Claudin-1 and -2: Novel Integral Membrane Proteins Localizing at Tight Junctions with No Sequence Similarity to Occludin

Mikio Furuse,* Kohji Fujita,*‡ Takashi Hiiragi,* Kazushi Fujimoto,§ and Shoichiro Tsukita*

*Department of Cell Biology, ‡Department of Neurosurgery, and §Department of Anatomy, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Abstract. Occludin is the only known integral membrane protein localizing at tight junctions (TJ), but recent targeted disruption analysis of the occludin gene indicated the existence of as yet unidentified integral membrane proteins in TJ. We therefore re-examined the isolated junction fraction from chicken liver, from which occludin was first identified. Among numerous components of this fraction, only a broad silver-stained band \(z22\) kD was detected with the occludin band through 4 M guanidine-HCl extraction as well as sonication followed by stepwise sucrose density gradient centrifugation. Two distinct peptide sequences were obtained from the lower and upper halves of the broad band, and similarity searches of databases allowed us to isolate two full-length cDNAs encoding related mouse 22-kD proteins consisting of 211 and 230 amino acids, respectively. Hydrophilicity analysis suggested that both bore four transmembrane domains, although they did not show any sequence similarity to occludin. Immunofluorescence and immunoelectron microscopy revealed that both proteins tagged with FLAG or GFP were targeted to and incorporated into the TJ strand itself. We designated them as “claudin-1” and “claudin-2”, respectively. Although the precise structure/function relationship of the claudins to TJ still remains elusive, these findings indicated that multiple integral membrane proteins with four putative transmembrane domains, occludin and claudins, constitute TJ strands.

1. Abbreviations used in this paper: GFP, green fluorescence protein; ORF, open reading frame; pAb, polyclonal antibody; TJ, tight junctions.
ponent of TJ. Overexpression of full-length occludin in cultured MDCK cells elevated their trans-epithelial resistance (TER) (McCarthy et al., 1996; Balda et al., 1996), and introduction of COOH-terminally truncated occludin into MDCK cells or Xenopus embryo cells resulted in increased paracellular leakage of small molecular mass tracers (Balda et al., 1996; Chen et al., 1997). The TER of cultured Xenopus epithelial cells was reduced by addition of a synthetic peptide corresponding to the second extracellular loop of occludin into the culture medium (Wong and Gumbiner, 1997). Furthermore, in transfected fibroblasts, occludin was reported to show some cell adhesion activity (Van Itallie and Anderson, 1997). In addition to these findings suggesting the involvement of occludin in the TJ barrier and cell adhesion, the TJ fence function was also shown to be affected, when COOH-terminally truncated occludin was introduced into MDCK cells (Balda et al., 1996).

On the other hand, some recent observations suggested that occludin is not the only integral membrane protein in TJ strands. The introduction of COOH-terminally truncated occludin into MDCK cells caused the reorganization of endogenous occludin in a dotted manner along the cell–cell border, whereas the continuous network of TJ strands was not affected (Balda et al., 1996). When a synthetic peptide corresponding to the second extracellular loop of occludin was added to the culture medium, it drove out the endogenous occludin from junctional areas of cultured epithelial cells without affecting the gross epithelial cell morphology (Wong and Gumbiner, 1997). Furthermore, endothelial cells in non-neuronal tissue and Sertoli cells in some species bore TJ but expressed only trace amounts of occludin (Hirase et al., 1997; Moroi et al., 1998).

Recently, we succeeded in targeted disruption of both alleles of the occludin gene in embryonic stem (ES) cells (Saitou et al., 1998). Surprisingly, when the occludin-deficient ES cells were differentiated into epithelial cells by embryoid body formation, well-developed TJ were found between adjacent epithelial cells. This finding conclusively indicated that occludin is not necessarily required for TJ formation itself, and that there are as yet unidentified TJ integral membrane protein(s) that can form strand structures without occludin. To understand the function and the molecular architecture of TJ in more detail, it is necessary to identify other integral membrane protein(s) of TJ strands. Identification of integral membrane proteins of TJ was originally regarded as difficult, but it is now possible to use occludin as a probe.

In this study, we examined the integral membrane proteins in the isolated junction fraction from chicken liver, which is highly enriched in TJ, and found that a broad ~22-kD band was detected with occludin through 4 M guanidine extraction as well as sonication, followed by stepwise sucrose density gradient centrifugation. Through peptide sequencing of this band and homology search, we isolated full-length cDNAs encoding mouse homologues of chicken occludin. This result indicated that occludin is not necessarily required for TJ barrier and cell adhesion, the TJ fence function was also shown to be affected, when COOH-terminally truncated occludin was introduced into MDCK cells (Balda et al., 1996).

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Isolated Junction Fraction from Chick Liver

The junction fraction was prepared from the livers of 1- to 2-d-old male chicks from the crude membrane and the bile canaliculi fractions according to the method described previously (Tsukita and Tsukita, 1989; Furuse et al., 1993). A total of 320 chicks yielded ~20 ml of sucrose solution of the junction fraction. For immunosassay of occludin, 100 µl of the isolated junction fraction was washed with 10 mM Tris-HCl solution, pH 7.5, by centrifugation, and then the pellet was suspended in 100 µl of SDS buffer (0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) by pipetting several times. After a 15-min incubation at room temperature, 100 µl of NP-40 buffer (2% NP-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) was added, followed by centrifugation at 15,000 g for 20 min. A 40-µl bed volume of protein A-Sepharose 4B (Pharmacia Biotech Sevrage, Uppsala, Sweden), which had been pre-incubated with 25 µl of rabbit anti-chicken occludin serum (F44) or control rabbit serum, was added to the supernatant and rotated for 2 h at 4°C. The beads were removed by brief centrifugation, and the supernatant was processed for SDS-PAGE.

To thoroughly extract peripheral membrane proteins from isolated junctions, 400 µl of the junction fraction was washed twice with 10 mM Tris-HCl solution, pH 7.5, by centrifugation (15,000 g, 5 min). The pellet was resuspended and incubated in 800 µl of a solution containing 4 M guanidine-HCl and 10 mM Tris-HCl, pH 7.5, on ice for 30 min, and then diluted with 700 µl of 10 mM Tris-HCl solution, pH 7.5. Extracted peripheral membrane proteins were removed by centrifugation (15,000 g, 20 min), and the pellet was used for analysis of integral membrane proteins by SDS-PAGE.

For fragmentation of isolated junctions, 1.8 ml of the junction fraction was washed twice and suspended in 600 µl of 10 mM Hepes solution, pH 7.5, and then sonicated for 10 s on ice four times using a Sonifier 250 (Branson Ultrasonics Co., Danbury, CT). The sonicated fraction was mixed with 50% (wt/wt) sucrose solution to make 38% (wt/wt) sucrose solution. This solution was then centrifuged through a discontinuous gradient consisting of 50%, 46%, 42%, 38% (containing sample), 34%, 30%, 25%, 20%, and 0% sucrose solutions (wt/wt) in an SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 40,000 rpm for 1.8 h at 4°C. Each interface was carefully collected. 0% to 20% and 42%-46% interfaces were mixed with 20%/25% and 42%-46% interfaces; these were called 0%/25% and 42%-50% interface fractions, respectively. 200-µl aliquots of each fraction were diluted with 800 µl of 10 mM Hepes solution, pH 7.5, and centrifuged in a TLA100.2 rotor (Beckman Instruments, Inc.) at 80,000 rpm for 1 h at 4°C. Pellets were processed for SDS-PAGE.

SDS-PAGE and Immunoblotting

One-dimensional SDS-PAGE (12.5%) was performed according to the method of Laemmli (1970), and gels were stained with a silver-staining kit (Wako Pure Chemicals, Osaka, Japan). For immunoblotting, proteins were electrophoretically transferred from gels onto nitrocellulose membranes, which were then incubated with the first antibody. Bound antibodies were detected with biotinylated secondary antibodies and streptavidin-conjugated alkaline phosphatase (Amersham Corp., Arlington Heights, IL). Nitroblue tetrazolium and bromochloroindolyl phosphate were used as substrates for detection of alkaline phosphatase.
Peptide Sequencing

The 4 M guanidine-insoluble fractions of isolated junctions were electrophoresed on a 12.5% polyacrylamide gel. The lower and upper halves of the broad 22-kD band (see Fig. 1, band 9) was then subjected to direct peptide sequencing as follows. For amino acid sequencing of the lower half, proteins separated by SDS-PAGE were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The PVDF membrane was stained with Coomassie brilliant blue R-250 (CBB), and the lower half of the transferred 22-kD band was excised and subjected to NH₄OH massie brilliant blue R-250 (CBB), and the lower half of the transferred 22-kD band was excised and subjected to NH₄OH.

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cDNA Cloning and Sequencing

Mouse liver total RNA was isolated according to the method described by Chomczynski and Sacchi (1987). Poly(A) + was prepared from the total RNA using oligo d(1-cellulose (New England Biolabs, Inc., Beverly, MA). First strand cDNA was prepared from this poly(A)+ RNA with SuperScript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and used for PCR. A 101 mouse liver cDNA library was constructed using a Time Saver TM cDNA Synthesis Kit (Pharmacia Biotech Sverage) and used for cDNA screening.

The cDNA encoding claudin-1 was isolated as follows (see Fig. 3). Similarity search using the GenBank/EMBL/DDJB databases with the MPscrch TPN program identified a human EST clone (these data are available under accession number AA305424) encoding a polypeptide showing significant similarity to peptide sequence-1 (see Fig. 3 a). Mouse liver first strand cDNA was thus subjected to PCR with two primers, 5′-ATGGCCGAACCCGGGGCTGCAGCTGTTGGG-3′ and 5′-CAGGATCAGCAAGGTCAAGGACGACGATGACAAGTAGATCT-3′, and 5′-AGATTCAGCAAGGTCAAGGACGACGATGACAAGTAGATCT-3′, and 5′-AGGAGTCAAGACTTT-3′. Mouse liver first strand cDNA was thus subjected to PCR with two primers, 5′-ATGGCCGAACCCGGGGCTGCAGCTGTTGGG-3′ and 5′-CAGGATCAGCAAGGTCAAGGACGACGATGACAAGTAGATCT-3′, and 5′-AGATTCAGCAAGGTCAAGGACGACGATGACAAGTAGATCT-3′, and 5′-AGGAGTCAAGACTTT-3′. One positive cDNA clone (IN01) with a 0.9-kb insert was obtained, and from this cDNA library the remaining 5′-noncoding region and 5′-noncoding region, this insert was interpreted to contain the 3′-end of the open reading frame (ORF) and 5′-noncoding region, but not the 5′-end of the ORF. Repeated searches of updated databases identified a mouse EST clone (these data are available from GenBank/EMBL/DDJB under accession number AA116709) encoding a polypeptide showing significant similarity to peptide sequence-1, indicating that this EST clone contained the 5′-noncoding region and 5′-end of ORF. Two primers, 5′-AGGAGTCAAGACTTT-3′ and 5′-AGGAGTCAAGACTTT-3′, were designed from the EST clone and IN01, respectively, and using them cDNA encoding the whole ORF was amplified by PCR from the mouse liver first strand cDNA. The amplified cDNA was subcloned into the pGEM-T Easy Vector (Promega), and the resulting construct was designated as pGTCL-1. To ensure no sequence errors occurred during PCR, the nucleic acid sequence was determined from three independently amplified fragments.

The cDNA encoding claudin-2 was isolated as follows (see Fig. 3). Similarity search using the GenBank/EMBL/DDJB database with the MPscrch TPN program identified a human EST clone (these data are available under accession number AA116709) encoding a polypeptide showing significant similarity to peptide sequence-2 (see Fig. 3 a). This clone appeared to contain the 3′-end of the ORF and 5′-end of ORF. Two primers, 5′-AGGAGTCAAGACTTT-3′ and 5′-AGGAGTCAAGACTTT-3′, were designed from the EST clone and IN01, respectively, and using them cDNA encoding the whole ORF was amplified by PCR from the mouse liver first strand cDNA. The amplified cDNA was subcloned into the pGEM-T Easy Vector (Promega), and the resulting construct was designated as pGTCL-2. To ensure no sequence errors occurred during PCR, the nucleic acid sequence was determined from three independently amplified fragments. DNA sequencing was performed using a Dye Terminator Cycle Sequence Kit (Applied Biosystems, Inc., Foster City, CA). For further plasmid construction, the insert was excised from pGTCL-2 by EcorI digestion, and then subcloned into pBlueScript SK(−) to make pSKCL-2.

Mammalian Expression Vectors and Transfection

Claudin-1 and -2 were tagged with FLAG-peptide or GFP at their COOH termini. To construct a FLAG-tagged claudin-1 expression vector (pCCL-1F), BamHI site was introduced at the stop codon of claudin-1 cDNA by PCR with primers 5′-GGGCAGCTATTGATGCCACACATGTG-3′ and 5′-CCGGGATCCTGCCATATGCTTCCTCCACT-3′. The EcoRI-BamHI fragment of this PCR product and SalI-EcoRI fragment excised from pGTCL-2 were simultaneously ligated into SalI-BamHI-digested pBlueScript SK(−) to construct pSKCL-1CB. An adapter DNA encoding a FLAG peptide was produced by annealing two oligonucleotides, 5′-GATCCGACTACAAGGAGCAAAGGTAGAAGATCT-3′ and 5′-AGAAGTCAGACCTGCTGCTGCTGCTGCTGCTTGAAGGCG-3′, followed by phosphorylation with T4 polynucleotide kinase. The plasmid pSKCL-1F was then subjected to direct peptide sequencing as follows. For amino acid sequencing of the lower half, proteins separated by SDS-PAGE were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The PVDF membrane was stained with Coomassie brilliant blue R-250 (CBB), and the lower half of the transferred 22-kD band was excised and subjected to NH₄OH.

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Figure 1. Identification of the occludin band and nine guanidine-insoluble bands in the isolated junction fraction from chick liver. (a) Immunoadsorption of occludin from the isolated junction fraction. The isolated junction fraction was solubilized with 0.1% SDS. After dilution, immunoprecipitation was carried out with rabbit anti-chicken occludin pAb (F44) or control rabbit serum, and then the supernatant was processed for SDS-PAGE. In silver-stained gels, a 60-kD band (arrow) that was detected in the non-treated (Junction Fr.) as well as control serum-treated (+control IgG Fr.) fraction, disappeared in the F44-treated fraction (+anti-Oc pAb). Thus, occludin was identified as a 60-kD band in the junction fraction by silver staining. Accompanying immunoblots with anti-occludin pAb (F44) confirmed this notion. (b) Guanidine extraction of isolated junction fraction. To thoroughly extract peripheral membrane proteins from isolated junctions, the fraction was treated with 4 M guanidine-HCl. The silver-stained banding pattern was compared between non-treated (Junction Fr.) and guanidine-treated (4 M guanidine-HCl) samples. Occludin in the non-treated fraction (arrow) was concentrated by guanidine treatment (occludin). In addition to the occludin band, nine guanidine-insoluble bands were identified (bands 1–9), the amounts of which were similar to or greater than that of occludin. Bars indicate molecular masses of 200, 116, 97, 66, 45, 31, and 21 kD, respectively, from the top.
containing an insert encoding FLAG-tagged claudin-1 was produced by ligating BamHI-digested FLAG adapter and BamHI–EcoRV-digested pSKCL-1CB. The insert was excised from pSKCL-1F by SacI–KpnI digestion followed by blunting with T4 polymerase, and then introduced into pCAGGSneodelEcoRI (Niwa et al., 1991), provided by Dr. J. Myazaki (Osaka University). The final expression vector for FLAG-tagged claudin-1 was designated as pCCL-1F. FLAG-tagged claudin-2 expression vector (pCCL-2F) was constructed in basically the same manner. A BamHI site was introduced at the stop codon of claudin-2 cDNA by PCR with primers 5'-ACGACAAGCAACAGGCTCCGAAG-3' and 5'-GGGGGATCCACATACCCAGTCAGG-3'. The SacI–XhoI fragment of pSKCL-2 and XhoI–BamHI fragment of the PCR fragment were ligated and subcloned into SacI–BamHI-digested pSKCL-2F to be tagged with FLAG peptide. The FLAG-tagged claudin-2 cDNA was subcloned into pCAGGSneodelEcoRI to make the expression vector pCCL-2F.

To construct the GFP-tagged claudin-1 expression vector pBCL-1G, GFP cDNA was excised from pQBI25 (Quantum Biotechnologies, Inc., Quebec, Canada) and an XbaI site and GGGGGGGGGGGGG (encoding four glysin residues) were introduced upstream of GFP in pBluescript (pGGLG4). The XbaI site was introduced at the stop codon of claudin-1 cDNA by PCR, and then ligated into the XbaI site of pGGLG4. The claudin-1–GFP fusion protein cDNA was introduced in the expression vector pBATEM2, from which E-cadherin cDNA was excised (Nose et al., 1988), to make the expression vector pBCL-1G.

MDCK II cells were used for transfection. To improve the transfection efficiency, cells on 35-mm dishes were cultured overnight in DME containing 50 μM Ca²⁺ (LCM) supplemented with 5% Ca²⁺-depleted FCS, and then washed twice with serum-free LCM. 1-μg aliquots of pCCL-1F, pCCL-2F, or pBCL-1G with 0.1 μg of pSTneoB were introduced into such pretreated cells in 1 ml LCM medium using Lipofectamine Plus (GIBCO BRL). After a 5-h incubation, 2 ml of DME containing 10% FCS was added. Cells were then cultured for 1 d, replated, and then cultured on two 9-cm dishes in DME containing 10% FCS and 300 μg of Geneticine (GIBCO BRL) to select stable transfectants. Geneticine-resistant colonies were isolated after 10–14-d selection. The clones expressing tagged proteins were screened by fluorescence microscopy with anti-FLAG antibody or GFP fluorescence.

Immunofluorescence Microscopy

Stable transfectants plated on glass coverslips were fixed with 1% formaldehyde in PBS for 10 min at room temperature. The fixed cells were treated with 0.2% Triton X-100 in PBS for 10 min and washed three times with PBS. After soaking in PBS containing 1% BSA, the samples were treated with primary antibodies for 1 h in a moist chamber. They were then washed three times with PBS, followed by a 30-min incubation with secondary antibodies. FITC-conjugated goat anti–rat IgG antibody (Bio-Source), rhodamine-conjugated goat anti–mouse IgG antibody (Chemicon, Temecula, CA), or Cy3-conjugated goat anti–rat IgG antibody (Amersham Pharmacia Biotech, Bucks, UK) were used as secondary antibodies.
antibodies. Cells were washed three times and then mounted in 90% glycerol-PBS containing 0.1% para-phenylenediamine and 1% n-propylgalate. Specimens were observed using a fluorescence microscope, Axioshot photomicroscope (Carl Zeiss, Inc., Thornwood, NY), or an MRC 1024 confocal fluorescence microscope (Bio-Rad Laboratories) equipped with a Zeiss Axioshot photomicroscope.

Electron Microscopy

Isolated junction fraction or sonicated samples were fixed overnight with a fixative containing 2.5% glutaraldehyde, 2% formaldehyde, 0.1% tannic acid, and 1% cacodylate buffer, pH 7.2. After washing with 0.1 M cacodylate buffer, pH 7.2, samples were then postfixed with 1% OsO4 in 0.1 M cacodylate buffer, pH 7.2, for 2 h, followed by en bloc staining with 1% uranyl acetate for 1 h. Samples were dehydrated and then embedded in Epon 812. Ultrathin sections were cut with a diamond knife, and stained doubly with uranyl acetate and lead citrate. Samples were examined with a JEOL 1200EX electron microscope at an accelerating voltage of 80 kV.

Immunoelectron microscopy to examine freeze-fracture replicas was performed as described (Fujimoto, 1995). Cultured transfectant cells were scraped with a rubber policeman followed by centrifugation, and the pellet was quickly frozen by being slammed against a pure copper block cooled by liquid helium gas (Heuser et al., 1979). The frozen samples were fractured at −10°C and platinum-shadowed unidirectionally at an angle of 45° in Balzers Freeze Etching System (BAF 400T; Balzers Corp., Hudson, NH). The samples were immersed in a sample lysis buffer containing 2.5% SDS, 10 mM Tris-HCl, and 0.6 M sucrose, pH 8.2, for 12 h at room temperature, and then replicas floating off the samples were washed with PBS. Under these conditions, integral membrane proteins were captured by replicas, and their cytoplasmic domain was accessible to antibodies. The replicas were incubated with the anti-FLAG mAb or anti-GFP pAb for 60 min, and then washed with PBS several times. They were then incubated with goat anti-mouse or anti-rabbit IgG coupled to 10-nm gold for 60 min, and then washed with PBS three times and then mounted in 90% glyceral-PBS containing 0.1% para-phenylenediamine and 1% n-propylgalate. The expression of claudin-1 and -2 in various mouse tissues was examined by Northern blotting using Mouse Multiple Tissue Northern Blot (Clontech). DNA fragments of claudin-1 (IN01) and claudin-2 (227–669) were radioiodinated with [32P]dCTP and used as probes for Northern blotting. Hybridization was performed in ExpressHybTM Hybridization Solution (Clontech) at 68°C for 12 h. The membranes were washed with 2× SSC containing 0.1% SDS at room temperature for 30 min, and then with 0.1× SSC containing 0.1% SDS at 50°C for 30 min. The membranes were exposed to imaging plates for 12 h, and the signals were visualized using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Results

Identification of Occludin and ~22-kD Band in SDS-PAGE of Isolated Junction Fractions from Chicken Liver

Occludin was first identified by mAb production using the isolated junction fraction from chicken liver as antigens (Furuse et al., 1993). However, it has not been determined whether occludin is present in the fraction in amount sufficient to be detected by silver staining. The isolated junction fraction was then solubilized with a solution containing 0.1% SDS, and occludin was immunabsorbed by anti-chicken occludin pAb (F44). When compared with the silver-stained banding patterns of non-treated and control IgG-treated isolated junction fractions, that of F44-treated junction fraction specifically lacked a band of ~60 kD (Fig. 1a). We thus concluded that occludin can be detected as a single 60-kD band in silver-stained gels of

**Figure 3.** Cloning and sequencing of cDNAs encoding claudin-1 and -2. (a) Peptide sequencing. The lower and upper halves of band 9 (see Fig. 1b) were separately subjected to the direct peptide sequencing, and yielded peptide sequence-1 and -2, respectively. Database searching identified human and mouse EST clones (these sequence data are available from GenBank/EMBL/DDJB under accession numbers AA305424 and AA116709, respectively), which encoded sequences showing significant similarity to peptide sequence-1 and -2, respectively. Identity and homology are indicated by asterisks and dots, respectively. (b and c) Nucleotide and deduced amino acid sequences of mouse claudin-1 and -2. Based on the above peptide sequences, two distinct cDNAs were isolated from a mouse liver cDNA library (for details, see Materials and Methods). Products encoded by these cDNAs were designated as claudin-1 and -2. Sequences homologous to peptide sequence-1 and -2 (a) are underlined. Since peptide sequence-1 was determined as the NH2-terminal sequence (for details, see Materials and Methods), the first ATG codon was conclusively determined in claudin-1 cDNA. Sequence similarity between claudin-1 and -2 (see Fig. 4a) then enabled us to estimate the first ATG codon in claudin-2 cDNA. Claudin-1 and -2 cDNAs encoded 211- and 230-amino acid polypeptides with predicted molecular masses of 22.9 kD and 24.5 kD, respectively.
the isolated junction fraction. This conclusion was confirmed by accompanying immunoblotting analysis with anti–chicken occludin pAb (Fig. 1a), although occludin was detected as multiple bands in immunoblots as reported previously (Sakakibara et al., 1997).

Occludin knockout analyses suggested the existence of as yet unidentified TJ integral membrane protein(s) (Saitou et al., 1998). As occludin-deficient epithelial cells still bore well-developed TJ strands, we expected that the content of the as yet unidentified TJ integral membrane protein(s) in native TJ strands would not be less than that of occludin. If this was the case, the as yet unidentified TJ integral membrane protein(s) should also be detectable as a silver-stained band or bands in the isolated junction fraction. We attempted to identify a band or bands in the isolated junction fraction, which could be co-isolated with the 60-kD occludin band during the following treatments.

First, to thoroughly remove peripheral proteins and leave only integral membrane proteins, we treated the isolated junction fraction with 4 M guanidine-HCl, pH 7.5. This treatment has been used to remove all peripheral membrane proteins from isolated desmosomes (Blaschuk et al., 1986). As expected, the 60-kD occludin band resisted guanidine extraction (Fig. 1b). In addition to the occludin band, nine other bands were detected in the guanidine-insoluble fraction in amounts similar to or greater than occludin. These bands were tentatively named bands 1–9 from the top of the gel.

We next sonicated the isolated junction fraction, and fragmented structures were recovered as a pellet by centrifugation. Electron microscopy revealed that in the pellet actin filaments became undetectable and that plasma membranes were fragmented into small vesicles, some of which appeared to contain fragmented TJ (Fig. 2a and b). The sonicated fraction was then fractionated by centrifugation in a stepwise discontinuous sucrose density gradient (Fig. 2c). Each interface was resolved by SDS-PAGE followed by silver staining, and the distribution of the occludin band was compared with those of the other guanidine-insoluble bands (bands 1–9). Occludin was abundantly recovered at the 25:30%, 30:34%, and 34:38% interfaces, but in only trace amounts at 0:25%, 38:42%, and 42:50% interfaces, which was confirmed by immunoblotting with anti-occludin mAb. Among bands 1–9, only band 9 was recovered with the same distribution pattern as occludin. In contrast, the other guanidine-insoluble bands 1–8 behaved differently from occludin; bands 1–6 and 8, and band 7 were absent at the 0:25% interface and 38:42%/42:50% interfaces, respectively.

We concluded that among the numerous bands in isolated junction fractions, only band 9 behaved similarly to occludin, not only after guanidine treatment, but also sonication followed by sucrose density gradient centrifugation. We speculated that the 22-kD polypeptide in the band 9 is a good candidate as an integral membrane protein comprising TJ strands together with occludin.

**Cloning of Full-Length cDNAs Encoding Mouse Claudin-1 and -2**

The broad band 9, ~22 kD in the guanidine-insoluble fraction, was tentatively divided into lower and upper halves, which were then subjected to direct peptide sequencing. As shown in Fig. 3a, one peptide sequence was obtained from each half of the band (peptide sequence 1 and 2, respectively). A similarity search identified two EST clones from each half of the band (peptide sequence 1 and 2, respectively). They showed 38% identity at the amino acid sequence level. (b) Hydrophilicity plots for claudin-1 and -2 prepared using the Kyte and Doolittle algorithm. The plot records the average hydrophilicity along the sequence over a window of eight residues. Hydrophilic and hydrophobic regions are in the lower and upper parts of the frames, respectively. The axes are numbered in amino acid residues. Both claudin-1 and -2 appeared to contain four major hydrophobic, potentially membrane-spanning regions (arrows), although the possibility was not excluded that five transmembrane domains were included in these molecules only from these plots as discussed in the text.
but not to occludin. We called these proteins claudin-1 and -2, respectively (the reason for this nomenclature will be explained in the Discussion session). Furthermore, hydrophilicity analysis predicted that, like occludin, both claudin-1 and -2 contained four transmembrane domains (Fig. 4 b). If this prediction is the case, their putative first extracellular loop appears to be rather hydrophobic, especially in claudin-2. Occludin was reported to bear glycine/tyrosine-enriched extracellular loops, but neither claudin-1 nor -2 showed such characteristics.

Expression and Distribution of Mouse Claudin-1 and -2

To examine the expression and distribution patterns of claudin-1 and -2, we attempted to produce specific mAbs or pAbs. As antigens, we used GST fusion proteins with the COOH-terminal cytoplasmic domains of claudin-1 and -2 (24 and 42 amino acids, respectively) produced in *Escherichia coli*, or synthetic peptides corresponding to their cytoplasmic domains conjugated to hemocyanin. However, it proved very difficult to obtain specific mAbs or pAbs.

We then introduced FLAG- or GFP-tagged claudin-1 and -2 into cultured MDCK cells, and MDCK transfectants stably expressing FLAG–claudin-1, FLAG–claudin-2, and GFP–claudin-1 with expected molecular masses were obtained. The subcellular distributions of these tagged proteins were then examined by confocal microscopy in comparison with those of occludin and E-cadherin. As shown in Fig. 5, both introduced FLAG–claudin-1 and FLAG–claudin-2 were precisely colocalized with occludin at the level of TJ. Computer-generated cross-sectional images revealed that FLAG–claudin-1 and FLAG–claudin-2 were precisely colocalized with occludin at the most apical part of the lateral membranes of confluent MDCK transfectants, whereas E-cadherin appeared to be localized more basally than the distribution patterns of claudin-1 or claudin-2 (Fig. 6). The same results were also obtained from GFP-tagged claudin-1–expressing MDCK cells, excluding the possibility that tag peptides affected the subcellular distribution of introduced proteins. These findings indicated that both claudin-1 and -2 were concentrated at TJ.

To examine whether these proteins were localized on the TJ strands themselves, MDCK transfectants expressing FLAG–claudin-1 (Fig. 7, a and b), GFP–claudin-1 (Fig. 7 c) and FLAG–claudin-2 (Fig. 8) were analyzed by the immuno-freeze fracture replica method using anti-FLAG mAb or anti-GFP pAb. In all cases, TJ regions were specifically labeled. Taking the intrinsic resolution of immunogold labeling (20–25 nm) into consideration, most of the immunogold particles were regarded to directly label the TJ strands themselves, indicating that both claudin-1 and -2 were directly incorporated into TJ strands.

Finally, we examined the expression of claudin-1 and -2 in various tissues by Northern blotting (Fig. 9). Claudin-1 mRNAs were detected as major 4.0-kb and minor 1.3-kb bands in all the tissues examined, and were seen in espe-

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Figure 5. Colocalization of FLAG-tagged claudin-1 and -2 with occludin in MDCK transfectants. Confluent cultures of MDCK transfectants expressing FLAG–claudin-1 or FLAG–claudin-2 (FLAG-tagged claudin-1 or FLAG-tagged claudin-2) were doubly stained with mouse anti-FLAG mAb (anti-FLAG) and rat anti-occludin mAb MOC37 (anti-occludin). Images were obtained at the focal plane of the most apical region of lateral membranes by confocal microscopy. Both FLAG–claudin-1 and FLAG–claudin-2 were precisely colocalized with occludin at tight junction regions. Bar, 10 μm.
cially large amounts in liver and kidney. In contrast, the expression of claudin-2 (major 4.0-kb and minor 2.0-kb mRNAs) was restricted to the liver and kidney (with small amounts in the brain).

Discussion

Occludin was the only integral membrane protein known to be localized at TJ strands (Furuse et al., 1993; Ando-Akatsuka et al., 1996; Saitou et al., 1997). However, occludin-deficient epithelial cells, differentiated from occludin gene double knockout ES cells, bore well-developed TJ (Saitou et al., 1998). To interpret this unexpected finding, we searched for as yet unidentified TJ integral membrane proteins. Initially, we expected the occurrence of isotypes of occludin, and performed RT-PCR using various combinations of primers and similarity searches of databases. However, this approach failed to find sequences that showed similarity to occludin. To identify the occludin-binding integral membrane proteins, yeast two-hybrid analysis as well as immunoprecipitation with anti-occludin antibodies were carried out, but again no good candidates were identified.

We then re-examined the isolated junction fraction from chick liver, from which occludin was first identified (Tsukita and Tsukita, 1989; Furuse et al., 1993). Among the numerous bands observed on SDS-PAGE of this fraction, we screened for bands that were co-partitioned with the occludin band through guanidine extraction and sonication; we identified a broad 22-kD band as a candidate. The broadness of this band was partially explained by the occurrence of multiple (at least two) similar but distinct ~22-kD polypeptides. Two peptides were obtained from the chicken 22-kD band, and cDNAs encoding their mouse homologues were cloned. These two proteins were structurally related (38% identical at the amino acid sequence level), but showed no sequence similarity to occludin. We did not succeed in obtaining mAbs or pAbs specific for these proteins, probably because of the small size and/or low antigenicity of extramembrane portions of these molecules. Instead, we introduced cDNAs encoding FLAG- or
GFP-tagged proteins into MDCK cells, and found that they exclusively targeted to TJ strands at both light and electron microscopic levels. We then designated these newly identified, related 22-kD proteins as “claudin-1” and “claudin-2” from the Latin word “claudere” (to close). This nomenclature is similar to that of TJ-associated MAGUK family members (Woods and Bryant, 1993; Kim, 1995; Anderson et al., 1995) such as ZO-1, ZO-2 and ZO-3 (Haskins et al., 1998).

No sequences similar to occludin were found in databases except for the occludin sequence itself. In contrast, several sequences similar to claudin-1 and -2 have been previously reported as ~22-kD four transmembrane domain proteins (Briehl and Miesfeld, 1991; Katahira et al., 1997) and also found in databases, although their physiological functions have not been elucidated. These findings point to the existence of a new multiple gene family which could be called the “claudin family” (Morita, K., M. Furuse, and Sh. Tsukita, manuscript in preparation). As reported previously, the levels of occludin expression in various types of cells are generally correlated well with the number of TJ strands (Furuse et al., 1993; Saitou et al., 1997). In contrast, as shown on Northern blots (see Fig. 9), no such correlations were found for the expression of claudin-1 or -2. For example, in the lung which shows well-developed TJ, occludin was abundant, whereas the levels of expression of claudin-1 and -2 were rather low. As occludin-deficient mice were born with normal TJ in various tissues (Saitou, M., K. Fujimoto, Y. Doi, M. Itoh, T. Fujimoto, M. Furuse, H. Takano, Sh. Tsukita, and T. Noda, manuscript in preparation), it is likely that members of the claudin family other than claudin-1 and -2 are also involved in the formation of TJ strands in various tissues.

Hydropilicity analysis predicted that both claudin-1 and -2 bore four transmembrane domains (see Fig. 4 b). Since the putative first extracellular loop is rather hydrophobic in the four transmembrane domain folding model of claudins, the possibility cannot be excluded that these molecules contain five transmembrane domains. However, considering that claudin-1 and -2 show sequence similarity to several proteins that were reported to have four transmembrane domains as discussed above (Briehl and Miesfeld, 1991; Katahira et al., 1997), and that other newly identified members of claudin family were also predicted to bear four transmembrane domains (Morita, K., M. Furuse, and Sh. Tsukita, manuscript in preparation), it appears to be reasonable to conclude that claudin-1 and -2 belong to the so called “four transmembrane domain proteins.” If this interpretation is the case, the hydrophobic character of the first extracellular loop of claudin-1 and -2 may be implicated in the barrier function of tight junctions.

In chicken liver, at least three proteins, occludin, claudin-1, and claudin-2, comprise TJ strands. Since among the guanidine-insoluble bands in isolated junction fraction, only the band ~22 kD behaved similarly to the occludin band, integral membrane proteins other than claudin family members or occludin do not appear to be major components of TJ strands. As epithelial cells derived from occludin-deficient ES cells still bore well-developed TJ strands (Saitou et al., 1998), we speculated that under specialized conditions the TJ strand itself can be formed solely from claudin family members without occludin. On the other hand, overexpression of occludin in insect Sf9 cells (Furuse et al., 1996) suggested that occludin alone can form TJ strand-like structures, although these were very short. These observations are similar to the polymerization of in-
termediate-sized filaments (for review see Fuchs and Weber, 1994; Heins and Aebi, 1994). The constituents of the different types of intermediate-sized filaments differ in amino acid sequence and molecular mass, but they all contain a structurally homologous central rod domain that forms an extended coiled-coil structure when hetero- or homodimers are formed, which allows them to be polymerized into filamentous structures collectively categorized as an intermediate-sized filament. Similarly, the integral membrane proteins with four transmembrane domains such as claudin family members and occludin may be polymerized into TJ strands as both hetero- and homo-polymers, although the efficiency of polymerization may differ depending on their combination. At present, it is still premature to discuss in what manner these integral membrane proteins with four transmembrane domains are integrated into TJ strands. Any model, however, must explain the thickness of TJ strands (∼10 nm), which is similar to the diameter of the gap junction channel (connexon) consisting of six connexin molecules that also bear four transmembrane domains (for reviews see Staehelin, 1973, 1974; Jiang and Goodenough, 1996; Kumar and Gilula, 1996).

ZO-1 has been thought to be associated with TJ strands through its direct interaction with occludin (Furuse et al., 1994). However, in epithelial cells differentiated from occludin-deficient ES cells, ZO-1 was still exclusively localized at TJ (Saitou et al., 1998). As ZO-1 is a multidomain protein containing one SH3, one GUK, and three PDZ domains (Itoh et al., 1993; Tsukita et al., 1993; Willott et al., 1993), it is possible that ZO-1 is associated not only with occludin but also with claudin family members directly or indirectly. Further analyses of the interactions of the cytoplasmic domains of claudin family members with TJ-associated peripheral membrane proteins are required to determine the molecular architecture of TJ in detail.

This study revealed that the molecular architecture of TJ strands is more complex than expected. We concluded that multiple integral membrane proteins with four transmembrane domains, occludin, and claudins, constitute TJ strands. Of course, the most significant limitation of this

Figure 8. Immunolabeling of freeze-fracture replicas of MDCK transfectants expressing FLAG-tagged claudin-2 with anti-FLAG mAb. TJ regions, but not other membrane domains such as desmosomes (asterisks in b), were specifically labeled with immunogold particles. Taking the intrinsic resolution of immunogold labeling (20–25 nm) into consideration, most of the immunogold particles appeared to directly label the TJ strands themselves, indicating that claudin-2 is incorporated into TJ strands. Bars, 0.1 μm.
study is that it has not been definitely proven that Claudins (alone or in combination with occludin) actually form TJ strands, but we recently found that the introduction of Claudin-1 and Claudin-2 cDNA fragments. Claudin-1 mRNAs were detected as major 4.0 kb and minor 1.3 kb bands in all the tissues examined, and were seen in especially large amounts in the liver and kidney. Claudin-2 expression (major 4.0 kb and minor 2.0 kb mRNAs) was restricted to the liver and kidney (with small amounts in the brain).

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