BIOCHEMICAL PHARMACOLOGY 75 (2008) 1639–1648

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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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ARTICLE INFO

Article history:
Received 17 November 2007
Accepted 21 December 2007

Keywords:
Clostridium perfringens enterotoxin
Tight junction
Claudin
Site-directed mutagenesis

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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doi:10.1016/j.bcp.2007.12.016

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.