Heparin II Domain of Fibronectin Uses α4β1 Integrin to Control Focal Adhesion and Stress Fiber Formation, Independent of Syndecan-4*

Received for publication, June 14, 2004, and in revised form, November 24, 2004 Published, JBC Papers in Press, November 30, 2004, DOI 10.1074/jbc.M406625200

Jennifer A. Peterson‡, Nader Sheibani§, Guido David‖, Angeles Garcia-Pardo**, and Donna M. Peters‡‡

From the Departments of Pathology and Laboratory Medicine and Ophthalmology and Visual Sciences, University of Wisconsin, Madison, Wisconsin 53706, Human Genetics, Flanders Interuniversity Institute for Biotechnology, University of Leuven, 3000 Leuven, Belgium, and Departamento de Immunologia, Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Científicas, 28006 Madrid, Spain

Co-signaling events between integrins and cell surface proteoglycans play a critical role in the organization of the cytoskeleton and adhesion forces of cells. These processes, which appear to be responsible for maintaining intracellular pressure in the human eye, involve a novel cooperative co-signaling pathway between α5β1 and α4β1 integrins and are independent of heparan sulfate proteoglycans. Human trabecular meshwork cells isolated from the eye were plated on type III 7–10 repeats of fibronectin (α5β1 ligand) in the absence or presence of the heparin (Hep) II domain of fibronectin. In the absence of the Hep II domain, cells had a bipolar morphology with few focal adhesions and stress fibers. The addition of the Hep II domain increased cell spreading and the numbers of focal adhesions and stress fibers. Cell spreading and stress fiber formation were not mediated by heparan sulfate proteoglycans because treatment with chlorate, heparinase, or soluble heparin did not prevent Hep II domain-mediated cell spreading. Cell spreading and stress fiber formation were mediated by α4β1 integrin because soluble anti-α4 integrin antibodies inhibited Hep II domain-mediated cell spreading and soluble vascular cell adhesion molecule-1 (α4β1 ligand)-induced cell spreading. This is the first demonstration of the Hep II domain mediating cell spreading and stress fiber formation through α4β1 integrin. This novel pathway demonstrates a cooperative, rather than antagonistic, role between α5β1 and α4β1 integrins and suggests that interactions between the Hep II domain and α4β1 integrin could modulate the strength of cytoskeleton-mediated processes in the trabecular meshwork of the human eye.

Adhesive interactions between cells and extracellular matrix molecules form highly specific yet very diverse signaling conduits that regulate a wide variety of biological processes associated with cell morphology, proliferation, differentiation, migration, and survival (1–5). These adhesive events are mediated mainly by the integrin family of receptors and lead to the formation of dynamic multi-molecular structures called focal adhesions, focal complexes, and fibrillar adhesions (6, 7).

The formation of focal contacts often depends on co-signaling events between integrins and cell surface proteoglycans. In fibroblasts and A375-SM melanoma cells plated on fibronectin, cooperative signaling between α5β1 integrin and syndecan-4, a cell surface heparan sulfate proteoglycan (HSPG), is needed to promote the formation of focal adhesions and stress fibers (8, 9). If fibronectin-null fibroblasts are plated on anti-β1 integrin antibodies or the RGD cell binding domain of fibronectin, cells bind but do not assemble the actin cytoskeleton. However, if soluble antibody directed against the ectodomain of syndecan-4 is added, the cells will assemble focal adhesions and stress fibers (10). A similar scenario has been documented for α4β1 integrin and cell surface chondroitin sulfate proteoglycans, supporting the idea that co-engagement of integrins and proteoglycans results in cooperative signaling (11, 12). These co-signaling events are often mediated by two different but adjacent sites within fibronectin. For instance, fibroblasts plated on the RGD cell binding domain of fibronectin can adhere, but they require additional signals from the heparin (Hep) II domain of fibronectin to form focal adhesions and stress fibers (13–15).

Co-signaling between integrins, in contrast, appears to result in antagonistic signaling. In melanoma cells and lymphocytes, the co-engagement of α5β1 and α4β1 integrins prevents the formation of focal adhesions and stress fibers that would normally form when only α5β1 integrin is engaged (16, 17). Likewise, α4β1 integrin suppresses metalloprotease expression transduced by α5β1 integrin in synovial fibroblasts (18), and in Chinese hamster ovary cells, the engagement of α1β3 integrin down-regulates the functions of α5β1 and α2β1 integrins (19).

The ability of co-signaling events to differentially mediate these processes lies in the fact that different integrins utilize different signaling mechanisms to trigger focal adhesion for-

* This work was supported in part by NEI Grants EY12515 (to D. M. P.) and EY13700 (to N. S.), National Fund for Scientific Research—Flanders Grant G.0239.01 (to G. D.), and Ministerio de Educacion y Ciencia (Spain) Grant SAF2003-00824 (to A. G.-P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Dept. of Pathology and Laboratory Medicine, Rm. 6590 MSC, 1300 University Ave., Madison, WI 53706. Tel.: 608-262-4626; Fax: 608-265-3301; E-mail: dmpeter2@facstaff.wisc.edu.

The abbreviations used are: HSPG, heparan sulfate proteoglycan; Hep, heparin; HTM, human trabecular meshwork; VCAM, vascular cell adhesion molecule; FAK, focal adhesion kinase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; mAb, monoclonal antibody; pAb, polyclonal antibody; Ab, antibody; FACS, fluorescence-activated cell-sorting; MES, 2-(N-morpholino)ethanesulfonic acid; TBS, Tris-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
A Novel Hep II-mediated Signaling Pathway Involving α5β1

mation. For instance, signaling events mediated by α5β1 integrin require a HSPG co-receptor such as syndecan and involve the activation of protein kinase Ca. In contrast, α4β1 integrin-mediated focal adhesion formation is independent of syndecans and protein kinase Ca activation (8). The cytoplasmic domain of α4 integrin also interacts directly with the signaling adapter protein paxillin, in contrast to α5β1 integrin, which does not directly interact with paxillin (20).

In this study, we investigated the need for co-signaling between integrins and syndecans in focal adhesion and stress fiber formation using primary diploid human trabecular meshwork (HTM) cells. HTM cells are a unique cell type found in the anterior chamber of the human eye. The cytoskeletal organization and adhesive forces of these cells play a key role in maintaining intraocular pressure. Chemical agents that disrupt the cytoskeleton or the signaling pathways that maintain the actomyosin network, such as H-7, cytochalasin, and latrunculins, cause a decrease in intraocular pressure (21–25). Therefore, understanding the signaling pathways that regulate these processes is critical for understanding the mechanisms by which intraocular pressure is maintained. Recently, the Hep II domain of fibronectin was shown to lower intraocular pressure in a human eye organ culture system, suggesting that cell-matrix signaling events mediated by the Hep II domain may be involved in controlling intraocular pressure (26). Using a recombinant Hep II domain and the type III 7–10 repeats of fibronectin, the present studies show that limited focal adhesion and stress fiber formation can be mediated by α5β1 integrin independently of a syndecan co-receptor and that α4β1 integrin signaling mediated by the Hep II domain can augment focal adhesion and stress fiber formation in HTM cells. This is the first report that α4β1 integrin can act as a co-receptor for α5β1 integrin and that the α5β1 and α4β1 signaling pathways converge to enhance focal adhesion and stress fiber formation. This is also the first time that the specific activation of α4β1 integrin by the Hep II domain of fibronectin has been demonstrated. This dual signaling through α5β1 and α4β1 integrins may serve to control the adhesive strength and contractility of HTM cells and may provide a mechanism by which these cells can regulate intraocular pressure.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Proteins**—Recombinant Hep II domain (type III 12–14 repeats of fibronectin) and the type III 7–10 repeats of fibronectin (Fig. 1) were made as described previously (26, 29). Recombinant type III 4–5 repeats of fibronectin (Hep III domain) was made as described previously (12). The mutated Hep II domain (Hep II/RK) was prepared using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions by mutating the residues Arg9 and Lys25 in the major heparin binding site (CATTAGCTGGAGAACCTCGACTGAGACGATCACTG-3′) (forward) and 5′-CCACAGAAGGGCTTCTGTGACAGATGCTACTG-3′ (reverse) (26). The changed nucleotides are underlined. Hep II/RK, which was produced in the bacterial expression vector pGEX 4T1, was then expressed as a recombinant Hep II domain and the type III 7–10 repeats of fibronectin in E. coli (Escherichia coli) or the serine/threonine kinase inhibitor H-7 (Sigma-Aldrich) was added 1 h after plating the cells.

To determine the involvement of HSPGs in Hep II domain-mediated cell spreading, cells were pretreated with 30 mM sodium chloride for 24 h in sultafate-free media and 15% sultafate-free serum, followed by an additional 24 h in the same medium without serum as described previously (12, 32, 33). As a control, cells were incubated with 10 mM sodium sulfate or 30 mM sodium chloride and 10 mM sodium sulfate. In other experiments, soluble heparin (50 μg/ml) was added as an inhibitor at the time of plating, or cells were treated with heparitinase and heparinase (0.0024 IU/ml; ICN Biomedicals, Inc., Irvine, CA). In these latter experiments, cells were incubated with these enzymes for 4 h before plating, with fresh enzyme added after 2 h and throughout the spreading assay (34).

To determine whether α4β1 integrin was involved in cell spreading, an α4 integrin blocking antibody (25 μg/ml; clone A4-PUJI; Upstate Group, Inc., Charlottesville, VA) was added at the time of plating in the presence and absence of 323 nM Hep II domain or 236 nM of recombinant vascular cell adhesion molecule (VCAM)-1 containing the seven extracellular domains in the spreading assay. VCAM-1 was kindly provided by Dr. Deane Mosher (University of Wisconsin, Madison, WI). HCAM-1, an integrin α4β1 competitor, was also added (34).

**Immunofluorescence Microscopy**—HTM cells were washed with 50 mM MES at pH 6.0, permeabilized for 2 min with 0.5% Triton X-100 in 50 mM MES, and fixed for 30 min with 4% paraformaldehyde in PBS, pH 7.4. Cells were blocked for 1 h with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (BSA/PBS). Blocked cells were incubated with anti-vinculin antibodies (Sigma-Aldrich) diluted 1:3000 in PBS/PBS for 1 h and then incubated with Alexa 488-conjugated goat anti-mouse secondary antibody (Ab) (4 μg/ml; Molecular Probes, Eugene, OR) and Alexa 488-conjugated phalloidin (0.67 unit/ml; Molecular Probes) in 0.1% BSA/PBS for 1 h. Coverslips were mounted onto slides using Immuno-mount (Shandon Lipshaw, Pittsburgh, PA). To visualize cell surface heparan sulfate, cells were washed with PBS, fixed, blocked as described above, and incubated with mouse (IgM) antibody 10E4, which detects an epitope present in most heparan sulfates (Seikagaku America, Inc., East Falmouth, MA) (35). Cultures were then labeled with a rabbit anti-mouse IgM (Zymed Laboratories, Inc., San Francisco, CA) followed by an Alexa 546-conjugated goat anti-mouse secondary Ab. All labeling was done for 1 h in 0.1% BSA/PBS, and coverslips were mounted as described above. Cell images were acquired using a Zeiss LSM-410 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) and mounted on a Zeiss Axioplan 2 Imaging fluorescence microscope together with Axiosvision version 3.1 software. In some experiments, the extent of cell spreading was determined by measuring cell width and length on the computer screen using the Axiosvision software. Measurements of cells were made from 8–12 different fields of view per coverslip (n = 40). Only cells with clear borders were measured. The ratio of cell width to length was compared with control cell ratios from four different experiments (n = 160; Fig. 3) or two different experiments (n = 80; Fig. 8). In some cases, the number of cells positive for stress fibers was also counted. Cell counts were made from 8–10 different fields of view per coverslip (n = 50). The number of stress fiber-positive cells was compared with control cells from two different experiments for analysis (n = 100).

**F-actin Detection**—HTM cells (3 × 104 cells/well) were plated onto 22-mm square glass coverslips pre-coated with the type III 7–10 repeats (47 nt) in the presence or absence of the Hep II domain (472 nt). After 3 h, the coverslips were transferred to new wells, washed with PBS, and fixed for 10 min with 4% paraformaldehyde/PBS followed by permeabilization for 10 min with 0.5% Triton X-100/PBS. Cells were incubated with Alexa 488-conjugated phalloidin (0.67 unit/ml) in 0.1% BSA/PBS. Cells were washed, and bound phalloidin was released by incubating the cells for 2 h at room temperature in humidified chambers with 2 ml of methanol under mild agitation (36). The entire sample from each well was collected, and phalloidin levels were detected using the FluoroMax-3 spectrofluorometer (Jobin Yvon Horiba, Edison, NJ) and Data Max v.2.2 software with excitation/emission wavelengths of 495/519 nm.
Immunoblotting—HTM cells were plated onto 10-cm tissue culture plates in the presence or absence of the Hep II domain (472 nm). All plates were pre-coated with 236 nm of the type III 7–10 repeats (8.5 μg/ml) and blocked with 1% heat-denatured BSA/PBS (85 °C for 10 min) for 1 h at room temperature. After 0.5, 1, 2, or 3 h, cells were washed with PBS and lysed for 10 min at 4 °C with 15 mM CHAPS in 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 mM NaF, and 10 μg/ml of pepstatin, leupeptin, and aprotonin. Lysate (10 μg) from the control and treated cells was separated on an 8% SDS-PAGE and transferred to Immobilon-P (Millipore, Billerica, MA). The membrane was blocked in 3% BSA/TBS and incubated with either folic acid adhesion kinase (FAK) polyclonal antibody (pAb; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or phosphospecific FAK pY397 pAb (Upstate Group, Inc.) in 1% BSA/TBS/0.1% Triton X-100 for 1 h. Membranes were then washed with TBS/0.1% Triton X-100 and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit Ab (Santa Cruz Biotechnology, Inc.). Bound antibody was detected with the ECL Plus Western blotting detection kit (Amersham Biosciences). The area of the bands from three different experiments was measured using Scion Image software (Scion Corp., Frederick, MD) and averaged together to determine fold induction in FAK pY397 phosphorylation.

Cell Adhesion Assay—Serum-starved HTM cells were plated in the presence of (0.5 μg/ml) or without fibronectin (10 μg/ml) for 30 min on ice. Cells were washed and before plating the cells. Unbound cells were removed by washing with PBS. Bound cells were fixed for 20 min with 4% paraformaldehyde/PBS and stained overnight with 0.5% toluidine blue in 4% paraformaldehyde/PBS. Bound dye was redissolved in 2% SDS and detected at 600 nm using a microplate reader as described previously (37). Rat β1 integrin Ab m13 and rat α5 integrin Ab m16 were both kindly provided by Dr. Steve Akiyama (National Institutes of Health, Research Triangle Park, NC). The β3 integrin monoclonal antibody (mAb) 1 and the αv integrin mAb M9 were purchased from BD Biosciences and Chemicon International, Inc. (Temecula, CA), respectively.

Cell-activated Cell-sorting (FACS) Analysis—Adherent cells were removed with 2 mM EDTA in 0.05% BSA/TBS and washed. If syndecan expression was being detected, cells were resuspended in 1% BSA/TBS containing fluorescein isothiocyanate- or Alexa 488-conjugated anti-mouse or anti-rabbit second- ary Ab (0.5 μg/ml adhesion blocking M) was added 1 h after plating the cells. Y-27632 (7.5 μM) was added 1 h after plating the cells (A–D). Y-27632 (7.5 μM) was added 1 h after plating the cells (A–D). Concomitantly as was stress fiber formation and the number and intensity of focal adhesions (Fig. 2, E–G). Comparable results are seen when cells were plated on intact fibronectin or when soluble fibronectin was added to cells plated on the type III 7–10 repeats (data not shown). As shown in Fig. 3A, the Hep II domain significantly increased the number of stress fiber-positive cells by 56% over control cells. Measurements of cell width and length verified the immunofluorescent images and showed that the Hep II domain significantly enhanced cell spreading by 76% compared with control cells (Fig. 3B). Comconitantly with the increase in spreading, measurements of F-actin levels

![Fig. 1. Model of fibronectin.](image)

**FIG. 1.** Model of fibronectin. Each fibronectin molecule consists of two subunits disulfide-bonded at their carboxyl termini. Rectangles indicate type I repeats; black ovals indicate type II repeats. Circles are numbered to indicate specific type III repeats. The α5β1 integrin binding site (RGD) is in the type III 10 repeat. The Hep II domain is comprised of the type III 12–14 repeats. The main heparin binding site is in the type III 13 repeat and can bind heparin or heparan sulfate proteoglycans such as syndecans. The Hep II domain also contains an α4β1/7 integrin binding site in the type III 14 repeat (IDAPS).

![Fig. 2. Hep II domain increases stress fiber formation in a Rho kinase-dependent manner.](image)

**FIG. 2.** Hep II domain increases stress fiber formation in a Rho kinase-dependent manner. HTM cells were plated on type III 7–10 repeats for 3 h in the absence (A–D) or presence (E–H) of soluble Hep II domain. Y-27632 (7.5 μM) was added 1 h after plating the cells (D and H). Cells were labeled for vinculin to visualize focal adhesions (A and E; red) and phalloidin to visualize actin stress fibers (B and F; green). C and G are merged images of A and B and E and F, respectively. Bar = 20 μm.

**RESULTS**

The Hep II Domain Induces Stress Fiber Formation and Cell Spreading—To determine whether focal adhesion and stress fiber formation in HTM cells involved co-signaling, HTM cells were plated on the type III 7–10 repeats of fibronectin containing the RGD integrin binding site in the absence or presence of soluble Hep II domain (Fig. 1) for 3 h. As shown in Fig. 2, A–C, when HTM cells were plated on the type III 7–10 repeats in the absence of the Hep II domain, cells were able to attach and spread. Morphologically, however, the cells exhibited a bipolar morphology with few stress fibers and focal adhesions, indicating that they were not completely spread. If soluble Hep II domain was added to the media, cell spreading was enhanced, as was stress fiber formation and the number and intensity of focal adhesions (Fig. 2, E–G). Comparable results are seen when cells were plated on intact fibronectin or when soluble fibronectin was added to cells plated on the type III 7–10 repeats (data not shown). As shown in Fig. 3A, the Hep II domain significantly increased the number of stress fiber-positive cells by 56% over control cells. Measurements of cell width and length verified the immunofluorescent images and showed that the Hep II domain significantly enhanced cell spreading by 76% compared with control cells (Fig. 3B). Comconitantly with the increase in spreading, measurements of F-actin levels...
HSPGs Are Not Involved in Cell Spreading—Previous studies have indicated that the Hep II domain, which contains several HSPG binding sites (14, 30, 31), uses syndecan-4 to mediate complete spreading of fibroblasts on the type III 7–10 repeats of fibronectin (10, 13–15). To determine whether HSPGs are involved in Hep II domain-mediated spreading of HTM cells, FACS analysis was first performed using antibodies against the ectodomains of all four syndecans to determine the syndecan expression profile of HTM cells. As shown in Fig. 4, HTM cells only express syndecan-1 at the cell surface. Syndecans-2, -3, and -4 were not found at the cell surface. Expression of syndecan-4 was further examined using immunofluorescence microscopy experiments. This study showed that a few HTM cells express syndecan-4 at the cell surface, and the level of expression was very low. In most of the cells, syndecan-4 was found intracellularly, mainly in the Golgi and endoplasmic reticulum (data not shown). The significance of this is unknown, but such low levels of cell surface syndecan-4 suggest that it is unavailable as a coreceptor in HTM cells. These results suggest that syndecans are not involved in the formation of focal adhesions in HTM cells because syndecan-1 has yet to be implicated in focal adhesion and stress fiber formation.

To rule out the possibility that the Hep II domain was interacting with syndecan-1 or -4 to control focal adhesion and stress fiber formation, cells were pre-treated with heparitinase and heparinase for 4 h before plating and throughout the cell spreading assay. Previous studies have indicated that removal of heparin sulfate moieties from the core protein of syndecan will abolish interactions between the Hep II domain and syndecans. As shown in Fig. 5, C and D, heparitinase and heparinase treatment had no inhibitory effect on Hep II domain-mediated cell spreading of HTM cells plated on the type III 7–10 repeats. To verify that heparitinase and heparinase treatment successfully removed heparan sulfate moieties, cells were labeled with the monoclonal 10E4 antibody that detects an epitope present in most heparan sulfates. Enzymatic treatment abolished 10E4 antibody labeling (Fig. 5, E and F), indicating the cleavage of heparan sulfate. Enzymatic treatment alone had no effect on cell morphology (Fig. 5B). In addition, soluble heparin had no inhibitory effect on Hep II domain-mediated spreading, nor did chlorate treatment, which blocks sulfation of heparan sulfate glycosaminoglycan chains (41) (data not shown). As a final approach, a Hep II/RK domain that had an arginine and lysine residue mutated to serine in the heparin
binding cationic cradle in the type III 13 repeat (30) was tested for its ability to mediate cell spreading. This Hep II/RK domain, which was no longer able to bind heparin (data not shown), mediated cell spreading to the same extent as intact Hep II domain (Fig. 7E). Taken together, the data support the idea that HSPGs are not necessary for Hep II domain-mediated cell spreading of HTM cells plated on the type III 7–10 repeats.

**Hep II Domain Mediates Cell Spreading via α4β1 Integrin**—In addition to having HSPG binding sites, the Hep II domain also has a potential α4β1 integrin binding site, IDAPS, in the type III 14 repeat (42, 43). As shown in Fig. 6, FACS analysis indicated that HTM cells express α4β1 integrin on their cell surface along with α2, α3, α5, β1, αvβ3, αvβ5, and α5β1 integrins. To determine whether α4β1 integrin could mediate cell spreading on the type III 7–10 repeats, HTM cells were incubated with soluble extracellular domain of VCAM-1, which is a known α4β1 integrin ligand (44). Similar to the Hep II and Hep II/RK domains (Fig. 7, C and E), soluble VCAM-1 induced spreading and increased stress fiber formation in cells plated on the type III 7–10 repeats of fibronectin (Fig. 7G), indicating that HTM cell spreading on type III 7–10 repeats is α4β1 integrin-dependent. To determine whether all α4β1 integrin binding sites found in fibronectin induce cell spreading, cells plated on the type III 7–10 repeats were also incubated with soluble molar equivalents (472 nM) of the type III 4–5 repeats (12, 45) and the IIICS domain (46, 47). These studies showed that whereas the IIICS domain was equally effective as the Hep II domain in promoting cell spreading, the type III 4–5 repeats were less effective than the Hep II domain in promoting cell spreading (data not shown).

To determine whether the Hep II domain was using α4β1 integrin to mediate cell spreading, soluble α4 integrin blocking antibodies were added at the time of cell plating in the absence or presence of the Hep II domain, the Hep II/RK domain, or the soluble VCAM-1 extracellular domain. As shown in Fig. 7, the anti-α4 integrin antibodies blocked cell spreading in the presence of these ligands. These cells had fewer stress fibers and were more elongated than cells treated with soluble peptide alone (Fig. 7, D, F, and H). Soluble α4 integrin blocking antibody alone (Fig. 7B) or control non-immune IgG had no effect on cell spreading (data not shown), suggesting that cell spreading mediated by the Hep II domain involved α4β1 integrin. Comparison of the cell width versus length ratios verified these images and showed that the Hep II domain, Hep II/RK domain, and VCAM-1 increased cell spreading by 80%, 86%, and 77%, respectively, over control cells (Fig. 8), and the addition of soluble α4 integrin blocking antibody significantly reduced this increased cell spreading to 14%, 5%, and 2% of control cell spreading, respectively.

Interestingly, all the α4β1 ligands except for VCAM-1 had to be presented as a soluble ligand in order for cell spreading to be induced. Co-coating coverslips with the type III 7–10 repeats and the Hep II domain or the IIICS domain for the III4–5 repeats did not induce cell spreading above that observed with coverslips coated with the III 7–10 repeats alone (data not shown). Co-coating experiments were not done with the IIICS domain.

To determine which integrin(s) the HTM cells were using to attach to the type III 7–10 repeats, cell adhesion assays were performed in the presence of integrin blocking antibodies. As shown in Fig. 9, the HTM cells adhered to the type III 7–10 repeats via α5β1 integrin because cell adhesion was inhibited with α5 and β1 integrin blocking antibodies by 85% and 92%, respectively (p < 0.001), whereas αv and β3 integrin blocking...
antibodies had no inhibitory effect on cell adhesion. Thus, the partial cell spreading observed on the type III 7–10 repeats was due to interactions with αβ1 integrin. Cell spreading was never observed in HTM cells plated on the Hep II domain (data not shown). Thus, cell spreading in the presence of the Hep II domain was the result of αβ1/α4β1 co-signaling.

**DISCUSSION**

In this study, we showed that cooperative signaling between αβ1 and α4β1 integrins regulated cell spreading in trabecular meshwork cells and that neither αβ1 nor α4β1 integrin signaling alone was sufficient to mediate complete cell spreading. Activation of this signaling pathway occurred in the absence of syndecan-4 and utilized the Hep II domain as an α4β1 integrin ligand. This signaling pathway is in contrast to previously reported studies in fibroblasts in which αβ1 integrin-mediated cell spreading required syndecan-4 as a co-receptor and the Hep II domain was used as a syndecan-4 ligand. This is a novel signaling mechanism to control focal adhesion and stress fiber formation, and it indicates that different cell types utilize different receptor signaling pathways to control similar biological processes.

This novel integrin signaling pathway suggests that αβ1 and α4β1 integrin signaling pathways can converge to enhance cell spreading. In the presence of only the type III 7–10 repeats, αβ1 integrin-mediated cell attachment resulted in only partial cell spreading, as evident by the formation of fewer focal adhesions and stress fibers. Concomitant with this partial activation of cell spreading, low levels of FAK phosphorylation were observed. Full cell spreading in these trabecular meshwork cells required additional signaling from αβ1 integrin, which resulted in a higher level of FAK phosphorylation and increased stress fiber formation. An increase in the intensity of focal adhesions was also observed, suggesting that additional signaling complexes may have been recruited to the sites of cell attachment as a result of the co-signaling pathway to activate cell spreading, focal adhesion, and stress fiber formation compared with that typically seen in fibroblasts and A375-SM cells.

The ability of co-signaling between αβ1 and α4β1 integrins to control cell spreading is in contrast to previously reported studies in fibroblasts and A375-SM melanoma cells (8, 13). In those studies, cell spreading was mediated by an αβ1 integrin/syndecan pathway, whereas in the HTM cells, it is mediated by an αβ1/α4β1 integrin pathway. In addition, cell spreading in fibroblasts and the A375-SM cells was protein kinase C-dependent, whereas in HTM cells, it was independent of protein kinase C activation. Thus, HTM cells appear to use a different co-signaling pathway to activate cell spreading, focal adhesion, and stress fiber formation compared with that typically seen in fibroblasts and A375-SM cells, even though all the cells are using the same fibronectin domains. The difference in these pathways may simply reflect differences in the expression of
A Novel Hep II-mediated Signaling Pathway Involving α4β1

A novel Hep II-mediated signaling pathway involving α4β1 integrin has been identified. This pathway is activated by the Hep II domain, which binds to the α4β1 integrin, leading to a conformational change in the integrin that enhances cell attachment. In HTM cells, the Hep II domain, when added to the cells, induced cell spreading, which was dependent on the Hep II domain and the expression of co-receptors such as chondroitin sulfate. This suggests that the Hep II domain can activate the α4β1 integrin by interfering with the Rho activation pathway, leading to a conformational change in the integrin that enhances cell attachment.

In addition to this direct activation, the Hep II domain also interacts with other cell surface receptors, such as syndecan-4, which may be present in aqueous humor and thus able to participate in intraocular homeostasis. This signaling pathway is not only important for cell attachment but also for the regulation of cell spreading and migration, which is crucial for the movement of aqueous humor through the eye. Thus, a signaling mechanism could be used to control the flow of aqueous humor outflow through the trabecular meshwork, helping to regulate intraocular pressure.

The cooperative signaling of α5β1 and α4β1 integrins in trabecular meshwork cells seems countervariant compared to the traditional roles of α5β1 and α4β1 integrins. The α4β1 integrin is normally thought to play a role in mediating cell migration by weakening cell contacts, and α5β1 integrin mediates cell attachment. It addition, the need for two β1 integrins to promote adhesion would seem redundant. However, dual signaling via these two integrins may serve to control the adhesive strength and hence contractility of the HTM cells.

Contractility of the actin cytoskeleton plays an important role in mediating the movement of aqueous humor through the anterior chamber of the human eye. At low levels of adhesiveness, weakly attached cells may not generate sufficient force to promote contraction of the trabecular meshwork and enhance movement of aqueous humor. At high levels of adhesiveness, strongly attached cells would generate too much contractility force, and it would be difficult to regulate changes in the movement of aqueous humor through the eye. Thus, a dual signaling mechanism of α5β1 and α4β1 integrins that could regulate the adhesiveness of the cell contacts could serve to adjust the contractility forces generated by the trabecular cells. Such a signaling mechanism could be used to control the flow rate of aqueous humor outflow through the trabecular meshwork in response to pressure changes in the anterior chamber.
