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Identification of the Active Site in the Heparin II Domain of Fibronectin that Increases Outflow Facility in Cultured Monkey Anterior Segments

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Abstract

PURPOSE—To determine the active site in the Heparin II (HepII) domain of fibronectin that regulates outflow facility in cultured anterior segments and disrupts the actin cytoskeleton in transformed human trabecular meshwork (TM-1) cells.

METHODS—Outflow facility was determined by two-level, constant-pressure perfusion in cultured anterior segments of rhesus and cynomolgus monkey eyes. One segment from each pair was exchanged with either the HepII domain or an integrin/syndecan binding peptide (IDAPS or PPRARI) from the HepII domain. To assay changes in the actin cytoskeleton, TM-1 cells were incubated for 24 hours with or without the HepII domain, PPRARI, or IDAPS. Changes were monitored with phase and immunofluorescence microscopy.

RESULTS—HepII domain (100 µg/mL) and PPRARI (500 µg/mL) increased outflow facility by $31\% \pm 13\%$ ($n = 9$, $P < 0.05$) and $24\% \pm 9\%$ ($n = 8$, $P < 0.05$), respectively in cultured anterior segments after an overnight infusion. Perfusion with IDAPS (500 µg/mL) had no effect on outflow facility. In TM-1 cultures, 250 µg/mL of the HepII domain or 4 mg/mL of PPRARI disrupted the assembly of actin filaments. A lower concentration of PPRARI (2 mg/mL) disrupted the actin cytoskeleton when used in combination with a nondisrupting concentration of the HepII domain (30–60 µg/mL). In contrast, IDAPS did not disrupt the actin cytoskeleton under any condition tested.

CONCLUSIONS—The active site in the HepII domain that regulates outflow facility in cultured anterior segments and disrupts the actin cytoskeleton in TM-1 cells is the syndecan/integrin binding sequence, PPRARI.

Aqueous humor drainage via the conventional outflow pathway accounts for one half to two thirds of the total aqueous outflow in a healthy human eye.^{1,2} One feature that contributes to outflow resistance via this pathway is the contractile property of the trabecular meshwork (TM). Thus, agents that disrupt the organization of the actin cytoskeleton, cell–cell junctions, and cell-matrix contacts that maintain tissue integrity tend to increase outflow facility in enucleated human and bovine eye organ perfusion cultures and in live monkey eyes.³ In contrast, agents that support actin cytoskeleton assembly increase resistance and reduce outflow facility.⁴

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It is well established that signaling events mediated by the extracellular matrix (ECM) play a critical role in maintaining tissue architecture by regulating the organization of the actin cytoskeleton and cell contacts. Hence, these signaling events could regulate outflow facility. Recent studies support this idea and show that the Heparin II (HepII) domain of fibronectin, an ECM protein found in the TM, increases outflow facility when perfused through cultured human anterior segments.⁵ Presumably, this domain increases outflow facility by mediating the disassembly of the actin cytoskeleton in TM cells.⁶

The HepII domain is a 30-kDa region of fibronectin that comprises the 12th to 14th type III repeats. It plays an important role in regulating the organization of the actin cytoskeleton by acting as a ligand for members of the syndecan and integrin family of receptors. Syndecans and integrins control the organization of the actin cytoskeleton by activating signaling pathways involving Rho GTPases.^{7,8} Integrins comprise a large family of heterodimeric receptors that usually bind ECM proteins through homologues of the tripeptide sequence, Arg-Gly-Asp (RGD). The HepII domain contains one RGD homologue (IDAPS) in the 14th repeat.⁹ Another integrin binding sequence, PPRARI, is also found within the 14th repeat.¹⁰ Both these sequences are believed to bind $\alpha 4 \beta 1$ integrins. Syndecans, on the other hand, are transmembrane heparan sulfate proteoglycans (HSPGs) that interact with ECM proteins via their sulfated glycosaminoglycan side chains. Binding of syndecans, especially syndecan-1 and -4, to fibronectin and the HepII domain is mediated by regions of positively charged residues located in the 13th repeat,¹¹ as well as by the sequence PPRARI.¹²

In this study, we investigated the role that the integrin/syndecan binding sites, PPRARI and IDAPS, in the HepII domain may play in regulating outflow facility. This study showed that PPRARI, but not IDAPS, increased outflow facility in monkey anterior segments *ex vivo* as well as disrupted the actin cytoskeleton of TM cells *in vitro*. This suggests that the active site in the HepII domain responsible for increasing outflow facility is the PPRARI sequence and that an integrin- and/or syndecan-mediated signaling pathway may be involved.

MATERIALS AND METHODS

Cell Culture

Immortalized human TM-1 cells were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Atlanta Biologicals, Inc., Norcross, GA), 2 mM L-glutamine, 2.5 μ g/mL amphotericin B, and 25 μ g/mL gentamicin, as previously described.¹³ For experiments, confluent cultures grown on glass coverslips were incubated overnight in serum-free DMEM containing 2.5 μ g/mL amphotericin and 25 μ g/mL gentamicin in the absence or presence of the HepII domain, PPRARI, PPRAAI, or IDAPS. Serum-free medium was used to avoid interactions between the HepII domain and serum factors such as plasma fibronectin.

Production of Recombinant Proteins and Peptides

The recombinant HepII domain was made as described previously.¹⁴ The peptides: PPRARI, IDAPS, PPRAAI, IEAPS, and EILDV, an $\alpha 4 \beta 1$ -integrin-binding peptide found in the V region of fibronectin,^{15,16} were synthesized at the Biotechnology Center of the University of Wisconsin-Madison. Peptides were synthesized on a 25-micromole scale by an automated synthesizer (model 432A; Applied Biosystems, Inc. [ABI], Foster City, CA). The cleaved peptides were precipitated with cold *t*-butylmethylether and their mass confirmed by electrospray ionization mass spectroscopy. The purity of the peptides was determined by HPLC. The activity of the PPRARI peptide has been attributed to the two argininy residues in the peptide. Deletion of either residue reduces the activity the peptide, but does not completely abolish it.¹⁰ A peptide lacking both argininy residues could not be used, since this

peptide was insoluble. Sequence homology between the HepII domains in human¹⁷ and rhesus monkeys (REFSEQ:accession XM_001083548.1) is 99%. IDAPS and PPRARI have 100% sequence homology between humans and monkeys.

Immunofluorescence Microscopy

TM-1 cells were permeabilized with 0.5% Triton X-100, fixed with 4% paraformaldehyde and then incubated with Alexa 488-conjugated phalloidin (0.67 U/mL; Invitrogen, Carlsbad, CA) in 0.1% BSA/PBS for 1 hour as previously described.^{6,18} Cells were labeled with Hoechst 33342 (Invitrogen) to localize nuclei. Coverslips were mounted (ImmuMount; Shandon Lipshaw, Pittsburgh, PA). All images were acquired using a digital camera (AxioCam HRm; Carl Zeiss Meditec, Inc., Thornwood, NJ) mounted on an epifluorescence microscope (Axio-plan 2 Imaging; Zeiss) equipped with image analysis software (Axio-Vision ver. 4.5; Zeiss).

Cytotoxicity Assay

A cell-viability assay (Live/Dead; Invitrogen) was used to evaluate cell viability in the absence or presence of increasing concentrations of PPRARI after 24 hours. The cells were lifted off the dish with a cell dissociation buffer (Sigma-Aldrich, St. Louis, MO), stained with calcein AM and ethidium homodimer-1, and analyzed by flow cytometry. In some experiments, cells were labeled while still attached to dishes and then examined by fluorescence and phase microscopy according to the manufacturer's protocol. TM-1 cells treated with 0.1% saponin in PBS for 10 minutes were used as a positive control of cell death.

Organ Culture and Outflow Facility

Anterior segments were obtained from either rhesus (*Macaca mulatta*; $n = 14$) or cynomolgus (*Macaca fascicularis*; $n = 9$) monkeys from the Wisconsin National Primate Research Center, Covance Inc. (Madison, WI), Dr. Kaufman's colony, or colonies from other investigators at the University of Wisconsin. The experimental protocol adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Eleven monkeys had had no prior ocular procedures and were euthanatized due to weight loss and chronic diarrhea. Four cynomolgus monkeys had their anterior chambers perfused >6 months before being euthanatized. Two cynomolgus monkeys underwent long-term treatments with echthiophate iodide to cause accumulation of ECM. These treatments ended 1 to 3 years before being euthanatized, and the intraocular pressures were normal. One rhesus had a bilateral iridectomy 8 months before being euthanatized and cholinergic agonist treatment 2 weeks before being euthanatized. Another rhesus had a single retinal laser lesion 8 months before being euthanatized. Other monkeys had been used in nonocular studies that included vaccine testing ($n = 2$), fetectomy ($n = 1$), ovariectomy ($n = 1$), kidney donor ($n = 1$), and cerebral infarction ($n = 1$). The anterior segments were placed in culture within 2 hours after death, as previously described.¹⁹ Anterior segments were cultured in high-glucose DMEM containing 0.584 g/L L-glutamine, 15mg/L-gentamicin, 100U/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B at 37°C with 5% CO₂. Media were infused with an infusion pump (PHD 2000; Harvard Apparatus, Holliston, MA) at a constant rate of 2.5 µL/min.

After 1 to 3 days of equilibration, baseline outflow facility was measured for 1 hour by two-level, constant-pressure perfusion.²⁰ Once a stable baseline was obtained, one of the paired segments was exchanged with 3 to 3.5 mL of media containing either the HepII domain or a peptide. The peptide concentration used was based on the concentration shown to give a maximum effect in vitro.¹² The contralateral segment was exchanged with the same volume of DMEM. The infusion was stopped for 2 to 4 hours to allow the HepII domain or peptides to competitively bind. Infusion with the corresponding solution was then continued overnight. The highest concentration of the HepII domain (833 µg/mL) was infused for only 4 to 5 hours, followed by an overnight vehicle infusion. Outflow facility was measured the next day.

To test the effect of the control peptides, the wild-type peptides were washed out with DMEM for 1 to 3 days, and a new baseline outflow facility was established. The initial control segments were then exchanged with the same dose of control peptides and the previous peptide-treated segments were exchanged with DMEM. Outflow facility was monitored as described above.

The effect of the HepII domain or peptides on outflow facility (exp) is expressed as the ratio of posttreatment outflow facility (Rx) compared to baseline (BL) and corrected for control (con) eye washout: $(Rx_{exp}/BL_{exp})/(Rx_{con}/BL_{con})$. The percentage of change in outflow facility was calculated as $[(Rx_{exp}/BL_{exp})/(Rx_{con}/BL_{con}) - 1] \times 100$. Statistical analysis was performed with a two-tail paired *t*-test. A Mann-Whitney U test was used to determine whether the treatments differentially affected rhesus and cynomolgus monkeys.

Light Microscopy

Monkey organ cultured anterior segments (MOCAS) were exchanged with 6 mL of 4% paraformaldehyde over 30 minutes. Each segment was then cut into quadrants and immersed in 4% paraformaldehyde. Quadrants were embedded in either resin (JB-4; Polysciences Inc., Warrington, PA) or paraffin. Sagittal sections, 4 to 5 μ m thick, were cut and stained with either toluidine blue (Polysciences Inc.) or hematoxylin and eosin. Sections were examined for the presence of TM cells, beams, and the integrity of Schlemm's canal. All four quadrants per anterior segment were examined.

RESULTS

Effect of HepII Domain and Peptides on Outflow Facility

Previous studies had shown that the HepII domain increased outflow facility in cultured human anterior segments (HOCAS).⁵ To determine whether the activity of the HepII domain involved one of the two known integrin/syndecan binding sites (IDAPS and PPRARI) in the HepII domain (Fig. 1), MOCAS were perfused with either the intact HepII domain or one of the synthetic peptides, PPRARI or IDAPS. A recombinant HepII domain containing mutations in these sites could not be used, because circular dichroism analysis indicated that these mutations affected the conformation of the HepII domain (data not shown). As shown in Figure 2 and Table 1, 100 μ g/mL of the HepII domain increased outflow facility by $31\% \pm 13\%$ ($n = 9$; $P < 0.05$) on the day after exchange. Among the nine pairs of MOCAS perfused with 100 μ g/mL of the HepII domain, two MOCAS did not respond, and two MOCAS showed a decrease in outflow facility. The effect of the HepII domain on outflow facility appeared to be dose dependent, although only two pairs of MOCAS were studied at each of the other concentrations. At a lower concentration (10 μ g/mL), the HepII domain did not have any effect on outflow facility ($n = 2$), whereas the higher concentration (833 μ g/mL), which previously increased outflow facility in HOCAS,⁵ decreased outflow facility by $80\% \pm 16\%$, 4 to 5 hours after exchange (data not shown). The decrease in outflow facility caused by the higher concentration was still present the next day despite an overnight infusion with media containing only vehicle (see Materials and Methods).

Overnight infusion with PPRARI also increased outflow facility by $24\% \pm 9\%$ ($n = 8$, $P < 0.05$), similar to that observed with the HepII domain. As shown in Figure 2 and Table 2, the mutant PPRAAI ($n = 6$), which has a lower activity (see Materials and Methods), did not significantly increase outflow facility ($12\% \pm 9\%$). Infusion with IDAPS, the mutant peptide IEAPS (Fig. 2, Table 3) or the $\beta 1$ integrin binding peptide EILDV (data not shown) had no significant effect on outflow facility. There were no statistically significant differences in outflow facility between rhesus and cynomolgus monkeys within any of the treatment conditions. Washout of the HepII domain or PPRARI after exchange or overnight infusion with plain medium resulted in the return of outflow facility to near baseline levels when

corrected for control eye washout (ratio treated/vehicle for baseline postwashout/baseline pretreatment = 1.15 ± 0.08 ; $n = 14$, not significant).

Morphologic Examination of Perfused MOCAS

Light micrographs show that gross changes were not observed in the juxtacanalicular tissue (JCT) or Schlemm's canal in MOCAS perfused with PPRARI, PPRAAI, or the HepII domain compared with control anterior segments (cf. Fig. 3A and 3B–D). In all the MOCAS, beams were intact, and cells were observed within the JCT and lining Schlemm's canal (Fig. 3).

Effect of PPRARI and IDAPS on the Organization of Actin Filaments

In TM-1 cultures incubated with 2 mg/mL of PPRARI a reduction in assembled actin filaments could be observed compared to nontreated cultures or cultures incubated with the PPRAAI mutant (Fig. 4). Neither the PPRAAI mutant nor IDAPS altered the assembly of the actin cytoskeleton compared to control cultures (Fig. 4). If PPRARI was used in combination with a low concentration (30 μ g/mL) of the HepII domain that did not trigger the disassembly of actin filaments (Fig. 4), assembled actin filaments were no longer observed, and large gaps between cells appeared (Fig. 4). As shown in Figure 5, the gaps were dependent on the concentration of the HepII domain.⁶ Gaps were also apparent when the HepII domain (60 μ g/mL) was used in combination with 2 mg/mL of PPRARI (Fig. 5G). Neither concentration alone caused a significant disruption in cell–cell interactions (cfs. Fig. 5B and 5F).

Cultures incubated with the PPRAAI mutant contained some actin filaments, but the filaments were smaller compared to control cultures and larger compared with cultures treated with PPRARI and the HepII domain (Fig. 4). These cultures also exhibited fewer gaps between cells compared to cultures incubated with PPRARI and the HepII domain (Fig. 4). This result supports previous findings that PPRAAI has a lower activity. The effect appeared to be specific for PPRARI and PPRAAI, because when IDAPS was used in conjunction with the HepII domain, actin filaments could still be observed (Fig. 4). Coincubation with IDAPS (2 mg/mL) and PPRARI (2 mg/mL) did not enhance the effect of PPRARI and assembled actin filaments were still observed (data not shown).

Treatment of TM-1 cultures with higher concentrations of PPRARI (4 mg/mL) in the absence of the HepII domain led to a disassembly of actin filaments (Fig. 4) and the appearance of gaps between cells compared to untreated control cultures (Fig. 5H). However, PPRARI was not as effective as 250 μ g/mL of the HepII domain which completely abolished the assembly of actin filaments (Fig. 4) and caused large gaps between cells (Fig. 5D). At 4 mg/mL, the PPRAAI mutant also reduced the assembly of actin filaments and caused gaps between cells (Fig. 4), but the gaps were smaller and fewer in number, suggesting that its activity was reduced compared to PPRARI. In contrast, TM-1 cultures treated with 4 mg/mL of IDAPS appeared similar to untreated controls and exhibited numerous actin filaments.

Treatment with PPRARI for 24 hours was not toxic to cultures at 500 μ g/mL, which was the concentration used in MOCAS and at 2 mg/mL. At these concentrations, the majority of TM-1 cells appeared healthy and well spread, similar to that observed in untreated controls (cf. Fig. 5A, 5E, 5F). The viability assay confirmed this observation and showed that less than 9% of the cells in cultures treated with 2 mg/mL PPRARI were nonviable compared to control cultures. Higher concentrations of PPRARI (4 mg/mL) appeared slightly toxic, as cell viability decreased by 18% compared with the control. Presumably, this decrease occurred because 4 mg/mL of PPRARI caused some cells to lift off the plates and undergo anoikis, which is a specific form of apoptosis that is induced when anchorage-dependent cells are detached from the surrounding ECM for a prolonged period (Fig. 5H).

DISCUSSION

This study demonstrates that the site in the HepII domain responsible for increasing outflow facility may be the integrin/syndecan-binding sequence, PPRARI. A synthetic peptide of this sequence was able to increase outflow facility by 24%, similar to the 31% increase in outflow facility caused by the HepII domain in cultured anterior segments. The effect was specific for PPRARI, since neither the mutated PPRAAI peptide nor the integrin-binding peptides (IDAPS, EILDV) were able to increase outflow facility to the same extent when perfused into cultured anterior segments. PPRARI also showed an ability to disrupt the assembly of actin filaments and cause large gaps between cells. A similar result was seen in TM-1 cultures when the HepII domain disrupted the assembly of actin filaments and cell-cell contacts.⁶ This suggests that the HepII domain and PPRARI may affect similar mechanisms.

Although the biological effects of PPRARI and the HepII domain were similar, the activity of PPRARI was much weaker. In MOCAS, the concentration of the HepII domain needed to increase outflow facility was 3 μ M compared with the concentration of 627 μ M needed for PPRARI. A higher concentration (5 mM) of the peptide was also necessary to disrupt the actin cytoskeleton of TM-1 cells. A lower concentration of PPRARI was able to disrupt the organization of the actin filaments but only if a low, inactive concentration of the HepII domain was also present. The reason for this is unclear. There may be an additive or synergistic relationship between PPRARI and the HepII domain. For instance, PPRARI and the HepII domain may activate different receptors, but the signals generated converge to induce the disruption of actin filaments in TM-1 cultures. However, it may simply be that the PPRARI sequence exists in a specific conformation in the HepII domain and that the smaller peptide, out of context, has a much lower affinity for its receptor and hence higher concentrations are needed. This has clearly been shown to be the case for other integrin binding peptides. For instance, the α 4 β 1 integrin-binding peptide LDV is 10 to 20 times less effective than the larger CS1 fragment (2700 μ M compared with 180 μ M)²¹ and a small α 9 β 1 integrin-binding peptide requires a concentration of 1000 μ M to inhibit cell adhesion by 50%.²²

The time course and concentration of the HepII domain required to elicit an effect in MOCAS differed from that previously observed in HOCAS. In HOCAS, the concentration of the HepII domain that increased outflow facility was eight times greater than that used in the MOCAS (833 μ g/mL versus 100 μ g/mL) and the increase in outflow facility was approximately four times greater (90% vs. 24%).⁵ The response in HOCAS was also faster and occurred within 3 hours. The reason for these differences is an interesting question. Both the HepII domain and PPRARI bind heparan sulfates on syndecans and other HSPGs.^{23–25} Thus, there may be differences in the relative density of HSPGs in MOCAS compared to HOCAS, resulting in higher doses of the HepII domain clogging the meshwork and lower doses taking longer to have an effect. MOCAS were also placed in culture sooner than the HOCAS. Thus, autolysis in the HOCAS could have weakened interactions in the TM, allowing the effects of HepII domain to occur more rapidly.

The percentage of MOCAS that responded to the HepII domain also differed from HOCAS; 55.5% of MOCAS responded to the HepII domain, whereas 90% of HOCAS responded.⁵ This result suggests that humans are more responsive to the HepII domain. Of note, HOCAS, unlike MOCAS, seemed to have two populations of responders: 50% of the HOCAS were low responders, showing a 20% to 50% increase in outflow facility, and 40% were high responders, showing a 101% to 400% increase.

The PPRARI sequence in the HepII domain has been reported to bind both α 4 β 1 integrins and syndecan-4.^{10,23} Thus, either of these receptors could be responsible for increasing outflow facility in MOCAS. Both receptors are found in the TM,^{26,27} and both activate signaling

pathways involving Rho GTPases^{8,28} which control outflow facility.^{29–33} At first glance, $\alpha 4\beta 1$ integrins would not seem to be the receptor for the HepII domain. In subconfluent TM cultures, the HepII domain used $\alpha 4\beta 1$ integrins to activate stress fiber formation,¹⁸ not to disassemble them. In addition, perfusion with the $\alpha 4\beta 1$ binding peptide IDAPS did not increase outflow facility or trigger the disassembly of actin filaments in TM-1 cultures. However, since IDAPS binds to the $\beta 1$ subunit of $\alpha 4\beta 1$ integrins and PPRARI is believed to bind to the $\alpha 4$ subunit,¹⁰ PPRARI and the HepII domain could be using another $\alpha 4$ integrin, such as $\alpha 4\beta 7$ which frequently shares common ligands with $\alpha 4\beta 1$.^{34–38}

Responses to cell-matrix signaling events are influenced by cross-talk from other ECM receptors and cadherins in cell-cell contacts.^{39–42} Hence, it is not surprising that confluent TM-1 cultures responded differently to the HepII domain, as opposed to subconfluent HTM cultures,^{6,18} by disassembling actin filaments. In the subconfluent studies,¹⁸ the only matrix present was two purified domains from fibronectin. In contrast, confluent TM-1 cultures¹³ contain type IV collagen, laminin, thrombospondin, and cell–cell contacts.⁴³ Which receptors cosignal with the HepII domain is not known.

In summary, this study demonstrates that a specific site in the HepII domain, associated with cell-matrix signaling events and involved in the regulation of cell contractility, is responsible for altering outflow facility. This is the first time a specific cell-matrix interaction has been shown to alter outflow facility and provides a glimpse into the possible mechanisms used by the ECM to regulate outflow facility. Understanding how the ECM functions in the TM should provide new pharmacologic targets for the control of intraocular pressure.

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FIGURE 1.

A schematic representation of fibronectin and the HepII domain. The HepII domain comprises the 12th to 14th type III repeats. The major binding site for HSPGs is through cationic binding sites in the 13th and 14th type III repeats (*box with pluses*). A PPRARI sequence in the 14th repeat is also reported to bind HSPGs and to mediate binding to $\alpha 4 \beta 1$ integrins. The IDAPS sequence in the 14th type III repeat is also thought to bind $\alpha 4 \beta 1$ integrins.

FIGURE 2.

Effect of the HepII domain and peptides on outflow facility. Outflow facility was monitored, and after overnight infusion, outflow facility was significantly increased by the HepII domain and PPRARI when compared with baseline and corrected for control eye washout. Other peptides did not produce a statistically significant effect on outflow facility. Significance was determined by the two-tailed paired *t*-test for ratios (Rx/BL)/(Con/BL) different from 1.0:

**P* < 0.05. Rx, treated; BL, baseline; Con, Control.

FIGURE 3.

Light micrographs of MOCAS treated with HepII domain or PPRARI. Anterior segments from rhesus monkeys were infused with DMEM (A), 100 µg/mL HepII domain (B), 500 µg/mL PPRARI (C), or 500 µg/mL PPRAAI (D). No morphologic changes were detectable in the treated MOCAS compared to the DMEM-infused control. All anterior segments contained an intact Schlemm's canal, organized trabecular beams, and a high degree of cellularity throughout the TM. Bar, 100 µm.

FIGURE 4.

Effect of the HepII domain, PPRARI or IDAPS on the actin cytoskeleton of TM-1 cultures. Incubations with the HepII domain (250 $\mu\text{g/mL}$) or PPRARI (2 mg/mL) plus the HepII domain (30 $\mu\text{g/mL}$) caused the disassembly of actin filaments. High concentrations (4 mg/mL) of PPRARI and PPRAAI also seemed to disrupt actin assembly. TM-1 cells were cultured and then incubated for 24 hours in the absence or presence of the HepII domain, PPRARI, PPRAAI, or IDAPS at the concentrations indicated in the figure. In some studies, an ineffective concentration (30 $\mu\text{g/mL}$) of the HepII domain (+HepII) was added to cultures incubated with PPRARI, PPRAAI, or IDAPS. *Arrowheads*: actin filaments; *arrows*: gaps between cells. Bar, 50 μm .

FIGURE 5.

Effect of PPRARI and the HepII domain on the cell morphology of TM-1 cultures. The effect of the HepII domain and PPRARI on cell–cell interactions and cell rounding was found to be dose dependent. TM-1 cells were cultured and incubated for 24 hours in the absence (**A**) or presence of 60 µg/mL (**B**), 125 µg/mL (**C**), or 250 µg/mL (**D**) of the HepII domain. Cultures were also treated with 500 µg/mL (**E**), 2 mg/mL (**F**), or 4 mg/mL PPRARI (**H**). In some cases, cultures were incubated with 60 µg/mL HepII domain and 2 mg/mL PPRARI (**G**). Bar, 50 µm.

TABLE 1

Effect of HepII Domain on Outflow Facility

Treatment	Donor	Species, Sex	Age (y)	OF (μL/min/mm Hg)				OF Ratio
				BLcon	BLexp	Rxcon	Rxexp	
100 μg/mL HepII	1	Cy, M	14	0.425	0.395	0.481	0.833	1.86
	2	Rh, M	6	0.434	0.262	0.669	0.434	1.07
	3	Cy, F	11	0.209	0.228	0.287	0.229	0.73
	4	Cy, F	10	0.166	0.232	0.183	0.382	1.49
	5	Rh, M	15	0.480	0.263	0.297	0.260	1.60
	6	Cy, F	9	0.200	0.191	0.129	0.217	1.76
	7	Rh, M	6	0.372	0.445	0.505	0.633	1.05
	8	Rh, M	6	0.475	0.320	0.448	0.409	1.36
	9	Rh, M	8	0.330	0.348	0.460	0.434	0.89
Mean ± SEM				0.34 ± 0.41	0.30 ± 0.03	0.38 ± 0.06	0.43 ± 0.06	1.31 ± 0.13*

BL, OF baseline for vehicle (con) and treated (exp) segments; con, vehicle; exp, treated; Rx, OF post vehicle or treatment; Cy, cynomolgus; Rh, rhesus.

* Ratio significantly different from 1.0 ($P < 0.05$)

TABLE 2
Effect of PPRARI and PPRAAI Peptides on Outflow Facility

Treatment	Donor	Species, Sex	Age (y)	OF ($\mu\text{L}/\text{min}/\text{mm Hg}$)				OF Ratio
				BLcon	BLexp	Rxcon	Rxexp	
500 $\mu\text{g}/\text{mL}$ PPRARI	1	Cy, F	5	0.360	0.197	0.523	0.377	1.32
	2	Rh, F	5	0.226	0.317	0.294	0.687	1.67
	3	Rh, M	3	0.393	0.303	0.404	0.450	1.44
	4	Rh, M	7	0.474	0.323	0.547	0.478	1.28
	5	Rh, F	29	0.177	0.288	0.229	0.417	1.51
	6	Rh, F	8	0.502	0.403	0.562	0.543	1.20
	7	Rh, F	19	0.312	0.475	0.353	0.525	0.98
	8	Cy, M	5	0.252	0.245	0.466	0.421	0.93
Mean $\pm$ SEM				0.34 ± 0.04	0.32 ± 0.03	0.42 ± 0.04	0.49 ± 0.04	1.29 ± 0.09
500 $\mu\text{g}/\text{mL}$ PPRAAI	1	Cy, F	5	0.406	0.679	0.369	0.942	1.53
	3	Rh, M	3	0.728	0.513	0.837	0.544	0.92
	4	Rh, M	7	0.651	0.698	0.889	0.835	0.88
	5	Rh, F	29	0.532	0.289	0.623	0.339	1.00
	7	Rh, F	19	0.623	0.333	0.650	0.460	1.32
	8	Cy, M	5	0.183	0.174	0.245	0.252	1.08
Mean $\pm$ SEM				0.52 ± 0.08	0.45 ± 0.09	0.60 ± 0.10	0.56 ± 0.11	1.12 ± 0.10

The same donors were used for PPRARI and PPRAAI as indicated. Abbreviations as in Table 1.

* Ratio significantly different from 1.0 ($P < 0.05$).

TABLE 3

Effect of Other $\alpha 4\beta 1$ Binding Peptides on Outflow Facility

Treatment	Donor	Species, Sex	Age (y)	OF ($\mu\text{L}/\text{min}/\text{mm Hg}$)				OF Ratio
				BLcon	BLexp	Rxcon	Rxexp	
500 $\mu\text{g}/\text{mL}$ IDAPS	1	Rh, F	6	0.428	0.434	0.484	0.647	1.32
	2	Cy, F	6	0.373	0.492	0.351	0.521	1.13
	3	Rh, M	10	0.728	0.682	0.881	0.741	0.90
	4	Cy, M	4	0.242	0.468	0.279	0.336	0.62
	5	Cy, M	4	0.542	0.424	0.570	0.535	1.20
	6	Rh, M	7	0.167	0.197	0.184	0.256	1.18
Mean $\pm$ SEM				0.413 ± 0.083	0.450 ± 0.063	0.458 ± 0.102	0.506 ± 0.075	1.06 ± 0.10
500 $\mu\text{g}/\text{mL}$ IEAPS	1	Rh, F	6	0.637	0.610	1.030	0.729	0.74
	2	Cy, F	6	0.679	0.280	1.059	0.454	1.04
	4	Cy, M	4	0.429	0.345	0.474	0.372	0.98
	5	Cy, M	4	0.828	0.699	0.842	0.792	1.11
	6	Rh, M	7	0.252	0.218	0.265	0.156	0.68
Mean $\pm$ SEM				0.565 ± 0.101	0.430 ± 0.095	0.734 ± 0.157	0.501 ± 0.117	0.91 ± 0.09

The same donors were used for IDAPS and IEAPS as indicated. Abbreviations as in Table 1.