Molecular Architecture of Tight Junctions of Periderm Differs From That of the Maculae Occludentes of Epidermis

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Occludin and claudins are tetraspan-transmembrane proteins in tight junctions. Maculae occludentes, which are less-developed tight junctions, occur in the granular cell layer of the epidermis. The periderm, which overlies the developing epidermis and functions as a protective layer for the embryo, carries developed tight junctions as observed in simple epithelia. In both periderm and epidermis, occludin is expressed at the cell–cell border. To determine the difference between tight junctions of periderm and epidermis, claudin-6 expression was examined in periderm and epidermis. Immunofluorescence staining showed claudin-6 expression at the cell–cell border of the periderm, but not in the epidermis.

Reverse transcription–polymerase chain reaction confirmed that claudin-6 was not expressed in mouse adult skin, whereas immunoelectron microscopy revealed that claudin-6 was localized at tight junctions of the periderm. Furthermore, L fibroblasts with stable expression of exogenous claudin-6 formed developed tight junctions at cell–cell borders. These findings indicate that molecular architecture of tight junctions of the periderm is different from that of the maculae occludentes of the epidermis, and that claudin-6 is important in the formation of tight junctions of the periderm. *Key words: claudin-6/ maculae occludentes/periderm/tight junctions. J Invest Dermatol 118:1073–1079, 2002*

Tight junctions (TJ) constitute a well-developed intercellular adhesion apparatus in simple epithelia, which are located at the most apical part of the lateral membranes (Farquhar and Palade, 1963). TJ seal the intercellular space between two adjacent cells, to create a permeability barrier against the diffusion of solutes across the cell sheet (barrier function) (Gumbiner, 1987, 1993; Schneeberger and Lynch, 1992). TJ also function as a boundary between the apical and basolateral membrane resulting in the polarization (fence function) (Rodriguez-Boulan and Nelson, 1989). Thin-section electron microscopy shows TJ as a series of discrete sites of apparent fusion, which involve the outer leaflet of the plasma membrane of adjacent cells. Freeze-fracture electron microscopy, on the other hand, shows TJ as a set of continuous, anastomosing intramembrane strands or fibrils in the P-face (the outward-facing cytoplasmic leaflet) with complementary grooves in the E-face (the inward-facing extracytoplasmic leaflet) (Staehelin, 1973). ZO-1 was first identified in 1986 as an undercoat constitutive protein of TJ with a molecular mass of 220 kDa (Stevenson *et al*, 1986). Subsequently, the peripheral membrane proteins, ZO-2 (Gumbiner *et al*, 1991), ZO-3 (Balda *et al*, 1993), cingulin (Citi *et al*, 1988), 7H6 (Zhong *et al*, 1993), and symplekin (Keon *et al*, 1996) were also identified. In contrast, the nature of TJ-associated integral membrane proteins was not clearly under-

stood until occludin had been identified (Furuse *et al*, 1993; Ando-Akatsuka *et al*, 1996). Unexpectedly, occludin knockout embryonic stem cells formed developed normally functioning TJ (Saitou *et al*, 1998). This suggested the existence of as yet unidentified TJ-integral membrane proteins, and two new integral membrane proteins of the TJ: claudin-1 and -2 were identified (Furuse *et al*, 1998a). At least, 20 genes similar in sequence to claudin-1 and -2 have been isolated, most of which were proven to be associated with TJ (the claudin multigene family) (Morita *et al*, 1999a, b; Simon *et al*, 1999). When claudins were exogenously introduced into a mouse fibroblast cell line (L fibroblasts), lacking TJ (Furuse *et al*, 1998b), well-developed TJ were formed at cell–cell borders (Furuse *et al*, 1998b; Morita *et al*, 1999b, c). Moreover, targeted disruption of the claudin-11/OSP gene showed slowed down central nervous system nerve conduction, hind limbs weakness, and male infertility (Gow *et al*, 1999). Genetic mutation of the claudin-14 gene in human causes hereditary deafness as a result of impairment of ion homeostasis in the inner ear (Wilcox *et al*, 2001). Claudin-16 was found to be involved in paracellular permeability of magnesium in renal tubules, and its mutation was associated with hereditary renal failure (Simon *et al*, 1999). These lines of evidence strongly suggest an important role for claudins in TJ.

In the epidermis, only the maculae occludentes (i.e., spots of TJ-like structures or focal strands) can be observed with freeze-fracture electron microscopy in the granular cell layer (Elias *et al*, 1977). The conclusion that maculae occludentes were identical in structure to TJ, was reached, because TJ-associated molecules (occludin, ZO-1, and ZO-2) were also localized there (Morita *et al*, 1998).

The periderm, on the other hand, which overlies the developing epidermis in embryonic skin, contains TJ as observed in simple epithelia, and functions as a protective layer for embryonic skin
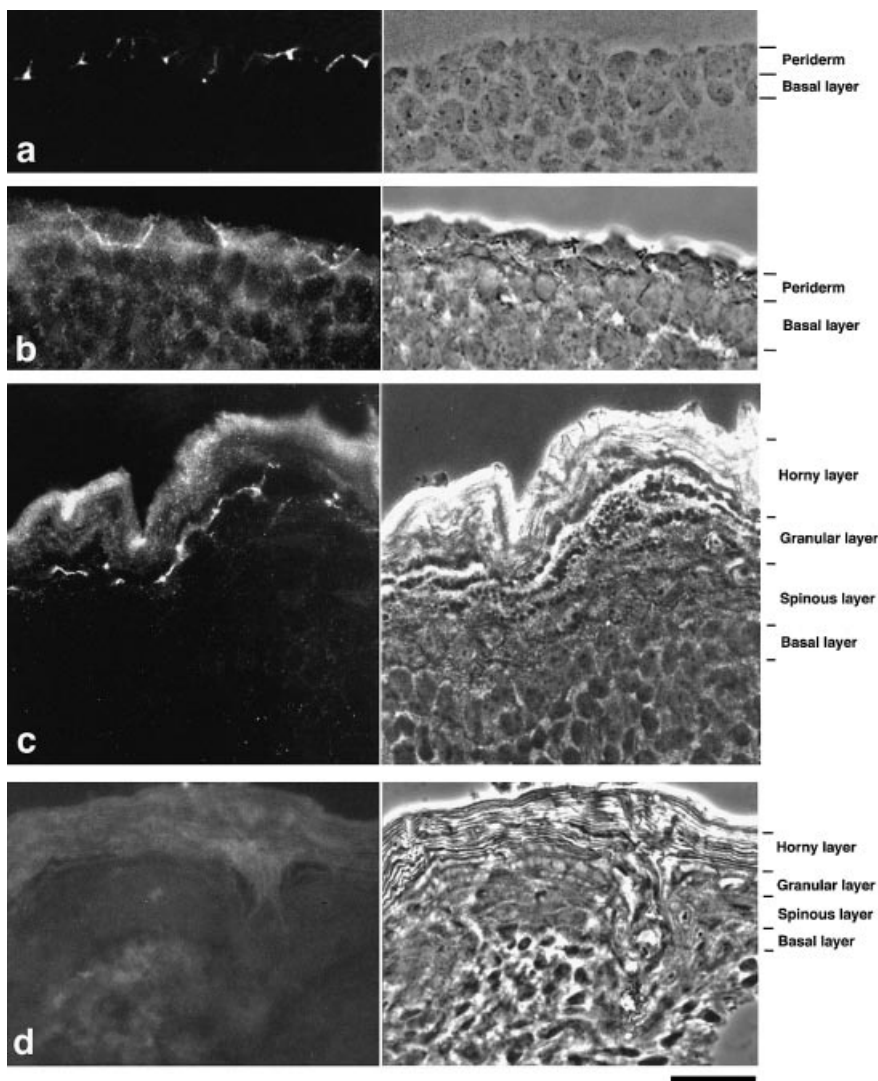
Figure 1. Claudin-6 shows differential subcellular distribution from occludin in periderm and epidermis. Immunofluorescence staining showed that occludin was expressed at the cell–cell border in the periderm of 13.5 d mouse embryo (*a*), as well as in the granular layer of newborn mouse epidermis (*b*). In contrast, claudin-6 was expressed at the cell–cell border in the periderm of 13.5 d mouse embryo (*c*), but not in newborn mouse epidermis (*d*). Each photograph shows fluorescence (left) and phase contrast (right). *Scale bar*: 20 μm.

(Holbrook and Odland, 1975; Weiss and Zelickson, 1975; Hurter and Behrens, 1980). The periderm sloughs off during late development of the epidermis. In the epidermis itself, the cornified layer and intercellular lipids are believed to fulfill an important barrier function. In TJ of both the periderm and the maculae occludentes, occludin is expressed at the cell–cell border (Morita *et al*, 1998). This naturally leads to questions about differences in molecular architecture among these TJ.

To answer the question, we focused on claudin-6. It has been suggested that embryos are rich in claudin-6, because northern blotting of mouse adult tissues produced no specific signals (Morita *et al*, 1999a). In good agreement, claudin-6 is reportedly expressed in epithelia of embryoid bodies and its expression is inhibited by Noggin (the bone morphogenic protein-signaling pathway inhibitor), which suggests that claudin-6 is regulated developmentally (Turksen and Troy, 2001). Thus, in this study, we examined expression of claudin-6 as well as of ZO-1 and ZO-2 in the periderm and epidermis. Furthermore, we assessed the ability of claudin-6 to form TJ in L fibroblasts with a lack of TJ.

MATERIALS AND METHODS

Antibodies and tissues Rat anti-mouse occludin monoclonal antibody (MOC37) (Saitou *et al*, 1997), rabbit anti-mouse claudin-6 polyclonal antibody (CL6pAb) (Morita *et al*, 1999c), mouse anti-mouse ZO-1 monoclonal antibody (T8-754) (Itoh *et al*, 1993), and rabbit anti-mouse ZO-2 pAb (ZO-2pAb) (Itoh *et al*, 1999) were used. All pAb were

affinity purified by using the respective recombinant proteins produced in *Escherichia coli*.

Male and female ddy mice were mated. The gestational days were counted from the day on which vaginal plugs were formed. On gestational day 11.5 or 13.5, pregnant female mice were anesthetized with diethylether, killed, and their embryo removed. Newborn mice were also killed after anesthesia with diethylether. The trunk skin of these mice was dissected away from the underlying muscle.

Immunofluorescence microscopy All samples were frozen with liquid nitrogen, cut into 8 μm thick frozen sections on a cryostat, mounted on glass slides, air-dried, and fixed in 95% ethanol at 4°C for 30 min followed by 100% acetone at room temperature for 1 min. They were then blocked with 1% bovine serum albumin/phosphate-buffered saline for 15 min, and incubated with primary antibodies. Having been washing with phosphate-buffered saline, the samples were incubated for 30 min with Cy2-conjugated goat anti-rat IgG (Amersham International, Amersham, U.K.), rhodamine-conjugated donkey anti-rabbit IgG (Chemicon International, Temecula, CA) or fluorescein isothiocyanate-conjugated sheep anti-mouse IgG (Amersham International). After another washing with phosphate-buffered saline, they were embedded and examined with a Zeiss Axiophot Photomicroscope (Carl Zeiss, Jena, Germany).

Reverse transcription–polymerase chain reaction (reverse transcription–PCR) The 11 d embryo total RNA (Clontech Laboratories, San Diego, CA) and adult skin poly(A)$^+$ RNA (OriGene Technologies, Rockville, MD) of Webster mouse were used. Reverse transcription–PCR was performed with a kit (Revertra Dash; Toyobo, Osaka, Japan), and first strand-cDNA was transcribed with the oligo-dT primer. PCR reaction (94°C, 1min; 60°C, 2 min; 72°C, 3 min; 30
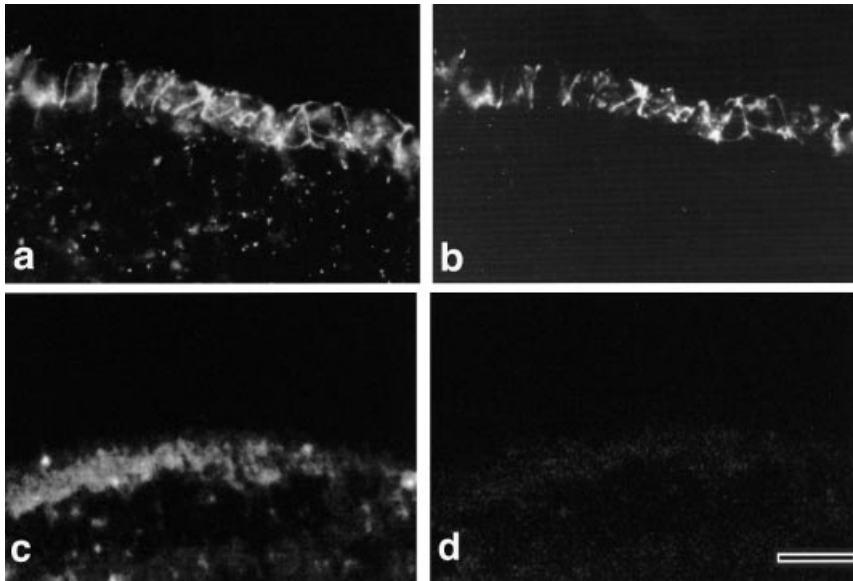
a: Reversetranscriptase (+)

b: Reversetranscriptase (-)

Figure 2. Reverse transcription–PCR shows mRNA of claudin-6 not expressed in mouse adult skin. In both mouse adult skin and 11 d mouse embryo, occludin-mRNA was detected as a 729 bp DNA band; however, claudin-6-mRNA was detected as a 660 bp DNA band in 11 d mouse embryo, but not in mouse adult skin. The positive control of both mouse adult skin and 11 d mouse embryo showed G3DPH mRNA as a 451 bp DNA band (*a*). Reverse transcription–PCR without reverse transcriptase showed no amplified bands (*b*). Amplified PCR fragments with claudin-6 primers in 11 d embryo were sequenced and confirmed to be mouse claudin-6 (*c*).

cycles) was performed with 100 pM primer sets of mouse occludin (5′-atgtccggccgatgctctct-3′, 5′-atattggacttatcataccg-3′) and mouse claudin-6 (5′-atggcctctactggtctgca-3′, 5′-tcacacataattcttggtgg-3′). Positive control PCR using a 10 pM primer set of the G3PDH (glyceraldehydes 3-phosphatase dehydrogenase) gene (Toyobo) was performed in the same procedure. Reverse transcription–PCR without reverse transcriptase was also performed. Amplified PCR fragments with claudin-6 primers were directly sequenced with mouse claudin-6 forward primer (5′-atgtccggccgatgctctct-3′).

Immunoelectron microscopy The samples were fixed with PLP (0.01 M NaIO$_4$, 0.075 M lysine, 0.0375 M phosphate buffer, 2% formaldehyde), washed with 0.1 M phosphate buffer (PB), and frozen in liquid nitrogen. Ten micrometer thick sections of the samples were cut with a cryostat and air-dried on glass slides, and rinsed in 0.1 M PB containing saponin and in 0.1 M PB. The sections were then incubated with CL6pAb for 2 d at 4°C. Having been washed with 0.005% saponin in 0.1 M PB, the samples were incubated overnight at 4°C with an anti-rabbit IgG antibody coupled with 10 nm gold particles (BB International, Cardiff, U.K), After which the sections were washed, and fixed with 1% glutaraldehyde in 0.1 M PB for 10 min, and postfixed with 0.5% osmium oxide in 0.1 M PB. After several washes with distilled water, the sections were stained with uranyl acetate for 30 min, dehydrated by passage through a graded series of ethanol (60, 70, 80, 90, 95, and 100%) and propylene oxide, and embedded in epoxy resin. From these samples, ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then observed with an electron microscope operated at an acceleration voltage of 83.0 kV (JEM-1200EX, JEOL, Tokyo, Japan).

Expression vector of mouse claudin-6 and transfection Generation of the expression vector for mouse claudin-6 under the CAG promotor was previously reported (Morita *et al*, 1999c). Mouse L fibroblasts were used for transfection. Aliquots (1 μm) of each expression vector were introduced into the L fibroblasts in 1 ml of Dulbecco's minimal Eagle's medium by means of Lipofectamine Plus (Gibco BRL, Gaithersburg, MD). As the expression vector for mouse claudin-6 contained a G418 resistant gene, it was transfected as a single entity. Geneticin resistant colonies were isolated after 10–14 d of selection, clones with stable transfection and expressing claudin-6 were screened by means of immunofluorescence microscopy with CL6pAb.

Freeze-fracture electron microscopy 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 three times with 0.1 M sodium cacodylate buffer, 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, Hudson, NH). The samples were then immersed in household bleach, and replicas floating off the samples were washed with distilled water, gathered on formvar–film covered grids, and examined with a JEOL 1200EX electron microscope (JEOL, Peabody, MA) at an acceleration voltage of 100 kV.

RESULTS

First, immunofluorescence staining was used to examine the expression of occludin and claudin-6, which are integral membrane

Figure 3. ZO-1 and ZO-2 are coexpressed at the cell–cell border of periderm in 11.5 d mouse embryo. Double immunofluorescence staining was performed on the periderm of 11.5 d mouse embryo. ZO-1 (*a*)and ZO-2 (*b*) were colocalized at the cell–cell border of the periderm. In (*a*) significant background staining represents interaction between endogenous mouse IgG and the second antibody, as observed in staining without the ZO-1 primary antibody (*c*). No specific signal was observed in staining without the ZO-2 primary antibody (*d*). *Scale bar*: 20 μm.
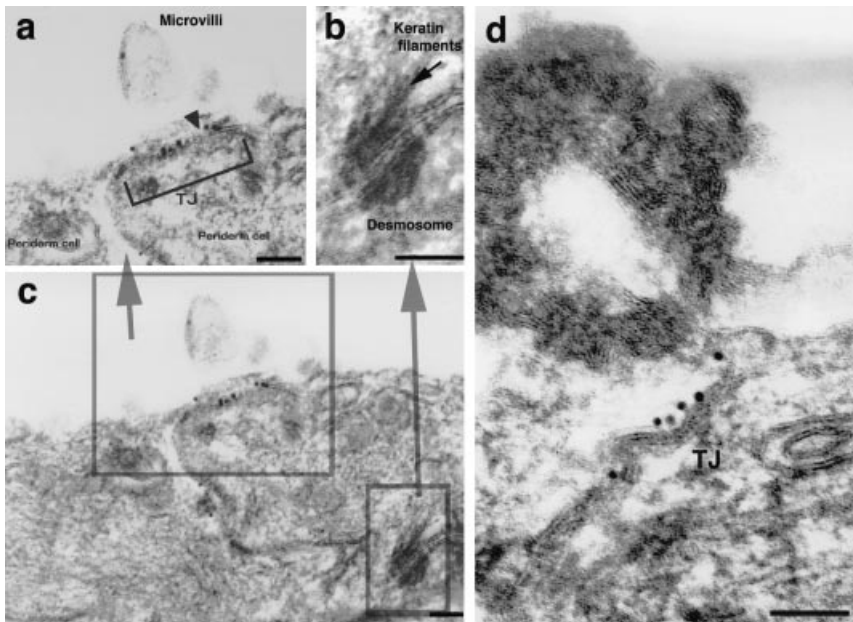
Figure 4. Subcellular localization of claudin-6 in periderm by immunoelectron microscopy. Immunoelectron microscopy using CL6pAb demonstrated that gold particles were observed at the TJ in the periderm of 13.5 d mouse embryo (*a*). A contact point of two adjacent plasma membranes is shown (*arrow* in *a*). Although keratin filaments were condensed at desmosome, gold particles were not detectable at desmosome (*b*). Cellular orientation and localization of **Fig 1**(*a*, *b*) between peridermal cells were shown in **Fig 3**(*c*). The other TJ in the periderm were also labeled moderately with gold particles (*d*). No labeling was detected in TJ without primary antibody (data not shown). *Scale bar*: 50 nm.

proteins at TJ, in the periderm and epidermis. As previously reported (Morita *et al*, 1998), occludin was localized at the cell–cell border in the mouse periderm of a 13.5 d embryo, as well as in the granular layer of newborn mouse epidermis (**Figs 1a**, **b**). Similarly, claudin-6 was seen to be localized at the cell–cell border in the periderm of mouse 13.5 d mouse embryo (**Fig 1c**); however, no specific claudin-6 signal was detected in the epidermis of newborn mouse (**Fig 1d**).

To exclude the possibility that CL6pAb was inaccessible to antigen in the epidermis, we performed reverse transcription–PCR on adult skin and 11 d mouse embryo. The result showed that mRNA of claudin-6 and occludin were detected in 11 d embryo as a 660 bp and a 729 bp band, respectively (**Fig 2a**). In mouse adult skin, on the other hand, mRNA of occludin was detected as a 729 bp DNA band, but mRNA of claudin-6 not at all (**Fig 2a**). Reverse transcription–PCR without reverse transcriptase showed no amplified bands (**Fig 2b**). Amplified PCR fragments with

claudin-6 primers in 11 d embryo were sequenced and were confirmed to be mouse claudin-6 (the sequence was shown partially in **Fig 2c**).

Next, the expression of undercoat proteins of TJ in periderm were examined by means of immunofluorescence staining. Similarly to occludin and claudin-6 in periderm, ZO-1 and ZO-2 were colocalized at the cell–cell border in the periderm of 11.5 d mouse embryo (**Fig 3a**, **b**).

Localization of claudin-6 in the periderm was then examined by means of immunoelectron microscopy using CL6pAb. It showed that claudin-6 was localized at the TJ in the periderm of 13.5 d mouse embryo (**Fig 4a**, **c**, **d**); however, no labeling of gold particles was observed at desmosomes (**Fig 4b**).

Finally, cDNA was introduced encoding claudin-6 into cultured L fibroblasts, which lacked TJ or claudins expression (Furuse *et al*, 1998b). Immunofluorescence staining of L fibroblasts expressing exogenous claudin-6 showed that claudin-6 was highly concen-
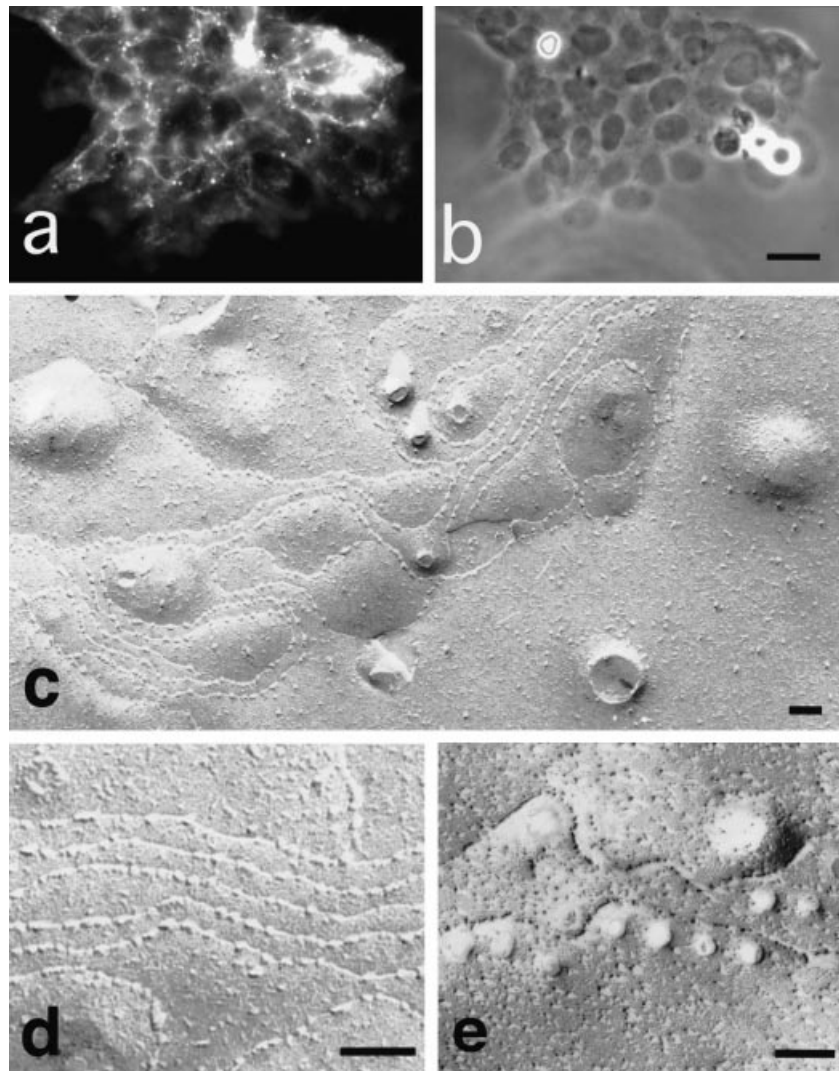
Figure 5. Claudin-6 stable transfectants form developed TJ. Immunofluorescence staining with CL6pAb showed that claudin-6 was highly concentrated at cell–cell contact sites in the form of planes or lines in claudin-6 stable transfectants (*a*) (*c* shows phase contrast image). Freeze-fracture frequently revealed TJ strands (*c*, *d*). The endogenous particles of these strands are localized discontinuously with similar frequency in the grooves not only on the E-face (*d*) but also on the P-face (*e*). *Scale bars*: (*a*, *b*) 20 μm, (*c–e*) 100 nm.

trated at cell contact sites in the form of lines or planes (**Fig 5a**, **b**). Endogenous claudin-6 expression in L fibroblasts was not observed by immunostaining (data not shown). Freeze-fracture of these transfectants frequently revealed TJ strands (**Fig 5c**). At higher magnification, the endogenous particles of the strands were seen to be localized discontinuously in the grooves on the E-face (**Fig 5d**). On the P-face, too, these particles are observed discontinuously in the grooves of TJ strands with virtually the same frequency as on the E-face (**Fig 5e**).

DISCUSSION

Although little attention had been paid so far to TJ in stratified epithelial cells such as the epidermis, subcellular distribution of occludin, ZO-1, and ZO-2 in the epidermis indicated that the maculae occludentes in the granular layer are in fact less-developed TJ (Morita *et al*, 1998). In the granular layer of the epidermis, occludin was expressed specifically and ZO-1/2 predominantly (Morita *et al*, 1998). These characteristic distributions of TJ-associated molecules have also been observed in other stratified epithelia, such as the cornea (Sugrue and Zieske, 1997).

Although the maculae occludentes of epidermis are less-developed TJ, keratinocytes in primary culture form were found to develop TJ in the same manner that simple epithelia do (Kitajima *et al*, 1983). This is probably because cultured keratinocytes form a simple layer (or a few layers at most) of cells in a culture dish, as

seen *in vivo* in simple epithelia, such as intestinal or vascular endothelia. For this reason, TJ are downregulated by keratinocytes of the epidermis.

The function of the maculae occludentes in the epidermis remains controversial. Elias *et al* (1977) reported that TJ in the epidermis were either absent or too fragmentary to constitute an effective barrier. On the other hand, Hashimoto (1971) reported that TJ were involved in the occlusion of granular cells. As barriers of the mammalian epidermis, cornified layers, and intercellular lipids have been identified as important (Elias *et al*, 1977; Elias, 1983).

In embryonic skin, simple epithelia, i.e., the periderm, covers the developing epidermis. The periderm is believed to function as a protective layer for the embryo (Holbrook and Odland, 1975). Periderm was found to carry a typical occludin-positive TJ (Morita *et al*, 1998). In this study, we showed that ZO-1 and ZO-2 were also colocalized at the cell–cell borders of the periderm. These results lead to the conclusion that occludin, ZO-1, and ZO-2 are localized at TJ of the periderm as well as the maculae occludentes. The question, therefore, naturally arises as to what molecules are involved in the functional and morphologic differences between TJ of the periderm and the maculae occludentes of the epidermis. To answer this question, we showed that claudin-6 was expressed at TJ in the periderm, but was not in epidermis. Furthermore, we showed that claudin-6 formed developed TJ when exogenously expressed in L fibroblasts. These findings suggest that claudin-6

fulfills important functions in the formation of TJ in the periderm and may be involved in developmental regulation of TJ in the epidermis.

Similarly, it has been shown that freeze-fracture of L fibroblasts, which exogenously express other claudin members, reveals developed TJ (Furuse *et al*, 1998b; Morita *et al*, 1999b, c). Freeze-fracture in glutaraldehyde-fixed L transfectants showed that endogenous particles of claudin-1-mediated TJ were associated with the P-face (Furuse *et al*, 1998b); however, those of claudin-5-mediated TJ were associated entirely with the E-face (Morita *et al*, 1999c). The current freeze-fracture in glutaraldehyde-fixed L fibroblasts, which expresses exogenous claudin-6, showed that TJ strand particles were associated with not only the E-face but also the P-face. *In vivo*, however, it has been shown that the extent of TJ strand particle association with the P- or E-face in glutaraldehyde-fixed samples varies depending on cell type. In intestinal epithelial cells and secretory cells, freeze-fracture images of TJ strands/grooves represented the P-face-associated type of TJ (Friend and Gilula, 1972; Hull and Staehelin, 1976). In endothelial cells in non-neuronal tissues or in Sertoli cells in the testes, the images of TJ strands represented the E-face-associated type of TJ (Nagano *et al*, 1982; Schneeberger, 1982). These findings favor the notion that the association of the P- or E-face in TJ of various tissues depends on the combination of the type and quality of claudins.

Finally, occludin should be discussed, which is another integral component of TJ in the periderm. Occludin has been shown to be a functional component of TJ. Transepithelial resistance and paracellular leakage in MDCK cells or *Xenopus* embryo are affected by overexpression of full-length or truncated occludin (Balda *et al*, 1996; McCarthy *et al*, 1996; Chen *et al*, 1997). Transepithelial Electrical Resistance in *Xenopus* epithelia is downregulated by addition to the culture medium of a synthetic peptide corresponding to the second extracellular loop of occludin (Wong and Gumbiner, 1997). On the other hand, it has become clear that the structure and functions of TJ cannot be explained in terms of occludin alone. When the occludin gene was disrupted in embryonic stem cells, visceral endoderms differentiated from these cells still contained well-developed TJ (Saitou *et al*, 1998). Thus, to understand the function of occludin in periderm, analysis of occludin knockout mice would be useful (Saitou *et al*, 2000). The finding that development of the skin was not impaired in occludin knockout mice (Saitou *et al*, 2000), suggests that functions of occludin in periderm are compensated with the other molecules, such as claudin-6.

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