Cis-Dimerization Mediates Function of Junctional Adhesion Molecule A

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Junctional adhesion molecule-A (JAM-A) is a transmembrane component of tight junctions that has been proposed to play a role in regulating epithelial cell adhesion and migration, yet mechanistic structure–function studies are lacking. Although biochemical and structural studies indicate that JAM-A forms *cis*-homodimers, the functional significance of dimerization is unclear. Here, we report the effects of *cis*-dimerization-defective JAM-A mutants on epithelial cell migration and adhesion. Overexpression of dimerization-defective JAM-A mutants in 293T cells inhibited cell spreading and migration across permeable filters. Similar inhibition was observed with using dimerization-blocking antibodies. Analyses of cells expressing the JAM-A dimerization-defective mutant proteins revealed diminished $\beta 1$ integrin protein but not mRNA levels. Further analyses of $\beta 1$ protein localization and expression after disruption of JAM-A dimerization suggested that internalization of $\beta 1$ integrin precedes degradation. A functional link between JAM-A and $\beta 1$ integrin was confirmed by restoration of cell migration to control levels after overexpression of $\beta 1$ integrin in JAM-A dimerization-defective cells. Last, we show that the functional effects of JAM dimerization require its carboxy-terminal postsynaptic density 95/disc-large/zonula occludins-1 binding motif. These results suggest that dimerization of JAM-A regulates cell migration and adhesion through indirect mechanisms involving posttranscriptional control of $\beta 1$ integrin levels.

INTRODUCTION

Junctional adhesion molecule-A (JAM-A) is a transmembrane component of tight junctions in epithelial and endothelial cells. In addition, JAM-A is expressed on the surface of blood cells, including leukocytes and platelets (Gupta *et al.*, 2000). JAM-A has been implicated in a diverse array of functions, including intercellular junction assembly (Liang *et al.*, 2000; Liu *et al.*, 2000), cell adhesion (Mandell *et al.*, 2005), leukocyte transmigration (Martin-Padura *et al.*, 1998; Del Maschio *et al.*, 1999; Ostermann *et al.*, 2002; Corada *et al.*, 2005; Khandoga *et al.*, 2005; Ostermann *et al.*, 2005), platelet activation (Kornecki *et al.*, 1990; Naik *et al.*, 1995; Sobocka *et al.*, 2000; Babinska *et al.*, 2002), and angiogenesis (Naik *et al.*, 2003a,b). Additionally, JAM-A has been shown to be a receptor for reovirus (Barton *et al.*, 2001; Forrest *et al.*, 2003; Prota *et al.*, 2003; Campbell *et al.*, 2005). Structurally, JAM-A consists of an extracellular domain with two immunoglobulin (Ig)-like loops, a membrane-spanning segment, and a cytoplasmic tail containing a C-terminal postsynaptic density 95/disc-large/zonula occludins-1 (PDZ) binding motif. The cytoplasmic tail of JAM-A has been reported to associate, either directly or indirectly, with PDZ domain-contain-

ing proteins, such as zona occludens-1 (Bazzoni *et al.*, 2000; Ebnet *et al.*, 2000), AF-6/Afadin (Ebnet *et al.*, 2000) and Par3/ASIP (Ebnet *et al.*, 2001; Itoh *et al.*, 2001), through characteristic hydrophobic residues (FLV) at the carboxy terminus. Evidence suggests that the cytoplasmic tail plays an important role in directing JAM-A localization to intercellular contacts (Ozaki *et al.*, 2000), formation of tight junctions (Rehder *et al.*, 2006), and transduction of intracellular signaling events (Bazzoni *et al.*, 2005; Mandell *et al.*, 2005; Naik and Naik, 2006). The extracellular domain of JAM-A can form homodimers through its N-terminal Ig loop (Prota *et al.*, 2003). Furthermore, the human JAM-A crystal structure predicts dimers forming between molecules on the same cell (*in cis*) (Prota *et al.*, 2003); however, the murine protein crystal structure predicts tetramer formation between the extracellular loops between cells (*in trans*) (Kostrewa *et al.*, 2001). Despite these intriguing observations, mechanistic studies linking dimerization of JAM-A to these functions are lacking.

JAM-A is abundantly expressed in polarized epithelia, yet its role in epithelial cell migration has not been studied. In endothelial cells, controversy exists concerning the functional role of JAM-A in the regulation of cell migration. In a study by Bazzoni *et al.* (2005) the absence of JAM-A in endothelial cells enhanced spontaneous and random cell motility by reducing the stability of microtubules and impairing the formation of focal adhesions (Bazzoni *et al.*, 2005). Transfection of full-length JAM-A, but not a C-terminal PDZ binding motif deleted JAM-A mutant restored random cell motility. Recently, JAM-A was shown to interact with integrin $\alpha v \beta 3$ and to enhance endothelial cell migration on vitronectin when overexpressed, as well as enhance phosphorylation of focal adhesion kinase and mitogen-activated protein kinase (MAPK) (Naik and Naik, 2006). None

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Abbreviations used: 6163, dimerization-defective JAM-A mutant E61A/K63A; DL1, dimerization-defective JAM-A mutant with deletion of the distal most immunoglobulin-like loop; FA, focal adhesion; JAM-A, junctional adhesion molecule A; QRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction analysis.

of the above-mentioned studies examined the role of JAM-A dimerization in mediating these effects on migration.

We recently reported that transient knockdown of JAM-A expression in epithelial cells resulted in decreased protein levels of $\beta 1$ integrin that correlated with altered cell shape and decreased cell adhesion (Mandell *et al.*, 2005). This study suggested that JAM-A may regulate cell adhesion by increasing integrin protein expression; however, the mechanisms for these JAM-A-mediated effects was not investigated and is the topic of this report.

Based upon these observations, we hypothesized that *cis*-dimerization of JAM-A plays a key role in regulation of cell migration. To test this hypothesis, we stably overexpressed wild-type and JAM-A with mutations in the putative dimerization domain in 293T cells, a human epithelial cell line that expresses low levels of JAM-A. Dimerization of the extracellular domain is mediated by the predicted formation of salt bridges in the membrane distal Ig loop D1. One dimerization-defective JAM-A mutant we studied has point mutations at two residues (E61A/K63A) predicted by the crystal structure to be required for dimerization (6163), and both mutations have been shown to disrupt dimerization *in vitro* (Mandell *et al.*, 2004). Mutation of either residue has been shown to result in JAM-A formation of only monomers as assessed by gel filtration (Guglielmi *et al.*, 2007). A second dimerization-defective construct that we tested consists of JAM-A with a deletion of the distal most immunoglobulin-like loop, which is necessary for dimerization (DL1). We observed that overexpression of both the 6163 and DL1 dimerization-defective mutants resulted in decreased 293T cell migration and spreading. We also determined that these cellular effects are mediated by decreased $\beta 1$ integrin protein levels. Notably, for the dimerization-defective constructs to have an effect, we determined that there must be a carboxy-terminal PDZ binding motif on JAM-A, suggesting that dimerization-defective mutants mediate their effects through sequestration of scaffolding proteins. These observations indicate that JAM-A dimerization indirectly regulates cell migration through signaling events that ultimately increase $\beta 1$ integrin protein levels resulting in increased cell adhesion, spreading, and migration.

MATERIALS AND METHODS

Cell Culture

293T human embryonic kidney epithelial cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU of penicillin, 100 μ g/ml streptomycin, 15 mM HEPES, and 1% nonessential amino acids (Cellgro, Mediatech, Herndon, VA). The cells were subcultured and harvested with 0.05% trypsin with EDTA in Hank's balanced salt solution (HBSS-) (Sigma-Aldrich, St. Louis, MO). SK-CO-15 cells, a transformed human colonic epithelial cell line (Lisanti *et al.*, 1989; Mandell *et al.*, 2005; Ivanov *et al.*, 2006) were cultured as described previously in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 15 mM HEPES, 1% nonessential amino acids, 40 μ g/ml penicillin, and 100 μ g/ml streptomycin, pH 7.4.

JAM-A Mutant Plasmid Production

Production of the plasmids encoding full-length JAM-A, the JAM-A truncation mutant lacking the N-terminal Ig-like loop (DL1), and a JAM-A point mutant containing substitutions at residues 61 and 63 (E61R/K63E, 6163) have been described previously (Mandell *et al.*, 2004). Briefly, the coding region along with ~1.5 kb of the 3' untranslated region was restriction enzyme digested and inserted into a pIRES-EGFP vector. Plasmids of all four constructs for transient transfections were amplified by polymerase chain reaction (PCR) (5'-ATATGGTACCAGCCACCATGGGGAC AAA-3'; 5'-ATATCTCGAGTCACACCAG GAATGACGAGGTCTG-3') and digested with KpnI and XhoI before ligation into pCDNA3.0. A construct lacking both the DL1 and last three amino acids (FLV) was made from the DL1 mutant by using PCR (5'-ATATGGTACCAGCCACCATGGGGACAAA-3'; 5'-ATATCTCGAGTCATGACGAGT CTGTTTGA-3'). PCR product was digested and ligated into pCDNA3.0 as described above.

Stable Cell Lines

293T cells were transfected with constructs and empty vector (pIRES2-EGFP) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The transfected cells were enriched using flow-cytometric-based cell sorting by gating on enhanced green fluorescent protein (EGFP) fluorescence. Single clones were grown under Geneticin (G418; Invitrogen) selection, and expression of EGFP in colonies was verified. Cell lines were verified for expression of JAM-A constructs by Western blot and immunofluorescence. Early passages were frozen in FBS and 10% dimethyl sulfoxide. No cell lines were used for >10 passages from transfection in these studies, and expression of EGFP was monitored before each experiment to ensure that cells used remained stably transfected.

Transient Cell Transfections

For plasmid transfections, the 293T cells were transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM I (Invitrogen) according to the manufacturer's protocol. For $\beta 1$ integrin overexpression, constructs containing full-length $\beta 1$ integrin cDNA in pCMV-XL6OriGene or the empty pCMV-XL6 vector were used at a concentration of 1.0 μ g of plasmid per ml, and assays were performed 48 h after transfection. SmartPool siRNA targeted to $\beta 1$ integrin was obtained from Dharmacon RNA Technologies (Lafayette, CO). For transient overexpression of JAM-A or JAM-A mutants, pCDNA3.0 with the JAM-A protein coding sequence or empty vector (pCDNA3.0) were used in the same manner as the $\beta 1$ integrin construct. siRNA transfections were performed in Opti-MEM I (Invitrogen) with HiPerFect (QIAGEN, Valencia, CA) according to the manufacturer's protocol using either a SmartPool for $\beta 1$ integrin or siRNA for cyclophilin B (a control gene), and the final concentration of siRNA was 50 nM. Three JAM-A siRNA sequences were used at a total concentration of 50 nM: 5'-AGGTGCACATGCCAATAAA-3', 5'-CAGTCTATTATTAACCTTA-3', and 5'-TCCCTCTAAGTAGACAGC-3'. Experimental time points for DNA and siRNA transfections were 48 and 72 h, respectively.

Antibodies

The murine monoclonal anti-JAM-A antibodies J10.4 and 1H2A9 were described previously (Liu *et al.*, 2000; Mandell *et al.*, 2004). All other antibodies were obtained commercially: murine anti- $\beta 1$ integrin (BD Biosciences PharMingen, San Diego, CA), rat anti- $\beta 1$ integrin (Mab13) (BD Biosciences PharMingen), rabbit anti- $\beta 4$ integrin (Santa Cruz Biotechnology), rabbit anti-phospho-paxillin (Tyr118) (Cell Signaling Technology, Danvers, MA), rabbit anti-Rap1 (Upstate Biotechnology, Charlottesville, VA), and murine anti-tubulin (Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), and fluorescently labeled secondary antibodies were obtained from Invitrogen.

Cell Migration Assays

Cells were washed in Hank's balanced salt solution without calcium (HBSS-), and then they were incubated in cell dissociation buffer (enzyme free, phosphate-buffered saline [PBS]-based) (Invitrogen) for 30 min to disrupt cell junctions. Cells were then washed in HBSS-, centrifuged, and resuspended in serum-free DMEM. Cells (1×10^5) were added on the top side of 8- μ m pore size Transwell (Corning Life Sciences, Acton, MA) inserts that had been coated with 10 μ g/ml fibronectin overnight on the underside of the transwell (Figure 2A). Cells were allowed to migrate across inserts toward the fibronectin-coated side for 3 h at 37°C. Inserts were then washed, fixed with ethanol, and stained with phalloidin. Confocal fluorescence microscopy was performed on the lower chamber side of inserts using a laser scanning confocal microscope (Axioplan2 microscope equipped with LSM510 Meta; Carl Zeiss, Thornwood, NY). Cells were counted from two randomly chosen fields of view from three separate inserts, and average counts with SEM were used to quantify the extent of migration. For the study on the effects of J10.4 and 1H2A9 antibodies on cell migration, cells were treated with 10 μ g/ml J10.4 or 1H2A9 at 4°C for 1 h before the addition of cells into Transwell inserts.

Cell Spreading

Cells (5×10^4) were added on coverslips coated with 10 μ g/ml fibronectin in 24-well plates, and then they were incubated for 1 h at 37°C. Phase-contrast microscopy was used to capture images at 5 $\times$ magnification for cell spreading assays. Two separate fields from each of three coverslips were averaged to assess the extent of cell spreading. Rounded cells with phase sharp edges and no protrusions were defined as rounded, and cells with protrusions and flattened morphology were defined as spreading. To study cell protrusion changes, 2.5×10^4 cells were incubated on 10 μ g/ml fibronectin coverslips at 37°C for 48 h, and then they were washed three times in HBSS+. Cells were then fixed in ethanol, blocked in 1% bovine serum albumin (BSA) in HBSS+, and stained with Alexa-488-phalloidin (1:1000) or Topro-3 (1:1000). Confocal fluorescence images were captured using a Zeiss laser-scanning microscope. The length of all cellular protrusions 30 high powered fields from three separate experiments were measured using the MetaMorph imaging program (Carl Zeiss) and reported as average length with SE of the mean.

Immunoblotting

Cells were homogenized in lysis buffer containing 20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.4. Lysis buffer was supplemented with protease and phosphatase inhibitor cocktails containing 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, pepstatin A, E-64, bestatin, leupeptin, aprotinin, microcystin LR, cantharidin, (-)-*p*-bromotetramisole, sodium vanadate, sodium molybdate, sodium tartrate, and imidazole (1:100 dilution; Sigma-Aldrich). Protein concentrations in lysates were quantified by bicinchoninic acid assay. Lysates were cleared by centrifugation and immediately boiled in SDS sample buffer. SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblots were performed by standard methods. Immunoblots were probed for tubulin to ensure equal protein loading.

Immunofluorescence Microscopy

Cells were grown on transwell filters or glass coverslips, fixed in 3.7% paraformaldehyde for 20 min at 25°C, permeabilized with 0.2% Triton X-100 for 10 min at 25°C, and blocked in 1% BSA in HBSS+ for 1 h. Primary antibodies were diluted in blocking buffer and incubated with cells for 1 h at 25°C. The cells were washed in HBSS+, and then they were incubated in fluorescently labeled secondary antibodies or Alexa fluorophore-conjugated phalloidin for 45 min at room temperature. Labeled cells were then washed and mounted in Prolong Antifade agent (Invitrogen). Confocal fluorescence images were captured using a laser-scanning microscope (Carl Zeiss).

Chemical Inhibitors

MG-132 (Calbiochem, San Diego, CA) was used at 10 μ M final concentration, whereas MG-262 (BIOMOL Research Laboratories, Plymouth Meeting, PA) was used at 20 μ M final concentration. Cyclohexamide (MP Biomedicals, Irvine, CA) was used at a final concentration of 50 μ g/ml.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. RT-PCR was performed by using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA) on iCycle iQ real-time PCR detection system (Bio-Rad) according to the manufacturer's instructions. A pair of PCR primers (5'-ATCCCAGAGGCT CCAAAGAT-3' and 5'-CTAAATGGGCTGGTGACAG-3') was used to amplify β 1 integrin. Primer pair (5'-CGGC-TACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3') targeting 18S RNA was used as internal control.

Rap1 Activity Assay

Active Rap1 was detected using a pull-down procedure (Franke *et al.*, 1997). Cells were lysed at 4°C in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5M NaCl, 1% Nonidet P-40, 2.5 mM MgCl₂, and 10% glycerol) supplemented with protease inhibitor cocktails (1:100; Sigma-Aldrich). The lysates were clarified by centrifugation, and then 200 μ g of protein from each lysate was incubated with 30 μ l of agarose beads conjugated with Ral GDS-Rap-binding domain (Upstate Biotechnology) for 45 min at 4°C. The beads were washed four times in lysis buffer, resuspended in 2 $\times$ reducing SDS sample buffer, and boiled for 15 min. The entire sample was then loaded into each well for separation by SDS-PAGE, and active Rap1 detected by immunoblot.

RESULTS

Expression of Dimerization-defective JAM-A Mutants Inhibits Migration of 293T Cells

To investigate the role of JAM-A *cis*-dimerization in cell migration, we used 293T human embryonic kidney epithelial cells that normally express low levels of JAM-A protein. We generated 293T stable cell lines expressing two different JAM-A mutants that either lack the distal extracellular Ig loop (DL1) or contain mutations in a characteristic dimerization motif within DL1 (6163). The 6163 mutant contains two point mutations at residues 61 and 63 that are involved in the formation of JAM-A homophilic dimer salt bridges (Figure 1A) (Prota *et al.*, 2003; Mandell *et al.*, 2004). Both mutants have been reported to be unable to form extracellular homophilic dimers as reported by gel filtration (Gugliemi *et al.*, 2007). Furthermore, in Western blots of JAM-A from cells expressing the 6163 mutant that were treated with a cell impermeable cross-linker, we observed no JAM-A dimerization in contrast to results from cells expressing only wild-type protein (Supplemental Figure 1). We also generated

Figure 1. Stable expression of JAM-A mutants in 293T cells. (A) Structure for endogenous JAM-A is shown and contains two Ig-like loops, a transmembrane domain, and a cytoplasmic tail with a carboxy-terminal PDZ binding domain. The star in the 6163 mutant highlights the region of mutations at amino acids 61 and 63 that forms a salt-bridge between two JAM-A molecules *in cis*. The DL1 mutant completely lacks the distal most Ig-like domain that mediates *cis*-dimerization. (B) Western blots demonstrating overexpression of wild-type and mutant proteins in 293T stable transfectants. Tubulin is shown as a protein loading control. A 10-s film exposure (B1) and a 2-min exposure (B2) are shown to demonstrate the presence of JAM-A overexpression in the stable cell lines and to highlight the presence of endogenous JAM-A, respectively. (C) Immunofluorescence labeling of 293T cells expressing mutant constructs for JAM-A protein demonstrating similar localization of endogenous JAM-A, exogenous JAM-A, and mutant JAM-A to cell-cell contacts.

two control cell lines, one line that overexpresses wild-type JAM-A and another line that contains the appropriate empty vector control (pIRES2-GFP). As shown in Figure 1, B and C, 293T cells normally express endogenous JAM-A, which localizes to cell-cell contacts. Expression levels of vector control, wild-type-overexpressing, and mutant JAM-A 293T cell lines were analyzed by Western blotting, and they are shown in Figure 1B1 after 10 s of exposure by enhanced chemiluminescence (ECL). Figure 1B2 highlights, by longer ECL exposure (2 min), endogenous JAM-A expression compared with that in cells stably transfected with JAM-A. Furthermore, immunofluorescence analyses indicated that in overexpressing cells, exogenous JAM-A localized to cell-cell contacts in a manner similar to endogenous, control JAM-A (Figure 1C).

Initially, we investigated the role of JAM-A dimerization on cell migration across matrix-coated permeable transwell filters. As shown in Figure 2A, transwell inserts with 8.0- μ m pores, which permit passage of 293T cells, were coated with fibronectin on the bottom of the transwell insert. Cells stably expressing the dimerization-defective JAM-A mutants or

Figure 2. JAM-A dimerization-defective mutant effects cell migration. (A) Schematic for cell migration assays. As detailed in the methods, 293T cells suspended in DMEM without serum were added to the upper chamber of transwell filters coated with 10 μ g/ml fibronectin on the bottom of the filters. To assess the extent of cell migration, cells were stained with phalloidin and confocal images were taken of the underside of the transwell filter. (B) Cell migration assays revealed that overexpression of DL1 and 6163, but not wild-type JAM-A resulted in decreased cell migration. Bar, 200 μ m. (C) The number of cells that migrated per square millimeter over 3 h was determined from three separate filters after assessing two photomicrographs of each filter and counting the number of cells by using MetaMorph software. Average counts $\pm$ SEM are shown. As can be seen, overexpression of 6163 and DL1 mutants significantly decreased cell migration (* $p < 0.05$).

controls were added to top chamber of the setup, and they were incubated for 3 h at 37°C. Cell migration across filters was quantified after phalloidin staining and immunofluorescence analysis by confocal microscopy. As shown in Figure 2, B and C, overexpression of dimerization-defective JAM-A resulted in significantly decreased transfilter migration (200–250 cells/mm²) compared with the empty vector control as well the wild-type JAM-A overexpression control (500–600 cells/mm²). These data suggest that the 6163 and DL1 JAM-A mutants, which prevent dimerization, have dominant-negative effects on JAM-A functions, as exemplified by the decreased levels of observed cell migration.

Dimerization-defective JAM-A Mutants Inhibit Cell Spreading on Fibronectin

To gain insight into mechanisms underlying inhibitory effects of the dimerization-defective JAM-A mutants on cell migration, we next analyzed mutant cells for effects on adhesion and spreading on extracellular matrix. Preliminary

Figure 3. JAM-A mutants alter 293T cell spreading. (A) Cell spreading assays reveal that dimerization-defective mutants 6163 and DL1 decrease cell spreading. Serum-starved 293T cells were added to 10 μ g/ml fibronectin-coated coverslips. After 1 h at 37°C, phase contrast images were taken (2 images each for 3 coverslips/sample). Black arrows point to spreading cells, and white arrows point to rounded cells. (B) Spreading versus rounded cells were counted manually for each image, and average $\pm$ SEM was plotted. As can be seen, expression of dimerization-defective JAM-A significantly decreases 293T cell spreading (* $p < 0.05$).

adhesion experiments suggested that a higher percentage of 293T cells adhered to collagen I, collagen IV, and fibronectin compared with laminin. Furthermore, adhesion was decreased after down-regulation of expression of JAM-A (Mandell *et al.*, 2005). Further experiments were performed using fibronectin-coated surfaces. Serum-starved control and stably transfected cells were seeded on fibronectin-coated coverslips. After 1 h, phase contrast images were taken and analyzed as detailed in *Materials and Methods*. As shown in Figure 3, A and B, control 293T cell lines adhered and spread on fibronectin. In contrast, cells bearing either dimerization-deficient JAM-A mutant failed to spread and remained rounded. The decrease in cell spreading in JAM-A dimerization-defective mutants versus control cells was 90%.

We hypothesized that the observed decrease in spreading would correlate with altered length of cellular extensions after prolonged growth on matrix. Phalloidin labeling was used to highlight F-actin in 293T cells actively spreading on fibronectin for 2 d. Indeed, confocal analysis revealed extensive elongated protrusions in control cells with an average length of 25–35 μ m, whereas the length of cellular protrusions was significantly decreased in the dimerization-defective JAM-A-expressing cell lines with an average length of 10–15 μ m (Figure 4). Together, these data suggest that dimerization of JAM-A may be required for spreading and formation of peripheral membrane protrusions, which represents an important early step in cell migration.

Dimerization-defective JAM-A Cell Lines Have a Decreased Density of Focal Concentrations of Phosphorylated Paxillin

Because focal adhesion (FA) complexes are involved in cell attachment, spreading, and migration, we hypothesized that expression of dimerization-defective JAM-A cell lines influ-

Figure 4. JAM-A dimerization defective-mutants decrease the length of 293T cell protrusions. (A) Confocal microscopy revealed a decrease in both the length and number of cellular protrusions (white arrows) in 293T cells overexpressing the 6163 and DL1 mutants. Bar, 50 μ M. (B) All cell protrusions in eight images were measured using the program ImageJ. Bars are the average length in micrometers + SEM. As can be seen, cellular protrusions in JAM-A dimerization-defective cells are significantly shorter than those in cells expressing wild-type JAM-A (* $p < 0.05$).

ences FAs. Experiments were performed to investigate whether overexpression of dimerization-defective JAM-A mutants resulted in decreased numbers affects the assembly of FAs in 293T cells. FAs were visualized by immunofluorescence labeling of the phosphorylated forms of their major protein component paxillin. Immunofluorescence staining revealed that PY118-paxillin was predominantly localized within discrete peripheral rod-like structures characteristic of FA in control cell lines. Interestingly, the number of these phospho-paxillin-based focal concentrations of phosphorylated paxillin per square millimeter was dramatically reduced in cells bearing either the 6163 or the DL1 JAM-A mutants (Figure 5A) from 2860 ± 680 and 2648 ± 435 for control lines to 1020 ± 240 and 812 ± 203 for the 6163 and DL1 cell lines, respectively (Figure 5B).

JAM-A Dimerization-defective Mutants Have Decreased $\beta 1$ Integrin Protein Levels

Because integrins concentrate at focal adhesions and mediate cell attachment to matrix during cell migration, we performed experiments to analyze the effect of JAM-A on integrins. The $\beta 1$ integrins are abundantly expressed in epithelial cells, and they are known to bind to extracellular matrix components, including collagen I, collagen IV, and fibronectin. We previously observed decreased $\beta 1$ but not $\beta 4$ integrin protein levels in cells after transient knockdown of JAM-A protein (Mandell *et al.*, 2005). We thus investigated whether stable overexpression of dimerization-defective JAM-A mutants results in altered expression of $\beta 1$ integrins. Densitometric analyses of Western blots revealed a 73% decrease in $\beta 1$ integrin protein levels in mutant cell lines compared with controls in subconfluent, ac-

Figure 5. The number of PY118-paxillin focal concentrations is decreased by overexpression of 6163 and DL1 mutants in 293T cells. (A) Staining with phalloidin, anti-PY118 paxillin, and Topro revealed that overexpression of both the 6163 and DL1 mutants significantly decreased the number and density of focal contacts as determined by PY-118 paxillin staining. Bar, 20 μ m. (B) PY118-paxillin containing rod-shaped structures were counted with MetaMorph software based on staining with anti-PY118 paxillin. Five slides were counted per sample, and average + SEM/mm² was reported (* $p < 0.05$).

tively spreading cells (Figure 6A). Conversely, there was no effect on $\beta 4$ integrin levels.

Decreased Protein Levels of $\beta 1$ Integrin in Dimerization-defective JAM-A-expressing Cells Are Not Due to Transcriptional Effects

The dimerization-defective JAM-A-induced decrease in $\beta 1$ integrin could be a result of either transcriptional inhibition or altered posttranscriptional steps in $\beta 1$ integrin biogenesis. To distinguish between these possibilities, we investigated whether dimerization-defective JAM-A mutants have decreased $\beta 1$ integrin mRNA levels. Primers that produced a single band of the appropriate size in RT-PCR were used for quantitative real-time RT-PCR analysis (QRT-PCR). QRT-PCR demonstrated no significant change in mRNA levels in dimerization-defective JAM-A expressing 293T cells compared with the controls (Figure 6B), suggesting that the expression of dimerization-defective JAM-A mutants did not result in down-regulated $\beta 1$ integrin gene transcription in 293T cells nor was there an effect on the stability of the

Figure 6. The $\beta 1$ integrin protein expression, but not mRNA levels is decreased in the 293T JAM-A dimerization-defective cell lines. (A) Immunoblotting revealed that 293T cells expressing 6163 or DL1 had dramatically decreased amounts of $\beta 1$ integrin protein levels, with no change in $\beta 4$ integrin or tubulin, which was used as a loading control. (B) Real-time PCR analysis from control and transfected cell lines demonstrating no significant change in $\beta 1$ Integrin mRNA for any of the overexpressing 293T cell lines.

mRNA. Conversely, these findings suggest that decreased $\beta 1$ integrin protein levels in the mutant cell lines are the result of posttranscriptional inhibition, or accelerated degradation.

Active Rap1 Is Decreased in JAM-A Dimerization-defective Cell Lines

Recently, we demonstrated a decrease in the active form of the small GTPase Rap1 after down-regulation of JAM-A expression in SK-CO15 cells (Mandell *et al.*, 2005). Furthermore, we demonstrated that down-regulation of Rap1 by small interfering RNA (siRNA) resulted in decreased $\beta 1$ integrin levels. Thus, we examined whether Rap1 levels were altered in the JAM-A dimerization-deficient cell lines. Using standard pull-down assays to isolate active, or guanosine triphosphate-bound Rap1, we analyzed the cell lines for activation status of Rap1. As shown in Supplemental Figure 2, the 6163 and DL1 mutant cell lines each had decreased active Rap1 levels compared with control cell lines. In concert with our previously reported findings, this observation suggests that active Rap1 may be a signaling link between JAM-A dimerization and $\beta 1$ integrin protein levels.

Inhibition of JAM-A Dimerization with Antibody Reduces Cell Migration and $\beta 1$ Integrin Protein Levels

Given that expression of dimerization-defective JAM-A mutants in 293T cells inhibited cell migration and decreased $\beta 1$ integrin protein level, we tested antibodies known to inhibit dimerization for effects on cell migration and $\beta 1$ integrins. We were particularly interested in the time course of effects of impaired dimerization of JAM-A, because the stable mutant cell lines represent long-term (chronic) effects. We have previously shown that the JAM-A monoclonal antibody

(mAb) J10.4 inhibits the formation of JAM-A dimers, whereas the JAM-A mAb 1H2A9 binds to the D1 Ig loop, but it does not inhibit dimerization at similar concentrations (Mandell *et al.*, 2004). 293T cells with endogenous levels of JAM-A were treated with 10 $\mu\text{g}/\text{ml}$ J10.4 or 1H2A9 before the migration assays. As shown in Figure 7, A and B, J10.4, but not 1H2A9, significantly inhibited 293T cell migration toward fibronectin compared with a murine IgG control. Cells treated with murine IgG or 1H2A9 migrated at a rate of ~ 590 cells per mm^2 per 3 h, whereas migration was decreased by treatment with J10.4 to a rate of ~ 310 cells per mm^2 over 3 h, representing a 47% decrease. These results suggest that acute disruption of dimerization of JAM-A inhibits migration in 293T cells.

We then examined $\beta 1$ integrin protein levels in cells treated with JAM-A mAbs. 293T cells were Ca^{++} depleted for 5 min to expose intercellular junctions followed by incubation with the dimerization-inhibiting mAb J10.4, the non-inhibitory JAM-A mAb 1H2A9, or mouse IgG for 3 h (10 $\mu\text{g}/\text{ml}$; 37°C) followed by analysis for $\beta 1$ integrin expression by Western blot and immunofluorescence. As can be seen in the Western blot (Figure 7C), total levels of $\beta 1$ integrin were not decreased after 3 h of antibody treatment; however, the immunofluorescence localization of $\beta 1$ integrin in Figure 7D demonstrates dramatic alterations after treatment with mAb J10.4 but not 1H2A9. Control antibody treated cells demonstrated a characteristic lateral expression pattern for $\beta 1$ integrin, whereas J10.4-treated cells showed $\beta 1$ integrin within cytoplasmic vesicles and very little staining at cell borders. These data suggest that mAb J10.4 induces internalization of $\beta 1$ integrin. To examine whether such stimulated endocytosis can lead to accelerated degradation of $\beta 1$ integrin, new protein synthesis was inhibited with cycloheximide, and cells were incubated for 4 h with mAbs J10.4, 1H2A9, or murine IgG1. As shown in Figure 7E, treatment with the dimerization-inhibiting antibody J10.4 accompanied by inhibition of de novo protein synthesis resulted in enhanced degradation of $\beta 1$ integrin protein (Figure 7E). These results, in concert with the mutant JAM-A data, suggest that JAM-A dimers stabilize $\beta 1$ integrin at the cell surface and that disruption of JAM-A dimers is likely to trigger internalization and subsequent degradation of $\beta 1$. Altered cell migration would then be an indirect consequence resulting from decreased cell surface expression of $\beta 1$ integrin.

Reduced Cell Migration in JAM-A Dimerization-defective Cell Lines Is Secondary to Decreased $\beta 1$ Integrin Protein Levels

We reasoned that decreased levels of $\beta 1$ integrin observed in the dimerization-defective JAM-A-expressing cell lines was linked to the observed JAM-A-mediated decreases in cell migration. We thus down-regulated $\beta 1$ integrin expression by using siRNA in 293T cells to determine whether similar inhibitory effects would occur as observed with the JAM-A dimerization-defective cell lines. Compared with control siRNA specific for cyclophilin B, $\beta 1$ integrin expression was virtually ablated in 293T cells as assessed by Western blot (Figure 8A). Importantly, reduction of $\beta 1$ integrin expression had no effect on JAM-A protein levels (Figure 8A). When such cells were tested in cell migration assays, there was significant inhibition of cell migration compared with siRNA-treated controls in a manner not significantly different from that observed with JAM-A mutants. Cells with down-regulated $\beta 1$ integrin protein levels migrated at a rate of 190 cells per mm^2 over the course of 3 h, whereas cells

Figure 7. Antibodies blocking JAM-A dimerization inhibits 293T cell migration and alters $\beta 1$ integrin localization. (A) JAM-A antibody J10.4 (inhibits JAM-A dimerization) inhibits 293T cell migration. Serum-starved 293T cells were treated with 10 $\mu\text{g}/\text{ml}$ mouse IgG, 1H2A9 (does not inhibit JAM-A dimerization), or J10.4 before migration assays. Confocal analyses revealed that J10.4 significantly inhibited 293T cell migration. Bar, 200 μm . (B) Blocking of JAM-A dimerization (J10.4) significantly reduced 293T cell migration compared with isotype (mouse IgG) and antigen-binding (1H2A9) antibody controls (* $p < 0.05$). (C and D) 293T cells were Ca^{++} depleted for 5 min to expose intercellular junctions followed by treatment with J10.4 (JAM-A dimerization blocking antibody), 1H2A9 (nondimerization blocking JAM-A antibody), or mouse IgG at 10 $\mu\text{g}/\text{ml}$ for 3 h in DMEM. Western blots of cell lysates were then probed for total levels of $\beta 1$ integrin (C), demonstrating no change in levels of $\beta 1$ integrin. However, immunofluorescence photomicrographs of such cells stained for $\beta 1$ integrin by using a rat anti- $\beta 1$ integrin antibody (D) reveal loss of lateral cell border staining after treatment with J10.4 but not 1H2A9 or mouse IgG. Note the appearance of $\beta 1$ integrin labeled cytoplasmic vesicles (white arrow) in J10.4-treated cells, suggesting internalization. (E) Concurrent treatment with J10.4 and Cyclohexamide results in decreased $\beta 1$ integrin.

with control siRNA treatment migrated at a rate of 470 cells per mm^2 (Figure 8C).

Figure 8. $\beta 1$ Integrin is the putative effector for JAM-A-mediated regulation of 293T cell migration. (A) Western blot demonstrating that transfection of 293T cells with siRNA specific for $\beta 1$ integrin results in significantly decreased $\beta 1$ integrin protein levels compared with transfection with siRNA targeting cyclophilin (CB). JAM-A protein levels were not changed. (B and C) Transfection of 293T cells with siRNA specific for $\beta 1$ integrin revealed significantly reduced 293T cell migration compared with controls treated with siRNA specific for cyclophilin B. Bar, 200 μm . Bar graph is average number of cells per field + SEM (* $p < 0.05$). (D) Western blot demonstrating increased $\beta 1$ integrin protein levels in JAM-A dimerization-defective cell lines transfected with a plasmid encoding $\beta 1$ integrin. JAM-A protein levels were not changed. (E and F)

We next overexpressed $\beta 1$ integrin in 293T cells stably transfected with the JAM-A dimerization-defective mutants to determine whether the cell migration defect in the JAM-A dimerization mutant cell lines could be rescued. As shown in Figure 8D, transfection resulted in increased protein levels of $\beta 1$ integrin as assessed by Western blotting, but it did not change the levels of JAM-A expression (Figure 8D). Furthermore, $\beta 1$ integrin overexpression resulted in increased cell migration from 120 cells/mm² for the DL1 mutants and 230 cells/mm² for the 6163 mutant cell lines to 470 cells/mm² for both cell lines with $\beta 1$ integrin expression restored. Thus, $\beta 1$ integrin overexpression resulted in increased cell migration to a level comparable with that of the control cell lines (Figures 8, E and F). Together, these results suggest a causal link between decreased $\beta 1$ integrin levels and reduced migration in JAM-A dimerization defective cells.

The PDZ Domain of JAM-A Is Necessary for the Dominant-Negative Effects of Dimerization-defective JAM-A Constructs

To clarify mechanisms of the dominant-negative effects on cell migration mediated by the dimerization-defective JAM-A mutants, we rationalized that these mutants are likely to sequester PDZ-containing JAM-A binding/signaling proteins away from native JAM-A dimers. To test this hypothesis, we created a modified DL1 mutant construct that lacks the C-terminal PDZ binding domain termed DL1-dFLV. Furthermore, we generated a JAM-A mutant lacking only the PDZ binding motif. Thus, if dimerization-defective JAM-A affects cell migration by sequestering PDZ domain-containing scaffolds, then DL1-dFLV should reverse the dominant-negative effect of DL1 on cell migration and the dFLV-only mutant should mimic the effects of dimerization-defective JAM-A by dimerizing with wild-type JAM-A and preventing endogenous JAM-A homodimerization.

The JAM-A mutants were transiently expressed in 293T cells (Figure 9A) with a transfection efficiency of 70–90%. Transfected cells were then used in cell migration assays on permeable filters as described above. In the passage of 293T cells used for this set of experiments, there was a higher rate of migration than observed in the passage of 293T cells clonally selected for the stable cell lines. In these transient transfections, we observed decreased $\beta 1$ integrin levels with 6163 and DL1, indicating that degradation of $\beta 1$ integrin due to interference with JAM-A dimerization occurs in a relatively short time frame of <48 h. In contrast, the DL1-dFLV double mutation had no effect on the levels of $\beta 1$ integrin protein. Furthermore, as shown in Figures 9, B and C, deletion of the C-terminal PDZ binding motif in the DL1 mutant (DL1-dFLV) completely reversed the dominant-negative effects on migration observed with DL1, and it restored migration to control levels. As predicted, transfection with a JAM-A mutant lacking only the PDZ motif also decreased the levels of $\beta 1$ integrin protein and cell migration. Overall, these data strongly suggest that dimerization-defective JAM-A mutants accelerated $\beta 1$ integrin degradation and decreased cell migration by sequestering PDZ-containing scaffolding protein from native JAM-A dimers at the plasma membrane.

Figure 8 (cont). Migration assays with mutant cell lines overexpressing $\beta 1$ Integrin. As can be seen, overexpression of $\beta 1$ integrin in the 6163 and DL1 mutant cell lines restores cell migration to that of control 293T cells. Transient transfection of a control plasmid had no effect on cell migration. Bar, 200 μ m (**p* < 0.05 for $\beta 1$ integrin-transfected cells vs. control).

Figure 9. The PDZ binding domain of JAM-A is critical for dominant-negative effects on cell migration and $\beta 1$ integrin expression by dimerization-defective mutants. (A) Expression of JAM-A mutant proteins and $\beta 1$ integrin after transient transfection. As can be seen in the Western blot, full-length (wild type), and the 6163 mutant of JAM-A have an *M_r* of 37 kDa, whereas constructs lacking the membrane-distal loop (DL1 and DL1-dFLV) have an *M_r* of ~25 kDa. Also shown are immunoblots for $\beta 1$ integrin after transient transfection with JAM-A constructs, demonstrating decreased expression with 6163, DL1, and dFLV, respectively. However, transfection with the DL1-dFLV double mutant results in no change in $\beta 1$ integrin protein expression. (B) Transient expression of DL1 or dFLV resulted in decreased cell migration, as measured by confocal analysis of Topro-3 nuclear staining, whereas expression of the DL1-dFLV construct had no effect. These findings suggest that the PDZ binding motif is required for regulation of $\beta 1$ integrin protein levels and cell migration by the DL1 mutant. (C) Average cells migrated $\pm$ SEM are shown. As can be seen, overexpression of 6163 and DL1 mutants significantly decreased cell migration, whereas expression of the double mutant DL1-dFLV had no effect (**p* < 0.05).

DISCUSSION

In previous studies, the N-terminal IgG loop of JAM-A has been reported as essential for homophilic dimerization and, presumably, function in intercellular junction assembly (Liu *et al.*, 2000; Mandell *et al.*, 2004). By using a DL1 mutant that lacks the N-terminal IgG loop as well as point mutations in the dimerization salt bridge (6163), we determined that loss of dimerization of the N-terminal IgG loop in JAM-A resulted in decreased cell transfilter migration, spreading, and altered cell shape. These observations suggest that the N-terminal IgG

loop, specifically the residues mediating dimerization, is important for JAM-A function in epithelial cells.

Although there are no studies on the role of JAM-A migration in epithelial cells, there are a few reports examining JAM-A and migration in endothelial cells. Bazzoni *et al.* (2005) reported decreased two-dimensional cell migration in JAM-A-deficient endothelial cells, by using scratch wound assays, that was restored after transfection with full-length JAM-A but not protein lacking the PDZ binding motif. Furthermore, Naik *et al.* (2003a) reported that overexpression of JAM-A increased two-dimensional cell migration in endothelial cells through interactions with $\alpha_v\beta_3$ integrin and activation of MAPK. However, neither of these studies addressed the role of JAM-A dimerization, nor did they provide a structural model for how JAM-A might regulate cell motility. In addition, there are no studies examining the role of JAM-A in models of cell migration in three dimensions.

We previously reported that transient down-regulation of JAM-A expression in intestinal epithelial cells by siRNA resulted in altered epithelial cell morphology, decreased cell-matrix adhesion, and decreased levels of β_1 integrin and Rap1 (Mandell *et al.*, 2005); however, mechanistic insight(s) was lacking. This study was directed at better understanding the mechanism linking structural determinants of JAM-A to β_1 integrin levels and cell migration. We determined that overexpression of JAM-A dimerization-defective mutants in 293T cells resulted in decreased cell migration across matrix-coated permeable filters, decreased spreading, and reduced length of cellular protrusions. The role of dimerization in cell migration was further confirmed through experiments demonstrating inhibition of cell migration after treatment with specific JAM-A dimer-disrupting antibodies. Furthermore, treatment with a dimerization-inhibiting antibody and cyclohexamide lead to degradation of β_1 integrin more quickly than treatment with cyclohexamide and IgG. Decreased cell migration in our assays correlated with decreased β_1 integrin levels, alterations in β_1 integrin protein localization, decreased levels of the active form of the small GTPase Rap1, and diminished numbers of focal concentrations of phosphorylated paxillin. An effector role for β_1 integrin in the observed JAM-A-mediated effects was supported by experiments with siRNA specific to β_1 integrin, demonstrating decreased cell migration in control cell lines after down-regulation of β_1 integrin protein levels. Furthermore, we observed increased cell migration comparable with that observed in control cell line levels after overexpression of β_1 integrins in cells expressing JAM-A dimerization-defective mutations.

Although the mechanism of decreased β_1 integrin in the JAM-A dimerization mutants remains unclear, our results provide important new insights. Our data obtained using two different approaches to disrupt JAM-A dimers (JAM-A mutants and antibodies) are consistent with a scenario in which disruption of JAM-A dimerization causes internalization and degradation of β_1 . This is consistent with the observation of no decrease in β_1 integrin mRNA levels in cells expressing dimerization-defective JAM-A. We tested whether increased proteosomal degradation might account for diminished β_1 integrins in JAM-A mutant cell lines. Treatment of the dimerization-defective JAM-A cell lines with the proteosome inhibitor MG262 failed to increase β_1 integrins despite increasing levels of ubiquitinated proteins (Supplemental Figure 3). This finding suggests that β_1 integrin degradation in our cell lines may not be mediated by the proteosome. Further studies are necessary to determine the mechanism for the degradation of β_1 integrin in the presence of the JAM-A dimerization-defective mutants.

The mechanism(s) behind regulation of β_1 integrin expression by JAM-A remain to be determined. Possibilities include direct interactions of β_1 integrins with JAM-associated scaffolding proteins; activation of signaling molecules that affect β_1 integrin turnover, such as the small GTPase Rap1; or sequestration of negative regulators of β_1 integrin stability by scaffolding complexes. In other studies, JAM-A has been reported to physically interact with $\alpha_v\beta_3$ (Naik and Naik, 2006) and β_2 integrin (Fraemohs *et al.*, 2004) and to regulate migration of endothelial cells (Fraemohs *et al.*, 2004; Naik and Naik, 2006); however, we have been unable to detect a direct association between JAM-A and β_1 integrin in coimmunoprecipitation experiments. These observations suggest that JAM-A mediates decreased β_1 integrin protein levels through an indirect mechanism(s).

A signaling link between JAM-A dimerization and β_1 integrin protein internalization/degradation is suggested by the correlation between decreased β_1 integrin and levels of the active form of the GTPase Rap1. We previously demonstrated a decrease in active Rap1 after down-regulation of JAM-A expression in SK-CO15 cells (Mandell *et al.*, 2005). Furthermore, we demonstrated that down-regulation of Rap1 by siRNA resulted in decreased β_1 integrin levels. Additionally, other studies have linked Rap1 activity and increased integrin protein levels and/or integrin activation (Reedquist *et al.*, 2000; Katagiri *et al.*, 2003; Shimonaka *et al.*, 2003). In concert with our findings in the dimerization-defective cell lines, it is thus likely that Rap1 is a signaling element between JAM-A and β_1 integrin. We speculate that JAM-A dimer-dependent activation of Rap1 may be required to prevent internalization and degradation of β_1 integrin.

Intriguingly, we observed that the dominant-negative effects of DL1 were abrogated after an additional mutation removed the PDZ binding domain. Because PDZ domains are responsible for interactions with scaffolding proteins, these results suggest that the dimerization-defective mutations may affect cell migration and β_1 integrin levels through sequestration of scaffolding proteins. This hypothesis is consistent with our data demonstrating that 293T cells transfected with JAM-A containing a mutation in the PDZ-binding domain (dFLV) led to decreased levels of β_1 integrin protein and decreased rates of cell migration. These data suggest that the effects on β_1 integrin and migration are mediated by dimerization of wild-type JAM-A with dFLV-JAM-A. Given that transfection of cells with dFLV resulted in much higher levels of expression of the mutant than endogenous JAM-A, it is likely that a majority of the endogenous JAM-A would dimerize with the dFLV mutant. Under such conditions, functional dimers of JAM-A would not be expected to form, resulting in effects similar to those observed with dimerization-defective mutants.

From these findings, we present a hypothetical model of JAM-A function (Figure 10). In the model, *cis*-dimerization of JAM-A brings into proximity two molecules of JAM-A. Each JAM-A molecule has a C-terminal PDZ binding motif that can interact directly or indirectly with scaffolding proteins such as ZO-1 or Afadin (Bazzoni *et al.*, 2000; Ebnet *et al.*, 2000). This scaffolding complex might interact with β_1 integrin via yet unidentified partners leading to stabilization of β_1 integrin at the plasma membrane. In other studies, JAM-A has been reported to physically interact with $\alpha_v\beta_3$ (Naik and Naik, 2006) and β_2 integrin (Fraemohs *et al.*, 2004) and regulate migration of endothelial cells (Fraemohs *et al.*, 2004; Naik and Naik, 2006); however, we have been unable to detect a direct association between JAM-A and β_1 integrin in coimmunoprecipitation experiments. Therefore, it is most

Figure 10. Model for effect of JAM-A dimerization on $\beta 1$ integrin levels. In this model, JAM-A dimers are required for close apposition of cytoplasmic tails bound to scaffolding complexes containing signaling elements. Under this scenario, disruption of *cis*-dimerization by either mutagenesis or a blocking antibody would result in loss of close apposition of these cytoplasmic tail complexes, inhibition of signaling, and subsequent internalization/degradation of $\beta 1$ integrin through an as of yet undetermined mechanism. Diminished cell surface expression of $\beta 1$ Integrin would then result in decreased adhesion to matrix and affect cell migration.

likely that scaffolding complexes associated with JAM-A dimers bind and regulate activity of some signaling and endocytic proteins such as Rap1, which can mediate internalization/trafficking of $\beta 1$ integrin. We speculate that the dimerization-defective JAM-A-expressing mutants disrupt these scaffolding complexes at endogenous JAM-A dimers by sequestering their certain components. This may activate/release yet unknown signaling cascade resulting in accelerated internalization and subsequent degradation of $\beta 1$ integrin.

The PDZ domain of JAM-A has been shown to bind to several scaffolding molecules, such as ZO-1, Cingulin, Occludin (Bazzoni *et al.*, 2000), Afadin (Ebnet *et al.*, 2000), and MUPP-1 (Hamazaki *et al.*, 2002), among others. This suggests that the cytoplasmic tail of JAM-A is able to bind to different molecules and that the two tails in a JAM-A homodimer would not necessarily bind to the same scaffolding molecule, but they may likely bring different molecules with signaling capacity into proximity, thus forming scaffolding complexes as discussed above.

Importantly, our model is also compatible with JAM-A dimers interacting with one another in *trans*, as has been observed in the mouse, but not human crystal structures (Kostreva *et al.*, 2001; Prota *et al.*, 2003). Transinteracting JAM-A dimers would allow for the close apposition of multiple sets of PDZ binding domains and the formation of large scaffolding/signaling complexes. *Cis*-dimerization may thus only be a prerequisite for the changes described in this report to occur.

The physiological significance of formation of JAM-A *cis*-dimers is highlighted in the various functions described for JAM-A *per se*, which include regulation of cell migration, barrier function (Liu *et al.*, 2000), angiogenesis (Naik *et al.*, 2003a), cell adhesion (Mandell *et al.*, 2005), and determination of cell polarity (Itoh *et al.*, 2001). We recently demonstrated that loss of JAM-A leads to changes in basal intestinal permeability and increased sensitivity to dextran sulfate sodium-induced colitis in vivo (Laukoetter *et al.*, 2007). In this study, loss of JAM-A was shown to result in an altered

claudin expression profile. It is tempting to hypothesize that such changes in claudins may be due to altered JAM-A dimer-mediated signaling.

It is possible to speculate on pathophysiological conditions that would result in dissociation of JAM-A *cis*-dimers. It is likely that the formation of such complexes results from low-affinity interactions that would be very sensitive to changes in levels of JAM-A in the plasma membrane. Therefore, stimuli that decrease abundance of plasma membrane JAM-A by inhibiting protein expression or enhancing internalization would diminish *cis*-JAM-A dimers. Interestingly, we and others have shown that junctional proteins, including JAM-A are internalized and decrease after a variety of stimuli including exposure to inflammatory cytokines and oxidant stress (Bruewer *et al.*, 2005; Utech *et al.*, 2005). We have also observed similar internalization and diminished levels of junctional proteins and JAM-A in the mucosa from individuals with inflammatory bowel disease (Kucharzik *et al.*, 2001). It is thus tempting to speculate that loss of JAM-A dimers at the cell surface through inflammatory stimuli contributes to the altered permeability and pathophysiology of chronic intestinal inflammatory states. Clearly, more work is needed to fully understand the physiological relevance of JAM-A dimerization.

In summary, these results suggest that dimerization of JAM-A is required for regulating several aspects of cell migration through signaling events. Disruption of JAM-A dimerization presumably prevents the formation of scaffolding protein complexes that prevent and/or lead to signaling events that result in loss of $\beta 1$ integrin and decreased cell migration. Further studies are needed to better understand the mechanisms of JAM-mediated regulation of integrin expression and cell migration as well as identification of scaffolding complexes involved in JAM-A-mediated signaling events.

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