JEAP, a Novel Component of Tight Junctions in Exocrine Cells*

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Tight junctions (TJs) consist of transmembrane proteins and many peripheral membrane proteins. To further characterize the molecular organization of TJs, we attempted here to screen for novel TJ proteins by the fluorescence localization-based expression cloning method. We identified a novel peripheral membrane protein at TJs and named it junction-enriched- and -associated protein (JEAP). JEAP consists of 882 amino acids with a calculated molecular weight of 98,444. JEAP contained a polyglutamic acid repeat at the N-terminal region, a coiled-coil domain at the middle region, and a consensus motif for binding to PDZ domains at the C-terminal region. Exogenously expressed JEAP co-localized with ZO-1 and occludin at TJs in polarized Madin-Darby canine kidney cells, but not with claudin-1, JAM, or ZO-1 in L cells. Endogenous JEAP localized at TJs of exocrine cells including pancreas, submandibular gland, lacrimal gland, parotid gland, and sublingual gland, but not at TJs of epithelial cells of small intestine or endothelial cells of blood vessels. The present results indicate that JEAP is a novel component of TJs, which is specifically expressed in exocrine cells.

The cell-cell junctional complex of endothelial and epithelial cells consists of three compartments: TJs, 1 AJs, and desmosomes (1). TJs form an apical junctional structure and act as a “barrier” and a “fence” to control paracellular permeability and to maintain cell polarity, respectively (2–7). TJs comprise transmembrane molecules, such as claudin (8, 9), occludin (10, 11), and JAM (12–14), as well as peripheral membrane proteins, such as ZO-1 (15), -2 (16), -3 (17), cingulin (18), 7H6 (19), symplekin (20), Rab3B (21), ASIP/PAR-3 (22–24), PAR-6 (23, 24), and MAGI-1 (25, 26). Claudin and occludin constitute the backbone of TJ strands and are involved in the barrier function of TJs (6, 7). JAM is involved in cell-cell adhesion and/or junctional assembly of endothelial and epithelial cells, as well as in the transendothelial migration of monocytes induced by chemokines (12, 13). ZO-1, -2, and -3 are scaffold proteins containing PDZ domains and directly bind to claudin and occludin at the cytoplasmic surface of TJ strands (17, 27–29). ZO-1, -2, and -3 also bind F-actin and might regulate TJ functions, probably via cross-linking TJ strands and the actin cytoskeleton (30–32). JAM also interacts with ZO-1 (33, 34). Several other PDZ-containing proteins at TJs might also serve as landmarks to recruit cytoskeletal and signaling molecules to TJ strands (6, 7). As a non-F-actin-binding scaffold protein, MAGI-1/2/3 localizes at TJs (25, 26) and interacts with signaling molecules such as a tumor suppressor gene product, PTEN (35, 36), and a GDP/GTP exchange protein for Rap small G protein (37). ASIP/PAR-3 and PAR-6 are polarity-related molecules containing PDZ domains and interact with atypical protein kinase C (22–24, 38). ASIP/PAR-3 interacts with JAM (39, 40). Among peripheral membrane proteins devoid of PDZ domains at TJs, RhoB is involved in vesicular transport (21). Furthermore, cingulin, 7H6 antigen, and symplekin have been shown to localize at TJs (18–20). Cingulin has recently been shown to interact with ZO-1, -2, -3, occludin, AF-6, and JAM (33, 41).

To further characterize the molecular organization of intercellular junctions, we attempted here to screen for novel proteins localized at cell-cell junctions by the fluorescence localization-based expression cloning method in which cDNAs can be isolated based on the subcellular localization of their GFP fusion protein products (42). We have cloned several novel cDNA fragments, one of which specifically localizes at TJs. We named this protein junction-enriched- and -associated protein (JEAP) and characterized it.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfection—An endothelial cell line, MS-1, was obtained from American Type Culture Collection and cultured in DMEM with 5% fetal calf serum. MDCK cells were kindly supplied by Dr. W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany) and cultured in DMEM with 10% fetal calf serum. For generation of ectopic retrovirus competent MDCK cells (MDCK/EcoVR), the ecotropic virus receptor (EcoVR) cDNA was inserted into pCAGGS-puro (43) and transfected to MDCK cells using LipofectAMINE reagent (Invitrogen). The cells were then cultured for 24 h, replated, and selected with 5 μg/ml of puromycin (Invitrogen). Each clone was isolated and infected with pMXI-EGFPN recombinant retrovirus (44), and a high competent clone for infection was used for the following studies. EcoVR cDNA (45) and pMX (46) were kindly provided by Dr. T. Kitamura (Tokyo University, Tokyo, Japan). Claudi-L cells and JAM-L cells were kindly supplied by Dr. Sh. Tsukita (Kyoto University, Kyoto, Japan). L cells were transfected by use of LipofectAMINE.

Construction and Screening of the cDNA Library—To identify novel cDNAs that encode proteins localized at cellular junctions, we created a cDNA-GFP fusion library from MS-1, a mouse endothelial cell line, as described previously (42). The resulting library contained 3 × 10^5 independent clones and showed an average insert size of cDNA with 1,500 bp. The expression library was then co-transfected into 293/EBNA-1 cells (Invitrogen) with a packaging vector, pCL-Eco (Imgenex,
San Diego, CA), and converted to retroviruses. From a pilot experiment using VE-cadherin-EGFP fusion retrovirus with various cell lines, we found that MDCK/EcoVR cells were suitable for visual screening of junctional proteins because these cells are cuboid and showed bright signals. MDCK/EcoVR cells were, thus, infected with 2 ml of variously diluted retrovirus supernatants to obtain singly infected cells. The initial frequency of the EGFP-positive cells was 4% as determined by fluorescence-activated cell sorting (FACS) analysis. After expansion, EGFP-positive cells were sorted and cultured at 50 cells per well in 96-well plates. When the wells became confluent, we performed screening of 10 plates under the fluorescence microscope and selected 6 wells containing cells with junctional staining patterns. These cells were replated into 10-cm dishes and a single clone was obtained. Each clone was expanded in 24-well plates, and the integrated cDNA was recovered by PCR from the genomic DNA. Among the cDNAs showing the junctional staining pattern, we obtained a novel cDNA clone and analyzed it. Full-length JEAP cDNAs were obtained from a MS-1 cDNA library constructed with pmxII vector (44).

**Abs**—Rat mAbs were raised against the GST-JEAP fusion protein containing amino acid 808–882 of JEAP. Two clones (2E5–1 and 4E1–20) were used here. Rabbit anti-ZO-1 pAb, rabbit anti-claudin pAb, mouse anti-ZO-1 mAb, and mouse anti-occludin mAb were obtained from Zymed Laboratories Inc. Rat anti-E-cadherin mAb was obtained from Takara.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was performed as described (47–49). Briefly, MDCK/EcoVR cells were fixed with 3.7% formaldehyde in PBS at room temperature for 15 min. The fixed sample was treated with 0.2% Triton X-100 in PBS for 15 min and washed with PBS three times. After the sample was soaked with PBS containing 1% bovine serum albumin, the sample was incubated with various combinations of the anti-E-cadherin, anti-occludin, anti-claudin, anti-ZO-1, and anti-JEAP Abs and washed with PBS, followed by incubation with fluorescein isothiocyanate-, Cy3-, Cy5-labeled secondary Abs (Jackson). After incubation, the sample was washed with PBS, embedded in PBS containing 50% glycerol and 0.1% 1,4-diazobicyclo-(2,2,2)octane (DABCO), and analyzed by a LSM 510 confocal laser scanning microscope (Zeiss).

**Ca**\(^{2+}\) Switch Experiments—Ca\(^{2+}\) switch experiments using JEAP-expressing MDCK cells were done as previously described (26, 50). Briefly, the pmxII JEAP IRES-EGFP expression vector was constructed by inserting the full-length JEAP coding region amplified by PCR from the pmxII JEAP cDNA into pmxII IRES-EGFP (44). The expression vector was transfected into 293/EBNA-1 cells with a packaging vector, and the MDCK/EcoVR cells. JEAP-expressing MDCK cells were washed with PBS and cultured in DMEM without serum for 1 h. The cells were then transferred to DMEM with 5 mM EGTA and cultured for 2 h. After the culture, cells were washed with PBS and cultured either in DMEM without serum for 2 h or in DMEM with 100 mM TPA for 1 h.

**Miscellaneous Procedures**—Other procedures, including Western blotting, immunofluorescence microscopy of frozen sections, and immunoelectron microscopy, were performed as described (47–49).

**RESULTS**

**Identification of JEAP**—To identify novel cDNAs that encode proteins localized at cell-cell junctions, we created a cDNA-GFP fusion library from MS-1, a mouse endothelial cell line (Fig. 1A). The expression library was then co-transfected into 293/EBNA-1 cells with a packaging vector and converted to retroviruses. MDCK/EcoVR cells were infected with 2 ml of variously diluted retrovirus supernatants to obtain singly infected cells. We performed screening and selected 6 wells containing cells with junctional staining patterns. These wells were replated into 10-cm dishes, and a single clone was obtained. The integrated cDNA was recovered by PCR from the genomic DNA. Among the cDNAs showing the junctional staining pattern, we obtained a novel cDNA clone and further analyzed it.

The full-length cDNA clone was isolated from the MS-1 cDNA library. We named this protein JEAP because it localized at TJs as described below. The full-length clone of the JEAP cDNA encoded a protein with 882 amino acids with a calculated molecular weight of 98,444 (Fig. 2A). JEAP contained a polyglutamic acid repeat at the N-terminal region, a coiled-coil domain at the middle region (51, 52), and a consensus motif for binding to PDZ domains at the C-terminal region (53) (Fig. 2B).

To confirm whether the isolated clone encodes the entire coding region of JEAP, 293/EBNA-1 cells were transfected with the JEAP cDNA, and the expressed protein was detected by Western blotting. A single band of about 105 kDa was detected in the extract from the 293/EBNA-1 cells transfected with JEAP, but not in that from nontransfected 293/EBNA-1 cells (Fig. 2C). The size of the expressed protein was similar to that of endogenous JEAP in MS-1 cells. Therefore, we concluded that the isolated cDNA encodes the full-length of JEAP.

To further confirm the junctional localization of the full-length protein, full-length JEAP was expressed in MDCK/EcoVR cells as an EGFP fusion protein. The fusion protein localized at the
apical region of the lateral membrane of polarized MDCK cells (Fig. 3A). The distribution pattern of the fusion protein was similar to that of ZO-1, which localized at TJs in polarized epithelial cells (15, 54), but different from that of E-cadherin which showed broad distribution along the lateral membrane. To confirm the co-localization of JEAP and ZO-1, full-length JEAP was stably expressed in MDCK/EcoVR cells, and the localization of the expressed protein was compared with that of endogenous ZO-1. Exogenously expressed JEAP co-localized with ZO-1 (Fig. 3B). These results suggest that JEAP localizes at TJs.

**Localization of JEAP at TJs of Exocrine Glands**—We next examined tissue distribution of JEAP in various mouse tissues including liver, brain, lung, kidney, spleen, testis, ovary, and heart by Western blotting, but did not detect JEAP in any of these tissues (data not shown). We then examined tissue distribution immunohistochemically. JEAP was detected specifically in exocrine glands including pancreas, submandibular gland, lacrimal gland (Fig. 4A), parotid gland, and sublingual gland, but not in brain, heart, liver, kidney, spleen, gall bladder, or duodenum (data not shown). In exocrine glands, JEAP was expressed around the terminal portion of serous glands. In the terminal portion, JEAP showed a similar staining pattern to that of ZO-1. Although MS-1 cells expressed JEAP, we detected no staining signal for JEAP in endothelial cells in any organs. Immunoelectron microscopy revealed that JEAP indeed localized at TJs, but not at AJs or desmosomes, in the lacrimal gland (Fig. 4B).

**Incorporation of JEAP into Cell-Cell Junctions along with Other Components of TJs**—We next monitored the behavior of JEAP and other AJ and TJ components during the disruption and reformation of cell-cell junctions. For this purpose, we used a MDCK cell line stably expressing JEAP, because it has been shown that when MDCK cells are cultured at 2 mM Ca\(^{2+}\) for 2 h, AJs and TJs are disrupted, and the staining of the AJ and TJ components except nectin, afadin, and ZO-1 disappear from the plasma membrane, and that when the cells are recultured at 2 mM Ca\(^{2+}\) for 2 h, AJs and TJs are reformed where all the AJ and TJ components reconcentrate (26, 50). We have shown that claudin, occludin, JAM, nectin, and ZO-1, and afadin, but not E-cadherin, are concentrated there (26, 50). It has been shown that when MDCK cells, precultured at 2 mM Ca\(^{2+}\) for 2 h, are cultured with TPA at 2 mM Ca\(^{2+}\) for 1 h, a TJ-like structure is formed, although AJs are not formed (26, 50). We have shown that claudin, occludin, JAM, nectin, ZO-1, and afadin, but not E-cadherin, α- or β-catenin, are concentrated there (26, 50). JEAP was also recruited to the TPA-induced TJ-like structure (Fig. 5). Similarly, JEAP co-localized with occludin and claudin-1 at cell-cell junctions (data not shown). These results indicate that JEAP is incorporated into TJs along with other components of TJs.

**No Recruitment of JEAP to Claudin-based or JAM-based Cell-Cell Contact Sites**—We examined whether JEAP directly
interacts with claudin or JAM. For this purpose, cadherin-deficient L cells stably expressing claudin-1 or JAM (claudin-L and JAM-L cells, respectively) (8, 39) were transiently transfected with pMXII JEAP IRES-EGFP. In claudin-L cells, ZO-1 was concentrated at cell-cell contact sites, but transiently expressed JEAP was not concentrated there (Fig. 6). In JAM-L cells, ZO-1 was concentrated at cell-cell contact sites, but JEAP was not concentrated there (Fig. 6). Although we have not examined the in vitro binding of JEAP with claudin, JAM, or ZO-1, these results suggest that JEAP does not directly interact with any of these proteins.

DISCUSSION

In the present study, we have identified a novel component of TJs by visual screening with cDNA-EGFP fusion proteins expressed in the living cells. This strategy is fascinating for cloning of junctional proteins that are difficult to obtain from cell fractionation techniques (e.g. components may be lost during biochemical purification or dynamically shuttle among subcellular compartments and may be restricted in a small fraction of organelles, cell types, or tissues). In fact, we have isolated a novel TJ protein, JEAP, which is expressed in very restricted compartments, the terminal portion of exocrine glands.

JEAP is a novel type of a TJ component consisting of the coiled-coil domain and a consensus motif for binding to PDZ domains. Coiled-coil domains have been identified in a variety of cytoskeletal proteins and are involved in inter- or intramolecular protein-protein interactions (51, 52). The coiled-coil domain of JEAP shows weak similarity to the conserved domain of intermediate filament proteins and myosin tail as estimated by CD search (www.ncbi.nlm.nih.gov/Structure/ddi/ddi.shtml). In the case of TJ proteins, occludin and cingulin also have similar conserved coiled-coil domains (10, 29, 41, 55). Occludin interacts with ZO-1 via the coiled-coil domain (29). Another feature of JEAP is the C-terminal consensus motif for binding to PDZ domains (53). Integral membrane TJ proteins, claudin and JAM possess a PDZ-binding motif and interact with membrane-associated guanylate kinases (MAGUK) including ZO-1, -2, or -3, via the PDZ domain (6, 7). Although JEAP contains the potential protein-protein interaction domains, the mechanism of its specific localization at TJs remains unknown. The present results, however, suggest that JEAP does not directly interact with claudin-1, JAM, or ZO-1. JEAP may interact with other known or still unidentified molecule(s) and localizes at TJs through interaction with this protein(s). Identification of such a molecule would be important for our understanding of the mechanism of the specific localization of JEAP at TJs.

At present, the function of JEAP in the exocrine glands remains unknown. However, the presence of cell type- and tissue-specific peripheral membrane proteins at TJs suggests that specialized epithelial and/or endothelial cells possess unique junctional complexes depending on their functions. In this context, membrane proteins at TJs, claudins, and JAMs, are differentially expressed in various combinations in epithelial and/or endothelial cells (6, 7). Recently, it was reported that claudin-2 controls paracellular permeability of MDCK cells (56). It is tempting to speculate that JEAP also regulates assembly or integrity of TJs of exocrine-terminal portions in which TJs function as a barrier to prevent spilling of exocrine juice into organs.

A data base search has revealed two related proteins, KIAA0989 and angiomotin, suggesting that JEAP comprises a family. KIAA0989 has not been characterized but angiogotinin is a 72-kDa protein that is expressed selectively in capillary endothelial cells as well as in actively angiogenic tissues, such as placenta and solid tumors (57). It localizes at the lamellipodia of the leading edge of migrating endothelial cells and is implicated in the angiostatin-mediated regulation of cell motility and capillary formation. The primary structure, subcellular localization, and tissue distribution of angiomotin are, how-

FIG. 5. Incorporation of JEAP into cell-cell junctions along with other components of TJs. JEAP-MDCK cells were cultured at $2 \mu M$ Ca$^{2+}$ for 2 h and then incubated with $2 \mu M$ Ca$^{2+}$ for 2 h or with 100 nM TPA at $2 \mu M$ Ca$^{2+}$ for 1 h. The cells were doubly stained with the anti-JEAP mAb and anti-ZO-1 pAb. Bars, 20 μm. Representative results from at least three independent experiments are shown.

FIG. 6. No recruitment of JEAP to claudin- or JAM-based cell-cell contact sites. Claudin-L and JAM-L cells transiently transfected with pMXII JEAP IRES-EGFP were doubly stained with the anti-JEAP mAb and anti-ZO-1 pAb. Bars, 10 μm. Representative results from at least three independent experiments are shown.
ever, different from those of JEAP, suggesting that each member of the JEAP family has unique characteristics in terms of function and subcellular distribution.

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