

Occludin: A Novel Integral Membrane Protein Localizing at Tight Junctions

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Abstract. Recently, we found that ZO-1, a tight junction-associated protein, was concentrated in the so called isolated adherens junction fraction from the liver (Itoh, M., A. Nagafuchi, S. Yonemura, T. Kitani-Yasuda, Sa. Tsukita, and Sh. Tsukita. 1993. *J. Cell Biol.* 121:491–502). Using this fraction derived from chick liver as an antigen, we obtained three monoclonal antibodies specific for a ~65-kD protein in rats. This antigen was not extractable from plasma membranes without detergent, suggesting that it is an integral membrane protein. Immunofluorescence and immunoelectron microscopy with these mAbs showed that this ~65-kD membrane protein was exclusively localized at tight junctions of both epithelial and endothelial cells: at the electron microscopic level, the

labels were detected directly over the points of membrane contact in tight junctions. To further clarify the nature and structure of this membrane protein, we cloned and sequenced its cDNA. We found that the cDNA encoded a 504-amino acid polypeptide with 55.9 kD. A search of the data base identified no proteins with significant homology to this membrane protein. A most striking feature of its primary structure was revealed by a hydrophilicity plot: four putative membrane-spanning segments were included in the NH₂-terminal half. This hydrophilicity plot was very similar to that of connexin, an integral membrane protein in gap junctions. These findings revealed that an integral membrane protein localizing at tight junctions is now identified, which we designated as "occludin."

IN mammalian cells, intercellular junctions are categorized into four types: adherens junctions (AJ)¹, desmosomes (DS), gap junctions (GJ), and tight junctions (TJ). These junctions were originally identified and defined by electron microscopy (Farquhar and Palade, 1963; Stevenson and Paul, 1989), and their molecular organization and functions have attracted increasing interest among cell biologists studying cell-cell interactions and communication in multi-cellular organisms.

It is now apparent that specific types of integral membrane proteins are concentrated in AJ, DS, and GJ. The major integral membrane protein in AJ is cadherin which is responsible for calcium-dependent cell-cell adhesion (Takeichi, 1991). Cadherins are a family of glycoproteins that span once the plasma membrane. This family includes E-cadherin/uvomorulin (Nagafuchi et al., 1987; Ringwald et al.,

1987), N-cadherin/A-CAM (Hatta et al., 1988), P-cadherin (Nose et al., 1987), L-CAM (Gallin et al., 1987), and several other cadherins that have recently been identified (Suzuki et al., 1991; Takeichi, 1991). The desmosomal integral membrane proteins are called desmogleins and desmocollins (Buxton et al., 1993). Recent cDNA cloning has revealed that these molecules are similar in amino acid sequence to cadherins, and that they fall into the cadherin superfamily (Holton et al., 1990; Koch et al., 1990). Desmogleins and desmocollins show cell type-specific variations (isoforms) like cadherins.

The integral membrane protein in GJ is also well characterized (Loewenstein, 1987; Musil and Goodenough, 1990). This type of junction is a dense aggregation of multimeric channels, each of which consists of six identical proteins named connexins. A family of related connexin proteins has been reported (Willecke et al., 1991). It is now widely accepted that the connexin molecule consists of four transmembrane segments with both NH₂ and COOH termini exposed at the cytoplasmic surface of the membrane.

Despite intensive studies, there remains no information about the integral membrane proteins localizing at TJ (Gumbiner, 1987; Schneeberger and Lynch, 1992; Citi, 1993). TJ

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1. *Abbreviations used in this paper:* Ac-membrane, acid-extracted membrane fraction; AJ, adherens junctions; DS, desmosomes; GJ, gap junctions; ORF, open reading frame; PLP, proteolipid protein; TJ, tight junctions.

is an element of the epithelial and endothelial junctional complex. It seals cells to create the primary barrier to the diffusion of solutes through the paracellular pathway. It also works as a boundary between the apical and basolateral plasma membrane domains to create the polarization of epithelial and endothelial cells. In thin section electron microscopy, TJ appears as a series of discrete sites of apparent fusion, involving the outer leaflet of the plasma membrane of adjacent cells (Farquhar and Palade, 1963). In freeze-fracture electron microscopy of glutaraldehyde-fixed samples, this junction appears as a set of continuous, anastomosing intramembrane strands or fibrils in the P-face (the outwardly facing cytoplasmic leaflet) with complementary grooves in the E-face (the inwardly facing extracytoplasmic leaflets) (Staehelein, 1974). In unfixed samples, however, the intramembrane strands are reportedly seen as a linear series of individual intramembranous particles (Staehelein, 1973). There has been considerable debate about the chemical nature of these strands. It remains controversial whether the particles in the strands are predominantly lipidic in nature, i.e., cylindrical lipid micelles, or represent units of integral membrane proteins linearly aggregated (Pinto da Silva and Kachar, 1982; Kachar and Reese, 1982; Verkleij, 1984). However, given the detergent stability of TJ strands visualized by negative staining (Stevenson and Goodenough, 1984) and freeze fracture (Stevenson et al., 1988), it is unlikely that these elements are composed solely of lipids. Therefore, it is now widely accepted that the identification of the integral membrane protein localizing at TJ is an important breakthrough, because it opens the investigation of TJ to molecular approaches.

We developed an isolation procedure for AJ from the rat liver (Tsukita and Tsukita, 1989), and using this isolated AJ fraction, we identified some novel plaque proteins such as tenuin, radixin, α catenin, and 220-kD protein (Tsukita, Sh. et al., 1989; Tsukita, Sa. et al., 1989; Funayama et al., 1991; Nagafuchi et al., 1991; Itoh et al., 1991; Tsukita et al., 1992). Recent cDNA cloning revealed that this 220-kD protein is identical to ZO-1, which was originally thought to be exclusively localized just beneath the plasma membrane of TJ (Stevenson et al., 1986; Anderson et al., 1988; Itoh et al., 1993). This indicated that the 220-kD/ZO-1 protein is involved in both AJ and TJ, suggesting an intimate relationship between them. Furthermore, in most endothelial, and some epithelial cells such as those of liver, TJ is spatially intermingled with AJ. We thus speculated that the putative integral membrane protein associated with TJ is present in our isolated AJ fractions from liver cells. In other words, our isolated AJ fraction should offer a good system with which to search for the TJ membrane proteins. We have so far raised many monoclonal antibodies in mice using the membrane fraction prepared from isolated rat AJ as an antigen, but we failed to identify the integral membrane protein localizing at TJ (Itoh et al., 1991).

In this study, we first isolated the so called AJ fraction from the liver of the chick, which is evolutionally distant from mouse and rat. To escape confusion, we refer to this fraction not as "AJ fraction" but as "junctional fraction" in this study. Using the membrane preparation from the junctional fraction as an antigen, we then raised a monoclonal antibody in rats. We found three monoclonal antibodies which recognized one integral membrane protein with an apparent

molecular mass of 65 kD by immunoblotting. Immunostaining revealed that this membrane protein was exclusively localized at TJ both at the light and electron microscopic level. Furthermore, using these monoclonal antibodies, we cloned the cDNA encoding this antigen. Sequence analysis revealed no homology between this membrane protein and other proteins so far identified. An interesting feature of its predicted sequence was that like connexin, this membrane protein contains four major hydrophobic, potentially membrane-embedded domains. Therefore, we conclude that the integral membrane protein localizing at TJ is now identified.

Materials and Methods

Junctional Fraction from Chick Liver

The junctional fraction was prepared from the liver of newly hatched or 3-d-old chicks through the crude membrane and the bile canaliculi fractions according to the method described previously (Tsukita and Tsukita, 1989). The peripheral membrane proteins were removed from the junctional fraction as follows (Tsukita and Tsukita, 1989; Nagafuchi et al., 1991; Itoh et al., 1993). First, the junctional fraction was dialyzed against a low salt extraction solution (1 mM EGTA, 0.1 mM PMSF, and 2 mM Tris-HCl, pH 9.2) overnight at 4°C, followed by centrifugation at 100,000 g for 1 h. This extraction was repeated twice more. Next, the pellet was resuspended in 1 M acetic acid (pH 2.3) for 30 min on ice, followed by centrifugation at 100,000 g for 1 h. The final pellet was stored at -80°C until use for immunization.

Production of mAbs

mAbs were obtained essentially according to the procedure of Köhler et al. (1980) as previously described in detail (Tsukita et al., 1989). Fisher rats were immunized with the acetic acid-extracted AJ membranes and hybridomas were prepared by fusion between rat lymphocytes and mouse P3 myeloma cells. The culture supernatant of each hybridoma was assayed for the antigen concentration at the junctional fraction by immunoblotting using crude membrane, bile canaliculi, and junctional fractions (see Fig. 2 A). Hybridomas which produced mAbs specific for the antigens concentrated in junctional fractions were expanded, frozen, and stored in liquid nitrogen.

Gel Electrophoresis and Immunoblotting

One-dimensional SDS-PAGE (12.5%) was based on the method of Laemmli (1970) and the gels were stained with Coomassie brilliant blue R-250 or silver staining kit (Wako Pure Chemical Industries, Osaka, Japan).

For immunoblotting, after electrophoresis, proteins were electrophoretically transferred from gels to nitrocellulose sheets, which were then incubated with the first antibody. For antibody detection, a blotting detection kit (Amersham Corp., Arlington Heights, IL) was used.

Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was performed as described previously (Itoh et al., 1991). For various types of chick tissues, samples were frozen using liquid nitrogen and the frozen sections (~5 μ m) were cut in a cryostat, mounted on glass slides, air-dried, and fixed in 95% ethanol at 4°C for 30 min and in 100% acetone at room temperature for 1 min. The first antibodies were rat anti-chick occludin mAb Oc-1 or Oc-2, mouse anti-rat ZO-1 mAb (T8-754) (Itoh et al., 1991), and rat anti-mouse α catenin mAb (α -18) (Nagafuchi and Tsukita, 1994). The second antibody was FITC-conjugated goat anti-rat IgG (TAGO, Inc., Burlingame, CA) or FITC-conjugated sheep anti-mouse IgG (Amersham Corp., Arlington Heights, IL). Samples were examined using a fluorescence microscope, a Zeiss Axiopt photomicroscope (Carl Zeiss, Inc., Thornwood, NY).

Immunoelectron Microscopy

The preembedding labeling of bile canaliculi was performed essentially according to the method described previously (Tsukita et al., 1989). The fraction rich in bile canaliculi was suspended and incubated in PBS containing

1% BSA for 10 min and then mixed and incubated with mAb Oc-1 for 1 h at room temperature. Samples were washed three times by centrifugation at 10,000 g for 5 min in PBS containing 1% BSA. The final pellet was incubated with goat anti-rat IgG coupled to 10 nm gold (GARAIgG G10; Amersham, Corp., Arlington Heights, IL), which was diluted tenfold with PBS containing 1% BSA, for 2 h at room temperature. The samples were washed twice with PBS containing 1% BSA and once with PBS by centrifugation at 10,000 g for 5 min. The pellets were fixed in 1% tannic acid/2% formaldehyde/2.5% glutaraldehyde/0.1 M sodium cacodylate (pH 7.3) overnight at 4°C, washed twice with 0.1 M cacodylate buffer, postfixed with 1% OsO₄/0.1 M sodium cacodylate for 1 h on ice, washed once with distilled water, stained en bloc with 0.5% uranyl acetate, dehydrated with graded concentration of ethanol, and then embedded in Epon 812. Ultrathin sections were cut with a diamond knife and then stained doubly with uranyl acetate and lead citrate.

Immunoelectron microscopy using ultrathin cryosections was performed essentially according to the method developed by Tokuyasu (1980, 1989). Small species of small intestine from newly hatched chicks were fixed in 1% formaldehyde in 0.1 M Hepes (pH 7.5) for 15 min at room temperature. All the mAbs for occludin used in this study were very sensitive to aldehyde fixation. The staining ability of mAbs Oc-1 and Oc-3 was completely abolished by formaldehyde fixation. Only mAb Oc-2 was able to stain the 1%, but not the 4% formaldehyde-fixed sample. The fixed samples were infused with 2.0 M sucrose containing 10% polyvinylpyrrolidone at room temperature for 2 h, rapidly frozen using liquid nitrogen, and then ultrathin sectioned in the frozen state at -110°C using glass knives with an FC-4E low-temperature sectioning system (Reichert-Jung, Vienna, Austria). The sectioned samples were collected on carbon-coated formvar-film grids, washed three times with PBS containing 30 mM glycine (PBS-glycine), and incubated with PBS-glycine containing 2% goat serum for 3 min. The samples were then incubated with mAb Oc-2 for 1 h. After being washed with PBS-glycine three times, the samples were blocked with PBS-glycine containing 2% goat serum for 3 min and then incubated with goat anti-rat IgG coupled to 10 nm gold for 1 h. After being washed with PBS and distilled water, the samples were incubated with 2% uranyl acetate for 10 min and then with 2% polyvinylalcohol/0.2% uranyl acetate for 10 min and air dried. Samples were examined in an electron microscope (1200 EX; JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV.

cDNA Library Screening and DNA Sequencing

A λ gt11 expression cDNA library made from 7.5-d-old chick embryo brain poly(A)⁺ RNA (Hatta et al., 1988) was used. The initial cDNA clone, FH7 (see Fig. 6A), was isolated from the library using mAbs Oc-1 and Oc-3 according to the method previously described (Huynh et al., 1985). The FH7 fragment was then labeled by means of a DIG labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and used to screen the same cDNA library using a DIG detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cDNA clones FH1-14 and FH2-9 were thus isolated. Furthermore, FH20 and FH28 were obtained by screening with FH2-9 fragment as a probe (see Fig. 6A). Inserts of these clones were subcloned into pBluescript SK(-) and sequenced with the 7-deaza Sequenase Version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH) or with the Taq Dye-Deoxy™ Terminator Cycle Sequencing Kit (Applied Biosystem, Foster City, CA). Both strands of all clones were sequenced.

Isolation of RNA and Northern Blot Hybridization

Total RNAs from chick tissues were isolated according to the method described by Chomczynski and Sacchi (1987). The poly(A)⁺ RNAs were obtained from total RNAs using oligo-dT-conjugated latex beads (Oligotex dT-30; Takara Shuzo Co., Ltd., Kyoto, Japan). About 5 μ g of poly(A)⁺ RNAs from each tissue were separated in a formaldehyde/agarose gel and transferred to a nitrocellulose membrane. An RNA ladder (Bethesda Research Laboratories, Bethesda, MD) was used as a size marker. The fragment produced by combining FH1-14 and FH2-9 at their BglII sites was labeled with [α -³²P]dCTP using the Random Primer Labeling Kit (Takara Shuzo Co., Ltd., Kyoto, Japan), and used as a probe. Hybridization proceeded under conditions of high stringency (50% formamide/5 \times Denhardt's solution, 5 \times SSC, 0.5% SDS, 100 μ g/ml of denatured salmon sperm DNA).

Production of Fusion Proteins

Two parts of occludin polypeptides were expressed fused to maltose-binding protein. DNA fragment encoding amino acid residues 270-419 was ampli-

fied by polymerase chain reaction with primers (GCGGGATCCGTGGT-GCAGGAGGGCCTGAC) and (CGAGGTTCGACGGTATCG) from FH7 cloned into pBluescript(SK-). Another DNA fragment encoding amino acid residues 185-504 was amplified with primers (CGCGGATCCGTCAA-CCCAGGCGCAGAT) and (TCTAGAAC TAGTGGATC) from FH2-9 cloned into pBluescript(SK-). These fragments were digested with BamHI-HindIII and BamHI-PstI, respectively, and then introduced into pMAL-cRI (New England Bio-Labs, Beverly, MA). Fusion proteins were produced in XL-1/Blue with these constructs according to the procedure described by the manufacturer.

In Vitro Translation

Translations in vitro were performed as reported previously (Pelham and Jackson, 1975). RNA was synthesized from a cDNA fragment produced by combining FH1-14 and FH2-9 using an mCAP™ mRNA Capping Kit (Stratagene Corp., La Jolla, CA). Capped RNA was translated in a mRNA-dependent rabbit reticulocyte lysate in the presence of [³⁵S]methionine using an In Vitro Express™ Translation Kit (Stratagene Corp.). The [³⁵S]Methionine (1,300 Ci/mmol, Amersham, Corp.) was present at ~2.6 mCi/ml in a final reaction volume of 25 μ l. Each reaction product in the in vitro translation reaction was then analyzed using one-dimensional electrophoresis followed by autoradiography.

Results

Production of mAbs Specific for a ~65-kD Membrane Protein Enriched in Junctional Fractions

The isolation method for junctional fraction was originally developed using rat liver (Tsukita and Tsukita, 1989). In this study, to obtain a powerful antigen, we applied this isolation method to newly hatched or 3-d-old chicks. From the livers from ~5,000 newly hatched and 3-d-old chicks, we obtained the junctional fraction, dialyzed it against a low-salt alkaline solution, and completely removed the peripheral membrane proteins with 1 M acetic acid as previously reported (Itoh et al., 1991; Nagafuchi et al., 1991). This acid-extracted membrane fraction (Ac-membrane) should be mainly composed of lipids and integral membrane proteins. Using the Ac-membrane as an antigen, we attempted to raise mAbs in four rats, which recognize each constituent of the Ac-membrane. We obtained ~100 clones and froze them. In this study, we identified three clones (Oc-1, Oc-2, and Oc-3), all of which recognized several bands around 65 kD by immunoblotting (Fig. 1).

As shown in Fig. 1, the immunoblotting profile of isolated AJ with mAb Oc-1 was identical to that with mAb Oc-2; five bands at 66, 64, 62, 61, and 58 kD were recognized, the lowest of which was the most intensive. The mAb Oc-3 recognized the four upper bands but hardly detected the lowest. These results suggested that all the mAbs recognized the same antigen, and that the epitope for mAb Oc-3 is distinct from that for mAbs Oc-1 and Oc-2 (this interpretation will be proved to be the case later in this study through cDNA cloning of the antigen). Explanations why this antigen from the junctional fraction separated into 4-5 bands in SDS-PAGE are proposed in the Discussion section.

To biochemically determine whether this antigen is exclusively enriched in the junctional fraction, we separated fractions of liver cells, crude membranes, bile canaliculi, and junctions (each containing the same amount of total protein) on a one-dimensional gel, and immunoblotted it with mAb Oc-1. As shown in Fig. 2A, this antigen was barely detectable in the liver cell fraction and highly enriched in the junctional fraction. Furthermore, to check whether this antigen

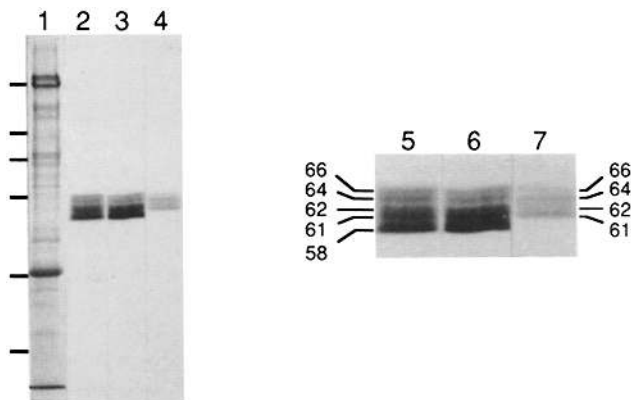


Figure 1. Monoclonal antibodies for the ~65-kD protein in the junctional fraction. Lane 1, Coomassie brilliant blue-stained gel (12.5%) of junctional fraction; lanes 2–4, accompanying immunoblots by the use of mAbs Oc-1, Oc-2, and Oc-3, respectively. Bars indicate molecular mass as 200, 116, 97, 66, 45, and 31 kD from the top. Close comparison reveals that the banding pattern with mAb Oc-3 (lane 7) is similar to that of mAbs Oc-1 (lanes 5) and Oc-2 (lane 6) but characteristically lacks the band around 58 kD. The same banding patterns were obtained, when the acid-extracted membrane fraction was used.

is an integral membrane protein or an undercoat-constitutive peripheral membrane protein, we examined its extractability from the bile canaliculi fraction with 1 M acetic acid (pH 2.3), 0.1 M NaOH, or low-salt alkali (1 mM EGTA, 2 mM Tris-HCl; pH 9.2) (without non-ionic detergent) (Itoh et al., 1991). As shown in Fig. 2 B, this antigen and L-CAM (the chicken homologue of E-cadherin) were hardly extracted from membranes with the acetic acid, whereas ZO-1, a peripheral membrane protein localizing at tight junctions in epithelial cells, was effectively removed. Both NaOH and

low-salt alkali gave the same results (data not shown). This antigen was hardly extracted from membranes with 0.1–1.0% NP-40, but completely extracted with 0.05% SDS. These strongly suggest that this antigen is not a peripheral membrane protein but an integral membrane protein.

Immunofluorescence and Immunoelectron Microscopic Localization of the ~65-kD Membrane Protein

Using the mAbs Oc-1, Oc-2, and Oc-3, we examined the distribution of the antigen in chick tissues by immunofluorescence microscopy. Basically, all three mAbs showed the same staining pattern. In intestinal epithelia, these mAbs exclusively stained the junctional complex region (Fig. 3). The degree of antigen concentration at the junctional complex region was similar to that of ZO-1, a tight junction-associated protein in epithelial cells, and much higher than that of α catenin, a cadherin-associated protein. Also in the liver, this antigen was highly concentrated at the junctional complex region along bile canaliculi (Fig. 4 A). As far as we examined with these mAbs in chick, all types of simple epithelial cells were clearly stained at their junctional area. In nervous tissues such as the brain, an intense signal was detected only from endothelial cells (Fig. 4 B). In the heart, endothelial cells were selectively stained, although to a significantly weaker degree than that in brain vessels (Fig. 4 C). Intercalated discs, which bear AJ but lack TJ, were not labeled. Taking these findings together, we speculate that this antigen is concentrated at TJ in epithelial and endothelial cells.

To confirm this hypothesis, using ultrathin cryosections of intestinal epithelial cells, we analyzed the distribution of this antigen in the junctional complex at the electron microscopic level. As shown in Fig. 5, A–C, this antigen was exclusively localized at the TJ zone. Next, the isolated bile canaliculi

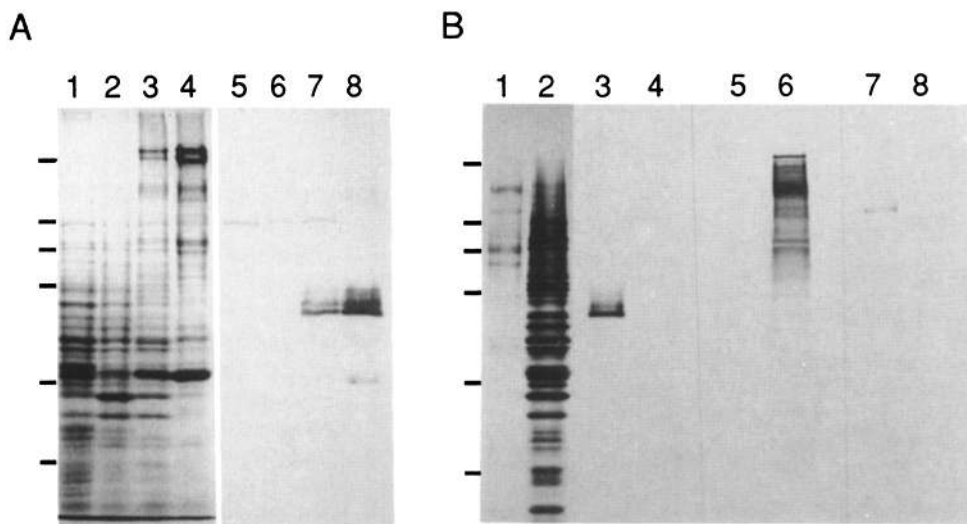


Figure 2. Identification and characterization of the ~65-kD integral membrane protein by immunoblotting. (A) Enrichment of the antigen in the junctional fraction. Coomassie brilliant blue-stained gels (lanes 1–4) and accompanying immunoblots with mAb Oc-1 (lanes 5–8). Fractions of liver cells (lane 1) crude membranes (lane 2), bile canaliculi (lane 3), and junctional fractions (lane 4) were loaded on one dimensional gels with the same amount of total protein. Lanes 5–8 clearly indicate that the antigen for mAb Oc-1 is highly concentrated at the junctional fraction. The weak staining around 110 kD is derived from a polypeptide recognized by the second antibody, which is not contained in the junctional fraction. (B) Acetic acid extraction experiments using the bile canaliculus fraction. Lanes 1, 3, 5, and 7, acetic acid-extracted bile canaliculus membranes; lanes 2, 4, 6, and 8, acetic acid extract. Lanes 1 and 2, Silver-stained gels (12.5%); lanes 3–8, accompanying immunoblots with mAb Oc-1 (lanes 3 and 4), anti-ZO-1 mAb (lanes 5 and 6), and anti-L-CAM mAb (lanes 7 and 8). The antigens for mAb Oc-1 and L-CAM are hardly extracted from the bile canaliculus fraction, suggesting that the antigen for mAb Oc-1 is an integral membrane protein like L-CAM. Bars indicate molecular mass as 200, 116, 97, 66, 45, and 31 kD from the top.

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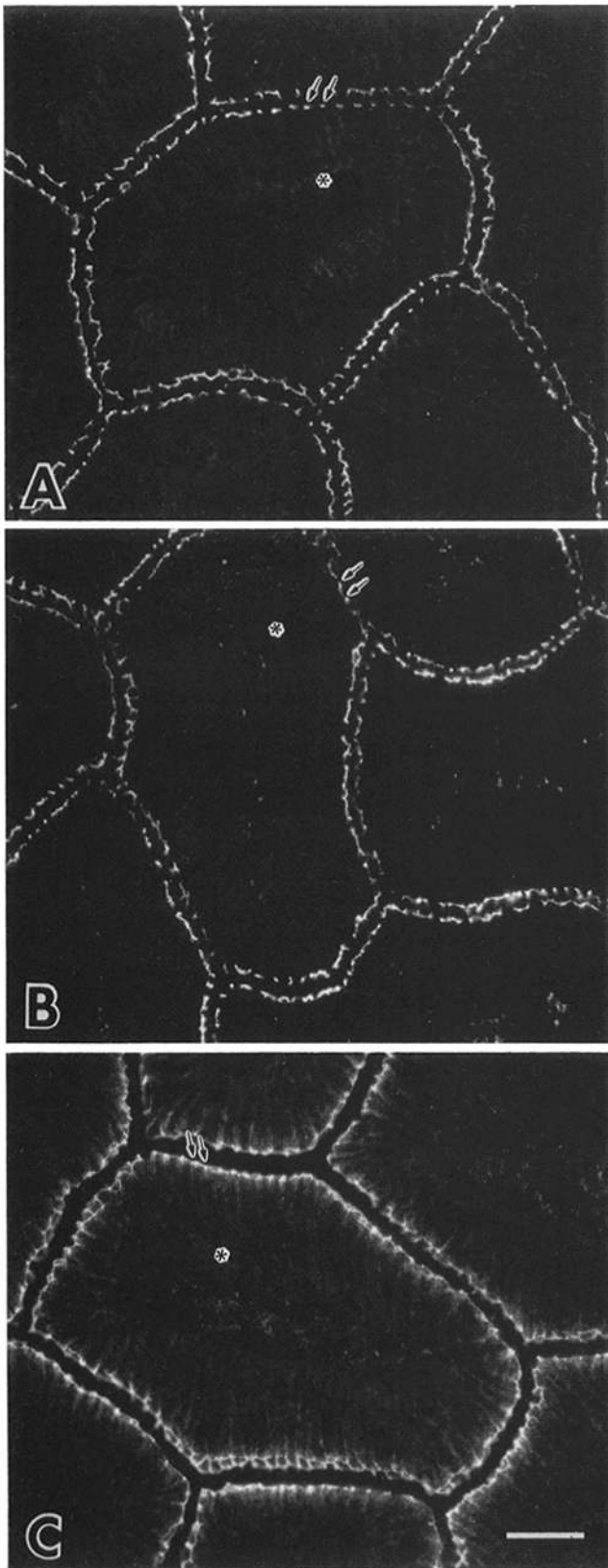


Figure 3. Immunostaining of a frozen section of chick intestinal epithelia with mAb Oc-1 (A), anti-ZO-1 mAb (B), and anti- α catenin mAb (C). Intestinal villi are cut transversely and each villus is covered with simple columnar epithelium (between *arrows* and *). The degree of concentration of the \sim 65-kD membrane protein

fraction was used to immunolocalize this antigen at the electron microscopic level. The cytoplasmic surface of TJ was specifically labeled (Fig. 5, D–H). Immunogold particles were characteristically detected directly over the points of membrane contact. No labeling was observed on membrane structures but TJ.

In summary, we conclude that the antigen recognized by the mAbs Oc-1, Oc-2, and Oc-3 is an integral membrane protein localizing at TJ in epithelial and endothelial cells. Accordingly, this protein is designated “occludin” (from the Latin word *occludere*).

Isolation and Sequencing of cDNA Encoding Occludin

Using the mAb Oc-1, we screened $\sim 5 \times 10^5$ plaques from a random-primed λ gt11 cDNA library made from the chick embryo brain as described in Materials and Methods, and cloned 21 positive phage recombinants. Since the epitope for mAb Oc-3 appeared to be distinct from that for mAb Oc-1 (see Fig. 1), we rescreened these Oc-1-positive plaques with mAb Oc-3, and found that the plaque from only one recombinant (FH7) was specifically recognized by mAb Oc-3. The FH7 plaque was not recognized by mAb Oc-2, indicating that the epitope for mAb Oc-2 is different from that for mAb Oc-1. To isolate the rest of the occludin cDNA, as described in Materials and Methods, the same library was rescreened, and five overlapping clones, including FH7, which together span 1,975 bp, were isolated and sequenced in both directions (Fig. 6 A).

Two criteria confirmed that these clones encoded occludin. Firstly, we generated fusion proteins encoded by FH7 and by FH2-9 (see Fig. 6 A). As stated above, the fusion protein from FH7 was specifically recognized by the mAbs Oc-1 and Oc-3, but not by Oc-2. As shown in Fig. 6 B, the fusion protein from FH2-9, which was obtained by hybridization with FH7, was recognized by the mAb Oc-2 as well as by Oc-1 and Oc-3. This indicates that the protein encoded by these clones is recognized by the mAbs Oc-1, Oc-2, and Oc-3, whose epitopes are distinct, meaning that these clones encode occludin. Secondly, we performed *in vitro* translation in a mRNA-dependent rabbit reticulocyte system, and the major band of the products was electrophoretically identical to the major 58-kD band of occludin (Fig. 6 C).

The complete nucleotide sequence encoded by the overlapping clones and the predicted amino acid sequence are shown in Fig. 7 A. In the 1,975 nucleotides sequence, the longest open reading frame (ORF), with 1,512 nucleotides, begins from an ATG codon at nucleotide 21 and ends with a TAA signal for translation termination at nucleotide 1533. This ORF encodes a 504 amino acid polypeptide with a predicted molecular mass of 55.9 kD. Judging from the length of the mRNA detected by Northern blotting (Fig. 8), the molecular mass of this protein in SDS-PAGE (\sim 65 kD),

at the junctional complex region (*arrows*) is similar to that of ZO-1 and much higher than that of α catenin. Although the staining for blood vessels is not clear in this figure, these antibodies more or less stain them locating at the center of intestinal villi. The signal from blood vessels with mAb Oc-1 is significantly weaker than that with anti-ZO-1 mAb, probably because, unlike TJ-specific mAb Oc-1, anti-ZO-1 mAb stains not only TJ but also AJ (see Itoh et al., 1993). *, basal membranes of epithelial cells. Bar, 20 μ m.

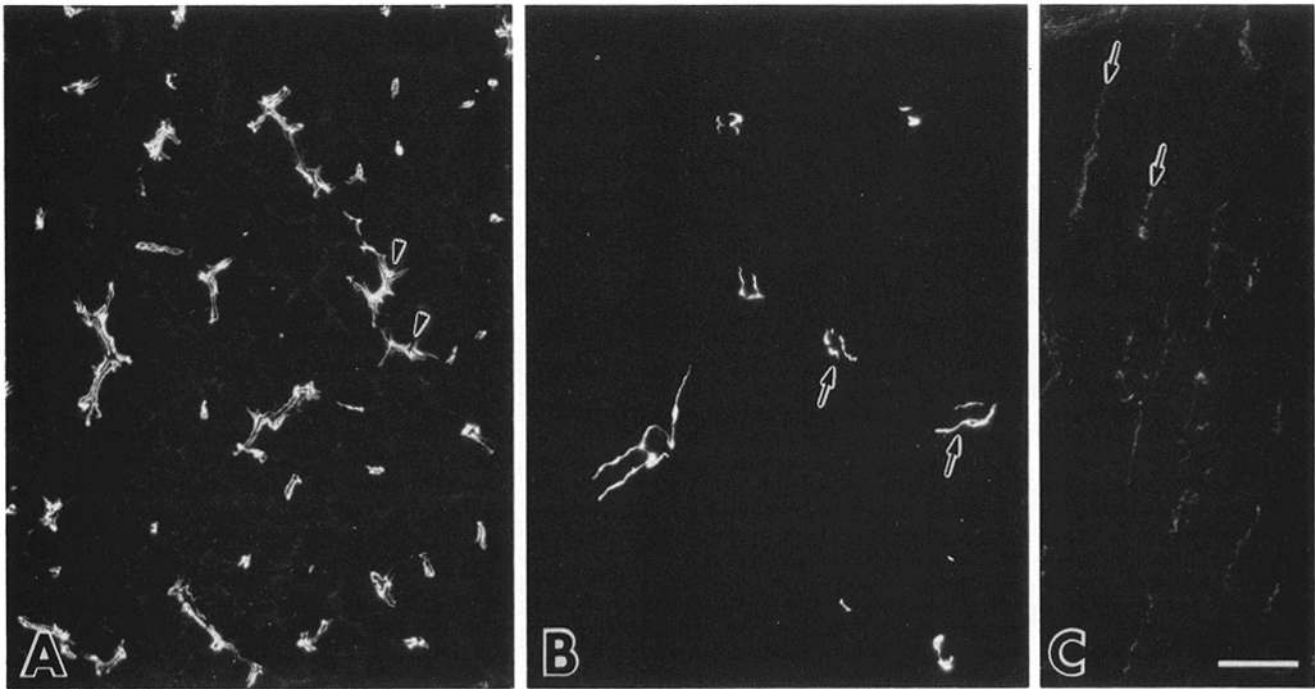


Figure 4. Immunostaining of frozen sections of chick liver (A), brain (B), and heart (C) with mAb Oc-2. In the liver, the beltlike junctional complex region is intensely stained with this mAb (arrowheads). In the newly hatched chick, more than two belts are often present along one bile canaliculus. Both in the brain and heart, the signal is detected only from endothelial cells (arrows). Note that the signal from endothelial cells is significantly stronger in the brain than in the heart. Bar, 20 μm .

and the data from the *in vitro* translation (see Fig. 6 C), we considered that the ATG codon in position 21 is the initiation site for translation. This interpretation is confirmed by the existence of the consensus context for the initiation of translation (Kozak, 1989) at nucleotides 15-23.

A search of the data bases identified no proteins with significant homology to occludin. In the deduced amino acid sequence of occludin, there appears to be no typical signal sequence at the NH_2 -terminal and no N-linked glycosylation site. The content of alanine, tyrosine, serine, and glycine residues is characteristically high (9.3, 9.1, 8.9, and 8.9%, respectively). The hydrophilicity plot for occludin predicts the hydrophobic characteristic of local regions of the sequence as shown in Fig. 7 B. At the NH_2 -terminal half, there are four major hydrophobic, potentially membrane-embedded areas: residues 58-80, 125-148, 159-183, and 228-249. The COOH-terminal 255 residues exhibit pronounced hydrophilicity and contain some clusters of charged residues; EEEEE at amino acid residues 347-351 and RRG-RRRRR at amino acids 363-370.

Using the occludin cDNA as probe, total RNAs from chick liver, lung, and brain were examined for homologous

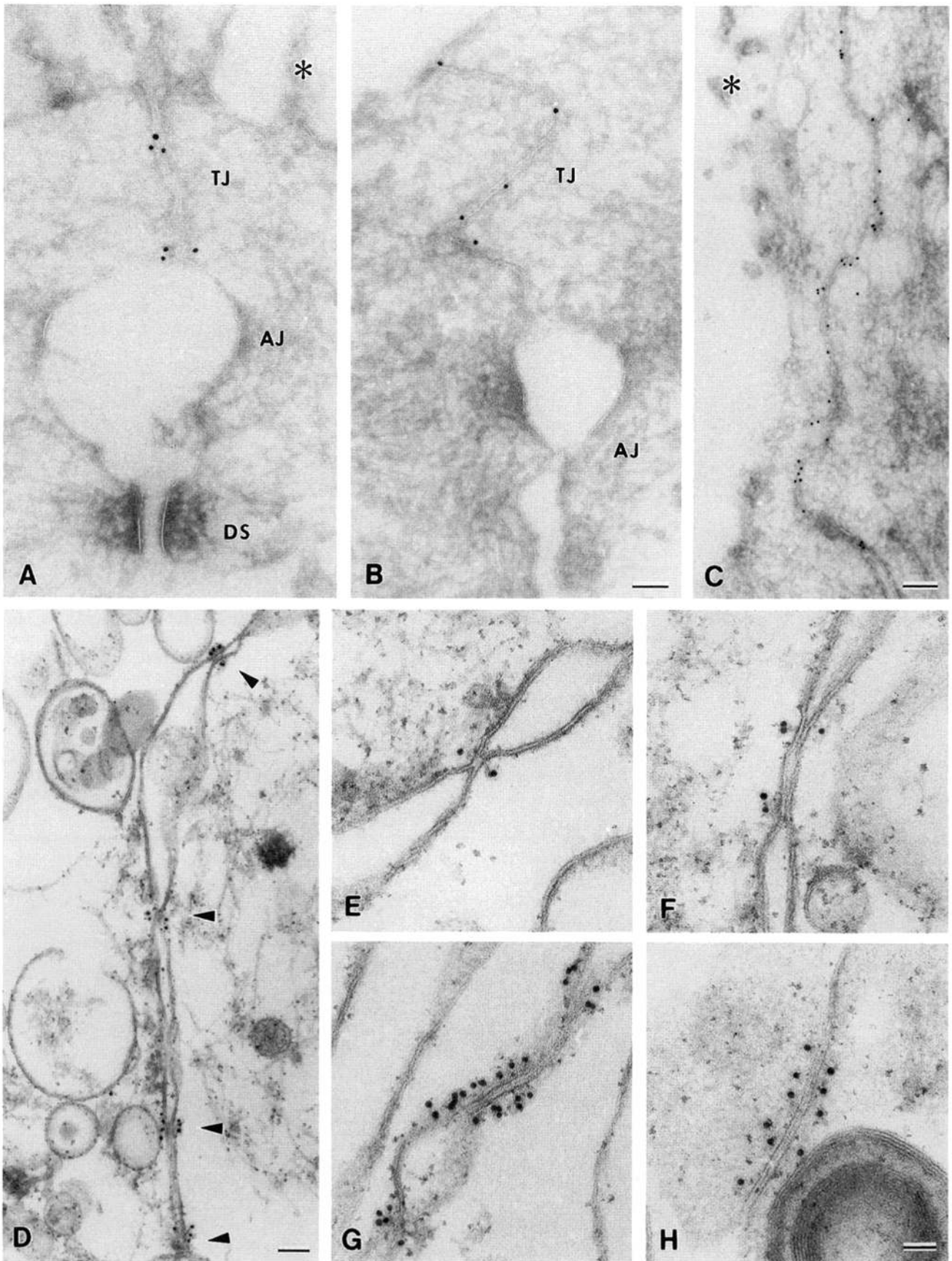
sequences by Northern blotting (Fig. 8). A single 2.3-kb band in all of these tissues hybridized, which is consistent with that of the cDNA cloned above.

Discussion

Occurrence of an Integral Membrane Protein at TJ

So far, the putative integral membrane protein consisting of TJ has not yet been identified, which has presented quite a challenge to cell biologists. In this study, taking advantage of the junctional fraction from chick which is expected to contain TJ components, we identified a ~ 65 -kD integral membrane protein (occludin) which is localized at TJ in both epithelial and endothelial cells. Light and electron microscopic evidence clarified the exclusive localization of occludin at TJ. We reasoned that occludin is an integral membrane protein because: (a) Treating the bile canaliculi fraction with 1 M acetic acid, 0.1 M NaOH, or low-salt alkali solution did not release occludin from plasma membranes. As far as we examined, occludin was not extracted from the plasma membrane without detergent; and (b) The amino

Figure 5. Ultrastructural localization of occludin in chick intestinal epithelial cells (A-C) and isolated bile canaliculi from chick liver (D-H). (A-C) Ultrathin cryosections of formalin-fixed intestinal epithelial cells were labeled with mAb Oc-2. Gold particles accumulated at the TJ region (TJ), and are hardly detected in the AJ (AJ) and DS (DS) regions. In A and B, AJ is artifactually opened, probably due to the formaldehyde fixation. In C, TJ was cut along its strand, which was heavily labeled with gold particles. *, microvilli. (D-H) Isolated bile canaliculi were labeled with mAb Oc-1, then with 10 nm gold particles conjugated with anti-rat IgG, and embedded in Epon. When the TJ strands were cut transversely (D-F), immunogold particles were detected directly over the points of membrane contact of TJ (arrowheads). Along the longitudinally cut TJ strands, many gold particles are aligned (G and H). Bars: (A and B) 0.1 μm ; (C) 0.2 μm ; (D) 0.1 μm ; (E-H) 50 nm.



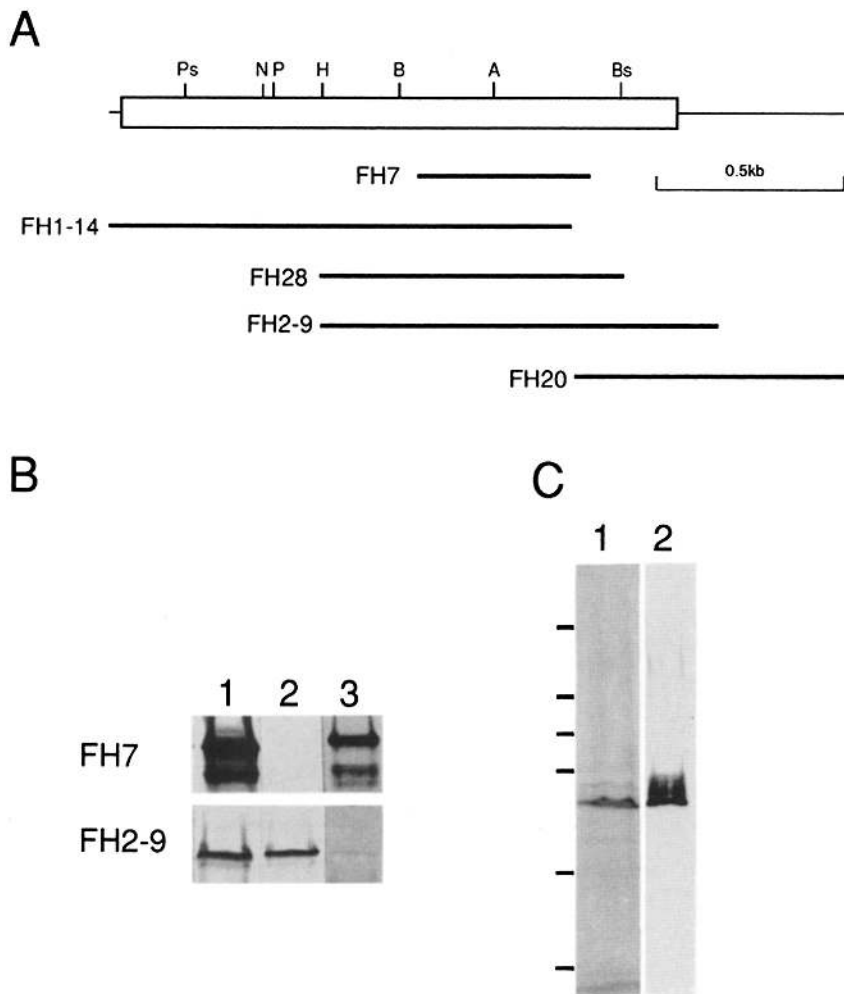


Figure 6. Isolation of cDNA encoding chick occludin. (A) Restriction map and cDNA fragments of the chick occludin. The open box indicates the coding region. FH7 was first obtained using mAbs Oc-1 and Oc-3. All clones were obtained from a random primed λ gt11 library made from the chick embryo brain. A, ApaI; B, BglIII; Bs, BstXI; H, HincII; N, NcoI; P, PvuII; Ps, PstI. (B) Fusion proteins generated in *E. coli* from FH7 (FH7) and FH2-9 (FH2-9) were electrophoresed and immunoblotted with mAb Oc-1 (lanes 1), mAb Oc-2 (lane 2), and mAb Oc-3 (lane 3). The expression level in *E. coli* of the FH2-9 fusion protein was very low, and the immunoblot staining with mAb Oc-3 was rather weak in general. Therefore, the signal from the Oc-3-stained FH2-9 protein was not intense but significantly positive. (C) In vitro expression of occludin from its composite cDNA from FH1-14 and FH2-9. The total reaction products obtained in vitro were separated on one-dimensional SDS-PAGE and detected by autoradiography (lane 1). Lane 2, immunoblotting of junctional fraction with mAb Oc-1.

acid sequence of occludin deduced from its cDNA nucleotide sequence was characterized by four major hydrophobic, potentially membrane-embedded domains.

This study provided an answer to the question as to whether or not the integral membrane protein occurs at TJ (Kachar and Reese, 1982; Verkleij, 1984). Of course, the identification of occludin in this study does not exclude the possibility that lipids also play a key role in the TJ formation, and it remains to be elucidated whether the expression of the single membrane protein, occludin, is sufficient for the formation of TJ and the establishment of the barrier and fence functions of TJ.

Structure of Occludin

The most striking feature of the primary structure of occludin is the four putative membrane-spanning segments at its NH₂-terminal half. So far, some integral membrane proteins have been reported to contain four potentially membrane-spanning segments; connexins, synaptophysin, proteolipid protein (PLP), and CD9, CD37, CD53, CD63, etc. (Paul, 1986; Kumar and Gilula, 1986; Leube et al., 1987; Dautigny et al., 1985; Milner et al., 1985; Horejsi and Vlcek, 1991). PLP is thought to be responsible for the fusion of the outer leaflets of apposed membranes in myelin formation (Lees and Brostoff, 1984): this function of PLP is very similar to the possible role of occludin in TJ formation. The

membrane arrangement of PLP remains to be elucidated, although several conflicting models have been so far proposed (Popot et al., 1991).

The membrane topology of connexin, the integral membrane protein of gap junctions, has been intensively analyzed. Isolated gap junctions immunolabeled with mAbs specific for defined connexin sequences resulted in a widely accepted model consisting of four transmembrane segments with NH₂ and COOH termini exposed at the cytoplasmic surface (Zimmer et al., 1987; Milks et al., 1988; Yeager and Gilula, 1992). Occludin shows no sequence similarity to connexins. However, in a sense, the possible barrier function of occludin is similar to the intercellular channel function of connexins. Occludin may be arrayed in a linear fashion to establish high electrical resistance between apical and basolateral extracellular spaces, while connexins are arranged in a circular manner, forming a channel that establishes high electrical resistance between the inside of the channel and the extracellular space (Loewenstein, 1987). Therefore, it is reasonable to apply the transmembrane orientation model for connexins to occludin (Fig. 9).

In this model, four membrane-spanning segments (M1~M4 in Fig. 9) correspond to amino acids residues 58-80, 125-148, 159-183, and 228-249 of occludin, respectively. This model is consistent with the present data on the localization of epitope for anti-occludin mAb Oc-1 in occludin. As shown

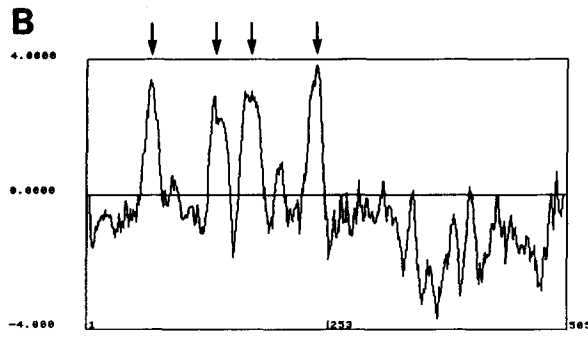
A

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120
M F S K K S Y D G P P A G Y G P P T G Y G A P T A D Y G Y G S P P P
34
CGGGCTCTACTACGTGGACAGCGCTCCGAGCTCTCTACAAAGTGAGCGTCCGCGCCGGCGGGTGCAGGCGGGGCTCCTCGTGTGTGATGCCATCTTCGGCTGGC
240
G S Y Y V D D A P Q L F Y K W T S P P G A V R G L Q A G V L V L C I A I F A C Y
74
TGCTTCCACGGTCCGCTGGATTACGGCTACGGCTCGGGGGGGTACGGCACCGGGTGGGGGGTTCTACGGCTCAACTACTAGCGCACGGGGCTGAGCTACAGCTACGGCTACG
360
A S T L A W D Y G Y G L G G A Y G T G L G G F Y G S N Y Y G S G L S Y S Y G Y G
114
GGGGCTACTACGGAGGGGTGAACAGCGCACGGGCAACGGCTTCATGATCGCATTGGCGTGTGCTTCTCTGCGCCAGCTGGGGCTGTGCTGGCGGCTCAAGCAATCCGGGGCCA
480
G Y Y G G V N Q R T A N G F M I A M A V L C F L A Q L G L L V A A L S K S G A T
154
CGGGCTCGGGCTCTTACCTGGCGTGTGTGCTGAGCGCGTGTGGCTCTGCTATGCTCATGGCTCCATGCTTACATCATGGGGCTCAACCGCACGGCGCAGATGTCCAGCG
600
R S R R F Y I A V L V L S A V L A F K R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R
194
GTACTACTACAGCCCTTGTGGCCATGTGCAGCAGCGCTACGGCAGCACTACCTCAACCAGTACATCTACCCTACTGCACCGTGGACCCCGAGGAGCTGTGGCTGTGCTGTG
720
Y Y Y S P L L A M C S Q A Y G S T Y L N Q Y I Y H Y C T V D P Q E A V A A V C C
234
GGTTCCTCATCGTCTCGCTCTGCTCTGCTCATCTGCTCTTCCGCCAGAAAGAGCGGCAAGATAGATCTGGCGCTACGGCAAGGCAACATCTACTGGGACCGCGCCCGCTGGTGCAGGAGG
840
F L I V I L L L C L I C F F A Q K T R S K I W R Y G K A N I Y W D R A P V V Q E G
274
GGCCTGACGTGGAGGATGGTGAAGAAGCGTGGGAGTGGGGCCAGCGCTGCAGGACAGAGCGCCACCGCTGCCTACTGGGAAGCCCAAGCGCTGTCCGCGCCCGCCCTACAGCT
960
P D V E E W V K N V A D G A S V Q D E T A T L A Y S E K P T S P V A A P P Y S Y
314
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1080
V P P P S A G Y Y P S G T Y S S R G D Q P D R A L S A S P V H G E E E E E K G K
354
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1200
D Q P S R R P P A R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R R
394
ACCAGGACAGTGGGCGAGTCTGTACCCCGCCATCAGCTCGGAGCGCGCCCGCCAGCGCTACAAGCAGGAGTTCGACACCGACTCAAGCGCTACAAGCAGCTCTGTGCTGAGATGGACA
1320
Q E Q W A S L Y P P I T S D G A R Q R Y K Q E F D T D L K R Y K Q L C A E M D S
434
GCATCAAGACCGCCTCAATCAGCTCAGCGGCGGCTGACAGCATCAGCAGGACAGCGCTCAATACCGAGATGGCGAGGAGTACAATCAGCTCAAGACCTGAAGCGGAGCGCCAG
1440
I N D R L N Q L S R R L D S I T E D S P Q Y Q D V A E E Y N Q L K D L K R S P D
474
ACTCAAAGCAAGAAGCAGGAGAGCAAGTGTGGCAACAAGCTTCCACATCAAGCGCATGTGTGAGCGCGCTAGCACAAGTGTGGGGGTAACGAGCAGCAGGAGGGGGGGGCA
1560
Y Q S K K Q E S K V L R N K L F H I K R M V S A Y D K V R G
504
CCCCACCCCCACCCAAAGACTGTGTGATTTTGTACTTCTTAAAGAAAACACACTCAATCAGCTCAGCCCGCCCGCAAGGGGTCACCCCGCCCAACCCCGCTTAAAC
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1800
AAGGGCTGAGGTCTGCAACAGCATCAGCGCGCCCTGGCAGCTGTGGGTACGAGATGTACCCTGAGCACAGCTCAAGTGGGGGGGGGAGCATCCCTTGGATGGGGGGGGGGGCA
1920
GTCCCTTGGGGGAGAGGGGGGGTCAAGGGCTTTTGGGAAGGCATTTTGAAGGC
1975

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Figure 7. Structure of chick occludin. (A) Nucleotide and deduced amino acid sequences of chick occludin. The ORF encodes a 504-amino acid polypeptide with a predicted molecular mass of 55.9 kD. The consensus context for the initiation of translation (Kozak, 1989) (*double-underlined*) precedes the ATG codon. These sequence data are available from EMBL/GenBank/DDBJ under accession number D21837. (B) Hydrophilicity plot for occludin from the Kyte and Doolittle program. The plot records the average hydrophilicity along the sequence over a window of 10 residues. Hydrophilic and hydrophobic residues are in the lower and upper part of the frame, respectively. The axis is numbered in amino acid residues. At the NH₂-terminal half of occludin, there are four major hydrophobic, potentially membrane embedded areas (*arrows in B; underlines in A*).



in Fig. 5, D-F, the mAb Oc-1 recognized occludin from the cytoplasmic side; the epitope for this mAb fell in the segment of amino acid residues 269-419, which is assigned to the COOH-terminal segment exposed to the cytoplasm in the model. When compared with the transmembrane orientation model for connexins, the extracellular loop domains of occludin (M1-M2 and M3-M4 loops) appear to be longer than the corresponding domains of connexins, indicating that the extracellular mass of occludin is larger than that of connexins. This appears to be inconsistent with the distance between apposed membranes in TJ (0 nm) and gap junctions (2 nm) (Farquhar and Palade, 1963; Staehelin, 1974). However, taking the following peculiar characteristics in amino acid sequence of the putative extracellular loops in occludin into consideration, we speculate that they are very important for TJ formation (Fig. 9): (a) The M1-M2 loop is characterized by a high content of tyrosine (25.0%) and glycine residues (36.4%), and the M3-M4 loop contains many tyrosine residues (18.1%). (b) The charged residues at neutral pH are mostly located at the putative cytoplasmic domains, and excluded from both extracellular loops.

We should discuss here reasons why in SDS-PAGE occludin from the junctional fraction separates into five bands

when detected by mAbs Oc-1 and Oc-2 and into four bands by mAb Oc-3 (see Fig. 1). Given that the epitopes for mAbs Oc-3, Oc-1, and Oc-2 would be located on the COOH-terminal cytoplasmic domain in this order from the COOH-terminal end and that between the epitopes of mAbs Oc-3 and Oc-1 there would be a sequence susceptible to proteolytic digestion leaving the 58-kD fragment on the membrane, the observation that the 58-kD band of occludin is detected by mAbs Oc-1 and Oc-2 but not by mAb Oc-3 can be explained. Also, during *in vitro* translation, the product would be mostly degraded into the 58-kD polypeptide (see Fig. 6 C). Of course, it is possible that these five bands are due to some kind of posttranslational modification.

Molecular Architecture of TJ

So far, mAbs have detected ZO-1 (220 kD), cingulin (140 kD), 7H6 (155 kD), and ZO-2 (160 kD) as undercoat-constitutive proteins localizing at TJ (Citi, 1993; Stevenson et al., 1986; Citi et al., 1988; Zhong et al., 1993; Gumbiner, 1991). Among these, ZO-1 and cingulin were well characterized. Immunolabeling has revealed that ZO-1 is located in the immediate vicinity of the plasma membrane and that cin-

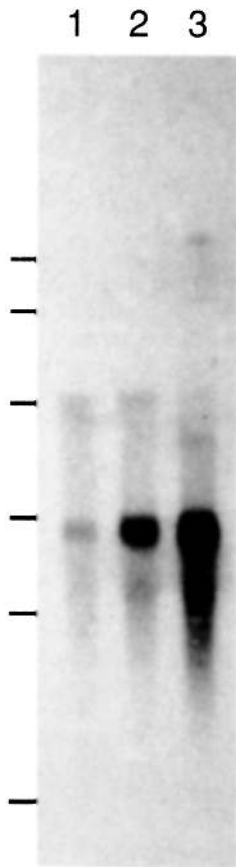


Figure 8. Northern blot of the poly(A)⁺ RNA isolated from the chick brain (lane 1), liver (lane 2), and lung (lane 3). The RNA (5 μg/lane) was probed at high stringency with the composite cDNA from FH1-14 and FH2-9. The bars on the left indicates the positions of RNA markers of 9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb (from the top).

gulin is distributed about three times farther from the plasma membrane than ZO-1 in TJ of epithelial cells (Stevenson et al., 1989). Therefore, cytoplasmic segments of the occludin molecule (amino acids 1-57, 149-158, 250-504) may directly

or indirectly interact with ZO-1 molecules. Since ZO-1 reportedly binds to spectrin, an undercoat-constitutive actin-binding protein (Itoh, 1991), this speculation may explain a previous observation of the intimate spatial relationship between TJ and actin-based cytoskeletons (Madara, 1987). These speculations must be evaluated both in vivo and in vitro in the near future.

The point the early ZO-1 studies made that ZO-1 is exclusively localized at the undercoat of TJ, has been reexamined (Itoh et al., 1991, 1993). The cDNA cloning and immunoelectron microscopy have revealed that ZO-1 precisely colocalizes with N- and P-cadherins in non-epithelial cells such as fibroblasts and cardiac muscle cells, and that only in epithelial cells ZO-1 and E-cadherins segregate into TJ and AJ, respectively (Itoh et al., 1993). As shown in this study, occludin appears to be specific for TJ, unlike ZO-1. Therefore, it is likely that the expression of occludin is involved in the molecular mechanism of the segregation of ZO-1 and E-cadherin in epithelial cells. Further studies on the expression of occludin from this perspective should provide a better understanding of the physiological functions of ZO-1 and of the relationship between TJ and AJ.

Tissue and Species Specificity of Occludin

TJ plays a crucial role in the physiological function of both epithelial and endothelial cells. The question has then naturally arisen as to whether or not the same integral membrane protein of TJ is shared by epithelial and endothelial cells. All our anti-occludin mAbs immunofluorescently stained both epithelial and endothelial TJ in various types of tissues. The signal from the vessels in muscle and intestine for endothelial TJ was clear but very weak, whereas a very intense signal was detected from endothelial cells in the brain. This is consistent with previous observations that TJ strands of

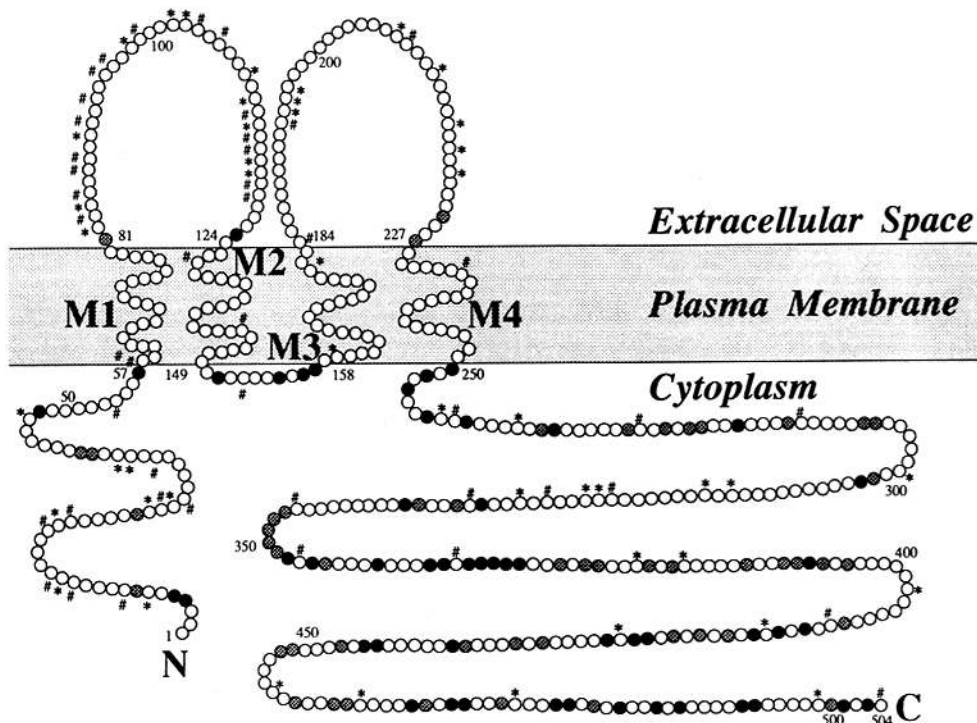


Figure 9. Folding model for occludin. A hydrophilicity plot predicted four membrane-spanning domains (M1-M4). The predicted locations of the extracellular, membrane and cytoplasmic regions are indicated. Both NH₂ and COOH termini are exposed at the cytoplasmic surface in this model. ● and ⊗ represent positively and negatively charged residues at neutral pH, respectively, and a non-charged residue is shown by ○. * and # represent tyrosine and glycine residues, respectively. Note that the M1-M2 extracellular loop is characterized by a high content of tyrosine and glycine residues, and that the charged residues are mostly located at the cytoplasmic domains.

brain endothelial cells are well developed (Simionescu et al., 1975, 1976; Shivers et al., 1984; Nagy et al., 1984). Furthermore, Northern blots revealed that in both the liver and the brain only a single major band of around 2.3 kb was detected as the occludin mRNA. Considering that TJ in liver and brain is mainly derived from epithelial and endothelial cells, respectively, we conclude that the same or highly related occludin molecules are expressed and localized at TJ in endothelial and epithelial cells.

Our anti-chick occludin mAbs, Oc-1, Oc-2, and Oc-3, did not recognize TJ in mammalian tissues. However, a mammalian homologue of occludin probably exists. Actually, in our recent transfection experiments using cultured epithelial cells (MDBK cells), the introduced chick occludin was exclusively concentrated at TJ, indicating that chick occludin is similar to structurally and functionally the bovine homologue (Furuse, M., T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, Sa. Tsukita, and Sh. Tsukita, manuscript in preparation). Considering that TJ is very important also from a medical perspective, human and mouse homologues of occludin should be identified. This is now being studied in our laboratory using chick occludin cDNA.

The precise characterization of the regulation mechanism of the permeability of endothelial and epithelial cells is an area of current active investigation. Unanswered questions include how TJ is involved in the blood-brain barrier system, how the permeability of endothelial cells is elevated during an inflammatory reaction, and how the permeability of intestinal epithelial cells is controlled during absorption. So far, the lack of information about the integral membrane protein at TJ made it impossible to answer these questions in molecular terms. However, now that we identified occludin and obtained its cDNA, we should be able to start dissecting the structure and functions of TJ at the molecular level.

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