A Novel Protein with Homology to the Junctional Adhesion Molecule

CHARACTERIZATION OF LEUKOCYTE INTERACTIONS*

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We have cloned a novel cDNA belonging to the Ig superfamily that shows 44% similarity to the junctional adhesion molecule (JAM) and maps to chromosome 21q21.2. The open reading frame of JAM2 predicts a 34-kDa type I integral membrane protein that features two Ig-like folds and three N-linked glycosylation sites in the extracellular domain. A single protein kinase C phosphorylation consensus site and a PDZ-binding motif are present in the short intracellular tail. Heterologous expression of JAM2 in Chinese hamster ovary cells defined a 48-kDa protein that localizes predominantly to the intercellular borders. Northern blot analysis showed that JAM2 is preferentially expressed in the heart. JAM2 homotypic interactions were demonstrated by the ability of JAM2-Fc to capture JAM2-expressing Chinese hamster ovary cells. We further showed that JAM2, but not JAM1, is capable of adhering to the HSB and HPB-ALL lymphocyte cell lines. Neutralizing mouse anti-JAM2 polyclonal antibodies provided evidence against homotypic interactions in this assay. Biotinylation of HSB cell membranes revealed a 43-kDa counter-receptor that precipitates specifically with JAM2-Fc. These characteristics of JAM2 led us to hypothesize a role for this novel protein in adhesion events associated with cardiac inflammatory conditions.

Intercellular contacts between endothelial cells are crucial for maintenance of vessel integrity. The various vascular segments display heterogeneity in their use of adherens, gap, and tight junctions (1). Contacts occur most apically at the tight junction; and in 1998, it was reported that a novel junctional adhesion molecule (JAM) co-localizes with this dynamic regulated structure (2). JAM belongs to the Ig-like superfamily of adhesion molecules. The mouse JAM protein is expressed in endothelium and epithelium, whereas the human homologue is additionally found on peripheral blood leukocytes and platelets (2–4).

Pro-inflammatory stimuli promote tethering, rolling, and subsequent firm adhesion of leukocytes to the luminal vessel wall (5). These effects are mediated by the concerted action of endothelial cell selectins and the Ig adhesion molecules VCAM and ICAM (6). The molecular mechanisms underlying the subsequent diapedesis of leukocytes through the endothelial monolayer are not yet fully understood, although a key role for PECAM has been demonstrated using neutralizing antibodies (7, 8). PECAM is also targeted to the lateral membranes of endothelia, but does not co-localize with junctional structures (9).

Most recent evidence supports a role for JAM in leukocyte transmigration and inflammation. A neutralizing monoclonal antibody is effective at reducing spontaneous and chemokine-mediated monocyte passage across endothelial monolayers (2). Furthermore, neutralization of JAM has proven effective in reducing not only monocyte, but also neutrophil transmigration across brain endothelium in a mouse model of meningitis (10). That JAM impinges on the inflammatory pathway is further substantiated by its redistribution to the endothelial cell surface in response to tumor necrosis factor-α interferon-γ (11).

PECAM and JAM share some structural and functional similarities. Both adhesion proteins belong to the Ig superfamily; they localize at intercellular clefts; and neutralizing antibodies raised against either protein are effective at inhibiting the paracellular movement of leukocytes. PECAM engages in homotypic interactions in addition to heterotypic binding to the α4β1 integrin and proteoglycans (12–16). To date, only homotypic binding has been described for JAM. Nevertheless, a counter-receptor on leukocytes is postulated since neutralizing antibodies do not affect JAM-JAM interactions (2, 10).

JAM possesses two V-type immunoglobulin domains, a novel structure for an Ig superfamily adhesion protein. We reasoned that JAM would belong to a family of adhesion molecules possessing similar structure and perhaps intercellular location. Using the public expressed sequence tag data base, we have identified a related putative adhesion molecule that shows preferential expression in the heart. For comparative purposes, we also isolated the human homologue to mouse JAM. We propose that the originally identified mouse JAM and its human counterpart be referred to as JAM1. In this report, we describe the novel JAM2 sequence, chromosomal localization, and expression characteristics. Furthermore, we show that JAM2 shows selective adhesion to immortalized T lymphocyte lines in vitro.

EXPERIMENTAL PROCEDURES

cDNA Cloning—JAM2 (V89915) was identified using the public expressed sequence tag (EST) data base. Sequences homologous to mouse JAM (U89915) were isolated using the tblastn program. AA406389 and AA912674 were assembled to form the 3′-end of human JAM2 (Fig. 1b). Rapid amplification of cDNA ends (RACE) was employed to obtain

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§ The abbreviations used are: JAM, junctional adhesion molecule; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; bp, base pairs; CHO, Chinese hamster ovary.
Identification and Cloning of Human JAM2

Expression in CHO Cells—The full-length clone of JAM2 with a C-terminal hemagglutinin tag was subcloned into pcDNA6 (Invitrogen, Carlsbad, CA). CHO-K1 cells were transfected with 10 μg of vector possessing either no insert or JAM2 using FuGENETM 6 reagent (Boehringer Molecular Biochemicals). Stable cell lines (control and JAM2) were selected with 5–10 μg/ml blasticidin. For Western blot analysis, cells were lysed in 1% Triton X-100 buffer in the presence of protease inhibitors (protease inhibitor mixture set III, Calbiochem). Approximately 36 μg of protein was electrophoresed through 10% polyacrylamide gels and probed with a 1:2000 times dilution of preimmune or a 1:200 times dilution of polyclonal serum. Specific bands were viewed using enhanced chemiluminescence with a 1:30,000 dilution of goat anti-mouse horseradish peroxidase (Fisher).

Expression in Insect Cells—For JAM2, the extracellular domain polymerase chain reaction product was digested with BamHI/KpnI and subcloned into a pFastBac1 vector (Life Technologies, Inc.) possessing the constant region of mouse IgG2a (17). For JAM1, the ectodomain was synthesized with sense (5'-ggatccGCGAGGCCCCAGAGGAGG-3') and antisense (5'-GATgtactACATTCCGCCCTC-3') primers with cycling as follows: one cycle at 95 °C for 120 s; 23 cycles at 95 °C for 20 s, 56 °C for 10 s, and 72 °C for 10 s; and one cycle at 72 °C for 300 s. Secreted recombinant JAM2 and JAM1 fusion proteins were purified from the media of infected SF21 cells using HiTrap protein A columns (Amersham Pharmacia Biotech).

Antibodies—For preparation of JAM2 immunogen, thrombin cleavage was used to release JAM2 from the Fe portion of the secreted recombinant fusion protein. The digest was reapplied to the protein A column to remove Fe. Female BALB/c mice (8 week olds; Harlan Sprague Dawley, Inc., Indianapolis, IN) were immunized and then boosted three times, 20 days apart, by intraperitoneal and subcutaneous injections of 100 μg of purified JAM2 extracellular domain emulsified with an equal volume of Freund’s adjuvant. Complete Freund’s adjuvant was used for the first immunization, and incomplete Freund’s adjuvant for subsequent injections. Serum was collected 10 days following each boost.

Immunofluorescence—CHO-K1 (control or JAM2-expressing) cells grown on glass slides to confluency were fixed with 1% paraformaldehyde and stained with a 1:100 dilution of either preimmune or mouse anti-JAM2 polyclonal serum. Goat anti-mouse-fluorescein isothiocyanate at 1:100 was used as a secondary antibody. Fluorescence was viewed using a Noran™ confocal laser-scanning microscope (Noran Instruments Inc., Middleton, WI) equipped with an argon laser and appropriate optics and filter module for fluorescein isothiocyanate detection. Digital images were acquired at ×400 using a 0.6 NA, Nikon ×20 lens. An axis motor attached to the inverted microscope stage was calibrated to move the plane of focus at 0.4-μm steps through the sample. Collected 12-bit gray scale images at 512 × 480 resolution, stored on a re writable optical hard disc, were volumetrically reconstructed using the Image-1/MetamorphTM three-dimensional module (Universal Imaging Corp., West Chester, PA).

Expression in Insect Cells—Ectodomain-Fc adhesion assays were performed in 96-well plates essentially as described previously (18). Briefly, 50 μl of goat anti-mouse IgG2a was coated at 5 μg/ml in phosphate-buffered saline and used to capture 4.8 pmol of JAM2-Fc, JAM1-Fc, VCAM-Fc, or mouse IgG2a (control). CHO and various leukocyte cell lines (i.e., T lymphocytes, HSB and HPB-ALL; B lymphocytes, RAMOS; monocyte cells, HL-60 and THP-1; and the erythroleukemic K562 line) were labeled with calcine (Molecular Probes, Inc., Eugene, OR) at 50 μM/ml for 25 min at 37 °C. Cell binding was performed for 90 min at 37 °C with 250,000 cells/well in binding buffer consisting of Tris-buffered saline plus 1 mM each of CaCl2, MgCl2, and MnCl2. Wells were washed three times and lysed with 50 μl Tris (pH 7.5), 5 mM EDTA, and 1% Nonidet P-40, and fluorescence was read in a Cytofluor with excitation at 485/20 nm and emission at 530/25 nm. Specific binding was calculated by removal of values obtained by capture of mouse IgG2a. When the various cell lines were compared, curves for calcine uptake were generated for each experiment to convert arbitrary fluorescence units into cell number.

For antibody inhibition, either JAM2-Fc protein captured on wells or HSB cells were incubated for 30 min at room temperature in binding buffer with 1:100 dilutions of preimmune (normal mouse serum) or mouse anti-JAM2 polyclonal serum. Following incubation, excess antibody was removed by washing three times prior to continuation of the assay.

Cell-surface Biotinylation/Counter-receptor Precipitation—HSB or K562 cells were surface-biotinylated using EZ-Link sulfo-NHS-biotin (Pierce) according to the manufacturer’s instructions. Cells (2.5 ×
10^6/ml) were washed three times following incubation with 0.5 mg/ml sulfosuccinimidobiotin for 30 min at room temperature. Cell lysis was achieved in Tris-buffered saline (pH 7.5), 1% Triton X-100, and 1 mM each MnCl₂, MgCl₂, and CaCl₂ with the inclusion of protease inhibitor mixture set III. Approximately 5 mg g⁻¹ of JAM-Fc fusion protein was added to 1 mg of lysate and incubated at 4 °C overnight. Proteins bound to JAM were precipitated with protein A-Sepharose (30 µl), boiled for 5 min with 10 mM dithiothreitol in SDS sample buffer, and separated on 9% SDS gels. Following transfer to polyvinylidene difluoride membrane, biotinylated proteins were detected using streptavidin-horseradish peroxidase (1:4000) and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

RESULTS

Fig. 2 depicts the complete cDNA and amino acid sequences of human JAM2. The open reading frame possesses 298 amino acids and features a putative signal sequence, an extracellular domain consisting of two Ig-like folds, a single transmembrane region, and a short intracellular domain. There are two possible cleavage sites for the signal peptide, i.e. VVA-LG (underlined) or AYG-FS (dash-underlined). In the extracellular region, there are three potential N-linked glycosylation sites (N-X(S/T)) at amino acids 98, 187, and 236. In the short intracellular tail, at amino acid 279, a protein kinase C phosphorylation consensus site ((S/T)X(R/K)) exists. At the extreme C terminus, a potential binding motif for PDZ domains is apparent (SFII). We were unable to identify an in-frame and upstream stop codon for human JAM2 in the 5'-untranslated sequence. That the designated ATG is the true translational initiation signal is supported by its context within a Kozak consensus sequence and alignment with JAM1 ATG (Fig. 3).

Identification and Cloning of Human JAM2

1. AAAAAGCAAGGCACCCACATCCCTGGGCTGGAGAGAACCCCTCTCCCTGGACGAGCTGGAAG - 60
   - KTEQPTIPLEDPELSQQLR
   61. AGGAGGCCGCGAGGAGGAGAAAAGCACAATCCATGAGCCCTCTCTCCCTCTGCCC - 120
   - RRPGGGEVRDISRAPAOPLPP
   121. CTCCTGGGCTGGAGAGAACCCCTCTCCCTGGACGAGCTGGAAG - 180
   - IPTLCSPFQAPVQYQQNL
   181. CTCCTGGGCTGGAGAGAACCCCTCTCCCTGGACGAGCTGGAAG - 240
   - PAGTLDLDRLRAAGCRPGKMA
   241. AGGAGGGCAGCCACCCGCTCTCTCTCTCTGCTGCTCACTGTTGTCGCCCTTGCGC - 300
   - RSRNRHLILGLLLRLYVLVAL
   301. TATCTTAAAGCTATGTTTCTGCTCAGGACGACGCCAGGAGGACCTGGCAAGCTGGAC - 360
   - 3XYSAPKDDQD.MinValue
   361. TACCAAGAGCCTATTATTTAGCCCGAAAACCAAAAGACGATTGCTCTCAGGATTAG - 420
   - 4 Q Y E A I L A C K P T K K T V S S R L E
   421. TGGAGGAAAGCAGGTGGTGATGCTCCCTCTCTGCTGCTCACTGTTGTCGCCCTTGCGC - 480
   - W K R L G R S V S F V Y Q Y Q T L Q Q D
   481. TTTTAATATCATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACAT - 540
   - FKR A E M I D F I N I S K N V T R S
   541. GATCCGGGGATAATCTTGTTGAAATCCTGCTCAGGAGGACCGGACCAAAAGCTGGCAAGCTGGAC - 600
   - D A G K Y R C E V S A P S E Q G Q N L E
   601. GGAGTACTACCTGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTAC - 660
   - B D T V L E L V L V I F A V F I G E V P
   661. TCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCTTCTCT
   - 720
   - SSALSAGTVVELRCDQKEGPN
   721. GCTCTTGATATAACTAGTTAAGGATGCTATGCTCCTGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTAC - 780
   - APEYTWFKDGIRLLENPRLG
   781. TCCAGAAGACACACACACGTCCTACACAGATGACATACAAAACAGGAGCTGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTAC - 840
   - SQTSTNSSYQFNTMNLTN
   841. AGCTTTCCAGACCTGATCAGGGAATATCTTGTTGAGGACGCCAGGACCGGACCAAAAGCTGGCAAGCTGGAC - 900
   - T V S K L D T Q R Y S C E A R S N V G Y
   901. GCAGTGGCCTGGGAAACCAGTACAGATGACATACAAAACAGGAGCTGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTAC - 960
   - R R C P G K R M Q V D D L N I S G I A
   961. GCAGTGGCCTGGGAAACCAGTACAGATGACATACAAAACAGGAGCTGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTAC - 1020
   - AVVYVGLVLSVCGGLVCYQAQ
   1021. AGAAAGGAGCTACCTTTTCCAAAGACCCACCTTGAGACGCAGGAGCTGGAAG - 1080
   - 1081
   - RKGYFSKTSFQKSNSBSBKA
   1081. AGCAACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTACATTTACATGCTGATTAC - 1131
   - TTSNFNKHTKFSII

Fig. 2. Human JAM2 cDNA and amino acid sequences. Highlighted are the N-linked glycosylation (amino acids 98, 187, and 236) and protein kinase C phosphorylation (amino acid 279) consensus sites. The signal sequence and transmembrane domain are underlined.

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FIG. 3. Alignment of human JAM2 and JAM1 amino acid sequences. Upper sequence, JAM2; lower sequence, JAM1. Sequences show 44% similarity and 35% identity. The asterisk denotes the conserved glycosylation site between JAM2 and JAM1. The protein kinase C consensus sites in both JAM2 and JAM1 are underlined, and the cAMP/cGMP consensus site in JAM1 is double-underlined.

revealed 44% similarity and 35% identity at the amino acid level (Fig. 3). Similarly, alignment with mouse JAM1 amino acid sequence showed 43% similarity and 35% identity (data not shown). The glycosylation motif at amino acid 187 is conserved between family members. However, JAM1 possesses the cAMP/cGMP consensus site in the transmembrane domain that is absent in JAM2.
JAM2 was mapped to chromosome 21 at position q21.2 using the public data base. Sequence was retrieved at 100% identity from two contiguous non-overlapping sequences of 100,000 bp each (GenBank™/EBI Data Bank accession numbers AP000087.1 and AP000086.1). The coding region of JAM2, which constitutes 897 bp, is distributed over 10 exons (Table I). The limits of the JAM2 cDNA sequence shown in Fig. 2 span ~74,853 bp of genomic DNA. Various exons were also found in AP000223 (coding exon 1), AP000225 (coding exons 2–6), and AP000226 (coding exons 6–10). Since the complete JAM2 transcript(s) is considerably larger than 897 bp (Fig. 4), further exons in the untranslated regions remain to be identified either up and/or downstream. All intron/exon boundaries conform to the consensus GT/AG rule (19).

Tissue expression of JAM2 was examined on normalized human multiple tissue Northern blots (Fig. 4A). Two transcripts of ~1.5 and <.4 kilobases were apparent under high stringency. These two species likely represent alternatively spliced products. Of the 12 tissues examined, human JAM2 appears to be preferably expressed in the heart. However, the transcript could also be detected to some degree in several other tissues, most notably the placenta. Fig. 4B shows a more detailed examination of the JAM2 transcript in the heart. A clear chamber-specific expression was not apparent. Relative to glyceraldehyde-3-phosphate dehydrogenase, there was somewhat lower expression in fetal heart. However, major differences in the aorta, atrium, and ventricles were not observed.

To facilitate study of this protein, we generated a JAM2-overexpressing CHO cell line and a mouse anti-JAM2 polyclonal serum raised against the ectodomain. Western blotting of CHO cell lysates estimated a 48-kDa protein (Fig. 5). This is ~14 kDa larger than the size predicted from the peptide sequence. Glycosylation of JAM2 on at least one of its three N-linked glycosylation consensus sites could explain this phenomenon.

For cellular localization, we stained confluent monolayers of both control and JAM2-expressing CHO cells with the anti-JAM2 antiserum. Fig. 6 shows that JAM2 mainly partitioned to sites of cell-cell contact, although some surface membrane fluorescence was observed. The border pattern of staining was identical to those shown by mouse JAM1 expressed in CHO cells and by endogenous human JAM1 expressed in human umbilical vein endothelial cells. For JAM1, this phenomenon contributed to the hypothesis that the extracellular domain forms homotypic interactions between cells.

To address the possibility of JAM2 homotypic binding, we utilized the recombinant ectodomain and tested its ability to capture the JAM2-expressing CHO cell line. Adhesion of calcine-loaded cells was performed under static conditions according to a previously established and well documented in vitro binding assay (18). In the same series of experiments, we examined JAM1 homotypic interactions and asked whether JAM2 could form heterotypic interactions with JAM1. Fig. 7 shows that JAM2 was clearly capable of capturing CHO cells expressing JAM2, but not control cells or those expressing JAM1. Furthermore, JAM1 was unable to maintain homotypic interactions under these conditions.

It has been demonstrated that JAM1 participates in leukocyte migration. Reasoning that JAM2 may also impinge on leukocyte adhesion and/or migration, we examined the capacity of the JAM2 extracellular domain to adhere to various leukocyte cell lines. Calcine-loaded cells were allowed to interact with JAM2-Fc captured in 96-well plates. Nonspecific binding of cells to captured mouse IgG2a was determined simultaneously and subtracted. JAM1-Fc was incorporated into the assay for comparative purposes. VCAM-Fc was included as a
positive control for all cell lines with the exception of K562, which does not express VCAM counter-receptors.

Fig. 8 shows that JAM2-Fc was able to capture the T lymphocyte cell lines HSB and HPB-ALL quite efficiently. The binding of these cells to IgG2a was always >2% of the binding to JAM2. In contrast, interactions with B lymphocytes (RAMOS), monocytic cells (HL-60 and THP-1), and the erythroleukemic K562 cell line were negligible. Most interestingly, JAM1-Fc was unable to adhere to any of these cell lines.

As demonstrated, JAM2 is capable of homotypic interactions. Thus, we next addressed whether the JAM2 ectodomain bound HSB cells through this mechanism. Fig. 4A shows that, unlike JAM1, JAM2 did not show expression in peripheral blood leukocytes. Nevertheless, to verify lack of expression in HSB cells, we used the mouse polyclonal serum to probe for JAM2 protein expression by Western blotting. No protein was detected (Fig. 5). As further proof, we compared the surface JAM2 expression levels using the following more sensitive test. The HSB and control and JAM2-expressing CHO cells were loaded with calcein and incubated with either normal mouse serum or anti-JAM2 serum. Cell surface-bound anti-JAM2 antibody was detected by cell capture in 96-well plates. Table II shows that although the anti-JAM2 serum was very effective at capturing CHO cells expressing the JAM2 protein, no HSB cell binding was apparent.

To extend these studies, we tested the ability of the mouse anti-JAM2 serum to neutralize HSB binding to recombinant JAM2. Antibody was used to block epitopes on recombinant JAM2 captured on 96-well plates. Table III shows that although preimmune serum was ineffective, anti-JAM2 serum successfully prevented HSB binding. Since relatively high levels of JAM2 were coated on these wells, we were confident that if low levels were expressed on HSB cells, the antibody should be capable of producing inhibition when incubated directly.

FIG. 5. Western blot analysis of JAM2. Cell lysate from control (lane 1) or JAM2-expressing (lane 2) CHO cells was probed with mouse anti-JAM2 extracellular domain polyclonal antibody. HSB cell lysate was probed with either preimmune (lane 3) or anti-JAM2 (lane 4) serum. Equivalent amounts of protein were loaded in all lanes.

FIG. 6. Localization of JAM2 expressed in CHO cells by immunofluorescence. Stable cell lines (expressing full-length JAM2 (A) or control (B)) were fixed with paraformaldehyde and stained with a 1:100 dilution of primary mouse anti-JAM2 antibody, followed by goat anti-mouse-fluorescein isothiocyanate. Shown is a single-angle view of cellular staining volumetrically reconstructed from 26 x 0.4-µm z axis planes. Working magnification, ×400. Digital contrast levels were not changed during image capture. Scale bar, 20 µm.

FIG. 7. JAM2 can mediate homotypic interactions. CHO cells (control (CHO-con) or expressing full-length JAM2 or JAM1) were loaded with calcein and added to immobilized mouse IgG2a and recombinant JAM2-Fc and JAM1-Fc adhesion proteins captured in 96-well plates. Wells were washed; retained cells were lysed; and fluorescence was quantitated with a fluorometer at 485 nm excitation and 530 nm emission. Bars represent mean ± S.E. (n = 6).

FIG. 8. Screening for JAM2 counter-receptors on various leukocyte cell lines. Calcein-loaded cells were added to immobilized adhesion proteins captured in 96-well plates. Wells were washed; retained cells were lysed; and fluorescence was quantitated with a fluorometer at 485 nm excitation and 530 nm emission. A, HSB; B, HPB-ALL; C, RAMOS; D, HL-60; E, K562; F, THP-1. Bars represent mean ± S.E. (n = 12).
TABLE II
Identification and Cloning of Human JAM2

| Cell type | Antibody | Mean ± S.E. |
|-----------|----------|-------------|
| HSB       | Pre      | 1877 ± 234  |
| CHO control | Pre      | 1135 ± 97   |
| CHO-JAM2  | Pre      | 1210 ± 63   |
| CHO-JAM2  | JAM2     | 1019 ± 44   |
| CHO-JAM2  | JAM2     | 2151 ± 287  |
| CHO-JAM2  | JAM2     | 112,329 ± 4457 |

with HSB cells. As predicted, under this experimental setup, the anti-JAM2 antibody was unable to inhibit HSB interactions with recombinant JAM2.

The studies thus far led us to postulate that HSB cells express a counter-receptor for JAM2. To strengthen this hypothesis and to gain a preliminary characterization of the protein, we performed precipitation experiments using JAM2-Fc. HSB cells were surface-biotinylated, washed, lysed, and incubated with JAM2-Fc. Bound proteins were precipitated using protein A and viewed on Western blots with avidin-horseradish peroxidase. Fig. 9 reveals that JAM2 could indeed specifically capture a surface protein from HSB cells of ~43 kDa. This band was not apparent in surface-biotinylated K562 cells, in agreement with the cell adhesion studies described above. Furthermore, JAM1-Fc, which was unable to bind calcein-loaded HSB cells, did not precipitate this protein.

Discussion
We describe a novel adhesion molecule that is most similar in primary sequence to the junctional adhesion molecule (2, 11). It possesses two immunoglobulin-like domains, and its amino acid sequence displays 35% identity to human JAM1. JAM2 transcripts show preferential expression in the heart. Although JAM1 is also found in the heart, expression is equally high for this protein in the lung, kidney (2), and liver.2 At the cellular level, JAM1 is found in epithelia, endothelia, leukocytes, and platelets (2–4, 20). Using our current antiserum, we were unable to characterize exactly which cells in the heart expressed JAM2. However, by Northern blotting, we were able to demonstrate lack of expression in peripheral blood leukocytes. While this manuscript was under review, a sequence identical to JAM2 was cloned, and its expression was localized exclusively to endothelial cells (21). Whether JAM1 and JAM2 show differing or overlapping patterns of expression within endothelial cells of the heart remains to be determined.

2 S. A. Cunningham, M. P. Arrate, and J. M. Rodriguez, unpublished observations.
cytoplasmic tail and other intracellular proteins following cell detachment. A similar phenomenon occurs for VE-cadherin, which is unable to promote aggregation of detached cells (30). Thus, either JAM2 ectodomains engage in higher affinity homotypic interactions, or they are less dependent upon cell attachment. Although our current data suggest that JAM2 does not form heterotypic interactions with JAM1, it is possible that the adhesion may be too weak for detection in this assay or reliant upon the factors discussed above.

Many adhesion proteins belonging to the immunoglobulin superfamily are able to engage with leukocyte counter-receptors. Furthermore, interactions are often studied using ectodomain-Fc fusions. Indeed, the extracellular domains of some adhesion proteins, e.g. selectins, VCAM, and ICAM, are released under certain conditions in vivo and correlate with disease (31–33). Using JAM2-Fc, we demonstrated that it is capable of adhering to the HSBind HPB-ALL T-cell lines. Although we were not able to detect interactions with other cell types, e.g. monocyte, adhesion specificity may not necessarily be specific to T-cells. A complete characterization of JAM2 binding to peripheral blood leukocytes under control and activated conditions is required to address this question. The adhesion of JAM1 to leukocytes has not yet been reported, and we were unable to demonstrate an interaction in this study.

Despite the ability of recombinant JAM2 to capture HSBind cells, we were unable to adhere HSBind CHO cell monolayers expressing JAM2. This is likely due to the intercellular location of JAM2. In an attempt to render JAM2-binding epistopes more accessible, we tried alternative methods employing immobilized HSBind cells with detached CHO cells. These efforts were also unsuccessful. The most likely explanation for these data is that the heterotypic interaction requires the clustering of JAM2, which would occur at cell-cell junctions, to increase the avidity of the adhesion. This would be lost in detached cells, but mimicked by capture of the JAM2 ectodomain to high density on 96-well plates.

To further verify that JAM2 specifically interacts with HSBind cells, we sought to identify a binding partner for JAM2 on HSBind CHO cell monolayers expressing JAM2. This is likely due to the intercellular location of JAM2. In an attempt to render JAM2-binding epitopes more accessible, we tried alternative methods employing immobilized HSBind cells with detached CHO cells. These efforts were also unsuccessful. The most likely explanation for these data is that the heterotypic interaction requires the clustering of JAM2, which would occur at cell-cell junctions, to increase the avidity of the adhesion. This would be lost in detached cells, but mimicked by capture of the JAM2 ectodomain to high density on 96-well plates.

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