# Identification and Characterization of Occludin:
# A Novel Adhesion Molecule of Tight Junctions

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**Abstract**

In multi-cellular organisms, establishment of compositionally distinct fluid compartments by epithelium and endothelium is crucial for the development and function of most organs. Tight junction (TJ), the most apical element of epithelial and endothelial junctional complexes in vertebrates, is directly involved in this compartmentation by sealing cells to create the primary barrier to the diffusion of solutes through the paracellular pathway. TJ is also thought to function as a boundary between the apical and basolateral plasma membrane domains, which differ in proteins and lipids composition and physiological functions, to create and maintain epithelial and endothelial cell polarity.

Other intercellular junctions such as adherens junctions, desmosomes, and gap junctions bear specific types of integral membrane proteins which play crucial roles in each junction. To clarify the structure and function of TJ at the molecular level, an integral membrane protein should be identified. In TJ, despite of intensive studies, an integral membrane protein remained elusive for quite some time. Here, by the use of the monoclonal antibody technique, I first identified an integral membrane protein localizing at TJ, which was designated as "occludin". Then I cloned its cDNA, which enabled us to clarify some unique functions of this molecule; its association with the some peripheral membrane proteins of TJ, its localization signal at TJ, and its cell adhesion ability.

In the Chapter 1, I identified an integral membrane protein of TJ and analyzed its primary structure by cDNA cloning. Recently, we found that ZO-1, a TJ-associated protein, was concentrated in the so called isolated adherens junction fraction from the liver (Itoh,M., A.Nagafuchi, S.Yonemu-

ra, 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, I obtained three monoclonal antibodies specific for a ~65kD 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 ~65kD membrane protein was exclusively localized at TJs of both epithelial and endothelial cells: At the electron microscopic level, the labels were detected directly over the points of membrane contact in TJs. To further clarify the nature and structure of this membrane protein, I cloned and sequenced its cDNA. I found that the cDNA encoded a 504 amino acid polypeptide with the calculated molecular mass of 55.9kD. 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 N-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 TJs is now identified, which we designated as 'occludin'.

In the Chapter 2, I investigated the roles of the COOH-terminal cytoplasmic domain of occludin using its cDNA. Immunofluorescence and laser scan microscopy revealed that chick full-length occludin introduced into human and bovine epithelial cells was correctly delivered to and incorporated into preexisting TJ. Further transfection studies with various deletion mutants showed that the long COOH-terminal cytoplasmic domain consisting of 255 amino acids(domain E), especially its COOH-terminal ~150 amino acids (domain E358/504), was necessary for the localization of occlu-

din at TJ. Secondly, domain E was expressed in E.coli as a fusion protein with glutathione-S-transferase(GST), and this fusion protein was shown to be specifically bound to a complex of ZO-1 (220kD) and ZO-2 (160kD) among various membrane peripheral proteins. In vitro binding analyses using GST-fusion proteins of various deletion mutants of domain E narrowed down the sequence necessary for the ZO-1/ZO-2 association into the domain E358/504. Furthermore, this region directly associated with the recombinant ZO-1 produced in E.coli. I concluded that occludin itself can localize at TJ and directly associate with ZO-1. The coincidence of the sequence necessary for the ZO-1 association with that for the TJ localization suggests that the association with underlying cytoskeletons through ZO-1 is required for occludin to be localized at TJ.

In the Chapter 3, I investigated the cell adhesion ability of occludin. Chick occludin was overexpressed in insect cells by recombinant baculo virus infection. When the cells were observed by confocal immunofluorescence microscopy, the majority of expressed chick occludin occurred inside cells. Thin section electron microscopy of these cells identified peculiar electron-dense membrane structures which consist of thin parallel or concentric lamellae. These multilamellar structure were shown to consist of many disk-like structure, each of which has a loop of membrane with its both ends. These structures were heavily labeled with anti-chick occludin monoclonal antibodies. These observation led us to speculate that the disk-like structure was transformed from each cisterna whose luminal space was completely collapsed by accumulation of occludin molecules. Furthermore I analyzed the arrangements of intramembranous particles in the multilamellar structures by freeze-fracture technic. Two distinct types of fracture images were observed: In one type, numerous particles ~10 nm in

diameter were densely packed in a ramdom or linear pattern, whereas in the other type short straight grooves were occasionally observed. I concluded that under the condition of this study, occludin shows a tendency to polymerize into a short strand inside the membrane. These findings provide the first evidence that occludin is an adhesion molecule working at TJ in a homophylic manner.

This study on identification and characterization of occludin, a novel adhesion molecule of TJ, opened a new way to analyze the structure and function of TJ at the molecular level. Further analyses of occludin will lead us to a better understanding how the permeability and polarization of epithelial and endothelial cell sheets are regulated in the near future.

# Chapter 1

## Occludin :A Novel Integral Membrane Protein Localizing at Tight Junctions

## Introduction

In mammalian cells, intercellular junctions are categorized into four types: adherens junctions (AJ), 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/uvomorlin (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 N- and C-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 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 glutaradehyde-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)(Staehlin, 1974). In unfixed-samples, however, the intramembrane strands are reportedly seen as a linear series of individual intramembranous particles (Staehlin, 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, $\alpha$ catenin, and 220kD 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 220kD 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 220kD/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 65kD 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

### Isolation of AJ from Chick Liver

The junctional fraction was prepared from the liver of newly-hatched or 3 day-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 ℃, followed by centrifugation at 100,000 xg 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 xg for 1 h. The final pellet was stored at -80℃ until use for immunization.

### Production of mAbs

Monoclonal antibodies 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.2A). 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 Coomasie 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 $\mu$m) were cut in a cryostat, mounted on glass slides, air-dried, and fixed in 95% ethanol at 4℃ for 30min and in 100% acetone at room temperature for 1min. 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 $\alpha$ catenin mAb ($\alpha$-18) (Nagafuchi et al., manuscript under submission). The second antibody was FITC-conjugated goat anti-rat IgG (TAGO, Inc, Bulmingame, CA) or FITC-conjugated sheep anti-mouse IgG (Amersham, Corp., Arlington Heights, IL). Samples were examined using a fluorescence microscope, a Zeiss Axiophoto 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 con-

taining 1% BSA for 10 min, then mixed and incubated with mAb Oc-1 for 1 h cat room temperature. Samples were washed three times by centrifugation at 10,000 xg 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 xg 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℃, washed twice with 0.1 M cacodylate buffer, postfixed with 1% $OsO_4$/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, then ultrathin sectioned in the frozen state at -110℃ using glass knives with an FC-4E low temperature sectioning system (Reichrt-Jung, Vienna, Austria). The sectioned

samples were collected on carbon-coated formvar-filmed 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, 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, then with 2% polyvinylalchol/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 100kV.

cDNA Library Screening and DNA Sequencing

A λgt11 expression cDNA library made from 7.5 day-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 (Boeringer Mannheim Biochemicals, Indianapolis, IN) and used to screen the same cDNA library using a DIG detection kit (Boeringer 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 DyeDeoxy$^{TM}$ Terminator Cycle Sequencing Kit (Applied Biosystem). 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; Roche). About 5 $\mu$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 Bgl II sites was labeled with [$\alpha$-$^{32}$P]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/5x Denhardt's solution, 5x SSC, 0.5% SDS, 100 $\mu$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 amplified by polymerase chain reaction with primers (GCGGGATCCGTGGTGCAGGAGGGCCTGAC) and (CGAGGTCGACGGTATCG) from FH7 cloned into pBluescript(SK-). Another DNA fragment encoding amino acid residues 185-504 was amplified with primers (CGCGGATCCGTCAACCCGCAGGCGCAGAT) and (TCTAGAACTAGTGGATC) from FH2-9 cloned into pBluescript(SK-). These fragments were digested with BamHI-HindIII and BamHI-PstI, respectively, then introduced into pMAL-cRI (New England Bio-Labs, Bevery, 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$^{TM}$mRNA Capping Kit (STRATAGENE). Capped RNA was translated in a mRNA-dependent rabbit reticulocyte lysate in the presence of [$^{35}$S]methionine using an In Vitro Express$^{TM}$ Translation Kit (STRATAGENE). The [$^{35}$S]Methionine (1300 Ci/mmol, Amersham, Arlington Heights, IL) was present at ~2.6 mCi/ml in a final reaction volume of 25 $\mu$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 ~65kD Membrane Protein Enriched in Isolated AJ 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-day old chicks. From the livers from ~5000 newly-hatched and 3-day 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 65kD 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 with apparent molecular masses of 66, 64, 61, 60, and 58kD 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 four-

five 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 AJ fraction. Furthermore, to check whether this antigen is an integral membrane protein or an undercoat-constitutive peripheral membrane protein, we examined its extractability from the bile canaliculi fraction with 1M acetic acid (pH 2.3), 0.1M NaOH, or low-salt alkali (1mM EGTA, 2mM Tris-HCl; pH 9.2) (without non-ionic detergent) (Itoh et al., 1991). As shown in Fig.2B, 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 ~65kD 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 $\alpha$ catenin, a cadherin-associated protein. Also in the liver, this antigen was highly concentrated at the junctional complex region along bile canaliculi (Fig.4A). 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.4B). In the heart, endothelial cells were selectively stained, although to a significantly weaker degree than that in brain vessels (Fig.4C). 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.5A-C, this antigen was exclusively localized at the TJ zone. Next, the isolated bile canaliculi fraction was used to immunolocalize this antigen at the electron microscopic level. The cytoplasmic surface of TJ was specifically labeled (Fig.5D-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 occlude ).

Isolation and Sequencing of cDNA Encoding Occludin

Using the mAb Oc-1, we screened ~$5x10^5$ plaques from a random-primed λgt11 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 1975 bp, were isolated and sequenced in both directions (Fig.6A).

Two criteria confirmed that these clones encoded occludin. Firstly, we generated fusion proteins encoded by FH7 and by FH2-9 (see Fig.6A). 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.6B, 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 58kD band of occludin (Fig.6C).

The complete nucleotide sequence encoded by the overlapping clones and the predicted amino acid sequence are shown in Fig.7A. In the 1975 nucleo-

tides sequence, the longest open reading frame (ORF), with 1512 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 55.9kD. Judging from the length of the mRNA detected by Northern blotting (see Fig.8), the molecular mass of this protein in SDS-PAGE (~65kD), and the data from the in vitro translation (see Fig.6C), 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 N-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 hydrophathic characteristic of local regions of the sequence as shown in Fig.7B. At the N-terminal half, there are four major hydrophobic, potentially membrane-embedded areas: residues 58-80, 125-148, 159-183, and 228-249. The C-terminal 255 residues exhibit pronounced hydrophilicity and contains some clusters of charged residues; EEEEE at amino acid residues 347-351 and RRGRRRRR 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 ~65kD 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 1M acetic acid, 0.1M 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. (b) The amino 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 N-terminal half. So far, some integral membrane proteins have been reported to contain four potentially membrane-spanning segments; connexins, synaptophysin, proteolipid protein (PLP), 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 aposed 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 N- and C-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) corre-

spond 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 in Fig.5D-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 C-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 aposed 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 C-terminal cytoplasmic domain in this order from the C-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 58kD fragment on the membrane, the obser-

vation that the 58kD 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 58kD polypeptide (see Fig.6C). Of course, it is possible that these five bands are due to some kind of post-translational modification.

Molecular Architecture of TJ

So far, monoclonal antibodies have detected ZO-1 (220kD), cingulin (140kD), 7H6 (155kD), and ZO-2 (160kD) 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 cingulin 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 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 et al., 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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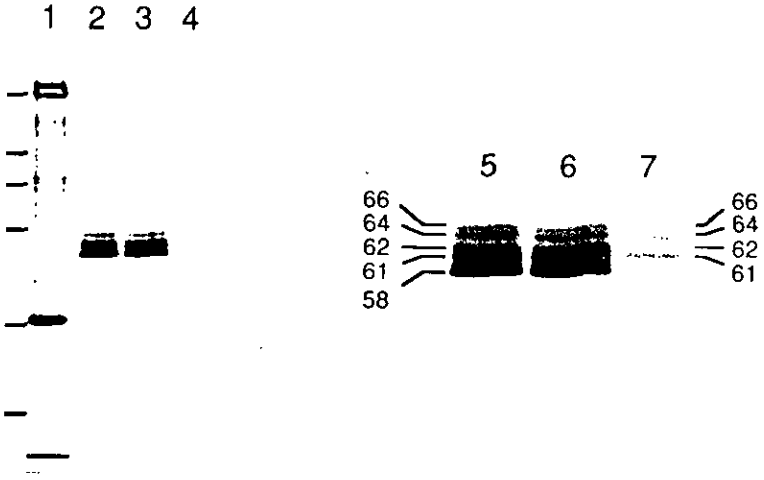


**Figure 1.** Monoclonal antibodies for the ~65kD protein in the junctional fraction. Lane 1, Coomassie brilliant blue-stained gel (12.5%) of junctional fraction; lanes 2,3, and 4, accompanying immunoblots by the use of mAbs Oc-1, Oc-2, and Oc-3, respectively. Bars indicate molecular mass as 200, 116, 97, 66, 45, and 31kD from the top. Close comparison reveals that the banding pattern with mAb Oc-3 (lane 7) is similar to that of mAbs Oc-1 (lane5) and Oc-2 (lane 6) but characteristically lacks the band around 58kD. The same banding patterns were obtained, when the acid-extracted membrane fraction was used.

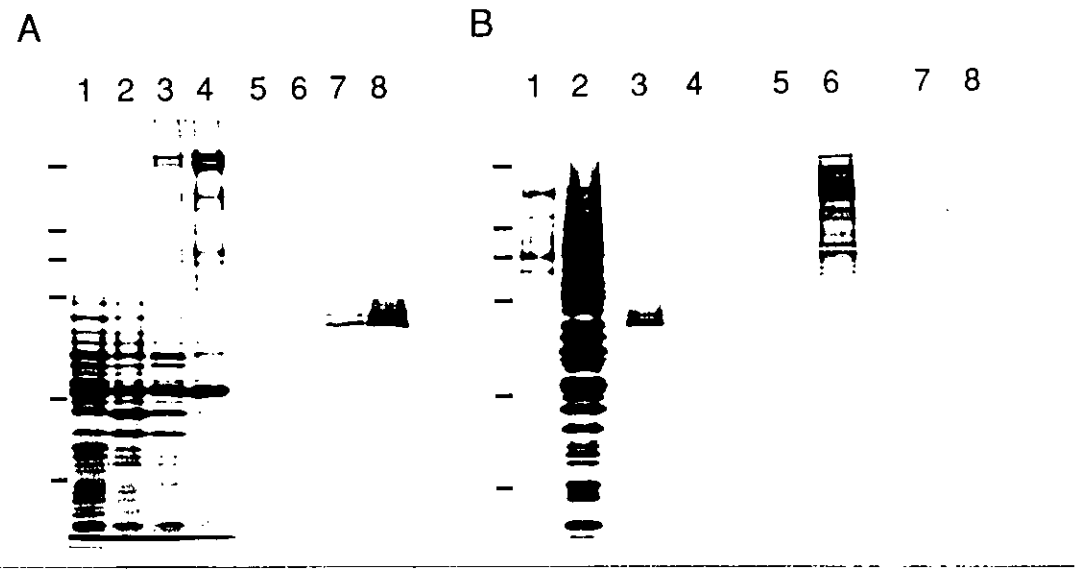


**Figure 2.** Identification and characterization of the ~65kD integral membrane protein by immunoblotting. (A) Enrichment of the antigen in the junctional fraction. Coomassie brilliant blue-stained gels (lanes 1-4) and accompanying immunoblots with mAb Oc-1 (lanes 5-8). Fractions of liver cells (lane 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 110kD 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, Coomassie brilliant blue-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 31kD from the top.

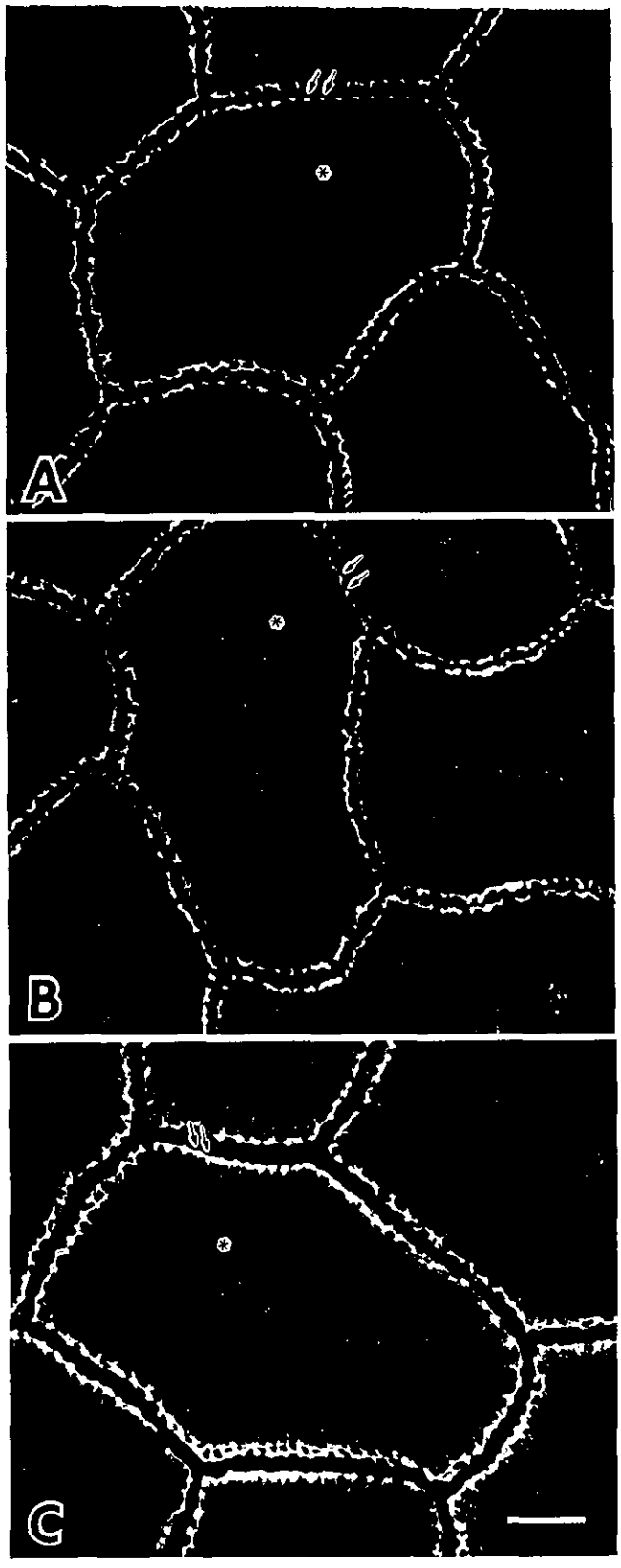


**Figure 3.** Immunostaining of a frozen section of chick intestinal epithelia with mAb Oc-1 (A), anti-ZO-1 mAb (B), and anti-$\alpha$ catenin mAb (C). Intestinal villi are cut transversely and each villus is covered with simple columnar epithelium (between arrows and *). The degree of concentration of the ~65kD membrane protein at the junctional complex region (arrows) is similar to that of ZO-1 and much higher than that of $\alpha$ 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$\mu$m.

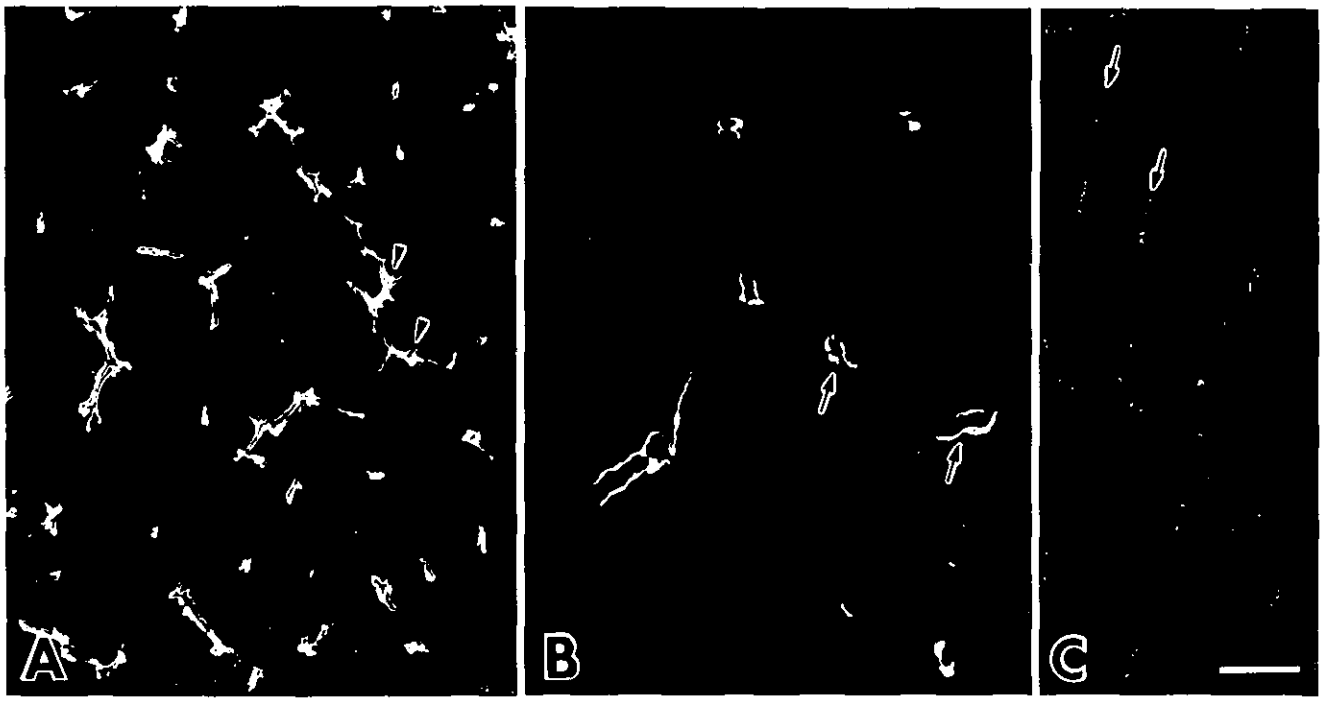


**Figure 4.** Immunostaining of frozen sections of chick liver (A), brain (B), and heart (C) with mAb Oc-2. In the liver, the beltlike junctional complex region is intensely stained with this mAb (arrowheads). In the newly-hatched chick, more than two belts are often present along one bile canaliculus. 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$\mu$m.

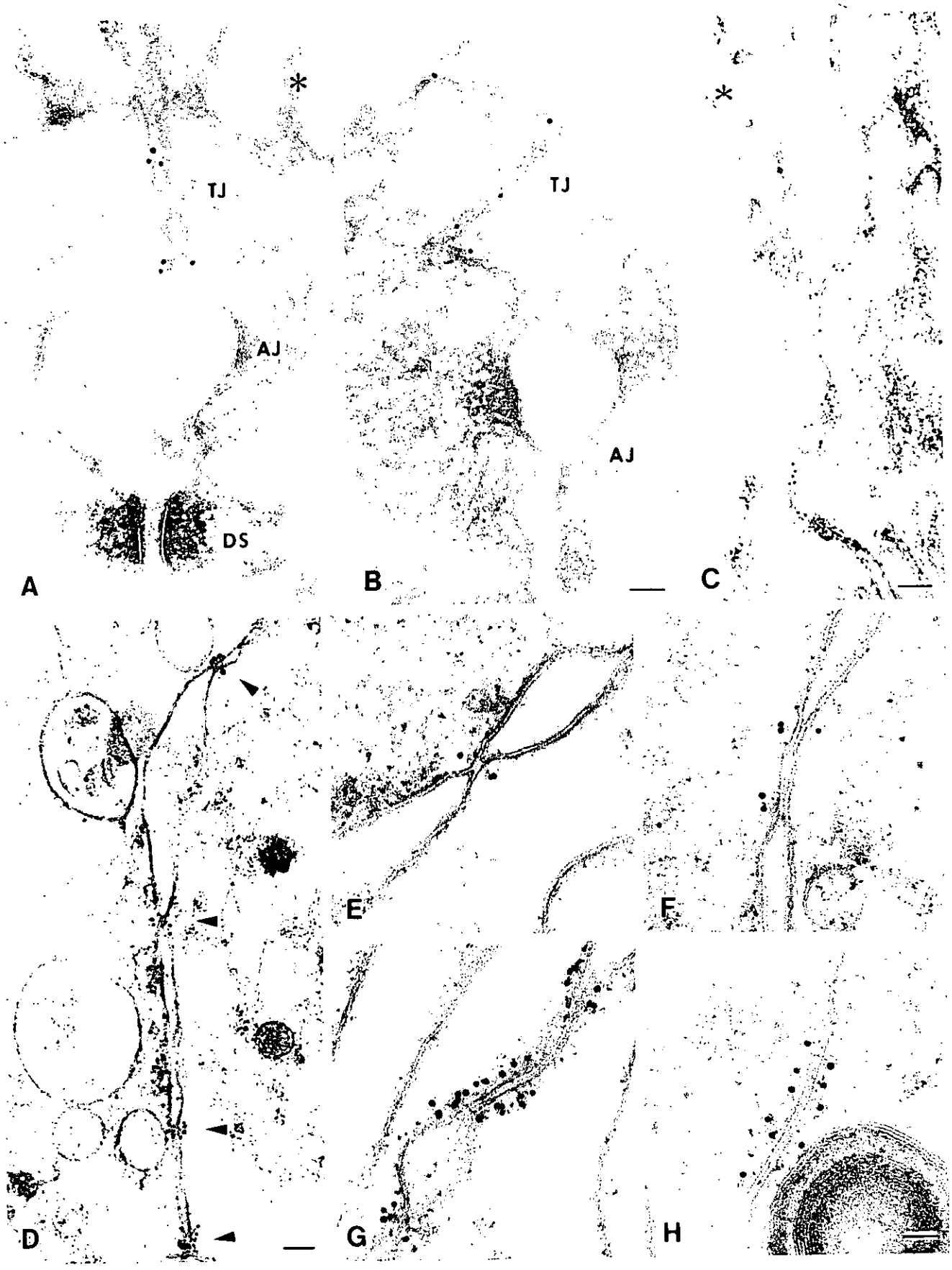
TJ
AJ
DS
A
TJ
AJ
B
C
D
E
F
G
H

**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. *, micorvilli. (D-H) Isolated bile canaliculi were labeled with mAb Oc-1, then with 10nm 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, H). Bars: (A,B) 0.1 $\mu$m; (C) 0.2 $\mu$m; (D) 0.1 $\mu$m; (E-H) 50 nm.

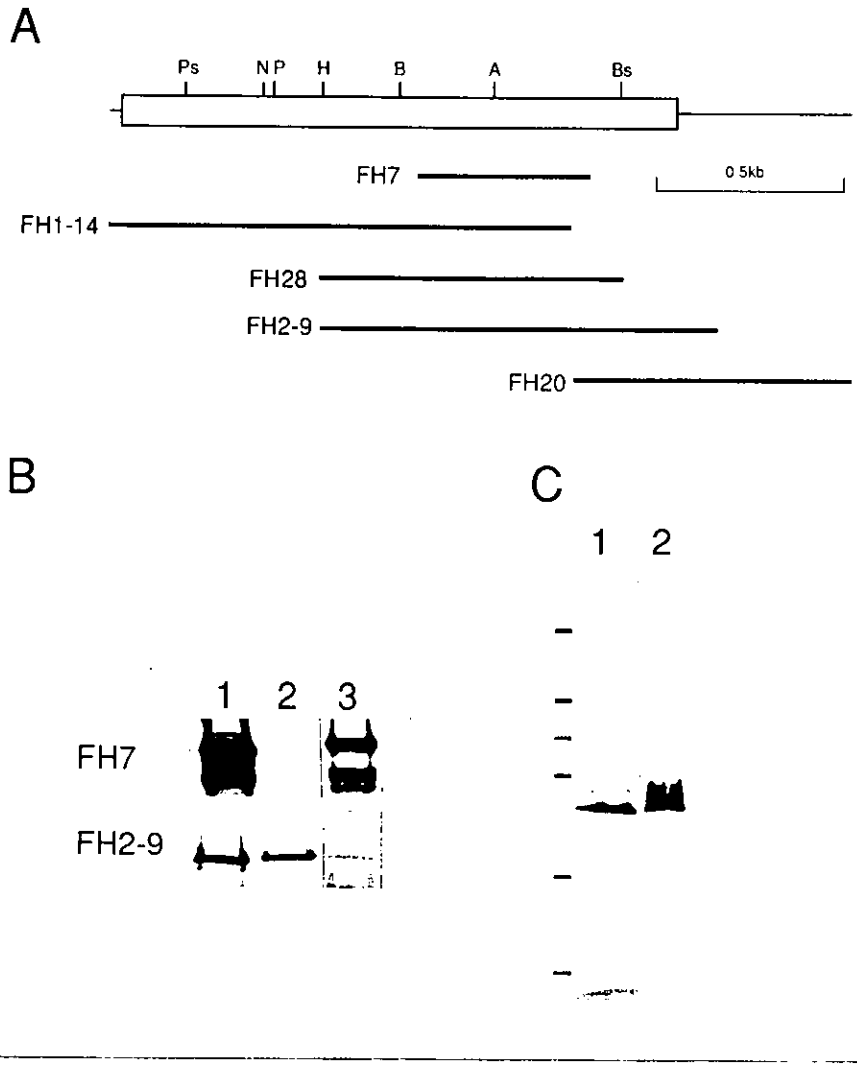


**Figure 6.** Isolation of cDNA encoding chick occludin. (A) Restriction map and cDNA fragments of the chick occludin. The open box indicates the coding region. FH7 was first obtained using mAbs Oc-1 and Oc-3. All clones were obtained from a random primed λgt11 library made from the chick embryo brain. A, ApaI; B, BglII; 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.

A

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CAAAGCGGCGGAGGGCCCACCATGTTCAGCAAGAAGTCCTACGACGGCCCCCCCCGCGGGGTACGGCCCCCCCCACGGGGTACGGCCCCCCCACGGCTGATTACGGCTACGGGTCTCCGCCGC   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

CGGGCTCCTACTACGTGGACGACGCTCCGCAGCTCTTCTACAAGTGGACGTCGCCGCCCGGCGCGGTGCGGGGGCTGCAGGCGGGGGTCCTCGTGCTGTGCATCGCCATCTTCGCCTGCG   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  V    74

TCGCTTCCACGCTCGGCTGGGATTACGGCTACGGCCTGGGGGGGGGCGTACGGCACCGGGCTGGGGGGGTTCTACGGCTCCAACTACTACCGCAGCGGGCTGAGCTACAGCTACGGCTACG   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

GGGGCTACTACGGAGGGGTGAACCAGCGGCACGGCCAACGGCTTCATGATCGCCATGGCCGTGCTGTGCTTCCTGGCCCAGCTGGGCCTGCTGGTGGCGGCGCTCAGCAAATCCGGCGCCA   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

CGCGCTCGCGGCGCTTCTACCTGGCCGTGCTGGTGCTGAGCGCCGTGCTGGCCTTCGTCATGCTCATCGCCTCCATCGTCTACATCATGGGCGTCAACCCGCAGGCGCAGATGTCCAGCG   600
  R  S  R  R  F  Y  L  A  V  L  V  L  S  A  V  L  A  F  V  M  L  I  A  S  I  V  Y  I  M  G  V  N  P  Q  A  Q  M  S  S  G  194

GTTACTACTACAGCCCCTTGTTGGCCATGTGCAGCCAGGCCTACGGCAGCACCTACCTCAACCAGTACATCTACCACTACTGCACCGTGGACCCCCAGGAGGCTGTGGCTGCTGTCTGTG   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  G  234

GGTTCGTCATCGTCATCCTGCTCTGCCTCATCTGCTTCTTCGCCCAGAAGACGCGCAGTAAGATCTGGCGCTACGGCAAAGCCAACATCTACTGGGACCGCGCGCGCCCGTGGTGCAGGAGG   840
  F  L  I  V  I  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

GGCCTGACGTGGAGGAGTGGGTGAAGAACGTGGCGGATGGGGCCAGCGTGCAGGACGAGACGGCCACGCTTGCCTACTCGGAGAAGCCCACCAGCCCTGTCGCCGCCCCCCCCCTACAGCT   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

ACGTGCCCCCCCCCCAGCGCTGGGTACTACCCCTCGGGCACCTACAGCAGCCGGGGCGACCAGCCGGACCGGGCCCTCAGTGCCAGCCCTGTGCATGGGGAGGAGGAGGAGCAGAAGGGGA  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

AGGATCAGCCCAGCAGACCGCCCGCCCGCCGGGGCCGCCGCCGCCGCCGTAACCCCGAGTTGGATGAGTCCCAGTATGAGACCGACTACACCACGGCCGTGGAGTCCAGTGATGAGCGGG  1200
  D  Q  P  S  R  P  P  A  R  R  G  R  R  R  R  R  N  P  E  L  D  E  S  Q  Y  E  T  D  Y  T  T  A  V  E  S  S  D  E  R  D  394

ACCAGGAGCAGTGGGCCAGTCTGTACCCCCCCATCACGTCGGACGGCGCCCCGCCAGCGCTACAAGCAGGAGTTCGACACCGACCTGAAGCGCTACAAGCAGCTCTGTGCTGAGATGGACA  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

GCATCAACGACCGCCTCAATCAGCTCAGCCGACGGCTGGACAGCATCACCGAGGACAGCCCTCAATACCAGGATGTGGCAGAGGAGTACAATCAGCTCAAAGACCTGAACCGGAGCCCAG  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

ACTACCAAAGCAAGAAGCAGGAGAGCAAAGTGCTGCGCAACAAGCTCTTCCACATCAAGCGCATGGTGACCGCCTACGACAAGGTGCGGGGGTAACCAGCACCAGCAGGAGGGGCGGGCA  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

CCCCCACCCCCCCCACCCAAAGACTGTTGTGCATTTTGTACCTTCTTCTTAAAAGAAAAAACACACTCAATCACATCCACCCCCCCCCCAAAGGGGTCACCCCCCCCCAACCCCCCCTTAAC  1680

CCCTCCCACTCCATCCACTGTGAATGCACCCACTGAGTGTTGGGGGCTGCCCCCCCCCCCCCGTGCTGTTATTATGGTAGTGGGGGGGGGGGGGGAGCTGTGTAAGGCCCACACCTCTGGG  1800

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GTGCCTTTGGGGGAGAGGGGGGGTCAGGGCTTTTTGGGAAGGCATTTTGTAAGGC                                                                      1975
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B

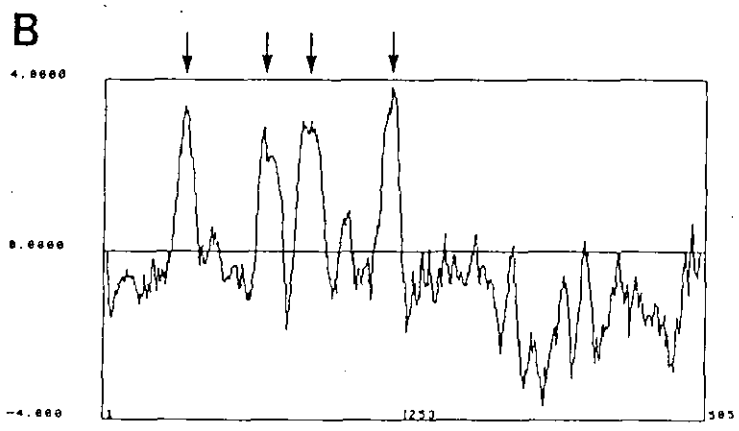


**Figure 7.** Structure of chick occludin. (A) Nucleotide and deduced amino acid sequences of chick occludin. The ORF encodes a 504 amino acid polypeptide with a predicated molecular mass of 55.9 kD. The consensus context for the initiation of translation (Kozak, 1989) (double-underlined) precedes the ATG codon. (B) Hydrophilicity plot for occludin from the Kyte and Doolittle program. The plot records the average hydropyilicity 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 N-terminal half of occludin, there are four major hydrophobic, potentially membrane-embedded areas (arrows in B; underlines in A).


**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$\mu$g/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).

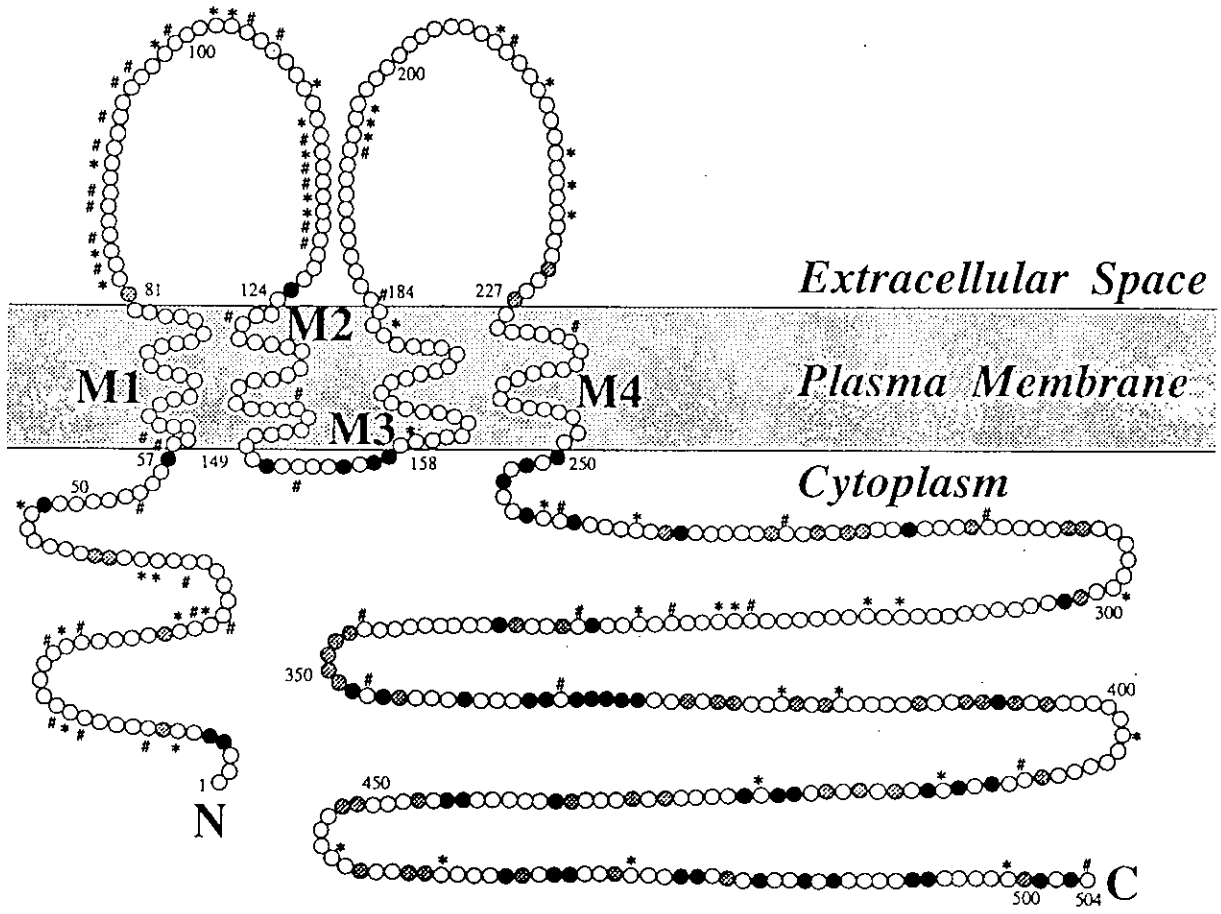


**Figure 9.** Folding model for occludin. A hydrophilicity plot predicted four membrane-spanning domains (M1-M4). The predicted locations of the extracellular, membrane and cytoplasmic regions are indicated. Both N- and C-termini are exposed at the cytoplasmic surface in this model. and represent positively 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.

# Chapter 2

## Direct Association of Occludin with ZO-1 and Its Possible Involvement in the Localization of Occludin at Tight Junctions

## Introduction

The establishment of compositionally distinct fluid compartments by epithelium and endothelium is crucial for the development and function of most organs. Tight junction (TJ), an element of epithelial and endothelial junctional complexes, is directly involved in this compartmentation by sealing cells to create the primary barrier to the diffusion of solutes through the paracelluler pathway (Schneeberger and Lynch, 1992; Gumbiner, 1987, 1993). TJ also functions as a boundary between the apical and basolateral plasma membrane domains, which differ in proteins and lipid composition and physiological functions, to create and maintain epithelial and endothelial cell polarity (Rodriguez-Boulan and Nelson, 1989). Therefore, TJ has been attracting increasing interest among cell biologists.

Accumulating evidence has shown that some unique proteins constitute TJ (Anderson et al., 1993; Citi, 1993). The first protein identified as a TJ constituent was ZO-1 with a molecular mass of 220kD (Stevenson et al., 1986; Anderson et al., 1988). This protein is a peripheral membrane protein that is localized in the immediate vicinity of the plasma membrane of TJ in epithelial and endothelial cells (Stevenson et al., 1986, 1989), whereas it is colocalized with cadherins in cells lacking TJ such as fibroblasts and cardiac muscle cells (Itoh et al., 1991, 1993; Howarth et al., 1992; Tsukita et al., 1992) with some exceptions (Howarth et al., 1994). As a ZO-1-binding protein, another peripheral protein called ZO-2 with a molecular mass of 160kD has been identified (Gumbiner et al., 1991). Unlike ZO-1, the distribution of this protein is restricted to TJ (Jesaitis and Goodenough, 1994). Both ZO-1 and ZO-2 reportedly show sequence similarity to the product of lethal(1)discs large-1 (dlg), one of the tumor

suppressor molecules in Drosophila (Itoh et al., 1993; Tsukita et al., 1993; Willot et al., 1993; Jesaitis and Goodenough, 1994). In addition to ZO-1 and ZO-2, two other TJ-specific peripheral membrane proteins have been so far identified; cingulin and the 7H6 antigen (Citi et al., 1988; Zhong et al., 1993). They are distributed more distantly from the membrane than ZO-1 (Stevenson et al., 1989; Zhong et al., 1993).

To clarify the structure and function of TJ at the molecular level, an integral membrane protein working at TJ should be identified. However, this integral membrane component remained elusive for quite some time. Most recently, we identified using mAbs an integral membrane protein named occludin, which was exclusively localized at TJ both in epithelial and endothelial cells (Furuse et al., 1993). The following structural characteristics of occludin molecules were clarified by cDNA cloning and sequencing (see Fig.2). (1)In the $NH_2$-terminal half, occludin contains four transmembrane domains, which segment the molecule into five domains (domains A-E). (2) A COOH-terminal half (domain E) consisting of ~250 amino acid residues resides in cytoplasm. (3) Charged amino acids mostly locate at domain E. (4) The content of tyrosine and glycine residues is very high in the extracellular domains (domains B and D).

Since occludin has been identified and its cDNA obtained, the following issues on the structure of TJ require resolution. How the newly-synthesized occludin molecules are delivered and localized at TJ; how occludin interacts with TJ-specific peripheral proteins such as ZO-1 and ZO-2, and whether or not the TJ strand is composed solely of occludin molecule. While studying these issues, we identified the importance of the COOH-terminal cytoplasmic domain (domain E) of occludin molecules. In this study, we showed that chick occludin introduced into human and bovine

epithelial cells was correctly delivered to and localized at TJ, and that domain E of occludin was necessary for the localization of the newly-synthesized occludin at TJ. In vitro binding using GST-domain E fusion protein revealed that occludin directly bound to ZO-1, and that domain E was necessary for the occludin-ZO-1 association. Furthermore, we narrowed down the sequences necessary for TJ localization and ZO-1 association, and found that both sequences fell within the same region in domain E. We believe that this type of study will lead us to further understanding of the structure and functions of TJ at the molecular level.

## Materials and Methods

### Cells and Antibodies

Madin-Darby bovine kidney (MDBK) cells and human intestinal epithelial cells (T84) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and the American Type Culture Collection (Rockville, Maryland), respectively. Human esophagus fibroblast primary culture cells (PF-7N) were provided by Dr.T.Iwasawa (Osaka University). MDBK and PF-7N cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. T84 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 5% FCS.

Rat anti-chicken occludin mAb (Oc-2) and mouse anti-rat ZO-1 mAbs (T8-754, T8-109) were obtained and characterized as described (Itoh et al., 1991; Furuse et al.,1993). Rabbit anti-ZO-2 pAb (R9989) was provided by Dr.D.Goodenough (Harvard Medical School). Mouse anti-c-myc mAb was purchased from Oncogene Science,Inc. (Uniondale,NY). Rabbit anti-bovine brain spectrin pAb was purchased from Chemicon International, Inc. (Temecula,Ca).

### Occludin Expression Constructs and Mutants

The expression plasmid (pBATOC) of full-length occludin driven by the chick $\beta$-actin promoter was constructed using two plasmids, pX1 and pBATEM2. To construct pX1, occludin cDNA containing the whole ORF was constructed by combining two cDNA fragments, FH1-14 and FH2-9, at the BglII site (Furuse et al.,1993), and then it was cloned into the EcoRI site of pBluescript SK(-). The BglII-SalI fragment of pBATEM2 (E-cadherin expression vector; Nose et al., 1988) that encodes full-length E-cadherin was replaced with

the BamHI-SalI 1.6kb fragment obtained from pX1 to construct pBATOC.

An epitope-tag of the partial sequence of c-myc (EQKLISEEDL) was linked to the COOH-terminal end of full-length or mutant occludin. For this purpose, we used a plasmid pCMYCB that was constructed by N.Funayama (National Institute for Physiological Sciences, Okazaki, Japan) as follows. An oligonucleotide encoding EQKLISEEDL followed with two stop codons and its complement oligonucleotide were synthesized and annealed. The EcoRI-EcoRV fragment of pBluescript SK(-) was replaced with this DNA fragment to produce pCMYCB.

Expression plasmids for full-length or mutant occludin with c-myc tag were constructed as follows (see Fig.2). DNA fragments encoding entire domain E of occludin or its deletion mutants were produced by PCR using appropriate primers. Using pCMYCB, the oligonucleotide encoding c-myc tag was linked to each PCR product. A fragment between BglII (Ile-255) and SalI (3' non-coding region) sites in pBATOC were replaced with each c-myc-tagged fragment from pCMYCB.

Proteins expressed by these constructs have an additional Glu-Ser before the c-myc tag. Furthermore, mOc/dN358 and mOc/d(445-474) products have additional Glu-Arg-Ser and Glu-Ser at their deletion sites, respectively. All DNA fragments in the plasmids amplified by PCR were sequenced using the Taq DyeDeoxy$^{TM}$ Terminator cycle Sequencing Kit (Applied Biosystem, Foster City, CA) to insure that no errors were introduced during PCR amplification.

DNA Transfections

MDBK and PF-7N cells were transfected with DNA using Lipofectin and Lipofectamine, respectively (GIBCO BRL, Gaitherburg,MD). Cells cultured on

coverslips were washed once with Opti-MEM (GIBCO BRL), and incubated for 5h with 1ml of Opti-MEM containing 1$\mu$g of plasmid DNAs and 10$\mu$l of the reagents followed by the addition of 3ml of normal medium containing FCS. Cells were then cultured until observation.

When T84 cells were transfected with DNA, the efficiency was improved by culturing the cells for over 48h on coverslips in Eagle's MEM containing 50$\mu$M $Ca^{2+}$ (LCM) in the presence of 5% FCS dialyzed against saline. Transfection was performed using Lipofectamine as described above in LCM and saline-dialyzed FCS instead of Opti-MEM and normal FCS, respectively. 24h after transfection, the medium was replaced with the normal medium.

Immunofluorescence Microscopy and Laser Scan Microscopy

Indirect immunofluorescence microscopy of transfected cells was performed as described previously (Itoh et al.,1991; Tsukita et al., 1989). Briefly, about 48h after transfection, cells were fixed with 1% formaldehyde in PBS for 10 min followed by soaking in 0.2% Triton X-100 in PBS for 10 min. The second antibodies were FITC-conjugated goat anti-rat IgG (TAGO,Inc., Burlingame, CA) for Oc-2, rhodamine-conjugated goat anti-mouse IgG (Chemicon,International,Inc.) for T8-754, and FITC-conjugated sheep anti-mouse IgG (Amersham International plc, Bucks,U.K.) for anti-c-myc mAb. Samples were examined using a fluorescence microscope, a Zeiss Axiophot photomicroscope, or a Zeiss Laser Scan Microscope LSM310 (Carl Zeiss, Inc.,Thornwood, NY).

Generation of Fusion Proteins

Occludin-domain E full-length or mutant cDNAs obtained by PCR were introduced into pGEX vectors, pGEX-2T or pGEX-3X (Pharmacia, Inc., Piscataway,

NJ) to express fusion proteins with glutathione-S-transferase (GST) in E-coli (see Fig.5). All constructs except plasmids for GST-OcE and GST-OcE/dN358 have an additional Glu-Phe-Ile-Val-Thr-Asp derived from pGEX vectors at their COOH-terminal ends of fusion proteins. Fusion proteins expressed by a plasmid for GST-OcE/d(445-474) have another additional Glu-Phe at its deletion site. All DNA fragments amplified by PCR were sequenced to insure that no errors were introduced during PCR amplification. Fusion proteins were produced in E.coli (XL-1/Blue) from these constructs according to the procedure described by the manufacturer.

The fusion protein of mouse ZO-1 with maltose-binding protein (MBP) was produced using an F22 fragment (190-1235a.a.) in pMAL-CRI (New England Bio-Labs, Beverly, MA) as described previously (Itoh et al.,1993).

In vitro Binding Assay

In vitro binding assays were performed using a column. Cultures of E.coli expressing GST-fusion proteins (100ml) were collected by brief centrifugation and resuspended in 6ml of solution K [140mM KCl, 10mM HEPES (pH7.5), 1mM $MgCl_2$, 2$\mu$g/ml leupeptin, 1mM p-amidinophenyl methanesulfonyl fluoride hydrochloride (pAPMSF)]. After sonication and centrifugation at 10,000g for 10 min, the supernatant was applied to a column containing glutathione-Sepharose 4B beads (Pharmacia, Inc., Piscataway, NJ), which was washed with 20 volume of solution K. Thereafter the low salt extract of chick junctional fraction or high salt extract of MDBK cells (see below) was applied onto the column. After washing with 40 volume of solution K, bound proteins were eluted with 50mM Tris-HCl buffer (pH8.0) containing 10mM glutathione. Fractions of 0.8ml were collected. Since almost all the bound proteins were eluted within the first 5 fractions, they were mixed

and used for SDS-PAGE and immunoblotting.

To detect $\alpha$-spectrin or MBP-ZO-1 fusion protein, binding assays were performed by means of the batch method. GST-fusion proteins of occludin were incubated with glutathione beads at 4℃ for 1h. After 5 washes with 10 volumes of solution K by brief centrifugation, the beads were incubated with the high salt extract of MDBK cells or the extract of E.coli containing MBP-ZO-1 fusion protein at 4℃ for 1h. The beads were then washed 5 times with solution K, and the excess solution was removed. Bound proteins were released from beads with SDS-PAGE sample buffer.

The low salt extract of chick junctional fraction was prepared as described previously (Furuse et al.,1993, Tsukita and Tsukita,1989). The extract from ~40 chicks was used in one experiment. The low salt extract was freeze dried and resolved in 1ml of solution K followed by centrifugation at 100,000g for 1h. The supernatant was used for the binding assay. The high salt extract of MDBK cells was prepared according to the method for purifying ZO-1 from mouse brain as described (Itoh et al.,1991). Confluent MDBK cells from two 15cm dishes were scraped and collected by brief centrifugation. They were homogenized in 1mM $NaHCO_3$ or in solution K with tight fitting Dounce homogenizer followed by centrifugation at 100,000g for 1h. The pellet was resuspended by sonication in 1ml of 10mM HEPES buffer (pH7.5) containing 1M KCl, 1mM EGTA, 2$\mu$g/ml leupeptin, 0.5mM PMSF, then incubated on ice for 1h. After centrifugation at 100,000g for 1 h, the supernatant was diluted with 10mM HEPES buffer (pH7.5) at a final concentration of 140mM KCl. Aggregated proteins were removed by centrifugation at 10,000g for 10 min, and the supernatant was used for the binding assay. The extract containing MBP-ZO-1 fusion protein was prepared by the same procedure as that for GST-occludin fusion

proteins.

Gel Electrophoresis and Immunoblotting

One-dimensional SDS-PAGE (12.5% gel) was based on the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue R-250 or using silver staining kit (Wako Pure Chemical Industries, Osaka, Japan). For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose sheets, which were then incubated with the antibodies. The antibodies were detected with a blotting detection kit (Amersham).

## Results

### Localization of Chick Occludin Expressed by a Full-length cDNA at Tight Junctions in Human and Bovine Epithelial Cells

Chick occludin cDNA clones were isolated and sequenced (Furuse et al., 1993). To construct the expression vector, a 1.6-kb cDNA encoding full-length occludin was assembled from two overlapping clones. This cDNA encodes a 55.9kD occludin polypeptide (Furuse et al., 1993). The complete cDNA was subcloned into a mammalian expression vector driven by the $\beta$-actin promoter, which was then introduced into cultured cells.

The bovine and human epithelial cell lines, MDBK and T84, were selected for our transfection studies. Both types of cells bear the typical junctional complex including TJ at the most apical portion of the lateral membranes. To detect the transiently-expressed chick occludin by immunofluorescence microscopy, we used rat anti-chick occludin mAb, Oc-2, which did not recognize human and bovine occludin (Furuse et al., 1993).

MDBK cells transfected with plasmids encoding full-length occludin displayed a characteristic pattern of fluorescence. In addition to the diffuse staining at perinuclear cytoplasm, the concentration of expressed chick occludin was detected in a linear fashion at the cell-cell border (Fig.1a). When the transfectants were doubly stained with anti-occludin mAb and anti-ZO-1 mAb, ZO-1 appeared to be colocalized with the expressed chick occludin concentrated at the cell-cell border (Fig.1b). Therefore, to precisely compare the distribution of occludin with that of ZO-1, we analyzed the doubly-stained T84 transfectants by laser scan microscopy (LSM) (Fig.1c,d). As shown in Fig.1e,f as overlaid computer-generating cross-sectional images, the expressed chick occludin and ZO-1 were precise-

ly colocalized at the most apical region of lateral membranes. In our LSM system, tight and adherens junctions can be resolved (Yonemura et al., 1994). All these observations together led us to the conclusion that the expressed chick occludin was correctly delivered to and concentrated at TJ in human and bovine epithelial cells.

The question naturally arose whether or not occludin expressed in non-epithelial cells lacking TJ were delivered to cell-cell contact sites. Chick occludin was then introduced into the human fibroblast, PF-7N. As reported (Itoh et al., 1993), ZO-1 was concentrated at cell-cell contact sites in fibroblasts. The introduced occludin was concentrated at some of these ZO-1-enriched cell-cell contact sites in cells expressing a large amount of occludin (Fig.1g,h).

Sequences in the COOH-terminal Cytoplasmic Domain of Occludin Necessary for Localization at Tight Junctions

To analyze the role of COOH-terminal cytoplasmic domain of occludin molecules (domain E;amino acid residues 250-504) in their localization at TJ, we performed transfections with various domain E deletion mutants. Since the efficiency of transfection was significantly higher in MDBK than in T84 cells, we used the former in the following mutant occludin expression studies. We designed an expression vector with two major considerations: Immunofluorescence microscopy would be used and the sequences encoding the antigenic determinant of the protein should not be lost during the construction of deletion mutants. To accomplish these goals, a 30-bp sequence encoding a portion of c-myc was added to the 3'end of each cDNA construct, allowing us to detect the expressed protein by anti-c-myc mAb.

To narrow down the sequence necessary for the TJ localization of occlu-

din from both COOH- and $NH_2$-terminal sides, we constructed several COOH- or $NH_2$-terminal truncations of domain E from full-length occludin with the c-myc epitope on their COOH-terminal end (Fig.2). As shown in Fig.3a, the c-myc-tagged full-length occludin (mOc) was transiently expressed and localized at TJ in MDBK cells, indicating that the tag peptide did not interfere with the concentration of occludin at TJ. All COOH-terminal truncations constructed here (mOc/dC474, mOc/dC444, mOc/dC414, and mOc/dC357) failed to localize at TJ (Fig.3b-e), indicating that the responsible sequence could not be narrowed down from the COOH-side. By contrast, the $NH_2$-terminal truncation, mOc/dN358, was clearly concentrated at TJ, although its localization efficiency was rather lower than that of mOc (Fig.3f). The further $NH_2$-terminal truncation, mOc/dN387, was by no means localized at TJ (Fig.3g).

Taking all results together, we concluded that amino acid residues 358-504 (domain E358/504) is necessary for the TJ localization of occludin, although detailed analysis of this domain remains to be performed. For example, mOc/d(445-474) was not concentrated at TJ (see Fig.2;Fig.3h).

Association of Tight Junction Peripheral Proteins with the COOH-terminal Cytoplasmic Domain of Occludin

Another possible function of the COOH-terminal cytoplasmic domain (domain E) of occludin is its association with TJ peripheral proteins such as ZO-1, ZO-2 etc. To test this association in vitro, domain E was expressed in E.coli as a fusion protein with GST (GST-OcE). E.coli lysate was incubated with glutathione-Sepharose beads on a column, and after washing, the low salt alkali extract of junctional fraction isolated from chick liver was applied onto the column. After incubation and washing, the

proteins associated with GST-OcE coupled to glutathione-Sepharose beads were eluted with a solution containing glutathione, then resolved by SDS-PAGE.

As shown in lane 2 of Fig.4A, this in vitro binding assay revealed two major bands with molecular masses of 220kD and 160kD bound to domain E of occludin. The 220kD band was specifically recognized by anti-ZO-1 mAb (Fig.4A, lane6). The molecular mass of the 160kD band indicated that it would be related to ZO-2, a ZO-1 binding protein identified in canine (Gumbiner et al., 1991). Since a mAb recognizing chick ZO-2 was not available, we prepared a high-salt extract from the membranes of cultured MDBK cells, from which ZO-2 is recognized by the pAb R9989 produced by Jesaitis et al. (1994). Occludin-binding proteins were recovered from the extract using the column system described above. Immunoblots of these proteins revealed that ZO-2 as well as ZO-1 were bound to domain E of occludin (Fig.4B). This suggests that the 160kD band from the chick junctional fraction is the chick homologue of ZO-2. Furthermore, as shown in Fig.4B, $\alpha$-spectrin from the high salt extract of MDBK cells, was also specifically trapped by the GST-OcE beads.

Sequences in the COOH-terminal Cytoplasmic Domain of Occludin Necessary for Association with ZO-1

The question has naturally arisen whether the association of occludin with ZO-1 is required for TJ localization of occludin. We attempted to narrow down the sequence necessary for the association of occludin with ZO-1 from both COOH- and $NH_2$-terminal sides, and evaluate whether or not this domain is included in or overlapped with the domain E358/504 which is necessary for TJ localization of occludin. We expressed several GST-fusion proteins containing COOH- or $NH_2$-terminal truncations of domain E in E.coli (Fig.5).

Using these fusion proteins and the extract of junctional fraction, we performed in vitro binding studies, in which the amount of ZO-1 molecules bound to a fixed quantity of GST-fusion proteins was evaluated by immunoblotting.

As shown in Fig.6, all COOH-terminal truncated fusion proteins (GST-OcE/dC474, GST-OcE/dC444, GST-OcE/dC414, and GST-OcE/dc357) exhibited no or remarkably weaker binding to ZO-1 than GST-OcE, indicating that the sequence necessary for the ZO-1 binding could not be narrowed down from the COOH-terminal side. On the other hand, the $NH_2$-terminal truncated fusion proteins, GST-OcE/dN358, strongly bound to ZO-1, whereas the further-truncated fusion proteins, GST-OcE/dN387, showed very poor binding.

Therefore, this bidirectional strategy identified amino acid residues 358-504 (domain E358/504) as the region necessary for the association of occludin with ZO-1. The same results were obtained, when the high salt extract from MDBK and T84 cells was applied onto the column (data not shown). This conclusion is similar to that of the analysis of sequences necessary for the TJ localization of occludin, but in sharp contrast, GST-OcE/d(445-474) strongly bound to ZO-1.

Finally, to evaluate whether ZO-1 is associated with occludin directly or indirectly, we performed the in vitro binding studies between various GST-OcE mutant proteins and a MBP-ZO-1 fusion protein produced in E.coli. As shown in Fig.7, the MBP-ZO-1 fusion protein bound strongly to GST-OcE, GST-OcE/dN358, and GST-OcE/d(445-474), but very weakly to the other GST-OcE mutant proteins. These data indicated that ZO-1 directly associates with the domain E358/504 of occludin.

**Discussion**

In our previous study, we identified a novel integral membrane protein with an apparent molecular mass of ~65kD called occludin, and showed by immunofluorescence and immunoelectron microscopy that it is localized exclusively at TJ of various types of epithelial and endothelial cells (Furuse et al., 1993). Preembedding electron microscopic immunolabeling of isolated bile canaliculi with anti-occludin mAb characteristically revealed immunogold particles directly over the points of membrane fusion of TJ, suggesting that occludin is a component of the TJ strand. In this study, we demonstrated by means of transfection, that the newly-synthesized chick full-length occludin was delivered to and incorporated into preexisting TJ in human and bovine epithelial cells. Also in fibroblasts lacking TJ, the introduced chick occludin was occasionally localized at cell-cell contact sites, although it remains to be checked electron microscopically whether or not TJ-like structures are formed there. Further analyses using these transfection systems will lead us to a better understanding how the cell polarity is formed and maintained in epithelial and endothelial cells.

In addition to the TJ localization mechanism, the isolation of occludin cDNA enabled us to analyze the interaction of occludin with TJ peripheral proteins at the molecular level. Based on the distance from the plasma membrane, TJ peripheral proteins can be subclassified into two categories (Anderson et al., 1993; Citi, 1993). The first class includes ZO-1, which is localized in the immediate vicinity of membranes (Stevenson et al., 1986; Anderson et al., 1988; Stevenson et al., 1989; Itoh et al., 1991, 1993). Immunoprecipitation studies have shown that ZO-1 forms a protein complex with another peripheral protein, ZO-2 (Gumbiner et al., 1991;

Jesaitis and Goodenough, 1994). Therefore, it is likely that ZO-2 is also localized just beneath the plasma membrane. The second class includes cingulin and the 7H6 antigen, which are localized over 40nm from the plasma membrane (Citi et al., 1988, 1993; Zhong et al., 1993; Stevenson et al., 1989). In this study, we showed that the GST-fusion protein (GST-OcE) of the COOH-terminal cytoplasmic domain of occludin (domain E) specifically associated with at least 220kD and 160kD bands among the various membrane peripheral proteins in the junctional fraction extract. Taking our immunoblotting data into consideration together with previous data, we concluded that the two bands corresponded to ZO-1 and ZO-2. Furthermore, we demonstrated that the MBP-ZO-1 fusion protein directly bound to the domain E of occludin, at least in vitro. Therefore, we concluded that ZO-1 is directly bound to the domain E of occludin, and that ZO-2 may be associated with occludin through ZO-1. This conclusion is highly consistent with the notion that ZO-1 and ZO-2 are localized just beneath the plasma membrane of TJ.

We showed that spectrin tetramers are associated with ZO-1 at ~10-20 nm from their midpoint (Itoh et al., 1991). Also in this study, spectrin from MDBK cells was specifically trapped by the GST-occludin column. Thus there may be a molecular linkage between occludin and actin filaments as shown in Fig.8, since an intimate spatial relationship between TJ and actin-based cytoskeletons has been observed (Madara, 1987). The reason why occludin has not been so far identified in ZO-1 immunoprecipitates is not clear, but it may be partly due to the insolubility of occludin against non-ionic detergents and partly due to the very low expression level of occludin in cells.

All the data obtained from two series of experiments with various do-

main E deletion mutants are summarized in Fig.9. The sequences in domain E (amino acid residues 250-504) necessary for the TJ localization or ZO-1 association of occludin were narrowed down from COOH- and $NH_2$-terminal sides. Neither TJ-localization nor ZO-1-association responsible sequences could be defined from COOH-terminal side, whereas both sequences were narrowed down as far as amino acid residue 358 from the $NH_2$-terminal side. This coincidence led to the speculation that the ZO-1 binding ability of occludin is required for its TJ localization. Actually, as far as we examined in a combination of in vivo transfection experiments and in vitro binding assays, all deletion mutants with significantly-reduced ZO-1 binding ability lacked TJ localization ability. However, the converse was not so. All deletion mutants bearing sufficient ZO-1 binding ability did not bear TJ localization ability. For example, an occludin mutant lacking amino acid residues 445-474, bound to ZO-1 in vitro, but were not concentrated at TJ. Therefore, we speculate that the association of not only ZO-1 but also other factors to the 358-504 a.a. sequence of domain E (domain E358/504) is required for the localization of occludin at TJ. At present, it is not clear why ZO-1 association is at least required for the TJ localization. It may be required for the targeting of occludin to membranes, assembly of occludin into TJ strands, or retention of occludin on membranes. These should be clarified in the near future.

Two distinct types of small rab GTPases, rab13 and rab3B, are reportedly concentrated at TJ in epithelial cells (Zahraoui et al., 1994; Weber et al., 1994). Considering that the rab family members are involved in membrane traffic in general (Bourne, 1988; Goud and McCaffrey, 1991; Zerial cand Stenmark, 1993), it is likely that these rab13 and rab3B play an important role in the assembly of TJ, that is, the targeting of occludin.

Therefore, we should evaluate whether or not these small rab GTPases can interact with the domain E of occludin directly or indirectly, and should further search for other factors that regulate the TJ localization of occludin by interacting with domain E. Also, we should obtain information about the functions of the other cytoplasmic domain of occludin (domains A,C), although they are somewhat shorter than the domain E. Identification of cytoplasmic proteins directly associated with occludin and assignment of their binding domains on occludin molecules will give us a wealth of information for the future studies not only on the structure but also on the functions of tight junctions. For example, it is possible that some of the deletion occludin mutants used in this study behave as dominant negative mutants and interfere with the functions of tight junctions. We believe that further analyses of the cytoplasmic domains of occludin will lead us to a better understanding of the structure and functions of TJ at the molecular level.

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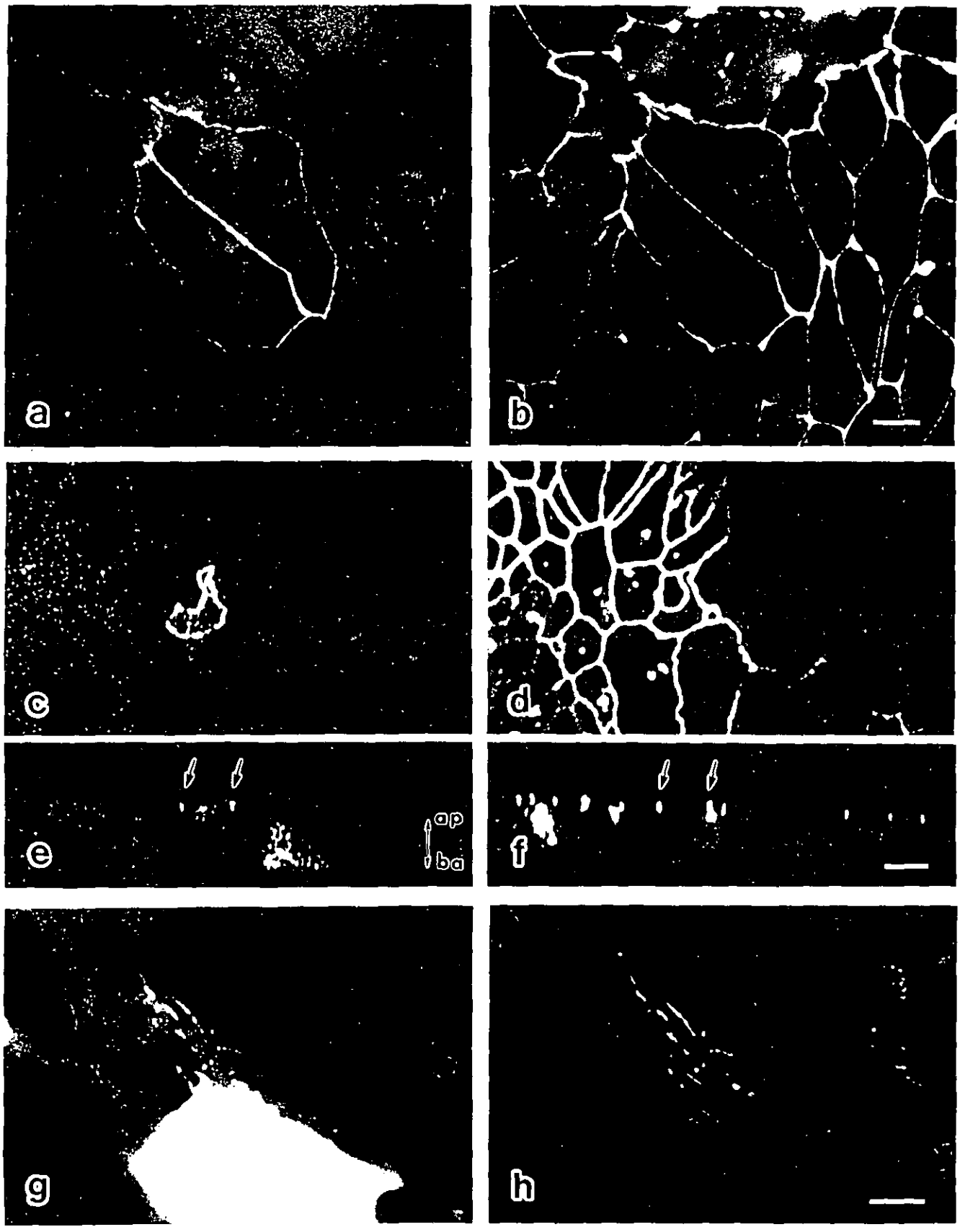
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**Figure 1.** Subcellular distribution of chick full-length occludin in transient transfectants. Bovine and human epithelial cells, MDBK (a,b) and T84 (c-f), respectively, or human fibroblasts, PF-7N (g,h) were transfected with plasmids encoding full-length occludin, and then doubly stained with anti-chick occludin mAb, Oc-2 (a,c,e,g) and anti-rat ZO-1 mAb, T8-754 (b,d,f,h). Oc-2 recognizes chick occludin but neither bovine nor human occludin, whereas T8-754 crossreacts with both human and bovine ZO-1. (a,b) Conventional immunofluorescence microscopic images of MDBK cells. The transiently-expressed chick occludin and bovine ZO-1 were co-concentrated at the cell-cell border. The cytoplasmic staining with anti-occludin mAb is specific, and this may be a result of overexpression of chick occludin. (c-f) Laser scan microscopic images of T84 cells. Optical sections at the level of the junctional complex (c,d) and computer-generated cross-sectional images (e,f). The expressed chick occludin and human ZO-1 were precisely colocalized at the most apical region of lateral membranes (arrows). ap, the level of apical surface; ba, the level of basal membrane. (g,h) Conventional immunofluorescence microscopic images of fibroblasts. Most of the expressed chick occludin was distributed in the cytoplasm, but some of them was colocalized with ZO-1 at cell-cell contact sites. Bar, 10$\mu$m.

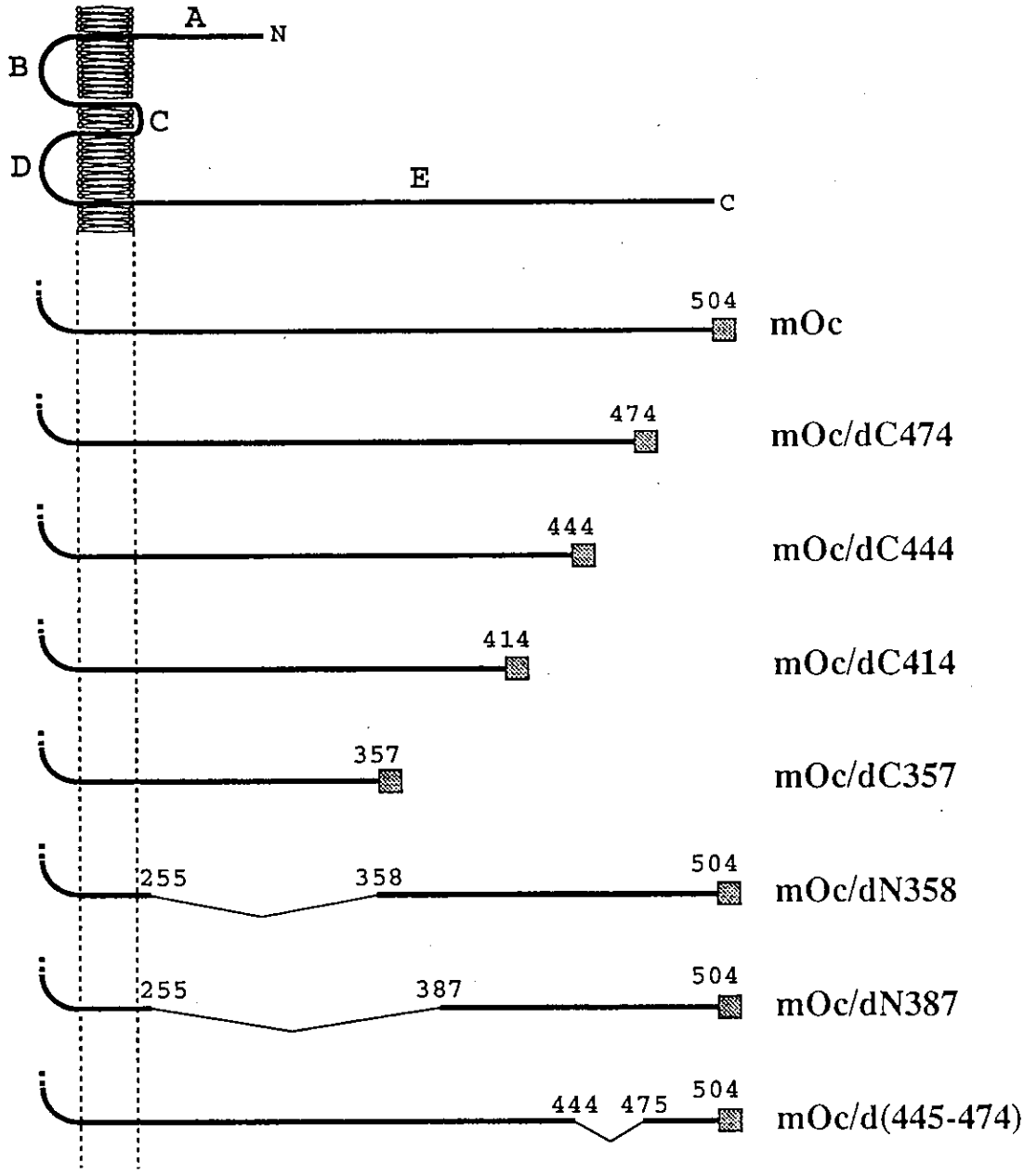


**Figure 2.** Full-length and truncated occludin constructs with the c-myc epitope on their COOH-terminal end ( ▩ ). Occludin is segmented into five domains (A-E) by four transmembrane domains, and both COOH- and $NH_2$-terminal truncations of domain E from full-length occludin were constructed.

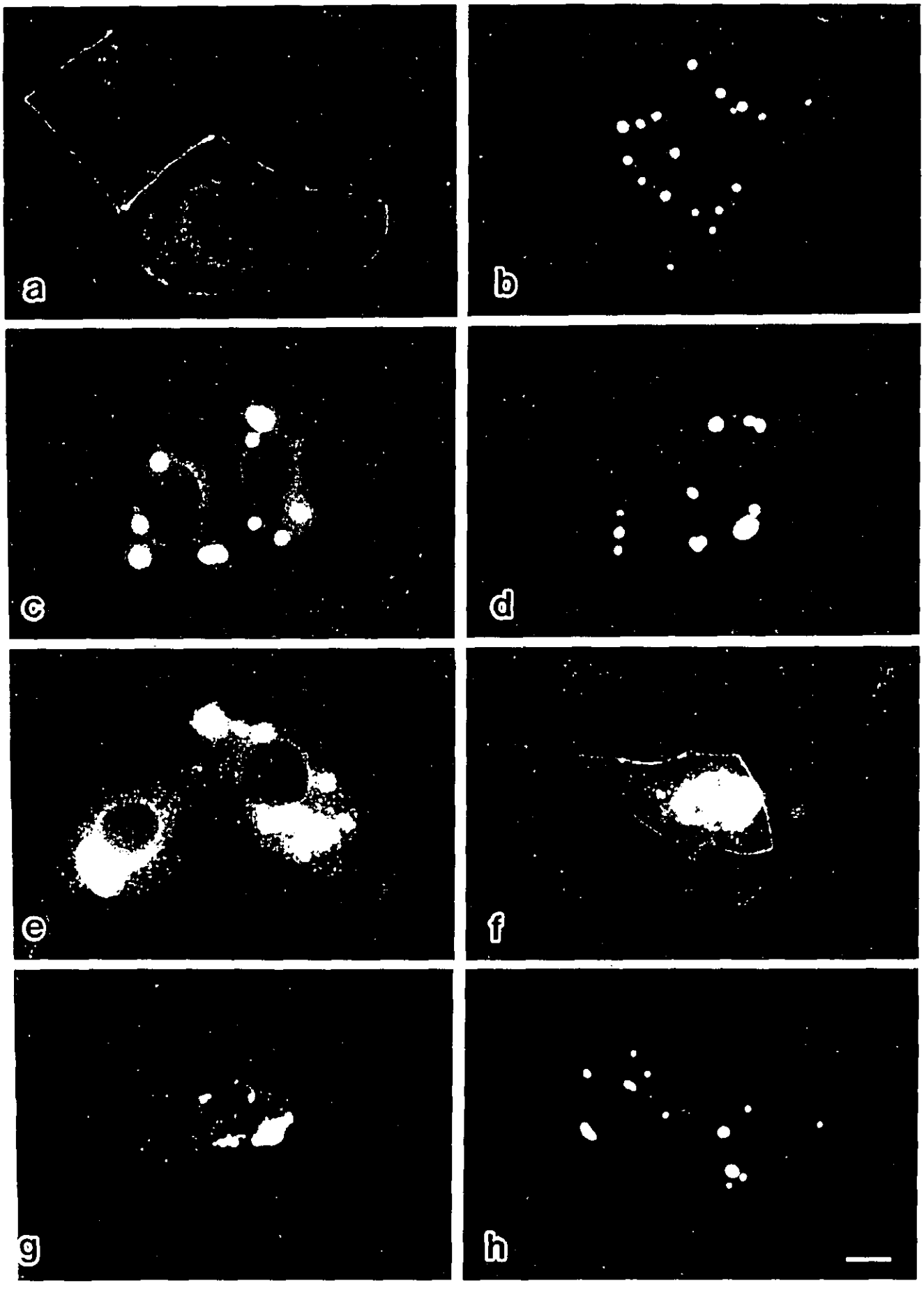
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**Figure 3.** Subcellular distribution of c-myc-tagged full-length and truncated chick occludin in transient MDBK transfectants. MDBK cells were transfected with plasmids of mOc (a), mOc/dC474 (b), mOc/dC444 (c), mOc/dC414 (d), mOc/dc357 (e), mOc/dN358 (f), mOc/dN387 (g), or mOc/d(445-474) (h), and then immunofluorescently stained with anti-c-myc mAb. Only in a and f, intense signal was detected from the cell-cell border. In the other transfectants, the expressed truncated occludin was concentrated at the cytoplasm, and formed large spheres. Even in the transfectants expressing a large amount of mOc and mOc/dN358 products, the products were concentrated not only in TJ but also in similar spheres in the cytoplasm (data not shown). Bar, 10$\mu$m.

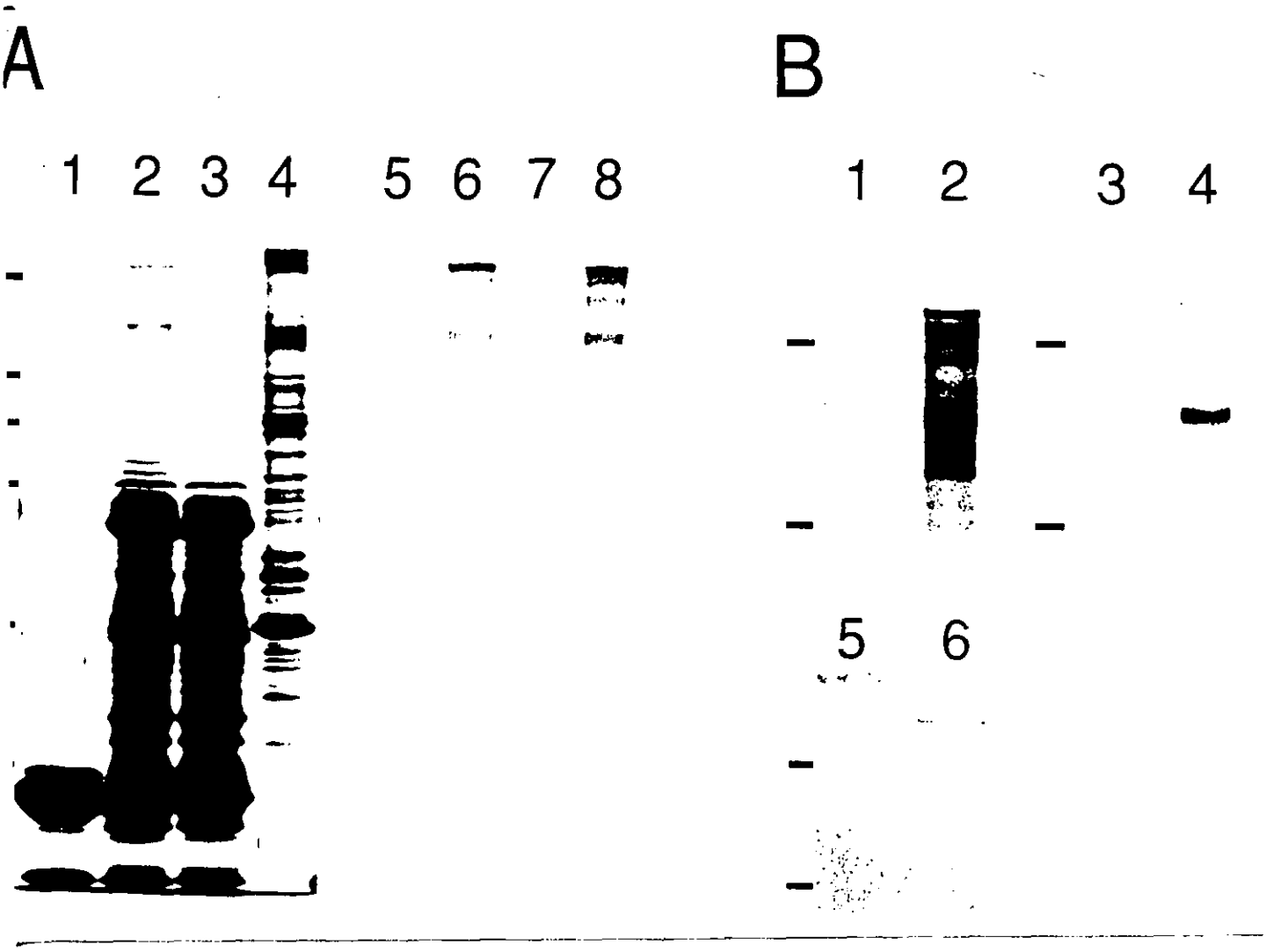


**Figure 4.** Association of tight junction peripheral proteins with domain E of occludin. (A) In vitro binding of GST-OcE fusion protein to proteins in the low salt alkali extract of junctional fraction isolated from chick liver (JF extract). Domain E of occludin was expressed in E.coli as a fusion protein with GST (GST-OcE), and E.coli lysate was incubated with glutathione-Sepharose beads on a column. After washing and application of JF extract onto the column, the proteins associated with GST-OcE were eluted with a solution containing glutathione. Silver-stained gel (lane 1-4) and accompanying immunoblot with anti-ZO-1 mAb, T8-754 (lane 5-8) of glutathione-eluate from GST column incubated with JF extract (lane 1,5), glutathione-eluate from GST-OcE column incubated with JF extract (lane 2,6), glutathione-eluate from GST-OcE column without the incubation with JF extract (lane 3,7), and JF extract (lane 4,8). Comparison between lanes 1-3 revealed that two major bands of ~220kD and ~160kD bound to domain E of occludin (arrowheads), and immunoblot analyses identified the former bands as ZO-1. The mobility of molecular mass markers is shown at the left (200, 116, 97, 66, 45, 31kD from the top). (B) In vitro binding of GST-OcE fusion protein to proteins in the high salt extract of MDBK cells. Immunoblots with anti-ZO-1 mAb, T8-754 (lane 1,2), anti-ZO-2 pAb, R9989 (lane 3,4) and anti-$\alpha$-spectrin pAb (lane 5,6) of glutathione-eluate from GST beads incubated with MDBK extract (lane 1,3,5) and glutathione-eluate from GST-OcE beads incubated with MDBK extract (lane 2,4,6). Note that $\alpha$-spectrin as well as ZO-1/ZO-2 were specifically trapped by domain E of occludin. The mobility of molecular mass markers is shown at the left (200, 116kD from the top).

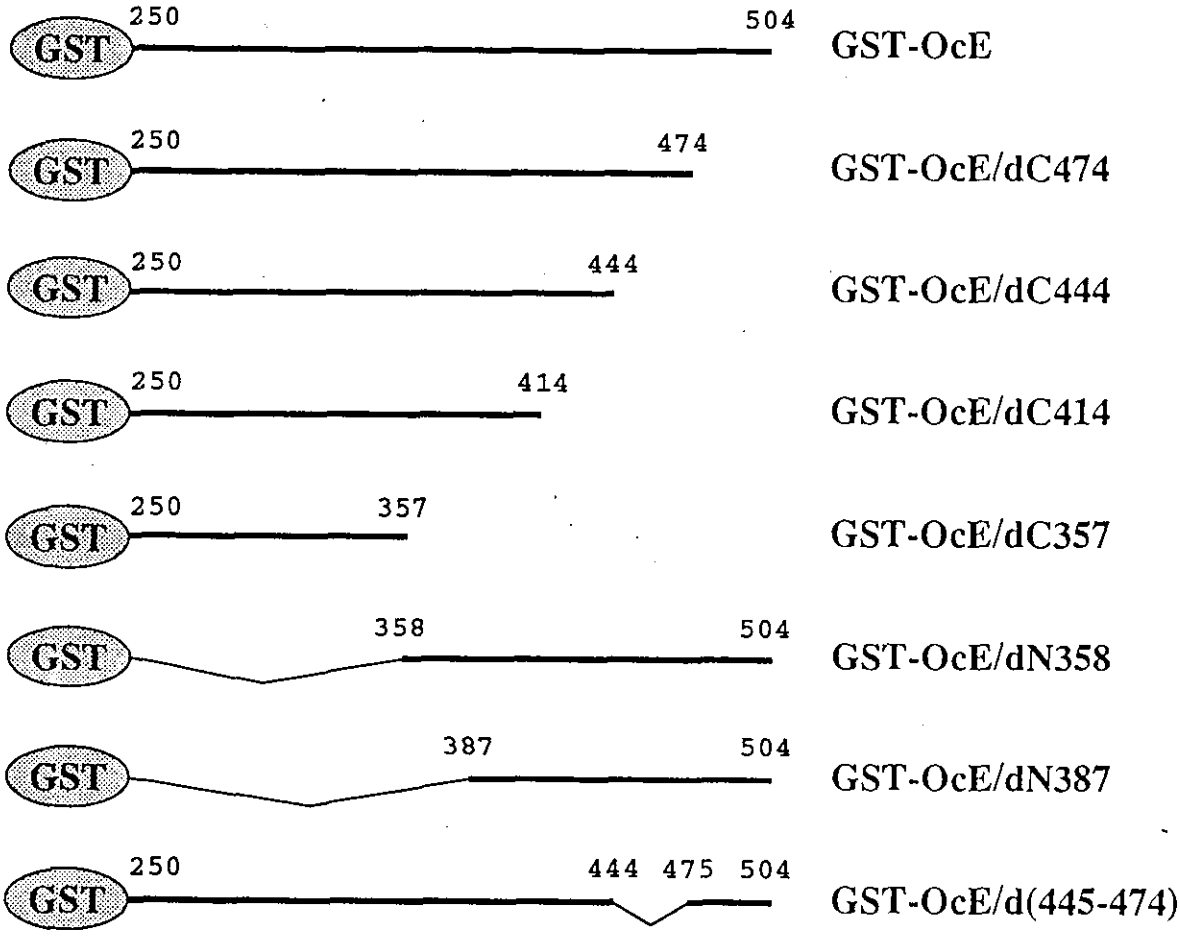


**Figure 5.** GST-fusion proteins containing normal or truncated domain E of occludin.

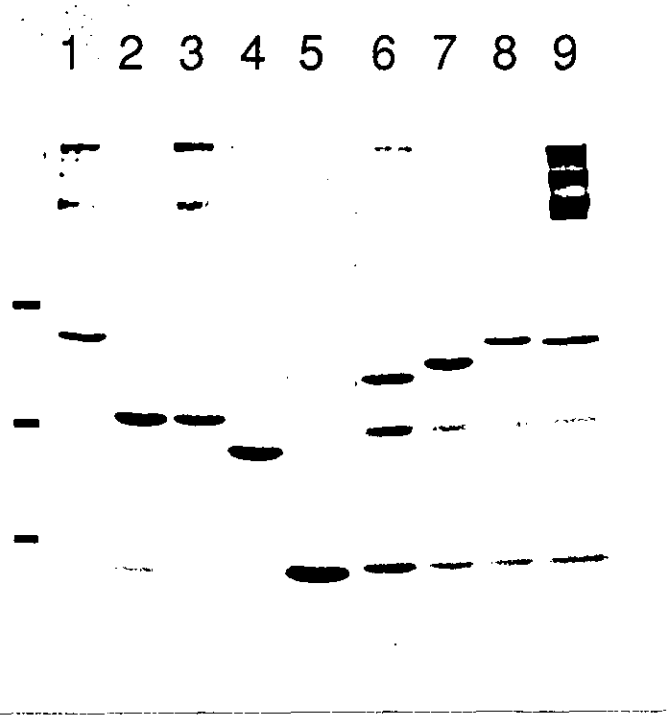


**Figure 6.** Association of normal and truncated domain E of occludin with ZO-1 in the low salt alkali extract of isolated junctional fraction from chick liver. Columns were constructed using normal or truncated GST-OcE fusion proteins (see Fig.5). After application of the low salt alkali extract, proteins trapped by each column were eluted with a solution containing glutathione. After each eluate was resolved by SDS-PAGE, the amount of GST-fusion protein and ZO-1 was evaluated by Coomassie brilliant blue staining (bottom) and immunoblotting with anti-ZO-1 mAb, T8-754 (top), respectively. (Lane 1) GST-OcE;(lane 2) GST-OcE/dC357;(lane 3) GST-OcE/dN358;(lane 4) GST-OcE/dN387;(lane 5) GST;(lane 6) GST-OcE/dC414; (lane 7) GST-OcE/dC444;(lane 8) GST-OcE/dC474;(lane 9) GST-OcE/d(445-474). Only GST-OcE/dN358 (lane 3) and GST-OcE/d(445-474) (lane 9) were strongly bound to ZO-1 to the same extent as GST-OcE (lane 1). GST-OcE/dN387, GST-OcE/dC414, and GST-OcE/dC444 (lanes 4,6,7) appeared to weakly trap ZO-1. The mobility of molecular mass markers is shown at the left (66, 45, 31kD from the top).

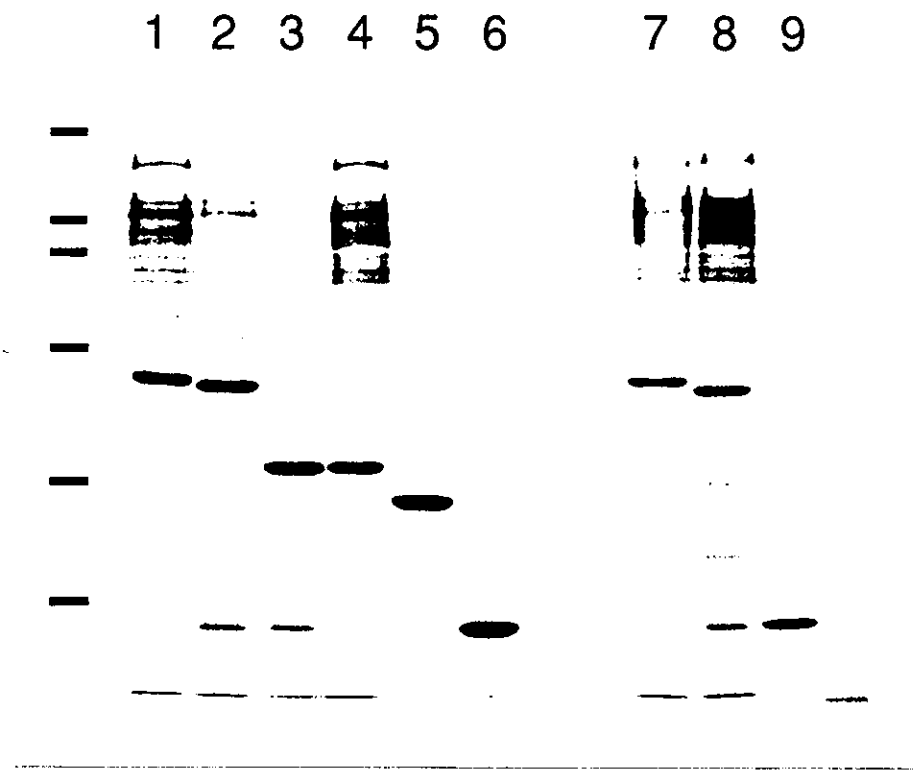


**Figure 7.** Association of normal and truncated domain E of occludin with MBP-ZO-1 fusion protein produced in E.coli. Glutathione-conjugated beads were incubated with normal or truncated GST-OcE fusion proteins (see Fig.5). After washing followed by incubation with the lysate from E.coli producing MBP-ZO-1 fusion protein, proteins trapped by beads were eluted with SDS-PAGE sample buffer. After each eluate was resolved by SDS-PAGE, the amount of GST-fusion protein and MBP-ZO-1 fusion protein was evaluated by Coomassie brilliant blue staining (bottom) and immunoblotting with anti-ZO-1 mAb, T8-109 (top), respectively. For Coomassie brilliant blue staining, one tenth or one fifth of the amount of each immunoblot sample was used in lanes 1-6 or lanes 7-9, respectively. (Lane 1) GST-OcE; (lane 2) GST-OcE/dC474; (lane 3) GST-OcE/dC357; (lane 4) GST-OcE/dN358; (lane 5) GST-OcE/dN387; (lane 6) GST; (lane 7) GST-OcE; (lane 8) GST-OcE/d(445-474); (lane 9) GST. Only GST-OcE/dN358 (lane 4) and GST-OcE/d(445-474) (lane 8) were strongly bound to MBP-ZO-1 fusion protein to the same extent as GST-OcE (lanes 1 and 7). GST-OcE/dC474 (lane 2) appeared to weakly trap MBP-ZO-1. The mobility of molecular mass markers is shown at the left (200, 116, 97, 66, 45, 31kD from the top).

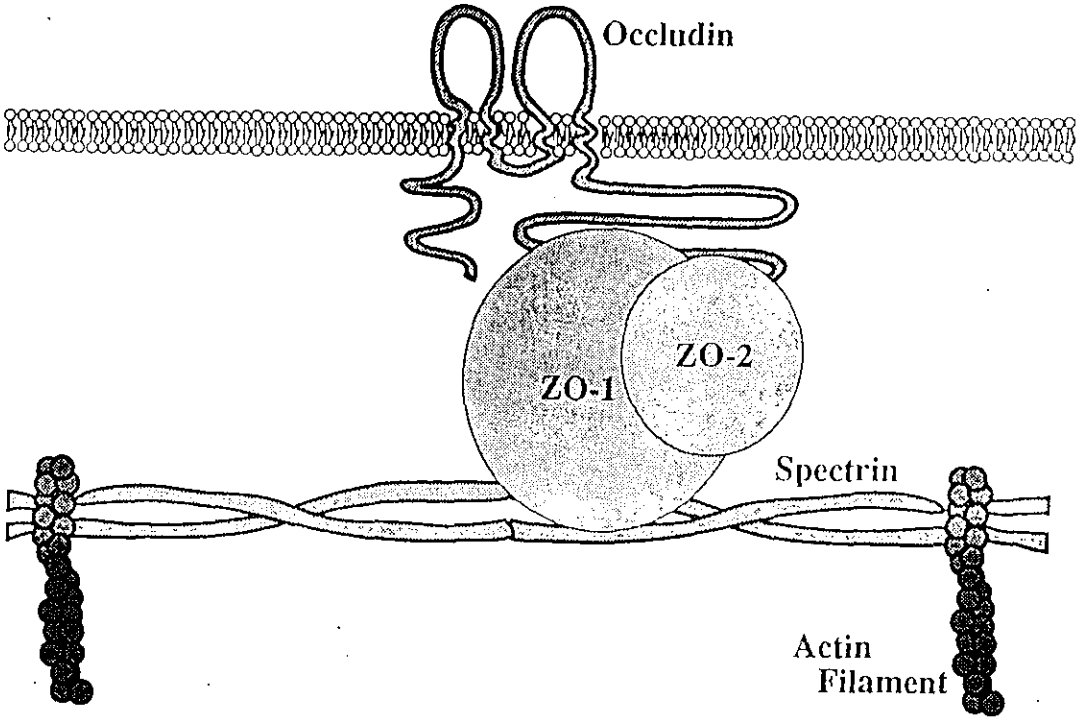


**Figure 8.** Schematic drawing of the possible molecular architecture of tight junctions. The direct association between ZO-1 and $\alpha$-spectrin was reported by Itoh et al. (1991) using the isolated junctional fraction. So far immunoprecipitation experiments with anti-ZO-1 antibodies from whole cell lysate have not detected this association (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994).

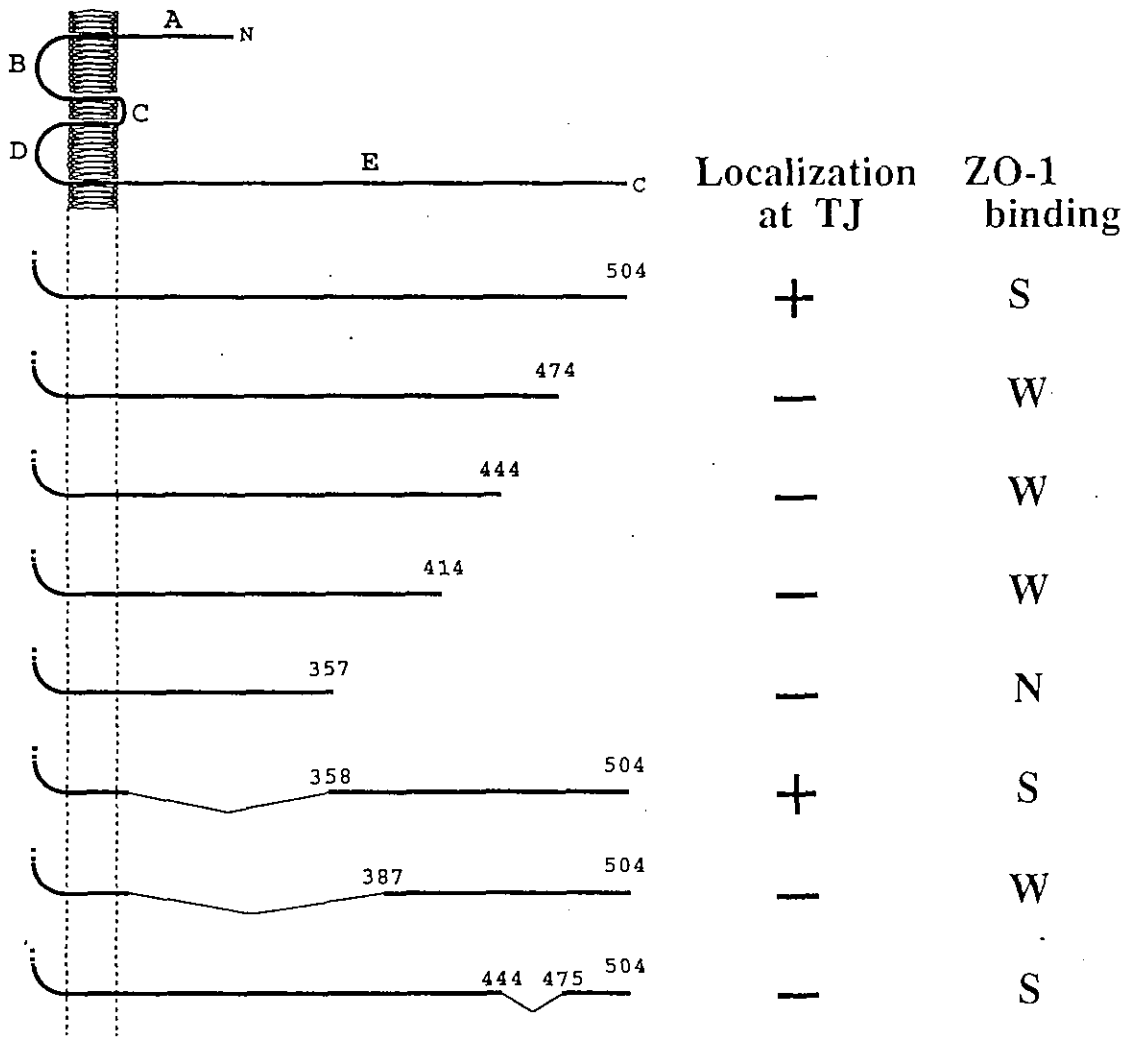


**Figure 9.** Comparison of the results obtained from transfection studies with those from in vitro binding studies. The constructs that were concentrated at tight junctions are marked with (+), and those that showed strong, weak, and no binding affinity to ZO-1 are represented by S, W, and N, respectively. Of course, it is possible that some of the constructs marked with (-) exhibit "weak" localization at tight junctions, which was hard to detect.

## Chapter 3

# Occludin, a Tight Junction Membrane Protein, is an Adhesion Molecule to Obliterate the Intercellular Space

## Introduction

In vertebrate cells, intercellular junctions are categorized into four types: adherens junctions (AJ), desmosomes (DS), gap junctions (GJ), and tight junctions (TJ). In epithelial and endothelial cells, the TJ seals cells to create the primary barrier to the diffusion of solutes across the cell sheet, and also works as a boundary between the apical and basolateral membrane domains to create their polarization[1-3]. It is now apparent that specific types of integral membrane proteins are concentrated and work as adhesion molecules in AJ, DS, and GJ (see the Introduction of the Chapter 1). However, the adhesion molecule working at TJ remained elusive for quite some time[4]. Here we show that occludin, an integral membrane protein of TJ, can work as an adhesion molecule to obliterate the intercellular space. When chick occludin was overexpressed in insect cells by recombinant baculovirus infection, they accumulated in the membranous cisternae of Golgi apparatus-like structures, followed by the complete collapse of these cisternae through the discontinuous fusion of the outer leaflets of the opposing membranes. These findings provide first evidence that occludin is an adhesion molecule working at tight junctions.

**Results and Discussion**

The chick occludin cDNA[5] was integrated into the baculovirus genome, and the recombinant virus containing the occludin cDNA was isolated and condensed. Then cultured insect cells, Sf9 cells, were infected with the recombinant virus. Immunoblot with anti-chick occludin mAb, Oc-2, revealed that chick occludin expressed in Sf9 cells was detected as broad bands around 65kD, although it was not detected by silver staining of the whole cell lysate (Fig.1a, lanes 1&2; see Fig.2c). This banding pattern was identical to that of occludin included in the junctional fraction isolated from chick liver[5] (Fig.1a, lane 3), suggesting that the processing of expressed occludin in Sf9 cells is the same as those of occludin in chick tissues.

When the Sf9 cells overexpressing chick occludin were observed by confocal immunofluorescence microscopy, the majority of expressed occludin occurred inside the cell, not on the cell surface (Fig.1b). Thin section electron microscopy of these cells identified peculiar electron-dense membrane structures inside the cell, which was never observed in Sf9 cells infected with normal baculoviruses (Fig.1c). At a higher magnification, this structure appeared to consist of thin parallel or concentric lamellae (Fig.1d).

Since this multilamellar structure was expected to be composed of chick occludin, we attempted to enrich them by homogenization followed by sucrose density gradient centrifugation. Thin section electron microscopy showed that the multilamellar structures were largely recovered at the 48/55% interface (Fig.2a). When this fraction was incubated with anti-chick occludin mAbs followed by second antibody-conjugated colloidal gold, the

multilamellar structures were heavily labelled (Fig2b). Furthermore, in the 48/55% fraction, occludin was directly recognized by silver staining as well as immunoblot (Fig.2c). These findings indicate that the multilamellar structures are mainly composed of expressed chick occludin.

Next, we examined the structural changes of membranous organelles after the virus infection, and were lead to speculate that the multilamellar structures were derived from the Golgi apparatus: The accumulation of occludin molecules in the Golgi apparatus for an unknown reason may result in the multilamellar structures. Then, how is the Golgi apparatus consisting of layered cisternae transformed into these multilamellar structures ? To answer this question, using the fraction rich in these structures fixed with glutaraldehyde containing tannic acid for contrast of the proteinous structures[6,7], we closely analyzed their ultrastructure (Fig.3). By the use of ultrathin sections <15nm thick, these multilamellar structures were shown to consist of many disc-like structures, each of which has a loop of membrane with its both ends. This indicates that each disc was transformed from each cisterna whose luminal space was completely collapsed (Fig.3a). On the cytoplasmic surface of each collapsed cisterna, electron dense protrusions ~12nm in length were densely and rather regularly arranged. By contrast, the outer leaflets of opposing membranes were appeared to be fused with no gaps, although this fusion occurred discontinuously.

Sequence analyses of occludin cDNA suggested that the occludin molecule consists of four transmembrane domains, a long carboxyl-terminal and a short amino-terminal cytoplasmic domains (255 and 57 a.a., respectively), and two extracellular loops (44 a.a. each)[5]. Therefore, the electron dense protrusion observed on the cytoplasmic surface may be the morphological counterpart of the cytoplasmic domains of occludin, and the extracellular

loops may be directly involved in the fusion of the outer leaflets of opposing membranes. This interpretation is highly consistent with the observation that these fused opposing membranes occasionally take apart with some luminal gaps where the protrusions on membranes were hardly detected (Fig.3a; inset). We are then led to the simple idea that occludin works in a homophilic manner as an adhesion molecule to fuse the outer leaflets of opposing membranes (Fig.3b). However, the direct interaction of the extracellular loops of each occludin with lipids in adjoining membranes is also possible.

Intercellular junctions such as adherens junctions, desmosomes, and gap junctions are characterized by their own specific intercellular distances, ~20, ~25, and ~2 nm, respectively [8,9]. These distances are determined by respective cell adhesion molecules, cadherins[10], desmogleins/desmocolins[11-13], and connexins[14-16]. In this sense, occludin is a very peculiar adhesion molecule which completely obliterates the intercellular space to form tight junctions. From the view point of the molecular mechanism of this obliteration, the roles of the extracellular loops of occludin with an extraordinarily-high content of glycine and tyrosine residues[5] must be clarified.

Another property expected for the tight junction adhesion molecule is the ability to form a linear polymer inside membranes[4], since in freeze-fracture the tight junction is seen to consist of a variable number of parallel interweaving strands of intramembranous particles in the cytoplasmic (P) half of the membrane and corresponding grooves in the outer (E) half[17-20]. Then, by freeze-fracture we analyzed the arrangements of intramembranous particles in the multilamellar structures. Two distinct types of fracture images were observed: In one type, numerous particles ~10 nm

in diameter were densely packed in a random or linear pattern (Fig.4a,b), whereas in the other type short straight grooves were occasionally observed (Fig.4c). Therefore, we conclude that under the expression conditions used in this study, occludin shows a tendency to 'polymerize' into a short strand inside the membrane. So far some cytoplasmic proteins[21-26] and membrane lipids[20,27] are considered to be involved in the formation of tight junctions. Therefore, the possible roles of these components in the polymerization of occludin should be examined in the future. Further analysis on the regulation mechanism of the occludin polymerization as well as its expression will lead us to a better understanding how the permeability and polarization of epithelial and endothelial cell sheets are regulated at a molecular level.

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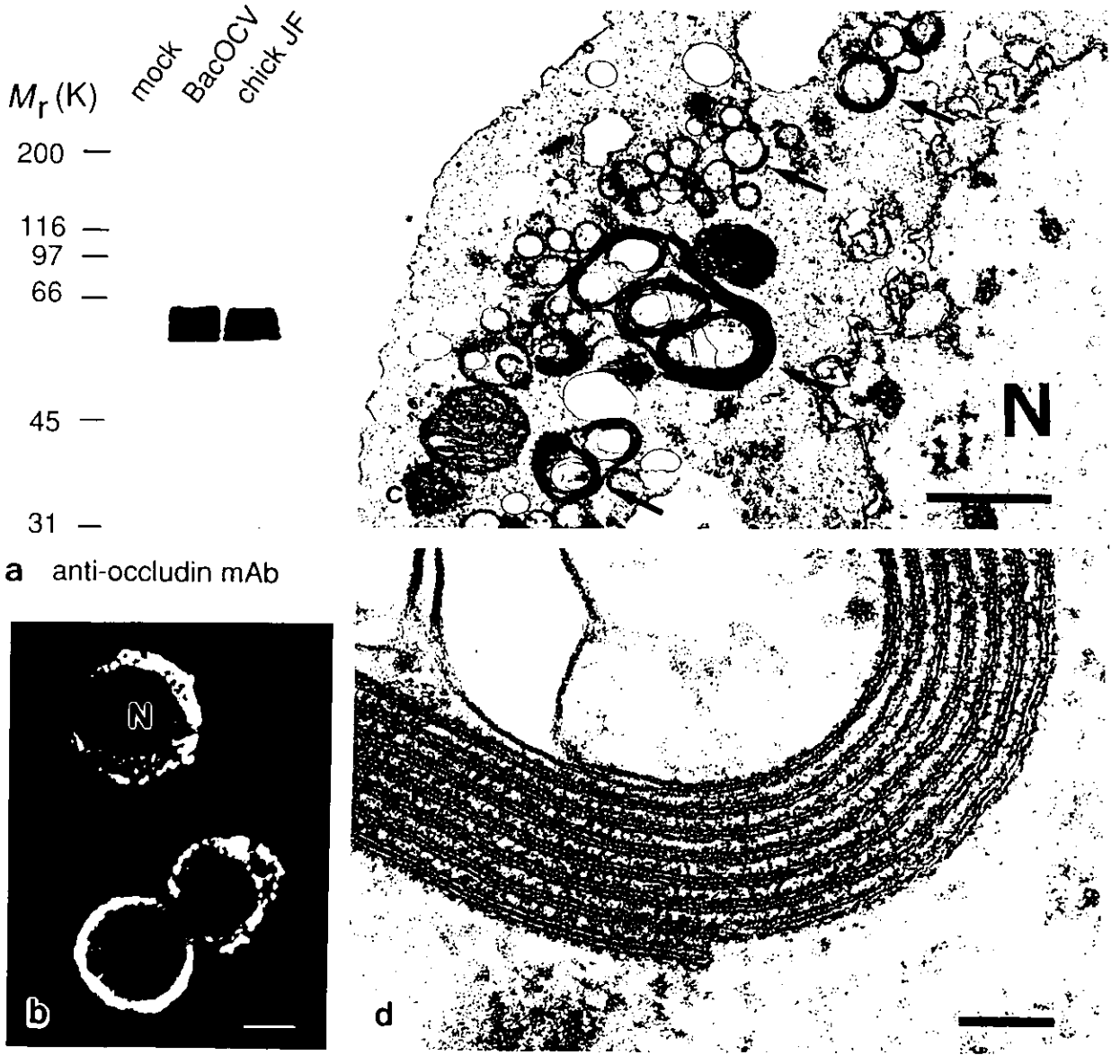
mock
BacOCV
chick JF
$M_r$ (K)
200
116
97
66
45
31
a anti-occludin mAb
N
b
c
N
d

Fig.1 Overexpression of chick occludin in insect Sf9 cells by recombinant baculovirus infection. a, Anti-occludin mAb (Oc-2) immunoblots of the whole cell lysate from non-infected Sf9 cells (mock), that from Sf9 cells infected with recombinant baculovirus carrying chick occludin cDNA (BacOCV), and isolated junctional fraction from chick liver (chick JF). b, Confocal immunofluorescence microscopic image of Sf9 cells overexpressing chick occludin. Cells were stained with anti-occludin mAb, Oc-2. Chick occludin was distributed inside the cytoplasm as granular structures. N, nucleus. c,d, Thin section electron microscopic images of Sf9 cells overexpressing chick occludin. Multilamellar structures (arrows in c; higher magnification in d) were accumulated in the cytoplasm. Scale bars and magnification: b, 10$\mu$m; c, 1$\mu$m; d 120nm.

METHODS. The recombinant baculovirus was isolated using a MAXBAC$^{TM}$ kit (Invitrogen, San Diego, CA) as follows. The chick occludin cDNA[5] was subcloned into the baculovirus transfer plasmid, pBlueBac2, using standard molecular biological techniques. This plasmid was co-transfected with the wild-type baculovirus, AcMNPV DNA, into Sf9 cells, and the recombinant virus, BacOCV, was isolated and condensed. The Sf9 cells were infected and incubated with BacOCV for 50h, and then processed for immunoblotting, immunofluorescence microscopy, or electron microscopy. For immunofluorescence microscopy, infected cells cultured on coverslips were fixed with 1% formaldehyde/PBS for 10min, permeabilized with 0.2% Triton X-100/PBS, then incubated with anti-occludin mAb, Oc-2. After incubation with FITC-conjugated goat anti-rat IgG (TAGO, Inc., Burlingame, CA), cells were cobserved with a laser scan microscope, Zeiss LSM310 (Carl Zeiss,Inc., Oberkochen, Germany). For electron microscopy, infected cells were fixed in 2% paraformaldehyde/2.5% glutaraldehyde/0.1 M cacodylate buffer (pH7.3) for 2h at room temperature and postfixed with 1% $OsO_4$ for 1h on ice. Samples were then stained en bloc with 1% uranyl acetate, dehydrated with graded concentration of ethanol, and embedded in Epon 812. Samples were examined in a JEOL 1200EX electron microscope.

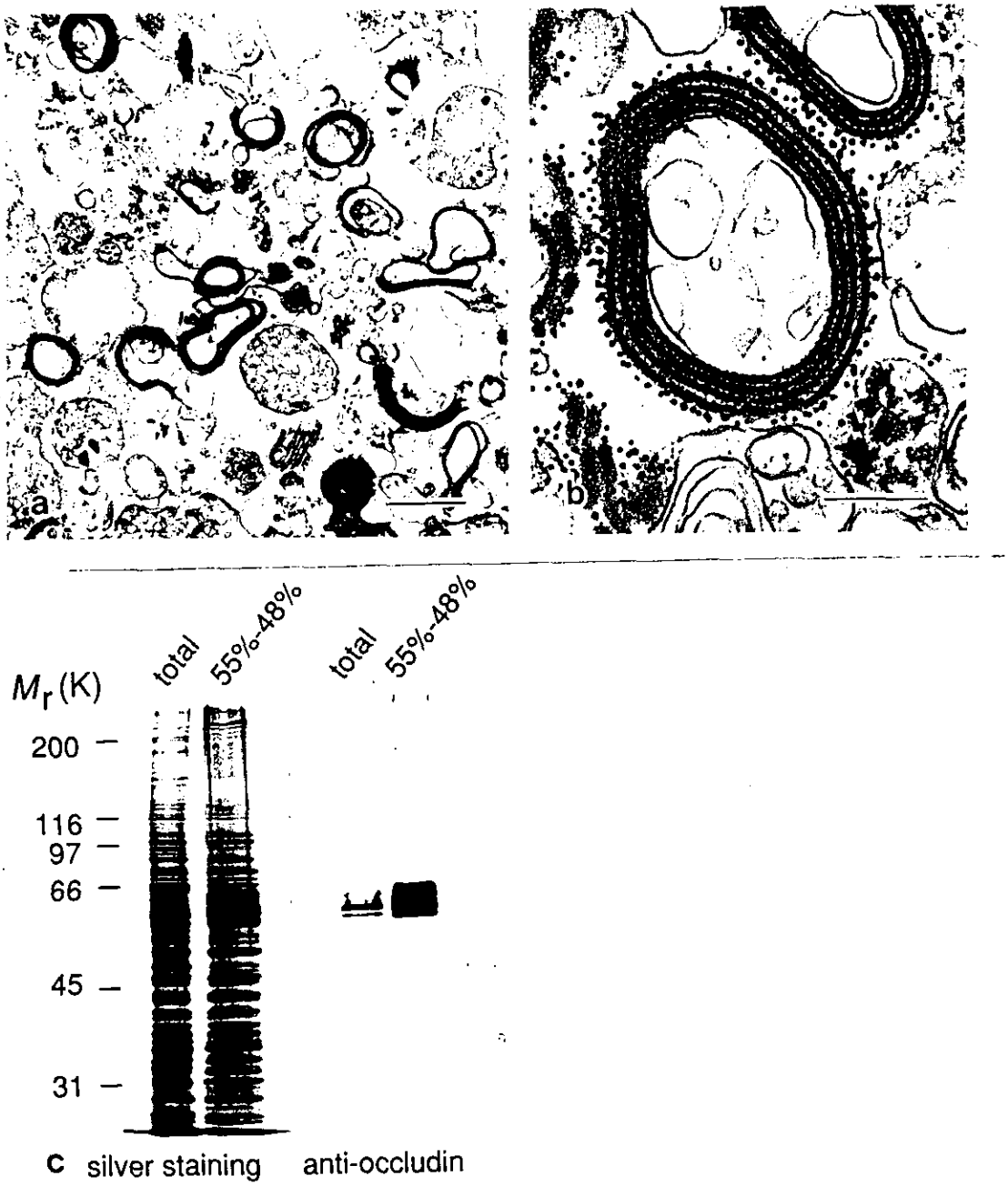


*Fig.2 Enrichment of multilamellar structures from Sf9 cells overexpressing* chick occludin by the sucrose density gradient centrifugation. a, Low power electron microscopic image of 48/55% interface. b, Immunoelectron microscopic localization of chick occludin in isolated multilamellar structures. Samples were incubated with the mixture of anti-occludin mAbs, Oc-1 and Oc-2, followed by second antibody-conjugated colloidal gold particles. c, Silver staining pattern of whole cell lysate from Sf9 cells overexpressing chick occludin (total) and the 48/55% interface fraction (55%-48%), and their accompanying immunoblots with anti-occludin mAb, Oc-2. Scale bars and magnification: a, 1$\mu$m; b, 240nm.

METHODS. Infected Sf9 cells ($2x10^7$) were collected and homogenized in 1 mM $NaHCO_3$ (pH 7.5)/1 mM PMSF by a tight-fitting Dounce homogenizer. The homogenate was added with sucrose to a final concentration of 48%(w/v), overlaid on 55% sucrose , and then centrifuged at 100,000xg for 2h in a swing rotor. The 48/55% band was collected, resuspended in 1 mM $NaHCO_3$ (pH 7.5)/1 mM PMSF, and then centrifuged. Pellets were analyzed by electron microscopy or SDS-PAGE/immunoblotting. For immunoelectron microscopy, the pellets were resuspended in 1 % BSA/PBS and incubated in first antibodies followed by the goat anti-rat IgG coupled to 10 nm gold (Amersham, Corp., Arlington Heights, IL). Samples were then fixed and processed for thin-section electron microscopy as described in Fig.1.

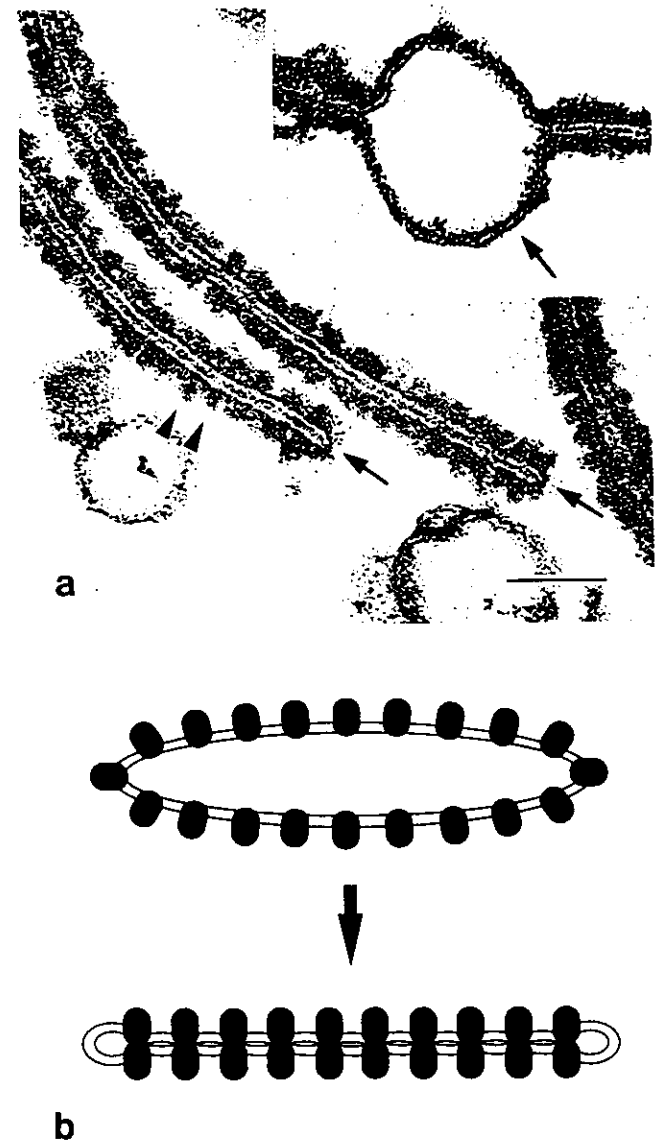


FIG.3 Ultrastructure of multilamellar structures. a, Ultrathin section (<15nm in thickness) electron microscopic image of the isolated multilamellar structures fixed with a fixative containing 0.1% tannic acid. Note the membrane loop structures (arrows) and electron dense protrusions on cytoplasmic surfaces (arrow heads). Fused opposing membranes are occasionally taken apart with some luminal gaps where the protrusions on membranes were hardly detected (arrow in inset). b, Schematic drawing to explain how the multilamellar structures are formed through the accumulation of occludin in membrane cisternae. Scale bar and magnification: a,b, 60nm.

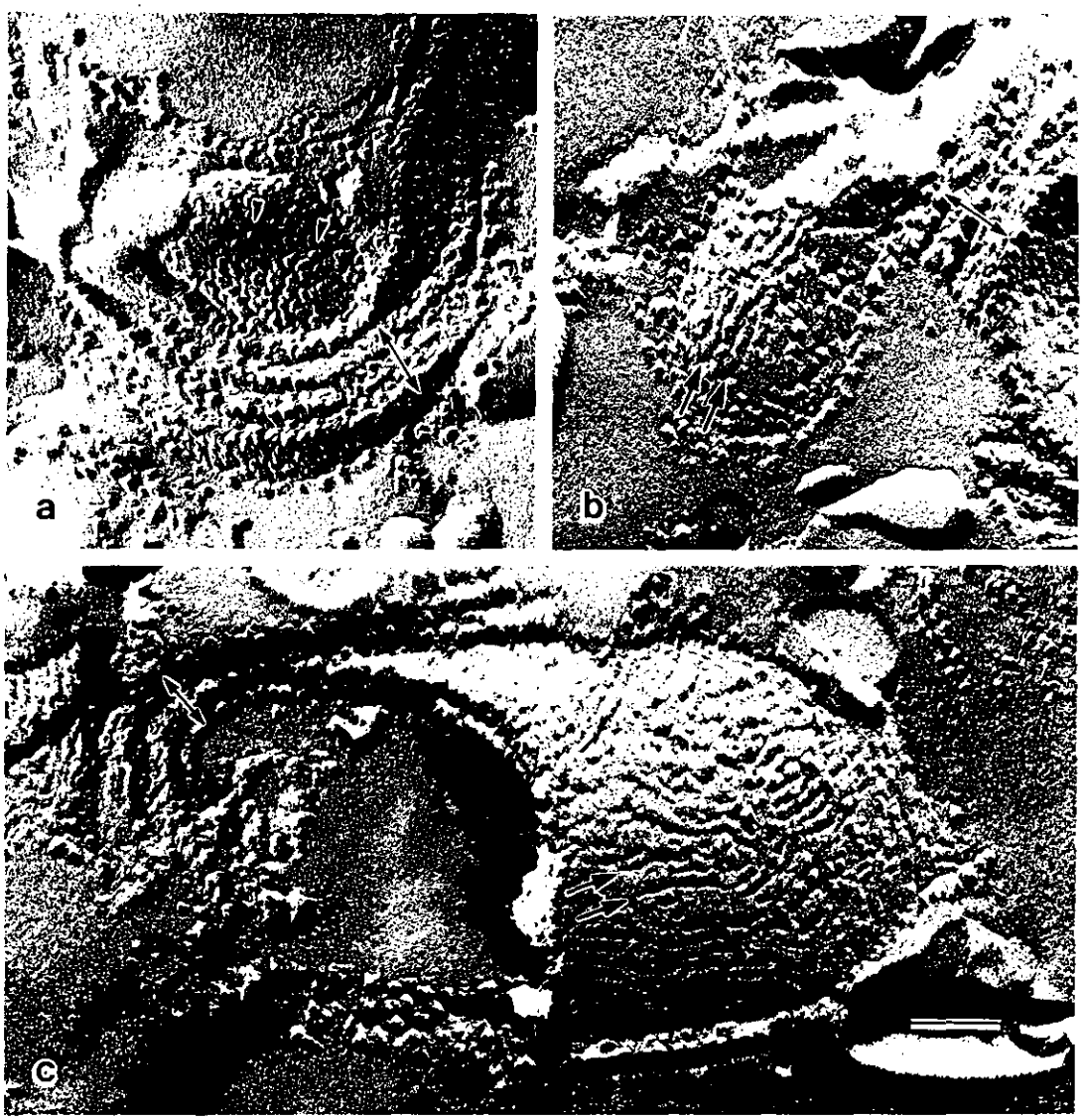


FIG.4 Freeze-fractured and unidirectionally shadowed replicas from isolated multilamellar structures. Two distinct types of fracture images were obtained: In a and b, numerous intramembranous particles ~10nm in diameter (arrowheads) were densely packed in a random or linear fashion (arrows), and in c, short straight grooves (arrows) were occasionally observed. Double arrows, transverse fracture images of multilamellar structures. Scale bar and magnification: 120nm.
METHODS. The membrane pellets rich in multilamellar structures were fixed and immersed in 20% glycerol/0.1 M cacodylate buffer (pH 7.4) at 4℃ for overnight. Specimens were then frozen in liquid nitrogen, fractured at -110℃, and plutinum-shadowed unidirectionally at the angle of 40° in the Balzers Freeze Etching System (BAF 400D; Balzers corp.,Hudson,NH). The samples were immersed in bleach, and the replicas floating off the sample were washed and picked up on grids.

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