

A Single Gene Product, Claudin-1 or -2, Reconstitutes Tight Junction Strands and Recruits Occludin in Fibroblasts

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Abstract. Three integral membrane proteins, claudin-1, -2, and occludin, are known to be components of tight junction (TJ) strands. To examine their ability to form TJ strands, their cDNAs were introduced into mouse L fibroblasts lacking TJs. Immunofluorescence microscopy revealed that both FLAG-tagged claudin-1 and -2 were highly concentrated at cell contact sites as planes through a homophilic interaction. In freeze-fracture replicas of these contact sites, well-developed networks of strands were identified that were similar to TJ strand networks in situ and were specifically labeled with anti-FLAG mAb. In glutaraldehyde-fixed samples, claudin-1-induced strands were largely associated with the protoplasmic (P) face as mostly continuous structures, whereas claudin-2-induced strands were discontinuous at the P face with complementary grooves

at the extracellular (E) face which were occupied by chains of particles. Although occludin was also concentrated at cell contact sites as dots through its homophilic interaction, freeze-fracture replicas identified only a small number of short strands that were labeled with anti-occludin mAb. However, when occludin was cotransfected with claudin-1, it was concentrated at cell contact sites as planes to be incorporated into well-developed claudin-1-based strands. These findings suggested that claudin-1 and -2 are mainly responsible for TJ strand formation, and that occludin is an accessory protein in some function of TJ strands.

Key words: tight junction • claudin-1 • claudin-2 • occludin • fibroblast

TIGHT junctions (TJs)¹ are thought to be directly involved in barrier and fence functions in epithelial and endothelial cells by sealing them to generate the primary barrier against the diffusion of solutes through the paracellular pathway and by acting as a boundary between the apical and basolateral plasma membrane domains to create and maintain cell polarity, respectively (for reviews see Gumbiner, 1987, 1993; Schneeberger and Lynch, 1992; Anderson and Van Itallie, 1995). On ultrathin section electron micrographs, TJs appear as a set of dis-

crete sites of apparent fusion involving the outer leaflet of plasma membranes of adjacent cells (Farquhar and Palade, 1963). On freeze-fracture electron micrographs of most epithelial cells, TJs appear as a set of continuous, anastomosing intramembranous particle strands (TJ strands) in the protoplasmic (P) face with complementary grooves in the extracellular (E) face (Staehelin, 1973, 1974).

There is accumulating evidence that some unique proteins constitute TJs (Citi, 1993; Anderson and van Itallie, 1995). The first protein identified as a TJ constituent was ZO-1 with a molecular mass of 220 kD (Stevenson et al., 1986). This protein is a peripheral membrane protein that is localized in the immediate vicinity of the plasma membrane of TJs in epithelial and endothelial cells (Stevenson et al., 1986, 1989). Other peripheral proteins called ZO-2 and ZO-3 with molecular masses of 160 and 100 kD, respectively, have been identified as ZO-1 binding proteins (Gumbiner et al., 1991; Balda et al., 1993; Haskins et al., 1998). All these TJ-associated peripheral proteins, ZO-1, ZO-2, and ZO-3, showed sequence similarity to the prod-

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1. *Abbreviations used in this paper:* TJ, tight junction; P face, protoplasmic face; E face, extracellular face; GFP, green fluorescent protein; HA, hemagglutinin; pAb, polyclonal antibody.

uct of lethal (*l*) 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; Haskins et al., 1998). In addition to these proteins, some other TJ-specific peripheral membrane proteins such as cingulin (Citi et al., 1988), 7H6 antigen (Zhong et al., 1993) and symplekin (Keon et al., 1996) have also been identified by mAb production.

Occludin, with a molecular mass of 60–65 kD, was first identified and characterized as an integral membrane protein localized at TJ strands in chicken (Furuse et al., 1993) and also in various mammalian species (Ando-Akatsuka et al., 1996; Saitou et al., 1997). Occludin bears four transmembrane domains in its NH₂-terminal half with both NH₂- and COOH-termini located in the cytoplasm, and its COOH-terminal ~150 amino acids specifically binds to ZO-1 (Furuse et al., 1994). Occludin is thought to be not only a structural component (Fujimoto, 1995; Furuse et al., 1996) but also a functional component of TJs; occludin has been shown to be directly involved in barrier functions (McCarthy et al., 1996; Balda et al., 1996; Chen et al., 1997; Wong and Gumbiner, 1997), in fence functions of TJs (Balda et al., 1996), and in cell adhesion (Van Itallie and Anderson, 1997).

Inconsistent with recent observations indicating the importance of occludin in structure and functions of TJs, however, targeted disruption of both alleles of the occludin gene in embryonic stem cells revealed that functional TJ strands could be formed without occludin (Saitou et al., 1998). As intensive efforts failed to identify isoforms of occludin, this knockout result suggested the existence of as yet unidentified TJ integral membrane protein(s). We recently identified two novel mouse integral membrane proteins, claudin-1 and -2, from isolated junctional fractions in which TJs are highly enriched (Furuse et al., 1998). Claudin-1 and -2 were structurally related (38% identical at the amino acid sequence level) and appeared to bear four transmembrane domains, but showed no sequence similarity to occludin. When FLAG-tagged claudin-1 and -2 were transfected into cultured MDCK cells, both were correctly targeted to and incorporated into TJ strands both at confocal immunofluorescence microscopic and immunoelectron microscopic levels. These findings indicated that multiple integral membrane proteins with four transmembrane domains, occludin, claudin-1, and -2, constitute TJ strands.

In this study, we examined the abilities of claudin-1, -2, and occludin to form strand structures within plasma membranes by introducing their cDNAs into mouse L fibroblasts. In stable L transfectants, introduced claudin-1 as well as claudin-2 were concentrated at cell contact sites as planes, and formed well-developed networks of strands, which were morphologically similar to TJ strand networks in situ. In contrast, occludin induced formation of only a small number of short strands, although it was also concentrated at cell–cell contact sites as dots. Interestingly, when L fibroblasts were cotransfected with claudin-1 and occludin cDNAs, expressed claudin-1 and occludin appeared to be copolymerized into well-developed strands at cell–cell contact sites. This study has provided a new way to analyze the structure and function of TJs, i.e., reconstitution of TJ strands in fibroblasts.

Materials and Methods

Antibodies and Cells

Rabbit anti-mouse occludin polyclonal antibody (pAb) (F4) and rat anti-mouse occludin mAb (MOC37) were raised and characterized as described previously (Saitou et al., 1997; Sakakibara et al., 1997). Mouse anti-FLAG mAb, rabbit anti-hemagglutinin (HA) pAb, and mouse anti-T7 mAb were purchased from Eastman Kodak Co. (New Haven, CT), MBL Co. (Nagoya, Japan), and Novagen (Madison, WI), respectively. Mouse L cells and their transfectants were cultured in DME supplemented with 10% fetal calf serum (Nose et al., 1988).

Mammalian Expression Vectors and Transfection

Claudin-1 and -2 were tagged with FLAG-peptide or GFP at their COOH-termini. Expression vectors for FLAG-claudin-1 and FLAG-claudin-2 were previously reported as pCCL-1F and pCCL-2F, respectively (Furuse et al., 1998). To construct the expression vector of GFP-claudin-1 (pCCL-1G), the claudin-1-GFP fusion protein cDNA (Furuse et al., 1998) was introduced into pCAGGSneodelecORI (Niwa et al., 1991), provided by J. Miyazaki (Osaka University, Osaka, Japan).

Occludin was expressed without a tag or with HA or T7 tag at its COOH terminus in L cells under the control of β -actin promoter. Expression vectors for occludin, HA-occludin, and T7-occludin (pBMOC, pBMOH1 and pBMOT, respectively) were constructed as follows. The full-length mouse occludin cDNA was cloned as described previously (Ando-Akatsuka et al., 1996). To construct a plasmid carrying HA-tagged mouse occludin cDNA (pMOH1), the DNA fragment encoding the HA peptide (YPYDVPDYA) was excised from pYT11 (Takita et al., 1995) by NheI digestion and subcloned into pBluescript SK(-) at the SpeI site, into which the mouse occludin cDNA with a BamHI site at the end of the open reading frame (ORF) was subcloned in frame using the BamHI site. Then a stop codon was introduced after the HA sequence by PCR. To obtain a plasmid carrying T7-tagged occludin cDNA (pMOT), the DNA fragment encoding T7 peptide (SMTGGQQMG) was excised from the pET3c vector (Novagen) by NheI and BamHI digestion. Four DNA fragments were linked in tandem and subcloned into pBluescript SK(-). A stop codon was introduced after the T7 sequence by SpeI digestion, into which mouse occludin cDNA with an EcoRI site at the end of the ORF was subcloned in frame using the EcoRI site. The expression vectors pBMOC, pBMOH1, and pBMOT were generated by subcloning of mouse occludin cDNA, HA-tagged mouse occludin cDNA excised from pMOH1 or T7-tagged occludin cDNA excised from pMOT into the expression vector pBATEM2 (Nose et al., 1988) from which E-cadherin cDNA had been removed.

DNA Transfection

Mouse L cells were used for transfection. 1- μ g aliquots of each expression vector were introduced into L cells in 1 ml of DME using Lipofectamine Plus (GIBCO BRL, Gaithersburg, MD). Since pCCL-1F, pCCL-2F, and pCCL-1G each contained a G418 resistance gene, they were transfected singly. pBMOC was cotransfected with pSV2hph (hygromycin resistant), whereas pBMOT and pBMOH1 were cotransfected with pSTneoB (G418 resistant). After a 24- or 48-h incubation, cells were replated and cultured in DME containing 10% FCS and 500 μ g of Geneticin (GIBCO BRL) or 200 μ g of hygromycin to select stable transfectants. Geneticin- or hygromycin-resistant colonies were isolated after 10–14 d of selection. Clones expressing claudins or occludin were screened by fluorescence microscopy with antibodies or for green fluorescent protein (GFP) fluorescence. The stable transfectants of L cells expressing FLAG-claudin-1, FLAG-claudin-2, GFP-claudin-1, occludin, HA-occludin and T7-occludin were designated as C1FL, C2FL, C1GL, OL, OHL and OTL, respectively. L cells overexpressing both occludin and FLAG-claudin-1 (OC1FL cells) were obtained by transfection of OL cells with pCCL-1F by the same procedure as described above. We isolated several stable clones for each transfection experiment. Since clone 8 of C1FL, clone 6 of C2FL, clone 1 of C1GL, clone 1 of OL, clone 28 of OTL, clone 29 of OHL, and clone 18 of OC1FL expressed relatively large amounts of the introduced molecules, we used these clones for the demonstrations in this paper.

Immunofluorescence Microscopy

Subconfluent cells plated on coverslips were fixed with 1% formaldehyde

in PBS for 10 min at room temperature. The fixed cells were then treated with 0.2% Triton X-100 in PBS for 10 min and washed three times with PBS. After soaking in PBS containing 1% BSA, the samples were treated with primary antibodies for 30 min in a moist chamber. They were then washed three times with PBS, followed by a 30-min incubation with secondary antibodies. FITC-conjugated goat anti-rat IgG antibody (Biosource, Camarillo, CA), rhodamine-conjugated goat anti-mouse IgG antibody (Chemicon, Temecula, CA), Cy3-conjugated goat anti-mouse IgG (Amersham-Pharmacia Biotech., Bucks, UK), and FITC-conjugated donkey anti-rabbit IgG (Chemicon) were used as secondary antibodies. Cells were washed three times with PBS and then mounted in 90% glycerol-PBS containing 0.1% para-phenylenediamine and 1% *n*-propylgallate. Specimens were observed using a fluorescence Zeiss Axiophot photomicroscope (Carl Zeiss, Inc., Thornwood, NY).

SDS-PAGE and Immunoblotting

One-dimensional SDS-PAGE (12.5%) was performed according to the method of Laemmli (1970), and proteins were electrophoretically transferred from gels onto polyvinylidene difluoride membranes that were soaked in 5% skimmed milk followed by incubation with the first antibodies. Bound antibodies were detected with biotinylated second antibodies and streptavidin-conjugated alkaline phosphatase (Amersham Corp., Arlington Heights, IL). Nitroblue tetrazolium and bromochloroindolyl phosphate were used as substrates for detection of alkaline phosphatase.

Freeze-fracture Electron Microscopy

For conventional freeze-fracture analysis, subconfluent L transfectants were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 3 h at room temperature, washed with 0.1 M sodium cacodylate buffer three times, immersed in 30% glycerol in 0.1 M sodium cacodylate buffer for 2 h, and then frozen in liquid nitrogen. Frozen samples were fractured at -100°C and platinum-shadowed unidirectionally at an angle of 45° in Balzers Freeze Etching System (BAF060; Balzers Corp., Hudson, NH). The samples were then immersed in household bleach, and replicas floating off the samples were washed with distilled water. Replicas were picked up on formvar-film grids, and examined with a JEOL 1200EX electron microscope (JEOL, Peabody, MA) or a Hitachi H-700 electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 100 kV. The immunoelectron microscopic technique for examining freeze-fracture replicas was described in detail previously (Fujimoto, 1995). Subconfluent sheets of L transfectants were quickly frozen by contact with a pure copper block cooled with liquid helium gas (Heuser et al., 1979). Frozen samples were then fractured and shadowed as described above. The samples were immersed in sample lysis buffer containing 2.5% SDS, 10 mM Tris-HCl, and 0.6 M sucrose, pH 8.2, for 12 h at room temperature, then replicas floating off the samples were washed with PBS. Under these conditions, integral membrane proteins were captured by replicas, and their cytoplasmic domains were accessible to antibodies. The replicas were incubated with primary antibodies for 60 min, then with secondary antibodies coupled to 10- or 15-nm gold (Amersham Int.). The samples were washed with PBS and observed as described above.

Results

L Transfectants Expressing Claudin-1 or -2

Since Northern blotting revealed that cultured mouse L fibroblasts did not express any members of the claudin family (data not shown) or occludin (Saitou et al., 1997), L fibroblasts were used as parent cells for transfection in this study. After the FLAG tag was fused with the COOH termini of claudin-1 and -2, we introduced their cDNAs into cultured L fibroblasts, and L transfectants stably expressing molecules with the expected molecular masses were obtained (C1FL and C2FL cells, respectively) (Fig. 1 *a*). The subcellular distributions of these tagged proteins were then examined by immunofluorescence microscopy with anti-FLAG mAb. As shown in Fig. 2 *a*, *b*, *d*, and *e*, both introduced FLAG-claudin-1 and FLAG-claudin-2 were highly concentrated at cell contact sites as planes. Interest-

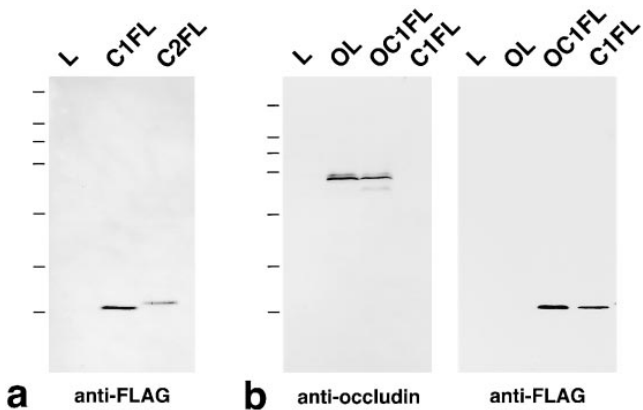


Figure 1. Stable L transfectants used in this study. Total lysates from the same number of parental L cells and stable transfectants were separated by SDS-PAGE and immunoblotted with anti-FLAG mAb or anti-occludin mAb. (*a*) Expression of FLAG-tagged claudin-1 and -2 in stable L transfectants in parental L cells (*L*), L transfectants expressing FLAG-claudin-1 (*C1FL*), and L transfectants expressing FLAG-claudin-2 (*C2FL*). FLAG-claudin-1 and FLAG-claudin-2 with expected molecular masses were detected. (*b*) Expression of occludin and/or FLAG-claudin-1 in parental L cells (*L*), L transfectants expressing occludin (*OL*), L transfectants coexpressing occludin and FLAG-claudin-1 (*OC1FL*), and L transfectants expressing FLAG-claudin-1 (*C1FL*). Occludin and FLAG-claudin-1 with expected molecular masses were detected. Bars indicate molecular masses of 200, 116, 97, 66, 45, 31, and 21 kD, respectively, from the top.

ingly, close inspection revealed that in the cell adhesion sites both proteins were not diffusely distributed but concentrated in an elaborate network pattern (Fig. 2, *c* and *f*).

Next, to examine whether these FLAG-claudins were concentrated at cell contact sites through homophilic interactions, we further obtained stable L transfectants expressing claudin-1 tagged with GFP (*C1GL* cells). Similarly to FLAG-claudin-1, this GFP-claudin-1 was also concentrated at cell contact sites as planes, when *C1GL* cells were cultured under subconfluent conditions. As shown in Fig. 2, *g* and *h*, when *C1GL* cells were cocultured with *C1FL* cells, GFP-claudin-1 was co-concentrated with FLAG-claudin-1 at the cell adhesion sites as planes between adjacent *C1GL* and *C1FL* cells. Furthermore, both proteins showed the same network pattern of concentration in the cell adhesion sites (Fig. 2, *i* and *j*). Since claudins were not concentrated at the cell contact sites between adjacent *C1FL* (or *C2FL*) and parent L cells in mixed culture (data not shown), we concluded that these molecules were concentrated at cell adhesion sites through homophilic interactions.

C1FL cells were then examined by freeze-fracture methods after glutaraldehyde fixation. In parent L cells TJ strand-like structures were not detected, whereas in *C1FL* cells large networks of strands/grooves were frequently observed (Fig. 3 *a*). Judging from their size, these networks may correspond to the network patterns at cell contact planes observed by immunofluorescence microscopy (refer to Fig. 2, *c*, *i*, and *j*). In these networks, the strands were largely associated with the P face, and mostly continuous with intervening spaces of various widths (Fig. 3, *a*

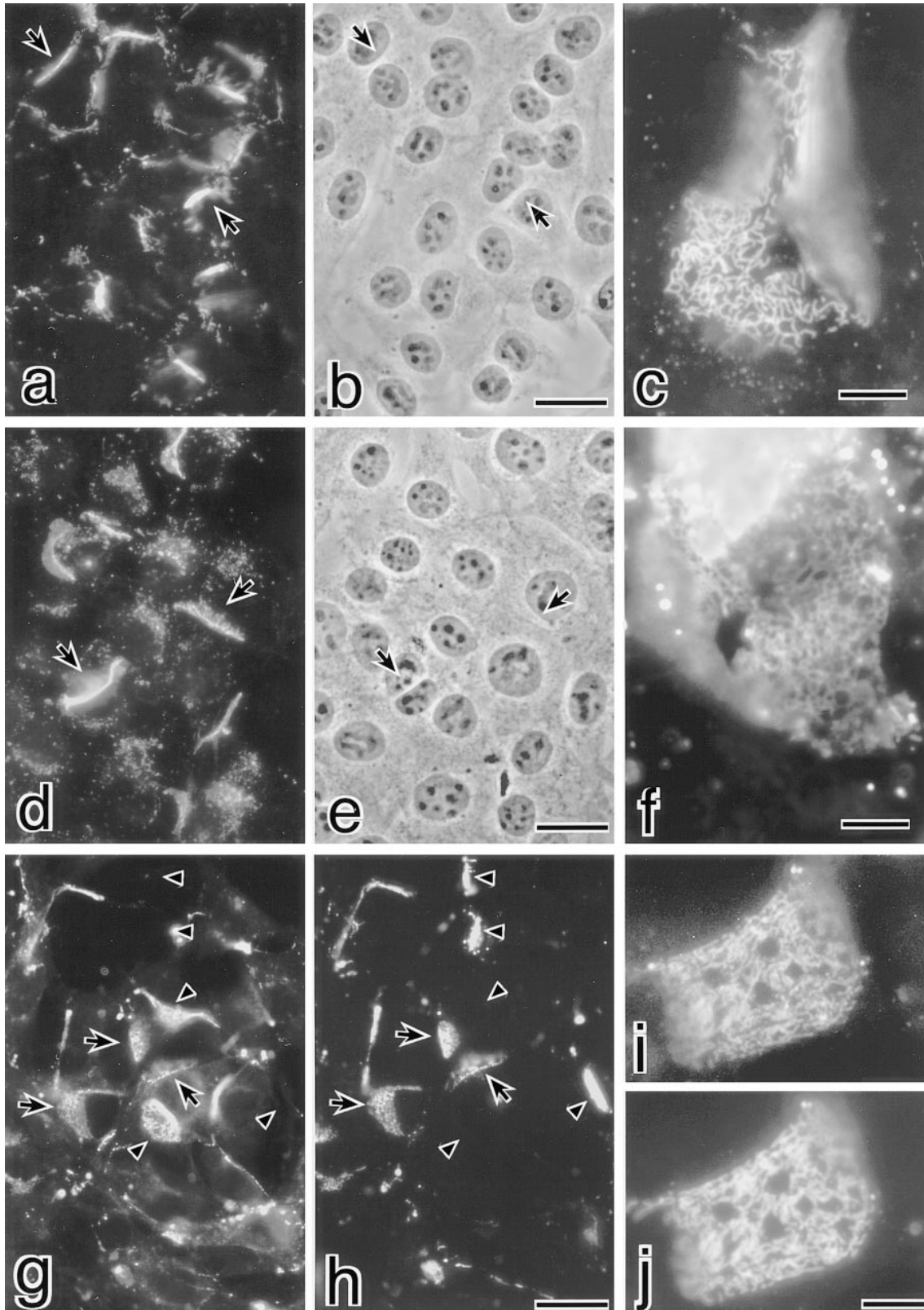


Figure 2. Concentration of claudin-1 and -2 at cell contact planes of stable L transfectants. (a–f) Stable L transfectants expressing FLAG-claudin-1 (C1FL cells) (a–c) or FLAG-claudin-2 (C2FL cells) (d–f) were immunofluorescently stained with anti-FLAG mAb (a, c, d, and f). (b and e) Phase-contrast images. Both FLAG-claudin-1 and -2 were highly concentrated at cell–cell borders as planes (arrows). At higher magnification, face or oblique images of these cell contact planes revealed the concentration of FLAG-claudin-1 (c) and FLAG-claudin-2 (f) in a network pattern. (g–j) Homophilic interaction of claudin-1 and -2. Stable L transfectants expressing GFP-tagged claudin-1 (C1GL cells) were cocultured with C1FL cells. Subcellular distributions of GFP-claudin-1 and FLAG-claudin-1 were visualized by GFP fluorescence (g and i) and immunofluorescence with anti-FLAG mAb (h and j), respectively. Both GFP-claudin-1 (g) and FLAG-claudin-1 (h) were concentrated at cell contact sites as planes between adjacent C1GL and C1FL cells (arrows), and either was detected at cell contact sites as planes between C1GL cells or C1FL cells (arrowheads). At higher magnification, the network pattern of GFP-claudin-1 concentration at the contact planes between adjacent C1GL and C1FL cells (i) was identical to that of FLAG-claudin-1 concentration (j). Bars: (a, b, d, and e) 20 μm ; (c and f) 5 μm ; (g and h) 20 μm ; (i and j) 5 μm .

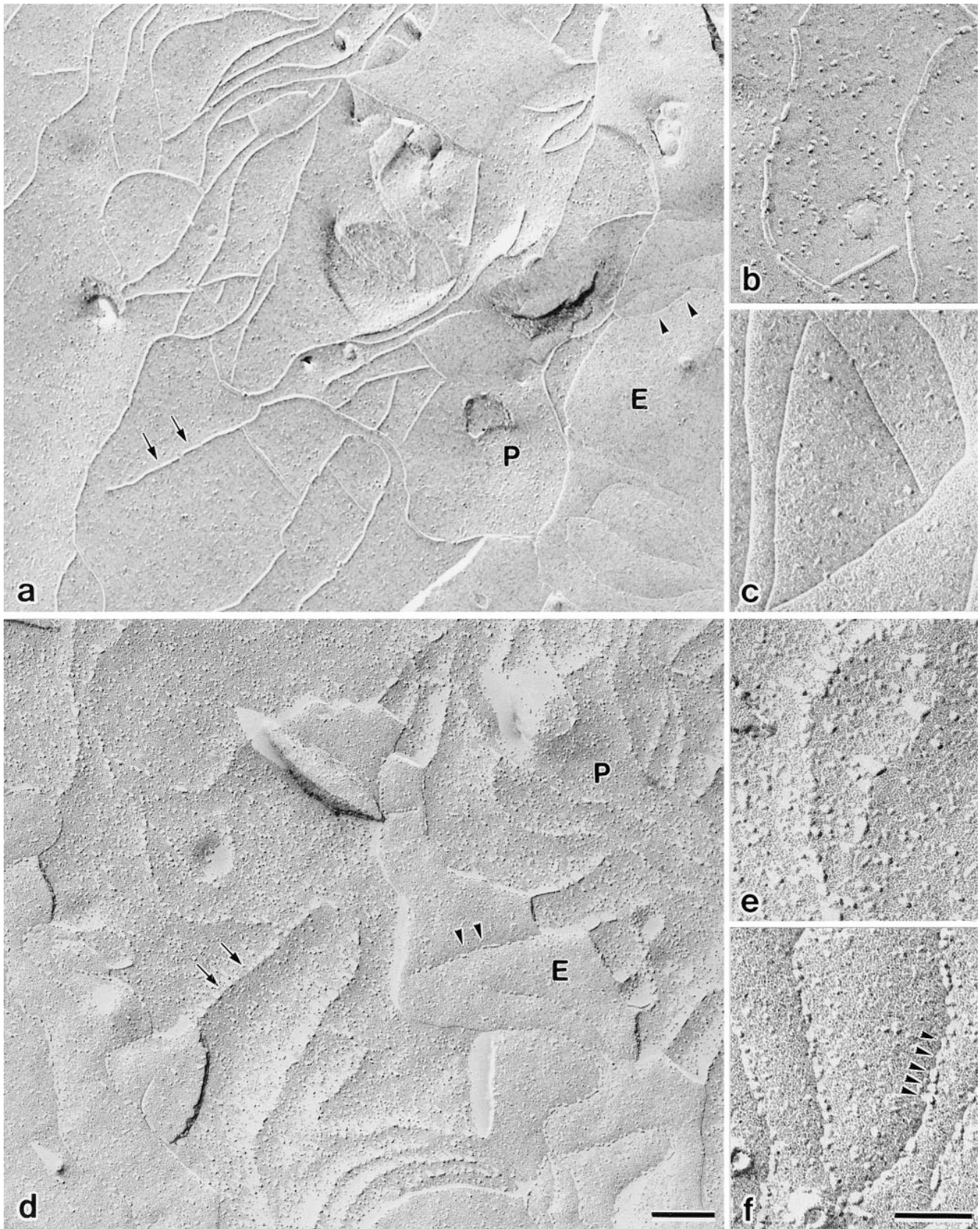


Figure 3. Freeze-fracture images of cell contact planes of stable L transfectants expressing FLAG-claudin-1 (C1FL cells) (*a-c*) and FLAG-claudin-2 (C2FL cells) (*d-f*). Cells were fixed with glutaraldehyde and then processed for freeze-fracture. At low magnification, large networks of strands/grooves were frequently observed. In *a* and *d*, the fracture plane jumped from one membrane (E face) (*E*) to another (P face) (*P*) maintaining the continuity of network pattern of strands (*arrows*) and grooves (*arrowheads*). These features were similar to those of TJ networks observed in situ. At higher magnification, in C1FL cells (*b* and *c*) strand particles were largely associated with the P face to form mostly continuous strands with intervening spaces of various widths (*b*), leaving complementary continuous grooves that were fairly occupied by only a small number of particles on the E face (*c*). In sharp contrast, in C2FL cells (*e* and *f*), strands on the P face were fairly discontinuous (*e*), and on the E face intramembranous particles (*arrowheads*) formed chains that occupied the grooves (*f*). Bars: (*a* and *d*) 0.2 μm ; (*b*, *c*, *e*, and *f*) 0.1 μm .

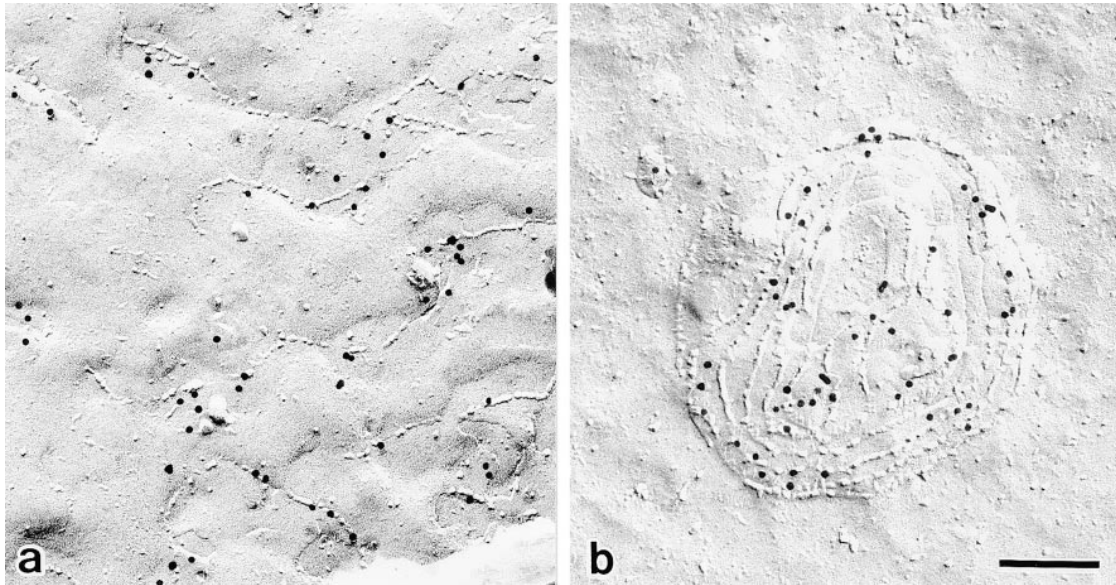


Figure 4. Immunolabeling of freeze-fracture replicas of L transfectants expressing FLAG-claudin-1 (*a*) or FLAG-claudin-2 (*b*) with anti-FLAG mAb. Cells were quickly frozen without chemical fixation, and then processed for freeze-fracture. In both cell lines, induced strands were specifically labeled with anti-FLAG mAb (10-nm gold particles). Note that without chemical fixation the difference in the extent of the association of strand particles with the P face was not clear between these two transfectants. Bar, 0.2 μm .

and *b*). On the E face, complementary continuous grooves were identified, containing only scattered small numbers of particles (Fig. 3, *a* and *c*). In Fig. 3 *a*, the fracture plane jumped from one membrane to another in a cell contact region, showing the E face of the nearer membrane and the P face of the further membrane with a narrow step-like discontinuity separating them. In these E and P faces, the strands and grooves appeared to constitute a continuous network, indicating that in these transfectants the extracellular space was completely obliterated at these strands/grooves. These findings indicated that the claudin-1-induced network was similar to the TJ network in situ.

Similar TJ-like networks were observed also from the cell adhesion planes of glutaraldehyde-fixed C2FL cells by freeze-fracture electron microscopy (Fig. 3 *d*). Again, judging from their size, these networks may correspond to the network patterns at cell contact planes observed by immunofluorescence microscopy (refer to Fig. 2 *f*). In marked contrast to C1FL cells, in the claudin-2-induced networks TJ strands were fairly discontinuous at the P face leaving particle-free ridges between them (Fig. 3, *d* and *e*), and at the E face intramembranous particles formed chains that occupied the grooves (Fig. 3, *d* and *f*). Since the pattern of P face strands appeared to be continuous with that of E face grooves, the claudin-2-induced network is

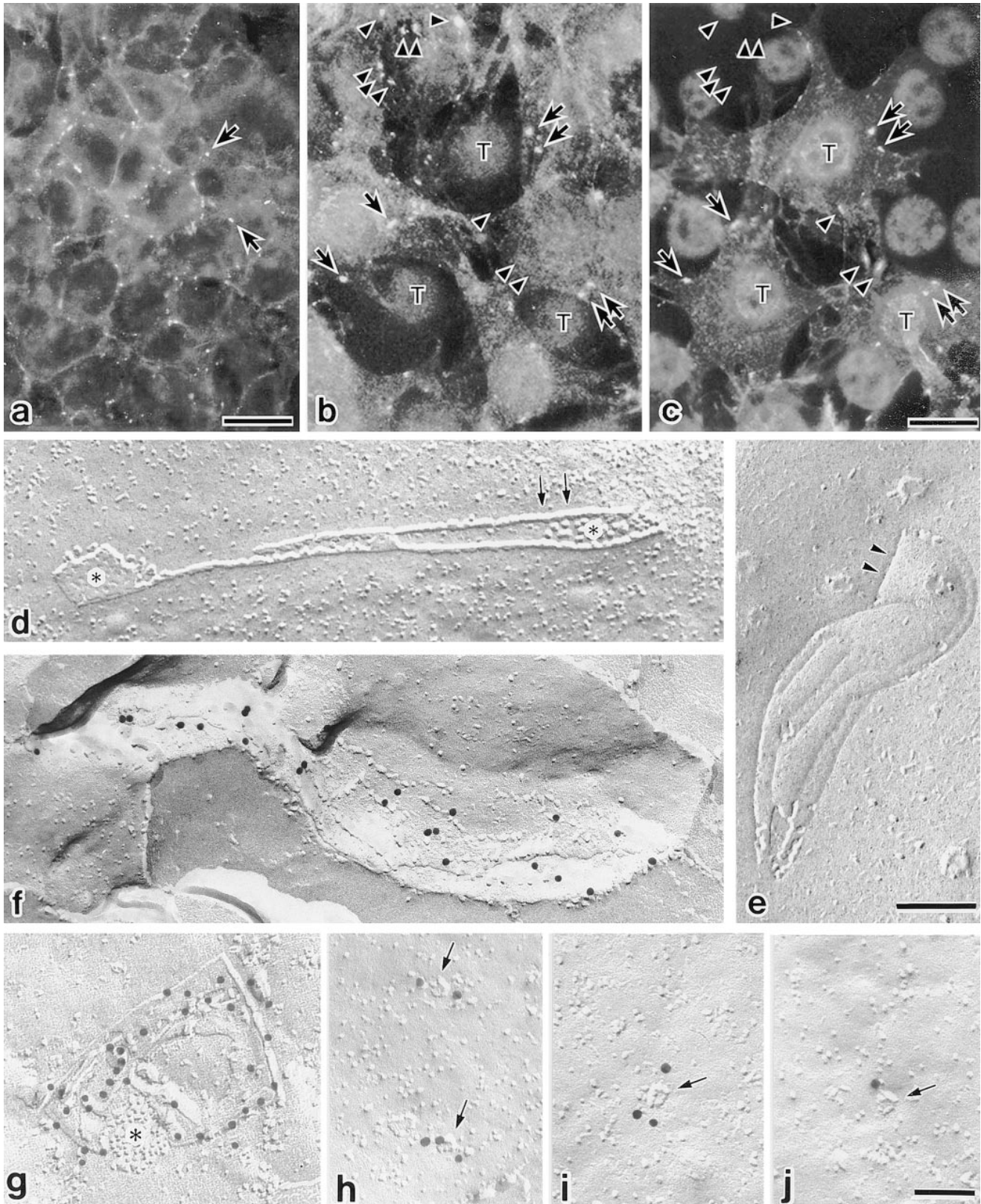
also concluded to function to obliterate the extracellular space (Fig. 3 *d*).

When C1FL and C2FL cells were examined by the freeze-fracture method without chemical fixation, the respective preferable associations of strand particles with P or E face became obscure (Fig. 4). Instead, both networks were characterized by ridges formed by aligned particles and short bars with intervening spaces on the P face. These P face strands from C1FL and C2FL cells were specifically labeled by anti-FLAG mAb, indicating that these claudins were directly involved in the formation of strand structures as major structural components (Fig. 4).

L Transfectants Expressing Occludin

Next, we transfected mouse occludin cDNA into L fibroblasts, and obtained stable L transfectants expressing occludin which was detected as a 65-kD band by immunoblotting with anti-occludin mAb (OL cells) (refer to Fig. 1 *b*). The subcellular distribution of introduced occludin was then examined by immunofluorescence microscopy with anti-occludin mAb. As shown in Fig. 5 *a*, occludin was concentrated at cell contact sites in a punctate pattern. Again, to examine whether this concentration was due to the homophilic interaction of occludin, L transfectants ex-

Figure 5. Concentration of occludin at cell contact sites of stable L transfectants expressing occludin (OL cells). (*a*) OL cells were immunofluorescently stained with anti-occludin mAb. In OL cells, occludin was concentrated at cell-cell borders in a punctate pattern (*arrows*). (*b* and *c*) Homophilic interaction of occludin. Stable L transfectants expressing HA-tagged occludin (OHL cells) and those expressing T7-tagged occludin (OTL cells) were cocultured, and subcellular distributions of HA-occludin and T7-occludin were detected by immunofluorescence with anti-HA pAb (*b*) and anti-T7 mAb (*c*), respectively. Three OTL cells (*T*) can be seen surrounded by many OHL cells. Both HA-occludin (*b*) and T7-occludin (*c*) were concentrated at cell contact sites as dots between adjacent OTL and OHL cells (*arrows*), whereas either was detected at cell contact dots between OTL cells or OHL cells (*arrowheads*). (*d* and *e*) OL cells were



fixed with glutaraldehyde and then processed for freeze-fracture. A small number of short TJ strand-like structures (*arrows*) were observed on the P face (*d*), and corresponding grooves (*arrowheads*) were detected on the E face (*e*). Strand particles were largely associated with the P face, and occasionally associated with gap junctions (*asterisks*). (*f-j*) Cells were quickly frozen without chemical fixation and processed for freeze-fracture, and then replicas were labeled with anti-occludin mAb (10-nm gold particles). In addition to the short strands on the P face (*f* and *g*), a large number of dimple-like structures (*arrows*) were specifically labeled (*h-j*). *Asterisk*, gap junction. Bars: (*a*) 20 μm ; (*b* and *c*) 10 μm ; (*d* and *e*) 0.1 μm ; (*f-j*) 0.1 μm .

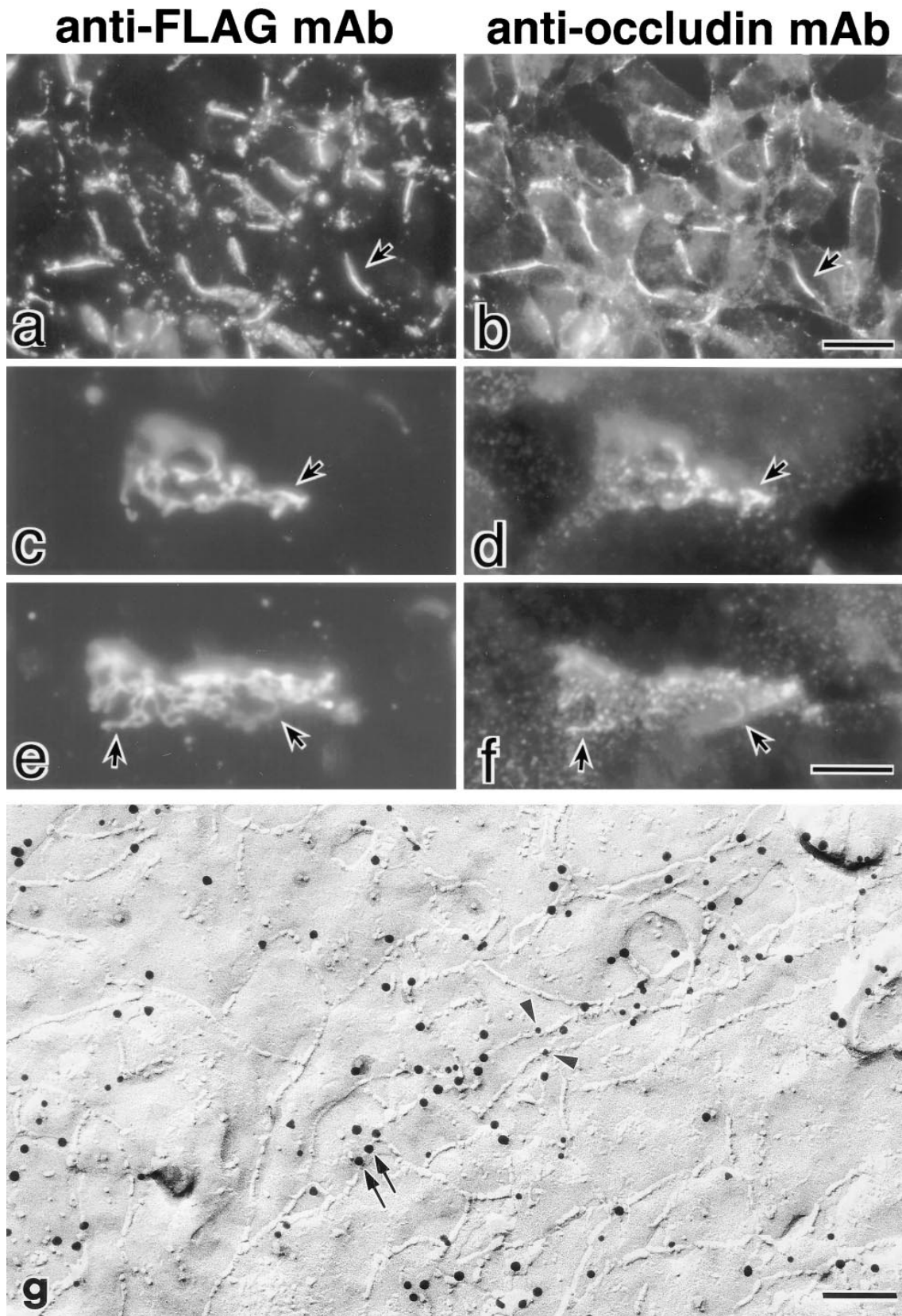


Figure 6. Cotransfection of FLAG-claudin-1 and occludin into L cells. (a–f) Stable transfectants coexpressing both molecules (OC1FL cells) were doubly stained with anti-FLAG mAb (a, c, and e) and anti-occludin mAb (b, d, and f). Similarly to C1FL cells, FLAG-claudin-1 was concentrated at cell contact sites as planes (a) in a network pattern (c and e). In contrast to OL cells in which occludin was concentrated at cell–cell borders in a punctate pattern (refer to Fig. 5 a), occludin was coconcentrated with FLAG-claudin-1 at cell contact sites as planes (arrow) in OC1FL cells (b). As shown in c–f (arrows), the pattern of occludin concentration (d and f) was included in the

pressing HA- or T7-tagged occludin were established (OHL and OTL cells, respectively). When these clones were co-cultured followed by double immunostaining with anti-HA pAb and anti-T7 mAb, HA- and T7-occludin were precisely coconcentrated in a punctate pattern between adjacent OTL and OHL cells (Fig. 5, *b* and *c*). We then concluded that similarly to claudins, occludin also showed a homophilic interaction resulting in concentration at cell contact sites.

In marked contrast to claudin-expressing cells, when glutaraldehyde-fixed OL cells were examined by the freeze-fracture method, large networks of strands/grooves were not detectable. Instead, small numbers of short and straight TJ strand-like structures (~10-nm thick and up to ~0.8 μm long) were observed (Fig. 5 *d*). These strands were largely associated with the P face (Fig. 5 *d*), and complementary grooves were found on the E face (Fig. 5 *e*). These structures were occasionally associated with gap junctions. Immunoreplica analyses with anti-occludin mAb for unfixed OL cells identified not only a small number of occludin-positive short strands (Fig. 5, *f* and *g*) but also a large number of occludin-positive dimple-like structures (Fig. 5, *h-j*). These dimple-like structures were too small to be interpreted in terms of P and E faces.

L Transfectants Coexpressing Claudin-1 and Occludin

Since claudins and occludin are thought to be localized on TJ strands in situ, we finally cotransfected L fibroblasts with cDNAs encoding FLAG-claudin-1 and occludin. As shown in Fig. 1 *b*, stable L transfectants coexpressing both proteins were obtained (OC1FL cells), and the subcellular distributions of FLAG-claudin-1 and occludin were compared by doubly staining these cells with anti-FLAG mAb and anti-occludin mAb (Fig. 6, *a-f*). Similarly to C1FL cells, FLAG-claudin-1 was concentrated at regions of cell contact as planes in OC1FL cells (Fig. 6 *a*). Interestingly, in contrast to OL cells where occludin was concentrated at cell-cell borders in a punctate pattern (refer to Fig. 5 *a*), occludin in OC1FL cells was concentrated with FLAG-claudin-1 as planes at cell contact sites (Fig. 6 *b*). Furthermore, close inspection revealed that in the cell contact planes occludin was recruited to the claudin-1-positive network, although it was not evenly distributed along the claudin-1-positive network (Fig. 6, *c-f*).

Glutaraldehyde-fixed OC1FL cells were then examined by the freeze-fracture method. Large networks of strands were frequently observed, and significant differences were not discerned in the morphology of P face strands and E face grooves between OC1FL and C1FL cells (data not shown; refer to Fig. 3 *a*). When freeze replicas from quickly frozen OC1FL cells were double labeled with anti-FLAG mAb and anti-occludin pAb, both labels were admixed along strands (Fig. 6 *g*). These findings indicated that FLAG-claudin-1 and occludin were copolymerized into strands in OC1FL cells.

Discussion

The structures of the elaborate TJ network revealed by the freeze-fracture technique has attracted increasing interest among cell biologists. In this study, we succeeded in reconstituting TJ-like network of strands/grooves in fibroblasts by introducing a single gene encoding claudin-1 or -2. Interestingly, the reconstituted networks were also detected by immunofluorescence microscopy. The claudin-induced network of strands/grooves was very similar to the in situ TJ networks in epithelial and endothelial cells (Staehein, 1973, 1974): intramembranous particle strands and complementary grooves were observed on P and E faces, respectively. These strands and grooves occasionally branched and fused to form an anastomosing network. Furthermore, the fracture plane frequently jumped from one membrane (E face) to another (P face) maintaining continuity of the network pattern, indicating that apposed strands obliterate the extracellular space completely. Since the pattern of the reconstituted networks varied significantly from the cell-cell contact planes to planes (see the claudin-2-induced networks in Fig. 3 *d* and Fig. 4 *b*), it was difficult to quantitatively compare them with the in situ TJ networks.

Whether the TJ strands are predominantly lipidic in nature, i.e., cylindrical lipid micelles, or represent units of integral membrane proteins aggregated linearly has been the subject of some debate (Pinto da Silva and Kachar, 1982; Kachar and Reese, 1982; Verkley, 1984). However, the detergent stability of TJ strands visualized by negative staining (Stevenson and Goodenough, 1984) and freeze-fracture (Stevenson et al., 1988) suggested that these elements were not composed solely of lipids. In this study, we showed that a single gene product, FLAG-claudin-1 or -2, induced TJ strand formation in fibroblasts, and that these strands were specifically labeled with anti-FLAG mAb by immunoreplica electron microscopy. As claudin-1 and -2 bear four transmembrane domains which allows their direct visualization as particles in freeze-fracture replicas, these results favor the notion that strands can be formed through polymerization of claudins within the plasma membrane. Furthermore, coculture experiments revealed that this polymerization was induced by homophilic interactions of claudins in adjacent cells. Of course, it is necessary to determine to what extent lipids contribute to the formation of strands in detail.

The most striking difference between claudin-1- and claudin-2-induced strands was found in the extent of the association of strand particles with the P face in glutaraldehyde-fixed samples: in L transfectants expressing FLAG-claudin-1, the strand particles were largely associated with the P face leaving continuous grooves occupied by a small number of particles in the E face. In contrast, in L transfectants expressing FLAG-claudin-2 the strands on the P face were fairly discontinuous, and in the E face particles formed chains that occupied the grooves. It has been

network pattern of claudin-1 concentration (*c* and *e*). (*g*) Double immunolabeling of freeze-fracture replicas of OC1FL cells with anti-FLAG mAb and anti-occludin pAb. Cells were quickly frozen without chemical fixation and processed for freeze-fracture, and then replicas were doubly labeled with anti-FLAG mAb (*arrowheads*, 10-nm gold particles) and anti-occludin pAb (*arrows*, 15-nm gold particles). Both 10-nm and 15-nm gold particles were admixed along the strands, indicating that FLAG-claudin-1 and occludin were copolymerized into strands. Bars: (*a* and *b*) 20 μm ; (*c-f*) 5 μm ; (*g*) 0.15 μm .

shown that the extent of TJ strand particle association with the P face in glutaraldehyde-fixed samples varies depending on cell type as well as their developmental stage (for reviews see Staehelin, 1973, 1974; Pinto da Silva and Kachar, 1982). For example, in intestinal epithelial cells and secretory cells, freeze-fracture images of TJ strands/grooves were similar to those of claudin-1-induced strands/grooves in L cells, i.e., the P face-associated type of TJs (Friend and Gilula, 1972; Hull and Staehelin, 1976). On the other hand, in endothelial cells in nonneuronal tissues or in Sertoli cells in the testes, the images of TJ strands/grooves were very similar to those of claudin-2-induced strands/grooves in L cells, i.e., the E face-associated type of TJs (Simionescu et al., 1975; Schneeberger, 1982; Naganano et al., 1982; Pelletier, 1988). These findings strongly suggested that the extent of TJ strand particle association with the P face is determined by the ratio of claudin-1 and -2 in TJ strands, or more correctly the combination ratio of claudin family members (Morita, K., M. Furuse, K. Fujimoto, and Sh. Tsukita, manuscript submitted for publication). As reported in situ, TJs (van Deurs and Luft, 1979; Pinto da Silva and Kachar, 1982), the extents of TJ strand association with the P face in L transfectants expressing claudin-1 and -2 were altered when they were observed without chemical fixation (refer to immunoreplica images in Fig. 4).

Recently, the extent of TJ particle association to the P face was demonstrated to be correlated well with the tightness of TJs in epithelial cells (Zampighi, G., R. Bacallao, L. Mandel, and M. Cerejido. 1991. *J. Cell Biol.* 115:479a) as well as endothelial cells (Wolburg et al., 1994; Kniesel et al., 1996). For example, cultured brain endothelial cells bore the E face-associated type of TJs and showed loose TJ barrier function, but when these cells were treated with astrocyte-conditioned medium to elevate their intracellular cAMP level the transendothelial electrical resistance was markedly increased with concomitant switching of TJs from the E face- to the P face-associated type (Wolburg et al., 1994). This correlation suggests that TJ strands consisting of claudin-1 are tighter than those consisting of claudin-2, and that the tightness of individual TJ strands in situ is determined by the combination of the members of claudin family in each TJ strand. This hypothesis will be evaluated by introducing the members of claudin family into cultured epithelial cells singly or in combination. Furthermore, the molecular mechanism behind the P face association of claudin-1 and behind the E face association of claudin-2 should also be clarified, paying special attention to the abilities of claudin-1 and -2 to interact with underlying cytoskeletal proteins.

In L transfectants expressing occludin (OL cells), occludin was concentrated at cell-cell contact sites in a punctate pattern through their homophilic interactions, but only a small number of short strands and grooves were formed on the P and E faces, respectively. Considering that overexpressed occludin formed short strands in multilamellar bodies in insect Sf9 cells (Furuse et al., 1996), occludin was concluded to also have a weak but significant ability to form TJ-like strands within the plasma membrane. Interestingly, these occludin-induced strands were occasionally associated with gap junctions. This is consistent with many previous observations that gap junctions were occasionally

associated with TJs in situ (Elias and Friend, 1976; McGinley et al., 1977). Furthermore, in this study we have shown that occludin can be copolymerized into claudin-based strands in L transfectants (OC1FL cells), which is consistent with our previous observations that occludin, claudin-1, and -2 were colocalized on TJ strands in situ (Furuse et al., 1998). The question has naturally arisen as to the physiological functions of occludin. Occludin-deficient epithelial cells were shown to still bear well-developed and functional TJs (Saitou et al., 1998). Occludin-deficient mice were also born normally, but their growth rate was significantly reduced in comparison to controls (Saitou, M., K. Fujimoto, Y. Doi, M. Itoh, T. Fujimoto, M. Furuse, H. Takano, Sh. Tsukita, and T. Noda, manuscript in preparation). Taken together, it is likely that occludin is involved in modification of some function of claudin-based TJ strands. To clarify the functions of occludin, further transfection experiments with occludin and claudin in combination are required.

Previously we demonstrated that in liver at least three four-transmembrane domain proteins, occludin, claudin-1, and -2, constituted TJ strands. Then in the present study, we found that among these, claudin-1 and -2, but not occludin, showed a strong ability to form TJ strands within plasma membranes. These studies suggested a way to reconstitute TJs at the cellular level, which will be important to understand the molecular mechanism of barrier and fence functions of TJs as well as their regulation. The TJ structures induced in L cells by claudin-1 or -2 transfection were not *zonula* but *puncta* or *fascia occludens*, i.e., they did not surround individual cells continuously. Determination of what factors would be required to alter these *punctae* to *zonulae occludens* and to localize them at the most apical region of lateral membranes will be interesting.

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