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Interaction of Junctional Adhesion Molecule with the Tight Junction Components ZO-1, Cingulin, and Occludin*

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Junctional adhesion molecule (JAM) is an integral membrane protein that has been reported to colocalize with the tight junction molecules occludin, ZO-1, and cingulin. However, evidence for the association of JAM with these molecules is missing. Transfection of Chinese hamster ovary cells with JAM (either alone or in combination with occludin) resulted in enhanced junctional localization of both endogenous ZO-1 and cotransfected occludin. Additionally, JAM was coprecipitated with ZO-1 in the detergent-insoluble fraction of Caco-2 epithelial cells. A putative PDZ-binding motif at the cytoplasmic carboxyl terminus of JAM was required for mediating the interaction of JAM with ZO-1, as assessed by *in vitro* binding and coprecipitation experiments. JAM was also coprecipitated with cingulin, another cytoplasmic component of tight junctions, and this association required the amino-terminal globular head of cingulin. Taken together, these data indicate that JAM is a component of the multiprotein complex of tight junctions, which may facilitate junction assembly.

Together with adherens junctions, tight junctions (TJ)[1] form apical junctional complexes in epithelial and endothelial cells (1–5), and play a central role in the control of paracellular permeability (6) and maintenance of cell polarity (7). TJ comprise transmembrane components, such as occludin (8) and claudins (9, 10), as well as cytoplasmic molecules, such as ZO-1 (11), ZO-2 (12), ZO-3 (13), cingulin (14), 7H6 (15), rab3B (16), rab13 (17), symplekin (18), and AF-6 (19). Interactions between the transmembrane and cytoplasmic molecules (together with the cytoskeleton) are likely to modulate the "barrier" and "fence" functions of TJ.

Conceivably, cytoplasmic molecules might bind the transmembrane proteins and target them to junctional areas, thus accounting for regulated formation and maintenance of TJ. In particular, ZO-1 might organize occludin at junctional sites, since transfected occludin usually colocalizes at cell-cell contacts with endogenous ZO-1, while remaining diffusely expressed at the cell surface in fibroblasts that do not target ZO-1 to the junctions (20). Conversely, neither junctional displacement (21) nor targeted gene disruption (22) of occludin affects the junctional localization of ZO-1. However, the upstream events that determine or enable proper targeting of these molecules are not completely understood. Both cytosolic signaling mediators and membrane docking proteins are likely involved in the assembly and sealing of TJ (23).

Analysis of the *de novo* assembly of TJ in epithelial cells using the "calcium-switch" assay indicates that E-cadherin-mediated intercellular adhesion is one of the determinants of TJ biogenesis (24). Additionally, cadherin-based cell-cell contacts are likely to stabilize TJ, since treatment of Madin-Derby canine kidney epithelial cells with E-cadherin blocking antibodies dissociates preformed TJ (25, 26). However, the observation that E-cadherin-null mouse blastocysts do form normal TJ (27) indicates that E-cadherin, albeit relevant, may not be the only integral membrane protein that contributes to the assembly and stabilization of TJ. Additionally, VE-cadherin null mutation in endothelial cells does not prevent JAM and other TJ components from being correctly localized at intercellular contacts (28). We have recently identified junctional adhesion molecule (JAM) as a novel integral membrane protein that colocalizes with TJ components (such as occludin, ZO-1, and cingulin) at the apical region of the intercellular cleft in epithelial and endothelial cells (29). To test whether JAM may play a functional role in the context of intercellular junctions, it is necessary to analyze possible relationships of JAM with TJ components. Here, we have examined the ability of JAM (i) to influence the molecular organization of intercellular junctions and (ii) to associate with cytoplasmic components of TJ.

EXPERIMENTAL PROCEDURES

Antibodies—For production of the anti-human JAM mAb BV16 (IgG1), BALB/c female mice were immunized with a fusion protein consisting of the extracellular domain of human JAM and the Fc portion of human IgGs. Splenocytes were fused with the SP2/0 myeloma cell line. Clones were screened for their ability to recognize the immunogen by enzyme-linked immunosorbent assay and to stain intercellular junctions in Caco-2 cells. Production of rat anti-murine JAM mAbs BV11 and BV12, as well as and rabbit anti-cingulin polyclonal antibody (pAb), has been described previously (14, 29). Anti-murine JAM mAb BV19 was produced by immunizing Lewis rats with an Fc-murine JAM construct. Rabbit anti-ZO-1 and anti-occludin pAbs were from Zymed Laboratories Inc. (San Francisco, CA).

DNA Constructs, Vectors, and Transfectants—The constructs GST-

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[1] The abbreviations used are: TJ, tight junction; CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; JAM, junctional adhesion molecule; pAb, polyclonal antibody; PDZ, PSD95/dlg/ZO-1; mAb, monoclonal antibody; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis.

JAM, GST-XC 1–378, and GST-XC 377–1368 encoded for GST fused with the cytoplasmic domain of JAM and an amino- and carboxyl-terminal fragment of cingulin, respectively. GST-JAM contains the cytoplasmic tail of murine JAM (residues 261–300). GST-XC 1–378 comprises most of the amino-terminal globular head, and GST-XC 377–1368 comprises a small part of the head (residues 377–439), the coiled-coil region (residues 440–1325), and the globular carboxyl-terminal tail (residues 1326–1368) of *Xenopus* cingulin (30).

A JAM deletion mutant lacking the carboxyl-terminal residues Phe^{298}-Leu^{299}-Val^{300} (JAM ΔFLV) was produced by polymerase chain reaction, using murine JAM cDNA in the pCDM8 vector as template, the sense oligonucleotide 5′-CCTGGTTCAAGGACGGGATATCCATGC-TTACAGC-3′ (which corresponds to nucleotides 491–524 and encompasses an *Eco*RV site) as forward primer, and the antisense oligonucleotide 5′-GAGCGGCCGC**TCA**CGACGAGGTCTGTTTGAATTC-C-3′ (which introduces a *Not*I site and a stop codon upstream of nucleotide 892) as reverse primer (the restriction sites are underlined, and the stop codon is highlighted in bold). The polymerase chain reaction product was cloned using the TA cloning kit (Invitrogen, Groningen, The Netherlands) and sequenced by dideoxy sequencing. The *Eco*RV-*Not*I fragment containing the mutation was inserted in the JAM/pCDM8 vector to replace the corresponding *Eco*RV-*Not*I fragment encoding for full-length JAM. Then, cDNAs for full-length JAM and mutated JAM ΔFLV were cloned as *Hin*dIII-*Not*I fragments into the PINCO retroviral vector (31) for transfection of the Phoenix packaging cell line (32), which were kindly donated by Drs. P .G. Pelicci (European Institute of Oncology, Milano, Italy) and G. P. Nolan (Stanford University, Stanford, CA). Supernatants of PINCO-transfected Phoenix cells were used to infect human Caco-2 cells, as described in detail (33). Surface expression of transfected JAM and JAM ΔFLV was tested by fluorescence-activated cell sorting analysis using anti-murine JAM mAb BV12.

Immunofluorescence Microscopy—Chinese hamster ovary (CHO) cells were transfected by calcium phosphate with 20 μg of pECE-JAM and/or pECE-occludin plus 2 μg of pB-SpacDp plasmids (34). Cloning of murine JAM has been described previously (29); cDNA encoding murine occludin was obtained from Dr. W. Risau (Max Planck Institute, Bad Nauheim, Germany). Transfectants were selected with puromycin and tested for JAM and occludin expression by immunofluorescence. Briefly, CHO cells were grown to confluence on glass coverslips and fixed in ice-cold methanol for 3 min at −20 °C, as described (35). For occludin staining, fixed cells were sequentially incubated with phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% saponin for 10 min and 0.5% saponin for additional 10 min.

Immunoprecipitation and Blot Analysis—Human intestinal epithelial Caco-2 cells were grown in Dulbecco's modified Eagle's medium containing 15% fetal calf serum. Confluent monolayers were lysed (for 30 min at 4 °C) with lysis buffer (pH 7.5) containing either 0.5% Triton X-100 or 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, and protease inhibitors. Following centrifugation of cell lysates (14,000 × *g* for 10 min), the supernatant ("soluble fraction") was separated from the pellet. The pellet was further incubated with 0.02% SDS in lysis buffer, resuspended by gentle pipetting, and centrifuged, and the resulting supernatant was collected ("insoluble fraction"). After preclearing, both fractions were incubated with antibodies for 60 min at 4 °C. Immunocomplexes were then absorbed with protein G-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Beads were washed five times, boiled with reducing sample buffer, and subjected to SDS-PAGE electrophoresis. Proteins were transferred onto nitrocellulose filters by electroblotting. Membranes were incubated with primary antibodies, washed, and incubated with HRP-conjugated secondary antibodies. Proteins were visualized using an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech, Buckingham, United Kingdom) and autoradiography. For ATP depletion, Caco-2 monolayers were preincubated (for 60 min at 37 °C) with 2 mM 2-deoxy-D-glucose and 10 μM antimycin A (Sigma) dissolved in Delbecco's-phosphate-buffered saline (36).

Reprecipitation experiments were carried out as described above, except that immune complexes were dissociated with 0.2% SDS for 10 min at 70 °C. Samples were then diluted 1:4 (v:v) in lysis buffer, incubated with the indicated antibody coupled to protein G-Sepharose for additional 60 min at 4 °C, and reprecipitated. For GST "pull-down" experiments, either GST-JAM or GST-cingulin fusion proteins were coupled to glutathione-Sepharose beads and added to the lysates. Precipitation was carried out as detailed above.

In Vitro Binding Assays—To prepare recombinant ZO-1 as fluid-phase ligand for binding assays, cDNA encoding full-length human ZO-1 under control of the T7 promoter in pBluescript SK^+ (kindly provided by Drs. A. S. Fanning and J. M. Anderson) was transcribed and translated *in vitro* using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI) as described (13). The nascent protein was labeled using Transcend biotin-lysyl-tRNA (Promega). Briefly, 25 μl of rabbit reticulocyte lysate, 2 μl of reaction buffer, 1 μl of T7 RNA polymerase, 0.5 μl of amino acid mixture minus leucine (1 mM), 0.5 μl of amino acid mixture minus methionine (1 mM), 40 units of RNasin, 1 μg of template DNA, 1 μl of Biotin-Lysyl-tRNA were mixed in a final volume of 50 μl, incubated for 90 min at 30 °C, and immediately used for the binding assays. As solid-phase ligands, we used either GST-JAM immobilized on glutathione-Sepharose beads or the carboxyl-terminal JAM peptide NH_2-KQTSSFLV (or reverse NH_2-VLFSSTQK control peptide) coupled to CNBr-activated Sepharose beads (Amersham Pharmacia Biotech). Aliquots (20 μl) of packed beads were diluted with binding buffer (140 mM KCl, 25 mM imidazole, pH 8.0, 1% Tween 20, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride) and incubated with 45 μl of the ZO-1 transcription and translation reaction in a final volume of 200 μl (overnight at 4 °C, with rotation). Beads were washed five times, resuspended with 20 μl of sample buffer, and boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and visualized using HRP-conjugated streptavidin (Biospa, Milano, Italy).

RESULTS

Expression of JAM in CHO Cells Induces Appearance of ZO-1 and Enhances Accumulation of Occludin at Intercellular Junctions—To investigate functional interactions of JAM with other junctional proteins, CHO cells (which do not form TJ) were transfected with JAM (and, for comparison, with occludin) and analyzed by immunofluorescence microscopy for expression of both transfected molecules and endogenous ZO-1. Although no occludin, JAM, or ZO-1 staining was detectable in untransfected CHO cells (Fig. 1, OCC^-/JAM^-), transfection of JAM resulted in the appearance of both JAM and ZO-1 at sites of cell-cell contact (Fig. 1, OCC^-/JAM^+). On the contrary, transfection of occludin induced junctional staining of occludin but not ZO-1 (Fig. 1, OCC^+/JAM^-).

Then, to evaluate whether concomitant expression of JAM and occludin might further increase ZO-1 expression levels, CHO were cotransfected with both JAM and occludin. In the double transfectants, ZO-1 staining was not brighter than in cells transfected with JAM alone (Fig. 1, compare OCC^+/JAM^+ with OCC^-/JAM^+). Remarkably, however, greater amounts of occludin were detectable compared with cells transfected with occludin alone (Fig. 1, compare OCC^+/JAM^+ with OCC^+/JAM^-), suggesting that JAM might help recruit both ZO-1 and occludin at sites of intercellular contacts.

Coprecipitation of JAM and ZO-1—A likely explanation for the observations reported above is that JAM and ZO-1 are components of a molecular complex involved in the formation of junctional structures. To dissect possible molecular interactions of JAM with other junctional molecules, JAM was immunoprecipitated with the anti-JAM mAb BV16 from epithelial Caco-2 cells. The immunoprecipitates were then resolved by SDS-PAGE and analyzed by Western blot with a ZO-1 pAb. Since most TJ components are linked to the actin cytoskeleton, both Triton X-100-soluble and -insoluble fractions were examined. In the Triton X-100-insoluble fraction, mAb BV16 coprecipitated a protein with an apparent relative mass of ~220 kDa, the predicted molecular mass of ZO-1, that was recognized by the ZO-1 pAb (Fig. 2*A*, *lane 2*). The coprecipitated band comigrated with ZO-1, when the latter was directly precipitated and blotted with the ZO-1 pAb (*lane 4*).

In the reciprocal experimental condition, ZO-1 was immunoprecipitated with the ZO-1 antiserum and analyzed by Western blot with mAb BV16. The ZO-1 pAb coprecipitated a ~40-kDa band (Fig. 2*B*, *lane 6*) that comigrated with JAM (*lane 8*). In both conditions, association of JAM with ZO-1 was only detectable in the Triton X-100-insoluble fraction of the lysate. Similar results were obtained when Nonidet P-40 was used as a deter-

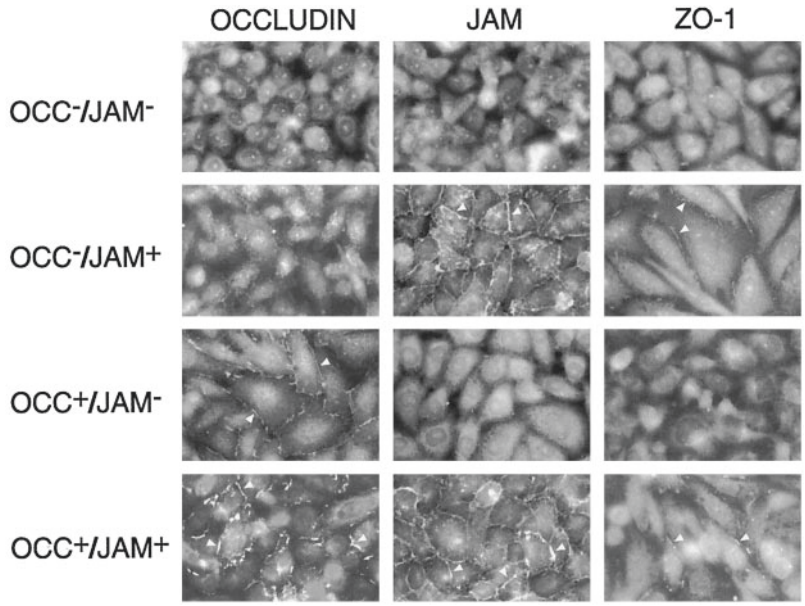

FIG. 1. **Expression of occludin, JAM, and ZO-1.** CHO cells were transfected with empty vector (OCC^-/JAM^-), JAM (OCC^-/JAM^+), occludin (OCC^+/JAM^-), or both JAM and occludin (OCC^+/JAM^+). Transfectants were stained with anti-ZO-1, anti-JAM, and anti-occludin pAbs. *Arrowheads* indicate staining at intercellular contacts.

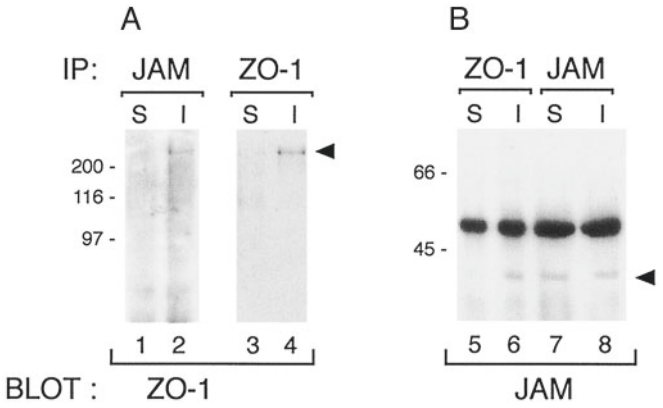

FIG. 2. **Coprecipitation of JAM and ZO-1.** *A*, anti-JAM mAb BV16 coprecipitates ZO-1; *B*, anti-ZO-1 pAb coprecipitates JAM. Triton X-100-soluble (*S*) and -insoluble (*I*) fractions of Caco-2 cells were immunoprecipitated with either mAb BV16 (*lanes 1*, *2*, *7*, and *8*) or anti-ZO-1 pAb (*lanes 3–6*). Proteins were separated by 7.5% SDS-PAGE under reducing conditions and analyzed by Western blot with either pAb anti-ZO-1 (*A*) or mAb BV16 (*B*). Molecular size standards are included on the *left* (kDa) of each panel. *Arrowheads* indicate the position of ZO-1 (*A*) and JAM (*B*).

gent instead of Triton X-100 (data not shown).

Association of JAM with ZO-1 Requires the Carboxyl Terminus of JAM—To identify molecular determinants of the association of JAM with ZO-1, we produced both molecules in recombinant form and tested their interaction in *in vitro* binding assays. The cytoplasmic domain of JAM (from Gly^{256} to the carboxyl-terminal residue Val^{300}) was expressed as a fusion protein with GST (GST-JAM) and immobilized on glutathione-Sepharose beads. Full-length ZO-1 was transcribed and translated *in vitro*, labeled with biotin, and used as fluid phase ligand. Bound ZO-1 was then eluted, analyzed by SDS-PAGE and blotting, and visualized with streptavidin-peroxidase. Like native ZO-1, recombinant ZO-1 displayed an apparent relative molecular mass of ~220 kDa (Fig. 3*A*, *lane 6*) and was recognized by the ZO-1 pAb (*lane 7*). As shown in Fig. 3*A*, recombinant ZO-1 bound GST-JAM (*lane 2*), but not GST alone (*lane 1*), indicating that the cytoplasmic domain of JAM is required for the association with ZO-1.

PSD95/dlg/ZO-1 (PDZ) domains mediate binding of several intracellular molecules (like ZO-1) to the cytoplasmic tail of transmembrane proteins (37). The presence of a putative PDZ-binding motif at the carboxyl terminus of JAM (see "Discussion") led us to investigate its involvement in the interaction with ZO-1. To this purpose, the peptide NH_2-KQTSSFLV (which corresponds to the last eight residues of JAM) was coupled to CNBr-activated Sepharose beads and incubated with *in vitro* transcribed/translated ZO-1. Recombinant ZO-1 specifically bound the JAM peptide (Fig. 3*A*, *lane 4*), but not the reverse peptide NH_2-VLFSSTQK (*lane 5*) or uncoupled beads (*lane 3*). The additional band with an apparent relative molecular mass of approximately 120 kDa is presumably due to proteolytic degradation of transcribed ZO-1.

To further analyze the role of the motif, a mutant form of murine JAM lacking the three carboxyl-terminal Phe^{298}-Leu^{299}-Val^{300} residues (JAM ΔFLV) was expressed in Caco-2 cells. The availability of mAbs specific for either human (mAb BV16) or murine (mAb BV12) JAM allowed us to directly compare endogenous human JAM (full-length) with transfected murine JAM ΔFLV. Preliminary fluorescence-activated cell sorting and immunofluorescence analysis showed that JAM ΔFLV was expressed at the cell surface and colocalized with endogenous ZO-1 at cell-cell contacts (data not shown). Both JAM and JAM ΔFLV were first immunoprecipitated from the Triton X-100-soluble and-insoluble fractions of Caco-2 transfectants using mAbs BV16 and BV12. Then, the association with endogenous ZO-1 was tested by Western blot with the ZO-1 pAb. As expected, ZO-1 was coprecipitated by mAb BV16 with wild-type JAM in the insoluble fraction (Fig. 3*B*, *lane 9*). In contrast, no ZO-1 was coprecipitated by mAb BV12 together with JAM ΔFLV (*lane 11*). No association was found in the soluble fractions immunoprecipitated with either mAb BV16 (*lane 8*) or BV12 (*lane 10*). The absence of detectable association of ZO-1 with JAM ΔFLV is not due to the inability of mAb BV12 to immunoprecipitate JAM ΔFLV, as JAM ΔFLV was immunoprecipitated by mAb BV12 in a way comparable to the immunoprecipitation of JAM with mAb BV16 (*lanes 13* and *15*).

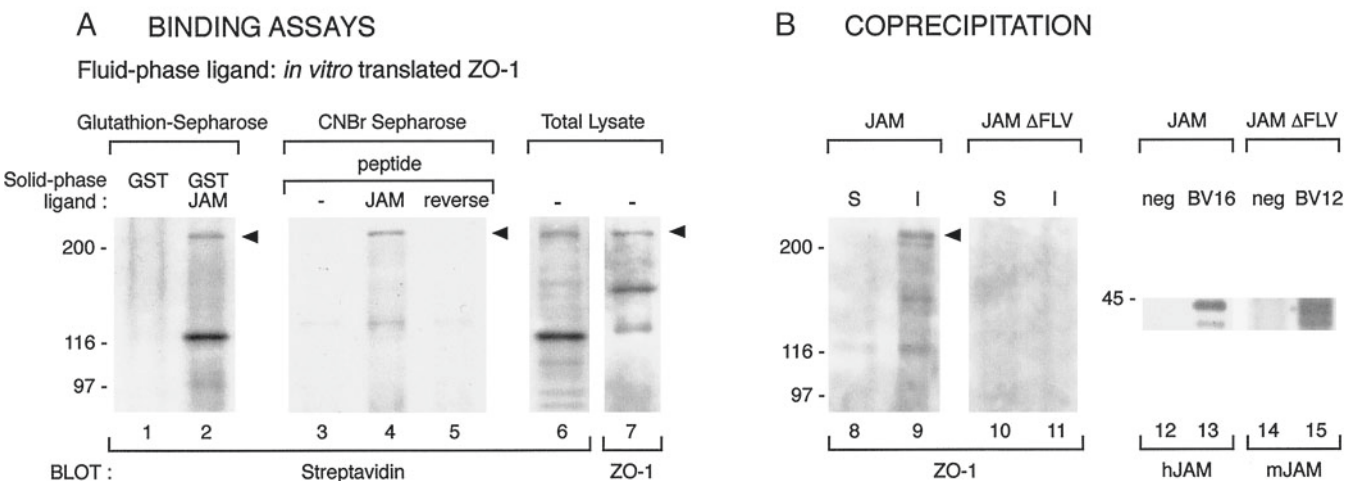

FIG. 3. **Association of JAM with ZO-1 requires the carboxyl terminus of JAM.** *A*, binding assays were performed using *in vitro* translated and biotin-labeled ZO-1 as soluble ligand and JAM fragments as solid-phase ligands. GST (*lane 1*) or GST-JAM (*lane 2*) were immobilized on glutathione-Sepharose beads. The carboxyl-terminal JAM peptide NH_2-KQTSSFLV (*lane 4*) or the reverse peptide NH_2-VLFSSTQK (*lane 5*) were immobilized on CNBr-activated Sepharose beads. Bound ZO-1 was eluted from the beads and analyzed by SDS-PAGE and electroblotting using HRP-streptavidin (*lanes 1–5*). Aliquots (2 μl) of transcribed lysate were analyzed in parallel with either HRP-streptavidin (*lane 6*) or pAb anti-ZO-1 (*lane 7*). *B*, coprecipitation of ZO-1 and JAM is abolished upon deletion of the carboxyl-terminal Phe^{298}-Leu^{299}-Val^{300} residues of JAM. Triton X-100-soluble (*S*) and -insoluble (*I*) fractions of Caco-2 cells were immunoprecipitated with either mAb BV16 or BV12, which recognize full-length human JAM (*lanes 8* and *9*) and mutated murine JAM ΔFLV (*lanes 10* and *11*), respectively. Coprecipitated proteins were analyzed by 7.5% SDS-PAGE and Western blot with pAb anti-ZO-1. Molecular size standards are included on the *left* (kDa) of each panel. *Arrowheads* indicate the position of ZO-1. As an additional control, JAM and JAMΔFLV were immunoprecipitated with mAbs BV16 (*lane 13*) and BV12 (*lane 15*), respectively. Mouse (*lane 12*) and rat (*lane 14*) anti-keyhole limpet hemocyanin mAbs were used as negative controls. Blot analysis was performed using either mAb BV16 (*lanes 12* and *13*) or anti-murine JAM mAb BV19 (*lanes 14* and *15*).

Coprecipitation of JAM and Cingulin—To test whether other junctional proteins could be coprecipitated with JAM, mAb BV16-immunoprecipitates of Caco-2 cells were analyzed by Western blot with an anti-cingulin pAb. In the Triton X-100-insoluble fraction, mAb BV16 coprecipitated a protein that was detectable with the cingulin pAb as a ~140-kDa band, the apparent relative molecular mass of cingulin (Fig. 4*A*, *lane 2*). The protein coprecipitated by mAb BV16 displayed the same electrophoretic motility of cingulin, when the latter was precipitated and blotted in parallel with the cingulin pAb (*lane 4*). Although cingulin was present in both fractions, mAb BV16 did not coprecipitate cingulin from the soluble fraction (*lane 1*).

Association of JAM with cingulin was confirmed in the reciprocal experiment, using the anti-cingulin pAb for immunoprecipitation and mAb BV16 for Western blot. The cingulin pAb coprecipitated a protein that was recognized by mAb BV16 as a band of ~40 kDa (Fig. 4*B*, *lane 6*) that comigrated with JAM (*lane 8*). Again, association of JAM with cingulin was only detectable in the insoluble fraction, even if a greater amount of JAM was present in the soluble fraction.

Coprecipitation of JAM with Cingulin Requires the Cytoplasmic Tail of JAM and the Globular Head of Cingulin—To further define the interaction of JAM with cingulin, GST-JAM was immobilized onto glutathione-Sepharose beads. Similarly to mAb BV16, GST-JAM (but not GST) coprecipitated cingulin in the Triton X-100-insoluble fraction of Caco-2 lysates, as assessed by Western blot analysis with the cingulin pAb (Fig. 5*A*, *lane 4*). The ~140-kDa band precipitated by GST-JAM comigrated with native cingulin, when the latter was precipitated and blotted in parallel with the cingulin antiserum (*lane 6*). Cingulin did not associate with GST-JAM in the soluble fraction (*lane 3*).

To define which region of cingulin is required for interacting with JAM, two GST fusion proteins containing either an amino-terminal (GST-XC 1–378) or a carboxyl-terminal (GST-XC 377–1368) fragment of cingulin were incubated with Caco-2 lysates. In order to reduce some aspecific background, the fraction of JAM bound to the GST-cingulin proteins was dissociated with 0.2% SDS (for 10 min at 70 °C), reprecipitated with mAb BV16, and finally analyzed by Western blot with mAb BV16. GST-XC 1–378 precipitated JAM in both TX-100-soluble and -insoluble fractions (Fig. 5*B*, *lanes 9* and *10*). By densitometry, the JAM band was from 1.5 to 1.8 times more intense in the insoluble than in the soluble fraction. The bands precipitated by either GST-XC 1–378 or mAb BV16 showed similar molecular mass and electrophoretic motility (*lanes 10* and *14*). In contrast, no JAM was found in samples precipitated by GST (*lanes 7* and *8*) or GST-XC 377–1368 (*lanes 11* and *12*), indicating that the amino-terminal globular head of cingulin is specifically required for associating with JAM.

ATP Depletion Decreases the Solubility of JAM in Triton X-100—The existence of two distinct subpopulations of JAM (a major Triton X-100-soluble pool, and a minor Triton X-100-insoluble pool that associates with cingulin and ZO-1) might reflect different degrees in the association with the cytoskeleton. To test this hypothesis, cells were subjected to depletion of ATP, a treatment that increases the association of junctional proteins with the cytoskeleton (23). Caco-2 cells were incubated with 2-deoxy-D-glucose and antimycin A (which inhibit glycolysis and oxidative phosphorylation) and then lysed in Triton X-100. JAM was immunoprecipitated and analyzed by Western blot with mAb BV16. The ratio of soluble to insoluble JAM was greatly reduced following ATP depletion (Fig. 6, compare *lanes 1* and *2* with lanes *4* and *5*), suggesting that the treatment increases the association of JAM with the actin cytoskeleton. In addition, when lysates were immunoprecipitated with the cingulin pAb, a greater amount of JAM was coprecipitated with cingulin upon ATP depletion (*lane 6*) compared with control conditions (*lane 3*). Thus, association with the cytoskeleton might shift the equilibrium between the two subpopulations of JAM toward the more insoluble state and facilitate the interaction of JAM with cingulin.

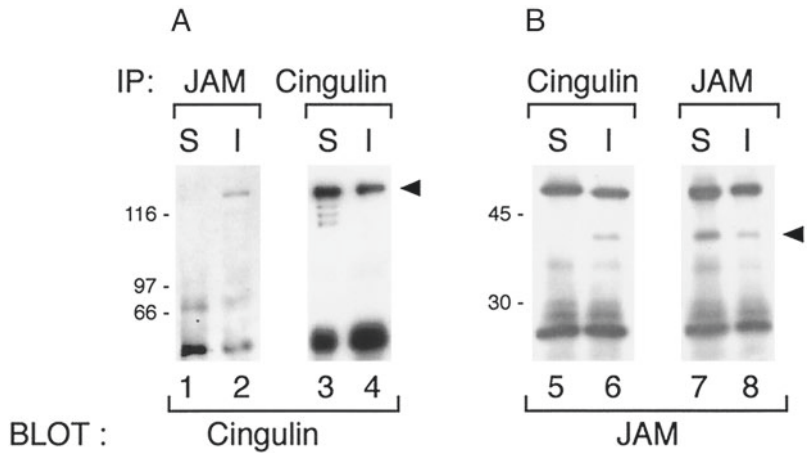

FIG. 4. **Coprecipitation of JAM with cingulin.** *A*, anti-JAM mAb BV16 coprecipitates cingulin; *B*, anti-cingulin pAb coprecipitates JAM. Triton X-100-soluble (*S*) and -insoluble (*I*) fractions of Caco-2 cells were immunoprecipitated with either mAb BV16 (*lanes 1*, *2*, *7*, and *8*) or anti-cingulin pAb (*lanes 3–6*). Proteins were resolved by 7.5% SDS-PAGE and analyzed by Western blot using either pAb anti-cingulin (*A*) or mAb BV16 (*B*). Molecular size standards are included on the *left* (kDa) of each panel. *Arrowheads* indicate the position of cingulin (*A*) and JAM (*B*).

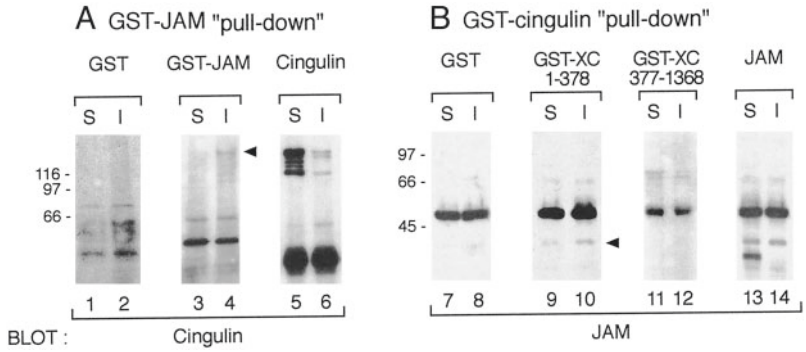

FIG. 5. **Coprecipitation of JAM with cingulin is mediated by the cytoplasmic domain of JAM (*A*) and the globular head of cingulin (*B*).** Triton X-100-soluble (*S*) and -insoluble (*I*) fractions of Caco-2 lysates were incubated with GST (*lanes 1*, *2*, *7*, and *8*), GST-JAM (*lanes 3* and *4*), the amino-terminal GST-XC 1–378 (*lanes 9* and *10*), or the carboxyl-terminal cingulin fragment GST-XC 377–1368 (*lanes 11* and *12*). As controls, proteins were also immunoprecipitated with either pAb anti-cingulin (*lanes 5* and *6*) or anti-JAM mAb BV16 (*lanes 13* and *14*). Proteins were analyzed by Western blot using either pAb anti-cingulin (*A*) or mAb BV16 (*B*). For the experiment reported in *B*, GST-bound material was treated with 0.2% SDS and then reprecipitated with mAb BV16.

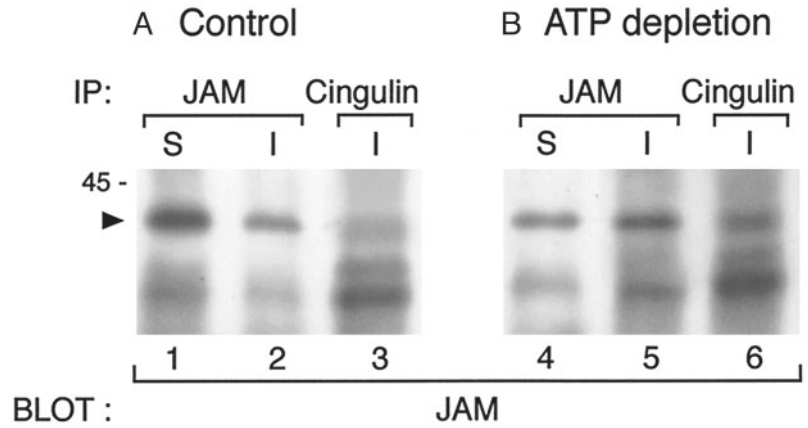

FIG. 6. **ATP depletion decreases the solubility of JAM in Triton X-100 and increases JAM association with cingulin.** Caco-2 cells were preincubated (for 60 min at 37 °C) with either culture medium (*A*) or a combination of 2-deoxy-D-glucose and antimycin A (*B*) and lysed with Triton X-100. Cell extracts were separated into the Triton X-100-soluble (*S*) and -insoluble (*I*) fractions and then immunoprecipitated with either anti-JAM mAb BV16 or pAb anti-cingulin. Proteins were separated by 7.5% SDS-PAGE and analyzed by Western blot with mAb BV16. *Arrowhead* indicates the position of JAM.

DISCUSSION

The present study was undertaken to define functional interactions of JAM with other junctional molecules. The major findings of this paper are: (i) JAM facilitates the junctional localization of ZO-1 and occludin in CHO transfectants, (ii) JAM can be coprecipitated with ZO-1 and cingulin in the insoluble fraction of Caco-2 cells, and (iii) the carboxyl terminus of JAM entails a putative PDZ-binding motif that plays a critical role in the association of JAM with ZO-1.

The ability of JAM to enhance the distribution of ZO-1 at cell-cell junctions complements our previous observation that transfection of JAM in CHO cells (which are normally not self-adherent) induces intercellular adhesion and reduces paracellular permeability (29). Thus, JAM might enable the junctional localization of ZO-1 by establishing functional intercellular adhesion. Similarly, transfection of the cell adhesion molecule Protein zero enhances the junctional expression of ZO-1 in HeLa cells (38), and cadherin-dependent intercellular adhesion (24, 39) is prerequisite to the formation of TJ. However, mere overexpression of transmembrane adhesive proteins may not be *per se* sufficient to recruit junctional molecules. For instance, in our hands, occludin did not induce detectable ZO-1 staining at the junctions, even if transfected occludin is efficiently transported to the cell surface in CHO cells (40) and confers adhesiveness to fibroblasts (20). This finding was somehow surprising, since ZO-1 has been shown to directly interact with occludin (41), even if it is possible that the amount of occludin associated with ZO-1 in our conditions was very low

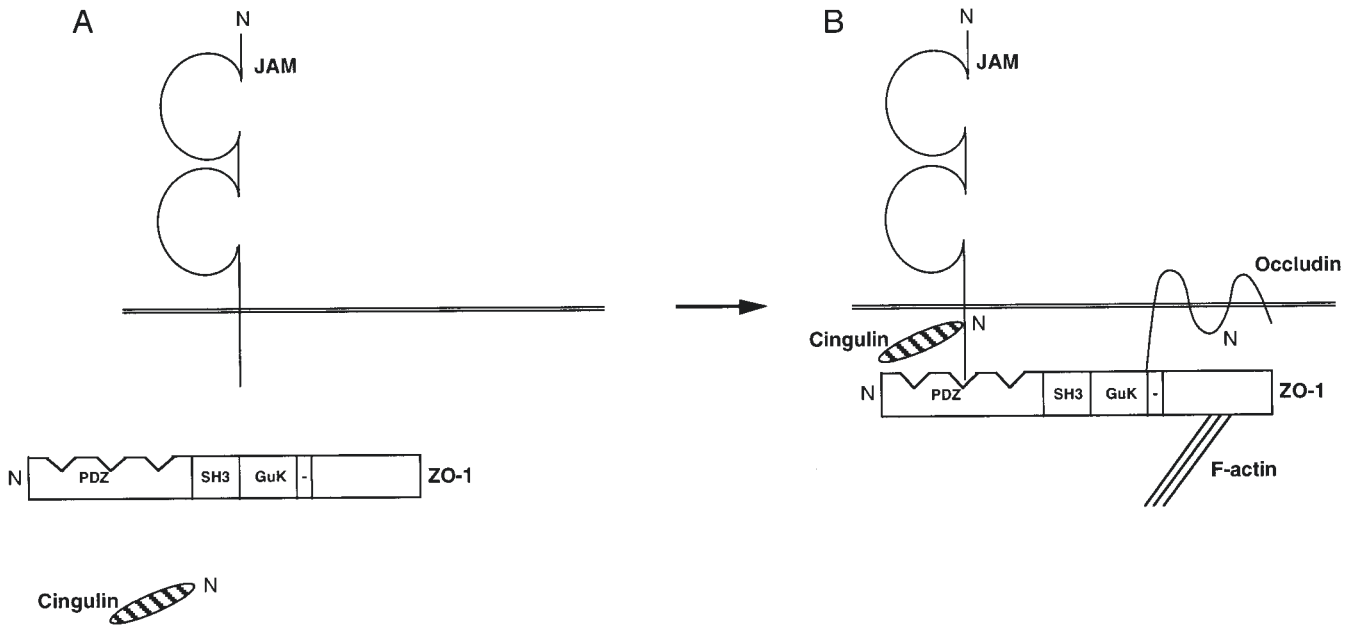

FIG. 7. **Hypothetical model of the role of JAM in the assembly of junctional complexes.** Results reported here suggest that JAM might bind both cingulin and ZO-1 (*A*) and that the resulting F-actin-bound (Triton X-100-insoluble) complex might help recruit occludin at junctional areas (B). It has not been determined yet whether JAM may directly bind cingulin. Association of a region of ZO-1 (corresponding to the guanylate kinase and/or acidic domain) with occludin and F-actin is based on published works (41, 48).

and below the sensitivity levels of immunofluorescence.

Besides recruiting ZO-1, JAM enhanced the junctional staining of occludin. JAM might directly bind and shuttle occludin to the lateral membrane in proximity of nascent junctional complexes, even if the carboxyl terminus of occludin is capable of autonomous targeting to the basolateral membrane (42). Alternatively, JAM-dependent recruitment of occludin at the junctions might be indirectly mediated by ZO-1, as suggested by the herein reported observation that JAM has the potential to associate with ZO-1. The latter is a junctional and multidomain protein, which may interact with several molecules and assemble them at intercellular contacts (1, 23). ZO-1 interacts with diverse cytoplasmic molecules, such as ZO-2 (41), ZO-3 (13), β-catenin (43), the Ras substrate AF-6 (19), heterotrimeric G-proteins (44), and an as yet unidentified serine kinase (45). Additionally, ZO-1 interacts with transmembrane proteins, such as the TJ components occludin (41, 46), claudins (47), and JAM (as shown here). The association between JAM and ZO-1 was demonstrated by reciprocal coprecipitation and was only detectable in the Triton X-100-insoluble fraction. Since ZO-1 binds F-actin (48) through its carboxyl-terminal half (41), one can envision a dynamic model of junction assembly in which JAM first forms soluble complexes with ZO-1 and occludin, which are then progressively recruited into more insoluble structures linked to the actin cytoskeleton. Since the last three residues of JAM (Phe^{298}-Leu^{299}-Val^{300}) fit the consensus sequence (Phe/Tyr-*X*-Val/Ile) identified in transmembrane proteins that bind type II PDZ domains (49, 50), ZO-1 might directly interact with the cytoplasmic tail of JAM. This hypothesis is reinforced by the herein reported observations that (i) *in vitro* transcribed and translated ZO-1 is specifically bound by either a GST fusion protein containing the cytoplasmic tail of JAM or a carboxyl-terminal JAM peptide, and (ii) coprecipitation of JAM with ZO-1 is lost upon deletion of the critical Phe^{298}-Leu^{299}-Val^{300} residues in JAM tail.

JAM was also coprecipitated with cingulin. The interaction required the carboxyl-terminal cytoplasmic tail of JAM and the amino-terminal globular head of cingulin, as indicated by studies with GST fusion proteins. Remarkably, the JAM-cingulin complex was almost exclusively detectable in the Triton X-100-insoluble fraction, despite the fact that JAM and cingulin were found in both detergent-soluble and -insoluble fractions. The lack of detectable association in the soluble fraction suggests that the JAM/cingulin association might require cytoskeletal proteins that are only present in the Triton X-100-insoluble fraction, even if we cannot exclude at the present a direct interaction of the two proteins. Several lines of evidence indicate that cingulin is associated with the actomyosin cytoskeleton. Cingulin, which was originally identified in the actomyosin fraction of intestinal epithelial cells (14, 51), was recently reported to colocalize with thick bundles of actin microfilaments during TJ assembly (52) and to interact with myosin (30). On the other hand, compared with ZO-1, cingulin is more easily extractable from membranes and is recruited to a lower extent into fodrin-rich insoluble complexes (53). Furthermore, cingulin (which is a highly asymmetric molecule with a contour length of at least 130 nm (Ref. 14)) is localized at about 40–60 nm distance from the membrane, farther than ZO-1 (14, 54). Hence, it is likely that cingulin may come into close association with the cytoskeleton, even if such association might be somehow weaker when compared with ZO-1. JAM association with cingulin might increase the linkage to the cytoskeleton of the JAM/cingulin complex, thus stabilizing further the junctional plaque. Consistent with this hypothesis, shifting the equilibrium of JAM toward the insoluble fraction upon ATP depletion substantially increased the amount of JAM coprecipitated with cingulin.

In conclusion, we propose that JAM might play a role in the molecular architecture of TJ by interacting with ZO-1 and cingulin and stabilizing occludin at the junctions. As schematically depicted in Fig. 7, the latter event could be accounted for by ZO-1-mediated bridging of the two transmembrane junctional molecules, since ZO-1 may bind both JAM (possibly via its PDZ domains) and occludin (via the guanylate kinase and/or acidic domain; Ref. 41). Further strengthening of the molecular scaffold might then be provided by JAM-dependent recruitment of cingulin into the developing junctional complex.

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