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Association of Junctional Adhesion Molecule with Calcium/calmodulin-dependent Serine Protein Kinase (CASK/LIN-2) in Human Epithelial Caco-2 Cells*

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We report here that junctional adhesion molecule (JAM) interacts with calcium/calmodulin-dependent serine protein kinase (CASK), a protein related to membrane-associated guanylate kinases. In Caco-2 cells, JAM and CASK were coprecipitated and found to colocalize at intercellular contacts along the lateral surface of the plasma membrane. Association of JAM with CASK requires the PSD95/dlg/ZO-1 (PDZ) domain of CASK and the putative PDZ-binding motif Phe-Leu-Val$_{COOH}$ in the cytoplasmic tail of JAM. Temporal dissociation in the junctional localization of the two proteins suggests that the association with CASK is not required for recruiting JAM to intercellular junctions. Compared with mature intercellular contacts, junction assembly was characterized by both enhanced solubility of CASK in Triton X-100 and reduced amounts of Triton-insoluble JAM-CASK complexes. We propose that JAM association with CASK is modulated during junction assembly, when CASK is partially released from its cytoskeletal associations.

Junctional adhesion molecule (JAM)[1] is an integral membrane protein that belongs to the family of immunoglobulin-like cell adhesion molecules (1) and mediates homophilic adhesive interactions (2). It is expressed at the intercellular cleft of epithelial and endothelial cells and colocalizes with the tight junction components occludin, cingulin, and ZO-1 (1). Several tight junction proteins may interact with each other and with cytoskeletal and signaling molecules (3). We recently found that JAM interacts with ZO-1 and cingulin and that such associations may favor the junctional recruitment of occludin (4). Also, JAM interacts with the junctional molecule all-1 fused gene on chromosome 6 (AF-6) (5).

While searching for potential molecular partners of JAM, we noticed that a sequence at the cytoplasmic carboxyl terminus of JAM fits a consensus motif for binding type II PSD95/dlg/ZO-1 (PDZ) domains (6). PDZ domains are protein-binding modules involved in the assembly of transmembrane molecules and signaling complexes at specialized domains of the plasma membrane (7). Membrane-associated guanylate kinases, a subfamily of modular PDZ domain-containing proteins, are found at intercellular junctions, neuronal synapses, and polarized membrane domains (8).

Based on a peptide library study that defined the binding specificity of several PDZ domains (6), we hypothesized that calcium/calmodulin-dependent serine protein kinase (CASK) might be a ligand for the carboxyl terminus of JAM. The membrane-associated guanylate kinase protein CASK is composed of a Ca^{2+}-calmodulin kinase, a PDZ, an SH3, and a guanylate kinase domain. Although predominantly expressed at the neuronal presynaptic membrane (where it binds neurexins), CASK is also expressed in epithelial cells (9). In nematodes, the CASK homolog LIN-2 is an essential component of a multiprotein complex that targets the Let-23 receptor tyrosine kinase to the basolateral membrane of vulval precursors (10). In vertebrates, CASK localizes at the lateral surface of epithelial cells and binds syndecan-2 (11). In the present study, we have examined the putative interaction of JAM and CASK in human epithelial cells.

MATERIALS AND METHODS

Antibodies and Cells—Mouse anti-human JAM mAb BV16, rat anti-murine JAM mAb BV12, and rabbit anti-JAM serum were produced as described previously (1, 4). Anti-CASK mAb C63120 and anti-AF-6 mAb A60520 were purchased from Transduction Laboratories, anti-CASK mAb 5230 was purchased from Chemicon International, and anti-ZO-1 serum was purchased from Zymed Laboratories Inc. mAb 6H (which recognizes the α1 subunit of Na^+,K^+-ATPase) and anti-cingulin serum were generous gifts of Drs. G. Pietrini (University of Milan, Milan, Italy) and S. Citi (University of Padova, Padova, Italy).

Human intestinal epithelial Caco-2 cells were cultured in DMEM plus 10% fetal calf serum and split 1:3 every week. For experiments with Caco-2 transfectants, the mJAM and mJAM ΔFLV constructs were cloned in the PINCO retroviral vector to transfect the Phoenix packaging cell line (gifts of Drs. P. G. Pelicci (European Institute of Oncology, Milan, Italy) and G. P. Nolan (Stanford University, Stanford, CA) and then to infect Caco-2 cells, as described previously (12). Production of mJAM and mJAM ΔFLV has been described previously (4).

Immunoprecipitation and Western Blot Analysis—Confluent Caco-2 cells were lysed with lysis buffer (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, and protease inhibitors (pH 7.5)). Lysates were precleared with protein A-Sepharose and immunoprecipitated with antibodies coupled to protein A-Sepharose. Immunocomplexes were separated by SDS-polyacrylamide gel electrophoresis and finally analyzed by Western blot, as described previously (4). To study protein partition-

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[1] The abbreviations used are: JAM, junctional adhesion molecule; GST, glutathione *S*-transferase; PDZ, PSD95/dlg/ZO-1; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium.

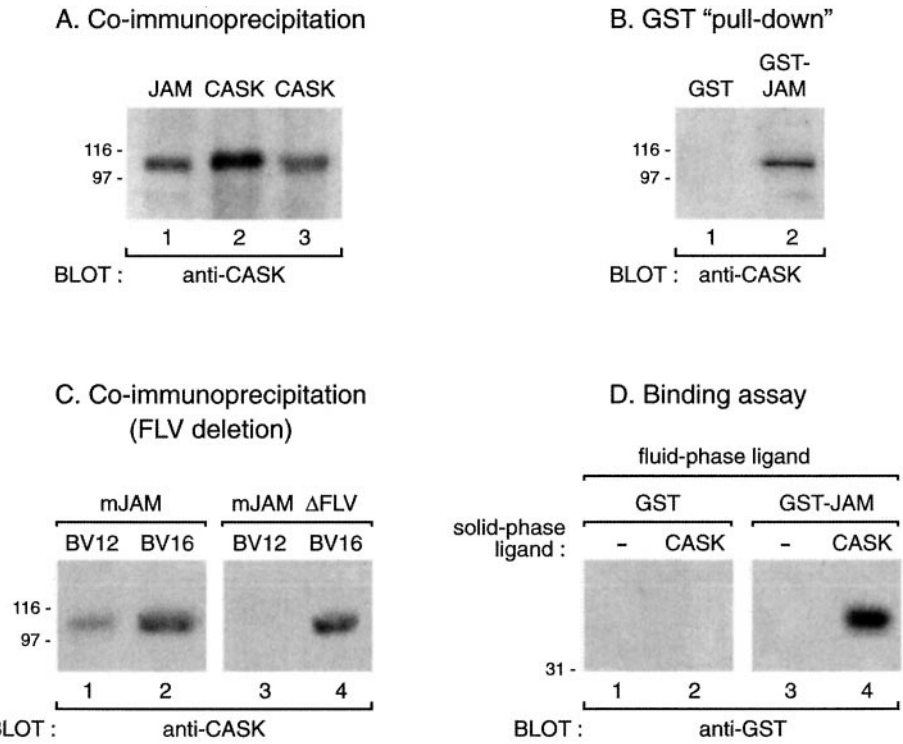

FIG. 1. **PDZ-dependent association of JAM with CASK.** *A,* anti-JAM mAb BV16 coprecipitates CASK. Caco-2 cell lysates were immunoprecipitated with mAb BV16 and analyzed by Western blot with anti-CASK mAb C63120 (*lane 1*). Lysates from Caco-2 cells (*lane 2*) and rat brain (*lane 3*) were used as positive controls. *B,* the cytoplasmic tail of JAM mediates the association with CASK. GST (*lane 1*) or GST-JAM (*lane 2*) was immobilized onto glutathione-Sepharose beads and incubated with Caco-2 lysates. *C,* deletion of the putative PDZ-binding motif FLV in the JAM tail abolishes the coprecipitation of CASK with JAM. Lysates from Caco-2 cells expressing murine JAM (either full-length murine JAM (*mJAM*) or a deletion mutant (*mJAM ΔFLV*)) were immunoprecipitated with either mAb BV12 or BV16, which recognizes transfected murine (*lanes 1* and *3*) and endogenous human (*lanes 2* and *4*) JAM, respectively. *D,* the cytoplasmic tail of JAM directly binds the PDZ domain of CASK. Fluid-phase GST (*lanes 1* and *2*) or GST-JAM (*lanes 3* and *4*) was incubated with either 6HisCASK-PDZ immobilized on Ni^{2+}-NTA beads (*lanes 2* and *4*) or beads alone (*lanes 1* and *3*). Proteins were eluted, separated by 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions, and analyzed by Western blot with either anti-CASK mAb C63120 (*A–C*) or anti-GST antibody (*D*). Molecular size standards (kDa) are indicated at the *left* of each panel.

ing in the Triton X-100-soluble and -insoluble fractions, in some experiments lysates were centrifuged (14,000 × *g* for 10 min) to separate the supernatant (soluble fraction) from the pellet. The pellet was incubated with 0.02% SDS in lysis buffer, resuspended by gentle pipetting, and centrifuged, and the supernatant was collected (insoluble fraction). In control experiments, Western blot analysis of the remaining pellet (*i.e.* material insoluble in 0.02% SDS) did not reveal the presence of JAM, whereas a minor fraction of CASK (between 5% and 10% of total CASK) was still detectable in the pellet, both in cells with mature junctions and during junction assembly.

Binding Assay—To produce the soluble PDZ domain of CASK, Caco-2 cell RNA was reverse-transcribed according to standard procedures. Human cDNA encoding the CASK PDZ domain was amplified by polymerase chain reaction using the sense oligonucleotide 5′-GCGGATCCAGAGTTCGGCTGGTACAGTTTC-3′ (which introduces the *underlined Bam*HI site upstream of nucleotide 1459) as forward primer and antisense oligonucleotide 5′-GGAAGCTTTCAGCGGTAACTTGGCACAA TCTTG-3′ (which introduces a stop codon and the *underlined Hin*dIII site downstream of nucleotide 1716) as reverse primer. The *Bam*HI-*Hin*dIII fragment was inserted in the pQE30 vector (Qiagen), which adds an amino-terminal hexahistidine sequence, and used to transform competent M15 *Escherichia coli*. The protein (6HisCASK-PDZ) was purified on a Ni^{2+}-nitriloacetic acid resin. For the binding assay, 6HisCASK-PDZ was immobilized on Ni^{2+}-NTA-Sepharose beads. After blocking with 1 mg/ml bovine serum albumin and 0.1% Tween 20, beads (20 μl) were diluted in binding buffer and incubated with either GST-JAM or GST as fluid-phase ligands (60 min at 4 °C, with rotation). Beads were washed five times, resuspended with 20 μl of sample buffer, and boiled for 5 min. Proteins were analyzed by Western blot using an anti-GST antibody (Biotech).

Immunofluorescence Microscopy—Cells were grown on glass coverslips and fixed with methanol. Mowiol 488-mounted coverslips were analyzed by confocal microscopy (with Kalman filtering), using an MRC 1024 Bio-Rad microscope equipped with a krypton/argon laser (13).

RESULTS

CASK Associates with the Cytoplasmic Tail of JAM—To study the association of JAM with CASK, JAM was first immunoprecipitated from Caco-2 lysates using the anti-JAM mAb BV16. Immunoprecipitates were then resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot with the anti-CASK mAb C63120. mAb BV16 coprecipitated a protein with an apparent relative mass of ~110 kDa that was recognized in Western blot by the anti-CASK antibody (Fig. 1*A, lane 1*). The coprecipitated protein displayed the same electrophoretic mobility of authentic CASK when the latter was analyzed in parallel by Western blot in lysates from either Caco-2 cells (*lane 2*) or rat brain (*lane 3*).

To define the molecular determinants of the JAM-CASK association, 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 onto glutathione-Sepharose beads. GST-JAM (but not GST) bound CASK in Caco-2 lysates as assessed by Western blot analysis with the anti-CASK antibody, indicating that the association of JAM with CASK is mediated the cytoplasmic tail of JAM (Fig. 1*B*).

JAM Association with CASK Is PDZ-dependent—As mentioned above, the cytoplasmic domain of JAM contains a putative PDZ-binding motif. To test its involvement in the association with CASK, we produced a deletion mutant of murine JAM lacking the carboxyl-terminal residues Phe^{298}-Leu^{299}-Val^{300} (mJAM ΔFLV). Caco-2 cells were then transfected with either mJAM ΔFLV or full-length murine JAM (mJAM). As evaluated by fluorescence-activated cell-sorting analysis, mJAM and

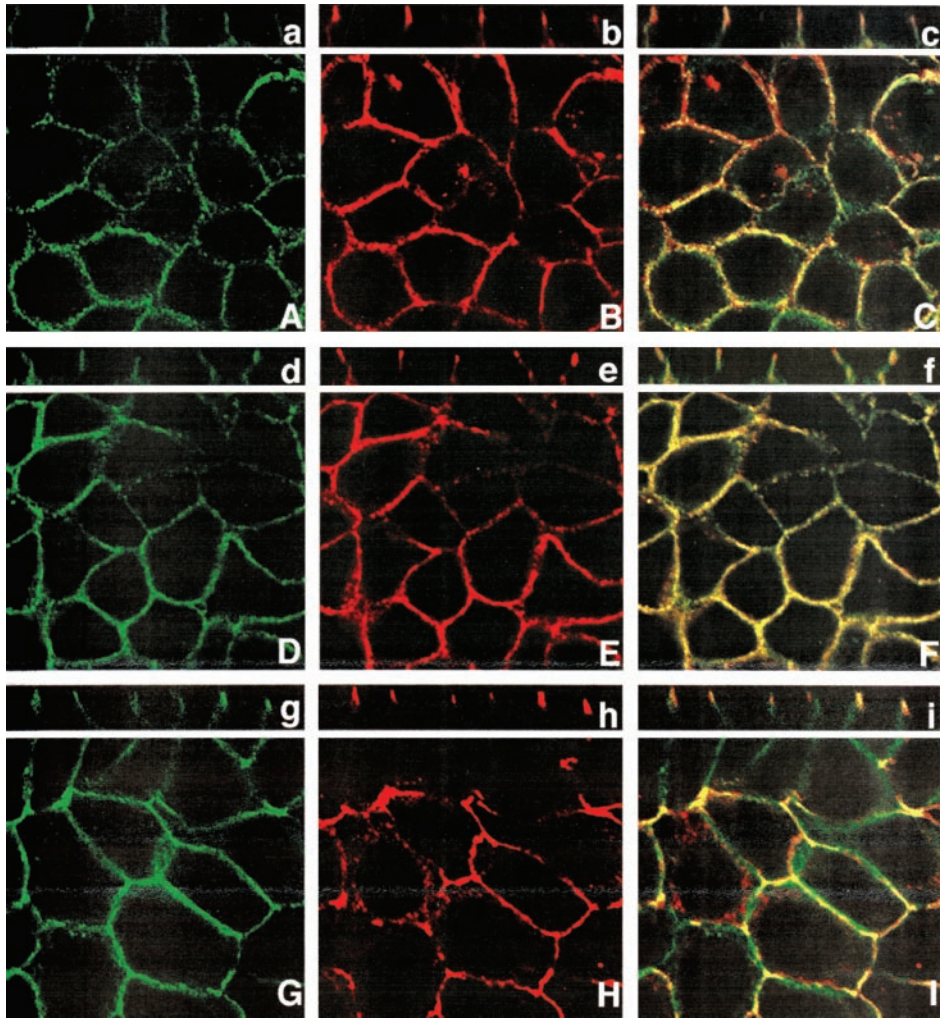

FIG. 2. **Immunofluorescence analysis of JAM and CASK distribution.** Caco-2 cells were stained with anti-CASK mAb 5230 (*A*), anti-JAM serum (*B* and *E*) and mAb BV16 (*G*), anti-Na^+,K^+-ATPase mAb 6H (*D*), and anti-cingulin serum (*H*). Confocal laser scanning micrographs of *x, y* (*A–I*) and *x, z* (*a–i*) optical sections (*first* and *second columns*), and merging of the two staining patterns (*third column*) are shown.

mJAM ΔFLV were expressed at comparable levels at the cell surface (26.4% and 26.9% positive cells, respectively). Endogenous human JAM and transfected murine JAM (both mJAM and mJAM ΔFLV) were first immunoprecipitated using anti-human BV16 and anti-murine BV12 mAbs, respectively. Then, the association with CASK was tested by Western blot using the anti-CASK mAb C63120 (Fig. 1*C*). As expected, mAb BV16 coprecipitated CASK together with endogenous human JAM in both mJAM (*lane 2*) and mJAM ΔFLV transfectants (*lane 4*). At variance, mAb BV12 only coprecipitated CASK in mJAM (*lane 1*) and not in mJAM ΔFLV transfectants (*lane 3*). Preliminary experiments had shown that the FLV deletion does not impair the ability of mAb BV12 to immunoprecipitate mJAM ΔFLV (4).

We then evaluated whether the association of JAM with CASK is mediated by the PDZ domain of CASK. To this end, the PDZ domain (from Arg^{487} to Arg^{572}) was produced as a polyhistidine-tagged soluble protein (6HisCASK-PDZ), immobilized onto Ni^{2+}-NTA-Sepharose beads, and used in a binding assay as solid-phase ligand. Either GST-JAM or GST alone was used as fluid-phase ligand. Bound GST proteins were then eluted and analyzed by SDS-polyacrylamide gel electrophoresis and Western blot using an anti-GST antibody. As shown in Fig. 1*D*, GST-JAM was specifically bound by immobilized 6His-CASK-PDZ (*lane 4*), whereas no binding of either GST to 6His-CASK-PDZ-coated beads (*lane 2*) or GST-JAM to beads alone (*lane 3*) was detectable.

Subcellular Distribution of JAM and CASK—The distribution of JAM and CASK in Caco-2 cells was analyzed by confocal

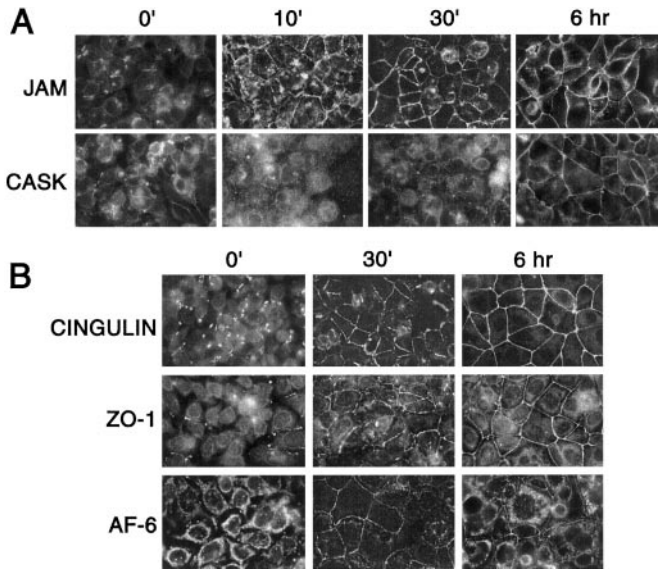

FIG. 3. **Temporal dissociation in the junctional recruitment of JAM and CASK.** Caco-2 cells were grown to confluence on glass coverslips in DMEM, incubated for 18 h with S-MEM to disrupt intercellular junctions, and finally switched back to DMEM. At the indicated time points, cells were fixed for staining with antibodies against JAM and CASK (*A*) as well as the other junctional molecules cingulin, ZO-1, and AF-6 (*B*).

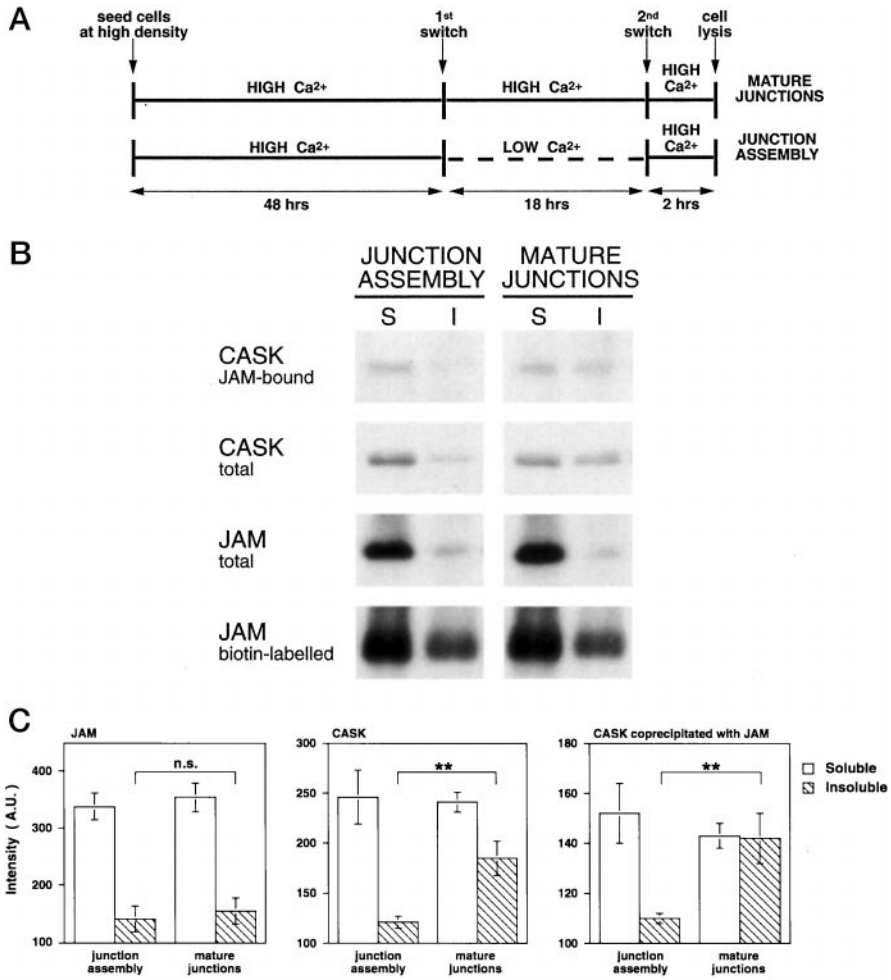

FIG. 4. **Effect of junction assembly on the solubility of JAM, CASK, and JAM-CASK complexes in Triton X-100.** *A,* schematic diagram of the experimental procedure. High and low calcium refer to DMEM (plus 10% fetal calf serum) and S-MEM (plus 0.1% bovine serum albumin), respectively. *B,* Triton X-100 lysates from Caco-2 cells were divided into Triton X-100-soluble (*S*) and -insoluble (*I*) fractions and analyzed by immunoprecipitation with a JAM antiserum followed by Western blot with anti-CASK mAb C63120 (*CASK JAM-bound*). Lysates were also analyzed by Western blot with either mAb C63120 (*CASK total*) or anti-JAM mAb BV16 (*JAM total*). Immunoprecipitation of cell surface JAM is shown (*JAM biotin-labeled*). *C*, densitometric analysis of the results from experiments similar to the one shown in *B*. Optical density values (mean ± S.D.) for JAM, CASK, and JAM-CASK complexes were determined in individual samples from five, four, and three independent experiments, respectively. **, $p < 0.01$, Student's *t* test, unpaired samples.

laser microscopy. As shown in Fig. 2, both CASK (Fig. 2*A*) and JAM (Fig. 2*B*) distributed and colocalized (Fig. 2*C*) at intercellular contacts. In vertical sections, CASK and JAM colocalized at the lateral region of the plasma membrane with minimal overlap at the apical-most portion of the latter (Fig. 2, *a–c*). Localization of either molecule at the apical surface was never observed. To define JAM distribution in greater detail, JAM was costained with either the basolateral marker Na^+,K^+-ATPase or the tight junction component cingulin. ATPase (Fig. 2, *D* and *d*) and JAM (Fig. 2, *E* and *e*) codistributed at intercellular contacts (Fig. 2*F*) and colocalized along the lateral surface (Fig. 2*f*), confirming the localization of JAM at the lateral domain of the plasma membrane. At variance, a subpopulation of intercellular JAM (Fig. 2*G*) localized to the apical-most region of the lateral membrane (Fig. 2*g*) and colocalized (Fig. 2, *I* and *i*) with cingulin, which exclusively decorated tight junctions (Fig. 2, *H* and *h*).

Association with CASK Is Not Required for Recruiting JAM to the Junctions—Because several cytoplasmic PDZ-proteins determine the subcellular localization of transmembrane partners (7), we tested whether association with CASK was required for recruitment of JAM to intercellular contacts. To this end, confluent monolayers of Caco-2 cells were incubated in the low calcium medium minimum essential medium for suspension cultures (S-MEM) to disrupt junctions and then switched back to DMEM, which contains physiological calcium concentrations, to induce junction reassembly. Junctional staining of both JAM and CASK was evaluated at different time points after calcium addition. We found that JAM was recruited to the junctions as early as 10 min after calcium addition, whereas CASK recruitment was only observed at 6 h (Fig. 3*A*). In additional experiments, junctional staining of CASK was not yet detectable at 2 h after calcium addition (data not shown), indicating that junctional recruitment of CASK occurs between 2 and 6 h under these experimental conditions. At variance with CASK, junctional staining of cingulin, ZO-1, and AF-6 was already detectable at 30 min after calcium addition (Fig. 3*B*). Temporal dissociation in the junctional recruitment of JAM and CASK suggests that JAM localizes to intercellular junctions independently of its association with CASK. The suggestion is further supported by the observation that the deletion mutant mJAM ΔFLV did localize to intercellular junctions in transfected Caco-2 cells (data not shown).

Modulation of the Interaction between JAM and CASK during Junction Assembly—To test the hypothesis that the association of JAM with CASK may be modulated by the state of maturation of intercellular adhesive complexes, confluent Caco-2 monolayers were first incubated (for 18 h) in low calcium medium to disrupt junctions and then incubated for additional 2 h in medium containing physiological calcium concentrations and finally lysed with 0.5% Triton X-100 (Fig. 4, *JUNCTION ASSEMBLY*). As a control, Caco-2 cells were maintained in the presence of physiological calcium levels throughout the assay (Fig. 4, *MATURE JUNCTIONS*). Upon lysis, both Triton-soluble and -insoluble fractions were analyzed for the presence of JAM and CASK, either alone (by Western blot with the respective antibodies) or in complex (by immunoprecipitation with anti-JAM and Western blot with anti-CASK antibodies). Compared with mature junctions, JAM distribution in the two fractions was unchanged during junction assembly, as assessed by Western blot analysis, with JAM being much more abundant in the soluble fraction. Notably,

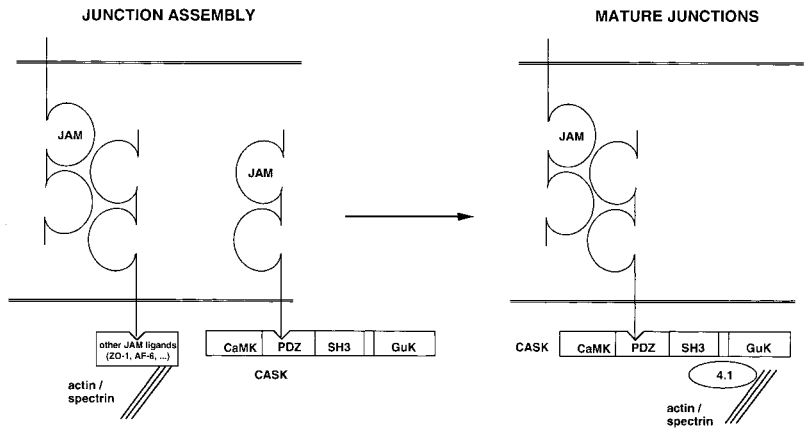

FIG. 5. **Regulation of the interaction between JAM and CASK during junction assembly.** Schematic model of the association between JAM and CASK in mature junctions (*right)* and upon junction assembly (*left*). See "Discussion" for a detailed description of the model.

detection of biotin-labeled JAM suggests that JAM was expressed at the cell surface in both fractions (Fig. 4*B*). In contrast, partitioning of CASK between the two fractions was severely affected because much lower amounts of CASK were detected in the insoluble fraction during junction assembly as compared with mature junctions. Similarly, the amount of CASK coprecipitated with JAM in the insoluble fraction was reduced during the assembly, as evaluated by immunoprecipitation and Western blot (Fig. 4*B*; see also Fig. 4*C* for a quantitative analysis).

DISCUSSION

The major findings of this study are: (i) JAM association with CASK requires the PDZ domain of CASK and the putative PDZ-binding motif F-L-V of JAM, (ii) the two proteins colocalize at the lateral surface in epithelial cells, (iii) association with CASK is not required for recruitment of JAM to the junctions, and (iv) junction assembly is accompanied by decreased amounts of JAM-CASK complexes in the Triton X-100-insoluble fraction of cell lysates. These data suggest that binding of JAM with CASK is dynamically regulated during junction assembly.

PDZ-dependent Association of JAM with CASK—The molecular association was determined by independent and complementary assays. First, CASK was coimmunoprecipitated with JAM. Second, CASK coeluted with the GST-JAM fusion protein. Third, the 6HisCASK-PDZ protein interacted with GST-JAM in a direct binding assay. Hence, the association requires the cytoplasmic tail of JAM (in particular, the carboxyl-terminal residues Phe-Leu-Val_{COOH}, as demonstrated by the inability of mJAM ΔFLV to coprecipitate CASK) and the PDZ domain of CASK. Crystal structure (14) and peptide binding studies (6) have established that CASK is a type II PDZ protein. Members of this class of PDZ domains bind transmembrane proteins bearing the carboxyl-terminal consensus sequence Phe/Tyr-*X*-Φ (*X*, any residue; Φ, hydrophobic). The preferred binding motif of CASK was predicted to be Phe-Phe-Val(Phe/Ala), and ligands known to interact with its PDZ domain, *i.e.* syndecan-2 (11) and neurexin (9), terminate in Phe-Tyr-Ala and Tyr-Tyr-Val, respectively. The data reported here confirm the prediction that JAM is a ligand for the PDZ domain of CASK and that binding requires the Phe-Leu-Val_{COOH} residues, which are conserved in all the sequences determined thus far, such as human, bovine (15), murine, and rat JAM (1), and which mediate JAM binding to the PDZ proteins ZO-1 (4) and AF-6 (5).

Subcellular Distribution of JAM and CASK—Molecular interaction of JAM and CASK is mirrored by their colocalization at the lateral surface of the plasma membrane. Whereas the lateral distribution of CASK (11) and human JAM (16) has been reported previously, murine JAM was shown to codistribute with tight junction-specific molecules in cell lines and to concentrate to the tight junction-containing segment of the intercellular cleft in tissue sections (1). Conceivably, differences in animal species, antibodies, cell type, and maturation state of cell junctions are likely determinants of such discrepancy. Data reported here indicate the existence of two distinct subpopulations of JAM in Caco-2 cells. The former decorates the lateral cell surface and colocalizes with the basolateral membrane marker Na^+,K^+-ATPase. The latter concentrates more apically and codistributes with the tight junction marker cingulin. Interestingly, the transmembrane protein occludin is also distributed both at basolateral membrane and at tight junctions, perhaps reflecting differences in the maturation of intercellular contacts (17). In our experimental system, CASK colocalized with both lateral JAM and (albeit to a much lesser extent) junctional JAM. Whereas a putative localization of CASK at epithelial tight junctions requires further analysis, it is noteworthy that CASK is a molecular component of the neuronal presynaptic plaque, another junctional specialization of the plasma membrane (18).

Association with CASK and Junctional Localization of JAM—Several PDZ proteins are instrumental in recruiting transmembrane ligands to specific domains of the plasma membrane (7). Among the immunoglobulin-like cell adhesion molecules known to interact with PDZ proteins, fasciclin II and nectin are recruited to neuromuscular and adherens junctions by the PDZ molecules discs-large and afadin, respectively (19, 20). However, a role for CASK in recruiting JAM is questioned by the observations that JAM localizes to the junctions earlier than CASK and that deletion of the CASK-interacting sequence Phe-Leu-Val_{COOH} does not abolish JAM recruitment to the junctions. CASK-independent targeting of JAM to intercellular junctions might be attributable to other intercellular molecules (such as ZO-1, cingulin, and AF-6) that, in contrast to CASK, localized to the junctions at early time points of junction assembly.

Modulation of JAM-CASK Complexes during Junction Assembly—A calcium switch protocol that induces disassembly and subsequent reassembly of cadherin-based intercellular junctions (21, 22) was used to evaluate whether junction assembly modulates the interaction of JAM with CASK. Junction assembly strikingly influenced partitioning of CASK (either alone or in association with JAM) between soluble and insoluble fractions of Triton X-100 lysates, with enrichment of CASK in the soluble fraction and reduction of JAM-CASK complexes in the insoluble fraction. Because CASK interacts with protein 4.1 (11), it is conceivable that the enhanced solubility reflects a transient loss of CASK interactions with the cortical F-actin cytoskeleton, even if the molecular interaction(s) primarily re-

sponsible for the observed effect remains unknown. Regardless of the precise molecular mechanism, we propose a dynamic model for the regulation of the JAM-CASK interaction during junction assembly (Fig. 5). In mature junctions, CASK is found in association with JAM and can be coprecipitated in both Triton X-100-soluble and -insoluble fractions of cell lysates. Triton-insoluble JAM-CASK complexes are likely to be associated with the actin cytoskeleton, possibly via protein 4.1 (11). At variance, upon junction assembly, CASK is released from its cytoskeletal associations, and JAM-CASK complexes are almost exclusively detectable in the Triton-soluble fraction outside of the junctions, whereas another subpopulation of JAM likely associates at the junctions with other proteins, such as AF-6 (5), ZO-1, and cingulin (4). We speculate that the interaction with JAM might be instrumental in preventing CASK from being prematurely assembled into nascent junctional complexes.

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Association of Junctional Adhesion Molecule with Calcium/calmodulin-dependent Serine Protein Kinase (CASK/LIN-2) in Human Epithelial Caco-2 Cells

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