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Role of Tyr306 in the C-terminal fragment of *Clostridium perfringens* enterotoxin for modulation of tight junction

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ABSTRACT

We previously reported that the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) is a novel type of absorption enhancer that interacts with claudin-4 and that Tyr306 of C-CPE plays a role in ability of C-CPE to modulate barrier of tight junctions. In the current study, to investigate effects of Tyr306 on the C-CPE activity, we prepared some C-CPE mutants substituted Tyr306 with Trp (Y306W), Phe (Y306F) and Lys (Y306K). We found that Y306W and Y306F mutants of C-CPE had claudin-4 binding affinities and effects on the barrier function of tight junctions, whereas both of these properties were greatly reduced with the Y306K mutant. Finally, the Y306K but not the Y306F and Y306W mutants had reduced abilities to enhance absorption in rat jejunum. These results indicate that aromatic and hydrophobic properties, not hydrogen bonding potential, of Tyr306 are involved in the interaction of C-CPE with claudin-4 and in the modulation of the tight junction barrier function by C-CPE.

1. Introduction

Recent dramatic progress in drug design based on genomic data has produced a large number of leads that must be analyzed by high-throughput screening, but delays in the development of drug delivery has slowed their clinical testing and application. Drugs can be delivered to their target tissues by two pathways: transcellular and paracellular. The paracellular route is thought to be the most reasonable for hydrophilic macromolecular drugs, such as peptides and

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Abbreviations: C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin; PSIF, protein synthesis inhibitory factor; TJ, tight junction; CPE, *Clostridium perfringens* enterotoxin; TER, transepithelial electric resistance; C-CPE-PSIF, C-CPE fused to PSIF; PCR, polymerase chain reaction; LDH, lactate dehydrogenase; FD-4, fluorescein-isothiocyanate-dextran with a molecular weight of 4000

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proteins, obtained by high-throughput screening because delivery through the transcellular route would require modification of each drug for recognition by specific transporters.

Tight junctions (TJs) between cells seal the paracellular route, preventing leakage of substances [1]. The TJs are complex biochemical systems in which claudins, transmembrane proteins with four membrane-spanning domains, play a pivotal role [2]. There are 24 members of the claudin family, each of which has different expression profiles and barrier functions. For instance, claudin-1 and claudin-5 contribute to the barrier functions of the epidermis and the blood–brain-barrier, respectively [3,4]. Tsukita and Furuse proposed that claudins function as a barrier by forming homo- and/or hetero-dimers [2] and that the many possible patterns of dimerization can explain the diversity in TJ barrier function [2,5,6]. These findings indicate that claudins could be targeted for the development of drug delivery systems.

Clostridium perfringens enterotoxin (CPE) causes the symptoms associated with *C. perfringens* food poisoning in humans [7]. CPE has two functional domains: an N-terminal cytotoxic region and a C-terminal binding region (C-CPE) [7]. C-CPE is involved in the interaction between CPE and claudin-3 or claudin-4 [8,9]. Interestingly, C-CPE modulates the barrier function of TJs in epithelial cell lines [9]. Therefore, using C-CPE as a claudin modulator, we previously investigated claudin as a target for the enhancement of drug delivery. We found that C-CPE was over 400-fold more potent at enhancing absorption than sodium caprate, an enhancer of drug absorption that is clinically used in Japan, Denmark, and Sweden [10]. Identification of the domain in C-CPE that mediates this effect should help in the development of modulators of other claudins. So far, we have narrowed the functional region of C-CPE mediating claudin-4 binding to the C-terminal 16 amino acids [10,11]. Very recently, we found that three tyrosine residues in the C-terminal 16 amino acids at position 306, 310, and 312 are responsible for the modulation of TJ barrier function by C-CPE, and Tyr306 is the most important residue among them [12]. In the current study, we try to address the reason why Tyr306 are involved in abilities of C-CPE to bind to claudin-4 and modulate TJ barrier function by site-directed mutagenesis.

2. Materials and methods

2.1. Materials

Anti-His-tag antibody and anti-claudin-4 antibody were obtained from Novagen (Madison, WI) and Zymed Laboratories (South San Francisco, CA), respectively. Ni-agarose resin was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell cultures

The human intestinal cell line Caco-2 was cultured in Dulbecoo's modified Eagle's medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37 °C. Passages 65–72 were used for experiments. Claudin-4-expressing mouse fibroblast L cells (CL4/L cells) were kindly provided by Drs. S. Tsukita and M. Furuse [9,13]. CL4/L cells were maintained in modified Eagle's medium containing 10% fetal bovine serum at 37 °C.

Table 1 – Primers used for site-directed mutagenesis

Primers	Sequences (5′ to 3′)
Common forward primer	ggaattc cat atg gaa aga tgt gtt tta aca gtt cca tct aca
Reverse primer for Y306A	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *agc* tga tga att agc ttt cat tac
Reverse primer for Y306F	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *aaa* tga tga att agc ttt cat tac
Reverse primer for Y306W	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *cca* tga tga att agc ttt cat tac
Reverse primer for Y306K	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg gta att tcc act *ctt* tga tga att agc ttt cat tac

The underline in forward primer and in reverse primer is *Nde*I site and *Bam*HI site, respectively. The italic letters in the reverse primer indicated the site of mutation.

2.3. Preparation of mutated C-CPEs

The indicated residues were mutated by polymerase chain reaction (PCR) using a forward primer containing *Nde*I site, a reverse primer containing a *Bam*HI site, and pET16bHiS_{10}C-CPE as a template [10]. The primer sequences are listed in Table 1. The resulting PCR products were ligated with *Nde*I/*Bam*HI-digested pET16b vector (Novagen), and the DNA sequence was confirmed. Each plasmid was transduced into *Escherichia coli* BL21 (DE3), and production of mutant C-CPEs were induced by addition of isopropyl-β-D-thiogalactopyranoside. The cells were harvested and 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) containing 8 M urea. The lysates were applied onto a Ni-NTA column, 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 (GE Healthcare Bio-Sciences Co., Piscataway, NJ). 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. Pull-down assay

Confluent Caco-2 cells were harvested and lysed in lysis buffer (1% Triton X-100, 0.2% SDS, 150 mM NaCl, 10 mM HEPES, pH 7.4, 2 mM EDTA, and 1% protease inhibitor cocktail [Sigma–Aldrich, St. Louis, MO]). C-CPE or mutant C-CPEs were incubated with the lysates for 30 min at 37 °C, after which Ni-agarose resin was added. After an additional 3-h incubation at 4 °C, the resin was washed with lysis buffer. The proteins

bound to the resin was separated by SDS-PAGE and analyzed by Western blotting using anti-human claudin-4 and anti-His tag antibodies. Bound primary antibodies were detected using a peroxidase-labeled secondary antibody and chemiluminescence reagents (GE Healthcare Bio-Sciences Co.).

2.5. Preparation of mutant C-CPE-PSIF

The plasmids expressing mutant C-CPE fused to protein synthesis inhibitory factor (C-CPE-PSIF) were prepared as follows. PSIF is an approximately 40 kDa fragment of the bacterial exotoxin (GenBank Accession No. K01397) derived from *Pseudomonas aeruginosa* (ATCC strain No. 29260). We cloned the cDNA for PSIF from *P. aeruginosa*, *Migula* by PCR using the primer set 5′-gat gat cga tcg cgg ccg cag gtg cgc cgg tgc cgt atc cgg atc cgc tgg aac cgc gtg ccg cag act aca aag acg acg acg aca aac ccg agg gcg gca gcc tgg ccg cgc tga cc-3′ and 5′-gat cga tcg atc act agt cta cag ttc gtc ttt ctt cag gtc ctc gcg cgg cgg ttt gcc ggg-3′. C-CPE fragments containing mutations of Tyr306 to Ala, Phe, Trp, and Lys and *Nco*I and *Not*I sites at the 5′- and 3′-ends, respectively, were amplified by PCR using a common forward primer (5′-cat gcc atg gcc gaa aga tgt gtt tta aca gtt cc-3′; *Nco*I site underlined), a common reverse primer (5′-ata gtt tag cgg ccg caa att ttt gaa ata ata ttg aat aag g-3′; *Not*I site underlined), and a pET16b plasmid containing mutant C-CPE as a template. The *Nco*I/*Not*I-digested mutant C-CPE fragments were inserted into *Nco*I/*Not*I-digested pY02-C-CPE-PSIF to generate pY02-Y306A-PSIF, pYO2-Y306F-PSIF, pYO2-Y306W-PSIF, and pYO2-Y306K-PSIF plasmids [14]. The sequence of the plasmids was confirmed. The C-CPE-PSIF and mutant C-CPE-PSIF plasmids were transduced into *E. coli* strain TG1. The cells were grown at 37 °C in 2YT medium containing 2% glucose to an optical density at 600 nm of 0.6–0.9, and the medium was changed to 2YT medium containing 1 mM isopropyl β-D-thiogalactopyranoside. After an additional 18-h of culture at 30 °C, the conditioned medium was collected and applied to an anti-FLAG M2 affinity column (Sigma–Aldrich). Bound proteins were eluted with FLAG peptide (Sigma–Aldrich). The buffer was exchanged with phosphate-buffered saline using a PD-10 column. Purification of mutant C-CPE-PSIF proteins was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-FLAG M2 antibody (data not shown). Protein levels were determined using a commercially available assay kit with bovine serum albumin as a standard (Bio-Rad).

2.6. C-CPE-PSIF-induced cytotoxicity

CL4/L cells were pretreated with C-CPE or mutant C-CPE at the indicated concentration for 1 h, and C-CPE-PSIF was added to the cells. After an additional 36 h of culture, the release of lactate dehydrogenase (LDH) from cells was assayed. CL4/L cells were treated with C-CPE-PSIF or mutant C-CPE-PSIF at the indicated concentration for 36 h. Next, the LDH release was measured using a CytoTox96 Non Radioactive Cytotoxicity Assay kit according to the manufacturer's protocol (Promega, Madison, WI). LDH release was calculated using the following equation: maximal LDH release (%) = 100 × (LDH in the cultured medium/total LDH in the culture dish).

Cytotoxicity of C-CPE-PSIF and mutant C-CPEs-PSIF was evaluated by measuring LDH release as described above.

2.7. TER assay

Caco-2 cells were seeded in Transwell chambers (6.5-mm diameter, 0.03 cm^2 area, 0.45-μm pore diameter; Nunc, Roskilde, Denmark) at a subconfluent density. The formation of TJ barriers in Caco-2 monolayers was monitored by measuring transepithelial electric resistance (TER) using a Millicell-ERS epithelial volt-ohmmeter (Millipore Corporation, Billerica, MA). When the TER values reached a plateau, Caco-2 monolayer cells were treated with C-CPE or mutant C-CPEs on the basal side of the chamber, and the TER values were measured. Treatment of the cells with C-CPE on the apical side did not disrupt TJ barriers (data not shown). Similar results were found in cytotoxic assay of CPE in Caco-2 cells [7]. The TER values were multiplied by the area of the Caco-2 monolayer. The TER value of a blank Transwell chamber was subtracted from the TER of cell monolayers.

2.8. In situ loop assay

Wistar male rats (250–280 g) were obtained from Animal and Material Laboratories, Inc. (Tokyo, Japan). The rats were maintained in an environmentally controlled room (23 ± 1.5 °C) with a 12-h light/12-h dark cycle and allowed access to standard rodent chow and water ad libitum. The rats were allowed to adapt for a week. The experimental protocol for the in situ loop assay was approved by the ethics committee of Showa Pharmaceutical University. Intestinal absorption of fluorescein-isothiocyanate-dextran with a molecular weight of 4000 (FD-4) was investigated by in situ loop assay as follows. 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 saline. A jejunal loop (5 cm in length) was prepared by closing both ends with sutures. A mixture of FD-4 and C-CPE proteins in 200 μl of PBS 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.

2.9. Statistical analysis

Statistical significance of differences was assessed using one-way analysis of variance followed by Dunnett's test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Interaction of Y306F, Y306W, and Y306K mutants with claudin-4

We previously found that C-CPE modulates the TJ barrier and interacts with claudin-4 through its C-terminal 16

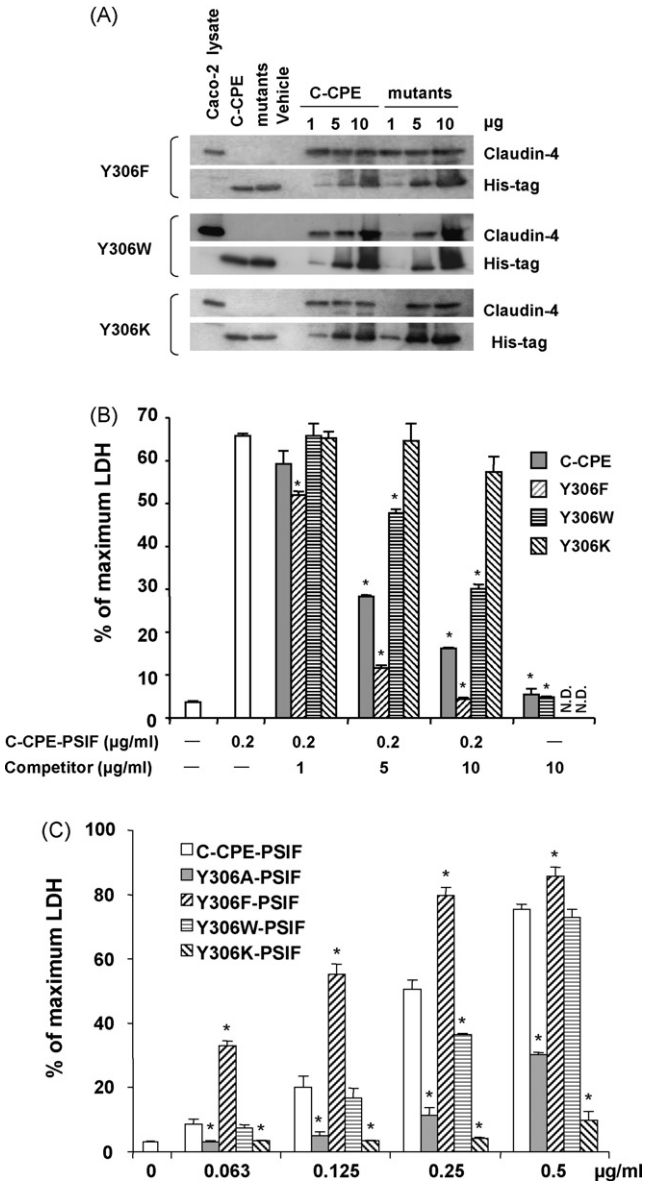

Fig. 1 – Effects of mutation of Tyr306 to Phe, Trp, and Lys on the interaction between C-CPE and claudin-4. (A) Pull-down assay. Caco-2 lysate was incubated with C-CPE or mutant C-CPE for 30 min at 37 °C. Ni-agarose was then added, and the mixture was incubated for 3 h at 4 °C. The resin was collected by centrifugation, and the bound proteins were separated by SDS-PAGE and analyzed by Western blotting with antibodies against the indicated proteins. The results are representative of three independent experiments. (B) Competitive effect of mutant C-CPEs on C-CPE-PSIF-induced cell death in CL4/L cells. CL4/L cells were treated with C-CPE or mutant C-CPE at the indicated levels for 1 h, followed by 0.2 μg/ml C-CPE-PSIF for 36 h, after which LDH release was assessed. Values are means ± S.D. ($n = 3$), and the results are representative of three independent experiments. Significant differences from C-CPE-PSIF-treated cells ($^{*}p < 0.05$). (C) Cytotoxicity of mutant C-CPE-PSIF. CL4/L cells were treated with C-CPE-PSIF or mutant C-CPE-PSIF proteins for 36 h at the indicated levels, after which LDH release was measured. Values are means ± S.D. ($n = 3$). The results are representative of three independent experiments. Significant differences from C-CPE-PSIF-treated cells ($^{*}p < 0.05$).

amino acids and the three tyrosine residues in the 16 amino acids at position 306, 310, and 312 are involved in the C-CPE activities [10–12]. Site-directed mutagenesis of Tyr to Ala revealed that Tyr306 plays a pivotal role in interaction of C-CPE with claudin-4 and modulation of TJ-barrier by C-CPE [12]. To further examine the contribution of Tyr306 to binding of C-CPE to claudin-4 and TJ modulation by C-CPE, we generated additional mutants, namely, Y306F, Y306W, and Y306K. To evaluate effect of substitution of Tyr306 with Phe, Trp, and Lys on the interaction of C-CPE with claudin-4, we performed a pull-down assay using Caco-2 lysates. As shown in Fig. 1A, substitution of Tyr with Phe (Y306F) did not cause a noticeable effect on claudin-4 binding; however, mutation of Tyr306 to Trp or Lys decreased the binding of claudin-4 in this assay. To confirm the interaction of C-CPE mutants with claudin-4, we performed an assay based on the cytotoxicity of a fusion of C-CPE and PSIF. PSIF is a fragment of *pseudomonas* exotoxin A that inhibits protein synthesis when it enters the cell [15–17]. PSIF cannot invade into cells and does not show any cytotoxicity because of lacking the cell binding domain. So it must be fused with a ligand molecule to be imported into the cell. We previously showed that the C-CPE-PSIF fusion protein is toxic to claudin-4-expressing cells [14]. We further examined whether mutant C-CPE can reduce the toxicity of C-CPE-PSIF in claudin-4-expressing cells. As shown in Fig. 1B, pretreatment of claudin-4-expressing cells with C-CPE, Y306F or Y306W, but not Y306K, dose-dependently inhibited the toxicity of C-CPE-PSIF. Especially, the affinity of the Y306F mutant was increased compared with that of C-CPE. Y306W had the moderate affinity for claudin-4. In contrast, substitution of Lys for Y306 almost eliminated the ability of C-CPE to interact with claudin-4. Then, we examined the effect of mutant C-CPE-PSIF fusion proteins on claudin-4-expressing cells. As shown in Fig. 1C, Y306A-PSIF was less toxic than C-CPE-PSIF, indicating that Tyr306 participates in the interaction between C-CPE and claudin-4. The data on Y306A corresponded to the data of Y306A in competition assay using C-CPE-PSIF [12]. We also found that substitution of C-CPE with Y306K in C-CPE-PSIF reduced its toxicity and displacement of C-CPE with Y306F in C-CPE-PSIF partly enhanced cytotoxicity of C-CPE-PSIF corresponded to their binding of claudin-4 (Fig. 1C). The order of affinity for claudin-4 is Y306F > C-CPE > Y306W > Y306A > Y306K.

3.2. *Effects of the Y306F, Y306W, and Y306K mutants on the TJ barrier function*

We next investigated the effects of Y306F, Y306W, and Y306K on the barrier function of TJs in Caco-2 monolayer cells. As indicated in Fig. 2A, all of the mutants decreased the TER values, although their abilities varied. Substitution of Tyr306 with Lys but not with Phe or Trp reduced the ability to modulate the TJ barrier function. We further examined the dose-dependency of the effects of Y306F and Y306W on the

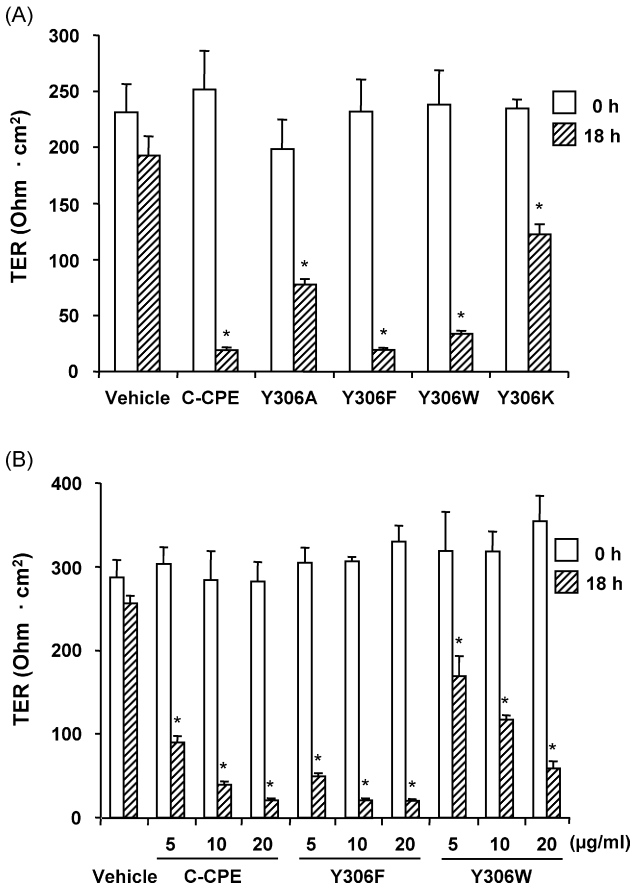

Fig. 2 – Effects of Tyr306 mutants of C-CPE on TJ barrier function. TJ-developing Caco-2 cells were grown in Transwell chambers and treated with vehicle or mutant C-CPEs from basal side of the Transwell chamber at 20 μg/ml (A) or at the indicated concentrations (B). After 18 h, TER values were determined. Values are means ± S.D. (n = 4), and the results are representative of three independent experiments. Significant difference from the value at 0 h ($^*p < 0.05$).

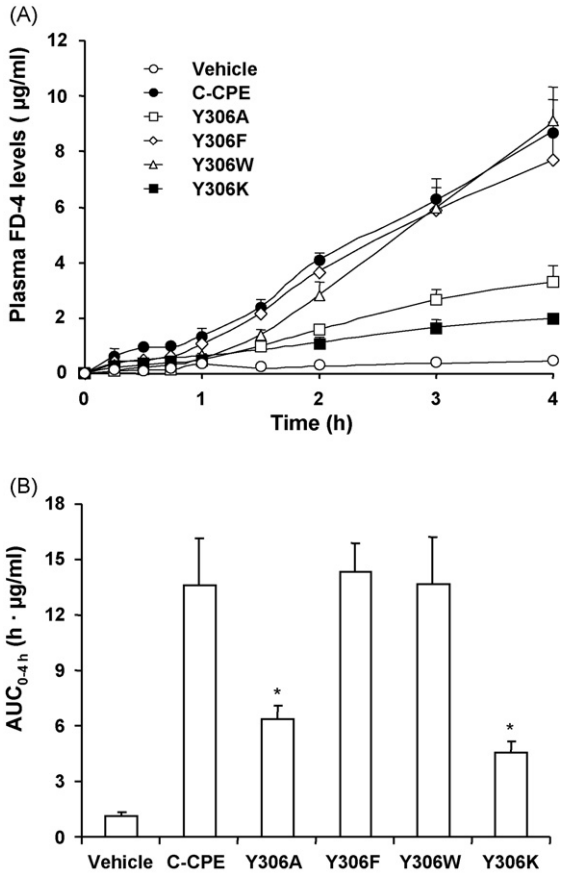

Fig. 3 – Effects of Y306 mutants on rat jejunal absorption. Rat jejunum was treated with FD-4 (10 mg/ml) with or without mutant C-CPEs (0.2 mg/ml). (A) The FD-4 levels in plasma collected from the jugular vein were determined at the indicated times. Values are means ± S.E.M. (n = 4), and the results are representative of three independent experiments. (B) The $AUC_{0-4\ h}$. Values are means ± S.E.M. (n = 4), and the results are representative of three independent experiments. Significant differences from C-CPE-treated group ($^*p < 0.05$).

TJ barrier function. As shown in Fig. 2B, at 5 and 10 μg/ml, Y306F had slightly stronger effects on the TJ barrier function than C-CPE, whereas Y306W was weaker than C-CPE. These results agree with those from our studies of claudin-4 binding (Fig. 1).

3.3. *Effects of Y306F, Y306W, and Y306K mutants on absorption in rat jejunum*

We previously reported that C-CPE enhances jejunal absorption in rats [10]. Therefore, we checked the effects of the mutations on the ability to enhance rat jejunal absorption. We used an in situ loop assay that employs FD-4 (fluorescein isothiocyanate-conjugated dextran) as a model drug absorbed via the paracellular route [18]. This assay allows investigation of the transport of a model drug from the intestine to the systemic circulation. Finally, we investigated the effects of the Y306F, Y306W, and Y306K mutants on jejunal absorption of FD-4 in rat. As shown in Fig. 3A and B, mutation of Tyr306 to Phe or Trp did not influence the absorption-enhancing activity of C-CPE, but mutation to Lys reduced the enhancing activity ($AUC_{0-4\ h}$ = 13.6 and 4.6 h μg/ml for C-CPE and Y306K, respectively). These results indicate that the aromatic ring of Tyr306 is important for the absorption-enhancing effect of C-CPE.

4. Discussion

Modulation of the barrier function of specific claudins is an attractive strategy for the development of a drug delivery system, but use of C-CPE is the only method that has been explored because it is the only molecule known to modulate the barrier function of a claudin [9]. We previously showed that modulation of claudin with C-CPE can be used to enhance drug delivery [10]. Identification of the functional domain of C-CPE should allow development of additional claudin-4 modulators. We previously found that Tyr306 is a key residue

for the disruption of the barrier function of TJs by C-CPE and for its interaction with claudin-4 [12], and we show here that aromatic and hydrophobic properties of Tyr at position 306 is partly important for the C-CPE activities.

Because CPE is a foodborne toxin in humans, its functional domains have been mapped in many studies. C-CPE is the C-terminal fragment of CPE and is responsible for binding of CPE to its receptor [19]. Hanna et al. showed that the C-terminal 30 amino acids of CPE mediates binding to its receptor, claudin-4 [20]. We previously show that deletion of the C-terminal 16 amino acids of C-CPE eliminates the TJ-modulating activity of C-CPE [11] and that the tyrosine residues (Tyr306, 310, 312) in the 16 amino acids of C-CPE are responsible for the C-CPE activity [12]. Substitution of Tyr306 with Ala reveals that Tyr306 is a pivotal residue for the abilities of C-CPE to bind claudin-4 and modulate the TJ barrier [12].

What means the attenuation of the C-CPE activities by mutation of Tyr306 to Ala in C-CPE? Tyr is a polar, hydrophobic and aromatic residue, but Ala is a non-polar, non-hydrophobic and non-aromatic residue. Therefore, we suspected that polar, hydrophobic and/or the aromatic property of Tyr is important for the activities of C-CPE. We prepared Y306F (aromatic and hydrophobic mutant), Y306W (aromatic, hydrophobic and polar mutant), and Y306K (polar and positive charged mutant) mutants and examined their abilities to modulate the TJ barrier function and bind claudin-4. Replacement of Tyr with Phe did not affect these activities, whereas replacement with Lys attenuated these activities, and mutation to Trp caused a partial reduction in both activities. These findings suggest that the aromatic and hydrophobic properties of Tyr at position 306 are important for the activities of C-CPE and hydrogen bonding potential of Tyr at position 306 is not essential for them.

How does Tyr306 contribute to modulation of the TJ barrier function and to the interaction with claudin-4? Considering the mode of action of C-CPE as a claudin modulator, this leaves the question of whether it is possible to separate the ability of C-CPE to modulate the TJ barrier function and its ability to interact with claudin. Deletion of the C-terminal region of C-CPE and substitution of Tyr306 with Ala in C-CPE eliminate these activities [10–12]. Similarly, we showed here that mutation of Tyr306 to Lys reduces both the ability to modulate the TJ barrier function and the ability to bind claudin-4. These findings suggest that the abilities of C-CPE to bind to claudin-4 and modulate TJ-barrier cannot be separated in Tyr306 mutants. Tsukita lab found that CPE interacted with claudin via extracellular loop domain of claudin and claudin was degraded by endocytotic pathway in C-CPE-treated cells [9,21]. We also found that loss of interaction of C-CPE with claudin-4 by deletion of the C-terminal C-CPE [10,11]. These findings indicate that interaction of C-CPE with claudin is the first step in the modulation of TJ-barrier by C-CPE. Tyr306 may be partly critical for interaction of C-CPE with claudin. Since Y306F mutant binds to claudin-4, interaction of C-CPE with claudin-4 may not be mediated by hydrogen bond. Mutation of Tyr to Trp at position 306 resulted in C-CPE with subtle differences in claudin-4 binding. In contrast, Y306K and Y306A mutants reduced binding to claudin-4. Taken together, the hydrophobicity at position 306 may be important for binding of C-CPE to claudin-4. An aromatic/polar amino acid at position 306 might be critical for the correct folding of C-CPE, which might expose other residues and allow them to reach their target site on claudin-4.

Determination of the three-dimensional structures of CPE and claudin is critical for elucidation of the precise mechanism of interaction between C-CPE and claudin-4, but, because these proteins are hydrophobic, this has not yet been accomplished. In the meantime, our findings should help to clarify how these two proteins interact and to prepare a novel claudin-modulator using C-CPE as a prototype.

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