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Domain mapping of a claudin-4 modulator, the C-terminal region of C-terminal fragment of *Clostridium perfringens* enterotoxin, by site-directed mutagenesis

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ABSTRACT

A C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) is a modulator of claudin-4. We previously found that upon deletion of the C-terminal 16 amino acids, C-CPE lost its ability to modulate claudin-4. Tyrosine residues in the 16 amino acids were involved in the modulation of claudin-4. In the present study, we performed functional domain mapping of the 16-amino acid region of C-CPE by replacing individual amino acids with alanine. To evaluate the ability of the alanine-substituted mutants to interact with claudin-4, we carried out a competition analysis using claudin-4-targeting protein synthesis inhibitory factor. We found that Tyr306Ala, Tyr310Ala, Tyr312Ala, and Leu315Ala mutants had reduced binding to claudin-4 compared to C-CPE. Next, we investigated effects of each alanine-substituted mutant on the TJ-barrier function in Caco-2 monolayer cells. The TJ-disrupting activity of C-CPE was reduced by the Tyr306Ala and Leu315Ala substitutions. Enhancement of rat jejunal absorption was also decreased by each of these mutations. The double mutant Tyr306Ala/Leu315Ala lost the ability to interact with claudin-4, modulate TJ-barrier function, and enhance jejunal absorption. These data indicate that Tyr306 and Leu315 are key residues in the modulation of claudin-4 by C-CPE. This information should be useful for the development of a novel claudin modulator based on C-CPE.

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Abbreviations: TJ, tight junction; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; CPE, *Clostridium perfringens* enterotoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; C-CPE-PSIF, C-CPE-fused protein synthesis inhibitory factor; LDH, lactate dehydrogenase; TEER, transepithelial electric resistance; FD-4, fluorescein isothiocyanate-dextran with a molecular weight of 4000.

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1. Introduction

Sequencing of the human genome has provided useful information about molecular targets for drug development and has enabled target molecule-based drug discovery. Many drug candidates, however, are eliminated during clinical development due to severe side effects caused by inadequate pharmacokinetic properties and biodistribution [1,2]. The ability of a drug to pass through epithelial and/or endothelial cell sheets is a critical aspect of its pharmacokinetics and biodistribution.

There are two routes by which drugs across epithelial and endothelial cell sheets: transcellular and paracellular. In the transcellular route, drugs are delivered by simple diffusion into the cell membranes and active transport via a receptor or transporter on cell membranes [3,4]. Transcellular delivery via transporters has been widely investigated, and the transporters involved in the influx and efflux of peptides and organic anions and cations have been identified [4–8]. The expression profiles of the transporters differ among tissues, and therefore methods for delivering drugs to a target tissue using a specific transporter may be promising; however, it may be necessary to modify the drug to target it to the appropriate transporter. Such modifications should not affect the pharmaceutical activity of the drug. Thus, the transcellular route is not always suitable for drugs created by genome-based high-throughput production.

In the paracellular route, a drug is delivered to cells by loosening the tight junctions (TJs), which normally restrict the movement of substances through the intercellular space in epithelial and endothelial cell sheets [9,10]. Therefore, to deliver drugs through the paracellular route, it is necessary to modulate the TJ-barrier function. TJ modulators have been developed as enhancers of absorption since the 1960s [11–13]. These absorption enhancers include chelators and surfactants, and they enhance absorption by dilating TJs, allowing drugs to enter the intercellular spaces of epithelial cell sheets [14]. Because opening of TJs is suitable for delivering a variety of molecules, absorption enhancers can be used for drug candidates created by genome-based high-throughput production; however their use is limited because they cause severe side effects, including exfoliation of the intestinal epithelium, irreversibly compromising its barrier functions, and because they have low tissue specificity [14–16].

As mentioned above, TJs form an intercellular seal and control solute movement through the paracellular route across epithelium and endothelium, thus maintaining the composition of the tissue interior [17,18]. There are some differences in the permeability of the TJ barrier in different types of epithelium and endothelium due to their specific physiological requirements [9]. This implies that a molecule regulating the tissue-specific barrier function of TJs should be useful for the delivery of drugs via the paracellular route.

Studies by Tsukita and co-workers have revealed that claudin plays a pivotal role in regulation of the TJ barrier [18]. Claudins are four-transmembrane proteins with molecular masses of ~23 kDa and form a large family with at least 24 members [19]. The expression of each claudin family member varies by cell type and tissue [17,20]. For instance, claudin-1 is ubiquitously expressed, whereas claudin-16 and claudin-6 are expressed in specific cell types and during specific periods of development, respectively [21,22]. Interestingly, the barrier functions of claudin are also tissue-specific. Deletion of claudin-1 and claudin-5 results in disruption of the epidermal and blood–brain barrier, respectively [23,24]. Moreover, claudins form homo/hetero-paired strands in the membrane between adjacent cells [25,26]. Because there are at least 24 claudin family members, many different strand pairs could be formed, which could give TJs a high degree of tissue specificity. The ability to modulate the barrier-function of claudin in a member-specific manner would allow tissue-specific delivery of drugs through the paracellular pathway.

Clostridium perfringens enterotoxin (CPE) causes food poisoning in humans. It consists of two functional domains, an N-terminal cytotoxic region and a C-terminal receptor-binding region (C-CPE) [27–29]. A receptor for CPE was identified in 1997 [30], and it was found to be identical to claudin-4 in 1999 [20]. Treatment of cells with C-CPE causes a decrease in intracellular claudin-4 levels and disruption of the TJ barrier in epithelial cell sheets [31]. Using C-CPE as a claudin modulator, we previously showed that it is possible to enhance drug absorption by 400-fold compared to sodium caprate, the only absorption enhancer used in the clinic [32]. Thus, claudin is a novel target molecule for drug delivery through the paracellular pathway.

Currently, C-CPE is the only known modulator of claudin. Functional domain mapping of C-CPE is useful for development of a claudin modulator using C-CPE as a prototype. Indeed, we previously found that upon deletion of the C-terminal 16 amino acids, C-CPE loses its ability to modulate claudin-4. Tyr306 is a key residue for claudin-4 modulation by C-CPE [32,33]. In the present study, we performed systemic analyses of each of the C-terminal 16 amino acids. We found that Leu315 in addition to Tyr306 is important for the ability of C-CPE to modulate the TJ barrier.

2. Materials and methods

2.1. Materials

Anti-His-tag and anti-claudin-4 antibodies were obtained from Novagen (Madison, WI) and Zymed Laboratories (South San Francisco, CA), respectively. Ni-NTA agarose and PD-10 columns were purchased from Invitrogen (Carlsbad, CA) and GE Healthcare Bio-Sciences Co. (Piscataway, NJ), respectively. The reagents used in this study were of research grade.

2.2. Cell cultures

Human intestinal Caco-2 cells at passages 68–80 were used for transepithelial electrical resistance (TEER) assays. Claudin-4-expressing mouse fibroblast cells (CL4/L cells) were kindly provided by Drs. S. Tsukita and M. Furuse (Kyoto University, Japan). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37 °C. CL4/L cells were maintained in modified Eagle's medium containing 10% fetal bovine serum at 37 °C.

2.3. Preparation of C-CPE mutants

Each of the C-terminal 16 amino acids of C-CPE were individually mutated to Ala by polymerase chain reaction (PCR) using the primers listed in Tables 1 and 2 for single and double mutants, respectively, and pET-H_{10}PER as a template as follows [30]. The resulting PCR fragments encoding the Ala-substituted mutants were ligated into the pET-16b vector (Novagen) via the NdeI/BamHI site, and the DNA sequence was confirmed. Each plasmid was transduced into *Escherichia coli* BL21 (DE3), and production of mutant C-CPEs was induced by addition of isopropyl-β-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris–HCl [pH 8.0], 400 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, and 1 mM β-mercaptoethanol), supplemented with 8 M urea when necessary. The lysates were applied to a HiTrap™ Chelating HP (GE Healthcare), and mutant C-CPEs were eluted with buffer A containing 100–1000 mM imidazole. The buffer was exchanged with phosphate-buffered saline by gel filtration using a PD-10 column. The concentrations of mutant C-CPEs were estimated using a protein assay kit with bovine serum albumin as a standard (Bio-Rad, Hercules, CA). The purification of mutant C-CPEs was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining of the gels with Coomassie Brilliant Blue (data not shown).

2.4. Competition assay

We previously prepared C-CPE-fused protein synthesis inhibitory factor (C-CPE-PSIF), which is a claudin-4-targeting molecule, and we showed that it is cytotoxic to CL4/L cells [34]. To assess the interaction between mutant C-CPEs and claudin-4, we evaluated the competitive inhibition of C-CPE-PSIF-induced cytotoxicity by mutant C-CPEs as follows. CL4/L cells were pretreated with C-CPE or mutant C-CPE at the indicated concentrations for 1 h, after which C-CPE-PSIF was added to the cells. After an additional 36 h of culture, the cytotoxicity of C-CPE-PSIF was assayed by the release of lactate dehydrogenase (LDH) using a CytoTox96 Non-Radioactive Cytotoxicity Assay kit according to the manufacturer's protocol (Promega, Madison, WI). The cytotoxicity was expressed according to the following equation: % maximum LDH release = 100 × (LDH released from the mutant-treated CL4/L cells/the total LDH content in the cells).

2.5. Pull-down assay

Confluent Caco-2 cells, which develop TJs, were harvested and lysed in lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1% protease inhibitor cocktail [Sigma, St. Louis, MO]). The resultant Caco-2 lysates were used for pull-down assay. Epithelial cell layers in rat jejunum were recovered and lysed in lysis buffer (25 mM Tris–HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail). The buffer of the rat lysates was changed into phosphate-buffered saline by gel filtration using a PD-10 column. The resultant lysates of rat jejunum were used for pull-down assay. C-CPE or mutant C-CPEs were incubated with the lysates for 30 min at 37 °C and then mixed with Ni-NTA agarose (Invitrogen). After an additional 3 h at 4 °C, the beads were washed, and bound proteins were analyzed by SDS-PAGE followed by Western blotting using anti-claudin-4 and anti-His-tag antibodies. The bound primary antibody

Table 1 – Primers used for alanine scan

Primers	Sequences (5′–3′)
Common forward primer	ggaattc catatg gaa aga tgt gtt tta aca gtt cca tct aca
Reverse primer for Ser304Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act ata tat *gca* att agc ttt cat tac aag aac
Ser305Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act ata *tgc* tga att agc ttt cat tac aag
Tyr306Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *agc* tga tga att agc ttt cat tac
Ser307Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc *agc* ata tga tga att agc ttt c
Gly308Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att *tgc* act ata tga tga att agc ttt
Asn309Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta *tgc* tcc act ata tga tga att agc
Tyr310Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata agg *tgc* att tcc act ata tga tga
Pro311Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga ata *tgc* gta att tcc act ata tga tga
Tyr312Ala	cg ggatcc tta aaa ttt ttg aaa taa tat tga *agc* agg gta att tcc act ata tga
Ser313Ala	cg ggatcc tta aaa ttt ttg aaa taa tat *tgc* ata agg gta att tcc act ata
Ile314Ala	cg ggatcc tta aaa ttt ttg aaa taa *tgc* tga ata agg gta att tcc act
Leu315Ala	cg ggatcc tta aaa ttt ttg aaa *tgc* tat tga ata agg gta att tcc
Gln317Ala	cg ggatcc tta aaa ttt *tgc* aaa taa tat tga ata agg gta att
Lys318Ala	cg ggatcc tta aaa *tgc* ttg aaa taa tat tga ata agg gta att
Phe319Ala	cg ggatcc tta *agc* ttt ttg aaa taa tat tga ata agg gta att

The underlined sequence in the common forward primer indicates the NdeI site. The underlined sequence in the reverse primers and the italicized codon indicate the BamHI site and the alanine-substituted residue, respectively.

Table 2 – Primers and templates used for preparation of double-alanine mutants

Primers or templates	Sequence (5′–3′)/template
Common forward primer	ggaattc catatg gaa aga tgt gtt tta aca gtt cca tct aca
Tyr306Ala/Leu315Ala	
Reverse primer	cg ggatcc tta aaa ttt ttg aaa *tgc* tat tga ata agg gta att tcc
Template	Tyr306Ala
Tyr310Ala/Leu315Ala	
Reverse primer	cg ggatcc tta aaa ttt ttg aaa *tgc* tat tga ata agg *ggc* att tcc
Template	Tyr310Ala
Tyr312Ala/Leu315Ala	
Reverse primer	cg ggatcc tta aaa ttt ttg aaa *tgc* tat tga *agc* agg gta att tcc
Template	Tyr312Ala

The underlined sequence in the forward and reverse primer indicate NdeI and BamHI sites, respectively. The italicized sequences in the reverse primers indicate the sites of mutation. The templates was pET-16b vector containing the indicated mutant C-CPE.

was detected with a peroxidase-labeled secondary antibody followed by visualization with chemiluminescence reagents (Amersham Bioscience, Piscataway, NJ).

2.6. TEER assay

Caco-2 cells were seeded in Transwell™ chambers (Corning, NY) at a subconfluent density. TEER of the Caco-2 monolayer cell sheets on the chamber, a sign of TJ integrity, was measured using a Millicell-ERS epithelial volt-ohmmeter (Millipore, Bedford, MA). When the TEER values reached a plateau and the TJs were well developed, the Caco-2 monolayers were treated with C-CPE or mutant C-CPEs on the apical side of the chamber, and the TEER values were measured. The TEER values were normalized by the area of the Caco-2 monolayer. The TEER value of a blank Transwell™ chamber (background) was subtracted.

2.7. In situ loop assay

The experimental protocol for the *in situ* loop assay was approved by the Ethics Committee of Showa Pharmaceutical University. Wistar male rats (250–280 g; Animal and Material Laboratories, Inc., Tokyo, Japan) were allowed at least a week to adapt in an environmentally controlled room. Fluorescein isothiocyanate-dextran with a molecular weight of 4000 (FD-4) was used as a model drug that passes across the intestinal epithelium mainly through the paracellular route [35–37]. Rats were anesthetized with thiamylal sodium (Mitsubishi Pharma Co. Ltd., Osaka, Japan). A midline abdominal incision was made, and the lumen of the jejunum was washed with phosphate-buffered saline. A jejunal loop (5 cm in length) was prepared by closing both ends with sutures. A mixture of FD-4 and C-CPEs was administered into the jejunal loop. Blood was collected from the jugular vein at the indicated time points. The plasma concentration of FD-4 was determined with a fluorescence spectrophotometer (Fluoroskan Ascent FL; Thermo Electron Corp., Waltham, MA). The area under the plasma concentration-time curve from 0 to 4 h (AUC_{0-4}) was calculated by the trapezoidal method.

2.8. Statistical analysis

Significant difference is evaluated by ANOVA followed by student *t*-test, and the significant difference is set at $p < 0.05$.

3. Results

3.1. Alanine scan of the C-terminal 16 amino acids in C-CPE

We previously found that upon deletion of the 16 C-terminal amino acids, C-CPE loses its ability to disrupt the TJ barrier and to interact with claudin-4, and tyrosine residues in the 16 amino acids are involved in its modulation of claudin-4 [38]. However, systematic analysis of the 16 amino acids has never been performed. To examine the function of each of the 16 amino acids, we generated mutants in which the individual amino acids were replaced with alanine. Of the 16 mutants, all except for Phe316Ala, could be expressed in *E. coli* BL21 (DE3). Thus, we were not able to examine the role of Phe316.

We previously prepared the claudin-4-targeting cytotoxic recombinant protein C-CPE-PSIF [34]. To screen the interaction of the mutants with claudin-4, we examined their ability to reduce C-CPE-PSIF-induced cytotoxicity in claudin-4-expressing CL4/L cells. Treatment of the cells with C-CPE-PSIF induced the release of approximately 55–65% of the total LDH (data not shown). The inhibition of C-CPE-PSIF-induced LDH release by each mutant C-CPE is summarized in Table 3. These results suggest that substitution of Tyr306, Tyr310, Tyr312, or Leu315 with alanine resulted in a decrease in the binding of the mutant C-CPEs to claudin-4, whereas replacement of Ser304, Ser305, Ser307, Asn309, Ser313, or Lys318 by alanine slightly increased the binding to claudin-4.

Next, we examined the effects of each mutant on the TEER value, an indicator of the tightness of the TJs. The experiments were performed using monolayers of human intestinal Caco-2 cells grown on a membrane, a method commonly used for evaluating the TJ-barrier function. Treatment of the cells with C-CPE at 20 μg/ml for 18 h lowered the TEER value from 498 to 22 Ω cm^2 (data not shown). TJ-modulating activities were calculated as the ratio of the reduction in TEER for the mutant compared to C-CPE and are presented in Table 4. Only the Tyr306Ala and Leu315Ala mutants had weaker TJ-modulating activity than C-CPE (64.1 and 45.1% of C-CPE, respectively).

3.2. Effect of Leu315Ala substitution on the ability of C-CPE to interact with claudin-4

Because of these findings, we performed further studies of the Tyr306 and Leu315 mutants. We confirmed the interaction of Tyr306Ala and Leu315Ala with claudin-4 by a pull-down assay using lysate from Caco-2 cells. Claudin-4 was precipitated by C-CPE at 1 μg/ml, whereas claudin-4 was not precipitated by Tyr306Ala or Leu315Ala at the same concentration (Fig. 1A). An

Table 3 – Competitive inhibition of C-CPE-PSIF-induced LDH release by mutant C-CPEs

Wild-type or mutant C-CPE	Inhibitory ratio (% of C-CPE)
C-CPE	100
Ser304Ala	125.6 ± 0.7
Ser305Ala	126.8 ± 0.2
Tyr306Ala	63.8 ± 0.4
Ser307Ala	123.6 ± 2.2
Gly308Ala	99.1 ± 2.7
Asn309Ala	125.6 ± 1.8
Tyr310Ala	72.2 ± 2.3
Phe311Ala	114.9 ± 0.5
Tyr312Ala	73.1 ± 2.4
Ser313Ala	132.9 ± 0.8
Ile314Ala	94.3 ± 3.1
Leu315Ala	69.1 ± 2.7
Gln317Ala	96.8 ± 1.6
Lys318Ala	126.2 ± 2.1
Phe319Ala	111.5 ± 3.9

After a 1 h of treatment with C-CPE or mutant C-CPEs at 5 μg/ml, claudin-4-exressing L cells were treated with C-CPE-PSIF (0.2 μg/ml) for 36 h, and the release of LDH was determined. The results are shown as the percent of C-CPE-induced LDH release, and the values are the means ± S.D. ($n = 4$).

Table 4 – Effects of mutant C-CPEs on TJ barrier in Caco-2 cells

Wild-type or mutant C-CPE	Decreased ratio of TEER (% of C-CPE)
C-CPE	100
Ser304Ala	100.3 ± 0.5
Ser305Ala	100.1 ± 2.3
Tyr306Ala	64.1 ± 4.3*
Ser307Ala	100.4 ± 1.8
Gly308Ala	98.1 ± 0.2
Asn309Ala	104.4 ± 1.3
Tyr310Ala	93.8 ± 1.2
Pro311Ala	101.4 ± 2.3
Tyr312Ala	100.1 ± 1.5
Ser313Ala	104.6 ± 0.8
Ile314Ala	98.3 ± 1.4
Leu315Ala	45.1 ± 5.3*
Gln317Ala	98.8 ± 1.3
Lys318Ala	99.3 ± 0.8
Phe319Ala	103.5 ± 0.9

Caco-2 cells were seeded on a Transwell™. After development of the TJ barrier in Caco-2 cells, C-CPE or mutant C-CPEs was added at 20 μg/ml, and TEER was measured. The decrease in the ratio of TEER vs. C-CPE was calculated from the following equation: 100 × (difference in TEER between 0 and 18 h after treatment of the cells with each mutant C-CPE)/(difference in TEER between 0 and 18 h after treatment with C-CPE). The values are means ± S.D. ($n = 4$).

* Significant difference from C-CPE ($p < 0.05$).

additional band was observed below claudin-4 in the pull-down assay using C-CPE and mutants. This was due to nonspecific binding of the anti-claudin-4 antibody by His-tagged protein (data not shown).

To confirm the interaction between mutated C-CPEs and claudin-4, we also investigated the dose-dependence of Tyr306Ala and Leu315Ala in a competitive assay using C-CPE-PSIF. As shown in Fig. 1B, pretreatment of the cells with C-CPE at 10 μg/ml reduced LDH release by C-CPE-PSIF from 56.5 to 8.3% of the total LDH, whereas the Tyr306Ala and Leu315Ala mutants had much weaker inhibitory effect (50.0 and 40.5% release, respectively). Thus, the interaction of C-CPE with claudin-4 was weakened by substitution of Tyr306 or Leu315 with alanine. These results suggest that Leu315 in addition to Tyr306 is important in the interaction of C-CPE with claudin-4.

We previously found that rat jejunal absorption of FD-4, a model compound used for evaluation of paracellular absorption, is enhanced by C-CPE via an interaction with claudin-4

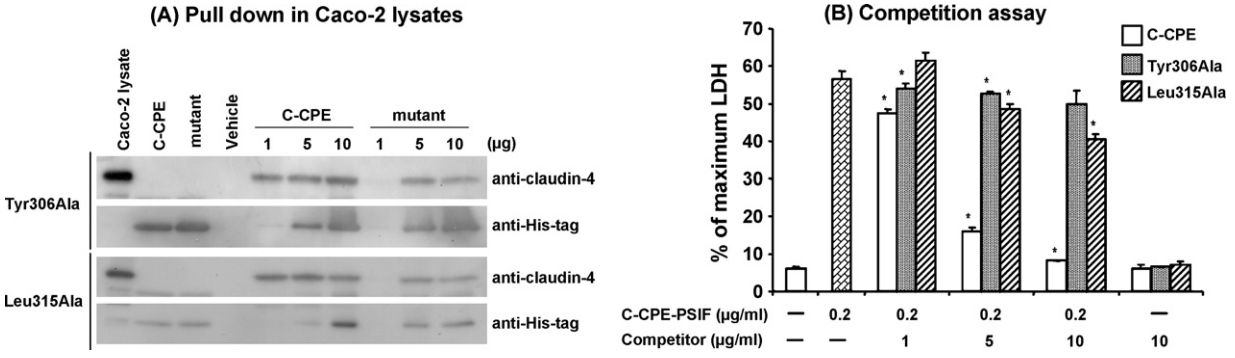

Fig. 1 – Role of Leu315 in the interaction between C-CPE and claudin-4. (A) Pull-down assay using Caco-2 lysates. TJ-developing Caco-2 cells were harvested and lysed in the lysis buffer. The lysate was incubated with vehicle, C-CPE, or mutated C-CPEs for 30 min at 37 °C. Ni-NTA agarose was added, and the mixture was incubated for 3 h at 4 °C. The Ni-NTA agarose was then precipitated, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting using anti-claudin-4 or anti-His-tag antibodies. The lanes containing Caco-2 lysate, C-CPE, and mutated C-CPE (mutant) served as positive controls for claudin-4, C-CPE, and mutant C-CPEs, respectively. The results are representative of three independent experiments. (B) Competitive inhibition of C-CPE-PSIF-induced cytotoxicity by mutant C-CPEs. Claudin-4-expressing CL4/L mouse fibroblasts were pretreated with C-CPE or mutant C-CPEs at the indicated concentrations for 1 h. The cells were then incubated with C-CPE-PSIF (0.2 μg/ml). After 36 h, LDH release was determined using a commercially available kit. The results are representative of three independent experiments. Values are means ± S.D. ($n = 3$). *Significant difference from C-CPE-PSIF-treated group ($p < 0.05$).

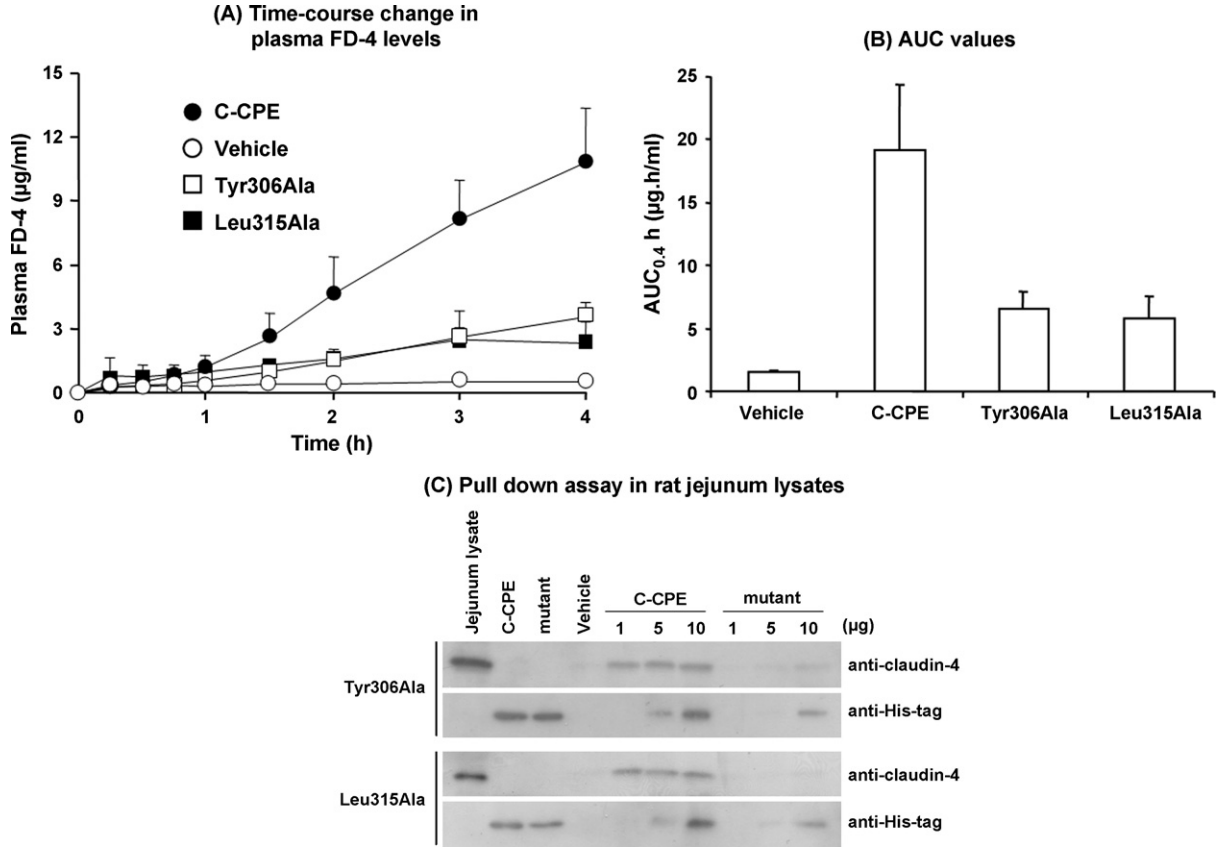

Fig. 2 – Role of Leu315 in the enhancement of absorption by C-CPE in rat jejunum. (A and B) Enhancement of jejunal absorption of FD-4 by C-CPE. The enhancement of absorption by mutant C-CPEs was evaluated by an *in situ* loop assay using rat jejunum. Briefly, rat jejunum was treated with FD-4 (10 mg/ml) in the presence of vehicle, C-CPE (0.2 mg/ml), or mutated C-CPE (0.2 mg/ml). The plasma FD-4 levels were determined at the indicated time (A), and the $AUC_{0-4\ h}$ values were calculated (B). Values are means ± S.E.M. (n = 4). (C) Pull-down assay in rat jejunum lysate. Mucosal epithelium in rat jejunum was recovered with a scraper and lysed in lysis buffer as described in Section 2. The lysates were incubated with C-CPE or mutated C-CPE for 30 min at 37 °C. Next, Ni-NTA agarose was added, and after a 3-h incubation at 4 °C, the precipitated Ni-NTA agarose was separated by SDS-PAGE and analyzed by Western blotting. The results are representative of three independent experiments.

[32]. We therefore examined the effect of Tyr306Ala and Leu315Ala on rat jejunal absorption of FD-4. As shown in Fig. 2A and B, C-CPE enhanced the absorption of FD-4 (12.2-fold compared to vehicle-treated controls), and Tyr306Ala and Leu315Ala had reduced abilities to enhance jejunal absorption (4.2- and 3.7-fold compared to vehicle-treated controls, respectively). To further evaluate the interaction of mutated C-CPEs with rat claudin-4, we performed a pull-down assay using rat jejunal lysate. Much less claudin-4 was precipitated by the Tyr306Ala and Leu315Ala mutants than by C-CPE, indicating that these two mutants have lower affinities for rat claudin-4 than C-CPE (Fig. 2C).

3.3. *The combination of Tyr306 and Leu315 is important for C-CPE modulation of claudin-4*

We found that ability of C-CPE to modulate claudin-4 is partially reduced by substitution of Tyr306 or Leu315 with alanine (Fig. 1 and Tables 3 and 4). We therefore evaluated whether the Leu315Ala mutation has synergistic effects by preparing double mutants Tyr306Ala/Leu315Ala, Tyr310Ala/Leu315Ala, and Tyr312Ala/Leu315Ala. Claudin-4 was precipitated by addition of Leu315Ala at 5 μg/ml but not by any of the double-alanine mutants at 10 μg/ml (Fig. 3A). Also, the double-substituted mutants did not affect C-CPE-PSIF-induced cytotoxicity even at 10 μg/ml (Fig. 3B), indicating that they lost their affinity for claudin-4.

Next, we investigated the absorption-enhancing effects of the double mutated C-CPEs using an *in situ* loop assay. Replacement of Tyr306 or Leu315 by alanine partially reduced the enhancement of absorption (Fig. 2A and B), whereas C-CPE with a combination of Leu315Ala and either Tyr306Ala, Tyr310Ala, or Tyr312Ala lost its absorption-enhancing effect (Fig. 4A and B). Similar results were obtained in the TEER assay using Caco-2 monolayers. Reduction of TEER was not observed for these double-substituted mutants (Fig. 4C). These data indicate that in addition to Tyr306, Leu315 is a key residue determining the potency of C-CPE as a modulator of the TJ barrier.

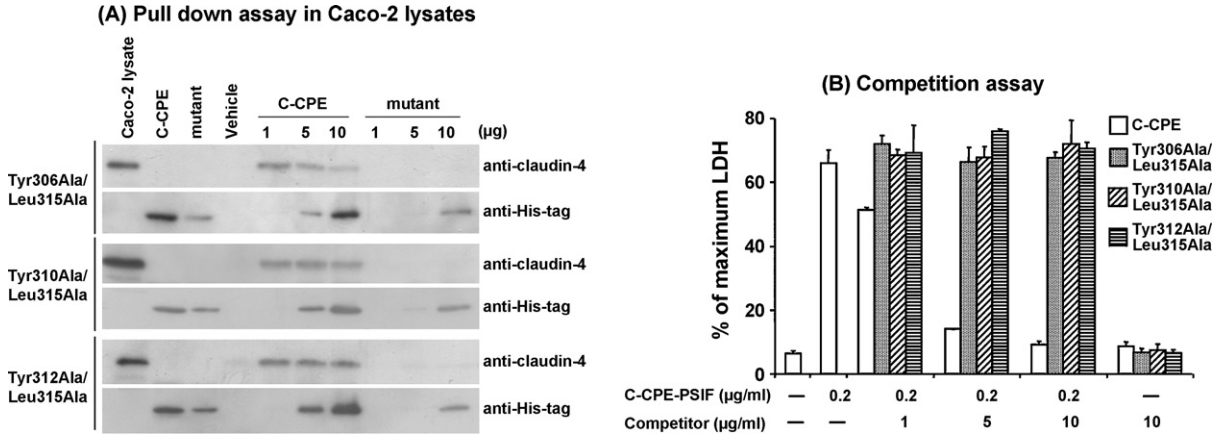

Fig. 3 – Interaction between double-substituted mutant C-CPE and claudin-4. Interaction of double-substituted mutant C-CPEs (Tyr306Ala/Leu315Ala, Tyr310Ala/Leu315Ala, and Tyr312Ala/Leu315Ala) with claudin-4 was evaluated by a pull-down assay using Caco-2 cell lysates (A) and by competitive inhibition of C-CPE-PSIF-induced cytotoxicity in CL4/L cells (B) as described in Fig. 1A and B, respectively. The results are representative of three independent experiments. In panel B, values are means ± S.D. (n = 3).

4. Discussion

Claudin is a promising target for developing a drug delivery system via the paracellular route. Our previous findings indicated that C-CPE is a potent modulator of claudin-4 and that the C-terminal 16 amino acids are pivotal for modulation of claudin-4 by C-CPE [32,38]. We have also evaluated the role of tyrosine residues within these 16 C-terminal amino acids by alanine scanning [33,39]. In the present study, we carried out a systematic functional domain mapping of these 16 amino acid residues. We found that Leu315 in addition to Tyr306 is pivotal for the interaction between C-CPE and claudin-4 and for modulation of the TJ barrier by C-CPE.

Functional domain mapping is needed for the development of claudin modulators based on C-CPE. We therefore screened for residues involved in binding of C-CPE to claudin-4 by a competition assay. We found that some mutants, such as Ser307Ala and Lys318Ala, had a slightly higher affinity for claudin-4 than C-CPE (Table 3). We did not focus on these mutants in the current studies, but it will be interesting to examine this further in future studies. We also found that substitution of Ser307 or Lys318 with alanine did not affect the abilities of C-CPE to modulate the TJ barrier and interact with claudin-4 in a pull-down assay (Table 4 and data not shown, respectively). Thus, at least, Ser307 and Lys318 do not appear to be essential for the activities of C-CPE. The results of our systematic domain mapping studies of the C-terminal 16 amino acids in C-CPE from the current study and from our previous report are summarized in Table 5 [33,38]. They suggest that multiple residues in C-CPE, especially Tyr306 and Leu315, are critical for the modulation of claudin-4.

Like Tyr306Ala, the Leu315Ala mutant had the reduced abilities to bind claudin-4, modulate the TJ barrier, and enhance absorption compared to those of C-CPE. The precise mechanism by which C-CPE disrupts the TJ barrier is still unclear. Sonoda et al. found that treatment of MDCK cells with C-CPE caused the disappearance of claudin-4 from TJs and a decrease in intracellular claudin-4 protein levels, indicating that claudin-4 may be degraded after its interaction with C-CPE [31]. Claudin-4 contains a signal sequence for sorting to clathrin-coated vesicles (a ALGVLL motif at amino acids 92–97 and a YVGW motif at amino acids 165–168) [40,41]. It is possible that C-CPE-bound claudin-4 is taken up by clathrin-mediated endocytosis. Indeed, Matsuda et al. showed that endocytosis of claudins occurs during the remodeling of TJs [42].

Do Tyr306 and Leu315 have the same function? Substitution of Tyr306 or Leu315 in C-CPE with alanine resulted in a partial reduction in the ability of C-CPE to modulate claudin-4, and the effects of double alanine-substitution were

Table 5 – Summary of functional domain mapping in the C-terminal 16 amino acids of C-CPE

Amino acid residue	Binding to claudin	Modulation of TJ barrier	Jejunal absorption	Source
Tyr306	Yes	Yes	Yes	This study, Harada et al. [33]
Tyr310	Yes	No	No	Harada et al. [33]
Tyr312	Yes	No	Yes	Harada et al. [33]
Leu315	Yes	Yes	Yes	This study

Binding to claudin was assessed using a competition assay using C-CPE-PSIF, modulation of TJ barrier was determined using a TEER assay using Caco-2 monolayer, and jejunal absorption based on an *in situ* loop assay using rat jejunum. Yes and No indicate important and not important for each function of C-CPE, respectively.

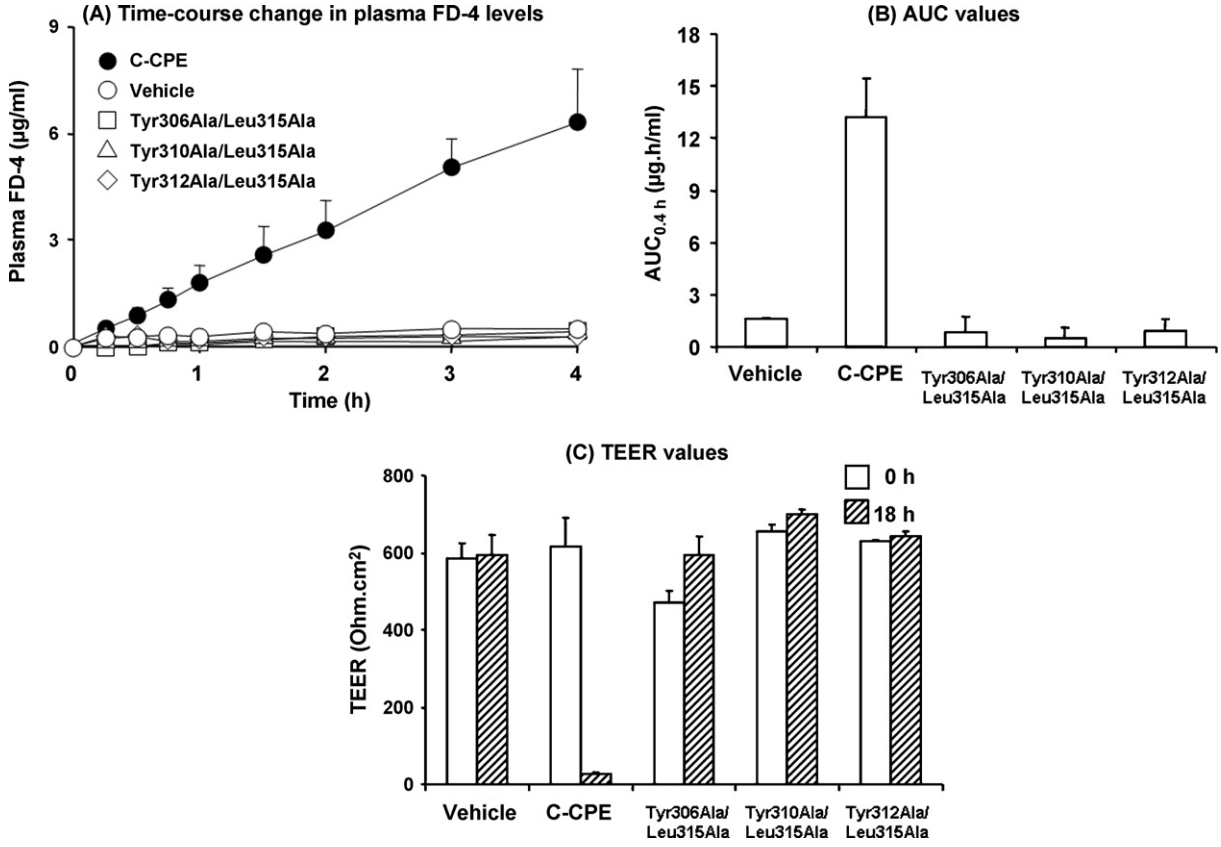

Fig. 4 – Modulation of TJ barrier by double-substituted mutant C-CPEs. (A and B) Enhancement of absorption by double-mutated C-CPEs in rat jejunum. Time-course of plasma FD-4 levels (A) and $AUC_{0-4\ h}$ values (B) were evaluated as described in Fig. 2A and B. Values are means ± S.E.M. (n = 4). (C) TEER assay in Caco-2 monolayer cells. Caco-2 cells were grown on Transwell™ filters. After TJs were developed, vehicle, C-CPE or mutated C-CPEs was added at 20 μg/ml, and TEER values were measured after 0 and 18 h. Values are means ± S.D. (n = 4).

additive (Fig. 4), suggesting that these two residues interact with claudin-4 at different sites or in different ways. Substitution of Tyr306 with Phe (aromatic and hydrophobic residue) and Trp (aromatic, hydrophobic, and polar residue) but not Lys (polar and cationic residue) did not affect the binding of C-CPE to claudin-4 or the modulation of the TJ barrier [39]. Taken together, these findings suggest that C-CPE interact with claudin-4 on the membrane through a hydrophobic cluster formed by the side-chains of Tyr at position 306 and Leu at position 315. Double substitution of Leu315 and Tyr310 or Tyr312 with alanine caused a loss of activities, indicating that the two residues equally and cooperatively contribute to modulation of claudin-4 by C-CPE. Indeed, triple- or quadruple-alanine mutants at Tyr306, Tyr310, Tyr312, and Leu315 lack activities like the double mutants (data not shown).

Other than the functional analyses of C-CPE, little is known about mode of action of C-CPE as a claudin modulator. Determination of the three-dimensional structure of claudin and C-CPE/CPE should help elucidate the mechanism by which C-CPE modulates claudin-4, but the tertiary structures of claudin and CPE have not yet been solved. Very recently, Van Itallie et al. reported structure of the C-terminal claudin-binding domain of CPE [43]. The structure has a nine strand β sandwich, and the claudin-4-binding site is on a loop domain between β8 and β9 strands. Tyr306, Tyr310 and Tyr312 exist on the loop domain, and Leu315 exists in the β9 strand. Our data is consistent with the putative claudin-4-binding domain by the structural analysis.

In conclusion, we carried out the complete fine mapping of the C-terminal 16 amino acids of C-CPE to determine their roles in claudin-4 modulation. We found that Leu315 plays a pivotal role in the modulation of claudin-4 by C-CPE. Together, our previous and current results indicate that Tyr at positions 306, 310, and 312 and Leu at position 315 of C-CPE participate in the modulation of claudin-4. These findings should help in the development of a novel claudin modulator based on C-CPE.

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REFERENCES

[1] White RE. High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. Annu Rev Pharmacol Toxicol 2000;40:133–57.
[2] Roberts SA. High-throughput screening approaches for investigating drug metabolism and pharmacokinetics. Xenobiotica 2001;31:557–89.
[3] Majumdar S, Duvvuri S, Mitra AK. Membrane transporter/receptor-targeted prodrug design: strategies for human and veterinary drug development. Adv Drug Deliv Rev 2004;56:1437–52.
[4] Mizuno N, Niwa T, Yotsumoto Y, Sugiyama Y. Impact of drug transporter studies on drug discovery and development. Pharmacol Rev 2003;55:425–61.
[5] Inui KI, Masuda S, Saito H. Cellular and molecular aspects of drug transport in the kidney. Kidney Int 2000;58:944–58.
[6] Koepsell H. Organic cation transporters in intestine, kidney, liver, and brain. Annu Rev Physiol 1998;60:243–66.
[7] Meijer DK, Hooiveld GJ, Schinkel AH, van Montfoort JE, Haas M, de Zeeuw D, et al. Transport mechanisms for cationic drugs and proteins in kidney, liver and intestine: implication for drug interactions and cell-specific drug delivery. Nephrol Dial Transplant 1999;14(Suppl. 4):1–3.
[8] Van Aubel RA, Masereeuw R, Russel FG. Molecular pharmacology of renal organic anion transporters. Am J Physiol 2000;279:F216–32.
[9] Powell DW. Barrier function of epithelia. Am J Physiol 1981;241:G275–88.
[10] Anderson JM, Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. Am J Physiol 1995;269:G467–75.
[11] Tidball CS, Lipman RI. Enhancement of jejunal absorption of heparinoid by sodium ethylenediaminetetraacetate in the dog. Proc Soc Exp Biol Med 1962;111:713–5.
[12] Engel RH, Riggi SJ. Effect of sulfated and sulfonated surfactants on the intestinal absorption of heparin. Proc Soc Exp Biol Med 1969;130:879–84.
[13] Aungst BJ. Intestinal permeation enhancers. J Pharm Sci 2000;89:429–42.
[14] Hochman J, Artursson P. Mechanisms of absorption enhancement and tight junction regulation. J Control Release 1994;29:253–67.
[15] Yamamoto A, Uchiyama T, Nishikawa R, Fujita T, Muranishi S. Effectiveness and toxicity screening of various absorption enhancers in the rat small intestine: effects of absorption enhancers on the intestinal absorption of phenol red and the release of protein and phospholipids from the intestinal membrane. J Pharm Pharmacol 1996;48:1285–9.
[16] Kondoh M, Takahashi A, Fujii M, Yagi K, Watanabe Y. A novel strategy for a drug delivery system using a claudin modulator. Biol Pharm Bull 2006;29:1783–9.
[17] Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. Annu Rev Physiol 2006;68:403–29.
[18] Furuse M, Tsukita S. Claudins in occluding junctions of humans and flies. Trends Cell Biol 2006;16:181–8.
[19] Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 1998;141:1539–50.
[20] Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 1999;96:511–6.
[21] Simon DB, Lu Y, Choate KA, Velazquez H, Al-Sabban E, Praga M, et al. Paracellin-1, a renal tight junction protein required for paracellular Mg^{2+} resorption. Science 1999;285:103–6.
[22] Turksen K, Troy TC. Claudin-6: a novel tight junction molecule is developmentally regulated in mouse embryonic epithelium. Dev Dyn 2001;222:292–300.
[23] Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 2002;156:1099–111.
[24] Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, et al. Size-selective loosening of the blood–brain barrier in claudin-5-deficient mice. J Cell Biol 2003;161:653–60.
[25] Furuse M, Sasaki H, Tsukita S. Manner of interaction of heterogeneous claudin species within and between tight junction strands. J Cell Biol 1999;147:891–903.
[26] Furuse M, Furuse K, Sasaki H, Tsukita S. Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 2001;153:263–72.
[27] Horiguchi Y, Uemura T, Kamata Y, Kozaki S, Sakaguchi G. Production and characterization of monoclonal antibodies to *Clostridium perfringens* enterotoxin. Infect Immun 1986;52:31–5.
[28] Hanna PC, Mietzner TA, Schoolnik GK, McClane BA. Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. The 30 C-terminal amino acids define a functional binding region. J Biol Chem 1991;266:11037–43.
[29] Hanna PC, Wieckowski EU, Mietzner TA, McClane BA. Mapping of functional regions of *Clostridium perfringens* type A enterotoxin. Infect Immun 1992;60:2110–4.
[30] Katahira J, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N. Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. J Cell Biol 1997;136:1239–47.
[31] Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, et al. *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. J Cell Biol 1999;147:195–204.
[32] Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, et al. A novel strategy for the enhancement of drug absorption using a claudin modulator. Mol Pharmacol 2005;67:749–56.
[33] Harada M, Kondoh M, Ebihara C, Takahashi A, Komiya E, Fujii M, et al. Role of tyrosine residues in modulation of claudin-4 by the C-terminal fragment of *Clostridium perfringens* enterotoxin. Biochem Pharmacol 2007;73:206–14.
[34] Ebihara C, Kondoh M, Hasuike N, Harada M, Mizuguchi H, Horiguchi Y, et al. Preparation of a claudin-targeting molecule using a C-terminal fragment of *Clostridium perfringens* enterotoxin. J Pharmacol Exp Ther 2006;316:255–60.
[35] Sallee VL, Wilson FA, Dietschy JM. Determination of unidirectional uptake rates for lipids across the intestinal brush border. J Lipid Res 1972;13:184–92.

[36] Utoguchi N, Watanabe Y, Shida T, Matsumoto M. Nitric oxide donors enhance rectal absorption of macromolecules in rabbits. Pharm Res 1998;15:870–6.
[37] Ohtake K, Natsume H, Ueda H, Morimoto Y. Analysis of transient and reversible effects of poly-L-arginine on the in vivo nasal absorption of FITC-dextran in rats. J Control Release 2002;82:263–75.
[38] Takahashi A, Kondoh M, Masuyama A, Fujii M, Mizuguchi H, Horiguchi Y, et al. Role of C-terminal regions of the C-terminal fragment of *Clostridium perfringens* enterotoxin in its interaction with claudin-4. J Control Release 2005;108:56–62.
[39] Ebihara C, Kondoh M, Harada M, Fujii M, Mizuguchi H, Tsunoda S, et al. Role of Tyr306 in the C-terminal fragment of *Clostridium perfringens* enterotoxin for modulation of tight junction. Biochem Pharmacol 2007;73:824–30.
[40] Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 2003;72:395–447.
[41] Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. Mol Biol Cell 2004;15:176–88.
[42] Matsuda M, Kubo A, Furuse M, Tsukita S. A peculiar internalization of claudins, tight junction-specific adhesion molecules, during the intercellular movement of epithelial cells. J Cell Sci 2004;117:1247–57.
[43] Van Itallie CM, Betts L, Smedley 3rd JG, McClane BA, Anderson JM. Structure of the claudin-binding domain of *Clostridium perfringens* enterotoxin. J Biol Chem 2008;283:268–74.