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Role of tyrosine residues in modulation of claudin-4 by the C-terminal fragment of *Clostridium perfringens* enterotoxin

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ABSTRACT

The C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) modulates the barrier function of claudin-4 via its C-terminal 16 amino acids. In the current study, we investigated the roles of tyrosine residues (Y306, Y310 and Y312) in this region in the modulation of TJs by C-CPE. Single mutations of Y306, Y310 and Y312 to alanine resulted in partial reduction of claudin-4 binding. We also prepared double mutants of C-CPE to further evaluate the roles of these tyrosine residues. Replacement of Y310 and Y312 with alanine (Y310A/Y312A) partly reduced the ability of C-CPE to bind to claudin-4. Double mutants Y306A/Y310A and Y306A/Y312A, however, lost the ability to bind to claudin-4 and to modulate the TJ barrier. We also found that a triple mutant (Y306A/Y310A/Y312A) lost the ability to bind claudin-4, modulate the TJ barrier, and enhance jejunal absorption in rats. These results indicate that tyrosines 306, 310, and 312 are critical for the interaction of C-CPE with claudin-4 and for the modulation of TJ barrier function by C-CPE. This study provides information that should help in the development of claudin modulators based on C-CPE.

1. Introduction

Tight junctions (TJs) play a central role in sealing the intercellular space in epithelial and endothelial sheets [1,2]. The key structure in this regard is the TJ strand, which lies within the plasma membrane. Each TJ strand associates laterally and tightly with a TJ strand on an opposing membrane of an adjacent cell to form a paired strand [3,4].

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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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Recent studies have revealed the molecular architecture of TJ strands and shown that TJs consist of three types of integral membrane proteins: occludin, junctional adhesion molecule, and claudins [5,6]. Occludin, an ~65 kDa integral membrane protein with four transmembrane domains, was the first component identified in TJ strands [7]; however, gene knockout analysis proved that it is not essential for forming TJ strands [8]. Junctional adhesion molecule has a single transmembrane domain and associate laterally with TJ strands, but it does not form TJ strands [9]. Thus, occludin and junctional adhesion molecule are not thought to be essential for the structure or function of TJs; however, claudin, an ~24 kDa integral membrane protein with four transmembrane domains, is thought to be essential for TJs. Overexpression of claudin in mouse L fibroblasts causes the formation of TJ strands and a TJ barrier [10,11]. In addition, claudin-based TJs have been shown to be directly involved in intercellular sealing [12–14].

There are more than 20 members of the claudin family, and the expression profiles and barrier function of each member differs by tissue [5,15]. For example, mice deficient in claudin-1 and -5 lose the barrier function of the epidermis and the blood-brain-barrier, respectively [16,17]. Each isoform of claudin can form homopolymers as well as heteropolymers with the other claudins, and each polymer laterally associates between adjacent cells [18]. One report proposed that the tightness of paired TJ strands is determined by the number and type of species of claudins and their mixing ratio in strands [5,18]. Thus, claudin family members are responsible for the barrier function of TJs, that is, the regulation of paracellular movement of water and solutes across epithelia [19]. A method to modulate the barrier function of claudins could therefore be a promising tool for understanding claudin function and for enhancing drug delivery.

Clostridium perfringens enterotoxin (CPE) is the substance that causes the symptoms of *C. perfringens* food poisoning in man [20]. The N-terminal half of CPE is responsible for toxicity, and the C-terminal half (C-CPE) plays a role in cell binding [20]. Interestingly, the CPE receptor is identical to claudin-4, and C-CPE has been shown to modulate the TJ barrier by binding to claudin-4 on the cell surface [12,21]. Furthermore, treatment of cells with C-CPE reduces claudin-4 levels in TJs, resulting in a disruption of the TJ barrier function [12]. To our knowledge, C-CPE is the only known modulator of claudin.

Based on this information, we suspected that we could create a claudin modulator using C-CPE as a prototype. Therefore, we previously identified the region of C-CPE necessary for modulating the TJ barrier and for binding to claudin-4. Deletion analysis revealed that the C-terminal 16 amino acids of C-CPE participate in modulation of the TJ barrier by C-CPE and for interaction between C-CPE and claudin-4 [22,23]. Previously, substitution of Tyr310 with Cys reduced binding of CPE to the brush border membrane in rabbits [24]. In the current study, we investigated roles of the tyrosines (Tyr306, Tyr310 and Tyr312) of C-CPE in claudin-4 binding and modulation of the TJ barrier. We also examined the effects of double and triple mutants of Tyr306, Tyr310, and Tyr312.

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-resin was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell cultures

Caco-2 human intestinal cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37 °C. Caco-2 cells (passages 65–72) were used for experiments. Claudin-4-expressing mouse fibroblast L cells (CL4/L cells) were kindly provided by Tsukita and Furuse [12,15] and were maintained in modified Eagle's medium containing 10% fetal bovine serum at 37 °C.

2.3. Preparation of mutant C-CPE

The indicated residues were mutated to Ala by polymerase chain reaction (PCR) using a forward primer containing *Nde*I site, a reverse primer containing a *Bam*HI site, and pET16b-His_{10}C-CPE as a template [23]. 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 using C-CPE as a claudin modulator 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. Preparation of mutant C-CPE-PSIF

Plasmids expressing mutant C-CPEs fused to protein synthesis inhibitory factor (PSIF) were prepared as described below. Mutant C-CPE fragments were amplified by PCR using primers 5′-catgccatggccgaaagatgtgttttaacagttcc-3′ (forward; *Nco*I site underlined) and 5′-atagtttagcggccgcaaatttttgaaataatattgaataagg-3′ (reverse; *Not*I site underlined) and with pET16b plasmids encoding each mutant C-CPE as templates. 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 mutant C-CPE-PSIF plasmids [25]. The sequence of the plasmids was confirmed. The C-CPE-PSIF and mutant C-CPE-PSIF plasmids

Table 1 – Primers used for site-directed mutagenesis

Primers	Sequences (5′–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 Y310A	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg *ggc* att tcc act ata tga tga att agc ttt c
Reverse primer for Y312A	cgggatcc tta aaa ttt ttg aaa taa tat tga *agc* agg gta att tcc act ata tga
Reverse primer for Y306A/Y310A	cgggatcc tta aaa ttt ttg aaa taa tat tga ata agg *ggc* att tcc act *agc* tga tga att agc ttt cat tac
Reverse primer for Y306A/Y312A	cgggatcc tta aaa ttt ttg aaa taa tat tga *agc* agg gta att tcc act *agc* tga tga att agc ttt cat tac aag
Reverse primer for Y310A/Y312A	cgggatcc tta aaa ttt ttg aaa taa tat tga *agc* agg *ggc* att tcc act ata tga tga att agc ttt cat tac
Reverse primer for Y306A/Y310A/Y312A	cgggatcc tta aaa ttt ttg aaa taa tat tga *agc* agg *ggc* att tcc act *agc* 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.

were transduced into *E. coli* strain TG1. The cells were grown at 37 °C in 2YT medium containing 2% glucose to a 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 recovered. The medium was applied to anti-FLAG M2 affinity gel, and the proteins bound to the gel were eluted with FLAG peptide. The buffer was changed to phosphate-buffered saline (PBS) using a PD-10 column (GE Healthcare Bio-Sciences Co., Piscataway, NJ). Purification of mutant C-CPE-PSIF was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-FLAG M2 antibody (data not shown). Protein levels were measured using a commercially available assay kit with bovine serum albumin as a standard (Bio-Rad, Hercules, CA).

2.5. Assay of cytotoxicity

The cytotoxicity of C-CPE-PSIF and mutant C-CPE-PSIF proteins was evaluated by measuring the release of lactate dehydrogenase (LDH) from cells. CL4/L cells were treated with C-CPE or mutant C-CPE-PSIF proteins at the indicated concentrations for 36 h. LDH release was then measured using a CytoTox96 Non Radioactive Cytotoxicity Assay kit according to the manufacturer's protocol (Promega).

2.6. Competition analysis

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, LDH release was assayed as described above.

2.7. Pull-down assay

Confluent Caco-2 cells were harvested and lysed in lysis buffer (1% Triton X-100, 0.2% sodium dodecyl sulfate, 150 mM NaCl, 10 mM HEPES [pH 7.4], 2 mM EDTA, and 1% protease inhibitor cocktail [Sigma, St Louis, MO]). C-CPE or mutant C-CPEs were incubated with the lysates for 30 min at 37 °C and then mixed with Ni-resin beads (Invitrogen, Gaithersburg, MD). After an additional 2 h at 4 °C, the beads were washed with lysis buffer, and bound proteins were analyzed by SDS-PAGE followed by Western blotting using anti-human claudin-4 (Zymed Laboratories, San Francisco, CA) and anti-His-tag antibodies (Novagen). The bound primary antibody was detected with a peroxidase-labeled secondary antibody followed by visualization with chemiluminescence reagents (Amersham Bioscience, NJ).

2.8. Transepithelial resistance (TER) assay

Caco-2 cells were seeded in Transwell chambers (Nunc, Roskilde, Denmark) at a subconfluent density. The formation of TJ barriers in Caco-2 monolayers was monitored by measuring the TER using a Millicell-ERS epithelial volt-ohmmeter (Millipore Co). When the TER values reached a plateau for continuous 3 days, the Caco-2 monolayers were treated with C-CPE or mutant C-CPEs on the apical side of the chamber, and the TER values were measured. The TER values were normalized by the area of the Caco-2 monolayer. The TER value of a blank Transwell chamber (background) was subtracted from the TER of cell monolayers.

2.9. 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 a week to adapt. 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-CPEs 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.

3. Results

3.1. Roles of Tyr306, Tyr310 and Tyr312 in the interaction between C-CPE and claudin-4

We previously found that the C-terminal 16 amino acids are responsible for ability of C-CPE to modulate the TJ barrier and to bind to claudin-4 [22,23] (Fig. 1). Kokai-Kun et al. showed that substitution of Tyr310 with Cys reduced binding of CPE to the brush border membrane in rabbits [24]. Here, we focused on three tyrosine residues in this region, namely Tyr306, Tyr310 and Tyr312. To evaluate the function of these tyrosine residues, we generated Tyr306, Y310A and Y312A mutants by site-directed mutagenesis. We then examined the ability of these mutants to inhibit the toxicity of C-CPE-PSIF, a molecule that specifically targets and is toxic to claudin-4-expressing cells [25]. In CL4/L cells, pretreatment with C-CPE attenuated toxicity of C-CPE-PSIF in a dose-dependent manner (Fig. 2A). The Y306A, Y310A and Y312A mutants had reduced abilities to inhibit C-CPE-PSIF-induced cytotoxicity. We also used a pull-down assay to examine the ability of these mutants to interact with claudin-4 in Caco-2 lysates, which have well-developed TJs [22]. Less claudin-4 precipitated with the Y306A and Y310A mutant than with C-CPE, but the Y312A mutant bound claudin-4 as effectively as C-CPE (Fig. 2B). The extra band below claudin-4 was observed in C-CPE, mutants and C-CPE/mutants-treated samples. The extra band was due to non-specific reaction of anti-claudin-4 Ab with histidine-tag (data not shown).

3.2. Interaction of double mutants of C-CPE with claudin-4

To evaluate the synergistic effects of Tyr306, Tyr310, and Tyr312 on the ability of C-CPE to interact with claudin-4, we generated double tyrosine to alanine substitution mutants (Y306A/Y310A, Y306A/Y312A, and Y310A/Y312A). As shown in Fig. 3A, we investigated the interaction of double mutants with claudin-4 in the C-CPE-PSIF competitive assay. Pretreatment of cells with C-CPE at 10 μg/ml inhibited LDH release to 18% of the vehicle-treated group, whereas treatment of cells with the Y306A/Y310A and Y306A/Y312A mutants did not affect C-CPE-PSIF-induced LDH release even at 10 μg/ml. Treatment of cells with Y310A/Y312A at 10 μg/ml partially attenuated the cytotoxicity of C-CPE-PSIF. A pull-down assay revealed that the Y306A/Y310A and Y306A/Y312A mutants completely lost the ability to bind claudin-4. Y310A/Y312A mutant had the partly reduced ability to bind claudin-4 (Fig. 3B). Thus, mutation of Tyr310 and Tyr312 to alanine reduced precipitation of claudin-4 in the pull down assay.

3.3. Effects of double mutants on TJ barrier function in Caco-2 cells

Next, we investigated the effects of the mutants on the TJ barrier function in Caco-2 monolayers grown in Transwells. Treatment of the cells with C-CPE, the Y310A and Y312A mutant for 18 h reduced the TER value, a marker of tightness in the TJs, from 295 to 30 and from 326 to 49 Ω cm^2, respectively (Fig. 4A). The Y306A and Y310A/Y312A mutants caused less of a reduction in TJ barrier function than C-CPE. The Y306A/Y310A and Y306A/Y312A mutants, however, had almost no effect on the TJ barrier function (from 291 to 261 and from 289 to 254 Ω cm^2, respectively; Fig. 4A).

We previously found that C-CPE enhances rat jejunal absorption of FD-4 by interacting with claudin-4 [23]. We further evaluated the ability of each mutant to enhance jejunal absorption of FD-4 (Fig. 4B and C). We found that the Y310A mutant enhanced absorption to a similar extent as C-CPE,

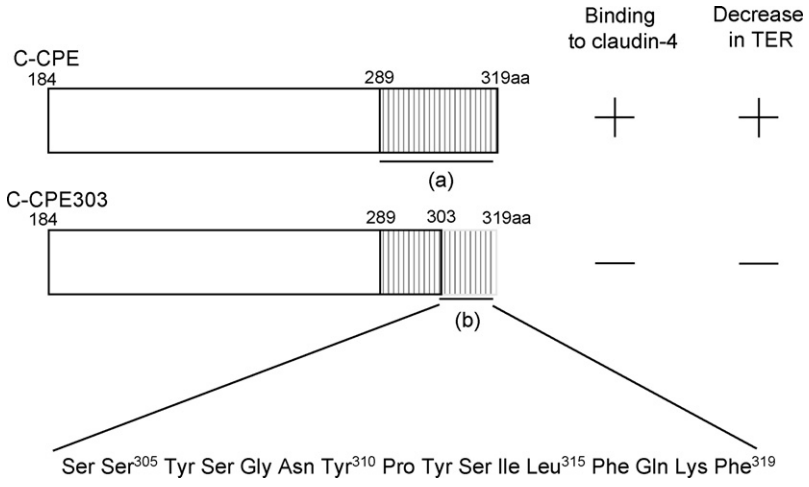

Fig. 1 – Diagram of C-CPE structure. C-CPE is the C-terminal fragment of CPE [21]. C-CPE binds to claudin-4 and decreases TJ barrier function as indicated by a decrease in TER [12]. The C-terminal 30 amino acids of CPE and C-CPE mediate interaction with the CPE receptor and claudin, respectively (a) [22,23,32]. Further analysis shows that the C-terminal 16 amino acids are responsible for the interaction of C-CPE with claudin and for its ability to decrease the TJ barrier function (b) [22,23].

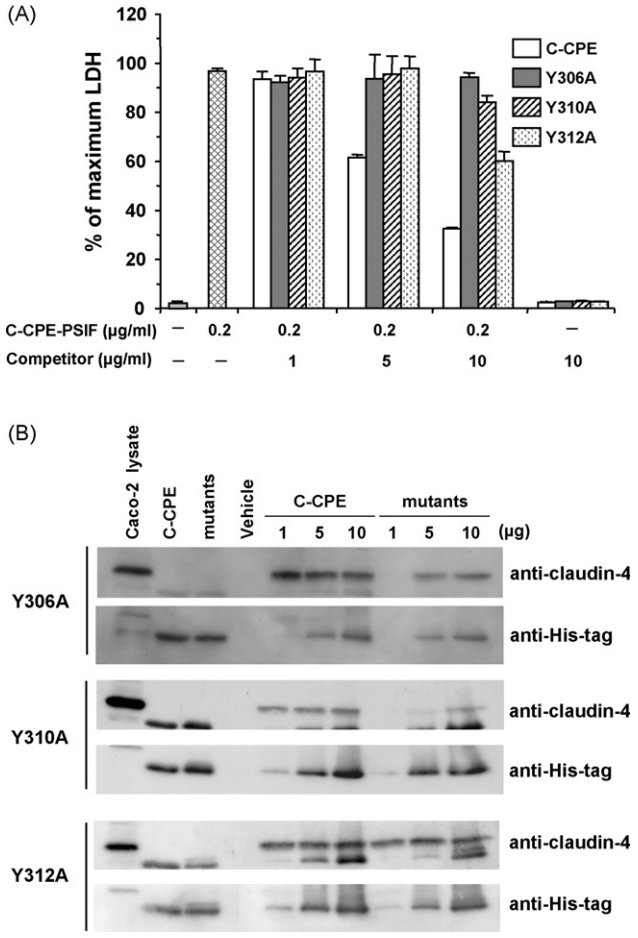

Fig. 2 – Effects of Tyr306, Tyr310 and Tyr312 on the interaction of C-CPE with claudin-4. (A) Competitive inhibition of C-CPE-PSIF-induced cytotoxicity by mutants of C-CPE. Claudin-4-expressing L (CL4/L) cells were pretreated with the indicated concentrations of C-CPE or C-CPE mutants for 1 h. The cells were then incubated with 0.2 μg/ml of C-CPE-PSIF. After 36 h, LDH release was assessed using a commercially available kit. The results are representative of three independent experiments, and the values are means ± S.D. (n = 3). (B) Pull-down assay. Confluent Caco-2 cells were harvested and lysed in lysis buffer. The lysate (10 μg) was incubated with vehicle, C-CPE, or mutants of C-CPE for 30 min at 37 °C. After addition of Ni-resin, the lysate was incubated for 3 h at 4 °C. The resin 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 lysates (10 μg), C-CPE (1 μg), and mutants of C-CPE (1 μg) were positive controls for claudin-4, C-CPE, and mutants of C-CPE, respectively. The results are representative of three independent experiments.

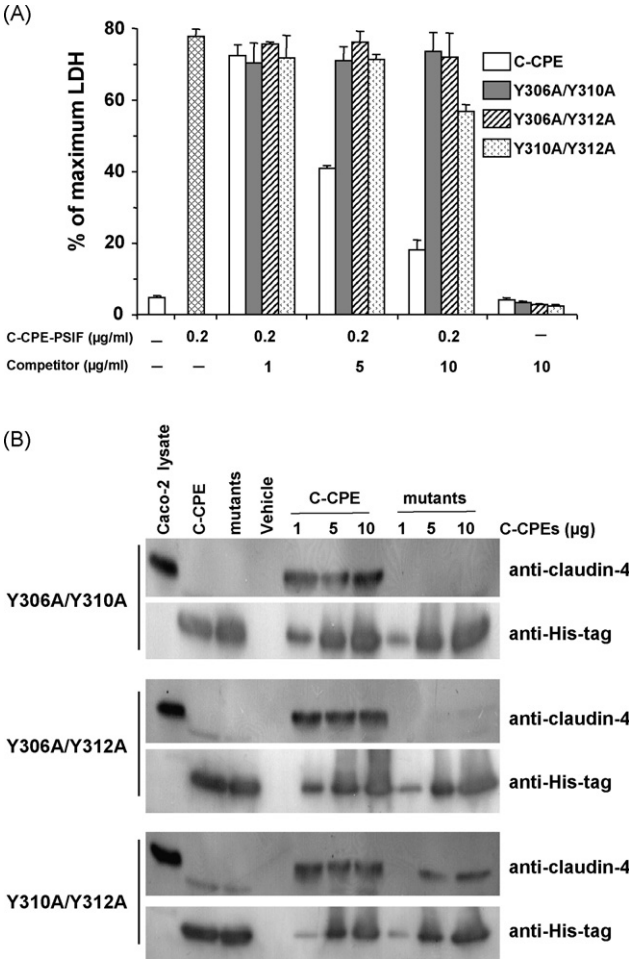

Fig. 3 – Interaction of double mutants at residues 306, 310, and 312 of C-CPE with claudin-4. (A) Competition assay. CL4/L cells were incubated with double mutants at the indicated concentration for 1 h and then mixed with C-CPE-PSIF (0.2 μg/ml). After 36 h, LDH release was assessed using a commercially available kit. The results are representative of three independent experiments. Values are means ± S.D. (n = 3). (B) Pull-down assay. Caco-2 lysates (10 μg) were incubated with double mutants (Y306A/Y310A, Y306A/Y312A, or Y310A/Y312A) for 30 min at 37 °C. Ni-resin was then added, and the lysate was incubated for 3 h at 4 °C. Next, the resin was precipitated, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting.

whereas the Y312A and Y306A mutants had weaker abilities to enhance absorption ($AUC_{0-4\ h}$ = 2.0 and 6.4, respectively). Double mutant Y306A/Y310A had a moderate absorption-enhancing activity, similar to the Y306A mutant. In contrast, the Y306A/Y312A and Y310A/Y312A double mutants lost the ability to enhance absorption. Thus, there are some differences between the effects of some of the mutants on the TER values and jejunal absorption.

3.4. *Effects of triple mutant Y306A/Y310A/Y312A on the ability to bind to claudin-4 and to modulate the TJ barrier function*

Finally, to clarify role of tyrosine residues 306, 310, and 312 in C-CPE function, we mutated all three to alanine, generating the Y306A/Y310A/Y312A triple mutant. To evaluate the

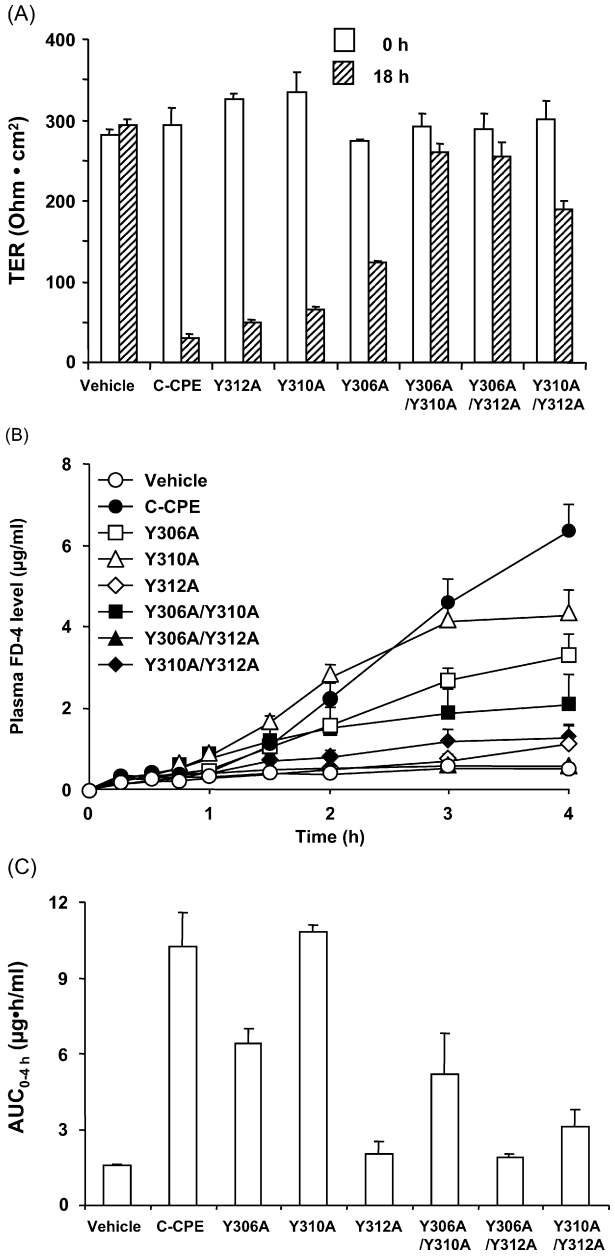

Fig. 4 – Effects of mutants of C-CPE on the TJ barrier function and jejunal absorption. (A) Effects of mutants on the TJ barrier function of Caco-2 monolayer cells. Confluent Caco-2 cells were cultured in Transwell chambers for 10–14 days. When TER values stabilized, C-CPEs were added to the basal side of the Transwell chamber at the indicated concentrations. TER values were measured after 0 and 18 h. Values are means ± S.D. (n = 4). The results are representative of three independent experiments. (B) Effects of mutants on jejunal absorption of FD-4 in rat. Rat jejunum was treated with FD-4 (10 mg/ml) in the presence of vehicle, C-CPE (0.2 mg/ml), or mutant C-CPEs (0.2 mg/ml). The FD-4 levels in plasma collected from the jugular artery were determined at the indicated times. Values are means ± S.E.M. (n = 4). (C) The $AUC_{0-4\ h}$ values were calculated from (B) and are means ± S.E.M. (n = 4). The results shown in (B) and (C) are representative of three independent experiments.

interaction of Y306A/Y310A/Y312A mutant with claudin-4, we performed a competitive assay using C-CPE-PSIF (Fig. 5A) and a pull-down assay using Caco-2 lysates (Fig. 5B). As indicated in Fig. 5A and B, the triple mutant lost the ability to interact with claudin-4. Furthermore, Fig. 5C–E shows that the triple mutant could not modulate TJ barrier function or enhance jejunal absorption.

4. Discussion

Claudins are critical for the barrier function of TJs in epithelia and endothelia [5]. This suggests that modulating the barrier function of claudins can be employed to deliver drugs, and we and Tsukita and colleagues have indeed shown that claudin is a promising target for the development of a drug delivery system [16,17,23,25]. Methods of modulating the barrier function of claudins are, however, very limited, and to our knowledge C-CPE is the only known modulator of a claudin (claudin-4) in TJs [12]. Therefore, we have sought to develop claudin modulators using C-CPE prototype and identified the part of C-CPE that modulates the TJ barrier function [22]. In the current study, we used site-directed mutagenesis and functional assays to clarify the roles of tyrosines 306, 310, and 312 of C-CPE. We found that these three tyrosine residues are critical for binding of C-CPE to claudin-4 and for modulation of the TJ barrier function.

Interestingly, the Y312A mutant had different effects in the pull-down assay using Caco-2 lysates than in the competitive assay using C-CPE-PSIF. Specifically, the Y312A mutant bound to claudin-4 in the pull down assay but it displayed reduced binding in the C-CPE-PSIF competitive assay. These conflicting results could be due to differences in the assay systems or the species of claudin-4: the former assay investigates the interaction of C-CPE with human claudin-4 in cell lysates, whereas the latter assesses the interaction with mouse claudin-4 on the cell membrane [25]. Claudin is tetra-transmembrane protein with two extracellular loop domains [5], and CPE and C-CPE interact with the second of these extracellular loops [22,26]. Examination of the amino acid sequence in the second extracellular loop domain of claudin-4 (EC2cld4) reveals, in fact, that they are different in mouse (GenBank accession no. AF087822; residues 145–163; RDFYNPMVASGQKREMGAS; underline indicates sequence differences with human claudin-4) and human (GenBank accession no. BC000671; residues 142–160; NIIQDFYNPI-VASGQKREM; underline indicates sequence differences with mouse claudin-4) [15].

The Y312A mutant also had different effects between TER assay and *in situ* loop assay. Although the Y312A mutant reduced TER values in Caco-2 monolayer cells, the Y312A mutant did not enhance rat jejunal absorption of FD-4. TER is a marker of tightness in the TJs, but we did not evaluate the permeability of FD-4 in Caco-2 monolayer cells. Several reports indicate that TER values did not reflect permeability of non-electrolytes [27,28]. Therefore, decrease in TER values may be inconsistent with influx of paracellular markers in Caco-2 cells. The amino acids sequences in EC2cld4 are also different in rat (GenBank accession no. NM_001012022; residues 145–163; RDFYNPIVASGQKREMGAS; underline indicates sequence

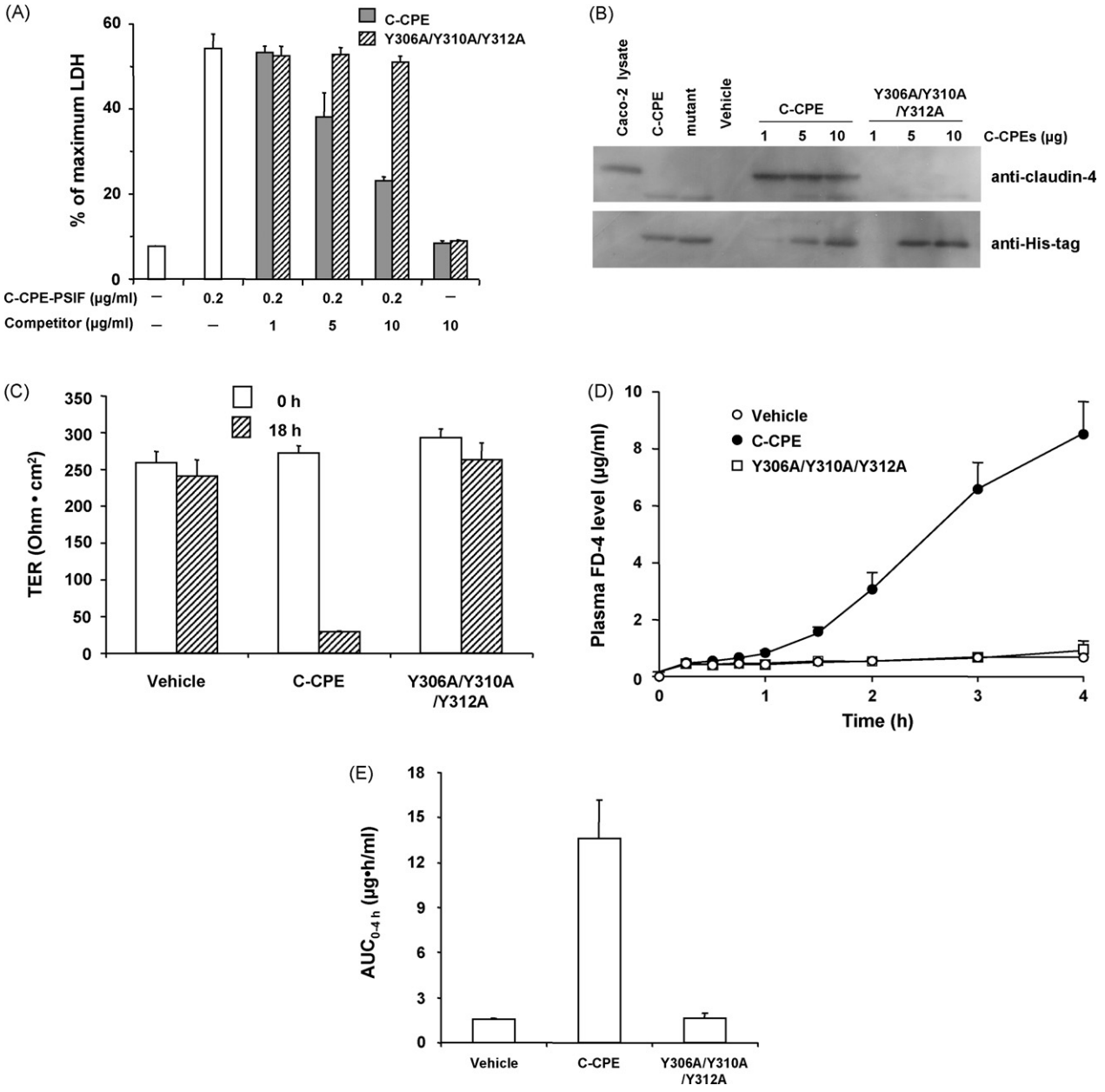

Fig. 5 – Effect of triple mutation on the activities of C-CPE. Interaction of the triple mutant with claudin-4 was examined using competitive assay with C-CPE-PSIF (A) and a pull-down assay (B) as described in Fig. 2A and B, respectively. Effects of the triple mutant on the TJ barrier in Caco-2 monolayer cells (C) and jejunal absorption in rat (D and E) were assayed as described in Fig. 4. The values in (B) and (C) are means ± S.D. (n = 4), and those in (D) and (E) are means ± S.E.M. (n = 4). The results are representative of three independent experiments.

differences with human claudin-4) and human (GenBank accession no. BC000671; residues 142–160; NIIQDFYNPI-VASGQKREM; underline indicates sequence differences with rat claudin-4) [15]. The differences of EC2cld4 between rat and human may contribute the contradiction of data on Y312A in TER assay and *in situ* loop assay.

We also found that mutation of Tyr310 to alanine reduced binding of C-CPE to claudin-4. This finding agrees with a previous report by Kokai-Kun et al. showing that replacement of Tyr310 with cysteine reduced the ability of CPE to bind to its receptor [24]. Tyr310 may participate in interaction of C-CPE with claudin-4 because the Y310A mutant had the ability to modulate the TJ barrier function in Caco-2 monolayers and to enhance rat jejunal absorption. Whether claudin-4 binding and modulation of the TJ barrier function can be separated is a critical question for the design of future claudin modulators. Mutation of Tyr306 to alanine reduced both activities suggested that they are mediated by the same functional domain on C-CPE. Results with the Y310A mutant, however, indicate that different residues mediate these two functions. In fact, the Y312A mutant did not enhance jejunal absorption in rats (Fig. 4B and C), although it bound claudin-4

in a pull-down assay using rat jejunal lysates (data not shown). Taken together, we cannot exclude the possibility that the two functions of C-CPE are mediated by distinct residues.

The next logical question is whether interaction of C-CPE with claudin-4 is sufficient for disrupting the TJ barrier. Although we did not examine the effect of C-CPE on the level of claudin-4 protein in TJs in the current studies, Sonoda et al. previously reported that treatment of cells with C-CPE causes a decrease in the level of claudin-4 protein in TJs, suggesting that its loss is essential for disruption of the TJ barrier by C-CPE [12]. This loss of claudin-4 could occur through clathrin-mediated endocytosis. Proteins are generally targeted to clathrin-coated vesicles by sorting signal sequences, including YXXØ or EXXXLL (where X is any amino acid and Ø is a bulky hydrophobic residue) [29], and claudin-4 contains an ALGVLL motif at amino acids 92–97 and a YVGW motif at amino acids 165–168 [30]. Indeed, Matsuda et al. showed that the endocytosis of claudins occurs during the remodeling of TJs [31]. The fact that the cytotoxicity of C-CPE-PSIF depends on its uptake into cytosol suggests that, in the CL4/L cells, a molecule bound to claudin-4 can be taken up into the cytosol [25]. In the current study, we found that the Y312A mutant interacts with claudin-4 in a pull-down assay using lysates of rat jejunum but that it does not enhance jejunal absorption. Therefore, Y312A may interact with claudin-4 on rat jejunal epithelial cells but not be taken up into the cytosol.

Single mutation of tyrosines 306, 310, or 312 to alanine resulted in partial reductions of C-CPE activities, whereas double or triple mutations of these tyrosines nearly eliminated the activities. For example, the Y306A/Y310A, Y306A/Y312A, and Y306A/Y310A/Y312A mutants did not bind to claudin-4 or modulate the TJ barrier, whereas the Y310A/Y312A mutant had a partial reduction in binding to claudin-4 and modulation of the TJ barrier function. Taken together, these results show that Tyr306 is a pivotal residue but that it is not the only residue important for the abilities of C-CPE to bind claudin-4 and modulate the TJ barrier. How these three tyrosine residues contribute to the interaction of C-CPE with claudin-4 and to modulation of the TJ barrier is not clear because the three-dimensional structures of CPE and claudin have not been determined. The different effects of C-CPE mutants on C-CPE activities may be due to differences in potency of the mutants or qualitative differences in the mechanism. Regardless, our results suggest that it may be useful to mutate tyrosines 306, 310, and 312 to develop claudin modulators. This information should also be useful for future structural studies on the interaction of CPE with claudin.

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