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

*Motoki Harada* [a,1], *Masuo Kondoh* [b,1,*], *Chiaki Ebihara* [a], *Azusa Takahashi* [a], *Eriko Komiya* [a], *Makiko Fujii* [a], *Hiroyuki Mizuguchi* [c,d], *Shin-Ichi Tsunoda* [e], *Yasuhiko Horiguchi* [f], *Kiyohito Yagi* [b], *Yoshiteru Watanabe* [a]

[a] *Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan*
[b] *Department of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan*
[c] *Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Ibaraki, Osaka 567-0085, Japan*
[d] *Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan*
[e] *Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation, Ibaraki, Osaka 567-0085, Japan*
[f] *Department of Bacterial and Toxinology, Division of Infectious Diseases, Osaka University, Suita, Osaka 565-0871, Japan*

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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].

* *Corresponding author*. Tel.: +81 6 6879 8176; fax: +81 6 6879 8199.
E-mail address: masuo@phs.osaka-u.ac.jp (M. Kondoh).

[1] These authors contributed equally to this work.

*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