The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein PAR-3: a possible role for JAMs in endothelial cell polarity

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Summary

Tight junctions play a central role in the establishment of cell polarity in vertebrate endothelial and epithelial cells. A ternary protein complex consisting of the cell polarity proteins PAR-3 and PAR-6 and the atypical protein kinase C localizes at tight junctions and is crucial for tight junction formation. We have recently shown that PAR-3 directly associates with the junctional adhesion molecule (JAM), which suggests that the ternary complex is targeted to tight junctions of epithelial cells through PAR-3 binding to JAM. The expression of JAM-related proteins by endothelial cells prompted us to test whether recruitment of the ternary complex in endothelial cells can occur through binding to JAM-2, JAM-3, endothelial cell-selective adhesion molecule (ESAM) or coxsackie- and adenovirus receptor (CAR). Here we show that the two JAM-related proteins JAM-2 and JAM-3 directly associate with PAR-3. The association between PAR-3 and JAM-2/-3 is mediated through the first PDZ domain of PAR-3. In agreement with the predominant expression of JAM-2 and JAM-3 in endothelial cells, we found that PAR-3 is expressed by endothelial cells in vivo and is localized at cell contacts of cultured endothelial cells. PAR-3 associates with JAM-2/-3 but not with the JAM-related Ig-superfamily members ESAM or CAR. In addition, we show that the tight junction-associated protein ZO-1 associates with JAM-2/-3 in a PDZ domain-dependent manner. Using ectopic expression of JAM-2 in CHO cells, we show that the junctional localization of JAM-2 is regulated by serine phosphorylation and that its clustering at cell-cell contacts recruits endogenous PAR-3 and ZO-1. Our findings suggest that JAM-2 affects endothelial cell junctions by its regulated clustering at intercellular contacts, and they support a role for JAM-2, and possibly JAM-3, in tight junction formation of endothelial cells.

Key words: Cell polarity, Endothelium, JAMs, PAR-3, Tight junction, ZO-1

Introduction

Endothelial and epithelial cells have distinct apical and basolateral membrane domains that differ in protein and lipid composition. The two domains are separated by tight junctions (TJs), where the outer leaflets of the plasma membranes of adjacent cells appear as series of fusions, the so-called TJ strands (Tsukita et al., 2001). These fusion points restrict the free diffusion of lipids and integral membrane proteins between the two compartments (fence function). TJs, therefore, are crucial in the generation and maintenance of cellular polarity in vertebrate endothelial and epithelial cells (Yeaman et al., 1999).

Three types of tight junction-associated integral membrane proteins have been identified so far. These are occludin (Furuse et al., 1993), the claudins (Furuse et al., 1998a) and several immunoglobulin (Ig) superfamily members, including junctional adhesion molecule (JAM-1) (Martin-Padura et al., 1998), endothelial cell-selective adhesion molecule (ESAM) (Nasdala et al., 2002) and the coxsackie- and adenovirus receptor (CAR) (Cohen et al., 2001). Among these, occludin and claudins seem to form the molecular basis of the tight junction strands, as antibodies against occludin exclusively label TJ strands and the intensity of occludin staining correlates with the number of tight junction strands (Saitou et al., 1997), and the expression of claudin-1 or claudin-2 in L cell fibroblasts results in the formation of tight junction strands (Furuse et al., 1998b). This is not the case when JAM-1 is expressed in L cells (Itoh et al., 2001), suggesting a function for JAM-1 that differs from the functions of occludin and claudins.

Recently, progress has been made in understanding the molecular mechanisms underlying the formation of TJs. Accumulating evidence supports the idea that a molecular complex consisting of the cell polarity proteins PAR-3 and PAR-6, as well as atypical protein kinase C (aPKC), plays a central role in the generation of TJs in vertebrate epithelial cells

(Ohno, 2001). These molecules are localized at TJs of epithelial cells and form a ternary complex in which PAR-3 and PAR-6 are linked through aPKC (Joberty et al., 2000; Lin et al., 2000; Suzuki et al., 2001). In addition, the small GTPases Cdc42 and Rac1 can be part of the complex through their association with PAR-6 (Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000; Qiu et al., 2000; Yamanaka et al., 2001). The requirement of this molecular complex for tight junction formation is suggested by the observations that first, overexpression of a PAR-6 mutant that lacks the aPKC binding domain leads to aberrant PAR-3 and aPKC (aPKC)ζ localization, as well as to mislocalization of TJ proteins like occludin, claudin-1 and ZO-1 (Yamanaka et al., 2001); and second, overexpression of a dominant-negative mutant of aPKC (aPKCkn) induces a mislocalization of PAR-3 and PAR-6 as well as occludin, claudin-1 and ZO-1. More importantly, the overexpression of both mutants disrupts the function of TJs as development of transepithelial electrical resistance (TER), paracellular permeability and membrane polarity are severely affected (Suzuki et al., 2001; Yamanaka et al., 2001). An intriguing finding in these studies, however, is that the effects of aPKCkn overexpression are observed in cells that are in the process of developing TJs but not in fully polarized cells, suggesting a central role for the PAR-3/PAR-6/aPKC complex in the biogenesis, rather than maintenance, of TJs (Suzuki et al., 2001; Yamanaka et al., 2001).

One component of the PAR-3/PAR-6/aPKC complex, PAR-3, directly associates with JAM-1 (Ebnet et al., 2001; Itoh et al., 2001). During cell contact formation JAM-1 colocalizes with E-cadherin and ZO-1 in primordial spot-like adherens junctions or puncta (Ebnet et al., 2001), indicating that JAM-1 is among the first tight junction-associated proteins appearing at cell-cell contacts during junction formation. PAR-3, as well as aPKC, appear after spot-like adherens junctions have been formed (Suzuki et al., 2002). This supports the idea that the PAR-3/PAR-6/aPKC complex is targeted to nascent cell-cell contacts through the association of PAR-3 with JAM-1. Although direct evidence is still missing, it seems conceivable that the concomitant activation of Cdc42 in response to E-cadherin-mediated cell adhesion (Kim et al., 2000) results in the activation of the complex-associated aPKC activity through the binding of active Cdc42 to PAR-6 (Yamanaka et al., 2001). The downstream targets of aPKC activity are still unknown. In this scenario, JAM-1 would play an important role in recruiting/localizing a signalling complex to sites of cell-cell adhesion and thus in promoting the formation of tight junctions from spot-like adherens junctions. Despite the evolutionary conservation of the PAR-3/aPKC/PAR-6 complex from *Caenorhabditis elegans* and *Drosophila* to vertebrates, integral membrane proteins through which the complex is targeted to the membranes in the former two species have not been identified.

JAM-1 belongs to a subfamily of the Ig superfamily, which is characterized by the presence of two Ig-like domains, a membrane-distal V-type and a membrane-proximal C2-type Ig-like domain, the CTX family (Aurrand-Lions et al., 2001a; Chretien et al., 1998). The closest relatives of JAM-1 are JAM-2 and JAM-3 (Arrate et al., 2001; Aurrand-Lions et al., 2000; Cunningham et al., 2000; Liang et al., 2002; Palmeri et al., 2000) (see footnote* for the nomenclature of JAM-2 and JAM-3); all three JAMs share a canonical type II PDZ domain targeting motif at their C-termini (Songyang et al., 1997). In multicellular tissues, JAM-1 is widely expressed by endothelial and epithelial cells (Liu et al., 2000; Martin-Padura et al., 1998; Ozaki et al., 1999), whereas JAM-2 and JAM-3 are largely confined to endothelial cells (Aurrand-Lions et al., 2001b; Liang et al., 2002; Palmeri et al., 2000), with JAM-3 also being identified in a squamous cell carcinoma cell line of epithelial origin (Aurrand-Lions et al., 2001a). The subcellular localization of JAM-2/-3 has not been analysed yet at the ultrastructural level. Ectopic expression of JAM-2 in MDCK (Madin-Darby Canine Kidney) epithelial cells results in colocalization of JAM-2 with ZO-1, suggesting that JAM-2 is TJ-associated (Aurrand-Lions et al., 2001b). The two other more distantly related members of the CTX family, which were described to be localized at tight junctions, i.e. ESAM and CAR, are expressed in endothelial cells or both endothelial and epithelial cells, respectively (Carson et al., 1999; Cohen et al., 2001; Nasdala et al., 2002). Despite a similar overall organization, the latter two molecules differ from JAM-1/-2/-3 in the size of the cytoplasmic domains and in their C-termini, which end in canonical type I PDZ domain targeting motifs (Bergelson et al., 1997; Hirata et al., 2001), suggesting differences in the nature of cytoplasmically associated proteins.

To date, peripheral membrane components of tight junctions associating with JAM-2 and JAM-3 have not been identified. The structural similarities between JAM-1 and JAM-2/-3 prompted us to address whether JAM-2 and JAM-3 associate with the cell polarity protein PAR-3. We report that PAR-3 strongly associates with JAM-2 and JAM-3 but not with CAR or ESAM. In addition, we found that the tight junction protein ZO-1 associates with both JAM-2 and JAM-3. The localization of JAM-2 at cell-cell contacts is regulated by serine phosphorylation, and JAM-2 at cell contacts recruits both PAR-3 and ZO-1. Our findings support the idea of a general role for all three members of the JAM family in the regulation of tight junction formation and cell polarity.

Materials and Methods

Cell lines, antibodies, reagents

CHO cells were maintained in HAM/F12 or α-MEM (modified Eagle's medium) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 100 U/ml penicillin/streptomycin (Life Technologies, Germany). CHO cell lines expressing JAM-2 or JAM-2 S281A (J-2 S281A) were generated according to established procedures (Aurrand-Lions et al., 2001a; Aurrand-Lions et al., 2001b). Transfected cells were selected using G418 at 1 mg/ml over ten days and flow cytometry cell sorting was used to select cells with a comparable amount of cell-surface protein expression. To avoid clonal variations, bulk sorted cells were used. Cell lines expressing JAM-2 or J-2 S281A had comparable expression levels as verified by fluorescence-activated cell sorting (FACS) analysis (data not shown). COS-7 cells and the murine rectal carcinoma cell line CMT were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine and 100 U/ml

*Different names have been assigned to the murine and human orthologues of JAM-2 and JAM-3. Mouse JAM-2 (Aurrand-Lions et al., 2000; Aurrand-Lions et al., 2001b) corresponds to human JAM3 (Arrate et al., 2001). Mouse JAM-3 (Aurrand-Lions et al., 2000) corresponds to human JAM2/VE-JAM (Cunningham et al., 2000; Liang et al., 2002; Palmeri et al., 2000). In this study we have applied the nomenclature for the murine orthologues of JAM-2 and JAM-3. According to a recent agreement on a new nomenclature for JAMs (Muller, 2003), murine JAM-2 and JAM-3 correspond to JAM-C and JAM-B, respectively.

penicillin/streptomycin (Life Technologies, Germany). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by collagenase treatment and were maintained in M199 supplemented with 20% FCS, 100 μg/ml endothelial cell growth supplement (ECGS; Sigma, Deisenhofen, Germany), 13.4 U/ml heparin (Sigma), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin.

Rabbit polyclonal antibodies against PAR-3 (C2-3) and AF-6 were described previously (Ebnet et al., 2000; Izumi et al., 1998). The anti-JAM-2 monoclonal antibody (CRAM XIXH36, rat IgG_{2a}) was purified from serum-free Ultroser HY 0.75% medium (Biosepra, France) by ammonium sulfate precipitation and protein G immunoaffinity column. A polyclonal antibody against JAM-2 (ke738) was generated by immunizing rabbits with a fusion protein consisting of the extracellular domain of JAM-2 fused to the Fc-part of human IgG. The antibodies were affinity-purified by adsorption on the same fusion protein covalently coupled to cyanogen bromide-activated sepharose beads (Amersham-Pharmacia Biotech, Freiburg, Germany), and antibodies directed against the Fc-portion were depleted by adsorption on human IgG coupled to cyanogen bromide-activated sepharose beads. The following commercially available antibodies were used: rat mAb against ZO-1 (Chemicon, Hofheim, Germany), rabbit pAb against ZO-1 (Zymed, Berlin, Germany), rat mAb against PECAM-1 and mouse mAb against the heat-shock protein HSP-90 (BD Pharmingen, Heidelberg, Germany); rabbit polyclonal antiserum against von Willebrand factor (DAKO, Hamburg, Germany) and rat mAb MECA-79 against peripheral node addressin (ATCC, Manassas, VA). Mouse anti-T7 tag mAb was purchased from Calbiochem-Novabiochem (Schwalbach, Germany). Secondary antibodies were purchased from Dianova (Hamburg, Germany).

Expression vectors

For the generation of GST fusion proteins pGEX expression vectors (Amersham Pharmacia Biotech) were used. GST-JAM-1 expression vectors were described elsewhere (Ebnet et al., 2000). Expression vectors encoding GST-JAM-2 and GST-JAM2Δ5 were generated by cloning the cytoplasmic tail (aa 261-310) or a C-terminal truncation mutant (aa 261-305) of JAM-2 in pGEX-5X-2 or pGEX-6P-2, respectively. Expression vectors encoding GST-JAM-3 and GST-JAM-3Δ5 were generated by cloning the cytoplasmic tail (aa 259-298) or a C-terminal truncation mutant (aa 259-293) of JAM-3 in pGEX-5X-2 or pGEX-6P-2, respectively. GST-ESAM was generated by cloning the cytoplasmic tail of ESAM (aa 278-394) into pGEX-KG (Nasdala et al., 2002). GST-CAR was generated by cloning the cytoplasmic tail of murine CAR (aa 259-345; GenBank accession number Y10320) into pGEX-4T-1. The expression vector encoding murine JAM-2 has been previously described (Aurrand-Lions et al., 2001a; Aurrand-Lions et al., 2001b). The point mutation S281A was generated by a PCR-based approach using PfuTurbo® DNA polymerase (Stratagene, Netherlands). Expression vectors encoding PAR-3 and truncation mutants of PAR-3 or ZO-1 were described previously (Ebnet et al., 2001).

Generation of GST fusion proteins and in vitro binding assays

The purification of GST fusion proteins and in vitro GST-pulldown assays were performed essentially as described previously (Ebnet et al., 2000; Ebnet et al., 2001).

In vivo labelling, phosphoamino acid analysis and phosphotryptic peptide mapping

CHO cells stably expressing JAM-2 wild-type or JAM-2 S281A were washed in phosphate-free DMEM and subsequently metabolically labelled for 12 hours in phosphate-free DMEM containing [^{32}P]-orthophosphate (0.5 mCi/ml). Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% (v/v) Triron X-100, 12.5 mM NaF, 10 mM NaPPi, 10 mM VO_4^{3-}, 0.07 trypsin inhibitory units/ml aprotinin, 1 mM PMSF (phenylmethyl sulphonyl fluoride), 1 mM dithiothreitol) and JAM-2 was immunoprecipitated using affinity-purified polyclonal rabbit antibodies. Phosphorylated proteins were resolved by SDS-PAGE. For phosphoamino acid analysis proteins were transferred to PVDF membranes and visualized by autoradiography. After excision of the bands corresponding to JAM-2, amino acids were released by acid hydrolysis and separated by two-dimensional electrophoresis on thin-layer cellulose plates using a Hunter HTLE 7000 apparatus. For phosphotryptic peptide mapping, the bands corresponding to JAM-2 were eluted from the polyacrylamide gels, digested, separated and visualized according to published protocols (Boyle et al., 1991).

Transient transfection

For transient transfection, COS-7 cells were grown to a density of approximately 80% confluency. Cells were incubated with a mixture of 2 μg/ml circular plasmid DNA and 12 μl/ml GeneJammer transfection reagent (Stratagene Europe, Amsterdam, The Netherlands) for 3 hours. Cells were then supplemented with complete medium and incubated under standard culture conditions. Forty hours after transfection cells were harvested and lysates were prepared as described (Ebnet et al., 2001).

Immunohistochemistry and immunocytochemistry

For cryosections, organs and tissues from Balb/c mice were embedded in Tissue Tek OCT compound (Miles, Elkhart, IN), snap frozen and stored at –80°C. Sections of 7 μm were cut on a freezing microtome, mounted on slides coated with poly-L-lysine (Menzel-Gläser, Nußloch, Germany) and dried. For immunoperoxidase staining, the sections were fixed in acetone for 10 minutes at 4°C; this was followed by a reduction of endogenous peroxidase activity with 0.1% hydrogen peroxide, 20 mM sodium azide, for 30 minutes at room temperature. Nonspecific binding was blocked by incubation with 2% bovine serum albumin in PBS for 30 minutes. Tissue sections were incubated with the primary antibodies diluted in PBS/1% bovine serum albumin for 1 hour, followed by incubation with affinity-purified peroxidase-conjugated secondary antibodies. After visualization of the reaction with 3-amino-9-ethylcarbazole the sections were counterstained with Mayer's hematoxylin and mounted. All steps were performed in a humidified chamber at room temperature. For control purposes sections were treated in the same way but with the primary antibodies being omitted; these controls consistently gave negative results.

For immunofluorescence analysis cells were grown on LabTec chamber slides (Nalge-Nunc, Wiesbaden, Germany). Alternatively, cells were plated at low density ($1\times10^3/cm^2$) on glass coverslips coated with matrigel 1/20 (Becton-Dickinson) and grown for four days. This results in islets of cells, which can be analysed individually for JAM-2 localization by immunocytochemistry. Stainings were performed as previously described (Ebnet et al., 2001).

Results

In vitro association of PAR-3 with the COOH termini of JAM-2 and JAM-3

Recently, we and others reported that JAM-1 binds in a PDZ domain-dependent manner to the cell polarity protein PAR-3 (Ebnet et al., 2001; Itoh et al., 2001). Given that all three JAMs end in type II PDZ domain targeting motifs we reasoned that JAM-2 and JAM-3 might bind to PAR-3 in a similar manner as JAM-1. To test this we performed GST binding assays using GST-JAM fusion proteins immobilized on glutathione-

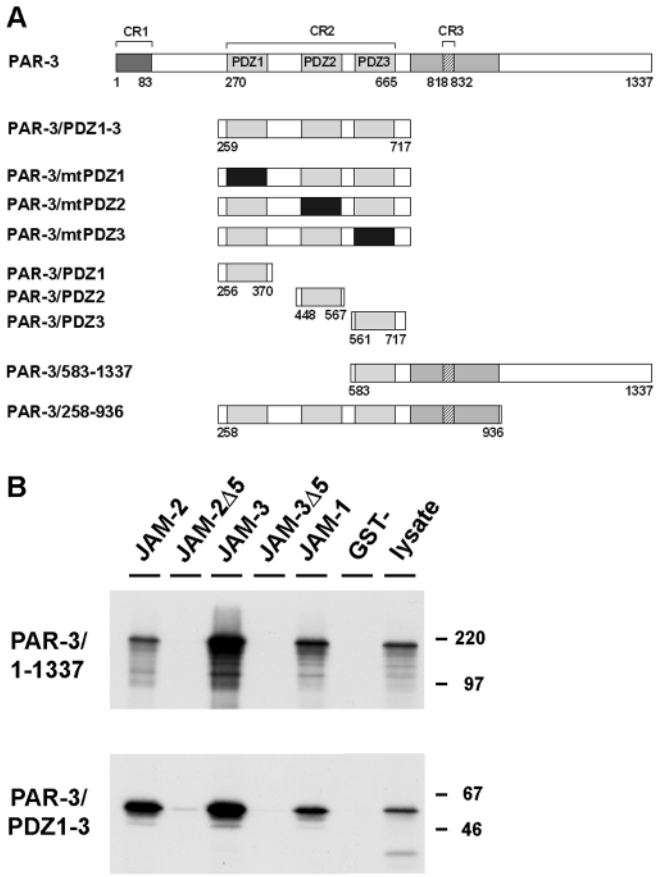

Fig. 1. PAR-3 associates directly with JAM-2 and JAM-3. (A) Schematic view of PAR-3 and PAR-3 expression constructs used in this study. The three conserved regions (CR) are indicated by brackets. The aPKC-binding region (aa 712-936) is illustrated as grey bar encompassing CR3. The three PDZ domains are indicated. The expression constructs used in this study are schematically illustrated. (B) Full-length PAR-3 (PAR-3/1-1337) and a PAR-3 fragment comprising the three PDZ domains (PAR-3/PDZ1-3) were generated by in vitro transcription/translation in the presence of [^{35}S]-methionine and incubated with GST-fusion proteins containing the cytoplasmic domains of JAM-2, JAM-3 and JAM-1. To analyse the requirement of the PDZ domain binding motif, the C-terminal five aa residues of JAM-2 and JAM-3 were deleted (JAM-2Δ5, JAM-3Δ5). As control for unspecific binding GST alone was used (GST-). In the lane marked with 'lysate', 7% of the transcription/translation reaction was loaded. PAR-3 binds to both JAM-2 and JAM-3 in a PDZ domain-dependent manner.

Sepharose beads and in vitro translated, [^{35}S]-methionine-labelled PAR-3 constructs comprising either full-length PAR-3 or a recombinant fragment containing the three PDZ domains of PAR-3 (Fig. 1B). PAR-3 bound to both JAM-2 and JAM-3. Deletion of the C-terminal five amino acids comprising the PDZ domain binding motif abrogated the association. These findings suggested that PAR-3 associates with all three JAM molecules in a PDZ domain-dependent manner in vitro.

PAR-3 associates with JAM-2 and JAM-3 through its first PDZ domain

PAR-3 contains three PDZ domains, for which binding partners have been described only for the first, i.e. JAM-1 and PAR-6 (Ebnet et al., 2001; Lin et al., 2000). Therefore, it seemed possible that JAM-2 and JAM-3 associate with PAR-3 through PDZ domains 2 and/or 3. To test this possibility, we generated individual PDZ domains of PAR-3 by in vitro translation and incubated these with GST-JAM fusion proteins immobilized on glutathione-Sepharose beads. As a positive control we used a PAR-3 construct comprising all three PDZ domains. PDZ1 domain of PAR-3 strongly bound to both JAM-2 and JAM-3; PDZ2 domain did not associate with either, whereas the PDZ3 domain weakly associated with both JAM molecules (Fig. 2). In all cases, the association was drastically reduced or abolished when the C-terminal five amino acids of the JAM molecules were deleted. These findings suggested that PAR-3 associates with JAM-2 and JAM-3 predominantly through PDZ1 domain and weakly through PDZ3 domain.

When we used PAR-3 fragments containing all three PDZ domains with individual PDZ domains inactivated by replacement with the inactive PDZ domain present in the secreted form of interleukin 16 (IL-16) (Ebnet et al., 2001; Muhlhahn et al., 1998), we found that the inactivation of the PDZ1 domain completely abolished the association between

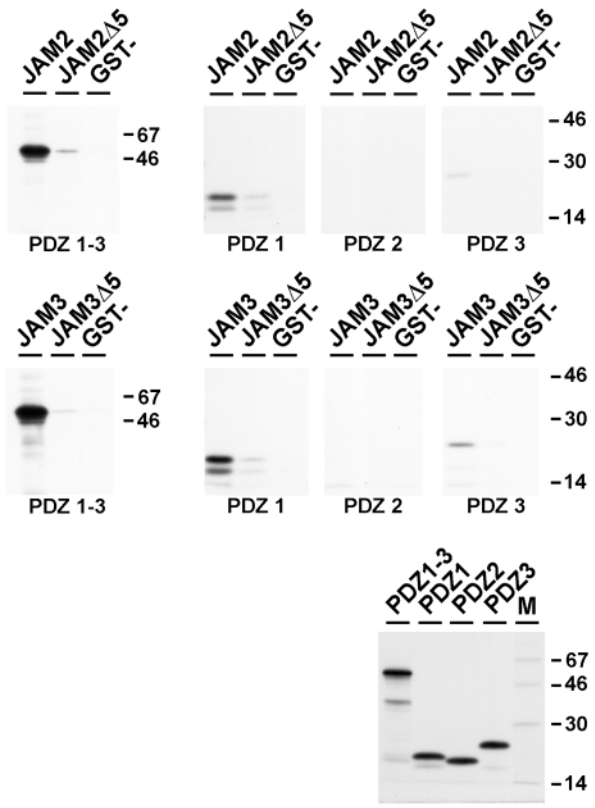

Fig. 2. PAR-3 associates with JAM-2 and JAM-3 through its first PDZ domain. Constructs comprising all three PDZ domains (PDZ 1-3) or individual PDZ domains of PAR-3 (PDZ 1, PDZ 2, PDZ 3) were generated by vitro transcription/translation and incubated with immobilized GST-fusion proteins as described in the legend to Fig. 1. From the three individual PDZ domains only PDZ 1 strongly bound to both JAM-2 and JAM-3; a weak association was observed with PDZ 3. As indicated in the lower panel all PDZ domains were generated with the same efficiencies.

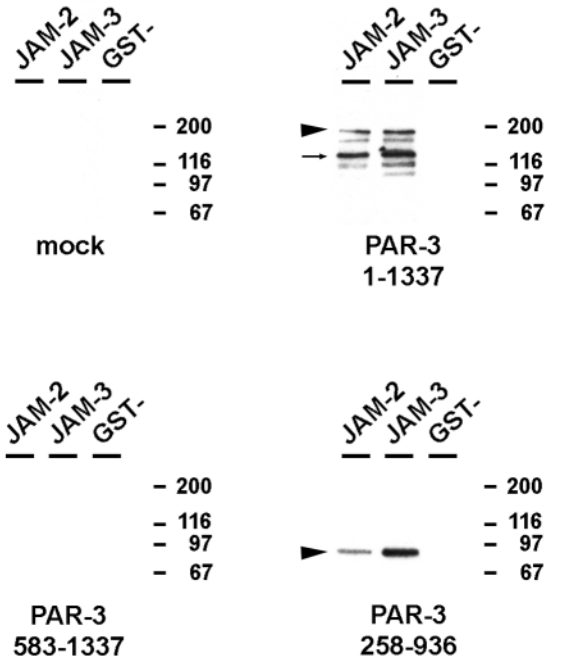

Fig. 3. PAR-3 generated in COS-7 cells associates with JAM-2 and JAM-3. Three T7 epitope-tagged PAR-3 constructs comprising either full-length PAR-3 (PAR-3/1-1337), or aa residues 583-1337 with the PDZ 3 and the aPKC binding domain (PAR-3/583-1337), or aa residues 258-936 with PDZ domains 1 to 3 and the aPKC binding domain (PAR-3/258-936) were transiently transfected into COS-7 cells. The lysates of transfected cells were incubated with immobilized GST-JAM fusion proteins and the resulting protein complexes were analysed by immunoblotting with antibodies against the T7 epitope. Arrowheads indicate the positions of recombinant PAR-3 molecules; the small arrow in the top right panel indicates PAR-3 degradation products. The two PAR-3 constructs containing PDZ domain 1 were efficiently affinity-isolated with both GST-JAM-2 and GST-JAM-3, whereas the construct lacking PDZ domains 1 and 2 did not bind to GST-JAM fusion proteins.

PAR-3 and JAM-2 or JAM-3, whereas the inactivation of the PDZ2 domain had no effect on the binding, and inactivation of PDZ3 domain reduced, but did not completely abolish the association (data not shown). These findings complemented the observation with individual PDZ domains and confirmed that PAR-3 associates in vitro with both JAM-2 and JAM-3 predominantly through PDZ1 domain and that PDZ3 domain might contribute to the association.

PAR-3 can be affinity-isolated from COS-7 cell extracts

To analyse whether JAM-2 and JAM-3 associate with PAR-3 generated in vivo, we transiently transfected COS-7 cells with PAR-3 expression vectors containing either full-length PAR-3 or truncated PAR-3 constructs comprising the C-terminal half of PAR-3, which includes PDZ3 domain and the aPKC-binding domain (amino acids 583-1337) or a central part of PAR-3, including PDZ domains 1-3 and the aPKC-binding domain (aa 258-936). The lysates of the transfected cells were then incubated with immobilized GST-fusion proteins containing the cytoplasmic domains of JAM-2 and JAM-3, and with immobilized GST alone. Bound proteins were detected by

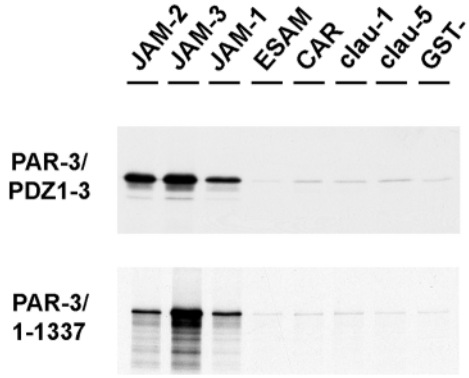

Fig. 4. PAR-3 associates exclusively with JAMs 1 to 3. GST fusion proteins with the C-terminal cytoplasmic domains of JAM-2, JAM-3, JAM-1, ESAM, CAR, claudin-1 and claudin-5 were incubated with [^{35}S]-methionine labelled PAR-3 constructs comprising PDZ domains 1 to 3 (PAR-3/PDZ1-3) or full length PAR-3 (PAR-3/1-1337) as described in the legend to Fig. 1. Both PAR-3 constructs efficiently associated only with JAMs 1 to 3.

western blot analysis using antibodies against the T7-tag fused to the PAR-3 constructs. Under these conditions full-length PAR-3, as well as the PAR-3 construct comprising all three PDZ domains (aa 258-936), could be affinity-isolated from COS-7 cell lysates, whereas the PAR-3 construct lacking PDZ domains 1 and 2 (aa 583-1337) could not be affinity-isolated (Fig. 3). These findings indicate that PAR-3 constructs generated in vivo associate with JAM-2 as well as with JAM-3 in vitro, and further support the notion that this association is mediated predominantly through the PDZ1 domain of PAR-3.

PAR-3 associates exclusively with members of the JAM family among tight junction-associated immunoglobulin-like transmembrane proteins

We have shown recently that among integral transmembrane proteins present at tight junctions, which include JAMs, occludin and claudins (Tsukita et al., 2001), PAR-3 associates exclusively with JAM-1 but not with occludin, claudin-1, claudin-4 or claudin-5 (Ebnet et al., 2001). Recently, two additional members of the immunoglobulin superfamily, ESAM (Hirata et al., 2001) and CAR (Bergelson et al., 1997), were described to be localized at tight junctions of endothelial cells and epithelial cells, respectively (Cohen et al., 2001; Nasdala et al., 2002). Both molecules carry canonical PDZ domain targeting motifs at their C-termini, which fit to the type I PDZ domain binding motif (Songyang et al., 1997). To address the possibility that PAR-3 binds to ESAM or CAR we performed GST binding experiments with GST-ESAM and GST-CAR fusion proteins and in vitro translated, [^{35}S]-methionine-labelled PAR-3 constructs comprising either the three PDZ domains of PAR-3 or full-length PAR-3. Both PAR-3 constructs associated exclusively with the three JAM molecules but not with ESAM or CAR (Fig. 4). As described recently (Ebnet et al., 2001), PAR-3 did not associate with claudin-1 or claudin-5. These findings suggest a striking selectivity of PAR-3 for the JAM molecules among all tight junction-associated integral membrane proteins.

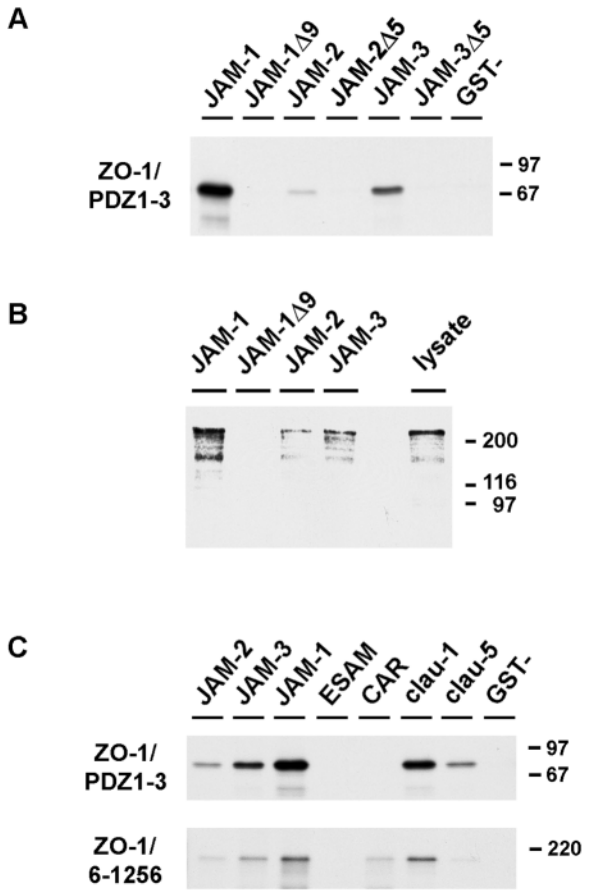

Fig. 5. JAM-2 and JAM-3 associate with ZO-1. (A) A ZO-1 construct comprising PDZ domains 1 to 3 of ZO-1 (ZO-1/PDZ1-3) was generated in vitro and incubated with immobilized GST-JAM fusion (JAM-1, JAM-2, JAM-3) proteins as described in the legend to Fig. 1. GST-fusion proteins lacking the C-terminal PDZ domain binding motifs were used as controls to analyse the PDZ domain-dependence of the association (JAM-1Δ9, JAM-2Δ5, JAM-3Δ5). All three JAMs bind to ZO-1 in a PDZ domain-dependent manner. (B) Lysates derived from CMT epithelial cells were incubated with immobilized JAM fusion proteins. The resulting protein complexes were subjected to SDS-PAGE and analysed by immunoblotting with antibodies directed against ZO-1; the lane marked with 'lysate' contains an aliquot of CMT lysates directly immunoblotted with ZO-1 antibodies. All three JAM molecules isolate ZO-1 from CMT lysates. (C) GST-fusion proteins containing the C-terminal cytoplasmic domains of JAM-2, JAM-3, JAM-1, ESAM, CAR, claudin-1 and claudin-5 were incubated with [^{35}S]-methionine-labelled ZO-1 constructs comprising PDZ domains 1 to 3 (ZO-1/PDZ1-3) or aa residues 6-1256 (ZO-1/6-1256) as described in the legend to Fig. 4. Besides JAMs 1 to 3, ZO-1 associates with claudin-1 and claudin-5; in addition, ZO-1 associates with CAR, probably in a PDZ-domain-independent manner.

JAM-2 and JAM-3 associate with ZO-1 in vitro

Besides PAR-3, JAM-1 associates with the tight junction-associated MAGUK (membrane-associated guanylate kinase) protein ZO-1 (Bazzoni et al., 2000; Ebnet et al., 2000; Itoh et al., 2001). To determine whether JAM-2 and JAM-3 also bind to ZO-1 we perfomed GST binding assays with immobilized GST-JAM fusion proteins and in vitro-generated ZO-1 fragments that comprise the three PDZ domains of ZO-1. As shown in Fig. 5A, both JAM-2 and JAM-3 bind to ZO-1. This association requires an intact C-terminal PDZ binding motif, suggesting a PDZ domain-dependent association. To further show an interaction between JAM-2 and JAM-3 with ZO-1, lysates derived from CMT epithelial cells were incubated with immobilized GST-JAM fusion proteins, and bound proteins were analysed by immunoblotting with antibodies directed against ZO-1. Similarly to JAM-1, both JAM-2 and JAM-3 precipitated a protein species of approximately 220 kDa that reacted with the ZO-1 mAb and that comigrated with a protein detected in the lysate of CMT cells by the same antibody (Fig. 5B). This protein band probably represents the 220 kDa isoform of ZO-1. We also analysed the interaction of ZO-1 with all integral membrane proteins of the immunoglobulin superfamily described so far to be present in tight junctions by GST binding assays. We found that both ESAM and CAR did not associate with a ZO-1 fragment comprising the three PDZ domains of ZO-1 (Fig. 5C). However, a ZO-1 fragment comprising aa residues 6 to 1256 bound to immobilized GST-CAR but not to immobilized GST-ESAM. These findings suggested that CAR might directly bind to ZO-1 in a non PDZ domain-dependent manner. In summary, these experiments indicated that ZO-1 binds to all three JAMs but, in contrast to PAR-3, ZO-1 associates with several other integral membrane proteins present at tight junctions, including CAR, claudins and occludin (Cohen et al., 2001; Furuse et al., 1994; Itoh et al., 1999).

PAR-3 localizes at cell-cell contacts of endothelial cells

So far, our data suggest that JAM-2 and JAM-3 associate with both PAR-3 and ZO-1 in a similar manner to JAM-1. A major difference between JAM-1 and JAM-2 or JAM-3 is in their expression patterns in multicellular tissues. JAM-2 and JAM-3 are predominantly expressed in endothelial cells, whereas JAM-1 is expressed by both endothelial cells and epithelial cells (Aurrand-Lions et al., 2001b; Liang et al., 2002; Martin-Padura et al., 1998; Palmeri et al., 2000). To determine whether PAR-3 is localized at cell-cell contacts of endothelial cells we analysed human umbilical vein endothelial cells (HUVEC) by indirect immunofluorescence with PAR-3 antibodies. As shown in Fig. 6A, PAR-3 localizes at cell-cell contacts of HUVEC in a similar way to AF-6 and ZO-1. Double immunofluorescence labelling indicated that PAR-3 colocalizes with JAM-2 in these cells when the stainings were performed within 48 hours after plating (Fig. 6B). Interestingly, the junctional staining for JAM-2 was lost over time, although the level of JAM-2 surface expression as analysed by flow cytometry was not changed (data not shown). This suggests that JAM-2 might be involved in the early events of interendothelial junction formation rather than in the stabilization of cell contacts. Taken together, these findings show that PAR-3 localizes at cell-cell contacts of endothelial cells and colocalizes with JAM-2 early during cell contact formation.

PAR-3 is expressed by endothelial cells in various tissues

To analyse PAR-3 expression by endothelial cells in vivo, cryostat sections of various mouse tissues were analysed by

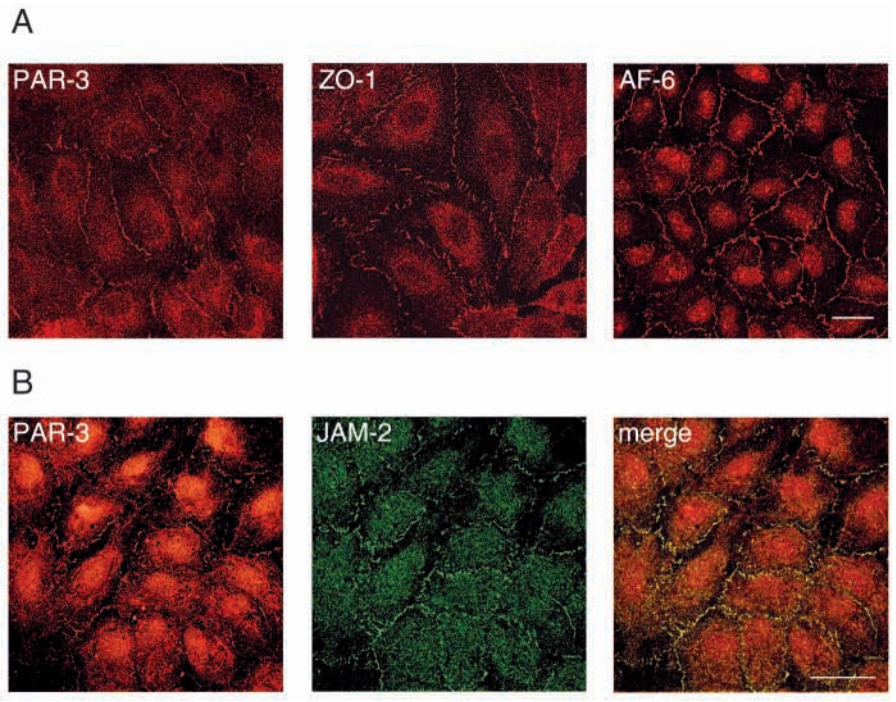

Fig. 6. PAR-3 localizes at cell-cell contacts of endothelial cells. (A) Human umbilical vein endothelial cells (HUVEC) were stained with antibodies against PAR-3, ZO-1 and AF-6. Bound antibodies were visualized with biotinylated donkey anti-rabbit IgG and Cy3-conjugated streptavidin. PAR-3 localizes at cell-cell contacts of HUVECs in a similar way to ZO-1 and AF-6. Bar, 20 μm. (B) Double-label immunofluorescence staining of HUVEC with antibodies against PAR-3 and JAM-2. Rat anti-JAM-2 antibodies were visualized with goat anti-rat FITC before further processing for incubations with rabbit anti-PAR-3 and goat anti-rabbit Texas Red in the presence of 0.2% of normal rat serum. PAR-3 colocalizes with JAM-2 at cell contacts of HUVECs. Bar, 25 μm.

immunohistochemistry. Endothelial cells were identified using endothelial cell-specific markers such as PECAM-1, von Willebrand factor or the MECA-79 epitope, which is selectively expressed in high endothelial venule (HEV) endothelial cells of peripheral and mesenteric lymph nodes. PAR-3 immunoreactivity was identified in endothelial cells lining capillaries in the tongue, the heart endocardium and the heart arteries (Fig. 7). By contrast, PAR-3 was absent in HEV endothelial cells. These data indicate that PAR-3 is expressed by endothelial cells in various organs but is absent from HEV endothelial cells.

PAR-3 and ZO-1 are recruited by JAM-2 to cell-cell contacts in CHO cells

To determine whether JAM-2 influences the subcellular distribution of PAR-3, we generated stable CHO cell lines expressing JAM-2. Surprisingly, only few of these cells showed JAM-2 localization at cell contacts, despite high levels of JAM-2 expression at the cell surface as analysed by flow cytometry (Fig. 8A, left panel). On the basis of this result and the observation of the regulated junctional localization of JAM-2 in HUVECs, we reasoned that the clustering of JAM-2 at cell-cell contacts may be affected by post-translational modifications such as phosphorylation. Therefore, we generated various mutants of JAM-2 with individual putative phosphorylation sites present in the cytoplasmic tail mutated into alanine residues. These mutants were used to generate stable CHO cell lines. One of them (aa residue 281 changed from serine to alanine, S281A JAM-2) showed strong JAM-2 localization at cell contacts (Fig. 8A, middle panel), although the overall surface expression level was comparable to that of wild-type JAM-2 as assessed by flow cytometric analysis (data not shown). Mutation of the threonine residue at postion 296 had no effect on junctional localization of JAM-2 (data not shown). Because the PDZ domain targeting motif at the C-terminus of JAM-2 (aa 306-310) was unaffected by the S281A mutation, we reasoned that endogenous PAR-3 and ZO-1 might be recruited to cell-cell contact sites with intensive JAM-2 staining. As shown in Fig. 8B, PAR-3 as well as ZO-1 colocalized with S281A JAM-2 at cell contact sites. HSP-90 was used as negative control and did not colocalize with S281A JAM-2 at cell-cell contacts. In cells transfected with wt JAM-2 the few cell contact sites positive for JAM-2 (Fig. 8A, left panel) were also positive for PAR-3 or ZO-1 (data not shown), indicating that the S281A point mutation affects the subcellular localization at cell-cell contacts of JAM-2 and does not influence the association between JAM-2 and PAR-3 and ZO-1. These findings have two implications: first, JAM-2 localization at cell contacts seems to be a regulated process, possibly through phosphorylation of the serine residue at position 281; second, JAM-2 actively recruits PAR-3 and ZO-1 to cell-cell contacts. The latter observation also points to an association between JAM-2 and both PAR-3 and ZO-1 in living cells.

JAM-2 is phosphorylated at the S281 residue in CHO cells

As outlined in the previous paragraph, the S281A mutation strongly increased the localization of JAM-2 at cell-cell contacts, suggesting that the junctional localization of JAM-2 is negatively regulated by phosphorylation of the S281 residue. This was further supported by the observation that when we mutated the S281 residue into aspartic acid, thus mimicking constitutive phosphorylation of S281 (JAM-2 S281D), JAM-2-positive cell-cell contacts were only sparsely observed and the frequency of junctional localization was comparable to wild-type JAM-2 (Fig. 8A, right panel). To determine directly whether JAM-2 is phosphorylated, we performed

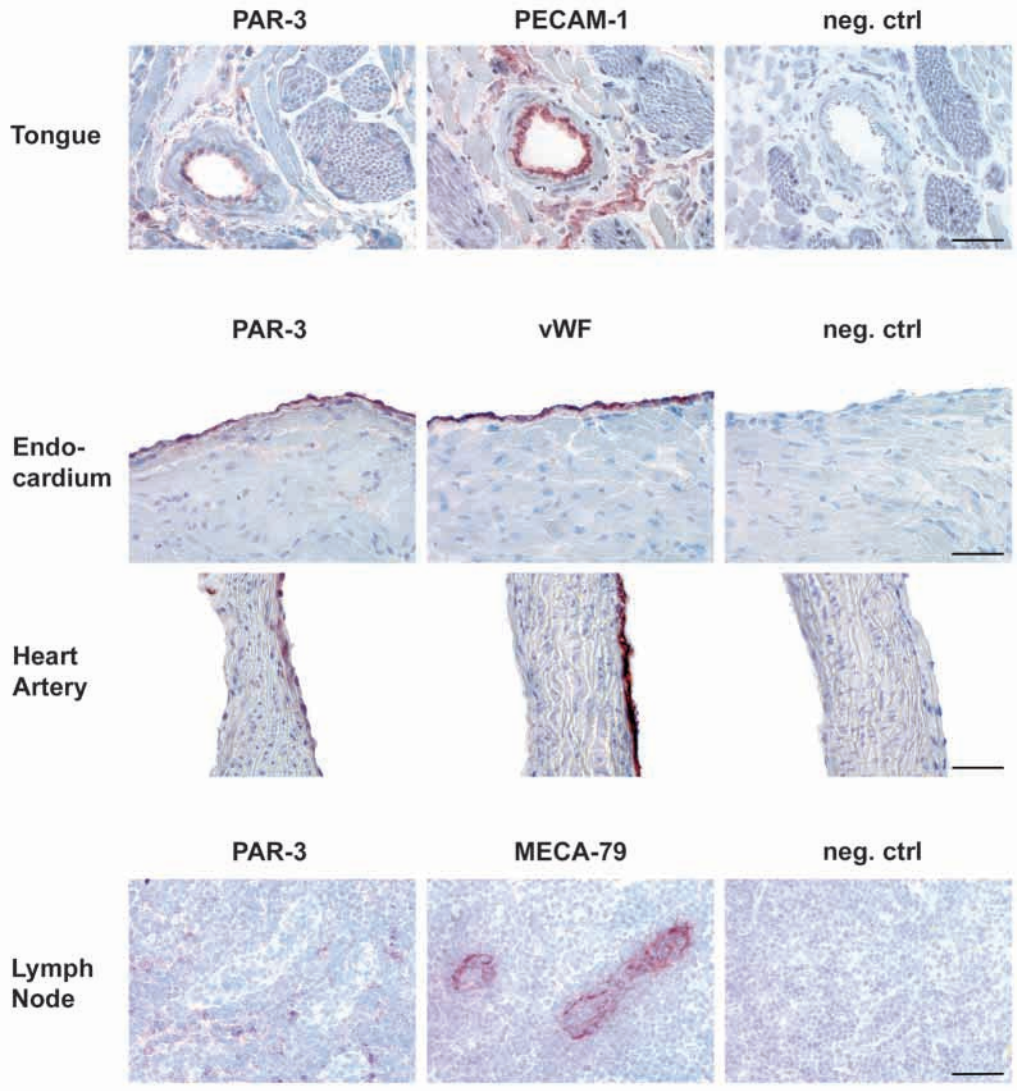

Fig. 7. PAR-3 is expressed by endothelial cells in various tissues. Cryostat sections of tongue, heart endocardium and a heart artery, as well as of mesenterial lymph node, were incubated with a polyclonal antibody against PAR-3. Bound antibodies were visualized by peroxidase-conjugated secondary antibodies. Antibodies against PECAM-1, von Willebrand factor (vWF) and the MECA-79 epitope were used as endothelial-specific markers. In negative control samples (neg. ctrl) the staining procedures were performed without primary antibodies. In the bottom panels, high endothelial venules appear as regions with lower cell densities. Note that PAR-3 is completely absent from high endothelial venule endothelial cells. Bars, 50 μm.

phosphoamino acid analyses of JAM-2 immunoprecipitated from stably transfected CHO cells. This revealed that both JAM-2 wt and JAM-2 S281A were phosphorylated exclusively on serine residues but not on threonine or tyrosine residues (Fig. 9A). A phosphotryptic peptide analysis revealed two phosphorylated peptides derived from JAM-2 wt (Fig. 9B, right panel). One of these two phosphopeptides was absent in tryptic digests derived from JAM-2 S281A (Fig. 9B, left panel). These findings indicate that JAM-2 is phosphorylated on S281 in CHO cells and make a strong case for a negative regulation of cell-cell contact localization of JAM-2 by phosphorylation of the S281 residue.

Discussion

Vertebrate epithelial and endothelial cells are highly polarized with distinct apical and basolateral plasma membrane domains. The two domains are separated by TJs, which restrict the free diffusion of integral membrane proteins and lipids between these domains. TJs, therefore, play a fundamental role in the generation of cell polarity in vertebrates. By freeze-fracture electron microscopy TJs appear as a continuous network of parallel and interconnected strands (Tsukita et al., 2001). It is now believed that claudins and – although the evidence is less direct – also occludin form the molecular basis of the TJ strands. Claudins exist as a family with more than 20 members of related proteins that associate through homotypic as well as heterotypic interactions (Tsukita et al., 2001). According to the current model, cell- and tissue-type specific differences in claudin expressions might account for the differences in the tightness and in the ion-selectivity of TJs observed in various cell types and tissues (Furuse et al., 1999; Van Itallie et al., 2001). Besides occludin and claudins, JAM-1 has been reported to be a component of TJs (Martin-Padura et al., 1998). JAM-1, however, is not incorporated into TJ strands and does not reconstitute TJ strands when ectopically expressed in fibroblasts (Itoh et al., 2001). JAM-1 mAbs block Ca^{2+}-depletion/repletion-induced recovery in TER and recruitment of occludin but not of E-cadherin or ZO-1 (Liu et al., 2000), suggesting that JAM-1 is involved in the regulation of TJ assembly and function rather than in the formation of cell-cell contacts per se. We and others have shown that JAM-1 associates directly with the cell polarity protein PAR-3 (Ebnet et al., 2001; Itoh et al., 2001) providing a putative molecular

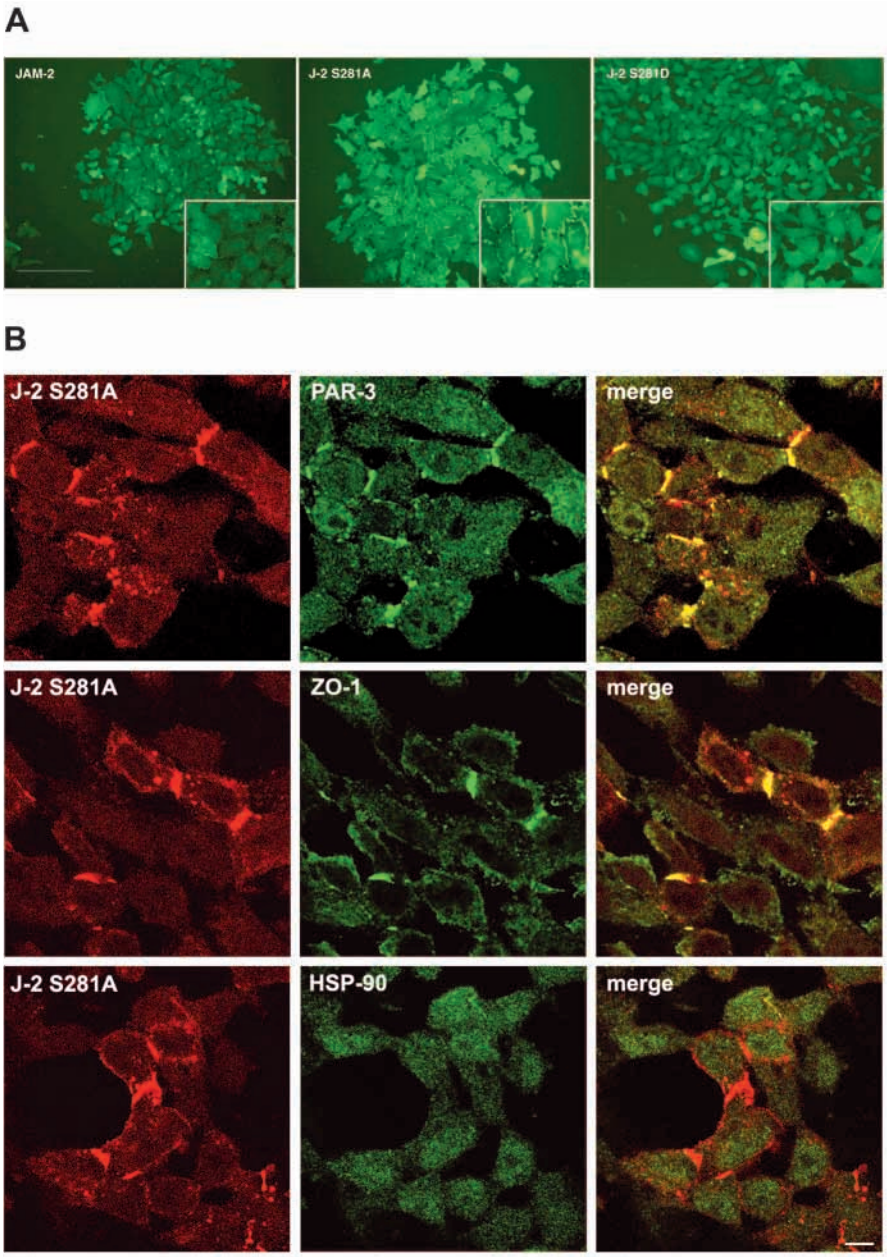

Fig. 8. JAM-2 recruits PAR-3 and ZO-1 in CHO cells. (A) CHO cells stably transfected with JAM-2 (JAM-2, left panel), the S281A mutant of JAM-2 (J-2 S281A, middle panel) or the S281D mutant of JAM-2 (J-2 S281D, right panel) were stained with a mAb against JAM-2. Wild-type JAM-2 is barely detectable at cell-cell junctions and appears as discrete punctate staining (small inset in left panel). By contrast, the S281A mutant of JAM-2 is predominantly clustered at intercellular contacts. The S281D mutant of JAM-2 behaves like wt JAM-2 and is rarely localized at cell-cell contacts. All three cell lines showed a comparable surface expression of the transfected constructs as analysed by FACS analysis (not shown). Bar, 100 μm. (B) CHO cells stably transfected with the S281A mutant of JAM-2 were simultaneously stained with antibodies against JAM-2 and either PAR-3, ZO-1 or HSP-90, followed by Cy-3-conjugated secondary antibodies to detect JAM-2 or Cy-2-conjugated secondary antibodies to detect PAR-3, ZO-1 or HSP-90. Both PAR-3 and ZO-1 were recruited by JAM-2 to sites of cell-cell contacts. Bar, 5 μm.

basis for the previous observations. These findings make a strong case for JAM-1 as a molecule that is relevant for TJ formation and thus for cell polarity in epithelial and endothelial cells.

In this study we report that PAR-3 associates with both JAM-2 and JAM-3. The association between PAR-3 and JAM-2/JAM-3 is PDZ-domain-mediated and involves predominantly the first PDZ domain of PAR-3. Thus, all three JAMs behave very similarly regarding the domain through which they associate with PAR-3. The physiological meaning of this similar behaviour is not clear, yet. Because, in some cell types, two or all three JAMs are simultaneously expressed [e.g. JAM-1 and JAM-3 are expressed by microvessels in the brain or by KLN205 epithelial cells (Aurrand-Lions et al., 2001a) and all three JAMs are expressed by glomerular endothelial cells in the kidney (Aurrand-Lions et al., 2001a)], the possibility that different tissues use different JAM molecules to regulate TJ formation can be excluded. Rather, it seems possible that all JAMs present in a given cell type are part of large molecular complexes involving the association of PAR-3 with all JAMs present. A similar scenario has been proposed for claudins. As in the case of JAMs, certain cell types express more than one claudin (e.g. endothelial cells express claudin-1 and claudin-5) (Liebner et al., 2000), and all claudins tested so far associate with ZO-1, ZO-2 and ZO-3 by a PDZ domain-mediated interaction through the first PDZ domains of the respective ZO proteins (Itoh et al., 1999). The same binding behaviour of all claudins towards ZO-1, ZO-2 and ZO-3 might result in a strong attraction of these proteins to TJs and thus perhaps in the formation of large protein clusters at the cytoplasmic plaque (Itoh et al., 1999).

PAR-3 associates exclusively with JAM-1/-2/-3

We have shown previously that PAR-3 does not bind to occludin or claudin-1, -4 or -5 (Ebnet et al., 2001). In this study we found that PAR-3 does not directly associate in vitro with the two Ig-like proteins ESAM or CAR. Both proteins are present in TJs of endothelial cells and/or epithelial cells. Their C-termini fit to the class I PDZ domain consensus binding sequence (Harris and Lim, 2001; Songyang et al., 1997), and therefore it is less likely that they associate with PAR-3 through a PDZ domain-dependent interaction because all PAR-3 PDZ domains are predicted to bind class II ligands (Izumi et al., 1998). However, we cannot exclude the possibility of an

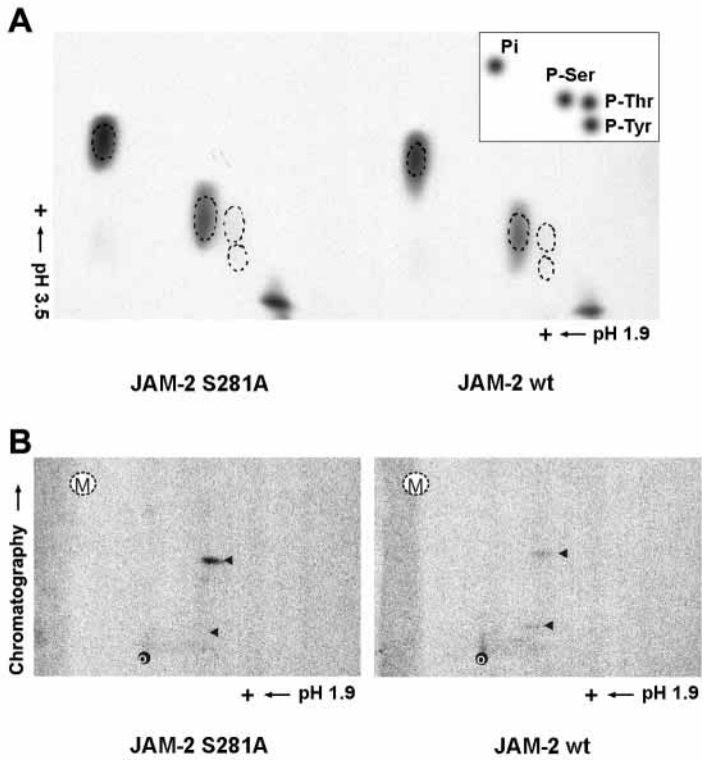

Fig. 9. JAM-2 is phosphorylated at serine residue S281 in CHO cells. (A) Phosphoamino acid analysis of JAM-2. CHO cells stably transfected with the S281A mutant of JAM-2 (JAM-2 S281A, left panel) or wild-type JAM-2 (JAM-2 wt, right panel) were metabolically labelled with [^{32}P]-orthophosphate. Immunoprecipitated JAM-2 was hydrolyzed and the resulting amino acids were subjected to two-dimensional electrophoresis. The broken circles indicate the positions of comigrating cold phosphoamino acids. The inset illustrates the relative positions of free phosphate residues (Pi), phospho-serine (P-Ser), phospho-threonine (P-Thr) and phospho-tyrosine (P-Tyr). JAM-2 is phosphorylated exclusively on serine residues in both cell lines. (B) Two-dimensional phosphotryptic peptide maps of [^{32}P]-labelled JAM-2 S281A and JAM-2 wt. Immunoprecipitated JAM-2 was subjected to trypsin digestion and the resulting peptides were subjected to electrophoresis and thin layer chromatography as indicated by the arrows. The origins of sample application are indicated by encircled black dots; the position of a marker dye for thin layer chromagtography is indicated by an encircled 'M'. The positions of phosphopeptides are indicated by arrowheads. From two phosphopeptides that are identified in wt JAM-2, one is missing in JAM-2 S281A indicating that JAM-2 is phosphorylated at the S281 residue.

indirect association in cells via other proteins. Thus, JAM-1/-2/-3 are the only currently known integral membrane proteins at tight junctions to which PAR-3 binds directly. This makes them distinct from the other proteins and further underlines their putative role in cell polarity formation.

ZO-1 associates with various integral membrane proteins in tight junctions including JAM-2 and JAM-3

We also found that ZO-1 associates with JAM-2 and JAM-3. ZO-1 belongs to the family of MAGUKs, which are associated with the plasma membrane (Anderson, 1996). ZO-1 associates with claudins through PDZ domain 1 (Itoh et al., 1999), with JAM-1 through PDZ domain 3 (Ebnet et al., 2000; Itoh et al., 2001) and with occludin through the guanylate kinase (GK) domain. The association of ZO-1 with all three families of integral membrane proteins in TJs (i.e. occludin, claudins and JAM-1) is mediated through nonoverlapping domains, which makes it conceivable that the association of ZO-1 with the various integral membrane proteins serves to cluster these at TJs.

As in the case of JAM-1, the association with JAM-2 and JAM-3 is PDZ domain mediated (Fig. 5A). We are currently in the process of identifying the PDZ domain of ZO-1 involved in binding to JAM-2 and JAM-3. We also found a weak association between ZO-1 and CAR. As described by others, ZO-1 co-immunoprecipitates with CAR, and ZO-1 is recruited to sites of homophilic CAR interaction in transfected CHO cells (Cohen et al., 2001). Our data support the view that ZO-1 and CAR can associate directly with each other. This association, however, is not mediated through one of the three ZO-1 PDZ domains because GST-CAR did not associate with the construct comprising the ZO-1 PDZ domains (Fig. 5C). This is in line with the prediction that all three ZO-1 PDZ domains do not bind class I PDZ domains ligands (Harris and Lim, 2001; Willott et al., 1993). We did not observe an association between ESAM and ZO-1/PDZ1-3 or ZO-1/6-1256 but we cannot rule out the possibility of a PDZ-independent association between ESAM and ZO-1 through a region in the C-terminal domain that is not present in the ZO-1/6-1256 construct.

PAR-3 is expressed by endothelial cells

Consistent with a predominant expression of JAM-2 and -3 in endothelial cells, we found that PAR-3 is localized at intercellular junctions of cultured HUVEC and is expressed by endothelial cells of certain tissues such as the tongue and the heart. The strong signal of PAR-3 in vessels of the heart and the endocardium correlates with JAM-2 and JAM-3 expression in the heart artery and endocardium, as well as with JAM-2 expression in cultured endothelial cells derived from the aorta (Arrate et al., 2001; Palmeri et al., 2000; Phillips et al., 2002). In other tissues such as skin or the brain, the expression of PAR-3 in vessels was less pronounced, which made it difficult to distinguish between specific staining in vessels and unspecific background staining (data not shown). By contrast, in endothelial cells lining the high endothelial venules in secondary lymphoid organs, PAR-3 expression was completely absent, although all three JAMs show expression in HEV endothelial cells (Aurrand-Lions et al., 2001a; Aurrand-Lions et al., 2001b; Malergue et al., 1998; Palmeri et al., 2000). This indicates that JAM expression does not necessarily correlate with PAR-3 expression in endothelial cells. The endothelium in HEVs is characterized by a high rate of constitutive lymphocyte transmigration, suggesting that the organization of

TJs is less complex than in the endothelium of other tissues. In fact, the complexity of interendothelial TJs varies along the vascular tree and the lowest complexity is found in postcapillary venules, the sites of leukocyte transmigration (Bowman et al., 1992; Schneeberger, 1982). So, it seems possible that the absence of PAR-3 expression in endothelial cells lining postcapillary venules such as the HEVs of secondary lymphoid organs helps to prevent the formation of highly complex TJs, thus allowing a high rate of paracellular transendothelial migration of lymphocytes. The expression of the three JAMs in HEV endothelial cells, despite the absence of PAR-3 expression, is in line with several reports describing a role for JAMs in the regulation of leukocyte-endothelial interactions by way of homophilic and/or heterophilic JAM/JAM interactions (Arrate et al., 2001; Del Maschio et al., 1999; Johnson-Leger et al., 2002; Liang et al., 2002; Martin-Padura et al., 1998; Ostermann et al., 2002).

The junctional localization of JAM-2 is regulated by serine phosphorylation

CHO cells stably expressing wt JAM-2 showed only sparse JAM-2 localization at cell-cell contacts (Fig. 8A). By contrast, a point mutation that abolishes phosphorylation of the S281 residue (S281A) dramatically increased JAM-2 localization at cell contacts, suggesting that JAM-2 localization is negatively regulated by phosphorylation. In addition to the S281 residue, we mutated the only threonine residue present in the cytoplasmic tail of JAM-2 into alanine (T296A), but this mutation had no effect on the junctional localization of JAM-2 (data not shown). Consistent with these findings, we found phosphorylation exclusively on serine residues (Fig. 9A). Interestingly, in addition to the peptide harbouring the S281 residue, we identified a second phosphopeptide of JAM-2, suggesting that additional serine residues can be phosphorylated. The identity, as well as the functional role, of this additional serine residue has not yet been analysed.

The mechanism underlying the enhanced localization of JAM-2 S281A at cell contact sites is not clear. The possibility that phosphorylation of JAM-2 influences the association with PAR-3 and ZO-1 is rather unlikely. This is based on our observation that, despite the sparse localization of JAM-2 at cell-cell contacts in JAM-2 wt-transfected CHO cells (Fig. 8A), the few cell-cell contacts positive for JAM-2 were also positive for PAR-3 and ZO-1, indicating that JAM-2 wt is as effective as JAM-2 S281A in associating with PAR-3 and ZO-1. The possibility that increased JAM-2 localization at cell-cell contacts is the result of an increased protein stability can also be excluded. This is based on two observations: first, both JAM-2 wt and JAM-2 S281A CHO cells had similar levels of JAM-2 surface expression as analysed by FACS analysis (data not shown); second, when cells were surface-biotinylated for 1 hour (‘pulse’) and analysed for the amounts of surface-expressed JAM-2 by immunoprecipitation at various time periods up to 48 hours after replating (‘chase’), we found no significant difference between JAM-2 wt- and JAM-2 S281A-transfected CHO cells (data not shown). Therefore, phosphorylation at S281 does not influence the stability of the protein at the surface, and it seems that the S281 phosphorylation specifically regulates the localization at sites of cell-cell contact in a negative manner.

A role for JAMs in cell polarity

One possible physiological relevance for the association between JAMs and PAR-3 is to anchor the PAR-3/aPKC/PAR-6 complex at TJs. As the PAR-3/aPKC/PAR-6 complex is localized at TJs of fully polarized epithelial cells (Johansson et al., 2000; Suzuki et al., 2001), and as no other membrane protein of TJs has been described yet for any of the three components of the complex, it is conceivable that the association between PAR-3 and JAM-1 serves to localize the whole complex to TJs. In addition to this function, the association between JAMs and PAR-3 might have a role that relates to TJ biogenesis. In the process of wounding-induced cell-cell contact formation JAM-1 appears together with E-cadherin and ZO-1 very early in primordial, spot-like adherens junctions (Ebnet et al., 2001). Spot-like adherens junctions or ‘puncta’ represent sites of initial cell-cell contact mediated by E-cadherin homophilic interactions at tips of filopodia (Adams et al., 1996; Yonemura et al., 1995). At this stage of cell contact formation, occludin or claudins are not present at cell contacts (Suzuki et al., 2002). Also, both aPKC and PAR-3 are absent from cell junctions at this stage (Suzuki et al., 2002). These observations open the possibility that early JAM-1 localization at spot-like structures is necessary to subsequently recruit the PAR-3/aPKC/PAR-6 complex, all components of which have been implicated in TJ formation (Nagai-Tamai et al., 2002; Suzuki et al., 2001; Suzuki et al., 2002; Yamanaka et al., 2001). Whether JAM-2 and JAM-3 are present at the tips of filopodia or lamellipodia and colocalize with VE-cadherin and ZO-1 in endothelial cells is currently being investigated in our lab. JAM-2 shows predominant cell-cell contact localization in HUVEC when cells are subconfluent and contact staining gradually decreases on contact maturation (Aurrand-Lions et al., unpublished observations). In addition, as suggested by our observations with the S281A JAM-2 mutant in CHO cells, the junctional localization of JAM-2 seems to be negatively regulated through phosphorylation of the S281 residue. One could envisage a scenario whereby nonphosphorylated JAM-2 is localized at cell contacts early during cell contact formation where it recruits PDZ domain-containing scaffolding proteins like PAR-3 and ZO-1, which are necessary for further junctional maturation. The simultaneous recruitment of serine kinases could lead to JAM-2 phosphorylation and its subsequent delocalization from cell-cell contact sites. Once the scaffolding complexes are recruited to cell-cell contacts they might be stabilized by other proteins constitutively present at cell-cell contacts, e.g. JAM-1, and JAM-2 would become dispensable. Taken together, these findings open the possibility that a regulated targeting of JAM-2 to nascent cell-cell contact sites might further promote TJ formation by recruiting the PAR-3/aPKC/PAR-6 complex to cell contacts.

In summary, our findings of a direct association between JAM-1/-2/-3 and the polarity proteins PAR-3 and ZO-1 make a strong case for JAMs as being involved in the formation and maintenance of TJ in epithelial and endothelial cells. Our findings further underline the functional dichotomy of JAM proteins as regulators of leukocyte recruitment as well as cell polarity formation.

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