Homophilic Interaction of Junctional Adhesion Molecule*

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Junctional adhesion molecule (JAM) is an integral membrane protein that belongs to the immunoglobulin superfamily, localizes at tight junctions, and regulates both paracellular permeability and leukocyte transmigration. To investigate molecular determinants of JAM function, the extracellular domain of murine JAM was produced as a recombinant soluble protein (rsJAM) in insect cells. rsJAM consisted in large part of noncovalent homodimers, as assessed by analytical ultracentrifugation. JAM dimers were also detected at the surface of Chinese hamster ovary cells transfected with murine JAM, as evaluated by cross-linking and immunoprecipitation. Furthermore, fluid-phase rsJAM bound dose-dependently solid-phase rsJAM, and such homophilic binding was inhibited by anti-JAM Fab BV11, but not by Fab BV12. Interestingly, Fab BV11 exclusively bound rsJAM dimers (but not monomers) in solution, whereas Fab BV12 bound both dimers and monomers. Finally, we mapped the BV11 and BV12 epitopes to a largely overlapping sequence in proximity of the extracellular amino terminus of JAM. We hypothesize that rsJAM dimerization induces a BV11-positive conformation which in turn is critical for rsJAM homophilic interactions. Dimerization and homophilic binding may contribute to both adhesive function and junctional organization of JAM.

Junctional Adhesion Molecule (JAM)[1] is a member of the immunoglobulin (Ig) superfamily and is composed of an extracellular domain (comprising two Ig loops), a single-pass transmembrane region, and a short cytoplasmic tail. JAM localizes at tight junctions in endothelial and epithelial cells (1), and its subcellular distribution is modulated by inflammatory cytokines (2). JAM is also expressed on circulating leukocytes (3) and platelets (4), as well as in lymphoid organs (5). A role for JAM in adhesion is supported by the finding that the anti-JAM monoclonal antibody (mAb) BV11 inhibits the transendothelial migration of leukocytes both *in vitro* and *in vivo* (1, 6), a multi-step process that involves several adhesive and de-adhesive interactions (7). In addition, when exogenously expressed in Chinese hamster ovary (CHO) cells, JAM establishes adhesive cell-cell contacts and decreases paracellular permeability. Interestingly however, JAM does not localize at intercellular junctions when the JAM transfectants contact non-transfected cells, suggesting that JAM may mediate intercellular adhesion in an homophilic manner (1).

At the present time, the mechanisms of both JAM-mediated adhesion and mAb BV11-dependent inhibition are unknown. *Homotypic* binding between JAM molecules expressed on adjacent endothelial cells might constitute a barrier for circulating leukocytes. Alternatively, *heterotypic* binding of endothelial JAM to leukocyte JAM might guide transmigrating leukocytes across interendothelial junctions. As a consequence, the anti-JAM mAb BV11 might inhibit transmigration by either *strengthening* homotypic or *weakening* heterotypic interactions between JAM molecules. Defining the molecular organization of JAM may help understand the adhesive function of JAM and the anti-migratory action of mAb BV11.

Here, we have characterized recombinant soluble JAM (rsJAM) and native transmembrane JAM by biophysical and biochemical methods. Additionally, we have mapped the BV11 epitope and evaluated the effect of the antibody on the structural properties of rsJAM. Results from this study support a model in which endothelial and leukocyte JAM adhere to each other to facilitate transmigration and suggest that mAb BV11 may inhibit JAM function by counteracting such homophilic interaction.

EXPERIMENTAL PROCEDURES

Antibodies—Production of rat anti-JAM mAbs BV11 and BV12 has been described in detail (1). Anti-JAM mAb BV20 was produced by immunizing Lewis rats with a fusion protein consisting of the extracellular domain of murine JAM and the Fc portion of human Igs. Antibodies were purified from spent hybridoma culture medium by affinity chromatography on rabbit anti-rat Ig Sepharose, followed by anion exchange chromatography and gel filtration. Fab fragments were generated by papain digestion and separated from Fc by anion exchange chromatography and gel filtration. The ability of Fab BV11 and Fab BV12 to bind rsJAM was confirmed to be identical to complete Igs, as measured by direct enzyme-linked immunosorbent assay.

Expression of rsJAM—cDNA encoding the extracellular domain of murine JAM was cloned into the pFAST BAC1 vector (Life Technologies, Inc.) as a *Kpn*I/*Hin*dIII restriction fragment coding for amino acids 1–238. Recombinant virus was generated in DH10 Bac1 cells (Life Technologies, Inc.). Sf9 cells were infected with selected virus clones

[1] The abbreviations used are: JAM, junctional adhesion molecule; BS³, bis(sulfosuccinimidyl)suberate; CAM, cell adhesion molecule; CHO, Chinese hamster ovary; ICAM, intercellular adhesion molecule; Ig, immunoglobulin; mAb, monoclonal antibody; rsJAM, recombinant soluble junctional adhesion molecule; SESIA, single epitope sandwich immunoassay; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

and cultured in TC100 medium (BioWhittaker) with 2% (v/v) fetal calf serum at 27 °C. Expression of rsJAM was monitored by immunoassay as described below.

Purification of rsJAM—Spent Sf9 medium obtained 72 h after infection with recombinant baculovirus was loaded onto an immunoaffinity column packed with the anti-JAM mAb BV12 bound to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). rsJAM was eluted with 0.1 M glycine (pH 2.8). The eluate was immediately neutralized with sodium bicarbonate and then buffered with 10 mM Tris-HCl to pH 8.0 and further purified by anion exchange chromatography (Resource Q, Amersham Pharmacia Biotech) eluting the protein with a linear gradient of 0–0.5 M NaCl. The main peak was analyzed by NH_2-terminal sequencing and matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis. A major fraction of rsJAM had its amino terminus at residue Lys[27], while a minor fraction started at residue Ser[22]. Gel exclusion chromatography (Superdex 200) in PBS showed a single size peak at about 50 kDa. For large scale rsJAM production, Sf9 cells were grown in 25-liter bioreactors to a density of 2–2.5×10^6 cells/ml prior to infection with recombinant baculovirus at a multiplicity of infection of 1–3.

Single Epitope Sandwich Immunoassay (SESIA) (8)—96-well Maxisorb immunoplates (Nunc International, Roskilde, Denmark) were coated with the "capture" anti-JAM mAb BV12 (10 μg/ml, in 100 mM sodium bicarbonate (pH 8.1)). The plate was blocked with blocking buffer containing 50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40 (v/v), 0.25% gelatin (w/v), and 1% BSA (w/v) for 2 h, and washed three times with 50 mM Tris-HCl, 140 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40 (pH 7.5). The rsJAM-containing samples were diluted, unless otherwise stated, with blocking buffer together with peroxidase-conjugated mAb BV12 (1:1,000) as detecting antibody. After 2 h, the plate was washed and the color reaction was started by addition of tetramethyl benzidine and hydrogen peroxide according to standard enzyme-linked immunosorbent assay protocols. The optical densities were read with a model 3550 Microplate Reader (Bio-Rad).

Analytical Ultracentrifugation—Sedimentation equilibrium runs were performed on an Optima XL-A analytical ultracentrifuge (Beckman, Palo Alto, CA) at 10,000 and $18,000 \times g$ at 20 °C. The concentrations used were 12 and 6 μM for rsJAM and (either BV11 or BV12) Fab fragments, respectively, in a buffer consisting of 10 mM phosphate and 150 mM NaCl (pH 7.0). The equilibrium absorbance profiles were recorded after 16 h at 280 nm. Absorbance of rsJAM and Fabs at 280 nm was determined in separate experiments. Analysis of the profiles was performed with the Beckman software package and with the program DISCREEQ (9), assuming a partial specific volume of 0.720 cm^3/g for rsJAM, according to the amino acid sequence, and 0.735 cm^3/g for Fab fragments. To control protein recovery, absorbance was measured both at the beginning (at slow rotational speed) and at the end (at the equilibrium) of each experiment. At the equilibrium, absorbance was obtained by computing the integral of the radial concentration from the meniscus to the bottom of the sedimentation cell. As the initial and the final absorbance measurements gave identical values in all the experiments, protein recovery was always complete.

Immunoprecipitation—Confluent monolayers of CHO cells (2×10^7) transfected with either full-length murine JAM or expression vector alone (1) were washed twice with PBS and incubated for 2 h at room temperature in PBS with 1.25 mM bis(sulfosuccinimidyl) suberate (BS^3), a non-cleavable and membrane-impermeable cross-linker (Pierce). In parallel, rsJAM (100 μg/ml) was treated similarly with BS^3. Cells were lysed (for 10 min at 4 °C) with lysis buffer containing 20 mM Tris-HCl, 1% Triton X-100 (v/v), 0.1% SDS (v/v), and protease inhibitors (pH 7.5). The lysates were centrifuged, diluted 1:10 with blocking buffer, and incubated with the anti-JAM mAb BV20 (coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech)) for 14 h at 4 °C on a rotating wheel. Beads were washed two times with 20 mM Tris-HCl, 0.1% Triton X-100 (pH 7.5), boiled with reducing sample buffer containing 1% SDS (w/v) and 1% β-mercaptoethanol (v/v), and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose filter by electroblotting. The filter was blocked with 5% delipidated milk in Tris-buffered saline plus Tween 20 and incubated overnight in the same buffer with horseradish peroxidase-conjugated mAb BV20. Blots were developed using the ECL peroxidase substrate (Pierce).

Self-assembly Assay—rsJAM was biotin-labeled with NHS-biotin (Pierce). As assessed by sedimentation equilibrium centrifugation, the amount of dimers was reduced to about one-half upon biotinylation. Another aliquot of rsJAM from the same batch was dissolved to 500 nM in sodium carbonate buffer (pH 9.6) and immobilized on 96-well Maxisorb plates during an overnight incubation at 4 °C. Wells were washed
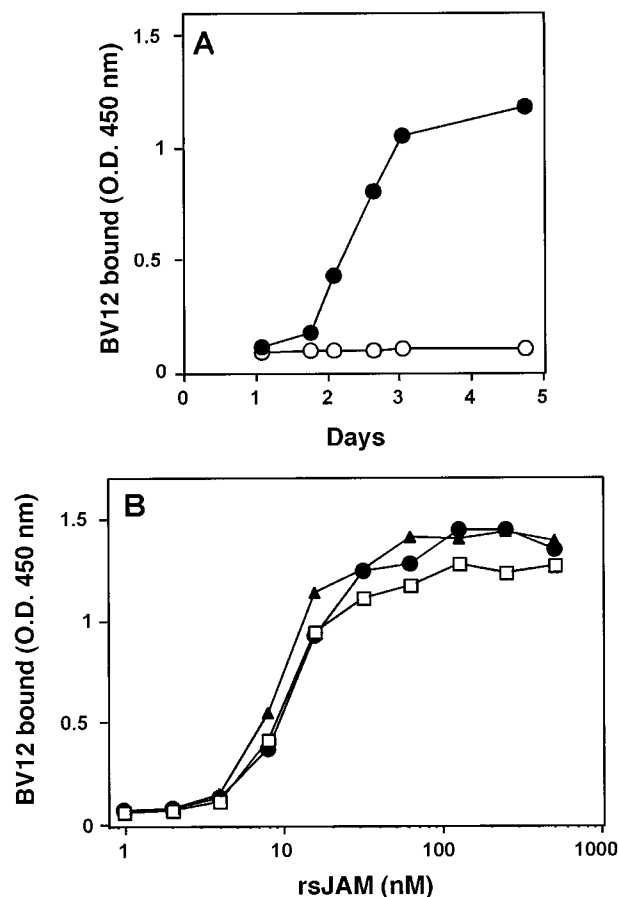
FIG. 1. **Production of rsJAM multimers by Sf9 cells.** *A*, culture medium of Sf9 cells was harvested at the indicated time points after cell infection and analyzed by SESIA (*closed circles*, baculovirus-infected cells; *open circles*, control cells). *B*, rsJAM was then purified and analyzed by SESIA. Samples were diluted in PBS plus 1% BSA in the presence of 10 mM EDTA (*open squares*), 2 mM $CaCl_2$ (*closed circles*), and 2 mM $MgCl_2$ (*closed triangles*). After pre-equilibration (18 h, 20 °C) SESIA was performed in the same medium.

twice with 0.2% (w/v) BSA in PBS (washing buffer), and blocked for 1 h with 2% BSA plus 0.05% Tween 20 in PBS (blocking buffer). Biotin-labeled rsJAM was then diluted in blocking buffer and added to the wells (for 60 min at 37 °C). Horseradish peroxidase-conjugated streptavidin was added and incubated for additional 60 min at room temperature. Absorbance at 450 nm of a *o*-phenylenediamine dihydrochloride solution (Sigma) was recorded to quantify the amount of biotin-labeled rsJAM bound to the wells. Data are shown as specific binding, which is obtained by subtracting aspecific binding (*i.e.* binding to BSA-coated wells) from total binding.

Epitope Analysis of mAbs BV11 and BV12—A series of truncated JAM proteins was expressed in *Escherichia coli* (10) and tested for reactivity with BV11 and BV12 by Western blot analysis. The following constructs were cloned into pDS56 (carrying a polyhistidine (His_6) tag and a thrombin recognition sequence upstream of the cloning site): Val[24]–Gly[238], Val[24]–Gly[169], Gly[26]–Gly[238], Gly[28]–Gly[238], Tyr[31]–Gly[238], Gln[38]–Gly[238], Leu[47]–Ser[132], Leu[47]–Cys[152]. Expression was confirmed by Western blot analysis of lysates with anti-polyhistidine antibody (Qiagen). To visualize immunoreactivity with mAbs BV11 and BV12, the filters were blocked with 5% (w/v) delipidated milk powder (Bio-Rad) in Tris-buffered saline plus Tween 20 and incubated overnight in the same buffer with antibody-horseradish peroxidase-conjugate. The blots were developed using the ECL peroxidase substrate (Pierce).

RESULTS

Analysis of rsJAM by SESIA—Murine rsJAM encompassing the leader sequence and the extracellular domain was cloned and expressed in Sf9 cells using the baculovirus system. To test whether rsJAM is produced in oligomeric form, culture medium from baculovirus-infected Sf9 cells was analyzed by SESIA. In

TABLE I

Effect of [NaCl] and pH on rsJAM dimerization

rsJAM (12 μM) was dissolved in 10 mM phosphate buffer and analyzed by sedimentation equilibrium centrifugation. Results from A, B, and C are obtained from three different experiments and are expressed as rsJAM concentration (μM) and percentage of total rsJAM (in parentheses). M, monomers; D, dimers; T, tetramers.

A. Effect of [NaCl] at constant pH (7.0)				
[NaCl]	M	D	T	D/M ratio
mM				
30	3.8 (32)	8.2 (68)	0.0 (0)	2.1
150	4.4 (37)	7.6 (63)	0.0 (0)	1.7
1,000	4.4 (37)	7.6 (63)	0.0 (0)	1.7

B. Effect of pH at constant [NaCl] (150 mM)				
pH	M	D	T	D/M ratio
8.0	3.6 (30)	8.4 (70)	0.0 (0)	2.3
7.0	4.4 (37)	7.6 (63)	0.0 (0)	1.7
6.0	6.0 (50)	6.0 (50)	0.0 (0)	1.0
5.0	8.3 (69)	3.7 (31)	0.0 (0)	0.4

C. Combined effect of pH and [NaCl]					
pH	[NaCl]	M	D	T	D/M ratio
	mM				
7.0	150	3.8 (32)	8.2 (68)	0.0 (0)	2.1
7.0	25	3.2 (27)	8.3 (69)	0.5 (4)	2.6
7.0	1,000	4.3 (36)	7.5 (62)	0.2 (2)	1.7
5.0	25	10.0 (83)	1.8 (15)	0.2 (2)	0.2
5.0	1,000	11.9 (99)	0.0 (0)	0.1 (1)	0.0

this assay, the antigen in solution (*e.g.* rsJAM) binds two distinct molecules of the same antibody (*e.g.* anti-JAM BV12), *i.e.* the "capture" antibody (which is immobilized on plastic and captures the antigen), and the horseradish peroxidase-labeled "detection" antibody (which is added in solution and detects the antigen). Only dimeric and multimeric antigens (with two and more solvent-exposed epitopes, respectively) can be recognized simultaneously by the capture and the detection antibody and produce a detectable signal (8). SESIA revealed the presence of rsJAM oligomers in culture medium of infected (but not control) Sf9 cells (Fig. 1*A*). Similar results were obtained when rsJAM was expressed in CHO cells (data not shown). rsJAM was then purified from Sf9 culture medium by immunoaffinity and anion exchange chromatography. Purified rsJAM produced a dose-dependent signal in the BV12 SESIA, with half-maximal response at a rsJAM concentration of 15 nM, which is suggestive of a high-affinity interaction. The SESIA signal was not significantly affected by 10 mM EDTA, 2 mM CaCl$_2$, or 2 mM MgCl$_2$ (Fig. 1*B*). The presence of excess rsJAM monomers (which may derive from either the dimer/monomer equilibrium or denaturation) may conceivably compete with multimers for binding the "capture" antibody and consequently quench the SESIA signal. Hence, these results indirectly indicate that the dissociation constant (for the dimer/monomer equilibrium reaction) is equal to (or smaller than) 15 nM, under these experimental conditions.

Analysis of rsJAM by Equilibrium Centrifugation—The oligomerization of purified rsJAM was further analyzed by equilibrium sedimentation centrifugation. When rsJAM (12 μM) was examined immediately after purification, two different components were observed, which accounted for 89% and 11% of the total protein. The two components displayed a molecular mass of 46 and 92 kDa, the theoretical weights of rsJAM dimers and tetramers, respectively. Remarkably, no 23-kDa fraction (*i.e.* rsJAM monomers) was detectable. Addition of 10 mM β-mercaptoethanol neither induced appearance of monomers nor substantially modified the relative amounts of dimers and tetramers (82% and 18%, respectively), indicating that oligomerization is not likely due to intermolecular disulfide bonds.
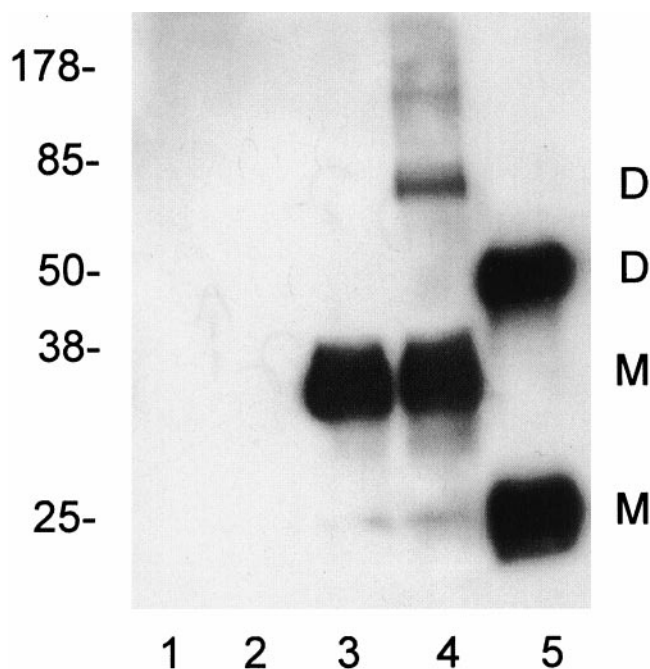

FIG. 2. **Detection of JAM dimers at the cell surface.** CHO cells transfected with either full-length murine JAM (*lanes 3* and *4*) or vector alone (*lanes 1* and *2*) were incubated with either BS^3 (*lanes 2* and *4*) or control buffer (*lanes 1* and *3*). In parallel rsJAM was treated with BS^3 in solution (*lane 5*). Samples were then immunoprecipitated with the anti-JAM mAb BV20 (coupled to CNBr-activated Sepharose) and analyzed by Western blotting with peroxidase-conjugated mAb BV20. Molecular markers are shown at the *left*. The position of JAM and rsJAM dimers (*D*) and monomers (*M*) is indicated at the *right*. Compared with native JAM, rsJAM has a lower apparent mass, which is due to the absence of both transmembrane and cytoplasmic domains in rsJAM.

Frozen and aged samples of rsJAM showed altered equilibrium between oligomers, with a nearly complete disappearance of tetramers and with the appearance of monomers. Additionally, the relative amount of dimers was reduced from 81% to 63%, with monomers accounting for the remaining 37%. This preparation was used to further investigate the sensitivity of rsJAM dimers to the ionic strength and pH of the buffer. When the NaCl concentration was either reduced from 150 mM to 30 mM or increased up to 1,000 mM (at a constant pH of 7.0), no major changes in the ratio of dimers to monomers were observed (Table I, part A). On the other hand, when the pH was stepwise shifted from 8.0 to 5.0 (at a constant NaCl concentration of 150 mM), the ratio of dimers to monomers was reduced in parallel, even if, at pH 5.0, dimers still accounted for more than 30% of the total protein (Table I, part B). However, the combination of 1,000 mM NaCl and pH 5.0 caused almost complete dissociation of dimers into monomers (Table I, part C), indicating that monomers are likely to associate within the dimers in a non-covalent manner.

Detection of JAM Dimers at the Cell Surface—We next evaluated whether native JAM could be detected as a dimer. For this purpose, CHO cells transfected with murine JAM were incubated with BS^3, a membrane-impermeable and homobifunctional cross-linker. CHO cells transfected with vector alone were used as control. After lysis, extracts were immunoprecipitated with the anti-JAM mAb BV20. Immunecomplexes were then resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with horseradish peroxidase-conjugated mAb BV20. In the absence of BS^3, mAb BV20 precipitated a protein with an apparent relative molecular mass of ~35 kDa (Fig. 2, *lane 3*). In the presence of 1.25 mM BS^3, besides the ~35-kDa monomeric JAM, mAb BV20 precipitated a minor molecular species with a nearly 2-fold larger mass of

A, homophilic interaction of rsJAM.

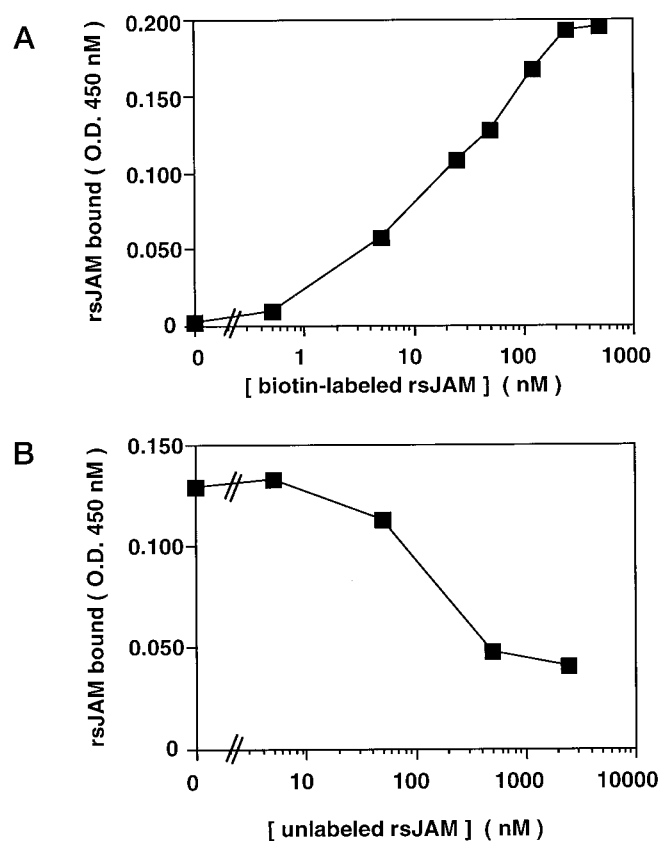B, homophilic interactions are inhibited by excess unlabeled rsJAM.

FIG. 3. *A*, homophilic interaction of rsJAM. Biotin-labeled rsJAM was added as fluid-phase ligand to 96-well plates that had been precoated with unlabeled rsJAM (500 nM). The amount of labeled rsJAM bound to immobilized rsJAM was quantified using peroxidase-conjugated streptavidin. *B*, homophilic interactions are inhibited by excess unlabeled rsJAM. Fluid-phase biotin-labeled rsJAM (125 nM) was added to immobilized unlabeled rsJAM in the presence of unlabeled rsJAM, used as a soluble competitor.
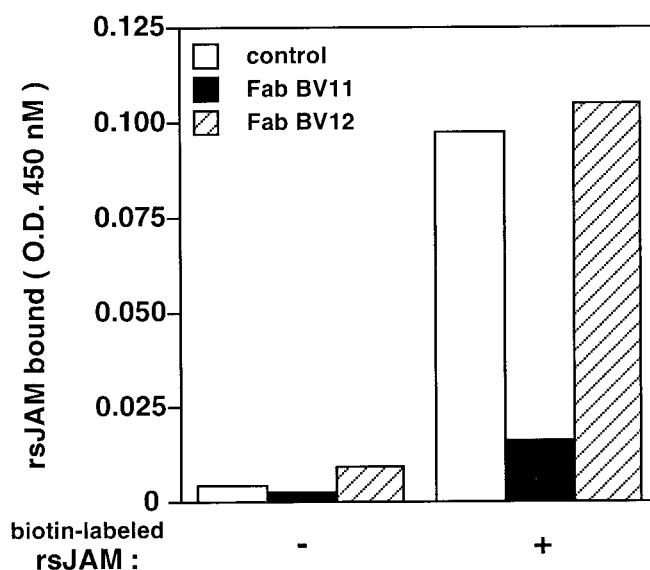

FIG. 4. **Homophilic interactions of rsJAM are inhibited by Fab BV11.** Biotin-labeled rsJAM (125 nM) was added to wells that had been precoated with unlabeled rsJAM, in the absence (control) and presence of either Fab BV11 or Fab BV12 (each at 50 μg/ml).

TABLE II
Binding of rsJAM to Fabs BV11 and BV12

rsJAM (12 μM) was dissolved in PBS (pH 7.0) in the absence (control) or presence of Fab BV11 or Fab BV12. Equilibrium sedimentation centrifugation was performed to measure the amounts of rsJAM that either remained free in solution (A) or bound to the Fabs (B). Results are expressed as rsJAM concentration (μM) and percentage of total rsJAM (in parentheses). M, monomers; D, dimers.

	(A) Free		(B) Fab-bound	
	M	D	M	D
Control	3.8 (32)	8.2 (68)	0.0 (0)	0.0 (0)
Fab BV11	4.4 (37)	0.0 (0)	0.0 (0)	7.6 (63)
Fab BV12	2.5 (21)	0.0 (0)	2.0 (17)	7.5 (62)

~75 kDa, that is suggestive of JAM dimers, and also trace amounts of even higher molecular mass, which likely correspond to JAM multimers (*lane 4*). Neither JAM monomers nor dimers and multimers were detectable when lysates from mock-transfected CHO cells were immunoprecipitated with mAb BV20 (*lanes 1* and *2*). Interestingly, rsJAM in solution could also be cross-linked by BS[3], yielding two molecular species that correspond to monomeric and dimeric rsJAM (*lane 5*). Hence, the occurrence of rsJAM dimers in solution is mirrored by the presence of native JAM dimers at the cell surface.

Homophilic Interactions of rsJAM—To gain insights into the adhesive properties of JAM, we tested whether rsJAM is capable of interacting homophilically. A solution of rsJAM was divided into two aliquots. The former was biotin-labeled and used as fluid-phase ligand, the latter was immobilized on plastic wells and used as solid-phase ligand. Homophilic recognition was quantified using peroxidase-conjugated streptavidin. When biotin-labeled rsJAM (0.5–500 nM) was added to rsJAM-coated wells, we detected a dose-dependent and saturable signal, which was half-maximal and maximal at concentrations of fluid-phase rsJAM of 15 and 250 nM, respectively (Fig. 3*A*). Additionally, excess unlabeled rsJAM (5–2,500 nM) dose-dependently inhibited binding of biotinylated rsJAM (125 nM) to immobilized rsJAM, with half-maximal inhibition at 200 nM and almost complete inhibition at 2,500 nM (Fig. 3*B*). Binding of biotinylated rsJAM (250 nM) to immobilized rsJAM was unaffected by EDTA (0.240 and 0.244 optical densities in the absence and presence of 5 mM EDTA, respectively). Hence,

rsJAM is capable of binding homophilically in a divalent cation-independent manner.

Homophilic Interactions of rsJAM Are Inhibited by Fab BV11—We next examined the effect of the blocking anti-JAM mAb BV11 on the homophilic binding of rsJAM. BV11 was compared with the non-blocking anti-JAM mAb BV12. Biotin-labeled rsJAM (250 nM) was added to immobilized rsJAM in the presence of either mAb BV11 or mAb BV12 (each at 100 μg/ml). As expected, both antibodies greatly increased the signal above the background (optical densities of 0.187, 1.335, and 1.363 at 450 nm in the presence of the negative control mAb HB151, mAb BV11, and mAb BV12, respectively). Since this effect is likely attributable to the ability of bifunctional mAbs to bridge fluid- and solid-phase antigens, monovalent Fab BV11 and Fab BV12 were used (each at 50 μg/ml). As reported in Fig. 4, Fab BV11 reduced the binding of biotin-labeled rsJAM to immobilized rsJAM, whereas Fab BV12 had no effect, indicating that Fab BV11 counteracts rsJAM homophilic interactions.

Fab BV11 Binds rsJAM Dimers but Not Monomers—The rsJAM preparations used for the binding assay contained both monomers and dimers. In an attempt to determine whether the BV11-inhibitable homophilic binding of rsJAM was mediated by either monomers or dimers (or both), we evaluated by sedimentation equilibrium centrifugation the interaction of Fab BV11 (and Fab BV12) with rsJAM monomers and dimers. First, solutions of purified Fab BV11 and Fab BV12 each appeared by analytical ultracentrifugation as individual molecular species with a molecular mass of 49 kDa. Assuming molec-

JAM Oligomerization

FIG. 5. **Mapping of BV11 (*A*) and BV12 (*B*) epitopes.** The binding of mAbs BV11 and BV12 to rsJAM from baculovirus-infected Sf9 cells (*lane 1*) and truncated JAM proteins expressed in *E. coli* (*lanes 2–8*) was analyzed by Western blot. Molecular size markers are indicated at the *left*. A schematic overview (*C*) of the proteins analyzed in *A* and *B* shows the first and last amino acid from the truncated proteins. The hydrophobic signal peptide and the transmembrane region are *shaded*. The larger size of *E. coli* proteins compared with rsJAM are due to the amino-terminal tag sequence.
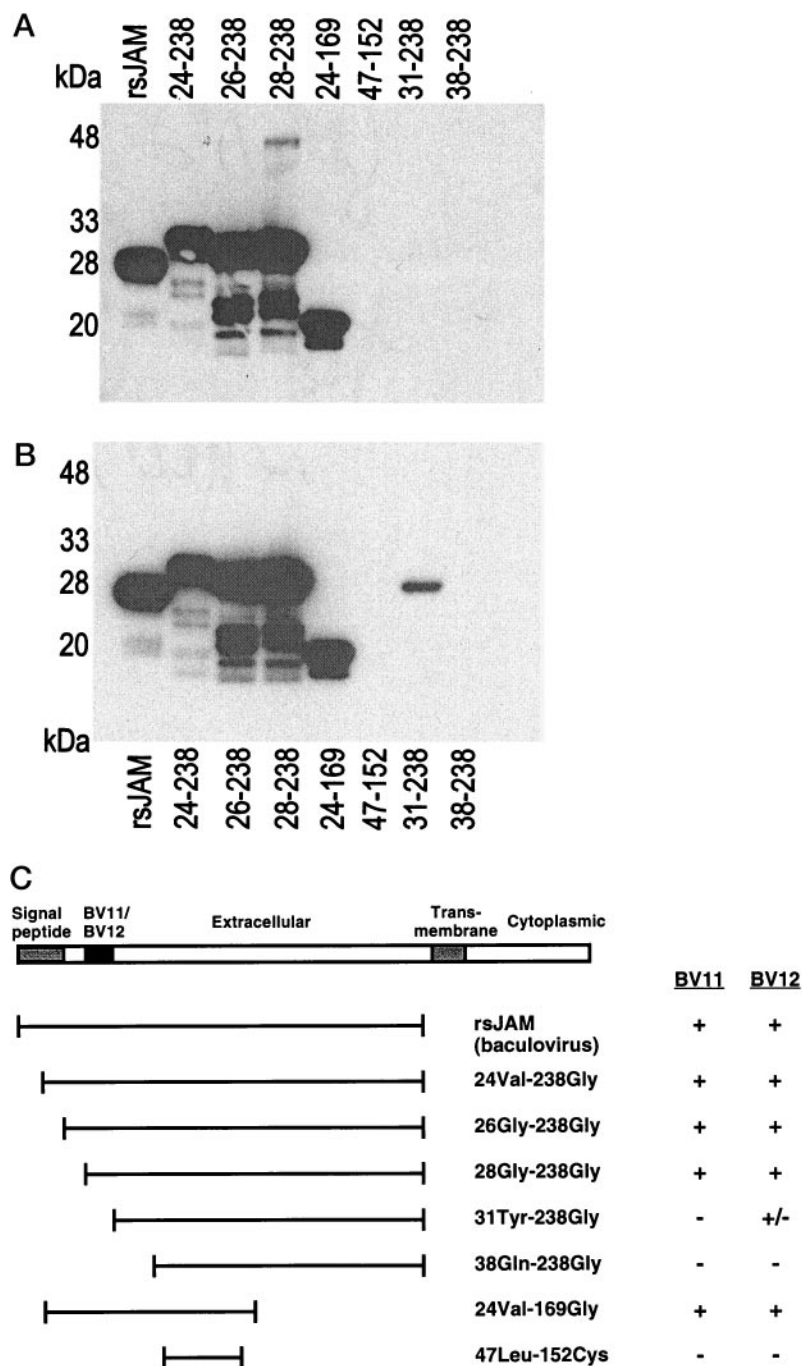
ular mass values of 23 kDa (rsJAM monomers), 46 kDa (rsJAM dimers), and 49 kDa (Fabs), formation of Fab-monomer and Fab-dimer complexes would result in the appearance of 72- and 95-kDa fractions, respectively. Absorbance profile of a solution of rsJAM with Fab BV11 showed two components of 23 and 95 kDa, which correspond to rsJAM monomers and to a complex consisting of Fab BV11 with rsJAM dimers, respectively. At variance, when rsJAM was mixed with Fab BV12, the absorbance profile revealed three species with molecular mass values of 23, 72, and 95 kDa, which correspond to rsJAM monomers and to two complexes consisting of Fab BV12 with either rsJAM monomers or dimers, respectively. Hence, while Fab BV12 associated with both monomers and dimers, Fab BV11 only associated with dimers, but not with monomers (Table II). These data indicate that BV11 discriminates between rsJAM dimers and monomers in solution and suggest that Fab BV11

may primarily inhibit rsJAM self-assembly by preventing dimer-mediated interactions.

Epitope Mapping of mAbs BV11 and BV12—In order to identify the BV11 and BV12 epitopes, a series of truncated JAM constructs (designed to cover the extracellular portion of JAM without the leader sequence) were expressed in *E. coli* and tested by Western blot for immunoreactivity with peroxidase-conjugated mAbs BV11 and BV12. Only the proteins Val[24]–Gly[238], Gly[26]–Gly[238], Gly[28]–Gly[238], and Val[24]–Gly[169] reacted with BV11, whereas Tyr[31]–Gly[238], Gln[38]–Gly[238], and Leu[47]–Cys[152] did not (Fig. 5*A*). Hence, mAb BV11 recognizes an epitope with and around the tripeptide sequence Gly[28]-Ser[29]-Val[30] at the penultimate amino terminus of the mature JAM after removal of the leader sequence. mAb BV12 gave similar results, but it showed in addition minimal reactivity with Tyr[31]–Gly[238], indicating that the BV12 epitope maps to a

slightly different site compared with BV11 (Fig. 5*B*). Interestingly, in initial attempts to map the BV11 epitope by overlapping decapeptides from the JAM sequence, peptides containing the Gly^{28}-Ser^{29}-Val^{30} sequence were not recognized by mAb BV11 (data not shown), suggesting strict requirements for secondary and/or tertiary structure elements at the amino terminus of the JAM molecule.

DISCUSSION

The major findings of this study are as follows. (i) rsJAM forms non-covalent dimers, (ii) rsJAM is capable of homophilic interactions, and (iii) the function-blocking mAb BV11 recognizes an amino-terminal epitope, which is associated with the dimeric state of JAM and is involved in the homophilic interactions.

JAM Dimerization—Multimerization of rsJAM was suggested by SESIA and confirmed by sedimentation equilibrium centrifugation, which identified a major fraction of rsJAM dimers and a minor (and more labile) fraction of tetramers. Dimers were resistant to thiol-reducing agents, but not to a combination of low pH and high ionic strength, indicating that dimerization is attributable neither to disulfide bridges nor to other types of covalent bond (11). On the other hand, the stability of rsJAM dimers at physiological pH and ionic strength suggests that the detection of rsJAM dimers in solution may bear relevance to the molecular organization of native JAM *in vivo*. In addition, the stability of rsJAM dimers suggests that, like several other known homodimers, JAM dimers might be permanent and obligatory complexes (12). In fact, even if we detected rsJAM monomers, we do not know whether monomers can exist in equilibrium with dimers as functional proteins or whether they reflect partial denaturation of dimers. Non-covalent dimerization has been reported for other cell adhesion molecules (CAM) of the Ig superfamily and is associated with increased surface and avidity of the binding sites (13–16). As assessed by three-dimensional structure analysis, dimerization of Ig-like CAM is mediated by either hydrophobic (*e.g.* ICAM-1 and CD8; Refs. 17 and 18), or charged residues (*e.g.* CD2 and CD2-CD58; Refs. 19 and 20).

To obtain evidence for dimerization of native JAM, non-covalent interactions between individual JAM proteins in close molecular contact at the cell surface were stabilized with a membrane-impermeable cross-linker (21–24). With this approach, putative JAM dimers could be identified at the cell membrane by immunoprecipitation and Western blot analysis. However, despite similarities between rsJAM and native JAM, we cannot rule out distinct mechanisms of dimerization. For instance, hydrophobic interactions between transmembrane helices might account for dimerization of native JAM within the context of the plasma membrane (as ICAM-1, glycophorin C, and the β_2 adrenoreceptor; Refs. 22, 25, and 26), but obviously not for dimerization of rsJAM in solution, which lacks the transmembrane domain.

Homophilic Interactions of rsJAM and Dimerization-dependent Epitopes—Several dimeric CAM display low affinity binding in three-dimensional solution-phase (27), yet mediate efficient adhesion when expressed in two-dimensional membranes (28). Here, to test rsJAM ability to interact homophilically, we reasoned that binding might be facilitated if the interaction would occur on a two-dimensional surface. Incubation of solid- and fluid-phase rsJAM showed that rsJAM is indeed capable of homophilic, saturable, and dose-dependent binding. Thus, like dimerization, homophilic binding is an intrinsic property of the extracellular domain of JAM.

It is still unclear whether the binding assay measured monomer-monomer, monomer-dimer, or dimer-dimer interactions (or combinations thereof). However, a prominent role for

dimers in rsJAM binding is indirectly supported by the observation that Fab BV11 (which efficiently blocked homophilic binding) only bound rsJAM dimers (and not monomers) in solution. Based on this evidence, we speculate that in our experimental conditions dimerization of rsJAM induces the formation of the BV11 epitope. In turn, the BV11-positive conformer might undergo facilitated homophilic binding, which could be inhibited by occupancy of the BV11 epitope. In this model, the BV11 epitope is not the dimerization domain itself, since Fab BV11 does not induce dissociation of rsJAM dimers. Neither is BV11 a combinatorial epitope (29) formed by the contact of two JAM monomers, since the antibody recognizes the epitope in the context of a single-chain linearized molecule. Rather, we favor the hypothesis that BV11 is a conformational epitope, which is associated (at least in the context of non-denatured rsJAM in solution) with the dimeric state of JAM. By analogy with ligand-induced binding sites induced by integrin ligands (LIBS epitopes; Refs. 30 and 31), we propose for the BV11 epitope the acronym "DIBS," for dimerization-induced binding sites.

Molecular Mechanisms of JAM Function and mAb BV11 Action—The homophilic binding of rsJAM, together with the intercellular localization of JAM at tight junctions (1), suggest that homophilic JAM-JAM interactions occur at and may contribute to the organization of tight junctions. Additionally, besides supporting a general model of homophilic interaction, the rsJAM binding data may provide mechanistic insights into the antimigratory action of mAb BV11 as well. The herein reported data do not support the hypothesis that mAb BV11 *strengthens* homophilic associations between JAM molecules expressed on adjacent endothelial cells, as mAb BV11 is an inhibitor of homophilic binding. On the other hand, it is also unlikely that mAb BV11 disrupts preexisting JAM-JAM interactions at the endothelial junctions, since the antibody would then affect the integrity of intercellular junctions and increase (rather than decrease) transmigration. Thus, we speculate that the antibody may block migration by *inhibiting* the homophilic interactions between endothelial JAM and leukocyte JAM, that dynamically ensue during leukocyte transmigration.

In conclusion, results from this study provide evidence for the dimeric organization of JAM, which is associated with acquisition of a conformation favorable to homophilic binding. The ongoing efforts aimed at solving the crystal structure of rsJAM are expected to cast light on the molecular basis of both JAM dimerization and the relationship between JAM dimers and the BV11 epitope.

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Homophilic Interaction of Junctional Adhesion Molecule

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