Junctional Adhesion Molecule (JAM) interacts with the PDZ domain containing proteins AF-6 and ZO-1

Klaus Ebnet*, Christian U. Schulz*, Maria-Katharina Meyer zu Brickwedde*, Gunther G. Pendl*, and Dietmar Vestweber*+

*Institute of Cell Biology, ZMBE, University of Muenster, Germany
+Max-Planck-Institute of Physiological and Clinical Research, Bad Nauheim, Germany

condensed title: JAM binds to AF-6 and ZO-1
Key words: JAM, AF-6, ZO-1, tight junctions, cell adhesion, immunoglobulin superfamily

Address correspondence to:
D. Vestweber
Institute of Cell Biology, ZMBE
Von-Esmarch-Str. 56
D-48149 Muenster
Germany
Tel.: ++49-251-8358617
FAX: ++49-251-8358616
e-mail: vestweb@uni-muenster.de

Abbreviations used in this paper:

aa, amino acid; GST, glutathione S-transferase; AF-6, ALL-1 fusion partner from chromosome 6; JAM, junctional adhesion molecule; MAGUK, membrane-associated guanylate kinase; PDZ, protein domain named for PSD-95, discs large, ZO-1; Ig-SF, immunoglobulin superfamily; RPTK, receptor protein tyrosine kinase; RA domain, ras-associating domain
Abstract

We have identified the PDZ domain protein AF-6 as an intracellular binding partner of the junctional adhesion molecule (JAM), an integral membrane protein located at cell contacts. Binding of AF-6 to JAM required the presence of the intact COOH-terminus of JAM, which represents a classical type II PDZ domain binding motif. Although JAM did not interact with the single PDZ domains of ZO-1 or of CASK, we found that a ZO-1 fragment containing PDZ domains 2 and 3 binds to JAM in vitro in a PDZ domain dependent manner. AF-6 as well as ZO-1 could be co-precipitated with JAM from endothelial cell extracts, demonstrating the association of the endogeneously expressed molecules in vivo. Targeting of JAM to sites of cell contacts could be affected by the loss of the PDZ domain binding COOH-terminus. Mouse full length JAM co-distributed with endogeneous AF-6 in human Caco-2 cells at sites of cell contact independent of whether adjacent cells expressed mouse JAM as an extracellular binding partner. In contrast, truncated JAM lacking the PDZ-binding COOH-terminus did not co-distribute with endogeneous AF-6 but was restricted to cell contacts between cells expressing mouse JAM. Our results suggest that JAM could be recruited to intercellular junctions by its interaction with the PDZ domain-containing proteins AF-6 and possibly ZO-1.
Introduction

Epithelial and endothelial cells are connected by intercellular junctions. Tight junctions, also called zonula occludens, form a regulated barrier between cells restricting the diffusion of small solutes across cellular sheets as well as the diffusion of proteins from the lateral to the apical membrane domain (1,2). Adherens junctions have evolved as signalling centers regulating tissue organization and morphogenesis (3,4). Both types of junctions are necessary to establish and maintain cellular polarity. Three integral membrane proteins or families of proteins have been identified in tight junctions. Occludin (5) and claudins (6,7) are four-transmembrane domain proteins that are involved in the formation of tight junction strands (8). Junctional adhesion molecule (JAM) is a newly described Ig-SF member of adhesion molecules that colocalizes with tight junction molecules in both epithelial and endothelial cells (9).

The organization of tight junctions requires the association of transmembrane proteins with cytoplasmic proteins. Recently, proteins containing PDZ domains have emerged as organizers of protein complexes at the plasma membrane (2,10). PDZ domains are 80 - 90 amino acids (aa) long and bind to five amino acid residue motifs at the carboxy terminus of their ligands (11). They are often found in multiple copies within a single protein and, therefore, PDZ domain-containing proteins are considered as scaffolding proteins that promote the clustering of protein complexes at specific subcellular sites (12).

In tight junctions four proteins containing one or several PDZ domains have been identified. These include ZO-1 (13), ZO-2 (14), ZO-3 (15), and AF-6 (16). Occludin directly binds to ZO-1 and ZO-3 (15,17). Several lines of evidence suggest that the proper targeting of occludin to the tight junctions requires ZO-1 and not vice versa. For example, the absence of occludin does not change the localization of ZO-1 (18) whereas the absence of ZO-1 prevents targeting of transfected occludin to cell-cell contacts in fibroblasts (19). In addition, connexin 32-occludin chimeras containing only the ZO-1 binding domain of occludin are properly targeted to tight junctional fibrils in MDCK cells (20). And finally, in occludin-deficient epithelial cells ZO-1 is properly targeted to tight junctions (21). These findings suggest that cytoplasmic proteins such as ZO-1 recruit occludin and act as an organizational scaffold for occludin and possibly other integral membrane proteins of tight junctions. This is substantiated by the recent findings showing that claudins directly associate with ZO-1, ZO-2, and ZO-3 (22).
Another junctional PDZ domain-containing protein is AF-6/afadin (23) that was described as associated with tight junctions (16) as well as with adherens junctions (24). AF-6 binds to cytoplasmic proteins as well as to integral membrane proteins. AF-6 binding to its cytoplasmic binding partners, the small GTPase ras, the tight junction protein ZO-1 and the vinculin-binding protein ponsin/SH3P12, does not involve the PDZ domain of AF-6 (16,25-27). In contrast, the two known integral membrane components that bind to AF-6, a subset of the EphB receptor protein tyrosine kinases (RPTKs) (28,29), and the poliovirus receptor-related protein PRR2/nectin (30) bind to AF-6 in a PDZ domain-dependent manner. The interaction with ras implicates that small GTPases like ras might regulate the organization and/or integrity of tight junctions. The importance of AF-6 for the generation and/or maintenance of cell-cell junctions is demonstrated by the phenotype of AF-6-deficient mice. The absence of AF-6 results in embryonic lethality at E9.5 most likely due to a disorganization of cell-cell junctions and to defects in the polarity of the embryonic ectoderm (31,32).

To identify proteins associating with JAM we performed yeast two-hybrid experiments. We have identified AF-6 as binding partner for JAM. JAM binds to the PDZ domain of AF-6 in vitro and associates with AF-6 in vivo. Microinjection of JAM expression vectors into cells expressing AF-6 in cell-cell junctions results in co-distribution of injected JAM with endogeneous AF-6. JAM lacking the PDZ domain target motif does not co-distribute with AF-6 and localizes exclusively at cell borders of two JAM-injected cells. We also found that JAM binds to ZO-1 in vitro and in vivo. Our results suggest that JAM is recruited to and maintained at the junctional complex by its interaction with the PDZ domain proteins AF-6 and ZO-1.
Materials and Methods

Cell lines, Antibodies, Reagents
The murine endothelial cell line bEnd.3 is derived from mouse brain capillaries (33) and was kindly provided by Dr. Werner Risau (Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim, Germany). Cells were maintained in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin (Life Technologies, Eggenstein, Germany). CHO cells were cultured in α-MEM medium supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine and penicillin/streptomycin. CMT cells were kindly provided by Dr. R. Kemler (Max-Planck-Institute for Immunobiology, Freiburg, Germany) and cultured in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine and penicillin/streptomycin. Caco-2 cells, kindly provided by Dr. Alexander Schmidt (Institute of Infectiology, ZMBE, University of Muenster, Germany) were cultured in Earle's MEM, supplemented with 10 % FCS, 2 mM L-glutamine, penicillin/streptomycin, 1% non-essential amino acids (Sigma, Deisenhofen, Germany).

To generate polyclonal rabbit antisera directed against JAM and murine AF-6, amino-terminal peptides derived from JAM (VQGKGSVYTAQSD; JAMN13) or from AF-6 (MSAGGRDEERRK; AF6N12) were coupled via cysteine at the NH2-terminal residue to ovalbumin and injected into New Zealand White rabbits. The antisera were affinity-purified with the synthetic peptides covalently coupled via bovine serum albumin to CNBr-activated sepharose (Amersham-Pharmacia Biotech Ltd, Freiburg, Germany). Monoclonal antibodies directed against AF-6, β-catenin, human ZO-1 (Pharmingen/Becton-Dickinson, Heidelberg, Germany), and mouse ZO-1 (Chemicon Intl, Hofheim, Germany) were obtained from commercial sources. A polyclonal antibody against ZO-1 was obtained from Zymed (Berlin, Germany). The monoclonal antibodies directed against murine and human JAM, BV11 (9) and BV16 were generously provided by Elisabetta Dejana, Istituto Mario Negri, Milan, Italy. The mAb H2O2-106-7-4 (34) against murine JAM was kindly provided by Michel Aurrand-Lions, CMU, Geneva, Switzerland and Philippe Naquet, INSERM-CNRS, Marseille, France. Secondary antibodies and fluorophor-conjugated reagents were purchased from Dianova (Hamburg, Germany).

Expression vectors
Expression vectors were generated by RT-PCR amplification of the respective fragments from total RNA derived from the mouse endothelial cell line bEnd.3 using standard techniques.
(35). Yeast expression vectors were constructed in pBTM116 (bait plasmids) and pGAD10 (prey plasmids) thereby generating fusion proteins between LexA and JAM or between the GAL4 activation domain and the prey protein, respectively. JAM bait vectors were generated by subcloning the cytoplasmic tail (aa 261-300) of murine JAM (pBTM116-JAM/cyt) or mutants of the cytoplasmic tail lacking the COOH-terminal aa V (aa 261-299, JAM/cytΔ1), FLV (aa 261-297, JAM/cytΔ3), or FKQTSSFLV (aa 261-292, JAM/cytΔ9). Vectors expressing only the COOH-terminal aa of JAM fused to LexA were generated by annealing complementary synthetic oligonucleotides comprising aa 290-300 (JAM/cyt11), aa 295-300 (JAM/cyt6), or aa 297-300 (JAM/cyt4) and cloning into pBTM116. GST fusion proteins were generated in pGEX-4T-1 or pGEX-6P-2 (Amersham-Pharmacia Biotech Ltd.). For JAM, glutathione-S-transferase (GST)- fusion protein expression vectors were generated in the same way as described above for LexA fusion proteins expression vectors. For expression in eukaryotic cells cDNA fragments were cloned into pcDNA3.1/mycHis (Invitrogen BV), or pKE081myc. pKE081myc contains a NH2-terminal myc tag including a Kozak consensus sequence for translation initiation and allows for expression of cDNA fragments representing endogenous protein sequences. The vector was constructed by cloning two sets of complementary oligonucleotides representing the myc tag (5’-CCCGCCATGGAGCAGAAGCTGATCAGCGAGGAGGACCTGA-3’) and a short multiple cloning site (5’-ACTCGAGGCGGCCACTGATATCTGCAATTCCAGCGTCGACTAAGTAG-3’ into PmeI-digested pcDNA3.1/mycHisA (Invitrogen BV, Groningen, Netherlands). The following constructs were generated in pKE081myc: myc-AF-6/PDZ (aa 946-1101 of mouse AF-6/afadin), myc-ZO1/PDZ1-3 (aa 1-575 of mouse ZO-1), myc-ZO1/PDZ2-3 (aa 146-575 of mouse ZO-1), myc-ZO1/PDZ1 (aa 1-151), myc-ZO1/PDZ2 (aa 146-304), myc-ZO1/PDZ3 (aa 407-575), myc-ZO1/PDZ1-2 (aa 1-304), myc-ZO1/PDZ2+ (aa 146-422), myc-ZO1/PDZ3 (aa 280-575), myc-ZO1/PDZ1-2+ (aa 1-413), myc-CASK/PDZ (aa 470-583). To express JAM in eukaryotic cells full length (aa 1-300, JAM) or COOH-terminal truncated JAM (aa 1-293, JAM/Δ9) were cloned into pKE081myc.

The murine homologues of l-afadin and s-afadin were cloned by RT-PCR with cDNA derived from bEnd.3 endothelial cells and primers derived from the rat cDNA sequences of l- and s-afadin (accession numbers U83230 and U83231) (24). The 5’-half of the cDNA common to l- and s-afadin was generated using the two primers AFA.13S (5’-GACATCATTACCACACTGGAAC-3’) and AFA.3AS (5’-TTGTCCAGGTACCTGGAGAAGC-3’) and cloned into pEGFP-C1 (Clontech Laboratories,
Heidelberg, Germany). The sequence corresponding to the first 14 aa was generated separately by annealing two complementary oligonucleotides corresponding to nucleotides 322 – 367 and subsequent cloning in front of the common 5'-region of afadin into pEGFP-C1 thereby generating the complete 5'-half of l- and s-afadin comprising nucleotides 322 - 3272. The 3'-domains were generated separately for l- and s-afadin using the primer pairs AFA.2S (5’-ACACGTTCCTCCAGGTACCTG-3’) and lAFA.1AS (5’-CTTGGTGTTGAGTTCCATTC-3’) for l-afadin or primer pairs AFA.2S and sAFA.1AS (5’-GAGGACCAT AACCTTTTCTTC-3’) for s-afadin. The PCR products were cloned into the pEGFP-C1 vectors containing the common 5'-domain of l- and s-afadin thereby generating the murine full length homologues of rat l- and s-afadin, called mlAF-6 (1816 aa) and msAF-6(a) (1643 aa), respectively. One additional splicing variant was isolated that contained a 36-bp deletion and instead a 22-bp insertion at position 5132 of the s-afadin cDNA thereby generating a shorter isoform of msAF-6/afadin (msAF-6(b)) containing 1604 aa (Fig. 1B). Expression vectors encoding mlAF-6 and msAF-6(a) were generated by subcloning the full length cDNAs into pcDNA3.1/mycHis (Invitrogen).

Two-Hybrid Screen
Two-hybrid screening experiments were performed essentially as described (36). Briefly, the Saccharomyces cerevisiae reporter strain L40 expressing a fusion protein between LexA and the cytoplasmic tail of JAM (aa 261-300) was transformed with 250 µg of DNA derived from a day 9.5/10.5 mouse embryo cDNA library (37) according to the method of Schiestl and Gietz (38). The transformants were grown for 16 h in liquid selective medium lacking tryptophan, leucine, and uracil (SD-TLU) to maintain selection for the bait and the library plasmid. The transformants were then plated onto synthetic medium lacking tryptophan, histidine, uracil, leucine, and lysine (SD-THULL) in the presence of 1 mM 3-aminotriazole (3-AT). After 3 days at 30°C large colonies were transferred to new plates, grown for additional 3 days on the same selective medium and assayed for β-galactosidase activity by a filter assay. His+LacZ+ colonies were grown in liquid selective medium and DNA was isolated by lysing the cells in yeast lysis solution (10 mM Tris-HCl pH8.0, 2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA) in the presence of acid-washed glass beads and subsequent phenol/CHCl₃ extraction and EtOH precipitation. To segregate the bait plasmid from the library plasmid, yeast DNA was transformed into E.coli HB101 and the transformants were grown on M9 minimal medium lacking leucine. Plasmid DNA was then isolated from E.coli HB101 and was reintroduced into L40 in combination with either the
In vitro binding of JAM to AF-6 and ZO-1

JAM GST fusion proteins were expressed in E.coli BL21 (Amersham-Pharmacia Biotech Ltd.). The bacteria were grown at 30°C in LB medium/2% glucose to an A<sub>600</sub> of 0.5. Expression of recombinant proteins was induced with 0.5 mM IPTG for 1 h. The bacteria were harvested by centrifugation, resuspended in 25 ml of lysis buffer (PBS, 1% Triton X-100, 1 mM MgCl<sub>2</sub>, 10 µg/ml DNase I, 10 µg/ml RNase A, 10 µg/ml leupeptin, 10 µg/ml PMSF, 10 µg/ml soybean trypsin inhibitor, 0.1 U/ml α2-macroglobulin), and lysed by passaging through a French pressure cell. GST-fusion proteins were isolated by affinity chromatography on glutathione-Sepharose 4B beads (Amersham-Pharmacia Biotech Ltd.) and dialyzed against 50 mM Heps-NaOH (pH 7.4). Protein solutions were adjusted to 50% (w/v) glycerol and stored at −20°C. Purified proteins were analyzed by SDS-PAGE and Coomassie brilliant blue staining.

For GST pulldown experiments the prey proteins were generated in vitro using the TNT T7-coupled reticulocyte lysate system (Promega Corp., Madison, WI) in the presence of 35S-methionine as described by the manufacturer. 10 µl of the translation reactions were incubated with 3 µg GST fusion proteins immobilized on glutathione-Sepharose 4B beads for 1 h at 4°C under constant agitation. Beads were washed five times with buffer B (10 mM Hepes pH7.2, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1% Triton X-100). Bound proteins were eluted by boiling for 5 min in SDS sample buffer, subjected to SDS-PAGE and analyzed by fluorography. GST-pulldown experiments from whole cellular lysates were done as follows: cells were lysed in lysis buffer (20 mM Tris-HCl pH7.2, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 0.3% Triton X-100, 1 mM PMSF, 100 mM benzamidine, 0.1 U/ml α2-macroglobulin) for 12 min on ice. The lysates were collected and centrifuged for 15 min at 4°C and 10,000g, then pre-cleared by incubation with glutathione-Sepharose 4B beads for 1 h at 4°C. The pre-cleared lysates were added to the GST fusion proteins immobilized on glutathione-Sepharose 4B beads and incubated for 1 h at 4°C. After washing for five times with buffer B bound proteins were eluted, subjected to SDS-PAGE, and blotted onto nitrocellulose membranes by semi-dry transfer (BioRad, München, Germany). The nitrocellulose filters were blocked in Blotto (TBS, 0.1% Tween, 5% dry milk powder) and incubated with AF-6 mAbs. Bound antibodies were visualized with biotinylated goat anti mouse IgG and polyHRP-conjugated streptavidin (RDI, Flanders, NJ) by enhanced chemoluminescence (ECL, Amersham-Pharmacia Biotech Ltd).
**Immunoprecipitation**

Confluent monolayers of bEnd.3 cells were rinsed three times with ice-cold PBS. Cells were incubated for 30 min on ice in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 0.1 U/ml α-macroglobulin, 1 µg/ml pepstatin A, 2 µg/ml leupeptin). Alternatively, cells were incubated in lysis buffer containing 0.02% SDS, or lysis buffer containing 0.02% SDS, 0.5% deoxycholate. Solubilized material was centrifuged at 13,000 g for 30 min to remove cell debris. The supernatant was pre-cleared by incubation with 15 µl protein G sepharose beads for 1 h. The pre-cleared lysates were then incubated with 2 µg rat anti mouse JAM mAb H2O2-106-7-4 106 (34) overnight, followed by 15 µl protein G sepharose beads for 2 h. Sepharose beads were washed five times in lysis buffer and bound proteins were solubilized by boiling for 5 min in SDS sample buffer. To detect co-precipitated proteins the eluted material was subjected to SDS-PAGE, and analyzed by western blot analysis with mAbs directed against AF-6 and ZO-1. Bound antibodies were visualized with HRP-conjugated donkey anti mouse IgG and goat anti rat IgG+IgM (Dianova, Hamburg, Germany), respectively, by ECL.

**Microinjection, fluorescence microscopy, and Laser Scanning Confocal Microscopy**

For microinjection experiments expression vectors were dissolved at 25 ng/µl in water, clarified by centrifugation (15 min, 14,000 rpm), then microinjected into the nucleus of cultured cells using a semi-automated microinjector (Eppendorf, Hamburg, Germany) and a micromanipulator (Luigs & Neumann, micromanipulation system LN SM1, Ratingen, Germany). After injection, cells were cultured for 24 – 48 h in a CO₂-buffered atmosphere, then analyzed by immunofluorescence microscopy. For immunofluorescence, injected cells were fixed with 4% paraformaldehyde in PBS. The fixed cells were permeabilized with 0.1% Triton X-100 in HBSS, washed with HBSS, and incubated with polyclonal or monoclonal antibodies against mAF-6, JAM, β-catenin, or ZO-1. The samples were washed with HBSS and incubated with either biotin-conjugated secondary antibodies and Cy3-conjugated streptavidin or with DTAF- or Cy5- or Cy3-conjugated secondary antibodies. After washing with HBSS the samples were mounted onto slides, embedded with fluorescent mounting medium (DAKO Corp., Carpinteria, CA) and visualized under a fluorescence microscope (Leica DM R) or analyzed by confocal microscopy (Leica TCS confocal imaging system, Leica, Heidelberg, Germany). To induce a polarized morphology cells were grown in some
cases on polycarbonate transwell filters (Costar, Bodenheim, Germany) for 4 days prior to fixation and analysis by confocal microscopy.

Results

Identification of AF-6/afadin as a binding partner for JAM

To identify molecules interacting with the cytoplasmic tail of JAM a LexA-based two-hybrid screen was performed with a size-selected murine embryonic cDNA library (36,37). In two independent screens, two overlapping clones of 471 bp and 352 bp were isolated. Sequencing revealed an open reading frame with 95% identity to the amino acid sequence of human AF-6 and 97% identity to rat AF-6 (afadin), respectively (Fig.1A). Both clones encompassed the PDZ domain of AF-6 suggesting a PDZ-domain dependent interaction between JAM and AF-6. To further analyze this interaction we cloned full length mouse AF-6 from bEnd.3 cells by RT-PCR using primers based on the rat sequence. Three isoforms of murine AF-6 were cloned corresponding to the splicing variants of rat and human AF-6 (24,39) (Fig. 1B).

PDZ domains recognize short COOH-terminal binding motifs of their ligands with the COOH-terminal residue being hydrophobic and bearing a free carboxylate group (11,40). Removal of the COOH-terminal residues abolishes the binding of the ligand to the hydrophobic cavity formed by the β-strand and the α-helix of the PDZ domain (41). To analyze the PDZ domain dependence of the JAM interaction with AF-6, COOH-terminal truncation mutants of the JAM cytoplasmic domain were tested for their binding capacities with the AF-6 PDZ domain (aa 931-1090) in yeast. Yeast strains expressing the intact cytoplasmic tail of JAM and the AF-6 PDZ domain grew on selective medium in the presence of 3-aminotriazole at concentrations up to 20 mM indicating a strong interaction between JAM and AF-6 (Table 1). Removing the COOH-terminal Val residue (JAM/cytΔ1) allowed growth on selective medium only in the absence of 3-AT and removing additional aa residues (JAM/cytΔ3, JAM/cytΔ9) completely abolished growth. These results indicated that the interaction between JAM and AF-6 is characteristic for PDZ domain containing proteins and their ligands. This was further substantiated by analyzing the minimal requirements for the interaction between JAM and AF-6. The last 11 aa residues (JAM/cyt11) as well as the last 6 aa residues (JAM/cyt6) of JAM fused to LexA were sufficient to bind to the AF-6 PDZ domain, whereas the last 4 aa residues (JAM/cyt4) bound only poorly and only in the absence of 3-AT (Table 1). The same results were obtained with X-gal assays (data not shown).
Together with the observation that the COOH-terminal aa residues of JAM match the binding motif predicted for group II PDZ domains (11) these results indicate that JAM binds to AF-6 in a PDZ domain-dependent manner.

**Specificity of the interaction of JAM with the PDZ domain of AF-6**

Both JAM and AF-6 have been described to be located at tight junctions. Since additional PDZ domain-containing proteins such as ZO-1, ZO-2, and ZO-3 are located in tight junctions we analyzed the specificity of the interaction of JAM with the PDZ domain of AF-6 by GST pulldown assays. To this end we constructed GST-JAM fusion proteins containing either the full length cytoplasmic tail of JAM or truncated versions of it. GST-JAM fusion proteins bound to glutathione-Sepharose beads were incubated with in vitro translated and 35S-methionine labelled protein fragments comprising the PDZ domain of AF-6, the single PDZ domains 1, 2, or 3 of ZO-1, or the PDZ domain of CASK. Under these conditions, only the PDZ domain of AF-6 bound to GST-JAM while none of the other four PDZ domains bound with sufficient strength to allow affinity isolation (Fig. 2). Binding to AF-6 was reduced if the COOH-terminal valine residue of JAM was missing and was abolished when the COOH terminus was further truncated confirming the PDZ domain-dependence of the JAM/AF-6 interaction.

**JAM interacts with fragments of ZO-1 containing PDZ-domains 2 and 3.**

As outlined above JAM does not bind to any of the three individual PDZ domains of ZO-1 in vitro. However, when GST-JAM fusion proteins were incubated with in vitro translated, recombinant forms of ZO-1 comprising more than one PDZ domain, strong interactions were observed. ZO-1 fusion proteins containing all three PDZ domains (PDZ(1-3)) as well as a fusion protein containing PDZ domains 2 and 3 and the interconnecting segment (PDZ(2-3)) could be affinity isolated with GST-JAM (Fig. 3). In contrast, no binding was observed with a recombinant form of ZO-1 containing PDZ domains 1 and 2 (PDZ(1-2)) (Fig. 3). Binding to PDZ(1-3) or PDZ(2-3) was abolished if the critical COOH-terminal amino acids of JAM were removed. Since no binding had been observed to any of the single PDZ domains of ZO-1 (Fig. 2) we reasoned that regions flanking PDZ domain 2 and 3 were necessary for the interaction with JAM. To address this possibility we analyzed the binding of constructs containing the connecting sequence segment between PDZ domains 2 and 3 in addition to either PDZ domains 1 and 2 (PDZ(1-2+)), the single PDZ domain 2 (PDZ(2+)) or the single PDZ domain 3 (PDZ(+3)). Surprisingly, none of these recombinant forms of ZO-1 bound to
JAM (Fig. 3). We conclude that JAM-binding to ZO-1 is not dependent on PDZ domain 1, but requires the two PDZ domains 2 and 3 as well as the connecting sequence segment between these two domains.

**Interaction of GST-JAM with native, endogeneous AF-6 from epithelial and endothelial cells**

Since JAM is expressed in epithelial and endothelial cells (9) we analyzed whether mouse AF-6 endogeneously expressed in epithelial CMT cells and endothelial bEnd.3 cells could be affinity isolated with a GST-JAM fusion protein. Three major protein bands that reacted in immunoblots with the monoclonal AF-6 antibodies were precipitated from detergent extracts of both cell lines with the GST-JAM affinity probe (Fig. 4). These proteins were not detected in affinity isolation experiments using truncated GST-JAM proteins lacking one, three or nine of the COOH-terminal amino acids of JAM (Fig. 4). The precipitated, anti-AF-6 reactive proteins most likely represent different isoforms of AF-6, possibly resulting from alternative splicing as described for rat and human AF-6 (16,24,39). In addition to the two bands marked with arrowheads in Fig. 4, a higher molecular weight band running at about 210 kD was strongly detected in CMT cells and only barely visible in bEnd.3 cells. This band was strongly detected in lysates of bEnd.3 cells treated with the proteasome inhibitor ALLN, suggesting that it represents a ubiquitinated form of AF-6 (not shown). This is in agreement with similar results published for human AF-6 (42).

**AF-6 and ZO-1 can be co-precipitated with JAM from endothelial cell extracts**

To test whether JAM associates with AF-6 and ZO-1 in vivo we performed co-immunoprecipitation experiments. bEnd.3 endothelial cells were lysed under various conditions and subjected to immunoprecipitation with the JAM mAb H2O2-106-7-4 (34). The precipitated material was analyzed in immunoblots for the presence of AF-6 and ZO-1. The JAM mAb co-precipitated AF-6 from cells lysed in lysis buffer (Fig. 5, lane 1) but not from cells lysed with lysis buffer containing 0.02% SDS (lane 2) or lysis buffer containing 0.02% SDS and 0.5% deoxycholate (lane 3) indicating a detergent-sensitive interaction between JAM and AF-6. The JAM mAb also co-precipitated ZO-1 and, similar to AF-6, the association between JAM and ZO-1 was sensitive to detergent treatment. Longer exposure of the film revealed that the presence of 0.02% SDS in the lysis buffer reduced but did not abolish the association between JAM and ZO-1 whereas the presence of 0.5% deoxycholate in
addition to 0.02% SDS disrupted the interaction completely (data not shown). Together, these findings indicate that JAM associates with AF-6 and ZO-1 in vivo.

**JAM is colocated with AF-6 in endothelial and epithelial cell contacts**

We next examined the localization of JAM and AF-6 in bEnd.3 endothelial cells and in CMT epithelial cells by laser-scanning confocal microscopy. The cells were double stained with antibodies against mouse AF-6 and JAM and images were generated from optical sections. In post-confluent CMT epithelial cells, immunoreactivity of AF-6 was associated with a strong cytoplasmic staining, but was not detectable at sites of cell contact (not shown). In contrast, in grow-out zones and areas where cells had just recently reached confluency, AF-6 was specifically localized at sites of cell-cell contacts with a belt-like staining pattern in addition to nuclear stain as reported previously (24) (Fig. 6A). No immunoreactivity was observed in areas where cells did not contact neighbouring cells (not shown). A similar result was obtained for bEnd.3 cells with the exception that AF-6 was always detected at sites of cell-cell contact independent of the state of confluency of the cells. Merging of the pictures indicated that the staining of JAM at cell-cell contacts overlapped with that of AF-6 in both cell lines (Fig. 6A). Our results are in agreement with previously published findings demonstrating that both molecules colocalize with tight junction components such as cingulin, occludin, or ZO-1 in epithelial cells (9,16). It was, however, also reported that AF-6 can be found at both tight and adherens junctions (31,43) and even exclusively at adherens junctions (24). In order to determine the subcellular localization of AF-6 in more detail, we performed Z-scan analyses of the cells as shown at the bottom of Fig. 6A. Unfortunately, the cells were not sufficiently polarized and JAM as well as AF-6 was found almost all along the lateral surface of the flat cells. Polarization was much improved, when CMT cells were grown to high confluency on polycarbonate filters (pore size 5 µm). As shown in Fig. 6B, ZO-1 as well as JAM colocalized at the apical top of the lateral surface of the cells, while β-catenin was found all along the lateral surface. However, at this high confluency, AF-6 was not detectable at cell contacts (not shown, see also Fig. 8C). Recently confluent CMT cells as well as fully confluent bEnd.3 cells even on filters were not sufficiently polar for this analysis (not shown). Thus, these cells do not allow to decide whether AF-6 is predominantly localized in tight junctions or adherens junctions or both.
JAM can be recruited to cell junctions by homophilic JAM interactions

We analyzed whether the COOH-terminal 9 amino acids of JAM are necessary for its localization to sites of cell-cell contact. To this end CHO cells were microinjected with cDNA constructs coding either for full length JAM or for truncated JAM lacking the last 9 amino acids at its COOH-terminus (JAM/Δ9). When CHO cells were microinjected with full length JAM, staining of JAM was observed only in areas of cell contacts where neighbouring cells were also microinjected, but not at cell borders to non-injected cells (Fig. 7A). Surprisingly, truncated JAM also appeared at cell contact sites between cells that both expressed truncated JAM (Fig. 7B). Staining for endogeneous AF-6 revealed that AF-6 was diffusely localized in the cytoplasm around the nuclei, more weakly within the nuclei, but not at cell-cell contact sites. We conclude that JAM can be recruited to and maintained at cell-cell contact sites in an AF-6- and PDZ-domain-independent manner. This is most likely due to the interaction in trans mediated through homophilic interactions between JAM molecules expressed by neighbouring cells.

JAM recruitment to cell junctions in grow-out zones of Caco-2 cells correlates with AF-6, but not with ZO-1 staining

We next performed microinjection experiments with human Caco-2 cells. These cells express endogeneous JAM, (detected with mAb BV16, not shown), that is not recognized by the mAb BV11 directed against mouse JAM (not shown). As described above for CMT cells, staining with the polyclonal AF-6 antibody resulted in diffuse cytoplasmic staining pattern in post-confluent cells (see Fig. 8C). In grow-out zones and recently confluent cells, AF-6 was localized to the nucleus as well as in areas of cell-cell contacts with a circumferential rim staining typical for junctional components such as ZO-1 (Fig. 8A and B). When full length JAM was microinjected into recently confluent Caco-2 cells, JAM co-distributed with AF-6 and was recruited to cell-cell contact sites irrespective of JAM expression in the neighbouring cell (Fig. 8A). In contrast, when truncated JAM (JAM/cΔ9) was microinjected into recently confluent Caco-2 cells a clustering of JAM was only observed at cell contacts between injected cells, despite the fact that the injected cells showed a typical circumferential rim staining for AF-6 (Fig. 8B). These findings suggest that JAM can be recruited by AF-6 and point to a role of AF-6 for the recruitment of JAM during the generation of cell contacts. This was further supported by experiments where full length JAM was injected in postconfluent Caco-2 cells that showed a typical circumferential rim staining for ZO-1, but not for AF-6 (Fig. 8C). Injected JAM did not colocalize with ZO-1 but only clustered at contact zones
between injected cells. This indicates that the presence of ZO-1 is not sufficient to recruit JAM to cell-cell contact sites. Taken together, these findings suggest that JAM can be recruited to sites of cell-cell contacts by two mechanisms: a) by homophilic interaction of two JAM molecules on neighbouring cells, and b) by a PDZ domain-dependent interaction possibly requiring junction-associated AF-6 in the same cell. They also suggest that endogeneous ZO-1 in post-confluent cells is not sufficient to recruit JAM.

Discussion

Searching for cytoplasmic binding partners of the junctional adhesion molecule JAM, we have identified the PDZ domain protein AF-6 by a yeast two-hybrid screen. The interaction between JAM and AF-6 requires an intact COOH-terminus of JAM which represents a classical type II PDZ domain binding motif (11) indicating a PDZ domain-dependent interaction. JAM was also found to bind to ZO-1, and our results suggest that the interaction between JAM and ZO-1 involves PDZ domains 2 and/or 3 but not PDZ domain 1 of ZO-1. Each of the two PDZ domain proteins were demonstrated to bind to JAM in vivo in endothelial cells. We could show that the PDZ domain binding COOH-terminus of JAM can influence the localization of JAM to sites of cell-cell contact. Injecting an expression vector coding for full length mouse JAM into human epithelial Caco-2 cells resulted in codistribution of JAM with AF-6 at sites of cell contacts even if the adjacent cell lacked expression of mouse JAM. Truncated mouse JAM lacking the PDZ domain binding did not co-distribute with endogeneous AF-6 but was always restricted to sites between cells that both expressed mouse JAM. Our findings suggest that AF-6 and ZO-1 represent cytoplasmic binding partners of JAM that could be involved in the recruitment of JAM to intercellular junctions.

Reports on the subcellular distribution of JAM and AF-6 have localized JAM at tight junctions while AF-6 has been described as an adherens junction- as well as a tight junction-associated component (16,24). This dual localization of AF-6 has been verified in more recent studies. In wild-type ectoderm of mouse embryos, AF-6 colocalizes with both ZO-1 and E-cadherin (31). In addition, the subcellular localization seems to depend on the cell type used. For example, Sakisaka et al. describe that in cultured MDCK cells tight junctions and adherens junctions are not well separated from each other and l-afadin (the long isoform of AF-6) and ZO-1 were observed at both adherens junctions and tight junctions (43).
Unfortunately, a clear assignment of AF-6 to junctions was not possible in the cells used here. Although well polarized CMT cells (Fig. 6B) or Caco-2 cells (not shown) on filters allowed to co-localize ZO-1 and JAM at the apical border of lateral cell contacts, AF-6 was not detectable at cell contacts in such post-confluent cell cultures (Fig. 8C). The endothelial cells, on the other hand, did not sufficiently polarize to allow identification of well defined subareas of lateral cell contacts. While the interaction of JAM with ZO-1 is likely to occur at tight junctions, the JAM/AF-6 interaction could either occur at tight or at intermediate junctions, depending on cell type, growth conditions, and cell density. There is evidence that proteins of tight junctions do associate with proteins not being present in tight junctions. For example, ZO-1 associates with tight junction components such as occludin, ZO-2, and ZO-3 in epithelial cells (44), but with adherens junction components such as α-catenin in fibroblasts (45). During the formation of epithelial cell contacts after wounding ZO-1 co-localizes with E-cadherin/β-catenin at the initial stage of junction formation, but with occludin in established junctions (46). It is conceivable that JAM associates with AF-6 in a transient way during the formation of intercellular junctions. Whether AF-6 and JAM still associate in fully polarized epithelium in tissues still needs to be tested.

AF-6 and ZO-1 are both directly linked to the actin cytoskeleton (24,47). This allows JAM to be anchored to the actin filament system via its binding to AF-6 and ZO-1. Of the three integral membrane proteins described at tight junctions, occludin (5), claudins (6,8) and JAM (9), occludin has been reported to be linked to the actin cytoskeleton via ZO-1 (47) and claudins were recently described to associate with ZO-1, ZO-2, and ZO-3 by which they might be linked to actin filaments (22). Besides anchoring to the cytoskeleton, binding to PDZ domain proteins would enable junction proteins to interact with regulatory molecules. AF-6 is a target for the small GTPase ras (25,48-50). AF-6 can directly bind to ZO-1 (16,26) and the ras associating domain in AF-6 is identical to the binding site for ZO-1 (16). Activated ras has been described to inhibit the interaction between AF-6 and ZO-1 and overexpression of ras was shown to perturb cell-cell contacts and decrease the amount of both AF-6 and ZO-1 at the cell surface (16). Together with these findings our results suggest that the ras-induced disruption of cell contacts could be mediated in part through the JAM/AF-6 or JAM/ZO-1 complex. Although in post-confluent Caco-2 and CMT cells AF-6 is not available at junctions for such regulatory mechanisms, it is present at epithelial and endothelial junctions in tissue (16,24).
Depending on the cell type and the state of confluency that was analyzed we have found that microinjected JAM can be recruited to sites of cell contact by two mechanisms: the first is independent and the second is dependent on the PDZ domain targeting motif of JAM. The PDZ domain targeting motif-independent pathway was observed with full length as well as with truncated JAM in CHO cells that lack expression of junctional AF-6 and in post-confluent Caco-2 cells that have lost AF-6 at cell contacts. This mechanism most likely reflects the trans interaction of JAM. Although not the purpose of these studies, our results imply that human JAM endogenously expressed in Caco-2 cells is not able to recruit exogenously expressed mouse JAM to sites of cell contact. This might indicate that mouse and human JAM do not interact in trans. Alternatively, it is possible that the endogenous expression level of human JAM was not sufficient to match exogenously expressed mouse JAM. More importantly, our results allow to conclude that JAM can be targeted to the plasma membrane independent of interactions with PDZ-domain proteins and most likely can be retained at the cell surface due to trans interaction between JAM molecules. Another membrane protein, the γ-aminobutyric acid (GABA) transporter that is recruited to the plasma membrane via the binding to the PDZ-domain protein LIN-7 is also able to reach the cell surface independent of PDZ domain interactions. This protein, however, is not able to form homophilic interactions and requires PDZ domain interactions for its retention at the cell surface (51).

The second, PDZ domain targeting motif-dependent mechanism of JAM-recruitment to cell contacts was only observed in recently confluent, but not in post-confluent Caco-2 cells. Only recently confluent epithelial cells expressed AF-6 at sites of cell contact. Full length JAM codistributed with AF-6 in recently confluent cells independent of JAM expression by adjacent cells. JAM recruitment to such sites seemed to be dependent on the presence of AF-6, since densely confluent cells that lost AF-6 expression at the cell borders also lost expression of JAM at those sites. Interestingly, at the same sites ZO-1 was present, suggesting that ZO-1 is not sufficient to recruit JAM to, or retain it at cell contacts if the adjacent cell does not express JAM.

We cannot exclude the possibility that the presence of JAM at the cell membrane influences the distribution of endogeneous AF-6. In fact, in CHO cells we observed that the co-injection of AF-6 and JAM cDNAs lead to the recruitment of AF-6 to sites at cell contacts where JAM molecules were clustering as a result of trans-interactions, excluding AF-6 from cell contacts devoid of JAM expression (unpublished observation). However, since CHO cells do not
contain organized tight and adherens junctions our findings do not allow to extrapolate these results to epithelial or endothelial cells.

In two epithelial cell lines, CMT and Caco-2 cells, junctional localization of AF-6 was only found in areas of recently confluent cells or grow out zones, but not in densely confluent cell layers. This suggests that in these cells AF-6 may have a dynamic regulatory function in generating cell polarity and junction formation rather than a structural function in maintaining cellular polarity. This hypothesis is supported by the phenotype of AF-6-deficient mice developed independently by two different laboratories (31,32). Polarity of the embryonic ectoderm was strongly disturbed and the mice died at day 10.5 p.c. The analysis of AF-6 deficient mice revealed that AF-6 expression is restricted to a subset of epithelia during early embryogenesis with strong AF-6 expression only in areas where dynamic tissue rearrangements are ready to occur such as the primitive streak regions, the neural groove, and somites (31,32). This indicates that AF-6 is dynamically regulated and suggests a role for AF-6 in the formation, rather than in the maintenance of polarized cell layers. In the light of these results, it is interesting that we observed a cell type-specific difference in AF-6 localization. In contrast to epithelial cells, AF-6 was constitutively present in cell-cell contacts of endothelial cells independent of their state of confluency, reflecting differences in the stability of junctions of the two cell types. In endothelial cells, tight and/or adherens junctions are expected to be subject to dynamic regulation in order to allow the migration of circulating leukocytes from the blood to subendothelial compartments. In the light of this special function of endothelial cells one might speculate that AF-6 could be involved in the reversible process of opening and closing interendothelial cell junctions. In this context it is intriguing that antibodies against JAM can block monocyte migration through endothelial cell layers in vitro and in vivo (9). It may be worthwhile to analyze whether the binding of JAM to AF-6 and the binding of the small GTPase ras to AF-6 are involved in this process.

We were surprised to find that no single PDZ domain of ZO-1 was able to bind to JAM while recombinant ZO-1 with all three PDZ domains as well as native ZO-1 bound efficiently. Binding required the presence of PDZ domains 2 and 3 and the interconnecting flanking region, but was not dependent on the first PDZ domain. Even PDZ domain 2 or 3 alone, combined with the flanking region was not sufficient for binding. Additional experiments will be necessary to identify which of the two PDZ domains directly binds the COOH terminus of JAM. Our results demonstrate that single PDZ domains are not necessarily sufficient for
ligand binding. In agreement with this also full length AF-6 interacts more efficiently with JAM than the PDZ domain of AF-6 alone (not shown).

The interaction of JAM with the two junction-associated PDZ domain proteins AF-6 and ZO-1 could be relevant for the recruitment of JAM to tight junctions, for its association with the actin cytoskeleton and/or for clustering of JAM with other proteins that form and regulate intercellular junctions. Since AF-6 and ZO-1 bind to the same site of JAM these complexes are mutually exclusive and each complex could preferentially be involved in different functions. For example, ZO-1 could be more important for the retention of JAM at tight junctions and cytoskeletal anchoring, while AF-6 could be more important for recruiting JAM to junctions in the process of junction formation or for clustering JAM with signalling molecules. On the other hand, binding of ZO-1 to AF-6 allows the formation of higher order complexes and a network of sub-membrane proteins linking JAM molecules with each other. The fact that ras can compete for AF-6 binding to ZO-1 allows for a regulatory mechanism that could control the formation of such networks and might influence the stability of cell contacts. PDZ domain proteins are organizers of protein complexes (2) at the plasma membrane and AF-6 seems to play a crucial role in the formation of junctions and the establishment of cell polarity (31,32). The interaction of JAM with AF-6 might be transient in some cells, as is suggested by the transient expression of AF-6 that we observed at cell contacts in two cultured epithelial cell lines. AF-6 might be more important for the formation of junctions than for the maintenance of stable junctional complexes. The permanent expression of AF-6 at cell contacts between cultured endothelial cells independent of their state of confluence might reflect the plasticity of endothelial junctions that are able to open and close to allow leukocytes to transmigrate. Future studies are necessary to elucidate whether AF-6 is involved in this process.
Acknowledgements:
We thank Dr. Jürgen Behrens, Max Delbrück Center for Molecular Medicine, Berlin, Germany, for providing reagents and valuable advice for yeast two-hybrid experiments. We also thank Dr. Elisabetta Dejana and Dr. Gianfranco Bazzoni, Istituto Mario Negri, Milan, Italy, for providing JAM monoclonal antibodies, for helpful comments on the co-immunoprecipitation experiments and for critically reading the manuscript. We wish to thank Dr. M. Aurrand-Lions, Geneva, Switzerland, and Dr. Philippe Naquet, INSERM-CNRS, Marseille, France, for providing JAM monoclonal antibodies, and Dr. Volker Gerke, Institute of Medical Biochemistry, Muenster, Germany, for critically reading the manuscript.
References
1. Mitic, L. L., and Anderson, J. M. (1998) Annu Rev Physiol 60, 121-42
2. Fanning, A. S., and Anderson, J. M. (1999) J Clin Invest 103(6), 767-72
3. Yap, A. S., Briher, W. M., and Gumbiner, B. M. (1997) Annu Rev Cell Dev Biol 13, 119-46
4. Gumbiner, B. M. (1996) Cell 84(3), 345-57
5. Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., and Tsukita, S. (1993) J Cell Biol 123(6 Pt 2), 1777-88
6. Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., and Tsukita, S. (1998) J Cell Biol 141(7), 1539-50
7. Furuse, M., Sasaki, H., Fujimoto, K., and Tsukita, S. (1998) J Cell Biol 143(2), 391-401
8. Tsukita, S., and Furuse, M. (1999) Trends Cell Biol 9(7), 268-73
9. Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D., and Dejana, E. (1998) J Cell Biol 142(1), 117-27
10. Ponting, C. P., Phillips, C., Davies, K. E., and Blake, D. J. (1997) Bioessays 19(6), 469-79
11. Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chisti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) Science 275, 73-77
12. Pawson, T., and Scott, J. D. (1997) Science 278(5346), 2075-80
13. Stevenson, B. R., Siliciano, J. D., Mooseker, M. S., and Goodenough, D. A. (1986) J Cell Biol 103(3), 755-66
14. Gumbiner, B., Lowenkopf, T., and Apatira, D. (1991) Proc Natl Acad Sci USA 88(8), 3460-4
15. Haskins, J., Gu, L., Wittchen, E. S., Hibbard, J., and Stevenson, B. R. (1998) J Cell Biol 141(1), 199-208
16. Yamamoto, T., Harada, N., Kano, K., Taya, S., Canaani, E., Matsuura, Y., Mizoguchi, A., Ide, C., and Kaibuchi, K. (1997) J Cell Biol 139(3), 785-95
17. Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., and Tsukita, S. (1994) J Cell Biol 127(6 Pt 1), 1617-26
18. Wong, V., and Gumbiner, B. M. (1997) J Cell Biol 136(2), 399-409
19. Van Itallie, C. M., and Anderson, J. M. (1997) J Cell Sci 110(Pt 9), 1113-21
20. Mitic, L. L., Schneeberger, E. E., Fanning, A. S., and Anderson, J. M. (1999) *J Cell Biol* **146**(3), 683-93
21. Saitou, M., Fujimoto, K., Doi, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., Noda, T., and Tsukita, S. (1998) *J Cell Biol* **141**(2), 397-408
22. Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M., and Tsukita, S. (1999) *J Cell Biol* **147**(6), 1351-63
23. Prasad, R., Gu, Y., Alder, H., Nakamura, T., Canaani, O., Saito, H., Huebner, K., Gale, R. P., Nowell, P. C., Kuriyama, K., and et al. (1993) *Cancer Res* **53**(23), 5624-8
24. Mandai, K., Nakanishi, H., Satoh, A., Obaishi, H., Wada, M., Nishioka, H., Itoh, M., Mizoguchi, A., Aoki, T., Fujimoto, T., Matsuda, Y., Tsukita, S., and Takai, Y. (1997) *J Cell Biol* **139**(2), 517-28
25. Kuriyama, M., Harada, N., Kuroda, S., Yamamoto, T., Nakafuku, M., Iwamatsu, A., Yamamoto, D., Prasad, R., Croce, C., Canaani, E., and Kaibuchi, K. (1996) *J Biol Chem* **271**(2), 607-10
26. Yamamoto, T., Harada, N., Kawano, Y., Taya, S., and Kaibuchi, K. (1999) *Biochem Biophys Res Commun* **259**(1), 103-7
27. Mandai, K., Nakanishi, H., Satoh, A., Takahashi, K., Satoh, K., Nishioka, H., Mizoguchi, A., and Takai, Y. (1999) *J Cell Biol* **144**(5), 1001-17
28. Buchert, M., Schneider, S., Meskenaite, V., Adams, M. T., Canaani, E., Baechi, T., Moelling, K., and Hovens, C. M. (1999) *J Cell Biol* **144**(2), 361-71
29. Hock, B., Bohme, B., Karn, T., Yamamoto, T., Kaibuchi, K., Holtrich, U., Holland, S., Pawson, T., Rubsam-Waigmann, H., and Strebhardt, K. (1998) *Proc Natl Acad Sci U S A* **95**(17), 9779-84
30. Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A., and Takai, Y. (1999) *J Cell Biol* **145**(3), 539-49
31. Zhadanov, A. B., Provance, D. W., Speer, C. A., Coffin, J. D., Goss, D., Blixt, J. A., Reichert, C. M., and Mercer, J. A. (1999) *Curr Biol* **9**(16), 880-888
32. Ikeda, W., Nakanishi, H., Miyoshi, J., Mandai, K., Ishizaki, H., Tanaka, M., Togawa, A., Takahashi, K., Nishioka, H., Yoshida, H., Mizoguchi, A., Nishikawa, S., and Takai, Y. (1999) *J Cell Biol* **146**(5), 1117-1132
33. Williams, R. L., Risau, W., Zerwes, H. G., Drexler, H., Aguzzi, A., and Wagner, E. F. (1989) *Cell* **57**(6), 1053-63
34. Malergue, F., Galland, F., Martin, F., Mansuelle, P., Aurrand-Lions, M., and Naquet, P. (1998) *Mol Immunol* **35**(17), 1111-9
35. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, 2 Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
36. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) *Cell* **74**(1), 205-14
37. Hollenberg, S. M., Sternglanz, R., Cheng, P. F., and Weintraub, H. (1995) *Mol Cell Biol* **15**(7), 3813-22
38. Schiestl, R. H., and Gietz, R. D. (1989) *Curr Genet* **16**(5-6), 339-46
39. Saito, S., Matsushima, M., Shirahama, S., Minaguchi, T., Kanamori, Y., Minami, M., and Nakamura, Y. (1998) *DNA Res* **5**(2), 115-20
40. Saris, J., and Heldin, C. H. (1996) *Trends Biochem Sci* **21**(12), 455-8
41. Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996) *Cell* **85**(7), 1067-76
42. Taya, S., Yamamoto, T., Kano, K., Kawano, Y., Iwamatsu, A., Tsuchiya, T., Tanaka, K., Kanai-Azuma, M., Wood, S. A., Mattick, J. S., and Kaibuchi, K. (1998) *J Cell Biol* **142**(4), 1053-62
43. Sakisaka, T., Nakanishi, H., Takahashi, K., Mandai, K., Miyahara, M., Satoh, A., Takaishi, K., and Takai, Y. (1999) *Oncogene* **18**(8), 1609-17
44. Wittchen, E. S., Haskins, J., and Stevenson, B. R. (1999) *J Biol Chem* **274**(49), 35179-85
45. Itoh, M., Nagafuchi, A., Moroi, S., and Tsukita, S. (1997) *J Cell Biol* **138**(1), 181-92
46. Ando-Akatsuka, Y., Yonemura, S., Itoh, M., Furuse, M., and Tsukita, S. (1999) *J Cell Physiol* **179**(2), 115-25
47. Fanning, A. S., Jameson, B. J., Jesaitis, L. A., and Anderson, J. M. (1998) *J Biol Chem* **273**(45), 29745-53
48. Watari, Y., Kariya, K., Shibatohge, M., Liao, Y., Hu, C. D., Goshima, M., Tamada, M., Kikuchi, A., and Kataoka, T. (1998) *Gene* **224**(1-2), 53-8
49. Linnemann, T., Geyer, M., Jaitner, B. K., Block, C., Kalbitzer, H. R., Wittinghofer, A., and Herrmann, C. (1999) *J Biol Chem* **274**(19), 13556-62
50. Quilliam, L. A., Castro, A. F., Rogers-Graham, K. S., Martin, C. B., Der, C. J., and Bi, C. (1999) *J Biol Chem* **274**(34), 23850-7
51. Perego, C., Vanoni, C., Villa, A., Longhi, R., Kaech, S. M., Frohli, E., Hajnal, A., Kim, S. K., and Pietrini, G. (1999) *Embo J* **18**(9), 2384-93
Legends

Figure 1. Schematic domain organization of human, rat, and mouse AF-6/afadin. A, Three splicing variants of human AF-6 (type I, II and III) and two splicing variants of rat AF-6 (s: short, l: long afadin), as they have been published, are shown with the ras binding domain (ras BD), the myosin V-like domain (myosin VD), and the PDZ domain (PDZ). Rat l-afadin contains a F-actin binding domain (F-actin BD). The two cDNA clones 128-24 and 155-61 (covering the sequences coding for amino acids 931-1090 or 981-1102, respectively, and comprising the PDZ domain of mouse AF-6) were identified as binding partners for JAM and isolated from a two-hybrid cDNA library. B, Three full length splicing variants of mouse AF-6 were cloned from bEnd.3 mouse endothelial cells. In analogy to the afadin nomenclature the short variants were named msAF-6(a) and msAF-6(b) (for mouse short AF-6 a and b) and the long variant containing the actin binding domain was called mlAF-6 (for mouse long AF-6).

Figure 2. Comparison of the binding of the PDZ-domains of AF-6, ZO-1 and CASK to JAM.

GST-JAM fusion proteins bound to glutathione-Sepharose beads were used to precipitate protein fragments synthesized in coupled in vitro transcription/translation reactions and containing either A, the PDZ-domain of AF-6 or one of the three PDZ-domains of ZO-1 (ZO1/1, ZO1/2, ZO1/3); or B, the PDZ-domain of CASK. The various GST-JAM fusion proteins contained either the full cytoplasmic tail of JAM (JAM/c) or truncation mutants lacking the carboxy-terminal aa residues V (JAM/cΔ1), FLV (JAM/cΔ3), or FKQTSSFLV (JAM/cΔ9), or contained only GST (GST/-) as indicated. 35S-methionine labeled PDZ domain-containing protein fragments were incubated with the immobilized GST fusion proteins, beads were extensively washed and bound proteins were eluted with SDS sample buffer, subjected to SDS-PAGE, and analyzed by fluorography. Only the PDZ domain of AF-6 bound to JAM and no binding was observed if the C-terminal three or nine amino acids of JAM were missing. Panel C, Equal aliquots of the PDZ-domain containing protein fragments synthesized in reticulocyte lysates, demonstrating that each fragment was synthesized with the same efficiency. Molecular mass markers (M) (in kD) are indicated.

Figure 3. JAM only interacts with protein fragments of ZO-1 containing PDZ-domains 2 and 3 as well as the connecting segment.
GST-JAM fusion proteins were used to precipitate fusion proteins containing various regions of the ZO-1 protein synthesized in vitro as described in Fig. 2. A, Schematic organization of the ZO-1 fusion proteins that were used in these experiments. Each construct was fused to a 10 amino acid myc-tag as indicated. The single PDZ-domain constructs that were used in the experiments shown in Fig. 2 are depicted at the bottom. B, The various GST-JAM fusion proteins used as affinity probes were the same as used in Fig. 2. The different ZO-1 fusion proteins that were analyzed are indicated at the left of each panel. The two lowest panels show equal aliquots of the various ZO-1 fusion protein synthesis reactions, demonstrating that each fragment was synthesized with similar efficiency. Molecular mass markers (M) (in kD) are indicated at the right.

**Figure 4. Interaction of GST-JAM with native endogeneous AF-6 from endothelial and epithelial cells.** GST pulldown experiments were performed with GST-JAM fusion proteins bound to glutathione-Sepharose beads. The fusion proteins contained either the full length cytoplasmic tail of JAM (JAM/c) or truncation mutants lacking the carboxy-terminal aa residues V (JAM/cΔ1), FLV (JAM/cΔ3), or FKQTSSFLV (JAM/cΔ9). Beads were incubated with detergent extracts of bEnd.3 endothelial cells or CMT epithelial cells, as indicated. After extensive washing, bound proteins were eluted from the beads with SDS sample buffer, subjected to SDS-PAGE, blotted onto nitrocellulose membranes and analyzed for binding to AF-6 mAbs. AF-6 was only detected in samples where the lysates had been incubated with a GST-fusion protein containing the full length cytoplasmic part of JAM. Arrowheads indicate two equivalent anti-AF-6 reactive bands detected in both cell lines. The higher molecular weight band at 210 kD most likely represents a ubiquitinylated form of AF-6.

**Figure 5. AF-6 and ZO-1 can be co-precipitated with native JAM from endothelial cells.** Cultured bEnd.3 cells were lysed with lysis buffer (lanes 1), lysis buffer containing 0.02% SDS (lanes 2), or lysis buffer containing 0.02% SDS, 0.5% deoxycholate (lanes 3). Lysates were subjected to immunoprecipitation using JAM mAb H2O2-106-7-4 106, and the precipitated material was analyzed by western blot analysis with mAbs directed against AF-6 (top panels) and ZO-1 (bottom panels). As a control for equal amounts of proteins extracted under the different lysis conditions aliquots of the lysates were directly subjected to western blot analysis without immunoprecipitation (right panels). Both AF-6 and ZO-1 were co-immunoprecipitated with JAM.
Figure 6. Confocal microscope analysis showing the distribution of JAM and AF-6 in cultured epithelial and endothelial cells. A, Cultured CMT epithelial cells and bEnd.3 endothelial cells (as indicated) were simultaneously stained with rabbit polyclonal antibodies against AF-6 and the rat monoclonal antibody BV11 against JAM. Primary antibodies were detected either with biotinylated donkey anti-rabbit IgG and Cy<sup>TM</sup>3-conjugated streptavidin or with DTAF-conjugated goat anti rat IgG antibodies, respectively. Optical sections were obtained using a laser scanning confocal microscope. JAM is shown in green, AF-6 is shown in red (as indicated). Images resulting from merging pictures are shown below. The yellow areas indicate the colocalization of JAM and AF-6. As reported earlier for different AF-6 antibodies (30) staining with our polyclonal rabbit AF-6 antibodies results in nucleoplasmic staining in addition to the staining at cell contacts. Z-scan analysis, performed at the region indicated by the arrowheads, is shown at the bottom of panel A. B, Z-scan images of CMT cells grown on polycarbonate filters, stained with the rat monoclonal antibody BV11 against JAM, a rabbit polyclonal antibody against ZO-1, and a mouse monoclonal antibody against β-catenin followed by biotinylated goat anti-rabbit IgG and Cy<sup>TM</sup>3-conjugated streptavidin, DTAF-conjugated goat anti rat IgG antibodies and Cy<sup>TM</sup>5-conjugated donkey anti mouse IgG, respectively. JAM is shown in green, ZO-1 in red, and β-catenin in blue. Bars, 5 µM.

Figure 7. JAM recruitment to cell junctions by homophilic JAM interaction. CHO cells were microinjected either with full length JAM cDNA A, or with a cDNA coding for a truncated form of JAM lacking 9 aa at the C-terminus (JAM/Δ9) B,. 24 h after injection cells were fixed and simultaneously stained with rat monoclonal antibody BV11 against JAM and rabbit polyclonal antibodies against AF-6, followed by biotinylated goat anti-rabbit IgG and Cy<sup>TM</sup>3-conjugated streptavidin and DTAF-conjugated goat anti rat IgG antibodies, respectively. JAM is shown in red, AF-6 is shown in green. No staining of AF-6 is seen at cell contacts. Both full length and truncated JAM localize at areas of cell-cell contact only between cells that both have been injected with a JAM cDNA. Arrowheads indicate localization of JAM. Bars, 5 µM.

Figure 8. PDZ domain-dependent recruitment of JAM to cell junctions in grow-out zones of Caco-2 cells correlates with AF-6 but not with ZO-1 staining. Recently confluent Caco-2 cells (A and B) and post-confluent Caco-2 cells (C) were microinjected with full length JAM cDNA (A and C) or a cDNA coding for truncated JAM lacking 9 amino acids at the C-terminus (JAM/Δ9) (B). 24 h after injection, cells were fixed
and simultaneously stained with rat monoclonal antibody BV11 against JAM, a rabbit polyclonal antibody against AF-6 and a mouse monoclonal antibody against ZO-1, followed by biotinylated goat anti-rat IgG and Cy\textsuperscript{TM}3-conjugated streptavidin, DTAF-conjugated goat anti rabbit IgG antibodies and Cy\textsuperscript{TM}5-conjugated donkey anti mouse IgG, respectively. JAM is shown in red, AF-6 is shown in green, and ZO-1 is shown in blue. Note that AF-6 was only seen in cell contact zones in areas of cells that became recently confluent, but not in areas of post-confluent cells, while ZO-1 was found at cell contacts in both areas. In recently confluent cells, full length JAM, but not truncated JAM, localized at cell contacts all around a cell independent of JAM expression in the neighbouring cell. In contrast, in post-confluent cells lacking AF-6 rim-staining full length JAM localized only at cell-cell contact sites where JAM was also expressed in the neighbouring cells, despite the circumferential rim staining of ZO-1. Arrowheads indicate colocalization of AF-6 and JAM, arrows indicate exclusive localization of AF-6 (B) or JAM (C). Bars, 15 \(\mu\text{M}\).
Table I. Interaction between various cytoplasmic domain constructs of JAM and the AF-6 PDZ domain (AA 946-1101) or full length CASK in yeast.

| 3-AT (mM) | -TL | -THULL |
|-----------|-----|--------|
| 0         | ++  | ++     |
| 1         | ++  | ++     |
| 2         | ++  | ++     |
| 3         | ++  | ++     |
| 4         | ++  | ++     |
| 5         | ++  | ++     |
| 7.5       | ++  | ++     |
| 10        | ++  | ++     |
| 15        | ++  | ++     |
| 20        | ++  | ++     |

Growth of yeast colonies was scored as – (no growth), +/- (colony diameter below 0.5 mm), + (colony diameter between 0.5 and 2 mm), ++ (colony diameter larger than 2 mm). The JAM constructs comprised the full length cytoplasmic tail of JAM (aa 261-300, JAM/cyt), or truncation mutants thereof lacking carboxy-terminal aa residues (aa 261-299, JAM/cytΔ1; aa 261-297, JAM/cytΔ3; aa 261-291, JAM/cytΔ9). Constructs containing only carboxy-terminal aa residues of JAM comprised aa residues 290-300 (JAM/cyt11), aa 295-300 (JAM/cyt6), and aa 296-300 (JAM/cyt4). Yeast strains co-expressing VE-cadherin and p120\textsuperscript{cas} or α-catenin and VE-cadherin were used as positive and negative controls, respectively.
Fig. 1

A

human AF-6

rat AF-6 / Afadin

ras BD  myosin VD  PDZ  F-actin BD

B

mouse AF-6

ras BD  myosin VD  PDZ  F-actin BD
Fig. 3

A

ZO-1

PDZ PDZ PDZ SH3 GUK

myc

PDZ (1-3)
PDZ (1-2)
PDZ (2-3)
PDZ (1-2+)
PDZ (2+)
PDZ (+3)
PDZ (1)
PDZ (2)
PDZ (3)

B

JAN/C JAN/CAS JAN/CAS9

PDZ1-3

PDZ1-2

PDZ2-3

JAN/C JAN/CAS JAN/CAS9

PDZ2+

PDZ+3

PDZ1-2+

PDZ1-3 PDZ1-2 PDZ2-3

PDZ2+ PDZ+3 PDZ1-2+
Fig. 4

JAM/c   JAM/cΔ1   JAM/cΔ3   JAM/cΔ9

bEnd.3

CMT
Fig. 5

| IP Blot | JAM | AF-6 |
|--------|-----|------|
|        |     |      |

| IP Blot | ZO-1 | ZO-1 |
|--------|------|------|
|        |      |      |
Fig. 6 A

CMT     bEnd.3

JAM

AF-6

merge

z-scan
Fig. 6 B

- JAM
- ZO-1
- β-Catenin
Fig. 7

A
AF-6  JAM

B
AF-6  JAM
Fig. 8

A

AF-6   JAM

B

AF-6   JAM

C

AF-6   JAM   ZO-1
Junctional Adhesion Molecule (JAM) interacts with the PDZ domain containing proteins AF-6 and ZO-1
Klaus Ebnet, Christian U Schulz, Maria-Katharina Meyer zu Brickwedde, Gunther G Pendl and Dietmar Vestweber

J. Biol. Chem. published online June 15, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002363200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts