Enhancement of the thermostability of mouse claudin-3 on complex formation with the carboxyl-terminal region of *Clostridium perfringens* enterotoxin improves crystal quality

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Tight junctions regulate substance permeation through intercellular spaces as a physical barrier or a paracellular pathway, and play an important role in maintaining the internal environment. Claudins, which are tetraspan-transmembrane proteins, are pivotal components of tight junctions. In mammals 27 claudin subtypes have been identified, each of which interacts with specific subtypes. Although the crystal structures of several subtypes have been determined, the molecular mechanisms underlying subtype specificity remain unclear. Here, mouse claudin-3 (mCldn3) was crystallized in complex with the C-terminal region of *Clostridium perfringens* enterotoxin (C-CPE) for the structural analysis of an additional claudin subtype. mCldn3 alone was difficult to crystallize, but complex formation with C-CPE enhanced the thermostability of mCldn3 and facilitated its crystallization. The introduction of an S313A mutation into C-CPE further improved its thermostability, and the resolution limits of the diffraction data sets improved from 8 Å for the wild-type complex to 4.7 Å for the S313A mutant complex.

1. Introduction

Multicellular organisms are composed of various tissues and organs, and maintaining a specific internal environment is essential for survival. Each compartment is sealed by epithelial cell sheets, the intercellular gaps in which are almost completely occluded by a cell-adhesion apparatus called the tight junction (TJ; Farquhar & Palade, 1963). TJs regulate the paracellular pathway of substance permeation as a physical barrier or a permselective channel (Tang & Goodenough, 2003). TJs have been visualized by freeze–fracture electron microscopy as net-like architectures, called TJ strands, on cell membranes (Staehelin, 1973). Claudins (Cldns), which are tetraspan-transmembrane proteins that play a key role in the adhesion functions of TJs, are the major components of TJ strands (Furuse, Fukita *et al.*, 1998; Furuse, Sasaki *et al.*, 1998).

27 Cldn subtypes have been identified in mammals (Mineta *et al.*, 2011) and exhibit tissue-specific expression patterns (Günzel & Yu, 2013). The adhesion or permeation properties of TJs depend on the Cldn subtypes that they contain (Tamura & Tsukita, 2014). Each Cldn subtype interacts with specific subtypes, not only homophilically but also heterophilically (Daugherty *et al.*, 2007). The subtype specificity may be regulated by the primary sequences of two extracellular segments that have variable regions depending on the subtype. A few structures of Cldn subtypes have been determined, but

Table 1

Micromolecule-production information.

Protein	mCldn3	C-CPE
UniProt accession No.	Q9Z0G9	Q4ZFR5
Source organism	Mouse	<i>C. perfringens</i>
Expression vector	pFastBac1	pGEX-4T
Expression host	Sf9 insect cells	<i>E. coli</i> BL21 (DE3)
Amino-acid sequence of the construct for crystallization†	GHMASGSMGLEITGTSLAVLGWLCTIVCCALPMWRVSAFIG SSIIITAQITWEGLMNVCVQSTGQMCKMYDSSLALPQDLQ AARALIVVSILLAAFGLLVALVGAQATNAVQDETAKAKITI VAGVLFLLAALLTLVPVSWASANTIIIRDFYNPLVPEAQKREM GAGLYVGWAAAAALQLLGGALLAAS	ESAAATERLNLTDALNSNPAGNLYDWRSSNSYPWTQKLNHLHT ITATGQKYRILASKIVDFNIYSNNFNVLVKLEQSLGDGVKD HYVDISLDAGQYVLVMKANSSYSGNYPYSILFQKF

† Non-native residues from the expression vector are underlined.

the molecular mechanism of the subtype specificity of Cldn remains elusive.

To elucidate this issue, we aimed to determine the structures of additional subtypes. Among the subtypes of unknown structure, we selected mouse claudin-3 (mCldn3) as the first candidate for structural analysis because mCldn3 acts as a barrier-forming TJ protein in various organs (Günzel & Yu, 2013) and was one of the first membrane proteins to be identified as a *Clostridium perfringens* enterotoxin (CPE) receptor (Briehl & Miesfeld, 1991; Katahira *et al.*, 1997; Morita *et al.*, 1999). CPE causes food poisoning by binding to specific Cldn subtypes and disrupting the TJs (Veshnyakova *et al.*, 2010). The C-terminal region of CPE (C-CPE) is able to bind to specific Cldn subtypes without cytotoxicity (Sonoda *et al.*, 1999). The binding of C-CPE to Cldn increases the hydrophilic part of the protein complex and is thus expected to facilitate crystallization.

Here, we report the expression, purification, crystallization and X-ray analysis of mCldn3 in complex with C-CPE. By forming its complex with C-CPE, we were able to crystallize mCldn3 for the first time. Furthermore, we found a mutation in C-CPE that improved the crystal quality.

2. Materials and methods

2.1. Expression and purification of C-CPE

The C-terminal region of *C. perfringens* enterotoxin (C-CPE; residues 203–319) was subcloned into a pGEX-4T plasmid (GE Healthcare; Table 1) and fused with an N-terminal GST tag and a thrombin cleavage site. The inserted DNA was confirmed by DNA sequencing. The recombinant plasmids were transformed into *Escherichia coli* strain BL21 (DE3) cells (Novagen). The bacteria were cultured in LB medium containing 100 µg ml⁻¹ ampicillin at 37°C with shaking until the OD₆₀₀ reached 0.5, and expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside. After culture for a further 5 h at 30°C with shaking, the bacteria were harvested by centrifugation at 5000g for 15 min at 4°C and washed with Tris-buffered saline (TBS; 20 mM Tris–HCl pH 7.5, 150 mM NaCl). The cell pellet was resuspended in TBS containing 10 µg ml⁻¹ DNaseI, 0.5 µg ml⁻¹ lysozyme and protease-inhibitor cocktail (Roche), incubated for 10 min at room temperature with stirring and disrupted by

sonication on ice. The insoluble debris was removed by ultracentrifugation at 40 000g for 25 min at 4°C. The supernatant was mixed with Glutathione Sepharose 4B (GE Healthcare) equilibrated with TBS and incubated for 2 h at 4°C. The resin was loaded into an open column and washed with TBS. The GST tag was then cleaved on the resin using thrombin overnight at 4°C. The tagless C-CPE was eluted with TBS, concentrated and loaded onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with TBS. Fractions containing C-CPE were concentrated to 30 mg ml⁻¹, flash-cooled in liquid nitrogen and stored at –80°C.

2.2. Expression and purification of mouse claudin-3

The mouse claudin-3 (mCldn3) gene was subcloned into a pFastBac1 vector (Life Technologies; Table 1) and the inserted DNA was confirmed by DNA sequencing. Baculoviruses were generated according to the manufacturer's instructions. Four cysteine residues in mCldn3 (Cys103, Cys106, Cys181 and Cys182) were substituted by alanine residues, and the last 36 C-terminal residues of the intracellular loop were removed. This construct was expressed with an N-terminal *Tobacco etch virus* (TEV) protease-cleavable hexahistidine tag and with dimerization-diminished enhanced green fluorescent protein (EGFP). For expression, Sf9 insect cells were infected with the recombinant baculoviruses and cultured in SF900III medium (Life Technologies) for 48 h at 27°C. The cells were harvested by centrifugation at 2000g for 15 min and disrupted with an EmulsiFlex-C5 (Avestin) at 103 MPa in TBS containing protease-inhibitor cocktail (Roche). The cell debris was removed by centrifugation at 2000g for 20 min at 4°C. The membrane fraction was collected by ultracentrifugation at 100 000g for 1 h at 4°C, resuspended in TBS using a homogenizer and solubilized with 1% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM; Anatrace) in TBS for 1 h at 4°C. The insoluble material was removed by ultracentrifugation at 100 000g for 45 min. The supernatant was mixed with TALON Superflow cobalt-affinity resin (Clontech), which was equilibrated with TBS containing 0.1% DDM and 3 mM imidazole, and incubated for 3 h at 4°C. The resin was washed with TBS containing 0.1% DDM and 10 mM imidazole, and EGFP-mCldn3 was eluted with TBS containing 0.1% DDM and 200 mM imidazole. The buffer of the eluate was exchanged to TBS containing 0.025% DDM using a PD-10 desalting column

(GE Healthcare). The tag was cleaved with TEV protease overnight at 4°C and removed by passage through TALON Superflow cobalt-affinity resin at 4°C. The purified mCldn3 was mixed with purified C-CPE in a ratio of 1:1.2(w:w) and incubated for 2 h at 4°C. The mCldn3–C-CPE complex was concentrated and loaded onto a Superdex 200 10/300 GL size-exclusion column pre-equilibrated with 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.025% DDM. Fractions containing the mCldn3–C-CPE complex were concentrated to 5 mg ml^{−1} for crystallization.

2.3. Crystallization and X-ray diffraction data collection

The purified mCldn3–C-CPE complex was crystallized by sitting-drop vapour diffusion. Drops were set up by mixing protein solution and an equivalent volume of reservoir solution using a Mosquito LCP robot (TTP Labtech) and were equilibrated against 55 µl reservoir solution in 96-well Viologam sitting-drop plates (AS ONE) at 20°C. For cryoprotection, the crystals were transferred into solutions containing all of the components of the crystallization solution and purification solution with four stepwise increments of PEG 400 from 0 to 6%. The crystals were transferred into cryoprotectant solution containing 6% PEG 400, mounted in a cryoloop and flash-cooled in liquid nitrogen. The diffraction

experiment was performed on beamline BL41XU at SPring-8, Japan or beamline BL2S1 at Aichi Synchrotron Radiation Center, Japan. Diffraction intensities were processed with *XDS* (Kabsch, 2010). The diffraction anisotropy was analyzed using the UCLA Diffraction Anisotropy Server (Strong *et al.*, 2006).

2.4. Thermostability assay

The thermostability of the purified protein was evaluated using a fluorescence-detection size-exclusion chromatography-based thermostability assay (FSEC-TS; Hattori *et al.*, 2012). Purified protein solution was diluted to 0.02 mg ml^{−1} in FSEC running buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% DDM). For heat denaturation, the protein solution was transferred into a polymerase chain reaction tube and incubated at different temperatures (4–80°C) for 30 min using a thermal cycler. Insoluble aggregation was removed by ultracentrifugation at 100 000g for 20 min at 4°C. The supernatant was loaded onto a Superdex 200 Increase 5/150 GL size-exclusion column pre-equilibrated with FSEC running buffer. The eluent was passed through a fluorometer (Kawate & Gouaux, 2006) that detected tryptophan fluorescence (excitation wavelength 280 nm, emission wavelength 330 nm; the mCldn3–C-CPE complex includes eight tryptophan residues). The peak height at which the native conformation was retained was normalized to that of the sample incubated at 4°C, and plotted by nonlinear regression using *SigmaPlot* (Systat). The apparent melting point (T_m) was calculated by curve-fitting to a sigmoidal equation.

2.5. Binding assay

Sf9 cells expressing mCldn3 were resuspended in TBS containing 2% DDM, 1 mM EDTA and protease-inhibitor cocktail. The suspension was incubated with rotation for 30 min at 4°C. The cell debris was removed by ultracentrifugation at 100 000g for 20 min at 4°C. The supernatant containing EGFP-mCldn3 was diluted in FSEC running buffer to approximately 200 pM EGFP-mCldn3, as estimated by EGFP fluorescence. EGFP-mCldn3 was incubated with purified GST-C-CPE at different concentrations (0–5 µM) for 2 h at 4°C. The samples were analyzed using FSEC by detecting EGFP fluorescence (excitation wavelength 488 nm, emission wavelength 509 nm). Complex formation was confirmed by a peak shift from a retention time of 4.2 min (EGFP-mCldn3) to a retention time of 3.6 min (EGFP-mCldn3–GST-C-CPE). Because mCldn3 was completely saturated at a concentration of 5 µM GST-C-CPE, the fraction of bound protein in this condition was taken as the maximum value. The apparent $K_{0.5}$ value (Saitoh *et al.*, 2015), which is the concentration of GST-C-CPE at which half of the Cldn bound to C-CPE, was calculated by curve-fitting using *SigmaPlot*.

Protein	Apparent T_m (°C)	ΔT_m (°C)
mCldn3	58.6	—
mCldn3–C-CPE _{WT}	62.1	3.5
mCldn3–C-CPE _{S313A}	63.9	5.3
C-CPE _{WT}	50.2	
C-CPE _{S313A}	49.8	

(b)

Figure 1
Thermostability of mCldn3, mCldn3–C-CPE_{WT}, mCldn3–C-CPE_{S313A}, C-CPE_{WT} and C-CPE_{S313A}. (a) Melting curves. (b) Apparent T_m values. ΔT_m represents the difference from the apparent T_m value of mCldn3 alone.

3. Results and discussion

For crystallization, we designed a construct of mCldn3 with the expectation that it would stably pack into a crystal. Based

on a sequence alignment of mCldn3 with the other subtypes for which structures have been determined, mCldn15 and mCldn19 (Suzuki *et al.*, 2014; Saitoh *et al.*, 2015), 36 residues at the C-terminal region were predicted to be disordered and were removed from our construct. We also mutated the four cysteines in the membrane-proximal domain to alanines to avoid heterogeneous palmitoyl modification. Although this construct was successfully expressed in insect cells and the mCldn3 was purified, no crystals could be produced using either vapour-diffusion or the lipidic cubic phase method in sparse-matrix screening of crystallization conditions.

A thermostability assay showed that the apparent T_m of the mCldn3–C-CPE complex was approximately 4°C higher than that of mCldn3 alone (Fig. 1); hence, complex formation with C-CPE was expected to improve the crystallization of mCldn3. The purification of the mCldn3–C-CPE complex was comparable to that of mCldn3 alone. In initial screening, we obtained needle-shaped crystals under several conditions with good reproducibility using the commercial screening kit MemGold (Molecular Dimensions), *e.g.* (i) 0.1 *M* Tris–HCl pH 8.0, 0.3 *M* magnesium nitrate, 23% PEG 2000 and (ii) 0.08 *M* sodium phosphate pH 6.2, 0.02 *M* sodium citrate tribasic, 18%

Figure 2 Crystals and representative diffraction patterns. (a) Crystal of mCldn3–C-CPE_{WT} grown under conditions consisting of 0.1 *M* Tris–HCl pH 7.5, 0.1 *M* sodium chloride, 0.9 *M* magnesium nitrate, 24.2% PEG 2000 MME. (b) Crystal of mCldn3–C-CPE_{S313A} grown under conditions consisting of 0.1 *M* Tris–HCl pH 8.0, 0.05 *M* sodium chloride, 0.9 *M* magnesium nitrate, 21.0% PEG 2000 MME. The scale bars represent 0.1 mm. (c) Representative diffraction pattern of an mCldn3–C-CPE_{WT} crystal obtained using a PILATUS3 6M detector on beamline BL41XU at SPring-8. (d) Representative diffraction pattern of an mCldn3–C-CPE_{S313A} crystal obtained using a PILATUS3 6M detector on beamline BL41XU at SPring-8.

Table 2
Crystallization.

Method	Sitting drop
Plate type	96-well
Temperature (°C)	20
Protein concentration (mg ml ⁻¹)	5
Buffer composition of protein solution	10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.025% DDM
Composition of reservoir solution	0.1 M Tris-HCl pH 8.0, 0.05 M sodium chloride, 0.9 M magnesium nitrate, 21.0% PEG 2000 MME
Volume and ratio of drop	200 nl protein solution, 1:1 ratio
Volume of reservoir (μl)	55

Table 3
Data collection and processing.

Diffraction source	BL41XU, SPring-8
Wavelength (Å)	1.00
Temperature (K)	100
Detector	PILATUS3 6M
Crystal-to-detector distance (mm)	600
Rotation range per image	0.1
Total rotation range	270
Exposure time per image	0.1
Space group	<i>P</i> 1 ₂ 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	92.45, 66.63, 111.51
α , β , γ (°)	90.00, 98.22, 90.00
Resolution range (Å)	47.23–4.67 (4.83–4.67)
Observed reflections	35674 (3166)
Unique reflections	7211 (657)
Completeness (%)	97.70 (90.85)
Multiplicity	4.9 (4.8)
Mean <i>I</i> / σ (<i>I</i>)	6.43 (1.58)
<i>R</i> _{merge} (%)	9.5 (129.9)
<i>R</i> _{p.i.m.} (%)	4.6 (63.2)

PEG 2000. However, the crystals only showed diffraction to approximately 20 Å resolution.

While optimizing the crystallization conditions, we found that increasing the concentration of magnesium nitrate in the reservoir solution increased the crystal size. Crystals with maximum dimensions of 100 × 50 × 20 μm with two slits at one end (Fig. 2*a*) were obtained in 0.9 M magnesium nitrate. Crystals of mCldn3–C-CPE_{WT} were exposed to X-rays on beamline BL41XU of SPring-8 and diffracted to only 8 Å resolution (Fig. 2*c*).

We also explored mutants of C-CPE to improve the crystal quality of the mCldn3–C-CPE complex. Structural analysis of mCldn19 was previously achieved using the S313A mutant (C-CPE_{S313A}; Saitoh *et al.*, 2015). The S313A mutation does not affect the formation of the mCldn3–C-CPE complex as the binding affinity of C-CPE_{S313A} for mCldn3 was similar to that for C-CPE_{WT} under purified conditions (Fig. 3*a*). Furthermore, the thermostability of mCldn3–C-CPE_{S313A} was slightly higher than that of mCldn3–C-CPE_{WT} (Fig. 1). Large crystals of mCldn3–C-CPE_{S313A} were also obtained under similar conditions to those for mCldn3–C-CPE_{WT} containing 0.9 M magnesium nitrate (Fig. 2*b*, Table 2). The appearance of the mCldn3–C-CPE_{S313A} crystal was identical to that of mCldn3–C-CPE_{WT}, except that it was slightly thicker. The crystals were confirmed to consist of the mCldn3–C-CPE complex by SDS-PAGE (Fig. 3*b*).

Crystals of mCldn3–C-CPE_{S313A} were exposed to X-rays on beamline BL41XU at SPring-8 and diffracted well: spots were observed to 4.2 Å resolution (Fig. 2*d*, Table 3). Despite an equivalent crystal size, the crystal quality of mCldn3–C-CPE_{S313A} was drastically improved from that of mCldn3–C-CPE_{WT}. The mCldn3–C-CPE_{S313A} data set was processed in space group *P*1₂1, with unit-cell parameters *a* = 92.45, *b* = 66.63, *c* = 111.51 Å, β = 98.22°. The diffraction pattern showed strong anisotropy, however, and diffraction data were only collected to 4.7 Å resolution in the *c**-axis direction. The initial phases could not be determined from the current diffraction data by molecular replacement with the mCldn19–C-CPE complex structure (PDB entry 3x29; Saitoh *et al.*,

Figure 3
Evaluation of the purified protein solution. (a) Binding affinity of C-CPE_{WT} and C-CPE_{S313A} for mCldn3. The apparent *K*_{0.5} values of C-CPE_{WT} and C-CPE_{S313A} are 2.5 and 0.9, respectively. (b) The final purified mCldn3–C-CPE_{S313A} protein solution (lane 1) and dissolved crystals of mCldn3–C-CPE_{S313A} (lane 2) were analyzed by SDS-PAGE and silver staining. Lane *M* contains molecular-mass markers (labelled in kDa).

2015). It is thought that 4.7 Å resolution is not sufficient to determine the phases. In the previously determined structure of mCldn19–C-CPE, two molecules are contained in the asymmetric unit and the Matthews coefficient (Matthews, 1968) is 5.36 Å³ Da^{−1}. Because of the detergent micelle, the crystals of membrane proteins usually have a high solvent content (Carpenter *et al.*, 2008). When it was assumed that two complexes are contained in the asymmetric unit of the mCldn3–C-CPE complex, the Matthews coefficient was calculated to be 5.46 Å³ Da^{−1}. Because of the similarity of the mCldn3 and mCldn19 proteins, this assumption seems to be reasonable.

In this study, we determined appropriate crystallization conditions for producing large crystals of mCldn3 in complex with C-CPE. Furthermore, the S313A mutant of C-CPE improved the diffraction quality. While the S313A mutation did not affect the binding affinity for mCldn3, it enhanced the thermostability of the mCldn3–C-CPE complex. Further improvement of the crystal resolution and removal of the diffraction anisotropy will enable determination of the crystal structure of mCldn3 and reveal the binding mechanism between mCldn3 and C-CPE. In addition, because of the stabilization effect, C-CPE_{S313A} could be an effective tool for the crystallization of CPE-sensitive claudin subtypes.

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