JAM2 Interacts with α4β1

FACILITATION BY JAM3*

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We have previously reported that junctional adhesion molecule 2 (JAM2) adheres to T cells through heterotypic interactions with JAM3. An examination of the cation dependence of JAM2 adhesion to HSB cells revealed a Mn2+-enhanced binding component indicative of integrin involvement. Using neutralizing integrin antibodies, we have defined an interaction between JAM2 and αβ1 in T cells. The interaction is readily amenable to drug intervention as demonstrated by the ability of TBC 772, an α4-specific inhibitor, to attenuate the Mn2+-enhanced component. Intriguingly, the engagement of αβ1 by JAM2 is only enabled following prior adhesion of JAM2 with JAM3 and is not detectable in cells where JAM3 expression is absent. Supporting this observation, we show that neutralizing JAM3 serum and soluble JAM3 ectodomain inhibit not only JAM2 binding to JAM3 but also prevent JAM2/αβ1 interactions in T cells. We further define the first Ig-like fold of JAM2 as being competent in binding both JAM3 and αβ1 counter-receptors. Mutagenesis of the only acidic residue in the C-D loop of this Ig fold, namely Asp-82, has no bearing on αβ1 interactions, and thus JAM2 deviates somewhat from the mechanism used by other immunoglobulin superfamilly cell adhesion molecules to engage integrin.

Convincing evidence suggests key roles for junctional adhesion molecules (JAMs)† in leukocyte transmigration, although the mechanisms by which they may facilitate this process remain largely unresolved (1, 2). While displaying differential tissue and cellular expression, all JAMs localize to endothelial sites of cell contact and as such are ideally situated to support leukocyte emigration (1, 3). Most recently we demonstrated that JAM3 was the 45-kDa T cell-expressed JAM2 (VE-JAM) counter-receptor. Up-regulation of JAM3 following T cell activation revealed a mechanism by which selective adhesion and/or emigration of lymphocytes may occur (7). The observation that JAM3 is also expressed on natural killer and dendritic cells and is capable of adhering to JAM2 (VE-JAM) extends the role of the JAM2 (VE-JAM)/JAM3 heterotypic interaction in inflammation (8).

The importance of integrins in adhesion and transmigration is paramount and well established (9). Several key IgSF cell adhesion molecules engage integrin and in so doing impact on the multistep paradigm of leukocyte emigration (10, 11). To help define how JAM fits into this sequential cascade, we sought a relationship between the JAM and integrin families. In this study we report an interaction between JAM2 and αβ1 that is facilitated by prior engagement of JAM2 with T cell-expressed JAM3.

The JAM nomenclature used throughout this report, and prior publications from this group, complies with the official names designated by the Human Genome Nomenclature Committee.

EXPERIMENTAL PROCEDURES

Adhesion Assays—JAM2-Fc adhesion to various calcein-acetoxy-methyl ester (Molecular Probes Inc.)-loaded leukocyte cell lines was performed by capture of fusion protein onto 96-well plates by either goat anti-mouse IgG or chicken anti-Myc antibodies as described previously (3, 7). Adhesion was performed in Tris-buffered saline (TBS) with various combinations of 1 mM EDTA, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2 for 90 min at 37 °C in 5% CO2. Adhered cells were lysed, and fluorescence was quantified in a CytoFluor plate reader with excitation at 485 ± 20 nm and emission at 530 ± 25 nm. For inhibitor studies, the JAM3 ectodomain was cleaved from the JAM3-Fc by thrombin and purified as described previously (7). The JAM3 ectodomain, neutralizing JAM3 serum, integrin antibodies, or the compounds TBC 772 (C′WLDVC′) and TBC 1194 (C′DLVWC′) were preincubated for 30 min at 37 °C with calcein-loaded cells prior to their incorporation into the adhesion assay.

Domain Constructs and Mutagenesis—For generation of the secreted JAM2-Fc-Myc fusion, sense 5′-GGGAGGCTTACATCATATAGGCCT-TGGGTGTTC-3′ and antisense 5′-GGGAGAGCTTTATACCGGGGATC-GGGAGAACCTC-3′ oligonucleotides incorporating HindIII and BglII sites, respectively, were used to amplify the JAM2-Fc, minus the signal peptide and stop codon, from a previously generated construct (3). Cycling was achieved with Pfu DNA polymerase (Stratagene) as follows: one cycle at 95 °C for 45 s; 25 cycles at 95 °C for 45 s, 59 °C for 45 s, and 72 °C for 120 s; one cycle at 72 °C for 600 s. The product was inserted into the APtag-5 vector (GenHunter Corp.) using HindIII and BglIII to generate the JAM2-Fc with further C-terminal tags of AP, Myc, and His combined with the Ig κ-chain secretion signal peptide.

For the JAM2 Ig fold domain 1 constructs, sense 5′-GCCCGGGGATC-CAAGATGGCGGAGAQG-3′ and antisense 5′-GGTACCTGCGTGGATC-GCAAATATAC-3′ primers that incorporated BglII and KpnI sites, respectively, were used. Cycling was with Takara Ex Taq DNA Polymerase (Panvera) as follows: one cycle at 95 °C for 120 s; 20 cycles at 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s; and one cycle at 72 °C for 300 s. For JAM2 Ig fold domain 2 constructs, sense 5′-GGTACCAT-CATGGAAAGTACC-3′ and antisense 5′-GGCTATGTGGGTTTCTCTG-GCC-3′ oligonucleotides were used to loop out the N-terminal Ig fold using Pfu DNA polymerase (Stratagene) and cycling as follows: one cycle at 94 °C for 240 s, 50 °C for 120 s, 72 °C for 600 s; 12 cycles at 94 °C for 60 s, 55 °C for 120 s, and 68 °C for 600 s. Individual domains were subcloned into pFastBac1 vector (Invitrogen) possessing the constant region of mouse IgG2a (3).

The QuikChange site-directed mutagenesis kit (Stratagene) was used for mutagenesis. Primers for JAM2-D82A were: sense 5′-CAGA-CCTTCAAGGGTGCTTTTAAAATCGAGCTG-3′ and antisense 5′-CAG-TGGCTAGTTTTTAAAAGCACCTTGAGCCTG-3′.

Protein Expression—JAM-Fc fusion proteins were generated as secreted proteins in SF21 cells as previously described or in COS cells (3).
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The divalent cation dependence of integrin function is well established. Coordination of divalent cations by integrin, and in particular Mn2+ reduces conformational changes within integrin extracellular domains resulting in exposure of epitopes required for ligand engagement (12, 13). Additionally, the ability of Ca2+ to counteract this effect is a general observation (11–14). Thus, our data suggested that JAM2 was not only able to adhere with T cell-expressed JAM3 but also possibly engage with an integrin counter-receptor on the same cell. As the first candidates to investigate, we considered the α4 integrins since they play a proven role in lymphocyte motility and appear particularly specialized to promote leukocyte migration (15–19).

The α4 subunit associates with β1 and β2 to form α4β1 and α4β2. Analysis of HSB cells by flow cytometry revealed expression of both α4 and β1, whereas the β2 subunit was not detectable (Fig. 1b). To probe for an interaction between JAM2 and α4β1, we used neutralizing antibodies raised against the individual α4 and β1 subunits to inhibit the JAM2 adhesion to HSB cells. Fig. 1c unequivocally demonstrates that JAM2 binds α4β1 in HSB cells. As expected, both neutralizing antibodies and isotype controls were without effect when adhesion was tested in TBS, the component assigned to the binding of JAM2 with JAM3 (7). In contrast both anti-α4 and anti-β1 greatly attenuated the Mn2+-enhanced component, reducing it to a level comparable to that obtained in TBS alone. Higher concentrations of antibody did not further attenuate adhesion (data not shown). The data thus demonstrate a mixed binding reaction in TBS + Mn2+ where molecules of JAM2 bind JAM3 and/or α4β1. VCAM-1, the classical IgSF binding partner for α4β1, also binds to α4β2 (20). Whether JAM2 can engage with α4β2 in other cell lines or under other conditions remains an open question. Just prior to completion of this study, Weber et al. (21) demonstrated the interaction of JAM1 with LFA-1. A β2 integrin binding component is not apparent in our JAM2 adhesion assays; although the β2 subunit is expressed on the HSB cell surface, the neutralizing antibody has no effect on JAM2 HSB cell adhesion (Fig. 1, b and c).

To extend and further validate this interaction, we asked whether TBC 772, a cyclic hexapeptide and potent antagonist of α4 integrins, could prevent the engagement of JAM2 with α4β1 (22). The dose-response curves show that although TBC 772 is ineffective when assessed in TBS, a clear attenuation is observed in the presence of Mn2+ (Fig. 1d). Following inhibition with compound, adhesion reached a level that approximated that obtained in TBS with an IC50 averaging 60 nM over three independent experiments. The specificity of inhibition is further demonstrated by the inability of TBC 1194, a control scrambled peptide, to reduce the Mn2+-enhanced component (Fig. 1d). These results convincingly demonstrate that the JAM2/α4β1 interaction is readily amenable to inhibition by small molecules and thus provides clear possibilities for future drug development.

The selective adhesion of JAM2 to T cells was previously discovered when performing binding assays in the presence of all three cations (3, 7). While these conditions are optimal for monitoring the JAM2/JAM3 adhesive event in this assay, the JAM2/α4β1 engagement that occurs with the defined cation requirements reported in Fig. 1a would have been overlooked. Thus, we reassessed JAM2 adhesion in the presence of Mn2+ to B cells (Ramos) and monocytic cells (HL60) that also express α4β1 but not JAM3 (7). The erythroleukemic K562 cells that express neither JAM3 nor α4β1 were included as a negative control. Surprisingly, Fig. 2a demonstrates that Mn2+-dependent JAM2 binding is restricted to JAM3-expressing T cells. Therefore, our assay conditions revealed a possible dependence

For the latter, cells were transfected with 6 μg of the various pcDNA6 JAM-Fc constructs and 18 μl of FuGENE 6 reagent (Roche Molecular Biochemicals). Serum-free media from either cell type was harvested on day 3 and purified over HiTrap Protein A HP columns (Amersham Biosciences).

**Flow Cytometry—**HSB cells (1 × 10⁶) were labeled with primary monoclonal antibodies in phosphate-buffered saline for 45 min followed by subsequent incubation with fluorescein isothiocyanate-conjugated secondary antibodies. Cells were analyzed in a Beckman Coulter Epics XL.

**Antibodies and Drugs—**The neutralizing integrin antibodies against α4 (clone P4C2), β1 (clone P5D2), and β3 (clone YFC118.3) were from Chemicon, and anti-β1 (clone E35-95) was from BD PharMingen. Chicken anti-Myc was purchased from Aves Labs, Inc. TBC 772 and TBC 1194 are drugs generated by Texas Biotechnology Corp. Neutralizing anti-JAM3 polyclonal serum was generated in female BALB/c mice. The purified JAM3 ectodomain was used as immunogen using procedures described previously (3).

**RESULTS AND DISCUSSION**

In characterizing the JAM2 interaction with T cell-expressed JAM3, we routinely performed adhesion in TBS plus all three of the cations Ca2+, Mg2+, and Mn2+ (binding buffer). An examination of the divalent cation dependence of adhesion revealed that JAM2 binding to HSB cells occurred independently of cation additions (Fig. 1a). Thus, binding in the presence of TBS plus 1 mM EDTA was comparable with that obtained in binding buffer. In contrast, a marked enhancement of JAM2 adhesion, above and beyond that obtained in binding buffer, was observed in the presence of TBS plus Mn2+. Subsequent analysis using cation combinations revealed that calcium was responsible for masking the enhancement of Mn2+ (Fig. 1a). In HSB and other T cell lines, we routinely recorded a 10-fold enhancement of adhesion.

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**FIG. 1. JAM2 binds α4β1 in HSB cells.** a, cation dependence of JAM2 adhesion to T cells. Calcium-loaded HSB cells were bound to JAM2-Fc captured by goat anti-mouse Ig2Ga. Adhesion was performed in the presence of the various cations shown and expressed as percentage of adhesion obtained in binding buffer that consists of TBS + 1 mM Ca2+/Mg2+/Mn2+. The data show average ± S.E., n = 6. b, analysis of α4, β1, β3, and β2 integrin expression on HSB cells by flow cytometry. Cells were labeled with integrin antibodies (•) and isotype controls (○) as shown. The results shown are from a representative analysis. c, JAM2-Fc-Myc tagged protein was captured to 96-well plates by chicken anti-Myc antibody. Adhesion was performed in binding buffer. In contrast a marked enhancement of Mn2+-enhanced component, reducing it to a level comparable to that obtained in TBS alone. Higher concentrations of antibody did not further attenuate adhesion (data not shown). The data thus demonstrate a mixed binding reaction in TBS + Mn2+ where molecules of JAM2 bind JAM3 and/or α4β1. VCAM-1, the classical IgSF binding partner for α4β1, also binds to α4β2 (20). Whether JAM2 can engage with α4β2 in other cell lines or under other conditions remains an open question. Just prior to completion of this study, Weber et al. (21) demonstrated the interaction of JAM1 with LFA-1. A β2 integrin binding component is not apparent in our JAM2 adhesion assays; although the β2 subunit is expressed on the HSB cell surface, the neutralizing antibody has no effect on JAM2 HSB cell adhesion (Fig. 1, b and c).

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**TABLE 1.** Summary of cation combinations used in adhesion assays. The cation combinations used in adhesion assays are listed in Table 1.
for the JAM2/αβ1 interaction upon co-expression of JAM3 within the same cell.

We next wished to determine what impact T cell-expressed JAM3 had on the JAM2/ integrin interaction. It seemed likely that binding of JAM2 to JAM3 facilitated interactions of JAM2 with αβ1. To test this hypothesis, we studied the effect of soluble JAM3 ectodomain (cleaved by thrombin from JAM3-Fc and purified), which is an efficient blocker of JAM3 mouse serum to attenuate JAM2 binding to HSB cells. A 10-fold molar excess of soluble JAM3 (+) was added to the adhesion reaction, and binding was compared with the control reaction (−). Fig. 2a shows that excess soluble JAM3 can completely prevent HSB cell adhesion to cell surface-expressed JAM3 (7). Fig. 2b shows that excess soluble JAM3 can completely prevent HSB cell adhesion to JAM2 regardless of buffer composition. Although this result supported our hypothesis, it might be postulated that JAM3 and αβ1 share a common binding site on JAM2. Upon soluble JAM3 binding to captured JAM2-Fc, epitopes for engagement with αβ1 may be masked. Therefore we used neutralizing anti-JAM3 serum to prevent the JAM2/JAM3 interaction while leaving JAM2 free for adhesion (Fig. 2c). The complete block of JAM2 binding under these conditions allows us to conclude that JAM2 must bind HSB cell-expressed JAM3 as a prerequisite to interactions with αβ1.

To explore the mechanism in more detail, we set out to determine the domain dependence of the specific adhesions. The N-terminal and C-terminal Ig folds were generated as each Ig fold is capable of supporting both cation-independent and Mn²⁺-enhanced adhesion and therefore possesses the primary sites of contact for both JAM3 and integrin binding. The other well described IgSF molecules that engage α4 integrins, namely VCAM-1 and MadCAM-1, also present dominant binding motifs in the most N-terminal Ig fold (23–26). In contrast, the second Ig fold of JAM1, which is located adjacent to the transmembrane domain, mediates binding to LFA-1 (21).

Commonly, IgSF molecules bind integrin through key residues found within the loop(s) intervening the C and D β-strands of the interacting Ig fold (11). Further, a consensus, (L/D/E/S/T/V)(P/S), has been identified from homologous sequences within VCAM-1, intercellular adhesion molecules, and MadCAM-1. Examination of the JAM2 sequence reveals only two acidic amino acids, Glu-62 and Asp-82, that fall within the sequence intervening proposed β-strands C and D of the N-terminal Ig fold (27). By analogy with JAM1, Glu-62 is predicted to be an integral part of the conserved dimerization motif R(V/I)L/E, namely mediating salt bridge formation between JAM2 monomers (27). Further, Glu-62 aligns well within the C’ β-strand. In contrast, Asp-82 is predicted to reside within the C’-D loop. While it does not fall within the context specified by other cell adhesion molecules, the QDG motif is reminiscent of the invariant RGD sequence common to many adhesions extracellular macromolecules. To explore its contributions to JAM2 adhesion, we mutated Asp-82 to alanine and studied its consequences. Surprisingly, Fig. 3b shows that JAM2 Asp-82 does not play a significant role, if any, in establishment of the JAM2-integrin complex. Less remarkable was the observation that loss of this charge does not attenuate JAM3 binding. JAM2 may utilize an acidic residue in a different loop or even possibly a β-strand to bind αβ1. As such, it deviates somewhat from the classical IgSF/integrin interaction. The alternative binding sites employed by JAM2 to achieve αβ1 engagement may underlie the apparent low affinity of this adhesive event.

The requirements for efficient adhesion of JAM2 and VCAM-1 with αβ1 differ under the same experimental conditions. VCAM-1 is fully capable of forming strong contacts in the presence of 1 mM Ca²⁺, Mg²⁺, and Mn²⁺, and clearly its interaction is independent of JAM3. On the other hand, under the conditions defined herein, the JAM2/αβ1 interaction does not appear sufficient to allow capture of HSB cells in itself but requires the participation of JAM3. Further, the inhibitory Ca²⁺ cation must be minimized in the assay. Since Mn²⁺ is by far the most potent stimulus for enabling the transition of the β1 subunit from the inactive to active conformation, our data would suggest that only the fully activated, Mn²⁺-bound αβ1 allows for JAM2 binding (28). Further, this conformation in itself is not sufficient but requires an event contributed by the JAM2 interaction with JAM3. While this may simply be facilitation of a lower affinity interaction between JAM2 and αβ1 by enabling more frequent, closer contacts between the two molecules, other more complicated scenarios can be envisaged. For example, it was postulated most recently from yeast two-hybrid studies that JAM1 could bind LFA-1 in both cis and trans (21). Although JAM2 does not appear to be a T cell-expressed molecule, a similar lateral interaction could be envisaged between α4 and JAM3 (7, 8). However, using the same yeast two-hybrid system, we have been unable to detect such a relationship between the cytoplasmic tails of α4 and JAM3 (data not show).
In the multistep paradigm of leukocyte emigration, provided that JAM2 remains primarily within the cell junctions, we would predict the JAM2/αβ1 event to occur more distal to that of the VCAM-1/αβ1 interaction as the leukocyte begins its emigration between endothelial cells. By definition, endothelial molecules that facilitate leukocyte extravasation are required to form only weak and/or transient interactions with the migrating cell. This first report, describing the characteristics of JAM2 adhesion to αβ1, presents a new interaction that may be particularly suited for this role and may provide a new target site for development of novel anti-inflammatory therapies.

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