Roles of immunoglobulin-like loops of junctional cell adhesion molecule 4; involvement in the subcellular localization and the cell adhesion

Makiko Tajima[1,2], Susumu Hirabayashi[1], Ikuko Yao[1], Madoka Shirasawa[1], Junichi Osuga[2], Shun Ishibashi[4], Toshiro Fujita[2,3] and Yutaka Hata[1,*]

[1]*Department of Medical Biochemistry, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan;*
[2]*Department of Metabolic Diseases and* [3]*Department of Nephrology and Endocrinology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan;*
[4]*Department of Internal Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-0498, Japan*

Abstract

***Background*: Membrane-associated guanylate kinase with inverted domain structure-1 (MAGI-1) is a scaffolding protein at tight junctions (TJs). We have recently identified junctional adhesion molecule 4 (JAM4) as a MAGI-1-interacting protein. JAM4 belongs to the immunoglobulin superfamily and mediates Ca^{2+}-independent adhesion. In this study, we examined the subcellular localization of JAM4 in various tissues and the involvement of JAM4 in the localization of MAGI-1. Moreover, we investigated into roles of immunoglobulin-like loops (Ig-loops) of JAM4.**

***Results*: JAM4 was localized at TJs but also on apical membranes of epithelial cells in jejunum, ileum, and renal proximal tubules. In Madine Darby canine kidney (MDCK) cells, the localization of JAM4 at TJs depended on the first Ig-loop and did not require the MAGI-1-interacting region. JAM4 determined the subcellular localization of MAGI-1 in MDCK cells. In ileum, however, MAGI-1 was localized at TJs where JAM4 was not detected. Both of Ig-loops were necessary for homophilic interactions, but *cis* interactions depended on the first Ig-loop.**

***Conclusion*: JAM4 may be primarily targeted to apical membranes, and subsequently recruited to TJs through the first Ig-loop–mediated molecular interaction. JAM4 determines the localization of MAGI-1 in MDCK cells, but the *in vivo* localization of MAGI-1 does not necessarily depend on JAM4.**

Introduction

Junctional cell adhesion molecule 1 (JAM1) belongs to the immunoglobulin superfamily and consists of two V-type immunoglobulin-like loops (Ig-loops), a single transmembrane region, and a short cytoplasmic tail (Martin-Padura *et al.* 1998; Ozaki *et al.* 1999). It is present at TJs in epithelial and endothelial cells. JAM1 mediates cell adhesion *via* homophilic interactions and regulates paracellular permeability (Martin-Padura *et al.* 1998; Liu *et al.* 2000). It is involved in the process of inflammation by mediating transmigration of monocytes and neutrophils through endothelial cells (Martin-Padura *et al.* 1998; Del Maschio *et al.* 1999). It is also expressed in peripheral blood platelets and thought to be involved in primary haemostasis (Naik *et al.* 1995; Sobocka *et al.* 2000; Naik *et al.* 2001). Subsequently, two homologs of JAM1 were reported and named JAM2 (also called VE-JAM and JAM-3) and JAM3 (also called JAM-2) (Cunningham *et al.* 2000; Palmeri *et al.* 2000; Aurrand-Lions *et al.* 2001a,b; Arrate *et al.* 2001). Like JAM1, JAM2 and JAM3 possess two Ig-loops, but the second Ig-loop proximal to the membrane is the C2-type instead of the V-type. JAM2 is preferentially expressed in the intercellular boundaries of the endothelium, particularly in heart and placenta, and function as an adhesive ligand for T, NK, and dendritic cells (Palmeri *et al.* 2000; Cunningham *et al.* 2000; Liang *et al.* 2002). JAM3 shows endothelial expression in many tissues, predominantly in

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**Correspondence*: E-mail: yuhammch@med.tmd.ac.jp

placenta, brain and kidney (Arrate *et al.* 2001). JAM3 is also found in T cells and is up-regulated by T-cell activation. JAM2 adheres to T cells through the heterotypic interaction with JAM3. The engagement of JAM2 with JAM3 facilitates the interaction between JAM2 and $\alpha_4\beta_1$ integrin in T cells (Cunningham *et al.* 2002). Thus, JAM2 and JAM3 are considered to take parts in leucocyte transmigration.

Recently, we have identified a novel protein which belongs to the immunoglobulin superfamily as a binding partner of membrane-associated guanylate kinase with inverted domain structure-1 (MAGI-1) (also called brain angiogenesis inhibitor 1-associated protein 1) (Dobrosotskaya *et al.* 1997; Shiratsuchi *et al.* 1998; Hirabayashi *et al.* 2003). Because the protein shows 14.0%, 10.4% and 13.5% amino acid homology to JAM1, JAM2 and JAM3, respectively, we named it JAM4. MAGI-1 has six PDZ domains, is localized at TJs in polarized epithelial cells and may be involved in the assembly of TJ components. Consistently, JAM4 is localized at TJs in Madine Darby canine kidney (MDCK) cells and mouse mammary gland epithelial cells. JAM4 contains two extracellular Ig-loops of the V-type and binds to the second and the fifth PDZ domains of MAGI-1 by its C-terminal PDZ-binding motif. JAM4 exhibits Ca^{2+}-independent cell adhesion activity and reduces the paracellular permeability in the monolayer of CHO cells. MAGI-1 strengthens the cell adhesion activity of JAM4. We also revealed that MAGI-1 is recruited to JAM4-based cell contacts in mouse fibroblastic L cells. In this model system, other TJ proteins such as ZO-1 and occludin are also accumulated to JAM4-based cell contacts. All these findings support the theory that the interaction between JAM4 and MAGI-1 is important for the organization of TJs.

For the previous study, we examined the subcellular localization of JAM4 in tissues of several organs including kidney, small intestine, and mammary gland. As in MDCK cells, JAM4 was detected at TJs in epithelial cells of small intestine and mammary gland ducts. In kidney, JAM4 was most concentrated in glomeruli and its localization at slit diaphragm was confirmed by immunoelectron microscopy. However, we also found the signal on apical membranes of epithelium of renal proximal tubules. We here extended the previous study to determine the localization of JAM4 in various tissues and compare it with that of MAGI-1. We attempted to elucidate the TJ-targeting mechanism of JAM4 and its role in the recruitment of MAGI-1. We are also interested in the structure of JAM4. The crystal structure determination of JAM1 revealed the structural basis for homophilic interaction (Kostrewa *et al.* 2001; Prota *et al.* 2003). Inspired by these studies, we have examined the role of each Ig-loop of JAM4 for the cell adhesion activity.

Results

JAM4 is localized at TJs in various tissues

In the previous study, we reported that JAM4 is localized at slit diaphragm in kidney glomeruli and confirmed the localization using immunoelectron microscopy (Hirabayashi *et al.* 2003). We also found that JAM4 is co-localized with ZO-1, a tight junction marker protein, in small intestine and mammary gland epithelial cells on the immunofluorescence level and concluded that JAM4 is a tight junction protein. However, JAM4 immunoreactivity was also detected on apical membranes of renal proximal tubular epithelial cells. These findings prompted us to perform a study to determine the subcellular localization of JAM4 in various rat tissues. First, we reexamined the localization of JAM4 in various portions of small intestines. JAM4 was co-localized with ZO-1 in duodenum, although some signal was detected on apical membranes (Fig. 1Aa). However, in jejunum and ileum, JAM4 was detected mainly on apical membranes (Fig. 1Ab and c). The absorption with the antigen abolished the apical signals, indicating that these signals are specific (Fig. 1B). Immunogold labelling of ultrathin sections of rat duodenal epithelium revealed that gold particles were accumulated at TJs, while they were absent from AJs and desmosomes (Fig. 1C). JAM4 was localized at TJs in epithelial cells of submandibular glands and eccrine sweat glands (Fig. 2, A and B). In kidney, JAM4 was detected at TJs of collecting ducts (Fig. 3A). JAM4 immunoreactivity on apical membranes in renal proximal tubular epithelial cells was also reproduced (Fig. 3B).

The first Ig-loop is required for the recruitment of JAM4 to TJs

Based on the *in vivo* localization of JAM4 on apical membranes and at TJs, we raised a question how JAM4 was targeted to TJs. To address this question, we prepared stable transformants of MDCK cells expressing various FLAG-tagged JAM4 proteins (Fig. 4). FLAG-JAM4-1 was concentrated at cell junctions, co-localized with ZO-1, but also slightly detected on apical membranes (Fig. 5A). FLAG-JAM4-2 lacking the first Ig-loop mostly appeared on apical membranes (Fig. 5B). We also confirmed the apical localization of FLAG-JAM4-1 and -2 using the surface protein biotinylation (Fig. 5E).

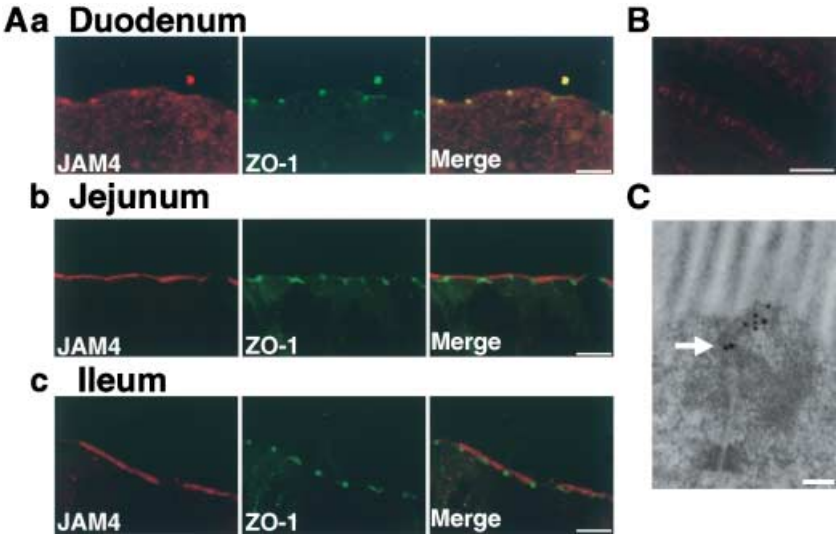

Figure 1 JAM4 in small intestine. (A) Immunohistochemical analysis of JAM4 in small intestine. (a) JAM4 and ZO-1 in duodenum. (b) JAM4 and ZO-1 in jejunum. (c) JAM4 and ZO-1 in ileum. Bar, 20 μm. (B) Immunofluorescence of ileum after absorption with the immunogen. Apical signals were abolished. Bar, 20 μm. (C) Immunogold analysis of JAM4 in rat duodenal epithelial cells. The ultrathin section of the adult rat small intestine was stained with rabbit polyclonal anti-JAM4 antibody, followed by immunoelectron microscopy with the immunogold method. Gold particles were detected at TJs (arrows) and also decorated the apical side to TJs. Bar, 100 nm.

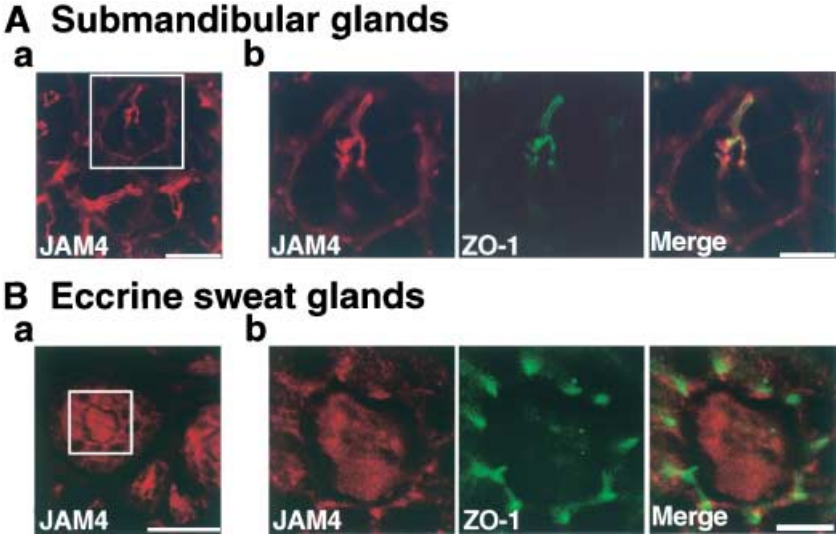

Figure 2 JAM4 in salivary and sweat glands. (A) JAM4 and ZO-1 in epithelial cells of submandibular glands. The demarcated area in (a) is shown at higher magnification in (b). (a) Bar, 20 μm (b) Bar, 10 μm. (B) JAM4 and ZO-1 in epithelial cells of eccrine sweat glands. The demarcated area in (a) is shown at higher magnification in (b). (a) Bar, 20 μm (b) Bar, 5 μm.

FLAG-JAM4-1 and -2 were biotinylated, only when the biotinylation reagent was added to the apical chambers. Like FLAG-JAM4-1, FLAG-JAM4-3 lacking the C-terminal PDZ-binding motif was mainly localized at TJs, although some of FLAG-JAM4-3 was detected on apical membranes (Fig. 5C). We also noticed that the junctional staining of FLAG-JAM4-3 appeared coarse, compared to that of FLAG-JAM4-1. FLAG-JAM4-4 lacking the second Ig-loop was localized at TJs, but was also distributed along lateral membranes outside TJs (Fig. 5D). These data imply that the targeting of JAM4 to TJs primarily depends on the first Ig-loop, and that the PDZ-binding motif, which is involved in the interaction with MAG-1, is not required.

Over-expressed JAM4 is localized on apical membranes and interferes with the localization of MAGI-1

In the next sets of experiments, we examined the effect of JAM4 on the localization of MAGI-1. Because FLAG-JAM4-1 was mostly co-localized with MAGI-1 in stable transformants of MDCK cells, we made use of FLAG-JAM4-2. First, we determined the localization of endogenous MAGI-1 in stable transformants of MDCK cells expressing FLAG-JAM4-2 (Fig. 6A). As described for Fig. 5B, FLAG-JAM4-2 was localized on apical membranes. MAGI-1 was still concentrated at TJs (arrowheads), but it was also detected on the apical membranes.

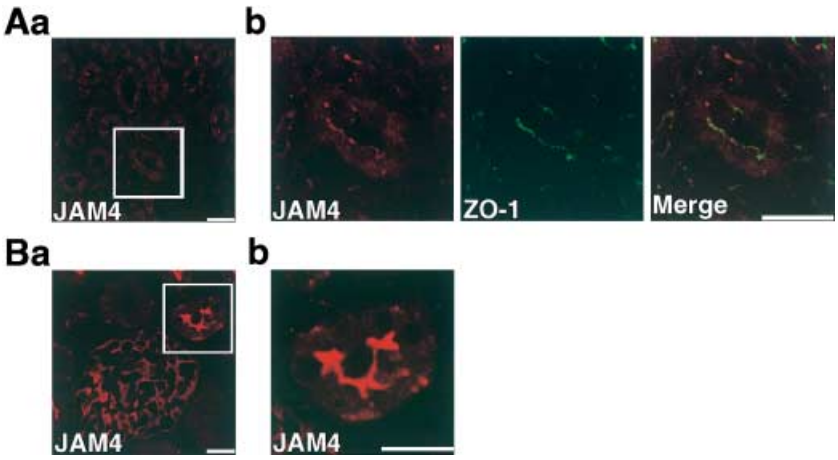

Figure 3 JAM4 in kidney. (A) JAM4 in collecting ducts. The demarcated area in (a) is shown at higher magnification in (b). Bar, 20 μm. (B) JAM4 in glomerulus and renal proximal tubular epithelial cells. The demarcated area in (a) is shown at higher magnification in (b). Bar, 20 μm.

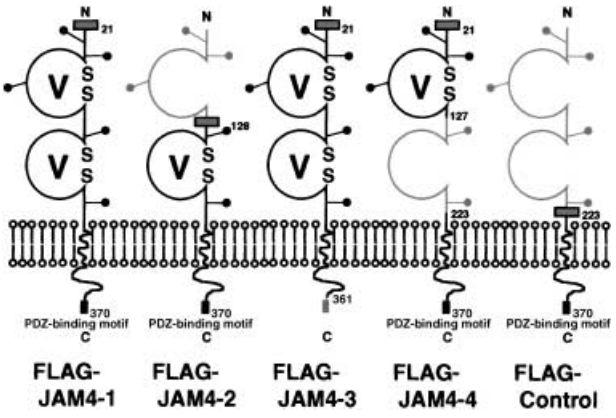

Figure 4 Schematic model for various FLAG-tagged JAM4 constructs. The extracellular region of JAM4 contains two domains with intramolecular disulphide bonds typical of immunoglobulin-like loops (Ig-loops) of the V-type. The closed rectangle at the C-terminus and the grey rectangle at the N-terminus of each construct indicate the PDZ-binding motif and the FLAG-tag, respectively. Black dots are putative N-linked glycosylation sites. The numbers show the first and the last amino acid residue number of JAM4 for each construct.

Transient expression of FLAG-JAM4-2 showed a similar but more remarkable effect on MAGI-1 (Fig. 6B). In MDCK cells expressing FLAG-JAM4-2, MAGI-1 was recruited to apical membranes, while it remained at TJs in parent MDCK cells (arrow). As described for Fig. 5C, FLAG-JAM4-3 without the MAGI-1-binding region mostly localized to TJs in stable transformant cells. However, when it was transiently over-expressed in MDCK cells, the expression level was much higher and FLAG-JAM4-3 was overflowed from TJs to be localized mainly on the apical membranes (Fig. 6C). Even so, it did not affect the localization of endogenous MAGI-1.

JAM4 recruits MAGI-1 to TJs in MDCK cells

To obtain another line of evidence that JAM4 determines the subcellular localization of MAGI-1, we employed stable transformants of MDCK cells expressing green fluorescent protein (GFP)-tagged MAGI-1 (MDCK-GFP-MAGI-1). GFP-MAGI-1 showed diffuse distribution in the cytoplasm and was slightly concentrated at cell contacts (Fig. 7A). Unlike endogenous MAGI-1, over-expressed GFP-MAGI-1 was localized along lateral membranes. When FLAG-JAM4-1 was transiently introduced to MDCK-GFP-MAGI-1 cells, FLAG-JAM4-1 was co-localized with ZO-1 at TJs (Fig. 7B). Simultaneously, the cytoplasmic signal of GFP-MAGI-1 disappeared. Moreover, the signal at cell contacts was accumulated exclusively to TJs (arrows). In contrast, FLAG-JAM4-3 lacking the MAGI-1-interacting domain did not affect the localization of GFP-MAGI-1 (data not shown). These findings overall suggest that JAM4 is a molecular determinant of the localization of MAGI-1 in MDCK cells.

MAGI-1 is localized at TJs in tissues where JAM4 is detected on apical membranes

Subsequently, we examined how MAGI-1 was localized in renal proximal tubules and ileum, where JAM4 showed apical localization. MAGI-1 was not detected in renal proximal tubular epithelium (data not shown) thus we could not evaluate how apical JAM4 affected the localization of MAGI-1. In ileum, we detected MAGI-1 on apical membranes (Fig. 7C, arrowheads). This is consistent with the results described for MDCK cells. However, MAGI-1 was also localized at TJs in ileum, although JAM4 was not detected at TJs (arrows). It suggests that the localization of MAGI-1 is not determined only by JAM4 *in vivo*.

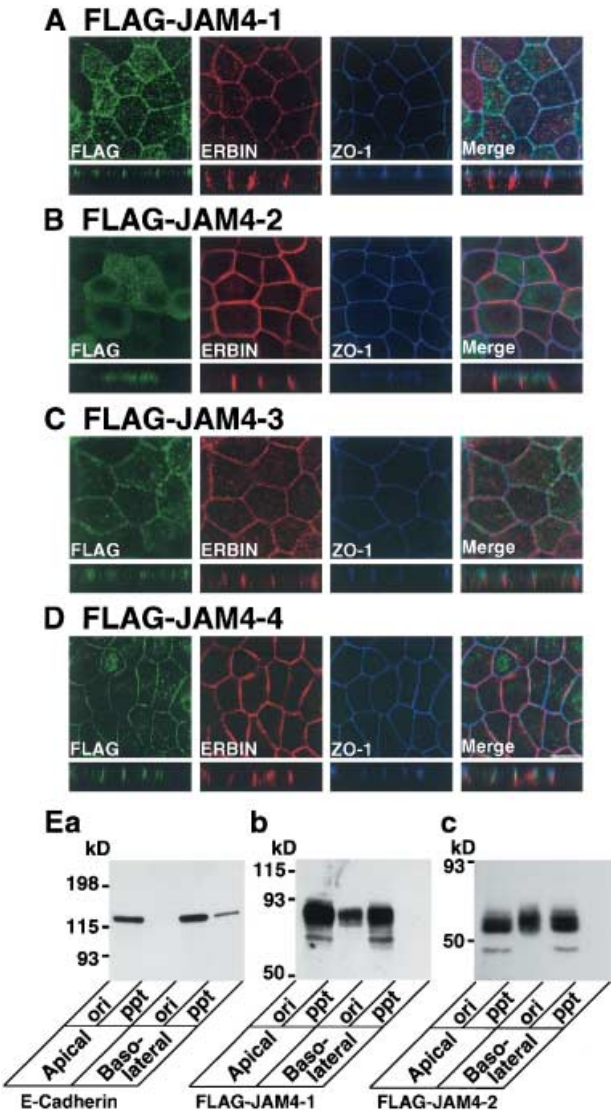

Figure 5 Subcellular distribution of various FLAG-JAM4 proteins in MDCK cells. Stable transformants of MDCK cells expressing various FLAG-JAM4 proteins were immunostained with mouse anti-FLAG, rabbit anti-ERBIN, and rat anti-ZO-1 antibodies. (A) FLAG-JAM4-1 was mainly co-localized with ZO-1, but some signal was also detected on apical membranes. (B) FLAG-JAM4-2 without the first Ig-loop was distributed on apical membranes. (C) FLAG-JAM4-3 without the C-terminal PDZ-binding motif was co-localized with ZO-1, but some signal was also detected on apical membranes. (D) FLAG-JAM4-4 without the second Ig-loop was localized on basolateral membranes and partially overlapped with both of ZO-1 and ERBIN. Bar, 10 μm. (E) Surface protein biotinylation of FLAG-JAM4-1 and FLAG-JAM4-2. MDCK cells expressing FLAG-JAM4-1 and FLAG-JAM4-2 were cultured on Transwell plates and biotinylated from the upper (Apical) and lower (Basolateral) chambers. Biotinylated proteins were precipitated with avidin-beads. Original cell lysates (ori) and precipitates (ppt) on avidin-beads were immunoblotted with anti-E-cadherin or anti-FLAG antibody. (a) Immunoblot with anti-E-cadherin antibody of the precipitates from MDCK-FLAG-JAM4-1 cells. Although we do not show the data, immunoblot of the precipitates from MDCK-FLAG-JAM4-2 cells gave a similar result. (b) Immunoblot with anti-FLAG antibody of the precipitates from MDCK-FLAG-JAM4-1 cells. (c) Immunoblot with anti-FLAG antibody of the precipitates from MDCK-FLAG-JAM4-2 cells. Protein standard markers are shown on the left.

Homophilic interactions of JAM4 depend on both of the first and the second Ig-loops

As described above, the first Ig-loop is essential for JAM4 to be recruited to TJs. We next examined the function of Ig-loops. We previously reported that JAM4 promotes Ca^{2+}-independent cell adhesion, and that this activity does not require the PDZ-binding motif (Hirabayashi *et al.* 2003). To evaluate how the Ig-loops are involved in this activity, we prepared stable transformants of L cells expressing FLAG-JAM4-1, -2, -3, -4 and -control (L-FLAG-JAM4-1, L-FLAG-JAM4-2, L-FLAG-JAM4-3, L-FLAG-JAM4-4 and L–FLAG-control cells). Aggregation assays demonstrated that L-FLAG-JAM4-2 and L-FLAG-JAM4-4 cells formed aggregates in a time-dependent manner, but these aggregates were consisted of fewer L cells and were smaller in size than those of L-FLAG-JAM4-1 and L–FLAG-JAM4-3 cells (Fig. 8A and B). Furthermore, L-FLAG-Control cells expressing a JAM4 variant with no Ig-loop also showed some aggregation activity, suggesting that the aggregation of L-FLAG-JAM4-2 and L-FLAG-JAM4-4 cells were mediated by the FLAG-tags rather than Ig-loops. All FLAG-proteins were expressed at similar levels in cells that we used for this experiment (Fig. 8C). Therefore, we concluded that JAM4 requires both of Ig-loops for the adhesion activity.

Cis interactions of JAM4 depend on the first Ig-loop

In the last sets of experiments, we investigated which Ig-loop is necessary for *cis* interactions of JAM4. We treated L cells expressing various JAM4 mutants with a chemical cross-linker and performed Western blotting to analyse the dimer formation. FLAG-JAM4-1 and -3 formed dimers when treated with a chemical cross-linker (Fig. 9). Consistent with this, the amount of FLAG-JAM4-1 and -3 monomers decreased. FLAG-JAM4-2 lacking the first Ig-loop did not form dimers, although the amount of FLAG-JAM4-2 seemed to decrease. We consider that this decrease may be due to the nonspecific cross-linking of FLAG-JAM4-2 with other proteins. FLAG-JAM4-4 lacking the second Ig-loop formed dimers.

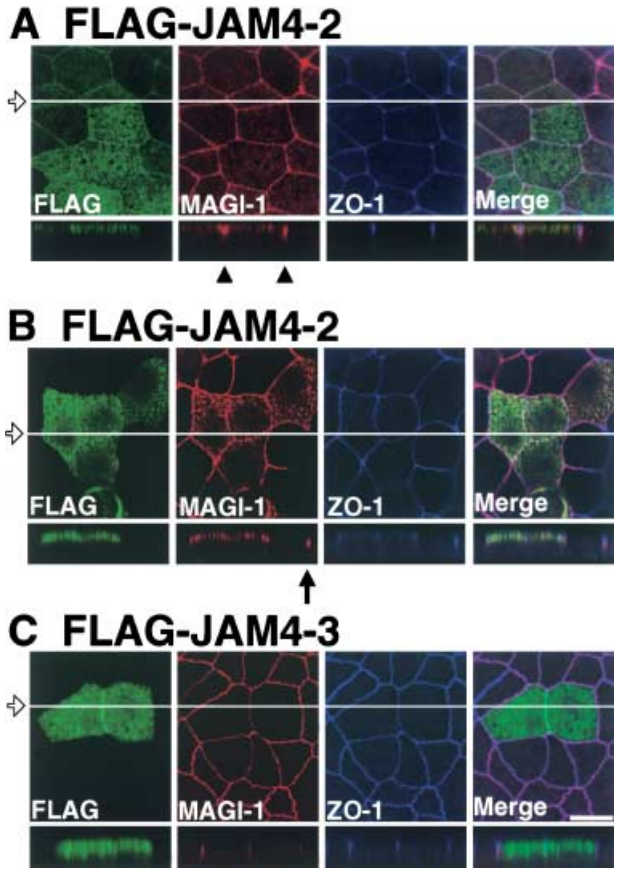

Figure 6 Effect of JAM4 on the subcellular localization of MAGI-1 in MDCK cells. (A) MAGI-1 in stable transformants of MDCK cells expressing FLAG-JAM4-2. FLAG-JAM4-2 was localized on apical membranes in MDCK cells. MAGI-1 was concentrated at TJs and was also distributed on apical membranes (arrow heads). Lines marked by open arrows indicate where the z-section was obtained. (B) MAGI-1 in MDCK cells in which FLAG-JAM4-2 was transiently introduced. Endogenous MAGI-1 moved to apical membranes and was co-localized with FLAG-JAM4-2. In contrast, MAGI-1 remained at TJs in MDCK cells that did not express FLAG-JAM4-2 (arrow). (C) MAGI-1 in MDCK cells in which FLAG-JAM4-3 was transiently introduced. FLAG-JAM4-3 lacking the PDZ-binding motif did not affect the localization of endogenous MAGI-1. Bar, 10 μm.

Discussion

We previously described the localization of JAM4 at slit diaphragm in kidney and at TJs in small intestine and mammary gland ducts (Hirabayashi *et al.* 2003). During the study, we detected JAM4 on apical membranes in renal proximal tubular epithelial cells. Moreover, we found that JAM4 was localized on apical membranes in jejunum and ileum, while it was concentrated at TJs in duodenum, eccrine sweat glands, and submandibular glands. Thereby, we consider that both of the localizations at TJs and on apical membranes are physiologically relevant. We tried to elucidate the mechanism that determines the subcellular localization of JAM4. Though JAM4 has not been detected in distal tubules of rat kidneys in the immunohistochemical study, we employed MDCK cells, a widely used epithelial cell line for analysis of TJ assembly and regulation. The mutant of JAM4 lacking the first Ig-loop was not recruited to TJs and was localized on apical membranes. Furthermore, even the full-length of JAM4, when over-expressed, was observed not only at TJs but also on apical membranes. The immunoelectron microscopy of small intestinal epithelium showed that gold particles were detected on the apical side to TJs. The surface protein biotinylation experiment also indicated that JAM4 was localized on the apical side to TJs in MDCK cells. N-glycans are known as apical sorting signals in epithelial cells and JAM4 is heavily modified with N-glycosylation (Scheiffele *et al.* 1995; Hirabayashi *et al.* 2003). Taken together, we speculate that JAM4 would be first targeted to apical membranes and then accumulated to TJs *via* the first Ig-loop–mediated molecular interaction. Because the first Ig-loop is involved in *cis* interactions, the recruitment of single JAM4 molecule to TJs would result in the further accumulation of JAM4. The molecule that directly tethers JAM4 to TJs is currently unknown. It should have the extracellular domain and may be a JAM4-like cell adhesion molecule that interacts with JAM4 in *cis*- or *trans*- interactions. In cells that lack the counterpart, JAM4 may remain on the apical membranes.

Another explanation is that JAM4 detected on apical membranes may be a splicing variant that lacks the signal sequence essential for the TJ targeting. One form of murine vascular cell adhesion molecule-1 (VCAM-1) called VCAMTM is expressed on basolateral membranes, whereas its splicing variant VCAMGPI is located on apical membranes (Pirozzi *et al.* 1994). Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) has two isoforms with different cytoplasmic domains and CEACAM1-L appears on both the apical and the lateral surfaces of epithelial cells, while CEACAM1-S is exclusively recruited to apical membranes (Sundberg & Obrink 2002). There may be a splicing variant of JAM4 that shows the apical localization in kidney and small intestine.

JAM4 recruits MAGI-1 to the cell contacts in L cells. Likewise, JAM4 accumulates the over-expressed GFP-MAGI-1 to TJs in MDCK cells. These data imply that JAM4 is a molecular determinant for the TJ localization of MAGI-1. However, in renal distal tubular epithelial

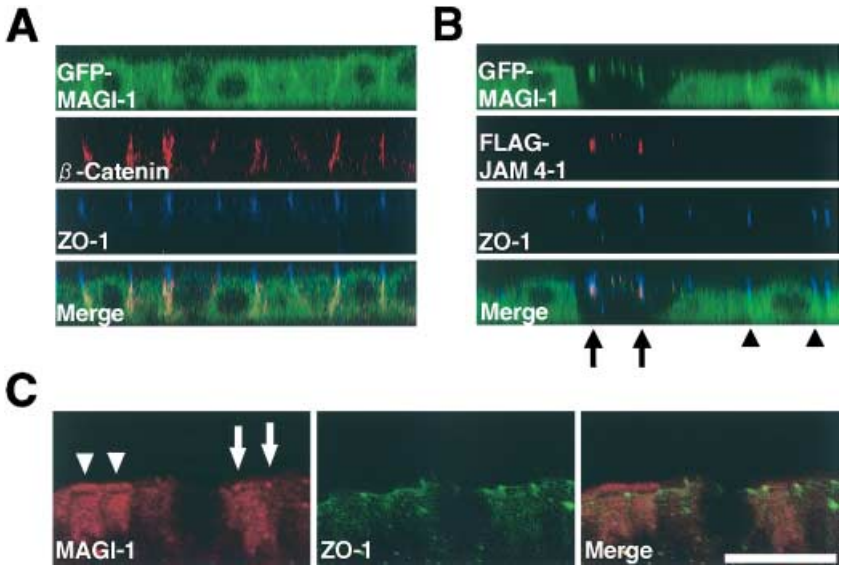

Figure 7 Recruitment of GFP-MAGI-1 by JAM4 to TJs. (A) GFP-MAGI-1 in MDCK cells. Stable transformants of MDCK cells expressing GFP-MAGI-1 (MDCK-GFP-MAGI-1) was double-stained with anti-β-catenin and anti-ZO-1 antibodies. GFP-MAGI-1 was localized along lateral membranes and was also detected in the cytosol. (B) FLAG-JAM4-1 was introduced into MDCK-GFP-MAGI-1 and stained with anti-FLAG and anti-ZO-1 antibodies. In cells with FLAG-JAM4, GFP-MAGI-1 was co-localized with ZO-1 (arrows). In MDCK-GFP-MAGI-1 cells without FLAG-JAM4-1, GFP-MAGI-1 was not concentrated at TJs (arrowheads). (C) MAGI-1 in epithelial cells of ileum. MAGI-1 was detected on apical membranes (arrow heads). However, some MAGI-1 was co-localized with ZO-1 (arrows). Bar, 20 μm.

cells, jejunum, and ileum, MAGI-1 is present even at TJs where JAM4 is absent. This fact rather contradicts to the model. There may be some other protein besides JAM4 that recruits MAGI-1 to TJs.

Recently, the roles of each Ig-loop in immunoglobulin-like cell adhesion molecules have been characterized. The first Ig-loop of JAM1 is responsible for *cis* interactions (Kostrewa *et al.* 2001). Concerning murine JAM1, homo-*cis*-dimers on the surface of one cell engage homo-*cis*-dimers on the adjacent cell and form the homo-*trans*-dimers. For human JAM1, a different model is proposed (Prota *et al.* 2003). The *trans*-dimerization of human JAM1 may depend on the presence of additional proteins, or a human JAM1 monomer may directly interact with a monomer on apposing cells and form homo-*trans*-dimers. In nectin, which has three Ig-loops, the first Ig-loop is involved in *trans*-dimerization, and the second one is necessary for *cis*-dimerization (Miyahara *et al.* 2000; Momose *et al.* 2002). In case of JAM4, both of two Ig-loops are indispensable for *trans* interactions of JAM4. On the other hand, only the first Ig-loop is necessary for *cis* interactions.

We conclude that JAM4 may be primarily targeted to apical membranes and subsequently recruited to TJs *via* the interactions of the first Ig-loops. We also proved that JAM4 determines the localization of MAGI-1 in MDCK cells, but the *in vivo* localization of MAGI-1 does not always depend on JAM4. The counterpart of JAM4 at TJs remains to be clarified. It is also an interesting question whether JAM4, like other JAM proteins, takes part in the process of inflammation, possibly as a mediator of leucocyte migration.

Experimental procedures

Construction of expression vectors pLN vector was previously described (Hirabayashi *et al.* 2003). pFLAG-CMV-1 was purchased from Eastman Kodak Company. The constructs of JAM4 used in this study are summarized in Fig. 4. They contain the following regions of JAM4; pFLAG and pLN FLAG JAM4, 21-370 of JAM4; pFLAG and pLN FLAG JAM4-2, 128-370 of JAM4; pFLAG and pLN FLAG JAM4-3, 21-361 of JAM4; pFLAG and pLN FLAG JAM4-4, 21-127 and 223-370 of JAM4; and pFLAG Control, 223-370 of JAM4.

Antibodies

Rabbit anti-ERBIN and anti-JAM4 antibodies were previously described (Ohno *et al.* 2002; Hirabayashi *et al.* 2003). Rabbit antibody against the N-terminal region of synaptic scaffolding molecule was used to detect MAGI-1 in immunohistochemical and immunocytochemical studies as in the previous study (Hirabayashi *et al.* 2003). Mouse anti-ZO-1 antibody (Zymed), mouse anti-β-catenin antibody (BD Transduction), mouse and rabbit anti-FLAG antibodies (Sigma-Aldrich Fine Chemicals), rat anti-ZO-1 antibody (Chemicon) and rhodamine-conjugated, FITC-conjugated and Cy5-conjugated second antibodies for dual labelling (Chemicon) were purchased from commercial sources.

Cells

Phoenix ampho, Madine Darby canine kidney II (MDCK) and mouse fibroblastic L cells were cultured in DME medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin under 5% CO_2 at 37 °C. MDCK cells were transfected with Effectene Transfection Reagent (Qiagen). Retrovirus system was used to generate stable transformants of MDCK and L

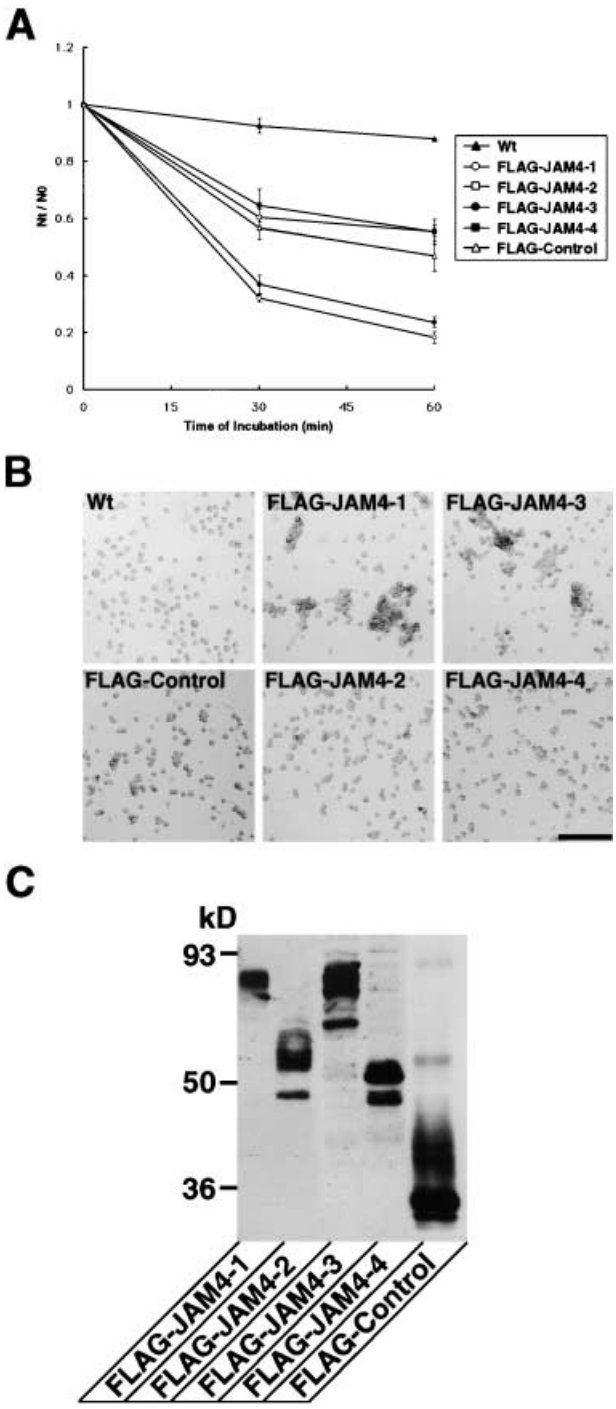

Figure 8 Cell adhesion activity of various FLAG-JAM4 proteins. (A) Cell aggregation assays were performed using wild-type L cells and stable transformants of L cells expressing various FLAG-JAM4 proteins. The extent of cell aggregation was represented by ratio of the total particle number at indicated time point (Nt) to the initial particle number (N0). The Nt/N0 values are indicated by the mean ± SE of three independent experiments. ▲ wild-type of L cells; ○ L-FLAG-JAM4-1; □ L-FLAG-JAM4-2; ● L-FLAG-JAM4-3; ■ L-FLAG-JAM4-4; Δ L-FLAG-Control. (B) Cell aggregates of wild-type of L cells (L-Wt) and various L-JAM4 cells (L-FLAG-JAM4-1, -JAM4-2, -JAM4-3, -JAM4-4 and -control) at 30 min in cell aggregation assay. Bar, 200 μm. (C) Immunoblottings by the anti-FLAG antibody of various L-JAM4 cell lysates. Protein standard markers are shown on the left.

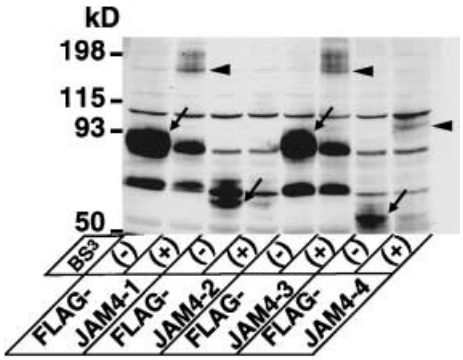

Figure 9 Chemical cross-linking of various FLAG-JAM4 proteins. Stable transformants of L cells expressing various FLAG-JAM4 proteins were treated with the mock or BS3 for cross-linking. The cell lysates were immunoblotted with anti-FLAG antibody. Arrows and arrowheads indicate monomers and dimers of FLAG-JAM4 proteins, respectively. Protein standard markers are shown on the left.

cells as described (Nishimura *et al.* 2000). Briefly, MDCK and L cells were infected with the retrovirus and cultured in the selective medium with 1 mg/mL G418 (Calbiochem). The resistant colonies were tested for the expression of each protein and cloned.

Immunocytochemistry and immunofluorescence microscopy

MDCK and L cells were fixed with 4% (w/v) formaldehyde in phosphate buffered saline (PBS) for 15 min, blocked with 50 mm glycine in PBS for 30 min, and incubated with 0.2% (w/v) Triton X-100 in PBS for 15 min and subsequently with 1% (w/v) bovine serum albumin (BSA) in PBS for 30 min. These processes were carried out at room temperature. The samples were incubated with various first antibodies and visualized with rhodamine-, FITC-, or Cy5-conjugated second antibodies. The images were obtained by a confocal microscopy (Zeiss LSM 510).

Immunohistochemistry

All procedures related to the care and treatment of animals were in accordance with institutional and National Institutes of Health guidelines. 4- to 6-week-old Wistar rats were deeply anaesthetized with sodium pentobarbital (60 mg/kg i.p) and perfused with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. For small intestines, skins and kidneys, tissues were removed, post-fixed in the same fixative, blocked with 50 mM glycine in 0.1 M

PB, immersed with 10% (w/v), 20% (w/v), and 30% (w/v) sucrose in 0.1 M PB sequentially and frozen at −80 °C. 5 μm sections were prepared, blocked with PBS containing 5% goat serum and 0.2% (w/v) Triton X-100 for 2 h and incubated with first antibodies at 4 °C overnight. After washed with PBS four times, the samples were incubated with second antibodies at room temperature for 2 h and bound antibodies were detected. Images were obtained by confocal microscopy (Olympus FV300-BX or Zeiss LSM 510). For submandibular glands, tissues were obtained from anaesthetized rats without perfusion and immediately frozen on powdered dry ice. 10 μm sections were then prepared directly from frozen tissues and fixed with −20 °C methanol for 15 min. Other procedures for the preparation of samples were the same as above. Images were obtained by using Olympus IX71 CCD microscope.

Immunoelectron microscopy

Small intestines from anaesthetized adult rats were fixed by perfusion of 0.5% (w/v) glutaraldehyde in PBS. Tissues were cut in small pieces and fixed in the same fixative for 1 h on ice, washed with PBS and dehydrated with a graded series of ethanol. Two mm cubes of intestinal epithelium were embedded in LR white resin (London Resin Company Ltd, London), polymerized with LR white accelerator and stored at −20 °C. Ultrathin sections were mounted on 300-mesh nickel grids and treated with 3% H_2O_2 for 10 min. After blocking with 1% (w/v) BSA in PBS for 30 min, grids were incubated overnight at 4 °C with affinity-purified rabbit anti-JAM4 antibody, washed with PBS, and incubated with 10-nm gold-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech) with the dilution of 1 : 20 for 1 h at room temperature. Grids were washed with distilled water and stained with uranyl acetate for 20 min. After air-drying at room temperature, the sections were photographed on Hitachi 7100 electron microscope at × 30 000 or × 8000 magnification.

Chemical cross-linking

Various L-FLAG-JAM4 cells were treated with 0.2% (w/v) trypsin and 1 mM EDTA at 37 °C for 5 min and dispersed by pipetting to obtain single-cell suspensions (1×10^6 cells/mL). Bis(sulfosuccinimidyl) suberate (BS^3) (Pierce) stock solution (10 mM BS^3 in PBS) was freshly prepared. Appropriate amount of the BS^3 stock solution was added to the cells to the final concentration of 1 mM and incubated at room temperature for 30 min. Reactions were quenched by the addition of 1 M Tris/HCl pH 7.4 to a final concentration of 20 mM. After 15 min, cells were homogenized in 40 μL of the buffer containing 20 mM HEPES/NaOH pH 7.4, 100 mM NaCl, and 1% (w/v) Triton X-100 and centrifuged at 100 000 ***g*** for 15 min at 4 °C. The supernatant was analysed in SDS-PAGE and immunoblotted with anti-FLAG antibody.

Cell surface biotinylation

2×10^6 cells were cultured on 24-mm Transwell (Costar) with uncoated polycarbonate filters at 37 °C in 5% CO_2. After 5 days when cells were grown to confluency, the medium was removed. Cells were rinsed with cold PBS-CM (PBS with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$) and biotinylated with 0.5 mg/mL sulfo-NHS-biotin (Pierce) in PBS-CM at 4 °C for 60 min. The reactions were quenched by the addition of 50 mM NH_4Cl. Cells were harvested, homogenized in 400 μL of the buffer containing 20 mM Tris/HCl pH 8.0, 100 mM NaCl, and 1% (w/v) NP-40 and centrifuged at 100 000 ***g*** for 15 min at 4 °C. The supernatant was incubated with avidin-agarose beads (Sigma-Aldrich Fine Chemicals), and the precipitated proteins were immunoblotted with appropriate antibodies.

Cell aggregation assay

Wild-type or various stable transformants of L cells were treated with 0.2% (w/v) trypsin and 1 mM EDTA at 37 °C for 5 min and dispersed by pipetting. Cells were resuspended in Hanks' balanced salt solution with either 1 mM $CaCl_2$ or 5 mM EDTA at 1×10^6 cells/mL, placed in 12-well plates precoated with BSA and rotated on a gyratory shaker at 37 °C for indicated periods of time. Aggregation was quenched by adding 2% (v/v) glutaraldehyde. The extent of aggregation was described as the ratio of the total particle number after incubation to the initial particle number before incubation. Images were obtained with Olympus IX70 at ×10 magnification with ×1.5 zoom and printed on papers with dimensions of 29 × 21 cm. Any cells that were in contact with another were regarded as one particle.

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