Increasing Paracellular Porosity by E-Cadherin Peptides: Discovery of Bulge and Groove Regions in the EC1-Domain of E-Cadherin

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Purpose. The objective of this work is to evaluate the ability of peptides derived from the bulge (HAV-peptides) and groove (ADT-peptides) regions of E-cadherin EC1-domain to increase the paracellular porosity of the intercellular junctions of Madin-Darby canine kidney (MDCK) cell monolayers.

Methods. Peptides were synthesized using a solid-phase method and were purified using semi-preparative HPLC. MDCK monolayers were used to evaluate the ability of cadherin peptides to modulate cadherin-cadherin interactions in the intercellular junctions. The increase in intercellular junction porosity was determined by the change in transepithelial electrical resistance (TEER) values and the paracellular transport of ^{14}C-mannitol.

Results. HAV- and ADT-peptides can lower the TEER value of MDCK cell monolayers and enhance the paracellular permeation of ^{14}C-mannitol. HAV- and ADT-decapeptides can modulate the intercellular junctions when they are added from the basolateral side but not from the apical side; on the other hand, HAV- and ADT-hexapeptides increase the paracellular porosity of the monolayers when added from either side. Conjugation of HAV- and ADT-peptides using ω-aminocaproic acid can only work to modulate the paracellular porosity when ADT-peptide is at the N-terminus and HAV-peptide is at the C-terminus; because of its size, the conjugate can only modulate the intercellular junction when added from the basolateral side.

Conclusions. Peptides from the bulge and groove regions of the EC1 domain of E-cadherin can inhibit cadherin-cadherin interactions, resulting in the opening of the paracellular junctions. These peptides may be used to improve paracellular permeation of peptides and proteins. Furthermore, this work suggests that both groove and bulge regions of EC-domain are important for cadherin-cadherin interactions.

KEY WORDS: E-cadherin; cell-cell adhesion; HAV peptides; ADT peptides; intercellular junctions; adherens junction; MDCK cell monolayers.

INTRODUCTION

Many peptides and proteins (i.e., synthesized or occurring naturally) have been found and developed as potential therapeutic agents. However, the utilization of such compounds as therapeutic drugs is often restricted by the difficulties of delivering them to target site(s) due to the presence of biological barricades such as the intestinal mucosa and the blood-brain barrier (BBB) (1,2). These barriers usually consist of cell membranes constructed from cells with intercellular junctions (1,3,4). Peptides and proteins cannot cross these barriers via transcellular pathways due to their size and hydrophilic properties. Alternatively, these molecules may be transported through paracellular pathways; unfortunately, the paracellular transport of peptides and proteins is limited by the presence of tight intercellular junctions. These tight junctions exhibit a minimal porosity (<11 Å), allowing only small molecules and ions to cross (5). Therefore, there is a need to develop methods to improve paracellular delivery of large hydrophilic molecules such as peptides and proteins. One way to accomplish this goal is by increasing the porosity of the intercellular junctions via the modulation of protein-protein interactions (i.e., those of E-cadherins) in the intercellular junctions (1,6,7).

We have evaluated the possibility of modulating the porosity of the tight intercellular junctions by inhibiting E-cadherin-E-cadherin interactions. These interactions can be disrupted using synthetic peptides derived from the sequence of contact (bulge and groove) regions of E-cadherins (6–8). E-cadherins are a family of transmembrane glycoproteins found in the *zonula adherens* (adherens junction), which is sandwiched between tight junctions (*zonula occludens*) and *desmosomes* (1,2,4,9,10). Homophilic interactions of E-cadherins are the primary force for cell-cell adhesion of the opposing cells (11), and the formation of tight junction is a secondary response to this primary interaction (12). The structure of E-cadherin contains an extracellular (EC) domain, a single membrane-spanning segment, and a relatively short cytoplasmic domain (Fig. 1) (1,2,13–15). The extracellular domain has five tandem repeats called EC1-to-EC5 domains, which bind to calcium ions (16). Calcium ions are located between the interconnections of EC repeats (i.e., between EC1 and EC2 domains) (17,18) that are important for the structural integrity and interactions of cadherins. The extracellular domain of E-cadherin forms a parallel *cis*-dimer (strand dimer) between two E-cadherin molecules in the same cells and an antiparallel *trans*-dimer (adhesion dimer) between E-cadherin molecules from opposing cells (Fig. 1) (17–20). There is emerging evidence that the *trans*-dimer formation is initiated by an EC1-to-EC1 interaction (Fig. 1a) followed by multiple interactions (EC1-to-EC4) as shown in Fig. 1b (21,22). Nonetheless, inhibition of cadherin-cadherin interaction maybe achieved by blocking the recognition sites for *cis*- or *trans*-cadherin interactions. The cytoplasmic tail anchors to cytoskeletal actin filaments through catenins (Fig. 1); the cytoplasmic cadherin-catenin binding is also important in regulating cadherin-cadherin interactions in the extracellular space (15).

Previously, we have found that His-Ala-Val (HAV) peptides derived from the EC1 domain of E-cadherin can inhibit E-cadherin-mediated cell-cell adhesion and modulate the intercellular junctions of cell monolayers (6–8). Mutations of the HAV sequence in E-cadherin have been shown to eliminate cell-cell contact (23). Residues flanking the HAV se-

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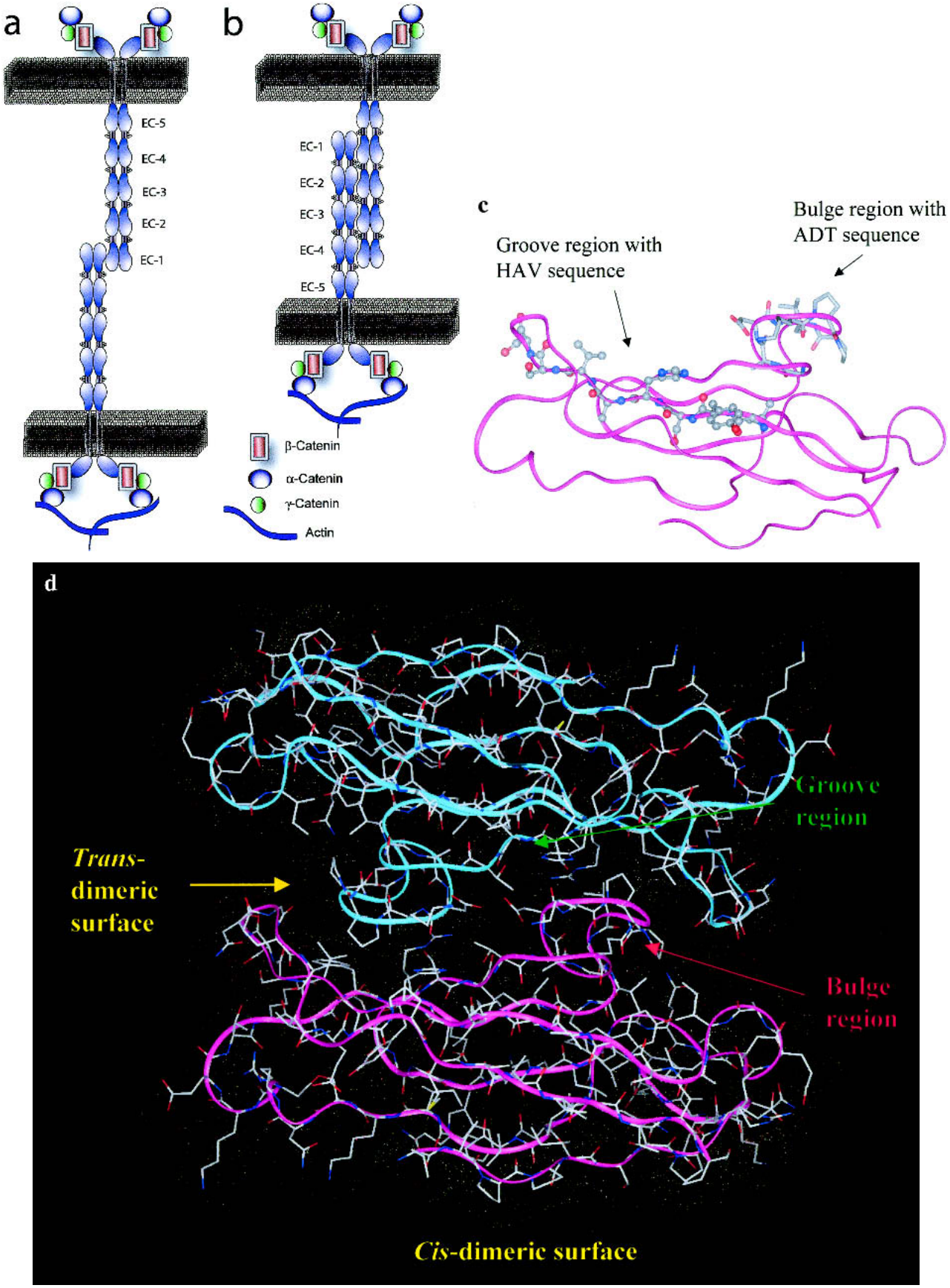

Fig. 1. The proposed *cis*- and *trans*-dimerization of E-cadherins in the intercellular junctions: (a) *trans*-dimerization mediated by EC1-to-EC1 interaction and (b) *trans*-dimerization mediated by multiple interactions of EC-domains (EC1-to-EC4). The black dots between the EC domains represent calcium ions. (c) The groove (SHAVSS) and bulge (ADTPP) regions in the EC1-domain of E-cadherin. (d) Model of *trans*-dimeric interaction between EC1-to-EC1 shows the bulge (ADTPP) region fits into the groove (SHAVSS) region of the EC1-domain.

quence are important for peptide selectivity to modulate the cellular junction of MDCK and bovine brain microvessel endothelial (BBME) cells (6–8). In addition, other possible interaction regions were identified using antibodies to E-cadherin (24,25).

In this work, we discovered a bulge region (QGADTPPVGV; Fig. 1c) counter to the HAV sequence in the groove region that is responsible for *trans*-cadherin interaction. This interaction between the groove and bulge regions was found by docking the x-ray structure of the EC1 domain with another EC1 domain of E-cadherins in a *trans*-dimer form (Fig. 1d) (17). To prove this hypothesis, we synthesized several peptides derived from the bulge region and evaluated their ability to modulate the intercellular junctions of MDCK cell monolayers. The biological activities of these bulge region peptides were compared to those of the groove region peptides (HAV peptides). Perturbation of intercellular junctions by peptides was followed using two different parameters, (a) the ability of peptides to decrease TEER and (b) the ability of peptides to increase mannitol flux via the MDCK monolayers. The ability of FITC-labeled peptides to bind directly to the intercellular junctions of MDCK monolayers is also evaluated.

MATERIALS AND METHODS

Materials

Fmoc-amino acids and resin were purchased from Bachem Biosciences (King of Prussia, PA, USA). HBTU was purchased from Peptides International (Louisville, KY, USA). Earle's balanced salts and lactalbumin enzymatic hydrolysate were obtained from Sigma (St. Louis, MO, USA), newborn calf serum was from Atlanta Biologicals (Norcross, GA, USA), and HBSS was from Cellgro Mediatech (Herndon, VA, USA). ^{14}C-mannitol was purchased from Life Science Products (Boston, MA, USA). All other reagents used in peptide synthesis and purification, as well as for cell culture experiments, were purchased from Fisher Chemical Co. (Pittsburgh, PA, USA), Aldrich Chemical Co. (Milwaukee, WI, USA), or Sigma (St. Louis, MO, USA).

Peptide Synthesis and Purification

All peptides used in this work (Table I) were acetylated and amidated at the N- and C-terminus. They were synthesized using a Rainin PS-3 peptide synthesizer by a solid-phase peptide synthesis (SPPS) method. Fmoc-amino acids and Fmoc-4-methoxy-4′-[γ-carboxypropyloxy]-benzhydrylamine-resin (DOD-resin) were used for the synthesis. [2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate] (HBTU) was added as the amino acid activator during the synthesis. The peptides were cleaved from the resin by trifluoroacetic acid at 30°C for 2 h in the presence of 7.5% v/v phenol as scavenger. The peptide was precipitated into cold ether and filtered out. In some cases, the peptide was extracted into 5% to 20% acetic acid solution from the ether solution followed by lyophilization. The crude peptides were purified by semi-preparative reversed-phase HPLC using a C18 column (Rainin, 21.4 × 250 mm, 12 μ, 300 Å) with a gradient of solvent A (5% acetonitrile in water containing 0.1% TFA) and solvent B (100% acetonitrile). The purity of peptide fractions was determined by analytical reversed-phase HPLC using a C18 column (4.6 × 250 mm, 5 μ, 300 Å) with same solvent system as in the preparative HPLC. The pure peptide fractions were combined, lyophilized, and confirmed by proton nuclear magnetic resonance (^{1}H-NMR) and FAB-MS.

Cell Culture

The MDCK cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) at serial passage 24. The cell line was subcultured several times; the cell cultures used in this work were from passages 48–67. Cells were grown in 150 cm^2 tissue culture-treated polystyrene flasks (Fisher Scientific, Pittsburgh, PA, USA) in a controlled atmosphere of 5% CO_2 and 95% relative humidity at 37°C. The culture medium consisted of 0.87 g/L Earle's balanced salt solution supplemented with 0.85 g/L sodium bicarbonate, 0.1 g/L penicillin G, 0.1 g/L streptomycin sulfate, 5 g/L lactalbumin enzymatic hydrolysate, and 5% newborn calf serum. The culture medium was replaced every other day for the first

Table I. Sequence of Synthetic Peptides

No	Peptide code	Sequence	MW	Origin
1	HAV-10	Ac-LFSHAVSSNG-NH_2	1059	groove region of EC-1 ^{h}Ecad
2	ADT-10	Ac-QGADTPPVGV-NH_2	981	bulge region of EC-1 ^{h}Ecad
3	HAV-6	Ac-SHAVSS-NH_2	628	groove region of EC-1 ^{h}Ecad
4	ADT-6	Ac-ADTPPV-NH_2	640	bulge region of EC-1 ^{h}Ecad
5	ADK-6	Ac-ADKPPV-NH_2	667	bulge region of EC-1 ^{m}Ecad
6	Amcap-1	Ac-ADKPPV-amcap-SHAVSS-NH_2	1349	
7	Amcap-2	Ac-SHAVSS-amcap-ADKPPV-NH_2	1349	
8	L-reversed	Ac-SSVAHS-NH_2	628	
9	D-reversed	Ac-SSVAHS-NH_2	628	
10	VVA	Ac-VVA-NH_2	329	
11	FITC-HAV-10	FITC-LFSHAVSSNG-NH_2	1406	
12	FITC-ADT-10	FITC-QGADTPPVGV-NH_2	1328	
13	FITC-ADT-6	FITC-ADTPPV-NH_2	987	

Notes. ^{h}Ecad, human E-cadherin; ^{m}Ecad, murine E-cadherin; Amcap, ω-aminocaproic acid; FITC, fluorescein isothiocyanate.

6 days, and every day thereafter. Nearly confluent cell monolayers (80–90% confluent) were trypsinized with 0.25% trypsin in 1 mM EDTA solution to give a cell suspension and one-fourth of the cell suspension was subcultured every 6–7 days.

For TEER measurements and paracellular transport studies, almost confluent cell monolayers were trypsinized and seeded on polystyrene filter inserts (0.4 μm pore, 10 mm diameter) inside 12-well Transwell™ plates (Costar, Cambridge MA, USA) at a density of 50,000 cells/cm^2. These cells were grown until confluency was reached on day eight. For fluorescence-localization of peptides, the cells were seeded on 48-well plates and used between days 6 and 8.

TEER Measurements

TEER values of cell monolayers were measured using an Evom epithelial voltohmmeter equipped with a STX-2 chopstick electrode (World Precision Instruments, Inc., Sarasota, FL, USA). Prior to peptide treatment, confluent MDCK cell monolayers were grown on polystyrene Transwell™ filters. The monolayers were then washed with pH 7.4 Hanks' balanced salt solution (HBSS) containing 10 mM Hepes, 1% glucose and 2 mM $CaCl_2$, followed by incubation in the same solution to allow the cells to equilibrate with the new medium. During incubation, the TEER values of the cell monolayers were measured at several time points until they reached a steady state. A stable TEER value was observed at 1–1.5 h; the measured value was used as an initial TEER. Only cell monolayers with an initial TEER value between 200 and 250 ohm·cm^2 were included in this experiment.

To examine the ability of peptides to change TEER values, 1 mM peptide solutions in HBSS, pH 7.4, were applied to the apical (AP) or basolateral (BL) sides, or simultaneously to both sides of cell monolayers. The TEER values were measured from 0 to 5 h at 1 h intervals after peptide addition. For each series of measurements, background resistance was measured using several unseeded wells a value of 80 ohm·cm^2; this value was used as a correction factor for each measurement. Each experiment was performed in triplicate to obtain the average and standard deviation.

Paracellular Transport Assay

Modulation of intercellular junctions by peptides was evaluated by measuring the enhancement of paracellular flux of [^{14}C]-mannitol across MDCK cell monolayers at 37°C. The cell monolayers were washed with pH 7.4 HBSS for 30 min, then treated with 1 mM peptide solution from the AP or BL side or from both sides simultaneously. After 1 h of peptide incubation, 10 μL of [^{14}C]-mannitol (NEN™, Life Science Products, Inc., Boston, MA, USA) was added to the AP side of each well. The accumulation of [^{14}C]-mannitol in the BL side was measured by counting the radioactivity of samples taken from the BL side using a Beckman LS-5801 liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). Aliquots of 30 μL were taken from each well at 0, 1, 2, 3 and 4 h time points after [^{14}C]-mannitol addition. After sampling, the wells were replenished with the same volume of HBSS to maintain a constant volume. Before scintillation counting, the sampled aliquots were diluted with 10 mL ScintiVerse (Fisher).

The flux (dQ/dt) of [^{14}C]-mannitol through MDCK cell monolayers was determined by plotting the concentration of accumulated [^{14}C]-mannitol in the BL chamber (Q) versus time (t). The apparent permeability coefficient (P_{app}) was calculated by the following equation: $P_{app} = Flux/D_o \cdot A$, where D_o represents the initial [^{14}C]-mannitol concentration in the AP chamber and A represents the cross-sectional area of the cell monolayers (1 cm^2). Triplicate experiments were performed to obtain the average and standard deviation.

Localization of Peptide Binding

Peptide localization and binding in the intercellular junctions were achieved using fluorescein isothiocyanate (FITC)-labeled peptide. FITC was conjugated to the N-terminal of LFSHAVSSNG-NH_2, QGADTPPVGV-NH_2, and ADTPPV-NH_2 peptides (Table I) by treating the peptides with FITC at a pH 9. The crude FITC-peptides were purified and analyzed by reversed-phase HPLC (see method above). The identities of FITC-LFSHAVSSNG-NH_2, FITC-QGADTPPVGV-NH_2, and FITC-ADTPPV-NH_2 were confirmed by mass spectrometry.

Prior to localization studies, the confluent cell monolayers grown on 48-well plates were washed 3 times for 10 min with HBSS pH 7.4 containing 2 mM Ca^{2+}. Following this washing, the cells were incubated with 3% bovine serum albumin (Sigma) in HBSS for 1 h to block the non-specific binding. The cell monolayers were washed again with HBSS and incubated with a 0.1 mM solution of FITC-labeled peptide for 1 h at 37°C. The cells were thoroughly washed with HBSS and then observed under a fluorescence microscope.

RESULTS

TEER Changes

Peptides from the Groove Region

The effect of HAV-10 (Fig. 2a) and HAV-6 (Fig. 2b) peptides on the modulation of tight intercellular junctions was monitored by observing the decrease in TEER values of MDCK cell monolayers. As negative controls, several different peptides were used, including L-reverse peptide (Ac-SSVAHS-NH_2), D-reverse peptide (Ac-ssvahs-NH_2), and an unrelated tripeptide (Ac-VVA-NH_2). Fig. 2a shows the effect of HAV-10 peptide on the TEER of MDCK monolayers when added from the AP or BL side or both sides simultaneously. The HAV-10 peptide was effective when the cells were treated from both sides (AP-BL). The peptide was also effective in reducing the TEER values when the monolayers were treated with HAV-10 from the BL side; however, it was slightly less effective than when simultaneous treatment from both sides was employed. In contrast, the peptide was not at all effective when administered from the AP side; this is due to the large size of the HAV-10 peptide, which cannot cross the tight junctions. As a negative control, the L-reverse peptide was not effective in modulating the tight junctions, and the result was similar to the blank.

HAV-10 was reduced to a hexapeptide (HAV-6) to test whether reducing the size of the peptide would affect its activity and ability to modulate the junctions from the AP side. HAV-6 reduced the TEER values when the MDCK cell

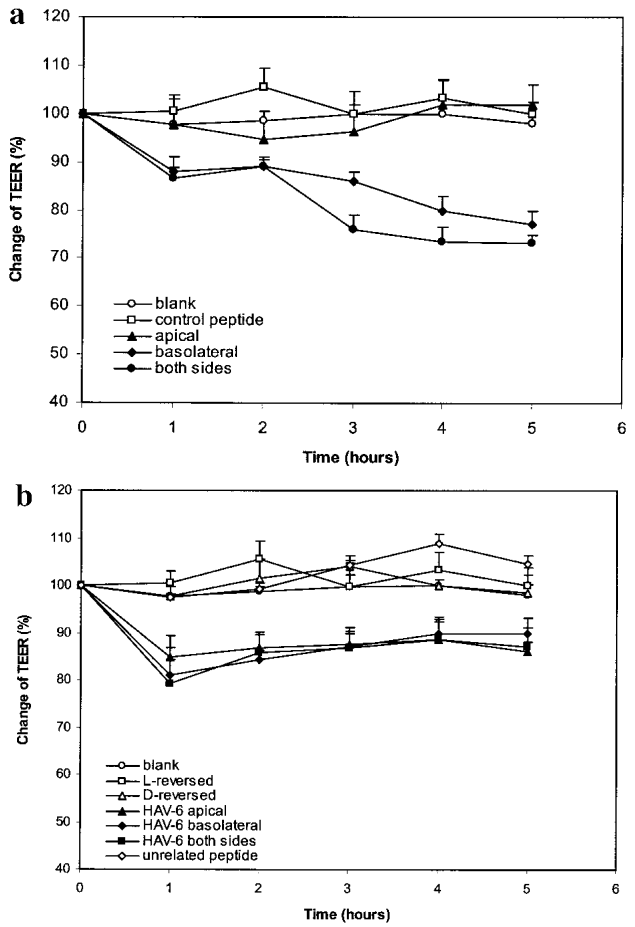

Fig. 2. Change in TEER values for MDCK cell monolayers incubated with 1.0 mM peptides from the apical (AP) or basolateral (BL) side or simultaneously from apical and basolateral (AP-BL) sides: (a) HAV-10 peptide and (b) HAV-6, L-reversed and D-reversed peptides. HAV-10 significantly decreased TEER when incubated from the BL and AP-BL sides, but did not lower TEER when incubated from the AP side. When reduced to a smaller peptide, HAV-6, it was significantly active whether applied from AP, BL, or AP-BL sides. The L-reversed and D-reversed peptides showed absolutely no activity and served as negative controls.

monolayers were treated from the AP or BL side or both sides simultaneously. The hexapeptide (HAV-6) can effectively modulate the junctions when applied to the AP side while HAV-10 cannot. The MDCK monolayers were also treated with L-reverse peptide, D-reverse peptide, and unrelated tripeptide from all sides (AP, BL, both sides); the results showed that these peptides were not effective. These peptides served as negative controls throughout the experiments.

Peptides from the Bulge Region

Modulation of intercellular junctions using peptides from the bulge region of EC1 (^{h}ADT-10, ^{h}ADT-6, ^{m}ADK-6; Table I) was evaluated on MDCK cell monolayers. ADT-10 and ADT-6 were deca- and hexapeptides derived from human E-cadherin sequence, and ADK-6 was derived from the mouse E-cadherin sequence (Table I). Figure 3a shows the activity of ADT-10 peptide when administered from the AP and BL sides and simultaneously from both sides. ADT-10 was very effective in lowering TEER values when administered from both sides simultaneously; the activity decreased when it was added from the BL side. Low activity was observed from the AP side due to the inefficiency of the peptide to permeate the tight junctions.

ADT-10 was reduced to a hexapeptide ADT-6 and a mutant peptide ADK-6 derived from mouse E-cadherin; the activities of these peptides were evaluated from the AP and BL sides, and from both sides simultaneously (Fig. 3b). ADT-6 was effective in lowering the TEER values of MDCK monolayers; treatment of the monolayers from BL side had the same effect as from both sides. Administration from the AP side still showed activity, but lower than that with treatment from the BL or both sides simultaneously. Thr3 in ADT-6 was mutated to Lys3 in ADK-6; the activity of ADK-6 was tested from both sides simultaneously. ADK-6 had a lower activity than ADT-6, suggesting that the selectivity of the peptide is sensitive to the peptide sequence.

Conjugation of Peptides from the Groove and Bulge Regions

ADK-6 and HAV-6 were conjugated via an ω-aminocaproic acid to give two different conjugates, Amcap-1 and Am-

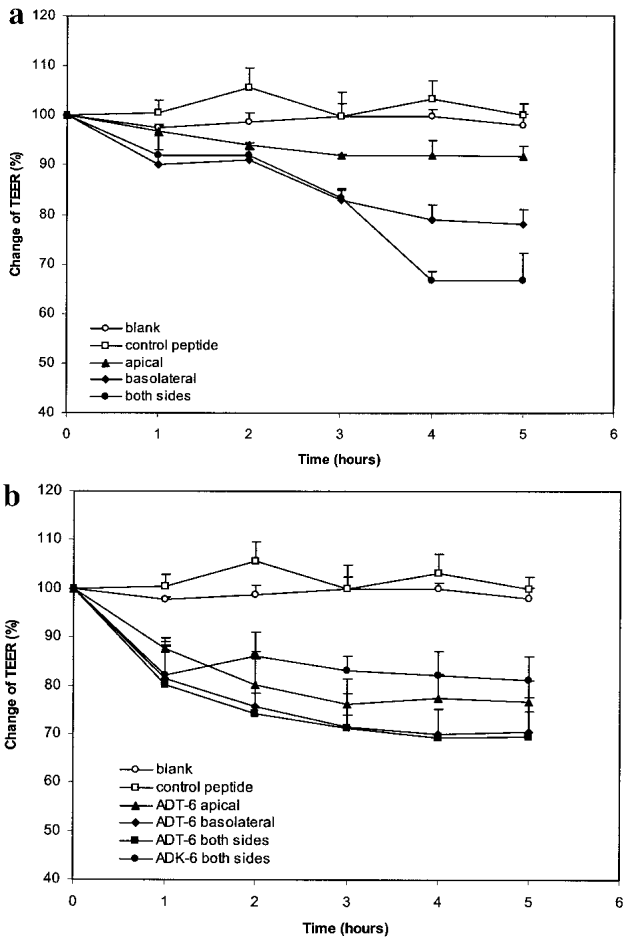

Fig. 3. Change of the TEER values for MDCK cell monolayers incubated with 1.0 mM peptides from AP, BL, AP-BL sides: (a) ADT-10 peptide and (b) ADT-6 and ADK-6 peptides. The ADT-10 lowers the TEER values when incubated from the BL and AP-BL, but is not very effective from the AP side. ADT-6 showed comparable activity whether applied from AP, BL, or AP-BL. When the Thr residue in ADT-6 was replaced with Lys to produce ADK-6, the activity was decreased.

cap-2 peptides; the ω-aminocaproic acid was used as a spacer between the two peptides (Table I). In Amcap-1 peptide, the ADK-6 and HAV-6 were conjugated to the N- and C-terminus of the aminocaproic acid, respectively. In Amcap-2, the ADK-6 and HAV-6 were conjugated to the C- and N-terminus of the ω-aminocaproic acid, respectively. Thus, the effect of ADK-6 and HAV-6 on the sequence of the conjugate could be evaluated. Figure 4a shows the effect of the Amcap-1 conjugate on TEER modulation; treatment from the BL and from both sides simultaneously can reduce the TEER of the MDCK cell monolayers. Due to the large size of Amcap-1, it is not effective in modulating the TEER from the AP side. On the other hand, Amcap-2 was only slightly effective when applied to both AP-BL sides and it was not effective from either the AP or BL side alone (Fig. 4b). This suggests that the arrangement of the conjugate is important on the activity of the conjugate.

Enhancement of ^{14}C-Mannitol Paracellular Transport by Peptides

Based on the TEER reduction results, we further evaluated the ability of the most potent peptides (HAV-10, HAV-6, ADT-10, ADT-6) to perturb the cadherin-cadherin interactions in the intercellular junctions (Table II). The peptide activities in increasing intercellular junction porosity were examined by measuring the enhancement of ^{14}C-mannitol paracellular transport in the AP-to-BL direction. As shown in Table II, BL and AP-BL treatment of the monolayers with HAV-10 peptide increased the transport of ^{14}C-mannitol across the monolayers; on the other hand, treatment of the MDCK monolayers from the AP side was less effective than AP-BL and BL treatments. Treatment of the monolayers using HAV-6 produced a result similar to that of HAV-10; however, AP treatment of the monolayers with HAV-6 produced a higher mannitol transport than did treatment with HAV-10. These results were consistent with the results of TEER modulations.

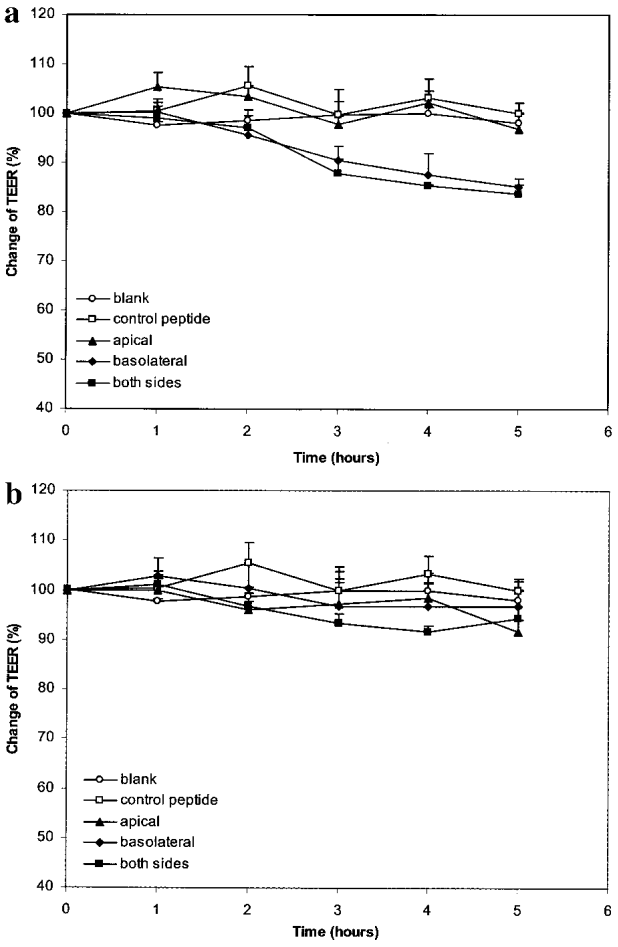

Fig. 4. The ability of 1.0 mM conjugated peptides (Amcap-1 and Amcap-2) to modulate intercellular junctions. (a) When added from the BL or AP-BL sides, the Amcap-1, with ADKPPV in the N-terminal side of SHAVSS as in the E-cadherin molecule, showed activity in decreasing the TEER of MDCK cell monolayers. (b) Amcap-2, with reversed position of the sequences, did not show activity. Both conjugates were ineffective when incubated on the AP side due to the large size of these molecules.

Peptides (ADT-10 and ADT-6) from the bulge region of the EC1 domain were examined for their ability to increase the porosity of the intercellular junctions (Table II). ADT-10 enhanced paracellular mannitol transport 1.7× , 4.5×, and 6.6× relative to control by treating the monolayers from AP, BL, and AP-BL sides; this is congruent with the TEER measurements. A smaller ADT-6 peptide also improved the mannitol transport when administered from the AP (3.2×) or BL (4.9×) or both sides simultaneously (5.1×) relative to control. Similar to the TEER measurements, ADT-6 was more effective than ADT-10 from the AP side; this is due to the size of these peptides. Overall, the mannitol flux results support our findings from the TEER measurements.

Intercellular Junctions Localization of the FITC-Labeled Peptides

The ability of FITC-labeled peptides (FITC-HAV-10, FITC-ADT-10, and FITC-ADT-6) to bind E-cadherins in the intercellular junctions was investigated by incubating the MDCK monolayers with the labeled peptide and observing the fluorescence emission from the FITC group. Fluorescence microscopy studies showed that the intercellular junctions of MDCKs monolayers were decorated by FITC-HAV-10, FITC-ADT-10, and FITC-ADT-6 peptides. The FTIC-labeled peptides showed up as punctate fluorescence spots at the cell borders. Figure 5 shows treatment of MDCK monolayers with FITC-labeled ADT-10 peptide observed under microscope using (a) normal light and (b) fluorescence light. These results were distinctly different than in the control where MDCK monolayers were treated with FITC alone; in that case, FITC reacted with the cell surface proteins and decorated the entire cells (data not shown).

Table II. Activity of Peptides in Enhancing Paracellular Transport

Peptide	P_{app} (cm/sec × 10^{-6}) [relative to control peptide]		
	Apical	Basolateral	Both sides
Control[a]	0.35 ± 0.04 [1.0×]	0.35 ± 0.04 [1.0×]	0.35 ± 0.04 [1.0×]
Blank[b]	0.37 ± 0.05 [1.0×]	0.37 ± 0.05 [1.0×]	0.37 ± 0.05 [1.0×]
HAV-10	0.61 ± 0.12 [1.7×]	1.31 ± 0.13 [3.7×]	1.88 ± 0.23 [5.4×]
HAV-6	1.01 ± 0.11 [2.9×]	1.59 ± 0.07 [4.5×]	1.71 ± 0.12 [4.9×]
ADT-10	0.60 ± 0.08 [1.7×]	1.57 ± 0.34 [4.5×]	2.31 ± 0.24 [6.6×]
ADT-6	1.12 ± 0.26 [3.2×]	1.70 ± 0.14 [4.9×]	1.80 ± 0.17 [5.1×]

[a] Unrelated peptide used as negative control.
[b] No peptide added to the system, only HBSS.

DISCUSSION

E-cadherins mediate cell-cell adhesion and play important roles in many biological processes, ranging from embryonal morphogenesis to the maintenance of the integrity of epithelial tissues in adult organisms, from bacterial entry to tumor metastasis (10,13,19,26–29). Understanding the molecular and atomic levels of E-cadherin-E-cadherin interactions is useful in designing molecules that can modulate various cellular functions mediated by E-cadherins. One application of the modulation of cadherin-cadherin interactions is the improvement of paracellular delivery of peptide and protein drugs via intercellular junctions (2,6,7,15). Therefore, our objective is to design peptides derived from the groove and bulge regions (Fig. 1c) of EC1 domain of E-cadherin to modulate the intercellular junctions. The ability of these peptides to modulate cadherin-cadherin interactions was evaluated in MDCK cell monolayers.

E-cadherins work as glue between the cellular junctions of biological barricades, including the intestinal mucosa (4,27) and the blood-brain (6,7) barriers as well as MDCK cell monolayers (30,31). Unfortunately, the exact mechanisms of E-cadherin-E-cadherin interactions between opposing cells are not well understood. It has been shown that the HAV sequence in the EC1 domain is critical for *trans*-dimer interactions (11,15,32), and that peptides containing the HAV sequence can inhibit cell-cell aggregation in BBMEC (6) and modulate the intercellular junctions of BBMEC monolayers (7,33). However, the counter-sequence, in which the HAV sequence (groove region) can interact in the EC1 or other domains, has not been identified. Therefore, we used the X-ray structure of the EC1 domain of E-cadherin to find the counter-sequence of the HAV peptide (17). Molecular docking experiments were performed to elucidate the possibility of HAV peptide binding site(s). The results show that the peptide can bind to a bulge region with a QGADTPPVGV sequence in the EC1 domain (8). Using this finding, we tried to model the EC1-EC1 antiparallel *trans*-dimerization; this fits the idea that the bulge region containing the QGADTPPVGV sequence interacts with the groove region containing the HAV sequence (Fig. 1d). It should be noted that this model assumes that the homophilic cadherin-cadherin interaction in *trans*-dimerization form occurred through interaction of EC1-to-EC1, as proposed by Nagar *et al.* (17). Thus, it does not rule out the possibility of interaction between EC1 and other EC domains (i.e., EC1-to-EC2 interaction) because the other EC domains (i.e., EC2) may have the same general structure as EC1. If this docking study is correct, the peptides from the bulge region as well as from the groove region should be able to modulate E-cadherin-E-cadherin interactions in the intercellular junctions.

To test this hypothesis, we synthesized peptides derived from the bulge (ADT-10 and ADT-6) and groove (HAV-10 and HAV-6) regions and evaluated their ability to modulate the E-cadherin-mediated intercellular junctions (Table I). The groove (HAV-10) and bulge (ADT-10) decapeptides lowered the TEER values of the MDCK monolayers when administered from the BL side and simultaneously from the AP and BL sides compared to control peptides (L-reverse, D-reverse, and unrelated tripeptide) (Fig. 2a and Fig. 3a). However, these decapeptides were not very effective in modulating the intercellular junctions from the AP side because they were too large to permeate via the tight junctions (*zonula occluden*) and reach the adherens junctions (*zonula adherens*) where E-cadherin is located. The HAV peptides (HAV-10 and HAV-6) have different sequences than the ADT peptides (ADT-10 and ADT-6), and yet, both HAV and ADT hexapeptides were more effective than the decapeptides when administered from the apical (AP) side. Therefore, the sequences of the peptides do not impede the apical penetration of the peptides; it must be their size.

Size reduction of these decapeptides to hexapeptides (HAV-6 and ADT-6) made them able to modulate the intercellular junctions regardless of their sequence (see below). On the other hand, the decapeptides were effective from the BL side due to the absence of tight junctions that keep the peptides from reaching the E-cadherins at the adherens junctions. The highest activity was found when the monolayers were treated simultaneously from both sides. These results suggest that decapeptides introduced from the AP side permeate more effectively to help the modulation of the intercellular junctions after some junction opening by the peptides from the BL side. The loosening of the intercellular junctions shown here was not due to cell death or damage because the cell viability was higher than 95% after incubation with peptide solution for 6 h. Thus, these results confirm the potential ability of the peptides to increase the porosity of the intercellular junctions.

To confirm the increase in porosity of the intercellular junctions by these peptides, the enhancement of the paracellular transport of ^{14}C-mannitol was examined via the decapeptide-treated MDCK cell monolayers (Table II). Interestingly, the decapeptides enhanced the mannitol flux when the peptides were added from the AP, BL or both sides. As in the TEER modulation, these decapeptides were very effective when used from the BL or both sides simultaneously and caused an increase in the mannitol flux of around 3.7 to 6.6 times compared to the control peptide. Although the decapeptides (HAV-10 and ADT-10) were not effective in lowering the TEER from the AP side, they were able to improve the mannitol flux 1.7$_\times$ compared to the control peptide (Table II). Therefore, the TEER value modulation results were congruent with the mannitol flux measurement results.

Because the decapeptide is unable to modulate the intercellular junctions from the AP side, it is unlikely that it will be useful in our goal of improving the paracellular drug delivery of peptide and protein drugs *in vivo* because the delivery of these therapeutic molecules is primarily in the AP-to-BL direction. Thus, the cadherin peptides have to surmount the tight junctions prior to working on the cadherin-cadherin interactions in the *zonula adherens*. To overcome this hurdle, we reduced the size of the decapeptides to hexapeptides (ADT-6 and HAV-6), hoping that these hexapeptides would be effective in modulating intercellular junction porosity when administered from the AP side of the monolayers (Table II). Interestingly, both hexapeptides were effective in modulating the TEER values when applied from AP side as well as from the BL or from both sides simultaneously (Fig. 2b and 3b); thus, they were able to penetrate the AP tight junctions (*zonula occludens*). To evaluate the effect of sequence on peptide activity, we synthesized the ADK-6 peptide derived from mouse E-cadherin; this peptide has a Lys3 instead of the Thr3 found in the ADT-6 peptide from human E-cadherin. The ADK-6 peptide showed lower activity than

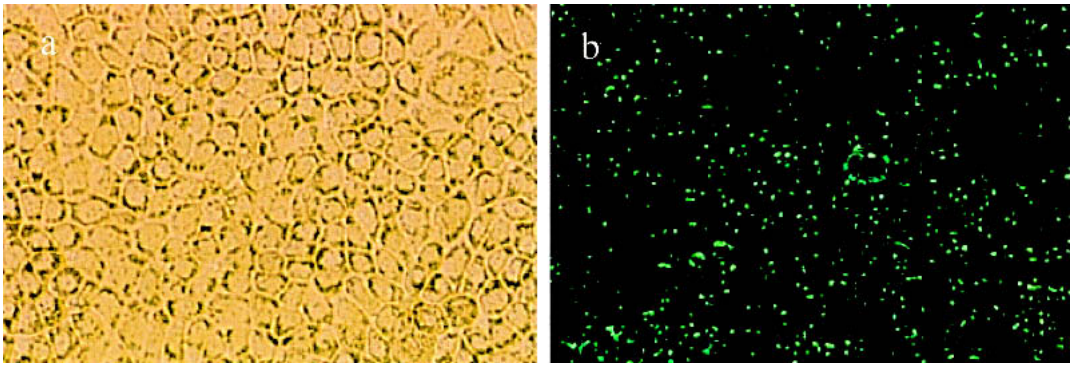

Fig. 5. MDCK monolayers treated with FITC-labeled ADT-10 peptide observed under a microscope using (a) normal light and (b) fluorescent light. The FITC-ADT-10 decorated the intercellular junctions of MDCK monolayers as punctate dots.

the ADT-6 peptide (Fig. 3b), suggesting that the activity of these peptides is sequence specific; in other words, the intercellular junctions of E-cadherins recognize Thr3 better than Lys3 in the hexapeptides. This also suggests that the E-cadherins in the MDCK monolayers may have a higher homology to the human than to the mouse E-cadherins, particularly at the bulge region.

The increase in paracellular porosity produced by the hexapeptides (ADT-6 and HAV-6) was also examined using ^{14}C-mannitol flux measurements (Table II). Similar to the decapeptides, these hexapeptides also enhanced the paracellular permeation of ^{14}C-mannitol when administered from the AP, BL and both sides. From the AP side, the hexapeptides improved the mannitol flux about threefold over the control peptide, which is about 1.8× higher than the effect of decapeptides.

The ability of hexapeptides (ADT-6 and HAV-6) to improve mannitol flux was compared to the respective decapeptides (ADT-10 and ADT-6) with a different mode of addition (Fig. 6). ADT-6 enhanced the mannitol flux better than ADT-10 when administered from the AP side (Fig. 6a); however, there was no difference in activity between ADT-6 and ADT-10 when administered from the BL side. ADT-10 has a better activity than ADT-6 in enhancing mannitol transport when added from both sides. HAV-6 was a better modulator than HAV-10 when added from the AP and BL sides but there was no difference in activity between HAV-6 and HAV-10 when added simultaneously from both sides (Fig. 6b).

To get more information on peptide-cadherin interactions, we examined the activities of L-reversed (Ac-SSVAHS-NH_2) and D-reversed (Ac-ssvahs-NH_2) hexapeptides. Besides serving as negative controls, these peptides gave additional information on the nature of binding between the HAV peptides (i.e., HAV-6 = Ac-SHAVSS-NH_2). Others have shown that retro-inverse peptides such as the D-reversed peptide may establish the nature of the involvement of the side chain and backbone interactions of the peptide with the protein receptor (34,35). If the D-reverse peptide binds to the receptor, this indicates that the peptide binds only to the receptors via the side chain without the involvement of the backbone interaction. In this case, the D-reversed peptide did not lower the TEER value of MDCK monolayers; thus, the D-reversed peptide did not bind to E-cadherin and modulate cadherin-cadherin interactions. This suggests that binding between the HAV-6 peptide and E-cadherins may involve both side chain and backbone interactions (i.e., hydrogen bond formations). The L-reversed peptide did not have any activity, suggesting the importance of sequence selectivity of the E-cadherin.

To evaluate the potential synergistic effect of both the groove and bulge regions, we evaluated the conjugated peptides Amcap-1 and Amcap-2, each of which contains both important sequences (Table II). The conjugated peptides were synthesized by linking SHAVSS and ADKPPV sequences via an ω-aminocaproic acid. The choice of an ω-aminocaproic acid linker was based on the approximately 6–10 Å distance between the SHAVSS and ADKPPV sequences in the X-ray structure of the EC1 domain. The distance between the N- and C-termini of ω-aminocaproic acid is about 7 Å. Amcap-1 lowered the TEER values of MDCK cell monolayers treated from the BL and AP-BL sides but not from the AP side (Fig. 4a). On the other hand, Amcap-2 was not effective in modulating the intercellular junctions regardless of the side of administration (Fig. 4b). This suggests that the conjugation position of ADKPPV and SHAVSS in the linker affects the binding of the conjugate to E-cadherin. These data indicate that the peptides bind to E-cadherin molecules in an antiparallel manner, as in native E-cadherin-E-cadherin *trans*-interaction. When the position of these sequences was reversed in Amcap-2, the peptide activity was completely abolished. Similar to HAV-10, Amcap-1 was not effective from the apical side due to the size of this molecule. Amcap-1 was less effective than the decapeptides (ADT-10 and ADT-10) and hexapeptides (ADT-6 and ADT-6) when incubated up to 5 h. However, Amcap-1 modulated the TEER values in a manner similar to HAV-6 when incubated about 7 h (data not show). This suggests several possible explanations. First, the SHAVSS and ADKPPV sequences may not work in a synergistic manner. Second, the Amcap-1 conjugate is too large to be effective in percolating through the *desmosomes* and *zonula adherens*. Third, the SHAVSS and ADKPPV sequences produced intramolecular peptide-peptide interactions that require energy to dissociate and produce an intermolecular interaction with the E-cadherin. In the future, the length of the linker will be adjusted to improve the synergistic effects of the Amcap-1 derivative; in addition, ADTPPV will be used in the future conjugates.

The direct localization of the hexapeptides and decapeptides in the intercellular junctions was examined using the FITC-labeled peptides (FITC-HAV-10, FITC-ADT-10, and

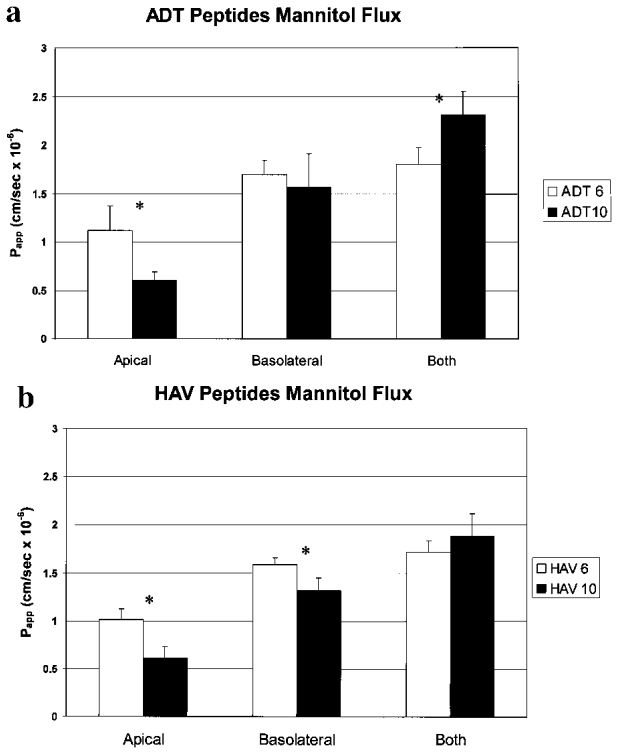

Fig. 6. Comparison between hexapeptides and their respective decapeptides in enhancing mannitol flux when administered from AP, BL, and both AP-BL sides. (a) ADT-6 and ADT-10. (b) HAV-6 and HAV-10. *Indicates statistical significance.

FITC-ADT-6). Since the intercellular junctions were not made permeable using EDTA, these peptides bound to the intercellular junctions as punctate fluorescence spots around the MDCK cell borders (Fig. 5b). The punctate fluorescence spots suggest that the peptide binds to E-cadherins at the adherent junctions not at the tight junctions. However, it is difficult to determine whether the peptides bind at the bicellular or tricellular junctions using fluorescence microscope data.

In conclusion, we have discovered another recognition site for E-cadherin-E-cadherin interactions, located in the bulge region of the EC1 domain of E-cadherin, which has an ADTPPV sequence. This sequence seems to recognize the SHAVSS sequence located in the groove region of the same domain in another E-cadherin molecule. We also found that the modulation of TEER values by the peptides is consistent with an increase in the paracellular transport of ^{14}C-mannitol. In the future, we will study several different parameters that are involved in modulation—the intercellular junctions, including pore size, time-dependent peptide activity, and the mechanisms of action of the peptides at the atomic level. Furthermore, we will synthesize and evaluate the derivatives of ADT-6 and HAV-6 for improving the modulator activity.

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