2+} to the cell culture medium, which prevents binding of 12G10 to the $\beta 1$ integrin, blocked the ability of the antibody to prevent cell attachment. This presumably occurred because the integrin was still available to interact with the HepII domain. The TS2/16 antibody, on the other hand, only slightly decreased cell binding to HepII, and its interaction with the $\beta 1$ integrin was not influenced by either cation, as previously shown [43]

HepII decreases collagen gel contractility by TM cells

Because $\alpha 4$ integrin activation decreases contractility of migrating lymphocytes and neutrophils [44], we investigated whether activation of the $\alpha 4$ integrin by the HepII domain would decrease contractility of TM cells. To assess changes in contractility, TM-1 cells were seeded at confluency onto collagen I gels and treated with varying concentrations of HepII. Gel diameter was then measured at 1, 6, 18, and 24 h after detachment from the well. A significant decrease in contractility was evident as early as 1 h in cells treated with 500 $\mu g/ml$ HepII and in as little as 6 h in cells incubated with 250 $\mu g/ml$ HepII (Figure 6). The greatest decrease in contractility relative to untreated cells was observed at 24 h in cells treated with 500 $\mu g/ml$ HepII.

To determine if the PPRARI sequence within the HepII domain was responsible for the decrease in contractility, the assay was also performed with 0.5, 1, 2, and 4 mg/ml of the PPRARI peptide. As expected from our previous studies [29], PPRARI was less effective than HepII and required higher concentrations to disrupt the contractility of the TM cells. At 1 h, a 20% decrease in contractility was observed in cells treated with either 500ug/ml or 1 mg/ml PPRARI, while no decrease in contractility was noticeable in cells treated with either the inactive PPAARI or IDAPS peptides (data not shown). However, a higher concentration (2mg/ml) of PPRARI decreased contractility by 60% indicating that PPRARI was the site in the HepII domain responsible for the decrease in contractility.

HepII increases outflow facility and expands area underneath Schlemm's canal in monkey organ cultured anterior segments (MOCAS)

Previous studies have shown that decreases in contractility of the TM are associated with an expansion of the space underlying Schlemm's canal in the TM of the eye and an increase in outflow facility [45]. To determine if the HepII domain alters the contractile properties of TM tissue, cultured anterior segments containing the TM were perfused with the HepII domain and outflow facility measurements before and after treatment with DMEM or the HepII domain were obtained. The anterior segments were also fixed before the HepII domain was washed out to permit observation of the region underlying Schlemm's canal. Table 1 shows that the HepII domain increased outflow facility in 2 out of 3 MOCAS after an overnight infusion, suggesting a decrease in the contractile properties of the TM (Figure 7A). Light micrographs of the anterior segments perfused with the HepII domain (Figure 7C) revealed an expansion of the space between the inner wall of Schlemm's canal and the trabecular collagen beams in the TM compared to control segments (Figure 7B). The MOCAS that did not respond to HepII treatment, however, did not show an expansion of the space underlying Schlemm's canal (data not shown). This further suggests that the HepII domain regulates outflow by altering the contractile properties of the TM.

HepII signaling involves collagen and $\alpha 1/\alpha 2$ integrins

Previous studies have shown that the HepII domain used $\alpha 4\beta 1$ integrins to activate formation of actin stress fibers in TM cells during cell attachment assays on the fibronectin [31]. Studies have also shown that type IV collagen is found in the extracellular matrix of confluent TM-1 cultures [32]. To determine if HepII signaling in confluent cultures was affected by the presence of type IV collagen, TM-1 cells were plated on 10 $\mu\text{g/ml}$ type IV collagen or plasma fibronectin and treated with 500 $\mu\text{g/ml}$ of the HepII domain. Cells plated on fibronectin were well-spread and displayed strong actin filaments (Figure 8A, B). The majority of cells plated on type IV collagen appeared similar to those plated on fibronectin, although a few cells appeared to be rounded. When treated with the HepII domain most cells plated on type IV collagen exhibited the rounded morphology and contained a disorganized actin cytoskeleton. In contrast, cells plated on fibronectin and treated with HepII did not differ in appearance from control cells, suggesting that the actin disrupting function of HepII in these cultures was dependent on the presence of type IV collagen.

We next wanted to identify which subunits were involved in binding TM-1 cells to type IV collagen, which is known to contain binding sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins [46]. Figure 8C shows that binding of TM-1 cells to 10 $\mu\text{g/ml}$ type IV collagen was not blocked by antibodies for $\alpha 1$, $\alpha 2$, or $\alpha \nu\beta 3$ integrins. However, incubating the cells with both $\alpha 1$ and $\alpha 2$ antibodies significantly decreased cell binding, suggesting that both of $\alpha 1$ and $\alpha 2$ integrins are involved in the attachment of TM-1 cells to type IV collagen.

DISCUSSION

This study shows that interactions between the PPRARI sequence in the HepII domain and a co-signaling pathway involving $\alpha 4\beta 1$ integrin regulate the contractile properties of TM cells. This co-signaling pathway occurs in the presence of both type I and IV collagens in the extracellular matrix, as activation of this HepII-mediated signaling pathway decreased the contractility of TM cells in collagen gels, of cell plated on type IV collagen, and in cultured anterior segments where both collagens are prevalent [47]. Taken together, these results suggest that the $\alpha 4\beta 1$ integrin may be part of a signaling mechanism along with an $\alpha 1/\alpha 2\beta 1$ collagen integrin signaling pathway that regulates TM contractility in the eye. This finding has special significance for the TM of the eye where changes in the composition of the extracellular matrix are often associated with increased intraocular pressure and glaucoma. Furthermore, it

demonstrates how critical the microenvironment of the cell is for the modulation of cell behavior via integrins.

These findings reflect a novel function for the PPRARI site in the HepII domain of fibronectin and for $\alpha 4\beta 1$ integrin in contractile tissues. $\alpha 4\beta 1$ integrin is usually found on non-adherent cells such as lymphocytes and melanomas where it plays a major role in regulating contractility during migration [48,49]. Although $\alpha 4\beta 1$ integrin has been reported to be expressed on adherent cells including TM cells, retinal ganglion cells, interstitial fibroblasts in the kidney, and smooth muscle cells [31,50–52], relatively little is known about the role of $\alpha 4\beta 1$ integrins outside of the immune system. These studies suggest $\alpha 4\beta 1$ signaling in adherent cells is similar to that which occurs in non-adherent cells and that $\alpha 4\beta 1$ signaling regulates contractile processes in tissues like the TM.

Our cell adhesion studies showed that TM cells used both $\alpha 1$ and $\alpha 2$ integrins to mediate adhesion to the collagen matrix which would suggest involvement of an $\alpha 4\beta 1$ and $\alpha 1/\alpha 2\beta 1$ integrin co-signaling pathway. To the best of our knowledge, this is the first time that this integrin co-signaling pathway has been observed. Such a co-signaling pathway would explain the apparent discrepancy with the previously reported actions of HepII. In the current study, the HepII domain decreased actin polymerization and contractility of TM cells on collagen substrates, whereas in previous studies the cells were plated on fibronectin or the RGD cell binding domain of fibronectin which interacts with $\alpha 5\beta 1$ integrin [31]. In those earlier studies,., Thus, the increase in actin stress fiber formation and contractility in the earlier studies involved an $\alpha 5\beta 1/\alpha 4\beta 1$ co-signaling pathway and not a distinct $\alpha 4\beta 1$ and $\alpha 1/\alpha 2\beta 1$ pathway as indicated by this study.

Interestingly, the PPRARI peptide, but not the IDAPS peptide, triggered the $\alpha 4\beta 1$ signaling pathway in TM cells and increased outflow facility in cultured anterior segments [29]. IDAPS is a RGD homologue located at the III₁₃-III₁₄ junction of the HepII domain and was originally identified as the $\alpha 4\beta 1$ binding site in the HepII domain [53]. More recent structural studies suggest that the PPRARI sequence within the III₁₄ repeat is the main $\alpha 4\beta 1$ integrin binding site and that IDAPS contributes indirectly to $\alpha 4\beta 1$ binding by forming a stabilizing hydrogen bond with the PPRARI site [22]. Alternatively, ligand-specific conformational differences in the $\alpha 4\beta 1$ active state may account for varying activities of the two peptides. PPRARI, which binds the $\alpha 4$ subunit, most likely induces an $\alpha 4\beta 1$ conformation that is unique from IDAPS, which binds to the $\beta 1$ subunit [22,53]. Such differences in the conformation of $\alpha 4\beta 1$ have been reported to regulate integrin function [54] and are consistent with our observations that the 12G10 induced conformation of $\alpha 4\beta 1$, but not the TS2/16 induced conformer, significantly blocked binding to the HepII domain. It is also consistent with previous studies in which the 12G10 antibody produced a conformation of $\alpha 4\beta 1$ that lead to the disruption of the cytoskeleton [12]. Furthermore, other $\alpha 4\beta 1$ ligands such as the QIDSP peptide from VCAM and the IIICS domain of fibronectin did not have a significant effect on TM cellular contractility (data not shown), suggesting that there is something unique about the activation state of $\alpha 4\beta 1$ integrins and their interactions with the PPRARI sequence.

Based on the observations with the 12G10 antibodies and the enhanced binding of TM-1 cells to HepII in the presence of Mn^{2+} , we propose that the PPRARI sequence in the HepII domain interacts with an activated conformation of $\alpha 4\beta 1$ integrins. We further suggest that this conformation of $\alpha 4\beta 1$ integrin only exists when a collagen signaling pathway is activated. Whether, this collagen signaling pathway is regulating the conformation of $\alpha 4\beta 1$ is unknown. This co-signaling pathway is likely to play a significant role in regulating intraocular pressure where the remodeling of the extracellular matrix that occurs in the eye under conditions of increased intraocular pressure would result in the release of fibronectin fragments.

The outflow study supports previous findings in which the HepII domain and the PPRARI peptide increased outflow facility in cultured human and monkey anterior segments [29,36]. Response to the peptides and the HepII domain were not uniform and some of the donor anterior segments did not respond to the HepII treatment. In the study by Gonzalez *et al.* [29] only five of nine monkey anterior segments responded to treatment with the HepII domain, and in cultured human anterior segments, 9 out of the 10 responded [36]. Once again, this raises the questions whether cooperative integrin signaling events are involved in regulating contractility and whether outflow facility in the eye and cultured anterior segments that did not respond are lacking a necessary co-signaling factor.

Activation of $\alpha 4\beta 1$ integrin by the HepII domain did not require HSPGs or the heparan sulfate binding activity of the HepII domain. Neither removal of heparan sulfates from the cell surface of TM cells nor treatment of TM cells with a mutant HepII domain that lacks the major heparan sulfate binding site affected the activity of HepII. In addition, siRNA specific knockdown of syndecan-4 expression did not abolish the actin disrupting activity of the HepII domain. Therefore, it appears that HepII acts strictly through an $\alpha 4\beta 1$ -dependent mechanism and does not require participation by syndecans or other cell surface HSPGs. This supports previous studies which showed that $\alpha 4\beta 1$ integrin, unlike $\alpha 5\beta 1$ integrin, does not require a proteoglycan as a co-receptor [55]. It also supports previous studies utilizing human skin fibroblasts and TM cells in which heparan sulfate interactions were shown to play a minor role in HepII-mediated cell spreading [56] and stress fiber formation [31], respectively.

Although HSPGs are not co-receptors for $\alpha 4\beta 1$ integrin in the TM, other protein-protein interactions may co-direct $\alpha 4\beta 1$ signaling. For example, it has been shown that the core protein of chondroitin sulfate glycosaminoglycan interacts directly with $\alpha 4\beta 1$ integrins [57]. Additionally, cooperative signaling in TM cells between $\alpha 4\beta 1$ and $\alpha v\beta 3$ has been reported to enhance the formation of cross-linked actin networks [58], and cross-talk between $\alpha 4\beta 1$ and $\alpha 5\beta 1$ in sub-confluent proliferating TM cells plated on fibronectin, augments cell attachment by increasing actin stress fiber formation and focal adhesion kinase (FAK) phosphorylation [31]. Thus, the $\alpha 4\beta 1$ pathway in confluent cultures is likely to involve cooperative signaling events with cell adhesion receptors which trigger the down-regulation of actin.

The finding that $\alpha 4\beta 1$ is involved in the down-regulation of actin assembly and contractility in TM cells was not completely unexpected. Studies using migrating lymphocytes, neutrophils, and melanoma cells have shown that the $\alpha 4$ subunit plays a key role in regulating contractility [44]. Protein kinase A phosphorylation of Ser⁹⁸⁸ on the $\alpha 4$ subunit blocks binding of the adaptor protein paxillin to the cytoplasmic tail of the $\alpha 4$ integrin subunit thereby preventing the establishment of connections with the actin cytoskeleton and activation of small GTPases such as Rac, CDC42, and Rho [59]. While these studies suggest that an $\alpha 4\beta 1$ integrin mediated signaling pathway down-regulates contractility in the TM and plays a role maintaining intraocular pressure, further studies examining how a collagen mediated signaling pathway affects $\alpha 4\beta 1$ integrin signaling in the TM are warranted.

Abbreviations

BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
FACS	fluorescence-activated cell-sorting
HepII	heparin II
HSPG	heparan sulfate proteoglycan

MAdCAM-1	mucosal addressin cell adhesion molecule-1
MLC	myosin light chain
PBS	phosphate-buffered saline
TBS	tris-buffered saline
TM	trabecular meshwork
VCAM-1	vascular cell adhesion molecule-1

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Figure 1. Knockdown of syndecan-4 does not inhibit HepII-mediated disruption of cytoskeleton
 (A) TM-1 cells were transfected with vehicle, non-targeting control siRNA, or syndecan-4 specific siRNA. Cells were labeled with the 8G3 anti-syndecan-4 antibody for FACS analysis. Solid black corresponds to isotype control and gray line represents population of cells expressing syndecan-4 on the cell surface. Each histogram has been normalized to its maximum counts (% of Maximum). (B) Transfected TM-1 cells were grown to confluence and treated with 500 μ g/ml of the HepII domain as described in *Materials and Methods*. Cells were then permeabilized, fixed, and labeled with Alexa 488-conjugated phalloidin to visualize F-actin. Arrows point to actin filaments and asterisks mark gaps in monolayer. Scale bar: 20 μ m.

Figure 2. Heparitinase III treatment does not prevent the disruption of TM-1 actin filaments by the HepII domain

Confluent monolayers of TM-1 cells were treated with vehicle or 0.01 IU/ml heparitinase III every 2 h for a total of 14 h. After the first 2 h of treatment, 250 μ g/ml of the HepII domain was added. Cells were either fixed, permeabilized, and labeled with Alexa 488-conjugated phalloidin to visualize F-actin, or they were fixed and labeled with the 10E4 antibody to detect cell surface heparan sulfate. Scale bars: 25 μ m (top row) and 50 μ m (bottom row).

Figure 3. Knockdown of $\alpha 4$ integrin inhibits HepII-mediated disruption of cytoskeleton
 (A) TM-1 cells were transfected with vehicle, non-targeting control siRNA, or $\alpha 4$ siRNA (Dharmacon). Transfected cells were labeled with the P1H4 antibody against the $\alpha 4$ integrin and untransfected TM-1 cells were labeled with the HB1.1 antibody to the $\beta 1$ integrin and the FIB504 antibody to the $\beta 7$ integrin. Solid black peak corresponds to isotype control and gray line represents specific antibody, as indicated. Histograms were normalized to maximum counts (% of Maximum). (B) At confluence, transfected TM-1 cells were treated with 500 $\mu\text{g}/\text{ml}$ of the HepII domain as described in *Materials and Methods*. Transfected cells were permeabilized fixed, and labeled with Alexa 488-conjugated phalloidin to visualize F-actin. Scale bar: 20 μm .

Figure 4. Knockdown of $\alpha 4$ integrins diminishes the ability of PPRARI to disrupt the actin cytoskeleton of TM cells

TM-1 cells were transfected with non-targeting control siRNA or siRNA targeting $\alpha 4$ integrin (Invitrogen). At confluence, cells were treated with vehicle, 4 mg/ml of the IDAPS peptide, or 4 mg/ml of the PPRARI for 19 h. Cells were then permeabilized, fixed, and labeled with Alexa 488-conjugated phalloidin to visualize F-actin, as described in *Materials and Methods*. Scale bar: 25 μ m.

Figure 5. Cell binding to HepII is enhanced by activation of $\alpha 4\beta 1$ via Mn^{2+}

(A) TM-1 cells were pre-incubated in the absence or presence of 2 mM of Ca^{2+} or Mn^{2+} for 30 min and plated on increasing concentrations of the HepII domain for 1 h. Attached cells were stained with 0.5% toluidine blue and absorbance of the solubilized dye was measured at 600 nM, as described in *Materials and Methods*. (B) TM-1 cells were also pre-incubated in the absence or presence of a divalent cation and 25 μ g/ml of the activating $\beta 1$ antibodies, 12G10 or TS2/16. Cells were then plated on 250 μ g/ml HepII for 1 h.

Figure 6. HepII decreases collagen gel contraction by TM cells

TM-1 cells were plated on rat tail collagen I gels for 24 h and were then incubated with 0, 100, 250, or 500 µg/ml of the HepII domain. Gels were released and their diameters were measured at 1, 6, 18, and 24 h. Values are expressed as mean percent contraction relative to untreated control cells (dashed line) from a minimum of three independent experiments. Error bars represent standard deviation. * $P < 0.005$ and ** $P < 0.0001$, compared with untreated group (Student's t -test).

Figure 7. HepII increases outflow facility and expands space underneath Schlemm's canal in MOCAS

(A) MOCAS were perfused with DMEM or 100 $\mu\text{g/ml}$ HepII. Facility change for each anterior segment is the ratio of outflow facility after HepII or DMEM perfusion (OF_{RX}) divided by the baseline facility (OF_{BL}). MOCAS from each donor were embedded in Epon 812 and semi-thin sections were stained with toluidine blue. Representative merged light micrographs for (B) DMEM and (C) HepII treated samples. TM, trabecular meshwork; SC, Schlemm's canal; brackets, region underlying Schlemm's canal. Scale bar: 100 μm .

Figure 8. HepII-mediated disruption of actin is dependent upon type IV collagen and not fibronectin

TM-1 cells were plated on 10 $\mu\text{g/ml}$ plasma fibronectin or 10 $\mu\text{g/ml}$ type IV collagen, grown overnight, and incubated with 500 $\mu\text{g/ml}$ of the HepII domain in serum free DMEM. (A) Phase images were acquired 24 h after HepII treatment. Scale bar: 100 μm . (B) Cells were also permeabilized, fixed, and labeled with Alexa 488-conjugated phalloidin to visualize F-actin, as described in *Materials and Methods*. Scale bar: 20 μm . (C) TM-1 cells were plated on 10 $\mu\text{g/ml}$ type IV collagen in the absence or presence of 25 $\mu\text{g/ml}$ antibodies against $\alpha 1$ (FB12), $\alpha 2$ (P1E6), $\alpha 4$ (P1H4), or $\alpha v \beta 3$ (LM609). Attached cells were quantified using a toluidine blue cell adhesion assay, as described in *Materials and Methods*. Values are mean percentage of

IgG control $\pm$ standard deviation. $*P < 0.005$, compared with untreated group (Student's t -test).

Table 1

Effect of HepII Domain on Outflow Facility

Donor	Treatment	OF _{BL}	OF _{Rx}	Facility Change	% Change
1	DMEM	0.711	0.700	0.98	77.6
	HepII	0.532	0.926	1.74	
2	DMEM	0.816	1.031	1.26	38.9
	HepII	0.472	0.826	1.75	
3	DMEM	0.125	0.339	2.71	-26.9
	HepII	0.243	0.482	1.98	

Facility change for each anterior segment is expressed as the ratio of outflow facility after HepII or DMEM perfusion (OF_{Rx}) divided by the baseline facility (OF_{BL}). The percent change in outflow facility induced by HepII treatment is expressed as $[(\text{OF}_{\text{Rx}}/\text{OF}_{\text{BL}})/\text{HepII}/(\text{OF}_{\text{Rx}}/\text{OF}_{\text{BL}})/\text{DMEM}] - 1 \times 100$.