

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

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Abstract. Occludin is an integral membrane protein localizing at tight junctions (TJ) with four transmembrane domains and a long COOH-terminal cytoplasmic domain (domain E) consisting of 255 amino acids. 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 domain E, especially its COOH-terminal ~150 amino acids (domain E358/504), was necessary for the localization of occludin at TJ. Secondly, domain E was expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase, and this fusion protein was shown to be specifically bound

to a complex of ZO-1 (220 kD) and ZO-2 (160 kD) among various membrane peripheral proteins. In vitro binding analyses using glutathione-S-transferase 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*. We 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.

THE establishment of compositionally distinct fluid compartments by epithelium and endothelium is crucial for the development and function of most organs. Tight junction (TJ),¹ 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 paracellular 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, lipid composition, and physiological functions, to create and maintain epithelial and endothelial cell polarity (Rodriguez-Boulant 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 220 kD (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 160 kD 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; Willott 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

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1. *Abbreviations used in this paper:* GST, glutathione-S-transferase; MBP, maltose-binding protein; MDBK, Madin-Darby bovine kidney cells; TJ, tight junction.

(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, using mAbs, we identified an integral membrane protein named occludin that 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). (a) In the NH₂-terminal half, occludin contains four transmembrane domains that segment the molecule into five domains (domains A–E). (b) A COOH-terminal half (domain E) consisting of ~250 amino acid residues resides in cytoplasm. (c) Charged amino acids mostly locate at domain E. (d) 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 has been 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 glutathione-S-transferase (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 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, MD), respectively. Human esophagus fibroblast primary culture cells (PF-7N) were provided by Dr. T. Iwazawa (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, Boston, MA). Mouse anti-*c-myc* mAb was purchased from Oncogene Science Inc. (Manhasset, 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 β -actin promoter was constructed using two plasmids, pX1 and

pBATEM2. To construct pX1, occludin cDNA containing the whole open reading frame was constructed by combining two cDNA fragments, FHI-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 1.6-kb 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 the 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' noncoding 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™ Terminator cycle Sequencing Kit (Applied Biosystems, Inc., 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, Gaithersburg, MD). Cells cultured on coverslips were washed once with Opti-MEM (GIBCO BRL), and were incubated for 5 h with 1 ml of Opti-MEM containing 1 μ g of plasmid DNAs and 10 μ l of the reagents, followed by the addition of 3 ml 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 >48 h on coverslips in Eagle's MEM containing 50 μ M Ca²⁺ (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. 24 h 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, ~48 h 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, UK) for anti-*c-myc* mAb. Samples were examined using a fluorescence microscope, an Axiopt photomicroscope, or a 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 Fine Chemicals, Piscataway, NJ), to express fusion proteins with GST in *Escherichia 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 (190-1235 aa) in pMAL-CRI (New

In Vitro Binding Assay

In vitro binding assays were performed using a column. Cultures of *E. coli* expressing GST-fusion proteins (100 ml) were collected by brief centrifugation and resuspended in 6 ml of solution K (140 mM KCl, 10 mM Hepes [pH 7.5], 1 mM MgCl₂, 2 μg/ml leupeptin, and 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride [pAPMSF]). After sonication and centrifugation at 10,000 *g* for 10 min, the supernatant was applied to a column containing glutathione-Sepharose 4B beads (Pharmacia Fine Chemicals), which was washed with 20 vol 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 vol of solution K, bound proteins were eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 10 mM glutathione. 0.8-ml fractions were collected. Since almost all the bound proteins were eluted within the first five fractions, they were mixed and used for SDS-PAGE and immunoblotting.

To detect α-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°C for 1 h. After five washes with 10 vol 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°C for 1 h. The beads were then washed five 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 1 ml of solution K, followed by centrifugation at 100,000 *g* for 1 h. 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 15-cm dishes were scraped and collected by brief centrifugation. They were homogenized in 1 mM NaHCO₃ or in solution K with tight-fitting Dounce homogenizer, followed by centrifugation at 100,000 *g* for 1 h. The pellet was resuspended by sonication in 1 ml of 10 mM Hepes buffer (pH 7.5) containing 1 M KCl, 1 mM EGTA, 2 μg/ml leupeptin, and 0.5 mM PMSF, and was then incubated on ice for 1 h. After centrifugation at 100,000 *g* for 1 h, the supernatant was diluted with 10 mM Hepes buffer (pH 7.5) at a final concentration of 140 mM KCl. Aggregated proteins were removed by centrifugation at 10,000 *g* 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 by using a 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 55.9 kD occludin polypeptide (Furuse et al., 1993). The complete cDNA was subcloned into a mammalian expression vector driven by the β-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 the 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. 1 *a*). 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. 1 *b*). Therefore, to precisely compare the distribution of occludin with that of ZO-1, we analyzed the doubly stained T84 transfectants by laser scan microscopy (Fig. 1, *c* and *d*). As shown in Fig. 1, *e* and *f* as overlaid computer-generated cross-sectional images, the expressed chick occludin and ZO-1 were precisely colocalized at the most apical region of lateral membranes. In our laser scan microscopy 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 as to whether or not occludin expressed in nonepithelial 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. 1, *g* and *h*).

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

To analyze the role of the 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 occludin from both COOH- and NH₂-terminal sides, we constructed several COOH- or NH₂-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. 3 *a*, 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

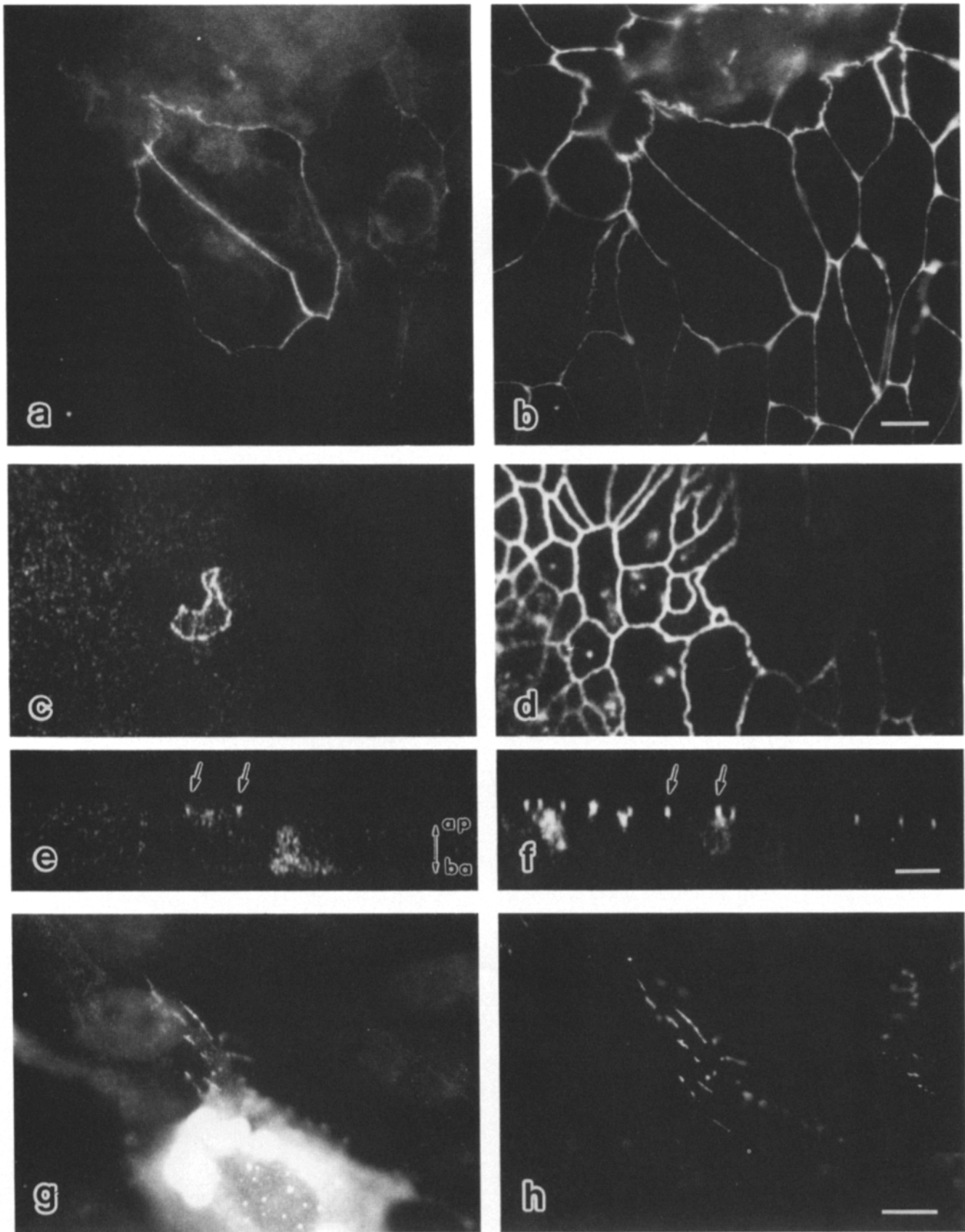


Figure 1. Subcellular distribution of chick full-length occludin in transient transfectants. Bovine and human epithelial cells, MDBK (*a* and *b*) and T84 (*c-f*), respectively, or human fibroblasts, PF-7N (*g* and *h*) were transfected with plasmids encoding full-length occludin, and were then doubly stained with anti-chick occludin mAb, Oc-2 (*a*, *c*, *e*, and *g*) and anti-rat ZO-1 mAb, T8-754 (*b*, *d*, *f*, and *h*).

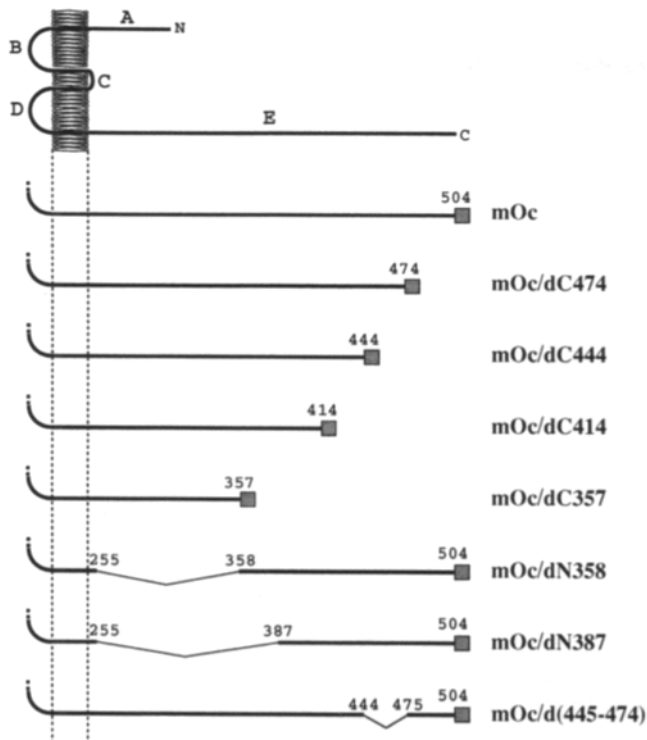


Figure 2. Full-length and truncated occludin constructs with the *c-myc* epitope on their COOH-terminal end (■). Occludin is segmented into five domains (A–E) by four transmembrane domains, and both COOH- and NH₂-terminal truncations of domain E from full-length occludin were constructed.

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. 3, *b–e*), indicating that the responsible sequence could not be narrowed down from the COOH side. By contrast, the NH₂-terminal truncation, mOc/dN358, was clearly concentrated at TJ, although its localization efficiency was rather lower than that of mOc (Fig. 3 *f*). The further NH₂-terminal truncation, mOc/dN387, was by no means localized at TJ (Fig. 3 *g*).

Taking all these 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 Figs. 2 and 3 *h*).

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, and were then resolved by SDS-PAGE.

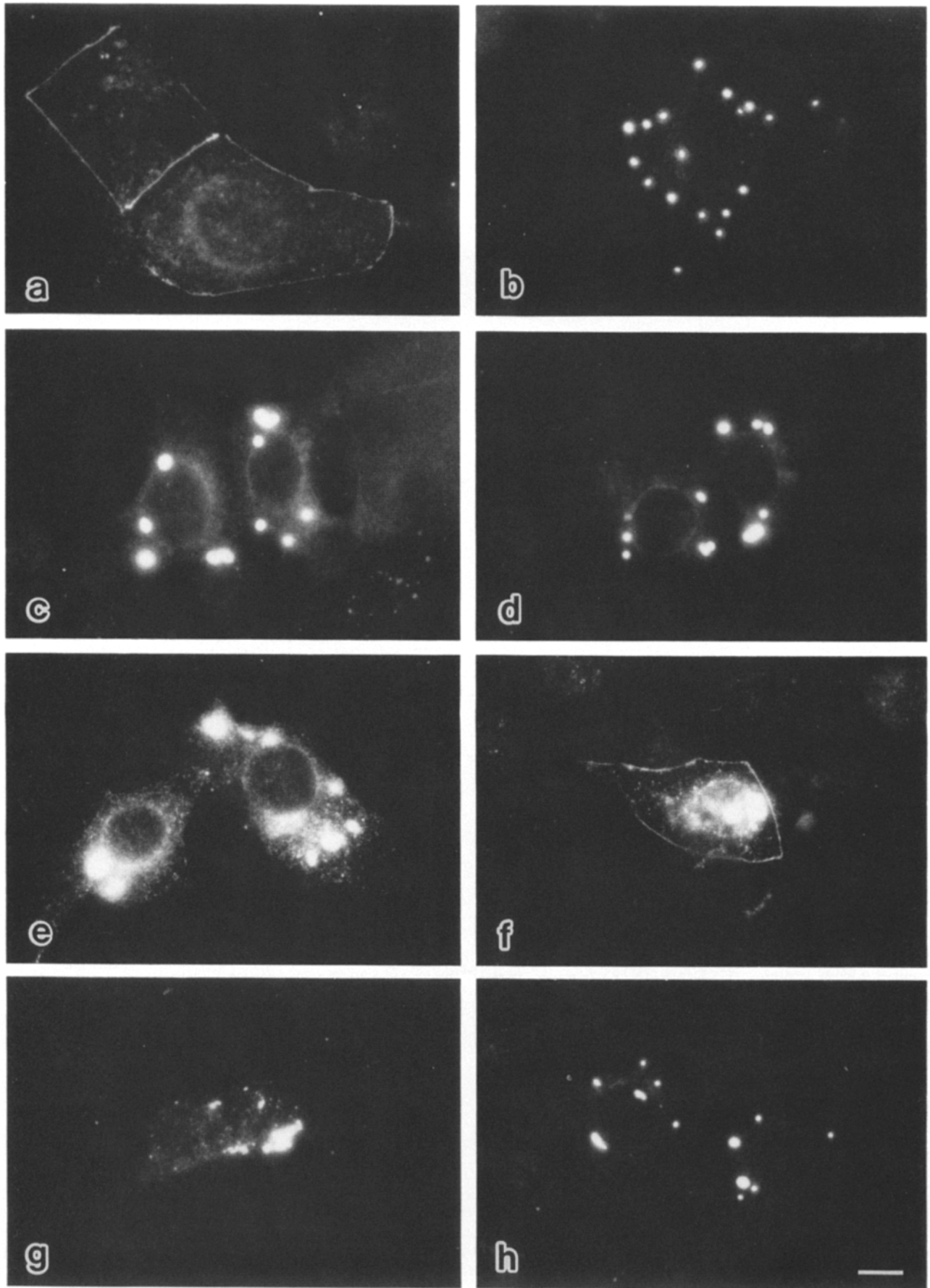
As shown in Fig. 4 *a*, lane 2, this *in vitro* binding assay revealed two major bands with molecular masses of 220 and 160 kD bound to domain E of occludin. The 220-kD band was specifically recognized by anti-ZO-1 mAb (Fig. 4 *a*, lane 6). The molecular mass of the 160-kD band indicated that it would be related to ZO-2, a ZO-1-binding protein identified in canines (Gumbiner et al., 1991). Since an 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 and Goodenough (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. 4 *b*). This suggests that the 160-kD band from the chick junctional fraction is the chick homologue of ZO-2. Furthermore, as shown in Fig. 4 *b*, α -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 NH₂-terminal sides, and to 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 NH₂-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 markedly weaker binding to ZO-1 than GST-OcE, indicating that the sequence necessary for the ZO-1 binding could not be nar-

Oc-2 recognizes chick occludin but neither bovine nor human occludin, whereas T8-754 crossreacts with both human and bovine ZO-1. (*a* and *b*) Conventional immunofluorescence microscopic images of MDBK cells. The transiently expressed chick occludin and bovine ZO-1 were coconcentrated 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* and *d*) and computer-generated cross-sectional images (*e* and *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* and *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 μ m.



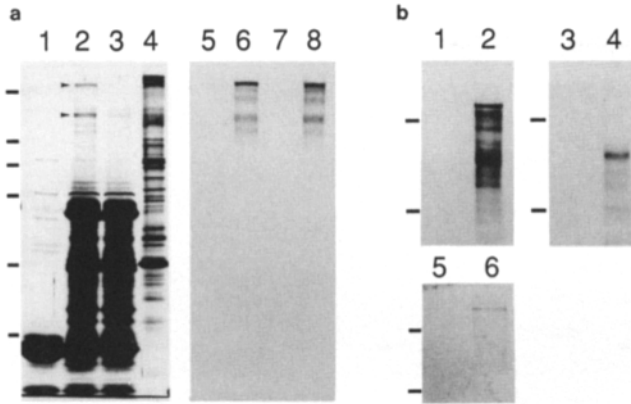


Figure 4. Association of tight junction peripheral proteins with domain E of occludin. (a) In vitro binding of GST-OcE fusion protein to proteins in the low salt alkali extract of junctional fraction isolated from chick liver (JF extract). Domain E of occludin was expressed in *E. coli* as a fusion protein 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 (lanes 1–4) and accompanying immunoblot with anti-ZO-1 mAb, T8-754 (lanes 5–8) of glutathione-eluate from GST column incubated with JF extract (lanes 1 and 5), glutathione-eluate from GST-OcE column incubated with JF extract (lanes 2 and 6), glutathione-eluate from GST-OcE column without the incubation with JF extract (lanes 3 and 7), and JF extract (lanes 4 and 8). Comparison between lanes 1, 2, and 3 revealed that two major bands of ~220 and 160 kD 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, and 31 kD 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 (lanes 1 and 2), anti-ZO-2 pAb, R9989 (lanes 3 and 4), and anti- α -spectrin pAb (lanes 5 and 6) of glutathione-eluate from GST beads incubated with MDBK extract (lanes 1, 3, and 5) and glutathione-eluate from GST-OcE beads incubated with MDBK extract (lanes 2, 4, and 6). Note that α -spectrin and ZO-1/ZO-2 were specifically trapped by domain E of occludin. The mobility of molecular mass markers is shown at the left (200 and 116 kD from the top).

rowed down from the COOH-terminal side. On the other hand, the NH₂-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

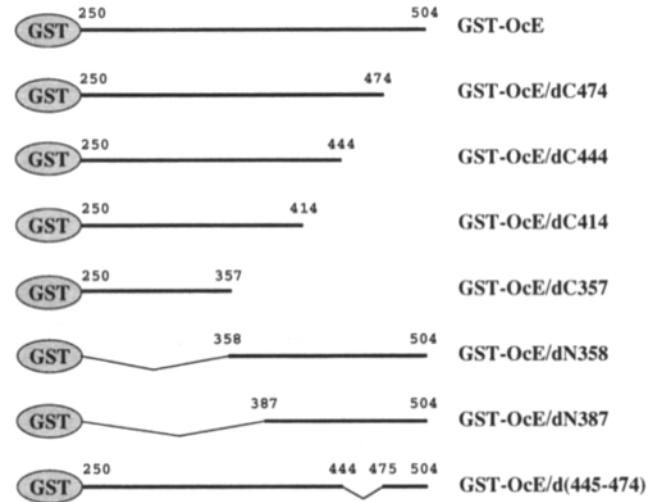


Figure 5. GST-fusion proteins containing normal or truncated domain E of occludin.

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 an 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 ~65 kD 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

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 were 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 it 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 μ m.

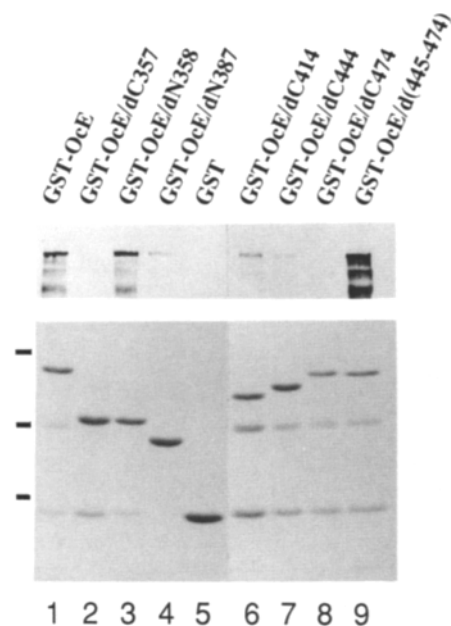


Figure 6. Association of normal and truncated domain E of occludin with ZO-1 in the low salt alkali extract of isolated junctional fraction from chick liver. Columns were constructed using normal or truncated GST-OcE fusion proteins (see Fig. 5). After application of the low salt alkali extract, proteins 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, and 7) appeared to weakly trap ZO-1. The mobility of molecular mass markers is shown at the left (66, 45, and 31 kD from the top).

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, 1989; Anderson et al., 1988; 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 more than 40 nm 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 at least with 220-

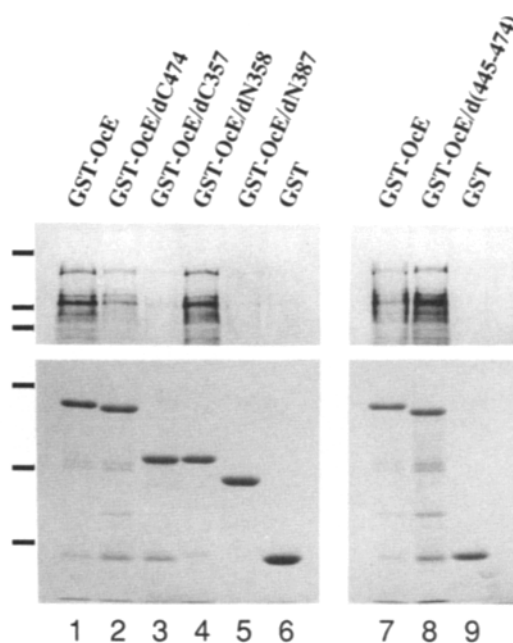


Figure 7. Association of normal and truncated domain E of occludin with MBP-ZO-1 fusion protein produced in *E. coli*. Glutathione-conjugated beads were incubated with normal or truncated GST-OcE fusion proteins (see Fig. 5). After washing followed by incubation with the lysate from *E. coli* producing 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 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, and 31 kD from the top).

and 160-kD 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 MDCK 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

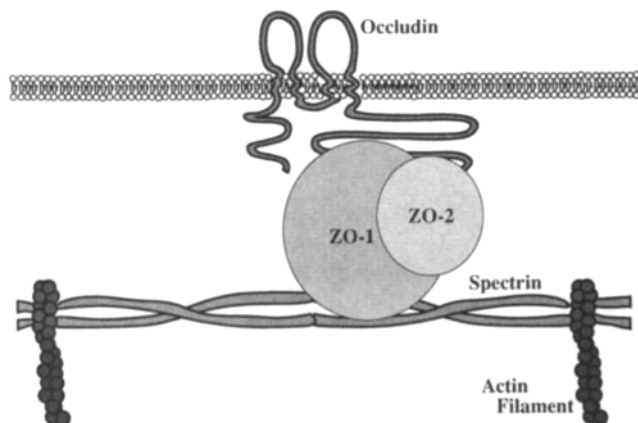


Figure 8. Schematic drawing of the possible molecular architecture of tight junctions. The direct association between ZO-1 and α -spectrin was reported by Itoh et al. (1991) using the isolated junctional fraction. So far, immunoprecipitation experiments with anti-ZO-1 antibodies from whole cell lysate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

far identified in ZO-1 immunoprecipitates is not clear, but it may be partly caused by the insolubility of occludin against nonionic detergents and partly caused by the very low expression level of occludin in cells.

All the data obtained from two series of experiments with various domain 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 NH₂-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 NH₂-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 was not concentrated at TJ. Therefore, we speculate that the association of not only ZO-1, but also other factors to the 358-504 aa 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 and 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

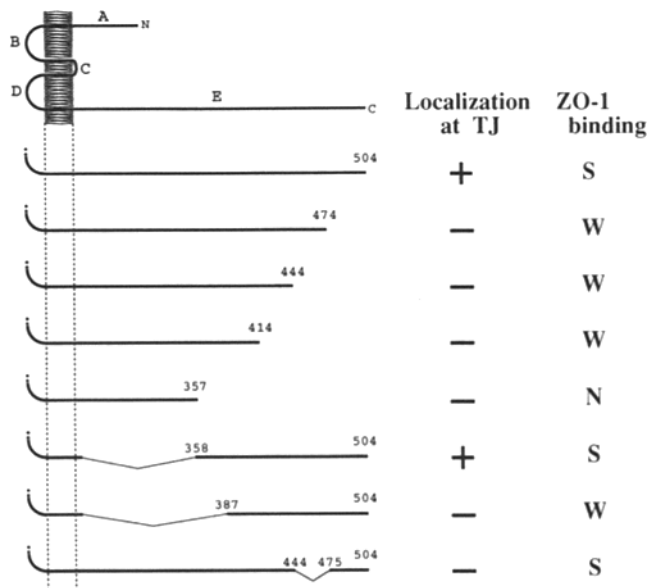


Figure 9. Comparison of the results obtained from transfection studies with those from in vitro binding studies. The constructs that were concentrated at tight junctions are marked with (+), and those that showed strong, weak, and no binding affinity to ZO-1 are represented by S, W, and N, respectively.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

E of occludin directly or indirectly, and we 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 and 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. It is possible, for example, 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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