A Transmembrane Tight Junction Protein Selectively Expressed on Endothelial Cells and Platelets*

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Searching for cell surface proteins expressed at interendothelial cell contacts, we have raised monoclonal antibodies against intact mouse endothelial cells. We obtained two monoclonal antibodies, 1G8 and 4C10, that stain endothelial cell contacts and recognize a protein of 55 kDa. Purification and identification by mass spectrometry of this protein revealed that it contains two extracellular Ig domains, reminiscent of the JAM family, but a much longer 120-amino acid cytoplasmic domain. The antigen is exclusively expressed on endothelial cells of various organs as was analyzed by immunohistochemistry. Immunogold labeling of ultrathin sections of brain as well as skeletal muscle revealed that the antigen strictly colocalizes in capillaries with the tight junction markers occludin, claudin-5, and ZO-1. Upon transfection into MDCK cells, the antigen was restricted to the most apical tip of the lateral cell surface, where it colocalized with ZO-1 but not with β-catenin. In contrast to JAM-1, however, the 1G8 antigen does not associate with the PDZ domain proteins ZO-1, AF-6, or ASIP/PAR-3, despite the presence of a PDZ-binding motif. The 1G8 antigen was not detected on peripheral blood mouse leukocytes, whereas similar to JAM-1 it was strongly expressed on platelets and megakaryocytes. The 1G8 antigen supports homophilic interactions on transfected Chinese hamster ovary cells. Based on the similarity to the JAM molecules, it is plausible that the 1G8 antigen might be involved in interendothelial cell adhesion.

The integrity of interendothelial cell contacts is vital for the physiological role of the endothelium as the interface between the blood and tissue structures. The control of vascular permeability, leukocyte extravasation, and the formation and outgrowth of blood vessels are dependent on the opening and closure or the dissociation and formation of interendothelial cell junctions (1, 2). Adherens junctions are essential for the integrity of endothelial cell contacts, and VE-cadherin, the most prominent transmembrane protein of adherens junctions, is directly involved in the maintenance of these contacts in vitro as well as in vivo (3–5). Paracellular permeability across endothelial or epithelial cell layers is determined by tight junctions that seal the intercellular space (6). Tight junctions show ion and size selectivity, and their barrier function varies in tightness between endothelial and epithelial cells and also between endothelia in different types of blood vessels and different tissues (7). To understand how the paracellular permeability of tight junctions is regulated and how they are formed, we need to know their molecular composition.

Several proteins have been identified that are associated with the cytoplasmic side of tight junctions, but only a few tight junction proteins are known that span the membrane (8). Occludin was the first tight junction membrane protein that was identified. It contains four transmembrane domains (9) and is found in epithelia as well as endothelia. It is likely to be a functional component of tight junctions, although junctional strands and functional tight junctions also form in the absence of the occludin gene (10). By contrast, members of the newly discovered gene family of the claudins are indeed essential for tight junction formation and can lead to the formation of tight junction strands upon transfection into fibroblasts (11–13). There are presently some 20 members of this family known. They are small tetraspanning membrane proteins with short cytoplasmic N and C termini. Some of the claudins have highly restricted expression patterns. Claudin-5 was found to be expressed by endothelia in a large range of blood vessels in different tissues including muscle and brain (14).

The third type of tight junction-associated membrane proteins is represented by the immunoglobulin supergene family (IgSF) member JAM (junctional adhesion molecule), a protein with two V-type Ig domains (15). JAM is expressed by endothelial as well as epithelial cells and was found to be enriched at tight junctions. JAM was also identified on mouse platelets and dendritic cells (16) and on human neutrophils, monocytes, subsets of lymphocytes, platelets, and red blood cells (17, 18). A mAb1 against JAM can block the extravasation of myeloid cells in two mouse inflammation models (15, 19). It is not yet known whether the inhibitory effect of the antibody is due to interference with control mechanisms that regulate the opening of interendothelial cell contacts or whether it is due to the inhibition of leukocyte-endothelial interactions possibly mediated by JAM. The antibody does not change paracellular per-
meability of endothelial cell monolayers; however, another mAB against human JAM was reported to inhibit transepithelial resistance recovery in epithelial cell monolayers (18). In combination with the recently found association of JAM with the PDZ domain protein ASIP/PAR-3 (20), an essential cytoplasmic factor for the establishment of cell polarity, it is conceivable that JAM is involved in the regulation or formation of tight junctions.

Recently two JAM-related proteins were identified, each containing one V and one C2 type Ig domain (21–23), for which the names JAM-2 and JAM-3 (24, 25) or VE-JAM (21) have been suggested. The subcellular localization at junctions was analyzed for JAM-1 by immunogold electron microscopy (15), revealing its localization at tight junctions and even its close spatial relationship with tight junction strands (26). Based on confocal laser scanning microscopy, JAM-2 was reported to be enriched at the apical site of intercellular contacts of transfected MDCK cells, suggesting its potential to be targeted to the area of tight junctions (23). As judged by immunohistochemistry, this JAM was restricted to endothelial cells in sections of mouse kidney and lymph nodes (23). VE-JAM was reported to be absent from human leukocytes and from epithelia in several human tissues and was found on endothelial cells of various blood vessels (21).

Based on a classical mAb approach, we have found two endothelially specific mAbs with which we identified a 55-kDa protein at interendothelial cell contacts. Identification of the purified protein by mass spectrometry revealed that it is an Ig-SF member containing one V-type and one C2-type Ig domain that is related to the JAMs although different in several aspects from the three known members of this family. Our antigen is identical to the recently described endothelial cell-selective adhesion molecule (ESAM) whose endothelial specificity has been analyzed so far on the RNA level by in situ hybridization (27). Analyzing a panel of mouse tissues by immunohistochemistry, we show here that the ESAM protein is indeed specifically expressed on endothelium and not on epithelium. Although a tagged recombinant form of ESAM had been reported not to colocalize with ZO-1 in transfected MDCK-II cells (27), we found that native ESAM does colocalize with ZO-1 but not with β-catenin in transfected MDCK-C7 cells. More importantly, ESAM was clearly colocalized with the three tight junction markers occludin, claudin-5, and ZO-1 in brain and muscle blood capillaries, as documented by immunogold electron microscopy. ESAM was not found on peripheral blood mouse leukocytes but was strongly expressed on megakaryocytes and activated platelets. The association of ESAM with endothelial tight junctions and its ability to support homophilic adhesion between transfected CHO cells suggest that ESAM might be involved in the control of interendothelial cell contacts.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**Monoclonal antibodies against mouse endothelial cell surface antigens were generated by immunizing rats with intact bEnd.3 mouse endothelioma cells and screening hybridoma supernatants for antibody binding in cell surface enzyme-linked immunosorbent assays as described (28). Positive antibodies were further screened for cell contact staining by immunofluorescence and for endothelial specificity by immunohistochemistry (see below). In this way two antibodies were selected: 1G8 (IgG2a) and 4C10 (IgG2a). Rabbit antibodies against the extracellular domain of ESAM (VE-19) were raised against an ESAM-IgG fusion protein, and rabbit antibodies against the cytoplasmic domain of ESAM except for the last 21 C-terminal amino acids. To purify the polyclonal antibodies, non-IgG proteins were removed from the sera by caprylic acid precipitation. Antigen-specific antibodies of serum VE-19 were affinity-purified on CNBr-Sepharose (Amersham Biosciences), and antibodies against the IgG1 Fc part were removed by incubation with immobilized human IgG1. Antibodies from antiserum VE-2 were affinity-purified on CNBr-Sepharose-immobilized MBP-ESAM fusion protein.

The following antibodies were commercially obtained: mouse mAb clone 14 (IgG1) against β-catenin (Transduction Laboratories, Lexington, KY); rat mAb RAM 34 (IgG2a) against mouse CD34 (Pharmingen, Heidelberg, Germany); rat mAb MECA-79 (29) against peripheral nodal addressin (ATCC, Manassas, VA); rabbit polyclonal antibodies against ZO-1 and occludin (Zymed Laboratories Inc., San Francisco, CA); rabbit serum against von Willebrand factor (DAKO, Hamburg, Germany); and labeled secondary antibodies (Cy5-conjugated goat anti-rat from Jackson Immunoresearch Lab., Inc. West Grove, PA; Alexa goat anti-rabbit from MoBiTec, Göttingen, Germany); gold-conjugated antibodies: goat anti-rat 5 nm, goat anti-rabbit 10 nm, and 15 nm (British Biocell Int., Cardiff, UK). Generation and affinity purification of rabbit polyclonal antibodies against mouse claudin-5 have been described (30).

**Immunos少不了的the IGBAntigen—**Confluent monolayers of bEnd.3 cells were rinsed twice with phosphate-buffered saline, collected by scraping in the same buffer supplemented with 2 mM dithiothreitol and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 20 μg/ml aprotinin, 20 μg/ml leupeptin), harvested by centrifugation, and extracted in lysis buffer (20 mM imidazole, pH 6.8, 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μM leupeptin). The lysates were centrifuged for 10 consecutive centrifugation steps (30,000 × g for 20 min; 90,000 × g for 2 h). The cell lysates were incubated with mAb 1G8 or 4C10 immobilized to CNBr-Sepharose. The immunocomplexes were washed four times with lysis buffer (protease inhibitors omitted), subjected to SDS-PAGE, and analyzed by silver staining.

**Protein Identification by Mass Spectrometry and Data Base Searching—**Gel-separated proteins were reduced, alkylated, and digested in gel according to Ref. 31 and subsequently identified by a two-tier mass spectrometric approach. In the first round, small aliquots (1–2%) of the generated peptide mixtures were analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry on a Bruker REFLEX III (Bruker Daltonik, Bremen, Germany) to yield a peptide mass map. A list of monoisotopic peptide masses was obtained from the spectrum, and this list was used to query a nonredundant protein sequence database (NRDB, ~650,000 entries) at the European Bioinformatics Institute (Hinxton, UK). Correlating the measured peptide masses with theoretical digests of all proteins present in the data base did not lead to the identification of a protein. Therefore, the isolated protein was subjected to quadrupole time-of-flight mass spectrometry (33). Protein digests were desalted and concentrated using microcolumns packed with approximately 100 nl of POROS R2 perfusion chromatography material (Perseptive Biosystems, Framingham, MA). Peptides were eluted with 60% MeOH/5% HCOOH directly into nanospray capillaries (MDS Proteomics, Odense, Denmark). Peptide sequencing by tandem mass spectrometry was performed using a nanoelectrospray tandem mass spectrometer (QSTAR, Sciex, Toronto, Canada). The sequence and mass information contained in the tandem mass spectra were assembled into peptide sequence tags (34) and used for queries in the NRDB and deEST (expressed sequence tags data base, NCBI). Proteins/ESTs were identified by comparing the retrieved sequences with the mass spectrometric data. All data base searches were performed using the program Pepsea (MDS Proteomics). This procedure led to the identification of ESTs that represent parts of the 1G8 sequence that were subsequently used for cloning. Based on these data, it was sufficient to perform MALDI mass spectrometry to identify the 4C10-immunoprecipitated protein as the 1G8 antigen. This second approach was done by TopLab (Martinsried, Germany).

**Cloning of the IGBAntigen—**Mass spectrometric data of immunos少不了的proteins were applied in EST data base searches, ESTs of interest were sequenced, and the retrieved sequences were aligned with each other, with mouse genomic sequences, or with sequences of homologous proteins. This led to the identification of an EST clone containing an open reading frame of 1,185 bp coding for a protein sequence that was covered to 49% by all MS generated sequence data. The sequence and its mass in bEnd.3 cells were confirmed by amplyifying the corresponding cDNA by reverse transcription-PCR from total RNA of bEnd.3 cells; using the sense primer (5′-GGC GTAGCC CTC CCT GGC TCC GGC CC-3′) and the antisense primer (5′-GGC AAGCTT ACA CAA GAG ACC CA CCT GAC T-3′) yielded a single 1.2-kilobase pair product that was subcloned into the pCR82.1/TOPO vector (Invitrogen) after the addition of 3′A overhangs with Platinum Taq (Invitrogen).
DNA sequence analysis on an ABI-377 automated DNA sequencer (Applied Biosystems, Foster City, CA) confirmed that the EST contained start and stop codons.

Expression Vectors—Full-length ESAM eukaryotic expression vectors were constructed by cloning the EcoRI/BstEII inserted cDNA of the EST (AA472096) into linearized pcDNA3 (pcMV5-ESAM) or pcDNA3-ESAM (pcDNA-ESAM) vector. C-terminally truncated ESAM (amino acid residues 1–389, referred to as pcDNA3-ESAM antigen/α5) was cloned by PCR using pcDNA3-ESAM as template and the sense primer (5'-GGC CCA TGG GAA GCA AGA CCT TGA GAG TGC-3') and antisense primer (5'-GGC TCT AGA CTA CTG ACT TGT GAG CAG CAC TCG-3'). The PCR product was subcloned into the multiple cloning site of the pCMV5 vector (Invitrogen) after the addition of 3'A overhangs with platinum Taq (Invitrogen). After digestion of the plasmid with EcoRV/XhoI, the insert was ligated into corresponding sites of pcDNA3-ESAM. Expression vectors encoding fragments of murine ZO-1 (ZO-1/1-3, ZO-1/6–1256), AF-6 (AF-6/full-length), and ASIP (ASIP/full-length) were described previously (20, 35). Expression vectors encoding the PDZ domains of ZO-3 (ZO-3/2–3) and ZO-3 (ZO-3/2–3) were generated by subcloning amino acid residues 1–636 of ZO-2 or amino acid residues 1–495 of ZO-3 into pSecTag (Invitrogen). The cloned cDNAs encoding canine ZO-2 and ZO-3 were kindly provided by Dr. B. Stevenson.

Construction of ESAM-IgG—A cDNA fragment coding for the extra-cellular part of ESAM covering amino acid residues 1–249 (bp 1–747) was amplified from BE3 and BE4 ESAM using the BamHI site containing sense oligonucleotide 5'-GGC GGATCC ATG ATT CTT CAG GCT GGA -3' and the EcoRI site containing antisense oligonucleotide 5'-GAGAATTC ATG ATG GCC CAC ACC-3'. The product was inserted in a pcDNA3 vector in frame and upstream of a fragment of human IgG1 covering bases 553–1803 (hinge, Cl, C2, C3). Prokaryotic expression vectors encoding the C termini of JAM, claudin-1, and claudin-5 fused to GST were described previously (20). GST-ESAM antigen was cloned by generating a cDNA fragment coding for the cytoplasmic part of ESAM (amino acid residues 278–394) into pGEX-KG (36). The respective fragment was obtained from total RNA of bEND.3 cells by reverse transcription-PCR using the XhoI site containing sense primer (5'-GGC CTCGAC AGC ACC TGA CTC GAT GTC-3') and the HindIII site containing antisense primer (5'-GGC AAACCT ACA CAA GAC ACC CA CAC GCT T-3'). For generation of rabbit antibodies against the cytoplasmic part of ESAM, prokaryotic GST and MBP (mamnose-binding protein) fusion proteins were generated that contained the truncated cytoplasmic part of ESAM lacking the C-terminal amino acids, which show high homology to mOCAR. For construction of GST-ESAM-21 and MBP-ESAM-21 (amino acid residues 278–343), the respective fragment of ESAM was amplified by polymerase chain reaction using the XhoI site containing sense primer (5'-GGC CTCGAC AGC ACC TGA CTC GAT GTC-3') and the HindIII site containing antisense primer (5'-GGC AAACCT ACA CAA GAC ACC CA CAC GCT T-3'). For generation of antibody rabbits against the cytoplasmic part of ESAM, prokaryotic GST and MBP (mamnose-binding protein) fusion proteins were generated that contained the truncated cytoplasmic part of ESAM lacking the C-terminal amino acids, which show high homology to mOCAR. For construction of GST-ESAM-21 and MBP-ESAM-21 (amino acid residues 278–343), the respective fragment of ESAM was amplified by polymerase chain reaction using the XhoI site containing sense primer (5'-GGC CTCGAC AGC ACC TGA CTC GAT GTC-3') and the HindIII site containing antisense primer (5'-GGC AAACCT ACA CAA GAC ACC CA CAC GCT T-3'). For generation of antibody rabbits against the cytoplasmic part of ESAM, prokaryotic GST and MBP (mamnose-binding protein) fusion proteins were generated that contained the truncated cytoplasmic part of ESAM lacking the C-terminal amino acids, which show high homology to mOCAR. For construction of GST-ESAM-21 and MBP-ESAM-21 (amino acid residues 278–343), the respective fragment of ESAM was amplified by polymerase chain reaction using the XhoI site containing sense primer (5'-GGC CTCGAC AGC ACC TGA CTC GAT GTC-3') and the HindIII site containing antisense primer (5'-GGC AAACCT ACA CAA GAC ACC CA CAC GCT T-3').

Cell Culture—The following cells were used: murine endothelium bEnd.3 derived from brain capillaries (37) provided by Dr. Werner Risau (Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim, Germany); murine endothelium myEnd from mouse myocard, a gift from Drs. N. Golenhofen and D. Drenckhahn (Julius-Maximilians-University, Würzburg, Germany); and murine retinal carci

Cell Transfection—CHO dhfr− cells were transfected with 20 µg of ESAM-IgG plasmid or pcDNA3 full-length ESAM by electroporation. Stable transfectants were selected with 800 µg/ml G418 (PAN Biotech, Aidenbach, Germany) and maintained in the continuous presence of the selecting drug. Stable transfectants of MDCK C7 expressing full-length ESAM or C-terminally truncated ESAM (ESAM/Δ5) were generated using the same protocol.

Flow Cytometry and Platelet Analysis—Mouse peripheral blood leucocytes were isolated and separated by density gradient centrifugation (Histopaque; Sigma) according to the manufacturer’s instructions. Platelet-rich plasma was obtained from 1 ml of acid citrate dextrose-anticoagulated blood by centrifugation at 125 g for 10 min at room temperature. Platelets were either left unstimulated or activated with thrombin (1 unit/ml) for 1 min at room temperature in the presence of glycine-proline-arginine-proline (1.25 µM) (Bachem) to prevent fibrin polymerization as described (40). The platelets were fixed with Cell Fix (Becton Dickinson, San Jose, CA) for 30 min at room temperature and washed with PBS. The staining was done with the following antibodies: RB40 (anti-mouse P-selectin), VE-2, VE-19 (rabbit polyclonal anti-mouse ESAM antibodies), 1G8, 4C10 (rat monoclonal anti-ESAM antibodies), fluorescein isothiocyanate-conjugated clone M6WReg30 (rat IgG1 mAb against mouse CD41), fluorescein isothiocyanate-conjugated clone R3–3 (iso-type standard rat IgG1), V7C7 (rat IgG2b mAb against mouse endomucin)/CMV75 (mouse ZO-1). All antibody incubations were used at a concentration of 10 µg/ml. As secondary antibodies rhodotoxirhizylin fluorescent and phycocerythrin-conjugated donkey anti-rabbit (H+L) and phycocerythrin-conjugated donkey anti-rabbit IgG (H+L) were used at a dilution of 1:100. All antibody incubations were performed for 30 min at room temperature.

Immunoprecipitation—Cells metabolically labeled with [35S]methionine/cysteine were washed with phosphate-buffered saline and lysed directly in the culture dish in lysis buffer (see above). Insoluble material was removed by two consecutive centrifugation steps (1300 × g × 10 min; 100,000 × g for 20 min). The cell lysates were incubated with affinity-purified antibodies coupled to protein G-Sepharose (Amersham Biosciences) for 1 h or overnight. Immunocomplexes were washed four times with lysis buffer (protease inhibitors omitted) (see above), subjected to SDS-PAGE, and analyzed by fluorography.

Immunoblot Analyses—Proteins of cell lysates or immunoprecipitates were separated on 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes. The filters were blocked in Tris-buffered saline, 0.1% Tween, followed by Blotto (10% dry milk powder in Tris-buffered saline, 0.1% Tween). The membranes were incubated with primary antibodies overnight at 4 °C. The membranes were washed for 10 min at room temperature, and, for control purposes, the first antibody was either omitted or replaced by an irrelevant polyclonal antibody. For stripping, the membrane was incubated in a 1:1 mix of 1% Tween, 0.1 M NaOH, and 1% Triton X-100 at 60 °C for 10 min, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. After removing unbound secondary antibodies by washing with the buffer described above, immunodetection was carried out with a chemiluminescent ECL kit (Pierce/Perbio) according to the manufacturer’s protocol.

In Vitro Binding Assays with GST Fusion Proteins—The GST fusion proteins and the in vitro target ligands used in the pulldown assays are described above, and purification of the GST fusion proteins as well as the GST pull-down assays were performed as described (35).

Immunohistochemical Staining of Mouse Organs—For cryosections, organs and tissues from Balb/c mice were embedded in Tissue Tek OCT compound (Miles, Elkhart, IN), snap frozen, and stored at –80 °C. Sections of 7–12 µm were cut on a freezing microtome, mounted on slides, coated with poly-l-lysine (Menzel-Gläser, Nuln, Germany), and dried. For immunoperoxidase staining, the sections were fixed in acetone for 10 min at 4 °C, followed by reduction of endogenous peroxidase activity with 0.1% hydrogen peroxide, 20 µm sodium azide, for 30 min at room temperature. Nonspecific binding was blocked with 2% bovine serum albumin in PBS for 30 min. The tissue sections were incubated for 1 h with the appropriate primary antibodies diluted in PBS containing 1% bovine serum albumin, followed by incubation with affinity-purified peroxidase-labeled donkey anti-rat IgG or goat anti-rabbit IgG. After the reaction was visualized with 3-amin-9-ethylcarbazole, the tissue sections were counterstained with Mayer’s hematoxylin and mounted. All of the reactions were performed in a humidified chamber at room temperature, and, for control purposes, the first antibody was either omitted or replaced by an isotype- or isotype-matched reagent. For immunofluorescence staining of brain and muscle capillaries, the sections were fixed for 5 min in ethanol at 4 °C followed by 1 min in acetone at room temperature and washed in TBS. Unspecific binding was minimized by incubation for 20 min in 5% (w/v) skimmed milk, 0.3% (w/v) Triton X-100 (Serva, Heidelberg, Germany), and 0.04% (w/v) NaN3 in TBS. The antibodies

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were diluted in the same solution and incubated overnight at 4 °C. Following several washes in TBS, the sections were incubated with secondary antibodies for 1 h at room temperature. The sections were mounted in 90% glycerol/10% TBS containing 1 mg/ml p-phenylene diamine (Sigma) as an anti-bleaching agent. For controls, the primary antibodies were omitted or substituted by unspecific IgG. For double labeling, the controls included cross-over incubation to exclude cross-reaction. For double fluorescent staining of lymphatic tissues, cryostat sections were incubated for 1 h with the biotinylated monoclonal antibodies 1G8 and MECA-79 diluted in PBS containing 1% bovine serum albumin, followed by incubation with Cy-3-labeled streptavidin and dichlorotriazinyl amino fluorescein-labeled goat anti-rat IgM µ chain.

Immunogold Labeling—Post-embedding immunogold labeling was performed on ultrathin sections of Lowicryl-embedded specimens. The tissues were fixed with 1% paraformaldehyde/Hanks’ modified salt solution for 15–30 min. Residual aldehyde was quenched using 50 mM sodium borohydride for 15 min. The specimens were snap frozen by injection into liquid propane at −180 °C in a Leica CPC cryo preparation chamber (Leica, Bensheim, Germany). The frozen specimens were subjected to freeze substitution and low temperature embedding using a Leica APS automatic freeze substitution device. The specimens were freeze substituted in anhydrous methanol containing 0.5% uranyl acetate and embedded in Lowicryl HM20 (Baltic Scientific, Parnawa, Lithuania) and Lowicryl K4M (Polysciences, Eppelheim, Germany), followed by low temperature polymerization using UV radiation. The sections were cut on a Leica Ultracut R at 50–70 nm and mounted on Formvar-coated nickel grids (SCI, Munich, Germany). For on-section labeling, grids were floated on drops of the following solutions and transferred using a nickel-coated copper loop. Initially, the grids were floated for 5 min on TBS followed by blocking buffer (5% (w/v) bovine serum albumin, 0.1% (v/v) cold water fish skin gelatin (Biotrend), 5% (v/v) normal goat serum (Biotrend), and 0.04% NaN₃ in TBS) for 30 min to minimize unspecific binding. The primary antibody was diluted in incubation buffer (0.8% (w/v) bovine serum albumin, 0.1% (v/v) cold water fish skin gelatin, and 0.04% NaN₃ in TBS), and the sections were incubated overnight at 4 °C. The sections were washed several times in incubation buffer and subsequently incubated for 1 h with secondary antibodies diluted 1:50 in incubation buffer. Following several washes in the same buffer and in TBS, the sections were postfixed with 2% glutaraldehyde and finally washed in distilled water. The sections were contrasted with 1% uranyl acetate and/or lead citrate. The specimens were observed and documented with a Zeiss EM 10 electron microscope.

Conventional Electron Microscopy—The specimens were immersion fixed in 2.5% glutaraldehyde in Hanks’ modified salt solution, postfixed in 1% OsO₄ in 0.1 M cacodylate buffer, and dehydrated in a series of ethanol steps. For contrast enhancement, the 70% ethanol was saturated with uranyl acetate. Dehydration was completed in propyleneoxide, and the specimens were embedded in Araldite (Serva, Heidelberg, Germany). Ultrathin sections were mounted on pioloform-coated copper grids and contrasted with lead citrate. The specimens were observed and documented with a Zeiss EM 10 electron microscope.

Immunofluorescence of bEnd.3 and Transfected MDCK Cells—The cells were plated on polycarbonate filters (Transwell™, 0.4-µm pore size, 6.5-mm diameter, from Costar, Corning, NY) coated with laminin (Sigma). The filters were rinsed twice in PBS and subsequently fixed in methanol for 10 min at −20 °C. After rehydration in PBS, the cells were blocked with 10 ng/ml bovine serum albumin in PBS to minimize unspecific binding. The cells were incubated with primary and secondary antibodies in blocking buffer for 20 min at room temperature, followed by three times washing with phosphate-buffered saline after each incubation. To visualize the primary antibodies, appropriate dichlorotriazinyl amino fluorescein- or Cy3-conjugated anti-rat, antimouse, or anti-rabbit secondary antibodies were used. Finally, the cells were mounted with fluorescent mounting medium (DAKO, Hamburg, Germany) and viewed with a TCS confocal imaging system (Leica, Bensheim, Germany). Controls included incubation of fixed cells with secondary antibodies alone. In addition, single label immunofluorescence for each antibody was performed as a control in double label immunofluorescence experiments.

RESULTS

Identification of a 55-kDa Endothelial Cell Contact Protein—To identify cell surface proteins potentially involved in the regulation of interendothelial cell contacts, we generated rat mAbs against intact mouse bEnd.3 endothelioma cells. In a sequential screening procedure mAbs were first tested for binding to intact bEnd.3 cells in a cell surface enzyme-linked immunosorbent assay, followed by immunofluorescence staining of bEnd.3 monolayers. Two mAbs, 1G8 and 4C10, were found that specifically stained cell contact areas of bEnd.3 cells, as shown for 1G8 in Fig. 1A. In immunoprecipitations of metabolically labeled bEnd.3 cells, both antibodies recognized a protein of 55 kDa, whereas no antigen was detected on the epithelial cell line CMT (Fig. 1B). The antigen was also detected with rabbit antibodies VE-19 and VE-2 in immunoblots on bEnd.3 and myEnd endothelioma cells (Fig. 1D and data not shown). These antisera were raised against distinct domains of the antigen upon cloning (see below). In preliminary studies on few tissues, we analyzed the distribution pattern of the 1G8 and

FIG. 1. Identification of a 55-kDa endothelial cell contact protein on bEnd.3 cells. A, indirect immunofluorescence staining of bEnd.3 cells with mAb 1G8 (left panel) or no antibody (right panel). B, bEnd.3 endothelioma and CMT carcinoma cells (as indicated below) were metabolically labeled with [35S]methionine/cysteine, and cell lysates were subjected to immunoprecipitations with a control mAb or the mAb 1G8 or 4C10, as indicated above. Immunoprecipitates were electrophoresed and visualized by fluorography. Molecular mass markers (in kDa) are indicated on the left. C, cell lysates of unlabeled bEnd.3 or CMT cells (as indicated below) were subjected to affinity isolation experiments with a subclass matched control mAb or the mAb 1G8 (as indicated above). Isolated proteins were electrophoresed and visualized by silver staining. Molecular mass markers (in kDa) are indicated on the left. D, cell lysates of bEnd.3 and myEnd cells were electrophoresed, transferred to filters, and immunoblotted with VE-19 (left panel) or an antibody against β-catenin (right panel).
the 4C10 epitopes on cryostat sections of mouse tongue. Endothelial cells were the only cells stained in this tissue (see below).

Cloning of the Endothelial Antigen Defined by mAb 1G8 and 4C10 Reveals Its Identity with the Recently Discovered ESAM—To clarify the identity of the 1G8 antigen, we purified it by immunopurification from lysates of bEnd.3 cells. Fig. 1C shows a silver-stained sample of a purification from 3.6 \( \times 10^7 \) bEnd.3 cells. Upon upscaling of the purification protocol and by using a CNBr-Sepharose-based antibody matrix, we obtained enough material for mass spectrometric analysis of tryptic peptides isolated from silver-stained SDS-PAGE gels. Simple peptide mass maps generated by MALDI MS failed to yield identification of the protein. Therefore, tandem mass spectra for partial peptide sequencing were acquired for a number of peptides from the unseparated tryptic peptide mixture. The information contained in tandem mass spectra was assembled into peptide sequence tags and searched against the NRDB. At the time of the experiment, no significant hit to any sequence in NRDB was found. The same data were then searched against the dBEST (NCBI), and three of the sequenced peptides unambiguously identified a noncommercially available EST (GenBank™ accession number AA520297). Further data base searches in combination with sequence alignments of homologous proteins (members of the cortical thymocyte Xenopus (CTX) family, especially mCAR) and mouse genomic sequences led to the identification of previously unconsidered mouse ESTs. The ESTs of interest were resequenced and aligned with each other, with homologous proteins, and with mouse genomic sequences in a second survey. One of the clones we retrieved, EST AA472099 contained the putative full-length cDNA of a novel cell surface protein. In total, 49% of the 1G8 antigen sequence was represented by all available MS data. We verified the existence and accuracy of the sequence by reverse transcription-PCR analysis using total RNA of bEnd.3 cells. A peptide mass map by MALDI MS of tryptic peptides of the immunoposlated 4C10 antigen revealed the identity of this antigen with the 1G8 antigen.

The cDNA encoding this molecule predicts a signal peptide, V-type and C2-type immunoglobulin domains, a single putative transmembrane region, and a cytoplasmic tail of 120 amino acids. The novel protein belongs to the cortical thymocyte Xenopus (CTX) subset of the immunoglobulin superfamily (41), which also contains the three members of the JAM subfamily. Upon completion of our cloning experiments Quertermous and co-workers (27) published a cDNA from human umbilical vein endothelial cells cloned by subtractive suppression hybridization in an attempt to identify genes preferentially expressed upon tube formation. They named this molecule and its mouse homologue ESAM. The sequence of the 1G8 antigen is identical to that of mouse ESAM.

Selective Expression of ESAM on Endothelial Cells of Various Tissues—Based on in situ hybridization analysis, the mRNA of ESAM had been reported to be expressed on endothelial cells (27). Using our mAbs we analyzed the tissue distribution of the ESAM protein by immunohistochemistry, extending our preliminary studies on mouse tongue to heart, kidney, intestine, and lymph nodes. Specimens were stained with 1G8 (Figs. 2 and 3) or 4C10 (data not shown), and both antibodies gave identical results. ESAM was found in all tissues examined, and its expression was exclusively detected on vascular endothelium. The results are shown for sections of tongue, heart, and kidney (Fig. 2). Endothelium of arterioles, venules as well as capillaries, was positive. In addition, capillaries of glomeruli as well as the endocard were specifically stained. Of special interest for us were high endothelial venules (HEV) in lymph nodes, which are the sites for lymphocyte migration into lymph nodes. As shown in Fig. 3, HEV were intensely stained by 1G8. The identity of these blood vessels with HEV was demonstrated by double label immunofluorescence with the mAb MECA-79 against peripheral node addressins (B and E), or no first antibody (C). First antibodies were detected with peroxidase-conjugated secondary antibodies. The bar represents 50 \( \mu \)m.

FIG. 2. Tissue distribution of the 1G8 antigen/ESAM. Cryostat sections of tongue (A–F), heart (G–M), and kidney (N–P) were incubated either with the mAb 1G8 (A, D, G, K, and N), polyclonal rabbit antibodies against von Willebrand factor (B, E, H, and L), a mAb against CD34 (O), or no first antibody (C, F, I, M, and P). First antibodies were detected with peroxidase-conjugated secondary antibodies. The bar represents 50 \( \mu \)m.

FIG. 3. ESAM is expressed on HEV of lymph nodes. Cryostat sections of mouse mesenteric lymph nodes were incubated with the mAb 1G8 (A and D), mAb MECA-79 against peripheral node addressins (B and E), or no first antibody (C). First antibodies were detected either with peroxidase-conjugated secondary antibodies (A–C) or with fluorescein isothiocyanate conjugated secondary antibodies (D–F). A merge of D and E is shown in F. The bar represents 50 \( \mu \)m.
ESAM Is a Tight Junction-associated Membrane Protein in Capillaries—Despite important structural differences (see below), ESAM is clearly related to the JAMs. Because JAM-1 has been demonstrated to be associated with tight junctions of epithelial cells (15, 26), we analyzed the subcellular localization of ESAM on endothelial cells of blood capillaries. First, the localization was analyzed by confocal laser scanning microscopy of cryostat sections of mouse brain and muscle. The sections were double stained with 1G8 (or 4C10) and polyclonal antibodies against the tight junction markers ZO-1, occludin, and claudin-5. As shown in Fig. 4, in each case staining for ESAM colocalized well with the respective tight junction molecule. However, because of the specific morphological characteristics of interendothelial junctions where tight and adherens junctions are intermingled (42), a possible tight junction association of an antigen can only be studied on the ultrastructural level. Therefore, we performed immunogold electron microscopy on ultrathin sections of mouse brain and muscle. The sections were double labeled with 1G8 (or 4C10) and rabbit antibodies against ZO-1, occludin, or claudin-5. Rat and rabbit antibodies were detected with secondary reagents conjugated to differently sized immunogold particles. The 5-nm gold particles labeling ESAM were observed in direct adjacency to the protein containing the cytoplasmic domain of ESAM except for the last 21 amino acids. The latter were omitted because they display substantial sequence homology to another member of the cortical thymocyte Xenopus family, the mouse coxsackie virus and adenovirus receptor (mCAR). A second antiserum (VE-19) was raised against an ESAM-IgG fusion protein containing the complete extracellular part of ESAM. Both antisera were affinity-purified on their antigen. In immunohistochemistry, affinity-purified antibodies gave results identical to those of the two mAbs, verifying the exclusive expression of ESAM on endothelium in the analyzed tissues (not shown). Both polyclonal antibodies specifically recognized a single protein of 55 kDa in immunoblots on mouse endothelioma cells, demonstrating the specificity of the antibody preparations (Fig. 1D and data not shown).

**Fig. 4.** ESAM colocalizes with ZO-1, claudin-5, and occludin in mouse capillaries, as judged by confocal laser scanning microscopy. Blood capillaries in cryostat sections of mouse brain (A–I) and mouse skeletal muscle (J–L) were double labeled either for ESAM (A, D, G, and J) or the tight junction markers ZO-1 (B and K), claudin-5 (E), or occludin (H). Merged images of double labeled sections are shown in the right panels (C, F, I, and L). Staining for ESAM and the three tight junction markers colocalized well (yellow). The bar represents 10 µm.

In ultrathin sections of mouse brain capillaries, as judged by double immunogold labeling, ultrathin sections of mouse brain capillaries were stained for ESAM with 5-nm gold particles (B–D) and for ZO-1 (B), claudin-5 (C), and occludin (D) with 10-nm gold particles. A, conventional ultrathin section of a capillary with tight junction kisses (arrowheads), L, lumen. The inset in B shows the framed area in greater detail. Scale bar in A represents 0.17 µm; scale bars in B–D represent 0.1 µm.
revealed colocalization of both antigens when the monolayer of the filter-grown cells was observed from the top (Fig. 7A). In contrast, the staining pattern of /H9252-catenin could clearly be resolved from that of ESAM and did not overlap at numerous sites (Fig. 7A). A similar result was obtained if the cells were analyzed in x-z views. ESAM was clearly detected at a more apical site than /H9252-catenin, whereas it colocalized well with ZO-1 (Fig. 7B). Interestingly a truncated form of ESAM lacking the last five C-terminal amino acids and thereby lacking its PDZ domain-binding motif was normally targeted to cell contacts (Fig. 7A). Furthermore, just like full-length ESAM, it colocalized with ZO-1 but not with /H9252-catenin, as revealed by viewing the cells in the x-y position (Fig. 7A) as well as in the x-z position (Fig. 7B). We conclude that native ESAM is targeted to the region of tight junctions between MDCK cells and that this targeting is independent of the PDZ domain-binding motif.

None of the PDZ Domain Proteins That Associate with JAM-1 Can Bind to ESAM—JAM-1 binds to the tight junction-associated PDZ domain proteins ZO-1, ASIP/PAR-3, and AF-6, and several claudins bind to ZO-1, ZO-2, and ZO-3. Because ESAM also contains a PDZ domain-binding motif, we tested whether any of those PDZ domain proteins would associate with ESAM. In GST pull-down experiments, we incubated a GST fusion protein containing the cytoplasmic domain of ESAM with various PDZ domain proteins that had been in vitro translated and labeled with [35S]methionine. In the case of AF-6 and ASIP, the full-length proteins were synthesized, whereas in the case of ZO-1, ZO-2, and ZO-3, large fragments containing all three PDZ domains were synthesized, and in the case of ZO-1 a second large fragment additionally containing the SH-3 and guanylate kinase domain was synthesized. As shown in Fig. 8, none of the PDZ domain proteins bound to the GST-ESAM fusion protein. In contrast a GST-JAM fusion protein containing the C terminus of JAM captured ZO-1, AF-6, and ASIP and GST fusion proteins containing the C termini of claudin-1 or claudin-5 bound well to ZO-1, ZO-2, and ZO-3. These interactions were specific because neither a GST-JAM-Δ9 fusion protein lacking the PDZ-binding motif nor GST alone associated with any of the in vitro translated proteins. Because our results with the GST-ESAM protein were negative, we had to confirm whether this fusion protein was indeed intact. Therefore, sub-

![Fig. 6. ESAM colocalizes with ZO-1 and claudin-5 in mouse skeletal muscle capillaries, as judged by double immunogold labeling.](image-url)
GST alone (ESAM). Beads loaded with the fusion proteins were incubated with GST (ESAM) or GST alone (GST). Beads loaded with the fusion proteins were incubated with in vitro translated and [35S]methionine-labeled recombinant protein fragments of ZO-1 containing the three PDZ domains (ZO-1/PDZ1–3), a longer fragment of ZO-1 containing the PDZ domains, the SH-3 domain, the guanylate kinase domain, and the alternatively spliced a-region (ZO-1/166–1256), a fragment of ZO-2 containing all three PDZ domains (ZO-2/PDZ1–3), an analogous fragment of ZO-3 (ZO-3/PDZ1–3), and the full-length proteins of AF-6 (AF-6/full-length), and ASIP (ASIP/full-length). Specifically bound proteins were eluted from the beads, electrophoresed, and visualized by fluorography. None of the analyzed PDZ domain proteins bound to the cytoplasmic tail of ESAM.

To test whether ESAM could support homophilic aggregation—To test whether ESAM could support homophilic interactions, we stably transfected full-length ESAM into CHO cells. Fig. 9 shows the monolayer of a mixture of untransfected and ESAM-transfected CHO cells. The subcellular distribution of ESAM in the merged image, ESAM clustered at sites of cell-cell contact between transfected cells (arrowheads), but not between transfected and nontransfected cells (arrows), indicating that homophilic interactions are required for cell contact recruitment of ESAM. The bar represents 20 μm.

**DISCUSSION**

With the help of two mAbs we have found a 55-kDa endothelial transmembrane protein, located at interendothelial cell contacts. Purification, identification by mass spectrometry, and cloning defined the protein as a member of the Ig supergene family related to the JAM subfamily. During the course of our study the same gene was found by a molecular genetic approach and named ESAM (27). Our major findings are as follows. First, the ESAM protein is indeed selectively expressed on endothelial cells in various organs. So far the evidence for endothelial specificity had been based on Northern analysis and in situ hybridization (27). Second, ESAM is located at tight junctions in close spatial relationship to ZO-1, occludin, and claudin-5, which is in contrast to what had been expected from published results on ESAM-transfected MDCK-II cells (27). Third, despite similarities to the JAMs with respect to structure and tight junction association, we found that none of the PDZ domain proteins that bind to JAM-1 can bind to ESAM. Fourth, ESAM is not significantly expressed on peripheral blood neutrophils, monocytes, and B and T cells but is strongly expressed by megakaryocytes and platelets.

Besides megakaryocytes, endothelial cells were the only cell type that was exclusively stained with our mAb by immunohistochemistry in various tissues. Importantly, this was verified with polyclonal antibodies against the cytoplasmic domain of ESAM, which rules out the possibility that other cell types might express ESAM carrying post-translational modifications that might mask the epitopes for the mAb. These results are largely in agreement with the Northern blot analysis and in situ hybridization results reported before that were mainly based on the analysis of embryonal tissue and cell lines (27). Adult tissues had only been successfully analyzed by Northern blot analysis and in situ hybridization (27). Second, ESAM is located at tight junctions in close spatial relationship to ZO-1, occludin, and claudin-5, which is in contrast to what had been expected from published results on ESAM-transfected MDCK-II cells (27). Third, despite similarities to the JAMs with respect to structure and tight junction association, we found that none of the PDZ domain proteins that bind to JAM-1 can bind to ESAM. Fourth, ESAM is not significantly expressed on peripheral blood neutrophils, monocytes, and B and T cells but is strongly expressed by megakaryocytes and platelets.

ESAM mediates homophilic interactions between transfected CHO cells. The subcellular distribution of ESAM in a mixed culture of untransfected and ESAM-transfected CHO cells was determined by indirect immunofluorescence staining (ESAM, red). To visualize all cells the same culture dish area was stained with antibodies against the intracellular antigen HSP-90 (HSP-90, green). As shown in the merged image, ESAM clustered at sites of cell-cell contact between transfected cells (arrowheads), but not between transfected and nontransfected cells (arrows), indicating that homophilic interactions are required for cell contact recruitment of ESAM. The bar represents 20 μm.

**FIG. 8.** ESAM does not bind to any of the known tight junction associated PDZ domain proteins. GST pull-down experiments were performed with GST fusion proteins containing either the complete cytoplasmic domain of JAM (JAM) or the truncated cytoplasmic domain lacking the last nine C-terminal amino acids (JAM9) or the cytoplasmic tails of claudin-1 (clau-1), claudin-5 (clau-5), ESAM (ESAM) or GST alone (GST). Beads loaded with the fusion proteins were incubated with in vitro translated and [35S]methionine-labeled recombinant protein fragments of ZO-1 containing the three PDZ domains (ZO-1/PDZ1–3), a longer fragment of ZO-1 containing the PDZ domains, the SH-3 domain, the guanylate kinase domain, and the alternatively spliced a-region (ZO-1/166–1256), a fragment of ZO-2 containing all three PDZ domains (ZO-2/PDZ1–3), an analogous fragment of ZO-3 (ZO-3/PDZ1–3), and the full-length proteins of AF-6 (AF-6/full-length), and ASIP (ASIP/full-length). Specifically bound proteins were eluted from the beads, electrophoresed, and visualized by fluorography. None of the analyzed PDZ domain proteins bound to the cytoplasmic tail of ESAM.

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Based on immunogold electron microscopy, we found ESAM directly associated with three tight junction markers, ZO-1, occludin, and claudin-5 in capillaries of brain and muscle. Importantly, we did not detect ESAM at sites devoid of any of these marker proteins, strongly suggesting the tight junction association of ESAM. The only other transmembrane protein that has been localized on the ultrastructural level at tight junctions in addition to occludin and the claudins is JAM-1. In contrast to our study, JAM-1 was analyzed in epithelial tissue (15) and in cultured epithelial cells (26).

The tight junction localization that we describe here for native ESAM in blood capillaries is in contrast to the subcellular localization that was reported for transfected ESAM in MDCK II cells (27) where recombinant ESAM with a C-terminal amino acids containing the PDZ-binding motif was used a FLAG-tag at the C terminus that might have disturbed the targeting to tight junctions. However, this is not the most likely explanation because a truncated form of ESAM lacking the last five C-terminal amino acids containing the PDZ-binding motif was efficiently targeted to tight junctions (this study). An alternative explanation for the different results might be that each study used different MDCK subclones. MDCK II cells are known to establish monolayers of low electrical resistance. By contrast, the MDCK subclone that we used were C7 cells, which were selected for high resistance (38, 39), similar to MDCK I cells. It is possible that differences between these two cell types in the molecular composition of tight junctions might be related to the different targeting of ESAM. Interestingly, it was shown recently that MDCK I and II cells both express claudin-1 and claudin-4, whereas only MDCK II cells additionally express claudin-2 (43). Transfection of MDCK I cells with claudin-2 converted the tight junctions of MDCK I cells from the tight to the leaky strand type (43). Whether such differences are related to the different localization of ESAM in these cells will be interesting to analyze in the future.

ESAM shares several important features with the three members of the JAM family. Beside clear structural similarities within the extracellular parts of the molecules, ESAM is associated with tight junctions similarly as JAM-1 (26) and probably also JAM-2 (23). For JAM-1 and JAM-2 this has only been studied in transfected epithelial cells (for JAM-2 only on the light microscope level) and for JAM-1 also in epithelia of sections of mouse duodenum (15). None of the JAMs has yet been analyzed for their subcellular distribution in endothelia. In light of the association of ESAM with tight junctions, it is remarkable that, in contrast to JAM-1, it is not expressed on epithelial cells in any of the tissues we analyzed. ESAM shares this lack of expression on epithelium with JAM-2 and JAM-3. With JAM-1, ESAM shares the prominent expression in megakaryocytes and platelets (16, 44, 45). The physiological function of JAM on platelets is unclear, but it was reported that antibodies against JAM-1 (also called the F11 receptor) can cause platelet activation (44, 45) and that activation of platelets can induce phosphorylation of JAM-1 (46). Interestingly, JAM-1 was reported to be constitutively expressed on the sur-

**FIG. 10. ESAM is expressed on megakaryocytes and platelets.** A–C, double immunofluorescence of a cryostat section of mouse spleen with fluorescein isothiocyanate-conjugated anti-GPIbIIa (A) and Cy-3-labeled anti-ESAM (B). The merged image is shown in C. The scale bar represents 50 μm. D and E, nonactivated and activated platelets were analyzed by flow cytometry for the surface expression of ESAM with polyclonal antibody VE-19 (D) and mAb 1G8 (E): ESAM staining of nonactivated platelets (green), ESAM staining of activated platelets (red), and staining with negative control antibodies of nonactivated platelets (black) and of activated platelets (blue). F, lysates of purified platelets (lanes 1 and 3) and of bEnd.3 cells (lanes 2 and 4) were analyzed in immunoblots with polyclonal antibody VE-19 (lanes 1 and 2) and secondary antibody only (lanes 3 and 4).
face of platelets and to be absent from granules (46). In contrast, we found for ESAM that it is up-regulated on the surface of platelets upon activation by thrombin. The rapid appearance of ESAM on the surface of platelets is most likely due to its storage in granules. It is surprising that a protein like ESAM that is constitutively expressed on the surface of endothelia is apparently targeted to storage granules inside of platelets. Other proteins such as P-selectin or von Willebrand factor are stored in granules in both cell types. In combination with its ability to support homophilic cell adhesion, ESAM could potentially participate in platelet aggregation.

The physiological role of the JAMs on leukocytes is not yet understood. In the mouse JAM-1 was found on B cells but not on T cells and myeloid cells (16), whereas human JAM-1 was found on most types of leukocytes (17, 18). JAM-3 (which is identical to JAM-2 named by Arrand-Lions et al. (23)) is absent from most human leukocytes but is expressed on phorbol 12-myristate 13-acetate-activated peripheral T lymphocytes (25). This JAM (23) and JAM-1 are able to mediate homophilic interactions, although these interactions are too weak to support cell-cell aggregation in transfected cells. Strong cell-cell adhesion activity was reported for the heterophilic interaction between JAM-2 and JAM-3 (25). In combination with the expression of JAM-3 on activated T cells (25), this allows the attractive hypothesis that such T cells interact with JAM-2 on endothelia during leukocyte extravasation. The inhibitory effect of an anti-JAM-1 antibody on the accumulation of neutrophils and monocytes in the cerebrospinal fluid in a meningitis model raised speculations about a heterophilic ligand on leukocytes (19). Because we did not detect ESAM on any type of leukocyte, it will be interesting to search for heterophilic ligands and to test whether ESAM can bind to any of the members of the JAM family.

Despite the similar localization of ESAM and JAM-1 at tight junctions, ESAM does not bind to any of the PDZ domain proteins that associate with JAM-1. This clearly indicates that ESAM differs in its functions from JAM-1 or at least mediates its functions via interacting with different partner molecules. The fact that it can mediate homophilic interactions similarly to JAM-1 suggests that it might also be involved in the regulation of interendothelial cell contacts.

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