Molecular Determinants of the Interaction between Clostridium perfringens Enterotoxin Fragments and Claudin-3

Received for publication, March 17, 2009, and in revised form, April 15, 2009 Published, JBC Papers in Press, May 8, 2009, DOI 10.1074/jbc.M109.008623

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Clostridium perfringens enterotoxin (CPE) binds to the extracellular loop 2 of a subset of claudins, e.g. claudin-3. Here, the molecular mechanism of the CPE-claudin interaction was analyzed. Using peptide arrays, recombinant CPE-(116–319) bound to loop 2 peptides of mouse claudin-3, -6, -7, -9, and -14 but not of 1, 2, 4, 5, 8, 10–13, 15, 16, 18–20, and 22. Substitution binding assays with claudin-3 mutant-transfected HEK293 cells lysates thereof demonstrated the involvement of Asn148 and Leu150 of full-length claudin-3 in the binding. CPE-(116–319) and CPE-(194–319) bound to HEK293 cells expressing claudin-3, whereas CPE-(116–319) bound to claudin-5-expressing HEK293 cells, also. This binding was inhibited by substitutions T151A and Q156E in claudin-5. In contrast, removal of the aromatic side chains in the loop 2 of claudin-3 and -5, involved in trans-interaction between claudins, increased the amount of CPE-(116–319) bound. These findings and molecular modeling indicate different molecular mechanisms of claudin-claudin trans-interaction and claudin-CPE interaction. Confocal microscopy showed that CPE-(116–319) and CPE-(194–319) bind to claudin-3 at the plasma membrane, outside cell-cell contacts. Together, these findings demonstrate that CPE binds to the hydrophobic turn and flanking polar residues in the loop 2 of claudin-3 outside tight junctions. The data can be used for the specific design of CPE-based modulators of tight junctions, to improve drug delivery, and as chemotherapeutics for tumors overexpressing claudins.

The clinical use of many promising drug candidates is impeded by unacceptable pharmacokinetics (1). The ability of a drug to pass through tissue barriers is a major determinant for its delivery. In epithelia and endothelia, the paracellular route is blocked by tight junctions (TJ).4 Different approaches have been used to enhance transcellular drug delivery. These include the use of influx transporters, blocking of efflux transporters, or receptor-mediated endocytosis (2). Alternative approaches aim to enhance paracellular permeation of drugs by loosening the TJ (3, 4). This strategy has the advantage that it could improve the delivery of structurally unrelated drugs, and the drug itself does not have to be modified. Although different TJ modulators have been described, most of these are based on surfactants or chelators (3). These often have low tissue specificity and cause severe side effects, e.g. exfoliation of cells, which irreversibly compromise the barrier functions (5, 6). Fewer side effects may be obtained by more specific modulation of a molecular key component of the TJ (7).

TJ consist of transmembrane proteins, mainly the tetraspan proteins of the claudin family, as well as occludin and tricellulin (8). Other molecules associated with TJ include membrane-bound scaffolding and signaling proteins (9). However, claudins (Cld) are the major functional constituent of TJ (10). Claudins tighten the paracellular space, selectively for tissue, size, and charge. The tissue-specific combination of the claudin subtypes present in heteropolymers is assumed to determine the permeability properties of TJ (11). It was therefore proposed that tissue-specific drug delivery via the paracellular route would be possible by modulation of the barrier-function of claudins in a subtype-specific manner (7).

A subset of claudins, e.g. Cld3 and -4 but not -1 and -2, have been shown to be receptors for Clostridium perfringens enterotoxin (CPE) with high association constants of about 108 M–1 (12). CPE causes one of the most common food-borne diseases (13). It consists of two functional domains, an N-terminal region that mediates the cytotoxic effect and the C-terminal region (CPE-(184–319)), which binds to extracellular loop 2 (ECL2) of Cld3 but not of Cld1 nor to the ECL1 of Cld3 (12). Treatment of epithelial monolayers with non-cytotoxic CPE-(184–319) increases paracellular permeability (14). CPE-(184–319) enhanced drug absorption in rat jejunum 400-fold relative to sodium caprate, which is in clinical use (15). Thus, CPE is a promising tool to specifically modulate claudins, the key constituents of TJ, and thereby to enhance paracellular drug delivery. In addition, some studies have suggested the use of CPE for the chemotherapy of tumors overexpressing claudins (16–18).

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.
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4 The abbreviations used are: TJ, tight junctions; CPE, Clostridium perfringens enterotoxin; ECL, extracellular loop; Cld, claudin; SPR, surface plasmon resonance; BLU, Boehringer light units; GST, glutathione S-transferase; GFP, cyan fluorescent protein; YFP, yellow fluorescent protein; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; HEK, human embryonic kidney; TEER, transepithelial electrical resistance.
Claudin-3-CPE Interaction

Cld1 and -5 are potential targets for transepidermal and brain drug delivery, respectively (19, 20). However, it has been reported that these claudins do not interact with CPE (12). Modification of CPE could enhance and/or shift its claudin-subtype specificity. Therefore, the design of CPE-based TJ modulators could permit efficient claudin subtype-specific modulation, which would also be tissue-specific modulation of TJ. To achieve this, an understanding of the molecular mechanism of the CPE-claudin interaction is a necessary prerequisite. In this study, we identify the residues within the ECL2 of Cld3 that are involved in interaction with CPE.

EXPERIMENTAL PROCEDURES

Plasmids—For construction of plasmids encoding GST-CPE-(116–319), GST-CPE-(194–319), and GST-CPE-(290–319) fusion proteins, cDNA of CPE (kindly provided by Dr. Y. Horiguchi, Osaka, Japan) was amplified by PCR and cloned into pGEX-4T1 (GE Healthcare) using EcoRI and Sall; GST-CPE-(194–309) was generated by site-directed mutagenesis. Plasmids encoding Cld5wt-CFP, Cld5wt-YFP, and mutant fusion proteins have been described previously (21). A pECPF-N1-plasmid containing the Cld3 sequence with a stop codon ahead of CFP was generated by subcloning full-length Cld3 with Sall and BamHI from pSK-Cl-3, kindly provided by Dr. M. Furuse (Kyoto, Japan). Similarly, Cld5wt was subcloned from pGTCL-5 (Dr. M. Furuse, Kyoto, Japan) into pEYFP-N1 using EcoRI. To generate the in-frame fusion Cld3wt-CFP, the stop codon in pECPF-N1-Cld3 was removed by side-directed mutagenesis. Cld3wt-YFP was generated by subcloning Cld3wt-CFP in pECFP-N1 using Sall and BamHI. The plasmids encoding mutants of Cld3 (Y147A, N148D, L150A, E153V, A154N, and Q155E, N148D/L150A) were generated by site-directed mutagenesis of pECPF-N1-Cld3wt, pEGFP-Cld4 was kindly provided by Dr. W. Hunziker (Singapore).

Antibodies—Rabbit anti-Cld3, rabbit anti-Cld4, rabbit anti-Cld5, HRP- and Alexa Fluor 488-conjugated anti-rabbit antibodies, HRP-, Cy3-, and Alexa Fluor 647-conjugated antimouse antibodies were obtained from Invitrogen. Mouse anti-GST (GST-2) was from Sigma. To detect CFP and YFP fusion proteins, mouse anti-GFP antibodies (Clontech) were used for Western blots.

Expression and Purification of CPE Constructs—CPE-(116–319), CPE-(194–319), CPE-(194–309), and CPE-(290–319) with N-terminal GST fusions as well as GST (control) were expressed in Escherichia coli BL21. Bacteria were grown to A₆₀₀ = 0.6–0.8, harvested by centrifugation, and lysed in PBS with 1% (v/v) Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, protease inhibitor mixture (Sigma) by using an Emulsiflex-C3 homogenizer (Avestin, Mannheim, Germany). To remove insoluble cell debris, the lysates were centrifuged at 40,000 × g for 1 h at 4 °C. The recombinant proteins were purified from supernatants using glutathione-agarose (Sigma) and eluted proteins were dialyzed against PBS buffer with Ca²⁺ and Mg²⁺.

Surface Plasmon Resonance (SPR)—Measurements were performed using a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). Claudin-3-ECL2 peptides (Cld3-(140–159), Cys-(6-aminoacaprico acid)-NTIRDYDPTVPEAQKREM; and Cld3-(140–159)N148D/L150T, Cys-(6-aminoacaprico acid)-NTIRDYDPTVPEAQKREM) were synthesized with N-terminal cysteine followed by a 6-aminoacaprico acid linker. Peptides were immobilized on a CM5 chip using the thiol coupling procedure, according to the supplier’s instructions (Biacore AB). To determine the binding affinities of GST-CPE-(116–319) and GST-CPE-(290–319), an amount of covalently coupled Cld3-(140–159) peptide, corresponding to a signal increase of ~1600 resonance units was used. For comparison of Cld3-(140–159) and Cld3-(140–159)N148D/L150T, 500 and 250 resonance units of Cld3 peptides were immobilized, respectively. Binding experiments were performed as described earlier (22). The analytes GST-CPE-(116–319), GST-CPE-(290–319), and GST (control) were injected in concentrations between 0.01 and 20 μM in running buffer (PBS, pH 7.4, supplemented with 2 mM dithiothreitol). For regeneration 10 mM NaOH solution with 1 mM NaCl was used. For every analyte concentration the maximal amount of GST-CPE-(116–319) and GST-CPE-(290–319) bound was calculated in femtomole (~1000 resonance units = 1 ng of protein bound).

Peptide Arrays—Arrays were generated by spot-synthesis using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry as described (23). The AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany) was used for automatic synthesis of 15–20-mer peptides on Whatman 50 cellulose membranes (C-terminal immobilization). The quality of synthesized peptides was evaluated by mass spectrometry of control spots. For binding experiments membranes were incubated with 10 μg/ml GST-CPE-(116–319) or GST in blocking buffer (Sigma) overnight at 8 °C, followed by incubations with mouse anti-GST and HRP-conjugated goat anti-mouse antibody in blocking buffer for 1.5 and 1 h at room temperature, respectively. The ECL Western blotting detection reagent (GE Healthcare) was applied and chemiluminescence intensities were measured as Boehringer light units (BLU) using a LuminoImager (Roche Applied Science). Determined BLU values of GST-incubated SPOTs were subtracted from BLU values of corresponding GST-CPE-(116–319)-incubated spots (ΔBLU). Background corrected ΔBLU values were normalized to the binding of GST-CPE-(116–319) on Cld3 spots (%Cld3).

Cell Culture and Transfection—Caco-2 human intestinal carcinoma cells and HEK293 cells (HEK cells) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and 1% l-alanyl-l-glutamine. Transient transfections of HEK cells were performed with Lipofectamine 2000 (Invitrogen).

Electrical Cell Substrate Impedance Sensing (ECIS)—Caco-2 cells were seeded into the wells of ECISTM 8W10E+ electrode arrays for ECISTM model 1600R (Applied Biophysics) at a density of 5 × 10⁵ cells per well and grown in a humidified CO₂ incubator with 5% CO₂. Functional resistance was measured every 5 min at 400 Hz frequency, as in a previous report (24). When monolayers reached maximum resistance (~1500 Ω) 10 μg/ml GST-CPE-(116–319) or GST were introduced into the medium.
Measurements of Transepithelial Electrical Resistance (TEER) and Paracellular Permeation—5 × 10⁵ Caco-2 cells were seeded into a 24-well transwell filter (Millipore, Eschborn, Germany). TEER was determined with Endohm electrodes (Millipore). After reaching stable TEER values, cells were incubated with GST-CPE-(116–319) or GST for 16 h. For permeation studies cells were incubated with 25 μg/ml fluorescein (Sigma) in Hanks’ balanced salt solution buffer on the apical side and Hanks’ balanced salt solution on the basal side for 10 min. 100-μl samples were removed from the basal compartment and the fluorescence units were measured using a fluorescence plate reader (Tecan, Crailsheim, Germany) to calculate the permeation coefficient.

Immunocytochemistry—Immunocytochemistry was performed as described (25). Briefly, 2–3 days after transient transfection, HEK cells were incubated with 10 μg/ml GST-CPE-(116–319), GST-CPE-(194–319), or GST for 20 min, 1 h, or 14 h at 37 °C. Cells were fixed with acetone, incubated with anti-GST antibodies, followed by incubation with anti-Cld3 or anti-Cld5 antibodies, and incubated with 4,6-diamidino-2-phenyindole, Cy3, or Alexa Fluor 647-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies. Cells were analyzed with the LSM 510 META confocal microscope (Zeiss, Jena, Germany).

Pull-down Assay—HEK cells seeded in 6-well plates were transiently transfected with Cld3wt, Cld3_mutants or GFP-Cld4. Two to 3 days after transfection, cells were washed with ice-cold PBS, with Ca²⁺ and Mg²⁺, scraped, harvested (300 × g, 5 min, 4 °C), and quick frozen in liquid nitrogen. The cells were lysed (1% Triton X-100 in PBS, EDTA-free protease inhibitor mixture (Roche), 0.1 mM phenylmethylsulfonyl fluoride), incubated on ice for 10 min, and centrifuged (5 min, 10,000 × g, 4 °C). The supernatants were incubated with GST, GST-CPE-(116–319), GST-CPE-(194–319), or GST-CPE-(194–309) bound to glutathione-Sepharose beads (GE Healthcare) in mini shaker at 4 °C. Beads were centrifuged (2 min, 500 × g) and the supernatants were eluted with Laemmli buffer (eluate). Eluate and unbound fractions were analyzed by SDS-PAGE and Western blot as described previously (26).

Cellular CPE-binding Assay—Two to 3 days after transfection, HEK cells expressing Cld3wt, Cld3_mutants, Cld3wt-CFP, Cld5wt, Cld5wt-YFP, or Cld5-YFP mutants were incubated with 1 or 10 μg/ml GST-CPE-(116–319), GST-CPE-(194–319), or GST for 1 h at 37 °C in 24- or 12-well plates. Cells were washed with ice-cold PBS supplemented with Ca²⁺ and Mg²⁺, scraped, and harvested (300 × g, 5 min, 4 °C). Cells were lysed with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1.0 mM EDTA, 1.0% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% SDS, EDTA-free protease inhibitor mixture (Roche, Mannheim, Germany) for 10 min on ice. After centrifugation (10,000 × g, 5 min, 4 °C), supernatants were analyzed by SDS-PAGE and Western blot. For each claudin mutant the ratios of the BLU values of the GST-CPE and the claudin signal were calculated relative to the ratio for the claudinwt (internal standard).

Structural Bioinformatics and Molecular Modeling—The ECL2 of claudins was predicted by alignment of sequences of mouse (human when indicated) Cld-(1–23) with the GCG program package (GCG Wisconsin package, Accelrys Inc., San Diego, CA). A homology model for Cld3-ECL2 was built on the model for murine Cld5-ECL2 as described (21). The Cld5-ECL2 model was based on part of Protein Data Bank structure 2BDV (Phage-related Protein BB2244 from Bordetella bronchiseptica). This resulted in a helix-turn-helix motif for ECL2, where the helices of the ECL2 are extensions of the predicted transmembrane helices 3 and 4 of Cld3. All manual reciprocal dockings, manipulations, and optimizations of ECL2 models were performed with the program Sybyl 7.3 (Tripos Inc., St. Louis, MO). The models were energetically minimized, using the AMBER95 force field.

Statistics—Unless stated otherwise, results are shown as mean ± S.E. Statistical analyses were performed by one-way analysis of variance and followed by an unpaired Student’s t test. p < 0.01 was taken as significantly different.

RESULTS

CPE-(116–319) Binds with Higher Affinity Than CPE-(290–319) to the ECL2 Peptide of Claudin-3—The C-terminal end of CPE-(290–319) is sufficient for interactions with Cld3 and Cld4 (27). In addition, further regions within CPE-(116–290) have been implicated in binding to Cld3/4 (28, 29). To determine whether CPE region 116–290 enhances the interaction of CPE with Cld3-ECL2, we compared binding of GST-CPE-(290–319) with the binding of GST-CPE-(116–319) to peptide Cld3-(140–159) (predicted ECL2, see “Experimental Procedures”). SPR spectroscopy using immobilized Cld3-ECL2-(140–159) showed that a 20-fold higher concentration of GST-CPE-(290–319) compared with GST-CPE-(116–319) was needed to obtain similar amounts of bound CPE (Fig. 1A). This demonstrates that GST-CPE-(116–319) binds much more strongly to Cld3-ECL2 than GST-CPE-(290–319). Thus, GST-CPE-(116–319) was used for further experiments. When confluent monolayers of Caco-2 cells were incubated with GST-CPE-(116–319), a decrease in junctional resistance, TEER, and an increase in paracellular fluorescence permeability compared with GST were detected (Fig. 1, B–D). Thus, GST-CPE-(116–319) binds to native claudins on living cells and influences paracellular tightness.

Substitution Analysis Identified the Motif NPLVP in Peptides of the ECL2 of Claudin-3 as Essential for Binding to CPE-(116–319)—To compare the binding ability of GST-CPE-(116–319) to the ECL2 of different claudins, peptide arrays were used. Peptides corresponding to the predicted ECL2 (11) of Cld1–22, except -17 and -21 were synthesized on membranes and incubated with GST-CPE-(116–319) or GST as control. GST-CPE-(116–319) specifically bound to ECL2 peptides of Cld3, -6, -7, -9, and -14 but not Cld1, -2, -4, -5, -8, -10–13, -15, -16, -18–20, and -22 (Table I).

For the identification of amino acids involved in the CPE-claudin interaction, additional peptide arrays were performed. Because GST-CPE-(116–319) did not bind to Cld5-ECL2 peptides (Table I), we tested whether introduction of defined amino acids of Cld3-ECL2 into Cld5-ECL2 enables binding to...
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To analyze the molecular determinants of the Cld3-CPE interaction in more detail, substitution mapping was performed. Here, peptide arrays were used that contained peptides with every possible single amino acid substitution for each position in Cld3-ECL2-(145–159) (Fig. 2). Cysteine was excluded because it leads to artificial covalent bonds during peptide synthesis. None of the substitutions at positions 145 to 147 had a considerable effect on the binding of GST-CPE-(116–319). In contrast, every substitution in the region N148D/L150A strongly reduced the binding of GST-CPE-(116–319). This indicates that the NPLVP motif is essential for the Cld3-CPE interaction. In addition, several substitutions at positions 154–158 also influenced the amount of GST-CPE-(116–319) bound. In particular, the introduction of negatively charged amino acids or proline at positions 154–157 strongly reduced the binding of GST-CPE-(116–319).

Interaction between Full-length Claudin-3 and CPE-(116–319) Is Affected by Amino Acid Substitutions in ECL2—To determine whether amino acid substitutions in ECL2 affect binding of GST-CPE-(116–319) to full-length Cld3, transfections of Cld3 in Tj-free HEK cells (21) were performed. Lysates of the cells were used for pull-down assays with GST-CPE-(116–319) (Fig. 3). Cld3 bound specifically to GST-CPE-(116–319) but not GST. Substitutions N148D and L150A in the ECL2 of Cld3 caused a strong reduction in the amount of Cld3 that bound to GST-CPE-(116–319). In contrast, substitutions A154N and Q155E did not affect the interaction of full-length Cld3 with GST-CPE-(116–319). Substitution E153V used to mimic a sequence element in Cld5 slightly reduced the amount of Cld3 bound to GST-CPE-(116–319) because it leads to artificial covalent bonds during peptide synthesis

FIGURE 1. A, CPE-(116–319) binds much more strongly to the ECL2 peptide of Cld3 than does CPE-(290–319). ECL2 peptide (amino acids 140–159) of mouse Cld3 was immobilized and superinfused with different concentrations of GST-CPE-(116–319) and GST-CPE-(290–319), respectively. The binding of GST was negligible (<1 fmol for <20 μM GST). The binding was analyzed by surface plasmon resonance spectroscopy. Mean ± S.E., n = 3 except for 18 μM GST-CPE-(290–319), n = 1. Incubation of Caco-2 cells with CPE-(116–319) reduces junctional resistance (B) and transepithelial electrical resistance, TEER (C), and increases the permeation coefficient (Pc) of fluorescein (D). For B, cells were incubated for 12 h with 10 μg/ml GST or GST-CPE-(116–319) and the junctional resistance relative to the value before GST or GST-CPE-(116–319) incubation was determined by electric cell-substrate impedance sensing. Mean ± S.E. (error bars); n = 9, *p < 0.001. For C and D, cells were incubated with 25 μg/ml GST or GST-CPE-(116–319) for 16 h and TEER determined relative to the value before incubation. Mean ± S.E. n = 6, *p < 0.001.

TABLE 1
GST-CPE-(116–319) binds to peptides corresponding to the extracellular loop two (ECL2) of claudin-3, -6, -7, -9, -14

| Peptide array incubated with | Sequence of ECL2 peptide | CPE bound (relative to Cld3) |
|-----------------------------|--------------------------|----------------------------|
| GST                         | GST-CPE-(116–319)        |                            |
| Cld3                        | NHKVEYFIPUPF151NARYF     | -0.09                      |
| Cld6                        | RDLRQYFPLC151NERYF       | 0.10                       |
| Cld7                        | N149KDYQ151NERYF         | 1.00                       |
| Cld8                        | NHKVEYFIPUPF151NARYF     | 0.04                       |
| Cld9                        | NVKVEYFIPUPF151NARYF     | 0.05                       |
| Cld10                       | HSIQYQ151NARYF           | 1.34                       |
| Cld11                       | HQIV151NARYF             | 0.55                       |
| Cld12                       | N149KDYQ151NERYF         | -0.02                      |
| Cld13                       | HAIQ151NARYF             | 1.15                       |
| Cld14                       | N149KDYQ151NERYF         | -0.09                      |
| Cld15                       | WAPPCARE151NARYF         | -0.08                      |
| Cld16                       | WAPPh151NARYF            | -0.07                      |
| Cld17                       | N149KDYQ151NERYF         | -0.01                      |
| Cld18                       | NTNQ151NARYF             | 0.63                       |
| Cld19                       | NVNQNYFPLC151NARYF       | 0.04                       |
| Cld20                       | VVTV151NARYF             | 0.02                       |
| Cld21                       | KMGY151NARYF             | 0.02                       |
| Cld22                       | LVQ151NARYF              | -0.01                      |
| Cld23                       | TXK151NARYF              | -0.09                      |
| Cld24                       | HMKTV151NARYF            | -0.08                      |

CPE. In contrast to single or certain multiple substitutions including E146D, V154E, S155A, and Y158R, specifically the double substitution D149N/T151L in Cld5-ECL2 peptides facilitated the binding of GST-CPE-(116–319) (Table 2, upper part). Similarly, substitution of S149N in Cld2-ECL2 corresponding to substitution of D149N in Cld5 enabled binding of GST-CPE-(116–319) (Table 2, lower part). The additional substi-
Claudin-3, incubated with GST-CPE-(116–319) or GST as a control, and the presence of bound GST-CPE in cell lysates tested by Western blots. GST-CPE-(116–319) bound specifically to Cld3-transfected but not to non-transfected cells (Fig. 4, left). This binding was not clearly affected by single amino acid substitutions N148D or L150A but strongly reduced by N148D/L150A double substitution in Cld3 (Fig. 4, middle). Similar results (Fig. 4, right) were obtained with GST-CPE-(194–319), which contains a CPE fragment of which the structure has been recently solved (30).

CPE Binds to Cld3 on the Surface of Transfected HEK Cells Outside Tight Junctions—The binding of GST-CPE to Cld3-transfected HEK cells was also analyzed by confocal microscopy. Cld3 was detected in the plasma membrane and intracellular compartments (Fig. 5, A–C). Marked enrichment of Cld3 at contacts between two Cld3-expressing cells was observed. This enrichment is known to correspond to the formation of TJ strands (21). No binding of GST to either Cld3 expressing or non-expressing cells was detected (Fig. 5A). In contrast, GST-CPE-(116–319) bound specifically to Cld3 expressing cells and did not bind to non-expressing cells (Fig. 5B). Interestingly, almost no GST-CPE-(116–319) signals were detected at the contacts between two Cld3-expressing cells, where Cld3 was strongly enriched. However, strong GST-CPE-(116–319) signals were detected on the surface of the cells outside the contacts. Similar results were obtained with GST-CPE-(194–319) (Fig. 5C). Cld3-N148D/L150A was enriched at contacts similarly to Cld3mut (Fig. 5D). This indicates that the amino acid substitutions do not alter plasma membrane targeting and ability for trans-interaction of Cld3 (21). Instead, binding of GST-CPE-(194–319) was strongly reduced. In addition, incubation of transfected HEK cells with GST-CPE-(194–319) for 14 h resulted in internalization of CPE and Cld3 (Fig. 5E, left). This was blocked by the N148D/L150A substitution (Fig. 5E, right). CPE-(116–319) but Not CPE-(194–319) Binds to Cld5-transfected HEK Cells—GST-CPE-(116–319) not only bound to Cld3 but also to Cld5-transfected cells (Fig. 6A). Cld3- and Cld5-YFP expression levels were detected via anti-YFP antibodies. Due to differences in the formation of SDS-resistant oligomers (31), the intensities of the Cld3- and Cld5-YFP bands were difficult to compare. Nevertheless, Cld5-YFP was expressed at least as high as Cld3-YFP, but gave a weaker signal for bound GST-CPE-(116–319). This indicates that GST-CPE-(116–319) binds more strongly to Cld3 than to Cld5. In contrast to GST-CPE-(116–319), GST-CPE-(194–319) bound to Cld3 but not to Cld5-transfected HEK cells (Fig. 6B).

Amino Acid Substitutions in ECL2 of Cld5 Affect the Binding of CPE-(116–319) to Cld5-transfected HEK Cells—To identify determinants in the ECL2 of Cld5 for the binding of CPE-(116–319), a set of Cld5-ECL2 mutants (21) was used. The plasma membrane targeting of these mutants was not affected. Transfection of most of the mutants resulted in binding of GST-CPE-

![Figure 2](image_url)
Claudin-3-CPE Interaction

Replication blots are shown. Representative blots are shown. Claudin-3 bound specifically to GST-CPE-(116–319) strongly, Y147A increased the amount of bound Claudin-3. The substitutions N148D and L150A reduced the binding unbound fractions were analyzed by SDS-PAGE and Western blot.

Binding of GST-CPE to Claudin-3-transfected HEK cells. Three days after transfection, cells were incubated with 1 μg/ml GST-CPE-(116–319) (left, middle) or GST-CPE-(194–319) (right) for 1 h at 37 °C, washed with PBS, and the amount of bound GST-CPE and claudin in the lysate analyzed by SDS-PAGE and Western blot using anti-GST (green) and anti-Cld3 (red) antibodies. Left, GST-CPE-(116–319) bound to Cld3wt-transfected (Cld3) but not to Cld3-negative/4,6-diamidino-2-phenylindole, and Cld3 (green) detected via CFP-fluorescence (A and B) or anti-Cld3 antibodies (C–E). Confocal images are shown. In contrast to GST (A, red), GST-CPE-(116–319) (B, red) and GST-CPE-(194–319) (C, red) bound to cells expressing Cld3 (green) but not to Cld3-negative/4,6-diamidino-2-phenylindole positive cells (violet arrows). GST-CPE-(116–319) and GST-CPE-(194–319) bound to Cld3 on the cell surface (B and C, arrow) but not to Cld3 in the tight junction area at cell-cell contacts (B and C, arrowhead). For the detection of Cld3, high (C, left and middle) or low (C, right) detector gain was used to visualize colocalization of GST-CPE-(116–319) and Cld3 (arrow) or enrichment of Cld3 at contacts between two Cld3-expressing cells (arrowhead), respectively. Cld3-N148D/L150A (D) showed enrichment at contacts between two Cld3-expressing cells similar to Cld3wt. Binding of GST-CPE-(116–319) to Cld3-N148D/L150A-transfected cells was barely detected. E, 14 h incubation with 10 μg/ml GST-CPE-(194–319) led to internalization (arrows) of Cld3 (green) and GST-CPE-(194–319) (red) for Cld3wt but not Cld3-N148D/L150A expressing cells. Z stacks, xy (quadrat), xz (top), and yz images (side) are shown and the position within the stack indicated by lines; 4,6-diamidino-2-phenylindole is shown for the xy image only. Equal detector gains were used for A and B, C, right, and D; E, left; and E, right. Bar, 4 μm.
trans-interaction (21), resulted in increased binding of GST-CPE-(116–319). The binding of CPE-(116–319) to Cld5wt and a marked reduction of this binding by substitutions T151A and Q156E decreased whereas F147A, Y148A, Y158A, and E159Q increased the amount of GST-CPE-(116–319) bound.

**DISCUSSION**

This study identifies amino acid residues and a sequence motif in the ECL2 of claudin-3 involved in the interaction with CPE by the use of biochemical and cellular approaches. Together with molecular modeling and literature data the results allowed us to deduce determinants of the interaction. The residues (Phe146, Cld3 ECL2 residues Asn148 and Lys156) created the consensus sequence and enabled binding of GST-CPE-(116–319) (Table 2). Substitution mapping showed that a central region (mouse 148–152) and the C-terminal flank, but not the N-terminal flank, of the loop are involved in interaction of GST-CPE-(116–319) with Cld3-ECL2 peptides (Fig. 2). Negative charges, proline, and other residues at positions 154–157 block binding of GST-CPE-(116–319). But especially, the motif created the consensus sequence NPL(V/L)(P/A). Introduction of Cld3-like residues in ECL2 peptides of Cld5 (D149N/T151L), Cld2 (S149N), or Cld4 (M151L/A153P) resulted in binding of GST-CPE-(116–319). This study identifies amino acid residues and a sequence motif in the ECL2 of claudin-3 involved in the interaction with CPE by the use of biochemical and cellular approaches. Together with molecular modeling and literature data the results allowed us to deduce determinants of the interaction.

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In the recently solved x-ray structure of CPE-(194–319), region 290–319 is part of a nine-strand β sandwich that forms the claudin-binding domain (30). This explains our SPR results (Fig. 1) showing that the ECL2 of Clid3 interacts more weakly with GST-CPE-(290–319) than with GST-CPE-(116–319), because the latter includes the whole binding domain.

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**FIGURE 6.** Binding of GST-CPE-(116–319) to Cld5-transfected HEK cells. Cells were incubated with 1 μg/ml GST-CPE-(116–319) (A) or GST-CPE-(194–319) (B) at 37 °C 3 days after transfection, washed with PBS, and the amount of bound GST-CPE and claudin in the lysate analyzed by SDS-PAGE and Western blot using anti-GST and anti-GFP (A) or anti-Cld3 and anti-Cld5 antibodies (B). Representative blots are shown. A, GST-CPE-(116–319) bound to Cld3-YFP- and Cld5-YFP-transfected cells. B, GST-CPE-(194–319) bound to Cld3 but not to Cld5-transfected cells. C, binding of GST-CPE-(116–319) to claudin-5 mutants. Transfected cells were incubated with 10 μg/ml GST-CPE-(116–319) for 1 h at 37 °C. Representative Western blots show that neither GST (n.t. + GST) nor GST-CPE-(116–319) (n.t. + GST-CPE-(116–319)) bound to non-transfected cells. GST-CPE-(116–319) (Cld5wt) but not GST (Cld5wt + GST) bound to Cld5mut, YFP transfected cells. D, quantification of the binding. The ratio of GST-CPE-(116–319) intensity to Cld5-YFP intensity relative to the ratio for Cld5wt-YFP (internal standard) was determined for each mutant (bound CPE). Substitutions T151A and Q156E decreased whereas F147A, Y148A, Y158A, and E159Q increased the amount of GST-CPE-(116–319) bound. Striped columns, substitutions that block interaction between claudins. Mean ± S.E. (error bars), n > 4. ***, p < 0.001 compared with Cld5wt.
double substitution N148D/L150A, but not by the single substitutions N148D and L150A (Figs. 4 and 5). This difference to the pull-down assay indicates that, in addition to amino acid substitutions, detergent solubilization decreases the affinity/avidity of the CPE-Cld3 interaction. This could be due to dissociation of multimeric claudin complexes (21) and/or destabilization of the claudin loop structure caused by solubilization.

Similar differences were found between membranous and non-membranous Cld5: GST-CPE-(116–319) bound to Cld5-expressing cells (Fig. 6 and supplemental Fig. S2) but not to solubilized Cld5 (data not shown) nor Cld5-ECL2 peptides (Table 2). Interestingly, GST-CPE-(194–319) did not bind to Cld5-transfected HEK cells (Fig. 6). In separate experiments, similar results were obtained with Cld5-transfected L-fibroblasts ruling out the possibility that this differential binding is due to unique properties of HEK cells. Preliminary experiments indicate that GST-CPE-(116–319) and GST-CPE-(194–319) as well as membranous and solubilized claudins differ in their oligomeric state. Hence, multivalent binding (32) between CPE-oligomers (33) and claudin-oligomers (34, 21) could enhance the presumably low affinity CPE-Cld5 interaction and promote binding between GST-CPE-(116–319) and Cld5 in the membrane, only.

In other studies, CPE-Cld5 interaction could not be detected (35). However, these authors used CPE-(184–319), a short fragment similar to CPE-(194–319), which, in contrast to CPE-(116–319), shows no binding to Cld5 in our study either. Fujita et al. (12) reported no high affinity binding of full-length CPE to Cld5. This does not rule out low affinity binding. Taken together, the data obtained with claudin peptides, full-length claudins, and from the literature, are consistent with the assumption of high affinity binding of CPE to Cld3 and low affinity binding of CPE to Cld5.

Previously, we characterized a set of Cld5-ECL2 mutants that were targeted to the plasma membrane in a similar manner to Cld5mut (21). To assess the CPE-Cld5 interaction in more detail, CPE binding to HEK293 cells transfected with these mutants was analyzed. In contrast to all other analyzed Cld5mutants,

FIGURE 7. Schemes of molecular interaction between CPE and the ECL2 of claudin-3. A, topology of claudins with ECL2 marked in a box. B, alignment of ECL2 of Cld3 and -5, with similarity of residues calculated with matrix blossom62 (dot = weak; colon = strong similarity; bar = identity). Secondary structure (SecStr) according to the model, helices (H), turn (t). C, homologous helix-turn-helix model for ECL2 of Cld3 based on the fragment of PDB code 2BDV in front view. N-terminal helix, orange; C-terminal helix, cyan. The hydrophobic residues 149PLVP (green) constituting the turn-fold of the ECL2 by hydrogen bridges (hydrogens in cyan). Residues Phe146, Tyr147, and Arg157 (yellow) correspond to aromatic residues Phe147, Tyr148, and Tyr158 in Cld5, which are found to be important for trans-interaction. Heteroatoms are red (oxygen) and dark blue (nitrogen). D, according to the results, CPE cannot bind to trans-interacting claudins (left), probably due to steric hindrance, but needs a free ECL2 for binding (right). E, supposed spatial regions of interaction between binding sensitive residues (Tyr306, Tyr310, Tyr312, and Leu315) visualized at the CPE-(194–319) x-ray structure (PDB code 2QUO) and the residues (149NPLVP, Gln155) identified in this study for Cld3.
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Cld5-T151A and Cld5-Q156E show strong reduction in binding of GST-CPE-(116–319) compared with Cld5wt. Interestingly, position Thr151 in Cld5 corresponds to Leu150 in Cld3, which is strongly involved in the interaction with CPE-(116–319). In addition, substitution T151L together with D149N enabled the binding of CPE-(116–319) to Cld5-ECL2 peptides. This underlines the involvement of position 150/151 in the CPE-Cld3/5 interaction. For Cld5, Q156E reduced but Q156A increased binding of CPE-(116–319) to transfected cells. The corresponding position in Cld3 peptides (Gln155) was the most sensitive one outside the NPLVP motif (Fig. 2). For example, Q155E or Q155D inhibited, whereas Q155A increased, binding of GST-CPE-(116–319) to Cld3 peptides. This indicates involvement of position Gln155 and Gln156 in the CPE-Cld3 and CPE-Cld5 interactions, respectively.

Structural Determinants Support an Interaction of the Helix-turn-helix Conformation of the ECL2 of Cld3 with CPE—The experimental binding data were combined with molecular homology modeling. Our previously generated helix-turn-helix homology model for the ECL2 of Cld5 indicates that mutations of exactly those residues that are supposed to stabilize the turn conformation led to folding defects (11). Because all of these stabilizing residues are identical (except a Asp → Asn exchange, which still preserves the same H-bonds) in Cld3, a comparable turn conformation could also be adapted for the ECL2 of Cld3 (Fig. 7). This is consistent with results of GST-CPE-(116–319) binding on ECL2 peptide arrays of Cld3 found in this study. For exactly those residues forming the turn ([148NPLVP), almost any possible substitution abolished CPE binding (Fig. 2). Substitutions at Val151 that preserved CPE binding were mainly hydrophobic. This is consistent with the suggested turn conformation, because Val151 forms the hydrophobic core of the turn between the two helices and is itself not supposed to be involved in the CPE binding. The hydrophobic property on the surface of this turn portion of our helix-turn-helix model is formed by NPLVP.

In CPE, Tyr306, Tyr310, Tyr312, and Leu315 have been shown to be involved in the CPE-Cld4 interaction (36, 37). Similarly, we found that deletion of amino acids 310–319 diminished binding of Cld3 and Cld4 to GST-CPE (supplemental Fig. S1). On the basis of the complementary properties and shape, direct interaction of the hydrophobic ECL2-turn with a hydrophobic pit on the surface of CPE formed by Tyr306, Tyr310, Tyr312, or Leu315 (30) is very likely. This is supported by mutations Cld3-L150A, which strongly reduced the binding (Fig. 3), and additionally by the peptide array data (Fig. 2). Substitution of N148D, flanking the hydrophobic turn, strongly reduced CPE binding also (Fig. 3). This also indicates that the amino group of Cld3-N148 is involved in interaction with CPE. In addition, double substitution N148D/L150A in Cld3 diminished binding of GST-CPE-(116–319) and GST-CPE-(194–319) (Figs. 4 and 5). Consistently, Cld5 whose turn (DPTVP) contains no amino group and a smaller hydrophobic property than that of Cld3 (NPLVP) binds more weakly to CPE than does Cld3.

CPE has been shown to bind to full-length Cld4 and Cld8 (12, 14). Indeed, we found that GST-CPE-(116–319) and GST-CPE-(194–319) bind to full-length Cld4 (supplemental Fig. S1). In contrast, we did not observe an interaction between CPE and synthesized ECL2-peptides of Cld4 and Cld8. Similar results have been reported by others earlier. They found that CPE peptides do not bind to claudin-4 fragments without transmembrane segments (38). Strikingly, Cld4 and -8 do not contain the conserved second proline implicated in the stabilization of the loop structure (11). In addition, Cld4 is the only CPE-binding claudin that contains a methionine, instead of leucine (Leu150 in Cld3), in the turn region. It is possible, that due to lack of the corresponding leucine and proline, the conformation of ECL2 peptides of Cld4 and -8 on the peptide mapping membrane differs more markedly from the native structure than it does for Cld3, -6, -9, or -14. Indeed, substitutions of M150L/A153P in the Cld4 ECL2 peptide enabled binding to GST-CPE-(116–319) (Table 2). Our results with ECL2 peptides and full-length claudins, as well as other reports (38), are consistent with the idea that transmembrane segments stabilize the native conformation of the ECL2 and, thereby, increase the affinity of the CPE-claudin interaction.

CPE Binds to the Free ECL2 of Claudins Outside of Tight Junctions—Aromatic residues in CPE (Tyr306, Tyr310, Tyr312) are involved in interaction with claudins (36) and formation of an aromatic core within an ECL2-dimer is involved in the transinteraction of claudins between two opposing cells (21). Others have speculated that aromatic residues in claudin could also interact with the aromatic residues in CPE (38). But interestingly, removal of the aromatic residues (F147A, Y148A, Y158A) in Cld5 resulted in an increase in binding of GST-CPE-(116–319) to transfected cells (Fig. 6). Similarly, substitution of Y147A in Cld3, which corresponds to Cld5-Y148A and also blocks transinteraction, increased the amount of Cld3 pulled down with GST-CPE-(116–319) (Fig. 3). Moreover, in our model, the aromatic residues in the ECL2 do not sterically match the aromatic residues in CPE (Fig. 7). These data indicate that residues in the turn region of the loop, but not aromatic residues in the flanking helices, are involved in the claudin-CPE interaction.

It has to be stressed that the aforementioned substitutions (F147A, Y148A, and Y158A in Cld5; Y147A in Cld3) and E159Q in Cld5 block transinteraction and strand formation of claudins (21) as well as increase binding of GST-CPE-(116–319). Consistently, confocal microscopy showed binding of GST-CPE-(116–319) and GST-CPE-(194–319) to Cld3 outside the tight junction area. These observations demonstrate that CPE binds to the free ECL2 of claudins that is not occupied by transinteraction and not incorporated in TJ strands.

Treatment of epithelial cells with CPE increases paracellular permeability. To explain this, two mechanisms have been discussed (14). Either 1) CPE binding to TJ strands leads to direct depolymerization of the claudins, or 2) CPE sequesters claudins in the plasma membrane, preventing their incorporation in the strands during the dynamic assembly of strands. Our results strongly support the sequestration mechanism and this explains our and other findings (37) in which CPE treatment needs hours for the TJ to be opened, despite high affinity interaction with claudins (12).

In conclusion, we have shown that the molecular mechanism of the CPE-claudin-3 interaction is different from the transinteraction between claudins. This improves the understanding
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of the molecular organization of TJ. In addition, the information gained is highly relevant for the design of CPE-based claudin modulators to improve drug delivery across tissue barriers (7) or treatment of tumors overexpressing claudins (16–18).

Acknowledgments—We thank Dr. Michael Beyermann for synthesis of ECL2 peptides and Angelika Ehrlich for excellent technical assistance.

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