Pilt, a Novel Peripheral Membrane Protein at Tight Junctions in Epithelial Cells*

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Tight junctions (TJs) serve as a barrier that prevents solutes and water from passing through the paracellular pathway, and as a fence between the apical and basolateral plasma membranes in epithelial cells. TJs consist of transmembrane proteins (claudin, occludin, and JAM) and many peripheral membrane proteins, including actin filament (F-actin)-binding scaffold proteins (ZO-1, -2, and -3), non-F-actin-binding scaffold proteins (MAGI-1), and cell polarity molecules (ASIP/PAR-3 and PAR-6). We identified here a novel peripheral membrane protein at TJs from a human cDNA library and named it Pilt (for protein incorporated later into TJs), because it was incorporated into TJs later after the claudin-based junctional strands were formed. Pilt consists of 547 amino acids with a calculated Mr of 60,704. Pilt has a proline-rich domain. In cadherin-deficient L cells stably expressing claudin or JAM, Pilt was not recruited to TJs and interacts with ZO-1 (16–18). As a non-F-actin-binding scaffold protein, MAGI-1/2/3 localizes at TJs (19, 20) and interacts with signaling molecules, such as PTEN (21, 22) and a GDP/GTP exchange protein for Rap small G protein (23). As cell polarity molecules, ASIP/PAR-3 and PAR-6 are concentrated at TJs (24, 25). Recently, ASIP/PAR-3 has been shown to interact with JAM (26, 27). Furthermore, several peripheral membrane proteins, cingulin, 7H6 antigen, and symplekin, have been shown to localize at TJs (28–30). Recently, cingulin has been shown to interact with ZO-1, -2, and -3 (31). Several molecules involved in intracellular vesicle trafficking, such as Rab3B small G protein and mammalian homologues of yeast SEC6 and -8 gene products, are also concentrated at TJs (32–34).

AJs consist of cell adhesion molecules and many peripheral membrane proteins including F-actin, F-actin-binding proteins, and non-F-actin-binding scaffold proteins. As a major cell adhesion molecule, cadherin plays a crucial role in the formation and maintenance of AJs (1, 2). Another cell adhesion molecule, nectin, localizes at AJs and regulates the formation of AJs in cooperation with cadherin (35, 36). At the cytoplasmic face, cadherin and nectin interact with F-actin-binding proteins, α-catenin and afadin, respectively (37, 38). α-Catenin furthermore interacts with other F-actin-binding proteins, such as vinculin and α-actinin (1, 2). A vinculin- and afadin-binding protein, ponsin, also localizes at AJs (39). As a non-F-actin-binding scaffold protein, hDlg/SAP97 localizes at AJs (40). In addition, growth factor receptors, such as the hepatocyte growth factor and epidermal growth factor receptors, are concentrated at AJs (41, 42).

hDlg has three isoforms, PSD-95/SAP90, PSD-93/chapsyn, and SAP102 (for reviews, see Refs. 43 and 44). Like ZO-1, -2, and -3, these isoforms belong to the MAGUKs (43, 44). The MAGUKs contain several PDZ domains, one SH3 domain, and one GK domain. Of these hDlg isoforms, PSD-95/SAP90, a neuron-specific isoform, has most extensively been characterized, and its many binding molecules have been identified. The PDZ domains of PSD-95/SAP90 interact with the N-methyl-D-aspartate receptor, K⁺ channels, neuroligins, synGAP, Citron, MAGUIN, APC, and CRIP (43–46). The GK domain interacts with SAPAP/GKAP/DAP and BEGAIN (44, 47, 48). hDlg is ubiquitously expressed including epithelial cells. Dlg1, a Drosophila counterpart of hDlg, plays a critical role in the formation of cell-cell junctions play crucial roles in various cell functions, including cell adhesion, growth, and polarization (for reviews, see Refs. 1 and 2). In polarized epithelial cells, cell-cell junctions form a specialized membrane structure, comprising TJs, AJ, and desmosomes, which is known as the junctional complex. These three junctional structures are aligned from the apical side to the basal side of the lateral membrane, although desmosomes are independently distributed in other areas. TJs function as a barrier preventing solutes and water from passing freely through the paracellular pathway (for reviews, see Refs. 3 and 4). TJs also serve as a fence between the apical and basolateral plasma membranes to form and maintain cell polarity (3, 4). Recent studies have revealed the molecular architecture of TJs (3, 4). TJs consist of transmembrane proteins and many peripheral membrane proteins. The peripheral membrane proteins include F-actin, F-actin-binding scaffold proteins, non-F-actin-binding scaffold proteins, and cell polarity and signaling molecules. As a major transmembrane protein, claudin forms TJ strands and plays a crucial role in the formation and maintenance of TJs (3, 4). Ocludin also forms TJ strands, but the physiological function remains to be clarified (3, 4). At the cytoplasmic face, claudin and occludin interact with F-actin-binding scaffold molecules, ZO-1, -2, and -3 (5–15). Another transmembrane protein, JAM, also localizes at TJs and interacts with ZO-1 (16–18). As a non-F-actin-binding scaffold protein, MAGI-1/2/3 localizes at TJs (19, 20) and interacts with signaling molecules, such as PTEN (21, 22) and a GDP/GTP exchange protein for Rap small G protein (23). As cell polarity molecules, ASIP/PAR-3 and PAR-6 are concentrated at TJs (24, 25). Recently, ASIP/PAR-3 has been shown to interact with JAM (26, 27). Furthermore, several peripheral membrane proteins, cingulin, 7H6 antigen, and symplekin, have been shown to localize at TJs (28–30). Recently, cingulin has been shown to interact with ZO-1, -2, and -3 (31). Several molecules involved in intracellular vesicle trafficking, such as Rab3B small G protein and mammalian homologues of yeast SEC6 and -8 gene products, are also concentrated at TJs (32–34).

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1 The abbreviations used are: TJ, tight junction; AJ, adherens junction; F-actin, actin filament; SH3, Src homology 3; GK, guanylate kinase; EGFP, enhanced green fluorescent protein; aa, amino acid(s); GST, glutathione S-transferase; MAGUK, membrane-associated guanylate kinase homologues; BSA, bovine serum albumin; Ab, antibody.

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of cell-cell junctions of epithelial cells and the polarity of neuro-epithelial cells in the embryo of Drosophila (49–51). Although the function of hDlg in mammalian epithelial cells is not clear, it could be a core protein in the formation of cell-cell junctions. However, little is known about hDlg-binding molecules or the mechanism of the localization of this molecule at AJs.

In this study, we attempted to identify an hDlg-binding protein using the yeast two-hybrid screening and identified a novel protein from a human cDNA library. However, this protein was a component of TJs rather than AJs. We also found that the protein was incorporated into TJs after TJ strands were formed, and therefore named it Pilt (protein incorporated later into TJs). We describe here the identification and characterization of Pilt.

**Experimental Procedures**

**Yeast Two-hybrid Screening**—A bait vector, pBTM116HA hDlg-2-2, was constructed by subcloning the insert encoding aa 581–926 of hDlg-2 into pBTM116HA (52). A mouse 11-day embryo yeast two-hybrid library was purchased from CLONTECH and screened as described previously (47, 48).

**Construction of Expression Vectors**—A cDNA of Pilt (NT2RF3003185; GenBank®/EMBL/DBJ accession no. AK024269) was kindly supplied from Dr. T. Isogai (Helix Research Institute Inc., Chiba, Japan). A cDNA of hDlg-2 was kindly supplied from Dr. T. Akiyama (Tokyo University, Tokyo, Japan). Various expression vectors were constructed in pcNeo Myc (53), pMXII-EGFP, and pGexT-1 (Amersham Biosciences, Inc.). pcNeo Myc was designed to express an N-terminal Myc-tagged protein, pMXII-EGFP was constructed by inserting a cDNA fragment encoding EGFP into pMXII (54) to express an N-terminal EGFP-tagged protein. Various constructs of Pilt and hDlg-2 contained the following aa: pMXIINeo Myc Pilt, aa 1–547 (full-length); pMXII-EGFP Pilt, aa 1–547 (full-length); pGexT-1 Pilt-2, aa 1–260; and pGexT-1 hDlg-2, aa 660–926 (SH3 and GR domains).

**Ab**—A rabbit polyclonal Ab was raised against GST-Pilt-2. A mouse monoclonal anti-human JAM Ab (55) was kindly supplied from Drs. T. Kita and H. Ozaki (Kyoto University, Kyoto, Japan). A mouse monoclonal anti-ZO-1 Ab (56) was kindly supplied from Drs. S. Tsukita and M. Itoh (Kyoto University, Kyoto, Japan). A rat monoclonal anti-nectin-2 Ab was prepared as described (57). Rabbit polyclonal anti-claudin-1, mouse monoclonal anti-Myc, and mouse monoclonal anti-PSD-95 family Abs were obtained from Zymed Laboratories Inc., American Type Culture Collection, and Upstate Biotechnology, respectively.

**Cell Culture and Transfection**—COS7, MTD1-A, and L cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. COS7 cells were transfected with the DEAE dextran method (57). MTD1-A cells stably expressing EGFP-Pilt were prepared using retrovirus-mediated gene transfection (54, 55). Claudin-1 and JAM-1 cells were kindly supplied by Drs. S. Tsukita, M. Furuse, and M. Itoh (Kyoto University, Kyoto, Japan).

**Affinity Chromatography**—COS7 cells on two 10-cm dishes were transfected with pcNeo Myc Pilt. The cells were then sonicated in 0.2 ml of Buffer A (20 mm Tris/Cl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 μM a-aminophenylmethanesulfonyl fluoride hydrochloride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) containing 1% (w/v) Triton X-100, followed by ultracentrifugation at 100,000 × g for 15 min. The supernatant was incubated with GST or GST-hDlg-2-2-4 (2 nmol each) immobilized on 50 μl (wet volume) of glutathione-Sepharose beads (Amersham Biosciences, Inc.). After the beads were extensively washed with Buffer A containing 3% (w/v) Triton X-100, the bound proteins were eluted by boiling the beads in an SDS sample buffer (60 mm Tris/Cl at pH 6.7, 3% SDS, 2% (v/v) 2-mercaptoethanol, and 5% glycerol). The sample was then subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-Myc Ab or protein staining with Coomassie Brilliant Blue.

**Wound Healing Assay of MTD1-A Cells**—Wound healing assay was performed as described (58–60). Briefly, about 60% confluent MTD1-A cells stably expressing EGFP-Pilt on 35-mm dishes were detached with a 10-μl syringe (Hamilton Co.) and further cultured for 3, 6, or 8 h.

**Miscellaneous Procedures**—Other procedures, including subcellular fractionation of rat liver (61), immunofluorescence microscopy of cultured cells and frozen sections (35, 38, 39), and immunoelectron microscopy (35, 38, 39), were performed as described. Protein concentrations were determined with BSA as a reference protein (62). SDS-PAGE was done as described (63). Prestained markers used in Western blotting were β-galactosidase (123 kDa), phosphorylase B (106 kDa), and BSA (77 kDa). Standard markers used in protein staining were phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carboxy anhydride (31 kDa).

**Results**

**Identification of Pilt as an hDlg-binding Protein**—To identify an hDlg-binding protein, we performed the yeast two-hybrid screening using the region of hDlg-2 containing the SH3 and GK domains (aa 581–926) as a bait. We obtained 31 positive clones from 1 × 10^8 clones of a mouse 11-day embryo yeast two-hybrid library. Twenty-three clones were overlapped and encoded an identical protein. These mouse cDNA clones had homology to the C-terminal portion of a human cDNA (NT2RF3003185; GenBank®/EMBL/DBJ accession no. AK024269) (74% identity of aa sequence). The full-length clone of this human cDNA encoded a protein composed of 547 aa and a calculated M, of 60,704 (Fig. 1A). We named this protein Pilt (protein incorporated later into TJs), because it was incorporated into TJs late after the claudin-based junctional strands were formed as described below. The aa sequences of the N- and C-terminal regions of Pilt were 43 and 51% identical to those of BEGAIN (48), respectively, but other regions showed no homology to BEGAIN (Fig. 1B). BEGAIN has been identified as a protein interacting with the GK domain of PSD-95/SAP90 (48). Like BEGAIN, the software COILS version 2 predicted a coiled-coil structure in the N-terminal region of Pilt. Pilt has a proline-rich domain, whereas BEGAIN does not. BEGAIN has
one nuclear localization signal, whereas Pilt does not. Pilt has no transmembrane segment.

To confirm whether the isolated cDNA encodes the full-length of Pilt, COS7 cells were transfected with pCIneo Myc Pilt. The extract was subjected to SDS-PAGE, followed by Western blotting with the anti-Pilt Ab. The other half was subjected to SDS-PAGE, followed by protein staining with Coomassie Brilliant Blue. A. Western blot analysis. Lane 1, cell extract of COS7 cells transfected with pCIneo Myc Pilt; lane 2, GST-immobilized beads incubated with the cell extract; lane 3, GST-hDlg-2–4-immobilized beads incubated with the cell extract. B, protein staining. Lane 1, GST-immobilized beads incubated with the cell extract; lane 2, GST-hDlg-2–4-immobilized beads incubated with the control buffer; lane 3, GST-hDlg-2–4-immobilized beads incubated with the cell extract. Arrow, Myc-Pilt; arrowhead, GST-hDlg-2–4; asterisk, GST.

Subcellular fractionation. Each fraction (25 μg of protein each) was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-Pilt or anti-hDlg Ab. Lane 1, the homogenate fraction; lane 2, the soluble fraction; lane 3, the pellet fraction; lane 4, the fraction rich in bile canaliculi; lane 5, the fraction rich in AJs and TJs.

Interaction of Pilt with hDlg.—To confirm the interaction of Pilt with hDlg, the extract of COS7 cells expressing Myc-tagged Pilt was incubated with a GST fusion protein of hDlg (SH3 and GK domains) immobilized on glutathione-Sepharose beads. After washing the beads, the bound proteins were eluted and the half of the eluate was subjected to SDS-PAGE, followed by Western blotting with the anti-Myc Ab. The other half was subjected to SDS-PAGE, followed by protein staining with Coomassie Brilliant Blue. Myc-tagged Pilt indeed bound hDlg (Fig. 2, A and B).

Tissue and Subcellular Distributions of Pilt.—Northern blot analysis using the full-length cDNA of Pilt as a probe detected ~3.0-kb mRNA in all the human tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and leukocytes (Fig. 3A). Subcellular fractionation analysis of Pilt in rat liver indicated that it was enriched in the fraction rich in AJs and TJs, where hDlg was also enriched (Fig. 3B).

Localization of Pilt at TJs in Epithelial Cells.—Because hDlg has been shown to localize to AJs (40), we examined by immunofluorescence microscopy whether Pilt colocalized with hDlg at AJs in MTD-1A cells. MTD-1A cells are mouse mammary tumor cells (64). Pilt and hDlg localized at cell-cell junctions, but the distribution pattern of Pilt was slightly different from that of hDlg (Fig. 4A). We therefore next examined whether Pilt colocalized with ZO-1 at TJs in MTD-1A cells. The two proteins colocalized at the cell-cell junctions (Fig. 4B). To further confirm the colocalization of Pilt and ZO-1 in MTD-1A cells, EGFP-tagged full-length Pilt was stably expressed in MTD-1A cells and the localization of the expressed protein was compared with that of endogenous ZO-1. Exogenously expressed Pilt colocalized with ZO-1 (Fig. 4C). Because ZO-1 localizes at TJs in epithelial cells (5, 7), these results suggest that Pilt localizes at TJs. It may be noted that Pilt was also stained at the perinuclear regions, most presumably the Golgi complex as estimated by the co-staining with Golgi 58-kDa protein (p58), a marker for the Golgi complex (65) (Fig. 4D).

To obtain the definitive evidence for the localization of Pilt at TJs, its localization was analyzed in small intestine absorptive epithelial cells, because TJs and AJs are well separated in this cell type (7). Immunofluorescence microscopy showed that Pilt colocalized with ZO-1 at TJs in small intestine absorptive epithelial cells (Fig. 5A). It was also stained at the perinuclear regions, presumably the Golgi complex. Immunoelectron microscopy revealed that Pilt exclusively localized at TJs and was absent from AJs and desmosomes (Fig. 5B).

No Recruitment of Pilt to Claudin-based or JAM-based Cell Contact Sites.—We examined whether Pilt directly interacts with claudin or JAM. For this purpose, we took advantage of cadherin-deficient L cells stably expressing claudin-1 or JAM (claudin-L and JAM-L cells, respectively) (27, 66). In claudin-L cells, claudin-1 and ZO-1 were concentrated at cell-cell contact sites, but Pilt was not concentrated there (Fig. 6A). Pilt was stained at the perinuclear regions. In JAM-L cells, JAM was concentrated at cell-cell contact sites, but Pilt was not concentrated there (Fig. 6B). Pilt was again stained at the perinuclear regions. It has been shown that ZO-1 colocalizes with JAM (27). Although we have not examined the in vitro binding of Pilt with claudin, JAM, or ZO-1, the results suggest that Pilt does not directly interact with these proteins.

Late Incorporation of Pilt into TJs.—When confluent cultures of MTD-1A cells are scratched with a needle, very thin cellular protrusions begin to emerge from the front edge of the wound at the initial stage of wound healing process. At the next stage,
small cell-cell junctions are formed at the tips of these cellular projections, which are regarded as spot-like primordial junctions as reported previously (59, 60). Nectin-2 is concentrated at these small contact sites (58). We confirmed this earlier observation (Fig. 7A). Pilt was not accumulated at the spot-like junctions, although it was stained at cell-cell junctions at the non-wounding regions. At the next stage of this wound healing process, the spot-like junctions begin to be fused to form short line-like junctions (58–60). Claudin was accumulated at this type of junction (Fig. 7B). Pilt was not concentrated at the line-like junctions. At the later stage of this process, the line-like junctions grow up to complete cell-cell junctions. Pilt was finally stained at these junctions (Fig. 7C). These results indicate that Pilt is incorporated into TJs at the very late stage.

**DISCUSSION**

We have isolated here a novel protein, named Pilt, as an hDlg-binding protein. However, the immunofluorescence and immunoelectron microscopic analyses indicate that Pilt localizes at TJs but not at AJs in epithelial cells including cultured MTD-1A cells and small intestine absorptive epithelial cells, whereas hDlg localizes at AJs. Furthermore, Pilt is incorporated into TJs at the very late stage of wound healing process, whereas hDlg is incorporated into spot-like junctions at the initial stage. These results suggest that Pilt is not a physiological binding partner of hDlg in epithelial cells, although we do not exclude the possibility that Pilt directly interacts with hDlg in cells lacking TJs. The Northern blot analysis indicates that Pilt is highly expressed in tissues, such as skeletal muscle and spleen, which are not rich in TJs. Conversely, the expression of Pilt is relatively low in tissues that are rich in TJs. It remains to be clarified why Pilt shows such tissue distribution patterns. It remains unknown, as well, whether Pilt colocalizes with hDlg in cells lacking TJs. Further studies are necessary to

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2 A. Fukuhara, K. Irie, and Y. Takai, unpublished observation.
Pilt is incorporated into TJs at the very late stage. It remains to be clarified how Pilt is recruited to TJs at the very late stage, but this recruitment may be closely correlated with the function of Pilt.

Several molecules involved in vesicle trafficking, such as Rab3B and mammalian homologues of yeast SEC6 and -8 gene products, are concentrated at the cytoplasmic face of TJs (32–34). As SEC6 and -8 products are involved in vesicular targeting required for polarized budding in yeast (67), it is proposed that TJs function as a site for vesicular targeting and fusion to establish and/or maintain epithelial cell polarity (34). We have shown here that Pilt localizes at the Golgi complex as well as at TJs. Therefore, Pilt may be involved in vesicle trafficking between the Golgi complex and TJs to establish and maintain cell polarity.

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