Junctional Adhesion Molecule A Interacts with Afadin and PDZ-GEF2 to Activate Rap1A, Regulate β 1 Integrin Levels, and Enhance Cell Migration

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Junctional adhesion molecule-A (JAM-A) is a transmembrane tight junction protein that has been shown to regulate barrier function and cell migration through incompletely understood mechanisms. We have previously demonstrated that JAM-A regulates cell migration by dimerization of the membrane-distal immunoglobulin-like loop and a C-terminal postsynaptic density 95/disc-large/zona occludens (PDZ) binding motif. Disruption of dimerization resulted in decreased epithelial cell migration secondary to diminished levels of β 1 integrin and active Rap1. Here, we report that JAM-A is physically and functionally associated with the PDZ domain-containing molecules Afadin and PDZ-guanine nucleotide exchange factor (GEF) 2, but not zonula occludens (ZO)-1, in epithelial cells, and these interactions mediate outside-in signaling events. Both Afadin and PDZ-GEF2 colocalized and coimmunoprecipitated with JAM-A. Furthermore, association of PDZ-GEF2 with Afadin was dependent on the expression of JAM-A. Loss of JAM-A, Afadin, or PDZ-GEF2, but not ZO-1 or PDZ-GEF1, similarly decreased cellular levels of activated Rap1, β 1 integrin protein, and epithelial cell migration. The functional effects observed were secondary to decreased levels of Rap1A because knockdown of Rap1A, but not Rap1B, resulted in decreased β 1 integrin levels and reduced cell migration. These findings suggest that JAM-A dimerization facilitates formation of a complex with Afadin and PDZ-GEF2 that activates Rap1A, which regulates β 1 integrin levels and cell migration.

INTRODUCTION

Junctional adhesion molecule-A (JAM-A) is member of a large family of transmembrane immunoglobulin superfamily molecules, most of which are expressed at cell–cell junctions and have a complex array of functions in epithelial and endothelial cells. In these cells, JAM-A has been reported to play a role in the regulation of epithelial barrier function (Mandell *et al.*, 2005; Laukoetter *et al.*, 2007), endothelial cell migration (Naik *et al.*, 2003a; Bazzoni *et al.*, 2005), angiogenesis (Naik *et al.*, 2003a), platelet aggregation (Babinska *et al.*, 2002), and leukocyte adhesion (Martin-Padura *et al.*, 1998; Del Maschio *et al.*, 1999). Recent reports suggest that JAM-A not only directly decreases paracellular permeability (Mandell *et al.*, 2005; Laukoetter *et al.*, 2007) but also promotes epithelial cell migration (Severson *et al.*, 2008), which is critical for the maintenance of intestinal barrier in health and disease (Lacy, 1988; Fenteany *et al.*, 2000). The relationship between these functions and JAM-A structure remains poorly understood.

The mature JAM-A protein is composed of two extracellular immunoglobulin (Ig)-like loops, a membrane-spanning segment, and a cytoplasmic tail ending in a postsynaptic density 95/disc-large/zona occludens (PDZ) binding motif.

The membrane distal most extracellular Ig loop mediates homodimerization between JAM-A proteins on the same cell (Prota *et al.*, 2003; Guglielmi *et al.*, 2006; Severson *et al.*, 2008) (*cis*) and potentially mediates interactions between JAM-A molecules on adjacent cells (Kostreva *et al.*, 2001) (*trans*). *Cis*-dimerization has been shown to be necessary for JAM-A regulation of epithelial cell invasion (Severson *et al.*, 2008) and epithelial barrier recovery (Liu *et al.*, 2000). The PDZ binding motif of JAM-A has been reported to associate with the PDZ domain-containing proteins Afadin and ZO-1 in endothelial and epithelial cells (Yamamoto *et al.*, 1997; Ebnet *et al.*, 2000). However, the signaling events downstream of JAM-A dimerization that mediate these functional effects are not understood.

Based on our previous findings (Severson *et al.*, 2008), we hypothesized that *cis*-dimerization of JAM-A results in close apposition of JAM-A PDZ binding motifs that facilitates interaction between scaffolding proteins, which in turn activates signaling molecules. However, biologically relevant scaffolding and signaling proteins downstream of the dimerized JAM-A complex have not yet been defined.

In this study, we validated the above-mentioned hypothesis that JAM-A dimerization and its association with specific scaffolding proteins results in activation of signaling molecules that regulate epithelial cell migration. We demonstrate 1) coassociation of the Rap1 guanine nucleotide exchange factor PDZ-GEF2 and Afadin with JAM-A; 2) Afadin and PDZ-GEF2 association requires JAM-A expression; 3) decreased expression of JAM-A, Afadin, PDZ-GEF2, or Rap1A results in analogous decreased β 1 integrin protein

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Table 1. siRNA targets used in this study

Protein	Sense sequence for target 1	Sense sequence for target 2
JAM-A	GAAGUGAAGGAGAAUUCAA	CGGGUGACCUUCUUGCCAA
Afadin	UGAGAAACCUCUAGUUGUA	GUUAAGGGCCCAAGACAU
Rap1a	GAAGUCUGCUCUGACAGUU	GCGAGUAGUUGGCAAAGAG
Rap1b	CAGUAUAAUGUCUUAGAUUAA	GACCUAGUGCGGCAAAUUA
PDZ-GEF1	CAGGCUGAAGAUACAAUAUA	CCGCUACAUAUGAUCAGUAA
PDZ-GEF2	CUGCAUGAAUUGUAACUGAA	CAGGAAGAAGGGACAAACAAA
β 1 Integrin	AAGUCUUGGAACAGAUUCUG	N.A.
ZO-1	CCAGUATCUGAUAAUGAAGAA	N.A.

N.A., not applicable.

levels and reduced cell migration; 4) and PDZ-GEF2 activates Rap1A to regulate β 1 integrin and cell migration. Our results support the hypothesis that JAM-A dimerization is required for the assembly of a protein complex containing PDZ-GEF2 and Afadin, which activates Rap1A, thereby stabilizing β 1 integrin protein levels and regulating cell migration. These findings demonstrate that JAM-A regulates cell migration and, by extension, implicates JAM-A as a key molecule that facilitates wound healing after physical or inflammatory injury.

MATERIALS AND METHODS

Cell Culture

SKCO-15 human colonic epithelial cells were grown in DMEM supplemented with 10% fetal bovine serum, 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 Hanks' balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO). For immunoblotting and differential interference contrast (DIC) imaging, cells were plated in T-25 flasks (Corning, Corning, NY) at a number such that their density was 25% the day of transfections. For filter based studies, cells were plated at a density of 1×10^5 cells/0.33 cm².

Immunoblots

Monolayers of epithelial cells that were between 60 and 90% confluent were homogenized in radioimmunoprecipitation assay (RIPA) 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 from Sigma-Aldrich (1:100 dilution). Protein concentrations in lysates were quantified by bicinchoninic acid assay. Lysates were cleared by centrifugation and immediately boiled in reducing SDS sample buffer. Additionally, isolated colonic epithelial cells from JAM-A-deficient and control mice were analyzed for β 1 integrin and Rap1 by immunoblot analysis for three animals. Distal colonic epithelial cell lysates were prepared after the serosa and external longitudinal layer of the muscularis propria were stripped away. Isolated epithelial sheets were subsequently lysed in RIPA buffer. Mice were genotyped and raised as reported previously (Laukoetter *et al.*, 2007). SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblots were performed by standard methods. Tubulin was used as a protein loading control. Each immunoblot shown is representative of at least three independent experiments.

Immunoprecipitations (IPs)

Cells were grown to between 60 and 90% confluence in T-25 flasks (Corning) and then lysed with 1 ml of RIPA buffer and dounced. Samples were then centrifuged to remove cell debris, and 100 μ l of supernatant was saved for input sample. The remaining supernatant was used for IPs. Sample was precleared for 45 min at 4°C with Sepharose beads followed by incubation for 1 h at 4°C with protein G-coupled to Sepharose (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom). Beads were washed three times with sample buffer. Beads were boiled for 15 min with SDS sample buffer, and the entire sample was loaded for analysis by immunoblot. Each IP was repeated at least three times.

Immunofluorescence (IF) Microscopy

Cells were grown on 0.3- μ m pore transwell filters (Corning Life Sciences, Lowell, MA), fixed in 100% ethanol at -20°C for 20 min, 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 incubated in fluorescently labeled secondary antibodies for 45 min at room temperature. Labeled cells were then washed and mounted in Prolong Antifade Agent (Invitrogen, Carlsbad, CA). A laser scanning microscope (Carl Zeiss, Jena, Germany) was used to capture confocal fluorescence images.

DIC Microscopy

Pictures were obtained using an Axiovert 35 light microscope at 5 $\times$ power by using the DIC 0.4 filter and saved using Axiomatic imaging software (Carl Zeiss). Images were then imported directly into Adobe Photoshop (Adobe Systems, Mountain View, CA) and saved as TIFF files for figures.

Antibodies

The murine monoclonal anti-JAM-A antibody J10.4 was described previously (Liu *et al.*, 2000), and the rabbit polyclonal anti-PDZ-GEF was a kind gift from Dr. N. Mochizuki (Sakurai *et al.*, 2006). Other antibodies were commercially purchased: polyclonal rabbit anti-JAM-A (Zymed Laboratories, South San Francisco, CA), monoclonal mouse anti-Afadin (BD Transduction Laboratories, Lexington, KY), monoclonal mouse anti-tubulin (Sigma-Aldrich), polyclonal rabbit anti-actin (Sigma-Aldrich), rabbit polyclonal Rap1 (Millipore, Billerica, MA), monoclonal rabbit anti- β 1 integrin (Novus Biologicals, Littleton, CO), monoclonal rabbit anti-JAM-A (Novus Biologicals), polyclonal rabbit ZO-1 (Zymed Laboratories), polyclonal rabbit anti-FLAG (Sigma-Aldrich), goat anti-rabbit-horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, West Grove, PA), and goat anti-mouse-HRP (Jackson ImmunoResearch Laboratories).

Small Interfering RNA (siRNA) Experiments and DNA Transfection

For siRNA protein targets, four to 10 oligonucleotides were designed for each target, and protein down-regulation was verified by immunoblot. For each candidate protein tested, two promising siRNA targets that led to effective protein down-regulation were identified and were then *separately* tested for effects on β 1 integrin levels and cell migration. Two separate oligonucleotides for each candidate molecule were tested to diminish the possibility of siRNA-mediated interferon and off-target effect and to increase the specificity of findings (Echeverri *et al.*, 2006). The two siRNA oligomers that resulted in the greatest decrease in protein expression were combined and used for the experiments presented in this report. siRNA oligonucleotides were obtained from Dharmacon RNA Technologies (Lafayette, CO) or QIAGEN (Valencia, CA). Controls included a scramble control from QIAGEN (AllStars Negative Control) as well as mock-transfected controls. All targets used are listed in Table 1. Every transfection was done at a total concentration of 50 nM siRNA. Transfections were performed using HiPerFect (QIAGEN) according to the manufacturer's instructions. Assays were performed 72 h after transfection.

DNA transfections in SKCO-15 cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol when the cells were 50% confluent and similar to previously published protocols (Severson *et al.*, 2008). Cells were used in assays 48 h after transfection.

Rap1 Activity Assay

A Rap1 activity assay was performed according to the manufacturer's instructions (Millipore). Briefly, cells were lysed in a Tris and Triton X-based lysis buffer at 4°C. Cell debris was removed by centrifugation, and 100 μ l of sample was saved as input to determine total Rap1 levels. Then, 50 μ g of protein for

each sample was incubated at 4°C for 45 min with Ral-GDS agarose beads to bind active Rap1. Beads were washed three times with the lysis buffer followed by boiling in SDS sample buffer. The entire sample was analyzed by immunoblot with detection using a polyclonal Rap1 antibody from Millipore. Each activity assay was repeated at least three times.

Surface Biotinylation

Cells were treated with siRNA as described above. At 72 h after treatment, cells were placed on ice, rinsed with phosphate-buffered saline, and then cells were treated with EZ-link-Sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, IL) according to the manufacturer's protocol. Cells were lysed as described above, and ImmunoPure Immobilized Monomeric Avadin (Pierce Chemical) was then used to isolate the labeled surface proteins. Proteins were removed from the Avadin beads by boiling in SDS sample buffer followed by Western blot and probing for β 1 integrin.

Scratch Wound Assay

Cells were seeded in 24-well plates at a density resulting in 50–70% confluence 24 h later. Cells were treated with siRNA 24 h after seeding. Wounds were made in confluent monolayers 48 h after siRNA treatment by using a pipet tip under vacuum suction. A straight line was drawn across the bottom of each well, and then pictures were taken with the line at the bottom of the viewing field. Wounds were measured from the exact vertical middle of each picture such that the initial measurement and the measurement 16 h later were taken from the same vertical spot in each well. The average starting wound width was $702 \pm 51 \mu\text{m}$, and there was no statistical difference in the sizes of initial wound widths in any of the experiments. Representative images are shown for each figure, and results are reported as percentage of wound closed. Percentage of wound closure is equal to (initial width – width at 16 h)/initial width. Measurements were taken in pixels, which directly correlate to distance in a linear manner. Data are from a representative experiment of three independent experiments with four sample replicates per experiment.

Constructs and Cloning

A PDZ-GEF2 construct was cloned from SK-CO15 cDNA. mRNA from SK-CO15 cells, which were 75% confluent, was isolated using TRIzol (Invitrogen). cDNA was then produced using the first-strand cDNA synthesis kit (Invitrogen). PDZ-GEF2 was amplified by polymerase chain reaction (PCR) with Phusion (New England Biolabs, Ipswich, MA) polymerase and an N-terminal FLAG tag, and BamHI and XhoI restriction sites were added in the primers. The forward primer containing the FLAG tag was as follows: 5'-aataagctt-gccaccATTGATTACAAGGACGACGATGACAAGATGAAGTCACTACCCGTGGA-CCCT-3'. The reverse primer was 5'-TTCAAGAAATGCTCTGTAAAGTT-3'. The PCR product was inserted into pCDNA3.0 by using the engineered restriction sites and sequence was verified.

Reverse Transcription (RT)-PCR

RT-PCR was performed using the SuperScript III one-step PCR kit from Invitrogen according to the manufacturer's instructions. The annealing T_m used for all primers was 57°C. The PDZ-GEF1 and two primers used have been described and verified previously (Schultess *et al.*, 2005).

Real-Time PCR

Quantitative real-time PCR was performed to determine mRNA levels. PCR amplification was performed using the GeneAmp 5700 sequence detection system (Applied Biosystems, Foster City, CA). PCR was run using the following protocol: initial activation at 94°C for 3 min, 40 cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 30 s. Each sample was performed in triplicate. Direct detection of PCR product was monitored in real-time by measuring the increase in fluorescence caused by the binding of SYBR Green I Dye (Invitrogen) to double-stranded DNA, by using the ABI Gene Amp 5700 sequence detection system (Applied Biosystems). Results of the real-time PCR data were represented as -fold change, which was determined by applying the formula $2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t$ of target gene – C_t of endogenous control gene (signal regulatory peptide), and $\Delta\Delta C_t = \Delta C_t$ of samples for target gene (siRNA treated) – ΔC_t of the control for the target gene (mock siRNA treated). The primers used were as follows: Rap1A.F: 5'-TGGATACTGCAGG-GACAGAGCAAT-3'; Rap1A.R: 5'-ACATCTTCCGTGCTCTTAACCCGT-3'; Rap1B.F: 5'-AGGCGTTGGAAAGTCTGCTTTGAC-3'; Rap1B.R: 5'-ATTGCTC-CGTTCCTGCAGTATCCA-3'; PDZ-GEF1.F: 5'-AAATTCGTCACGTTGGC-CGAATGG-3'; PDZ-GEF1.R: 5'-ACTCCGCCATTCTTCTTCCGAGT-3'; PDZ-GEF2.F: 5'-TGTTGACTCCATGTCTGCAGTCT-3'; and PDZ-GEF2.R: 5'-AC-CCAGGCCATGTTGACTATGAT-3'.

Statistics

For comparison of samples in experiments with only two groups, a Student's *t* test was used. One-way analysis of variance was used for comparisons in experiments with greater than two groups with post hoc analysis performed by GraphPad (GraphPad Software, San Diego, CA) to determine *p* values for

Figure 1. Association and colocalization of Afadin with JAM-A. (A) By IF, JAM-A (green) and Afadin (red) colocalize as seen in the merged images in the XY and XZ planes at the level of the apical junctional complex. Bar, 20 μm . (B) Coimmunoprecipitation of JAM-A with Afadin by using JAM-A-specific mAb J10.4. As can be seen, neither JAM-A nor Afadin immunoprecipitates with the isotype control antibody.

sample groups compared with controls. *p* < 0.05 was considered significant in either case.

RESULTS

JAM-A Regulates Epithelial Cell Migration through Afadin but Not ZO-1

Previous reports suggest that the PDZ binding motif of JAM-A is necessary for its function (Bazzoni *et al.*, 2000; Ebnet *et al.*, 2001; Severson *et al.*, 2008), and this presumably requires JAM-A interactions with PDZ domain-containing scaffolding proteins. To identify scaffolding proteins that mediate JAM-A function, we focused on the candidate proteins Afadin and ZO-1, which have previously been reported to associate with JAM-A (Ebnet *et al.*, 2000; Fukuhara *et al.*, 2002). Specifically, we investigated whether these proteins are required for JAM-A regulation of cell migration.

JAM-A, ZO-1, and Afadin were immunolocalized in a model intestinal epithelial cell line SKCO-15. ZO-1 is a tight junction (TJ) protein, but Afadin has been reported to associate with Nectin(s) in adherens junctions (AJs) (Miyahara *et al.*, 2000) and has also been observed to localize at both the TJs and AJs by electron microscopy (Yamamoto *et al.*, 1997) and subcellular fractionation experiments (Vogelmann and Nelson, 2005). As shown in Figure 1A and Supplemental Figure 1A, JAM-A, Afadin, and ZO-1 colocalized at TJs.

To evaluate whether JAM-A forms a protein complex with Afadin or ZO-1 in polarized intestinal epithelial cells, we performed coIP experiments. SK-CO15 cells were lysed in nonionic detergent containing solubilization (RIPA) buffer, and the supernatants were incubated with JAM-A monoclonal antibody (mAb) J10.4. We have reported previously that J10.4 binds to the membrane distal extracellular Ig domain of JAM-A (Liu *et al.*, 2000). Immunoprecipitated protein complexes were isolated using protein G-Sepharose followed by boiling in SDS-containing sample buffer. Supernatants were then subjected to SDS-PAGE followed by immunoblotting. Immunoblots were probed for JAM-A, ZO-1, and Afadin. As shown in Figure 1B Afadin coimmunoprecipitates with JAM-A, whereas ZO-1 was not detected in the protein com-

plex (Supplemental Figure 1B). Although the conditions used in this experiment may have disrupted a potential interaction between ZO-1 and JAM-A, these data suggest that JAM-A coimmunoprecipitates with Afadin in a manner independent of potential interactions between JAM-A and ZO-1 (Figure 1B).

It has been reported previously that JAM-A regulates epithelial and endothelial cell migration (Naik *et al.*, 2003a; Bazzoni *et al.*, 2005; Severson *et al.*, 2008). To confirm that JAM-A regulates cell migration in SKCO-15 cells, we evaluated the effect of decreased JAM-A protein levels on wound closure in a scratch wound assay. siRNA was used to decrease JAM-A protein expression in SKCO-15 cells, which are amenable to transfection. JAM-A siRNA treatment consistently resulted in a >80% decrease in JAM-A protein expression compared with mock- or scramble siRNA-transfected cells ($p < 0.01$) (Figure 2, B and C). These results are consistent with our previous observations demonstrating that overexpression of JAM-A dimerization-defective mutants or JAM-A mutants lacking the PDZ binding motif decreases the rate of epithelial cell migration across permeable filters (Severson *et al.*, 2008).

We next performed cell migration experiments to determine whether Afadin and/or ZO-1 were necessary for JAM-A regulation of cell migration. Because the JAM-A PDZ binding motif is required for effects on cell migration (Severson *et al.*, 2008), we reasoned that proteins which interact with this motif mediate the functional effects. To determine whether either Afadin or ZO-1 mediates the effects of JAM-A on cell migration, analogous experiments were performed using siRNA to decrease the expression of Afadin and ZO-1. As shown in Figure 2, siRNA-mediated down-regulation of Afadin expression, but not ZO-1, inhibited wound closure at 16 h ($p < 0.001$). These effects are likely due to changes in the rate of cell migration and not secondary to alterations in cell proliferation as the time course for these experiments (16 h) was sufficiently short as to minimize potential effects from proliferation (doubling time of SKCO-15 cells is 48 h). Because decreased expression of JAM-A and Afadin, but not ZO-1, decreased cell migration, and JAM-A was observed to associate in a protein complex with Afadin, these data are consistent with JAM-A signaling through Afadin to regulate cell migration.

JAM-A and Afadin Influence Cell Migration through Regulation of $\beta 1$ Integrin Protein Levels

We reported previously that JAM-A regulation of cell migration is mediated by effects on $\beta 1$ integrin protein levels. The decreased epithelial cell migration, which was observed after overexpression of dominant-negative JAM-A, was rescued by overexpression of $\beta 1$ integrin (Severson *et al.*, 2008). To evaluate whether Afadin regulates cell migration downstream of JAM-A, we analyzed $\beta 1$ integrin protein levels after decreased expression of either protein. Diminished expression of JAM-A or Afadin, but not ZO-1, resulted in decreased levels of $\beta 1$ integrin protein (Figure 3 and Supplemental Figure 1C). These findings suggest that JAM-A signals through Afadin to effect cell migration by regulating cellular levels of $\beta 1$ integrin protein.

JAM-A/Afadin Signal through the Small GTPase Rap1A to Regulate $\beta 1$ Integrin Protein Levels and Cell Migration

Recently, it was shown that decreased JAM-A expression results in reduced activation of the small GTPase

Figure 2. JAM-A and Afadin siRNA treatment both decrease the rate of cell migration. (A) Immunoblots demonstrating that treatment of SK-CO15 cells with JAM-A- (JA) or Afadin (AF6)-specific siRNA results in decreased levels of the targeted protein compared with scramble- (Scr) or mock-treated controls. (B and C) Scratch wound assays. Confluent monolayers of SK-CO15 cells were injured with a 10- μ l pipette tip by using vacuum suction, and the wound length was measured at 0 and 16 h after wounding. Wound closure in mock- or scramble-treated monolayers was nearly 100%, whereas JAM-A or Afadin down-regulated monolayers had only recovered by 65% (* $p < 0.01$) or 69% (** $p < 0.001$), respectively. Bar, 200 μ m.

Rap1 (Mandell *et al.*, 2005; Severson *et al.*, 2008). In *in vitro* studies, Rap1 has been reported to regulate integrin levels in epithelial cells (Mandell *et al.*, 2005) as well as integrin activation in leukocytes (Reedquist *et al.*, 2000). To verify these results *in vivo*, we determined the influence of JAM-A on $\beta 1$ integrin protein levels and Rap1 activation in intestinal epithelial cells derived from JAM-A knockout mice. In the colonic epithelial cells from JAM-A knockout mice, $\beta 1$ integrin protein levels were decreased by 50% compared with wild-type controls ($p < 0.05$) (Figure 4). Colonic epithelial cell lysates were incubated with Ral-GDS agarose beads to bind active Rap1, followed by SDS-PAGE. Immunoblots were then probed for Rap1. Total levels of Rap1 were unchanged compared with wild-type controls; how-

Figure 3. Down-regulation of Afadin or JAM-A expression results in decreased β1 integrin. (A) Immunoblots demonstrating that treatment of SK-CO15 cells with JAM-A- (JA) or Afadin (Af6)-specific siRNA results in decreased levels of targeted protein compared with scramble (Scr) siRNA or mock-treated controls. Tubulin represents the protein loading control.

ever, active Rap1 levels were decreased by 49% ($p < 0.05$) (Figure 4). These results suggest that JAM-A regulates Rap1 activity and β1 integrin levels both in vitro and in vivo.

Based on these findings, we hypothesized that, through Afadin, JAM-A regulates activation of Rap1, which in turn, affects β1 integrin protein levels. Indeed, decreased expres-

Figure 4. In vivo loss of JAM-A results in decreased levels of β1 integrin and active Rap1. (A and B) Immunoblots of colonic epithelial cell lysates from JAM-A^{-/-} (JA^{-/-}) C57/Bl6 mice compared with wild-type mice, demonstrating a 50% decrease in β1 integrin protein compared with controls (* $p < 0.001$). Equivalent protein loading is demonstrated by actin labeling. As can be seen, the ratio of active to total Rap1 is decreased by 49% in JAM-A^{-/-} mice compared with controls (* $p < 0.001$). For the densitometry graphs, gray bars represent control mice and black bars represent JAM-A knockout mice.

sion Afadin, like that observed after loss of JAM-A, lead to diminished levels of active Rap1 (Figure 5A). Analogous to reduced expression of JAM-A or Afadin, we observed that siRNA-mediated down-regulation of Rap1 resulted in reduced β1 integrin protein levels (data not shown; Mandell *et al.*, 2005). Because Rap1 consists of two closely related genes (paralogues), Rap1A and Rap1B, we performed experiments examining the individual roles of Rap1A and Rap1B in JAM-A/Afadin mediated regulation of β1 integrin levels and cell migration. To isolate the individual effects of Rap1A and Rap1B, siRNA specific to the mRNA sequences in the 3' untranslated region of Rap1A and Rap1B were used, because reliable and specific antibodies are not available. As shown in Figure 5B, siRNA-mediated down-regulation of Rap1A and Rap1B resulted in decreased expression of Rap1 as assessed by a pan-Rap1 antibody. By real-time RT-PCR, siRNA-mediated decreases in Rap1A or Rap1B reduced mRNA expression by 93 and 78%, respectively (Figure 5C). Interestingly, siRNA-mediated down-regulation of Rap1A, but not Rap1B, resulted in decreased β1 integrin levels. Furthermore, decreased expression of Rap1A, but not Rap1B, reduced cell migration compared with controls (Figure 5, D and E). These findings suggest that JAM-A and Afadin activate Rap1A, which in turn stabilizes β1 integrin levels and promotes cell migration.

PDZ-GEF2, but Not PDZ-GEF1, Regulates β1 Integrin Protein Levels and Epithelial Cell Migration

Overexpression of dimerization-defective JAM-A protein has been previously shown to decrease β1 integrin levels, Rap1 activity, and cell migration (Severson *et al.*, 2008). We proposed a model of JAM-A function involving dimerization of JAM-A that brings scaffolding proteins into proximity to facilitate downstream signaling. Because our results suggest that JAM-A coassociates with Afadin, and decreased expression of either protein has similar functional and biochemical effects, we hypothesize that JAM-A and Afadin are part of a complex that activates Rap1A. Interestingly, Afadin has been shown to directly associate with Rap1A (Linnemann *et al.*, 1999; Hoshino *et al.*, 2005). Furthermore, activation of Rap1A is dependent on guanine nucleotide exchange factors (GEFs). Given the dependence of JAM-A function on dimerization, we reasoned that a likely additional component of the JAM-A-associated signaling complex would be a Rap1-specific GEF. Experiments were thus performed to identify GEF molecules that contain PDZ binding domains and potentially coassociate with JAM-A. By RT-PCR (data not shown) and immunoblot, we observed expression of two Rap1-specific GEFs that contain PDZ binding domains, PDZ-GEF1 and PDZ-GEF2 (Rebhun *et al.*, 2000), in SKCO-15 cells (Figure 6A). We next used siRNA to selectively decrease expression of PDZ-GEF1 and PDZ-GEF2 to determine antibody specificity. As detected by immunoblot, decreased expression of PDZ-GEF1 or PDZ-GEF2 revealed a partial reduction in total PDZ-GEF expression (Figure 6A). Specificity of siRNA mediated down-regulation was confirmed by real time RT-PCR, which revealed that PDZ-GEF1 and PDZ-GEF2 mRNA were each specifically decreased by 77 and 90% respectively (Figure 6B). To determine whether either protein was involved in JAM-A signaling, we performed additional siRNA experiments. As shown in Figure 6, A, D and E, decreased expression of PDZ-GEF2, but not PDZ-GEF1, resulted in decreased β1 integrin levels and decreased cell migration. Interestingly, down-regulation of either PDZ-GEF1 or PDZ-GEF2 was associated with significantly decreased total Rap1 activity (* $p < 0.05$). Notably, a larger decrease in total Rap1 activity was observed when

Figure 5. Active Rap1A but not Rap1B regulates $\beta 1$ integrin protein levels downstream of JAM-A and Afadin. (A) Immunoblot of Rap1 activity assays demonstrating that loss of either JAM-A (JA) or Afadin (Af6) leads to decreased active Rap1. Densitometry from three independent experiments is shown for the ratio of active Rap1 to total Rap1. Values are normalized to 1.0 for the mock-treated sample. Note that JAM-A and Afadin siRNA significantly reduce the amount of active Rap1 (* $p < 0.05$). (B) Immunoblots with a pan-Rap1 antibody showing decreased total Rap1 levels after treatment with specific Rap1A (R1A) or Rap1B (R1B) siRNA. $\beta 1$ integrin levels are decreased after JAM-A (JA) or Rap1A siRNA treatment but not with Rap1B siRNA treatment. (C) Rap1A and Rap1B siRNA treatment leads to specific down-regulation of Rap1A or Rap1B mRNA as measured by real-time PCR. Values are reported as -fold change compared with mock siRNA control. White bars are Rap1A message levels, and gray bars are Rap1B message levels. (D and E) SK-CO15 cell monolayers with decreased Rap1A expression demonstrating a 50% ($p < 0.001$) decrease in wound closure after 16 h, whereas there was 100% wound closure in monolayers with decreased expression of Rap1B or control monolayers. Bar, 200 μ m.

expression of PDZ-GEF1 and PDZ-GEF2 were both simultaneously decreased (Figure 6C). These data suggest that PDZ-GEF2 and not PDZ-GEF1 activates Rap1A to stabilize $\beta 1$ integrin levels and promote cell migration, whereas PDZ-GEF1 may activate Rap1B.

Surface $\beta 1$ Integrin Protein Is Decreased after JAM-A, Afadin, PDZ-GEF2, and Rap1 Down-Regulation

Cell surface proteins were biotinylated and isolated using Avadin-Sepharose beads. Samples were then probed for $\beta 1$ integrin by immunoblot. Compared with the mock (NT) and scramble (SCR) controls, decreased expression of Rap1A, PDZ-GEF2 (PG2), JAM-A (JA), and Afadin (Af6) all resulted in decreased levels of $\beta 1$ integrin protein on the cell surface (Supplemental Figure 2).

JAM-A Coassociates with PDZ-GEF2

Given that PDZ-GEF2 contains a PDZ domain that has the potential to interact with the PDZ binding motif on JAM-A in concert with identical functional effects after decreased expression of either protein, experiments were performed to determine whether JAM-A and PDZ-GEF2 associate in a signaling complex. In particular, because similar phenotypes

were seen with loss of JAM-A, Afadin, Rap1A, and PDZ-GEF2, we hypothesized that PDZ-GEF2 may form a complex with dimerized JAM-A and Afadin that regulates activation of Rap1A. Decreased expression of any of the components of such a complex would be predicted to reduce the levels of active Rap1A and lead to the functional and biochemical changes observed in this report. To test this hypothesis, colocalization and coimmunoprecipitation studies of JAM-A with PDZ-GEF2 were performed.

As shown in Figure 7A, JAM-A and PDZ-GEF2 colocalize at cell-cell junctions as observed by immunofluorescence labeling in SKCO-15 cells at the XZ and XY planes. In experiments using JAM-A mAb J10.4, coimmunoprecipitation of JAM-A with endogenous PDZ-GEF was observed (Figure 7B). Furthermore, to determine whether Afadin was also associated in a complex with PDZ-GEF2, coimmunoprecipitation experiments with an anti-Afadin antibody were performed in SKCO-15 cells transfected with FLAG-tagged PDZ-GEF2. The model based on our findings predicts that the association of PDZ-GEF2 with Afadin would be dependent on JAM-A. To directly test this hypothesis, we treated cells with control siRNA or with siRNA targeting JAM-A expression. As shown in Figure 7C, Afadin coimu-

Figure 6. Down-regulation of expression of PDZ-GEF2 but not PDZ-GEF1 results in effects similar to those observed with loss of Rap1A or JAM-A. (A) Immunoblots of SK-CO15 cells probed for total PDZ-GEF demonstrating that transfection with siRNA for PDZ-GEF1 (PG1) and PDZ-GEF2 (PG2) results in decreased PDZ-GEF protein levels. Note that simultaneous down-regulation of PDZ-GEF1 and PDZ-GEF2 (PGt) results in a further decrease in protein expression. Immunoblots for $\beta 1$ integrin indicate that loss of PDZ-GEF2, but not PDZ-GEF1, leads to decreased protein expression. (B) PDZ-GEF1 (PG1) and PDZ-GEF2 (PG2) siRNA treatment of SK-CO15 cells leads to specific down-regulation of mRNA as measured by real-time RT-PCR. Values are reported as -fold change compared with mock siRNA control. White bars are PG1 message levels, and gray bars are PG2 message levels. Values are reported as -fold change compared with mock siRNA control. (C) Immunoblots of Rap1 activity assays demonstrating that loss of either PDZ-GEF1 or PDZ-GEF2 leads to decreased active Rap1, with a more pronounced loss of Rap1 activity after expressional down-regulation of both. Densitometry was performed on at least three separate experiments, which indicated that the PDZ-GEF1 and PDZ-GEF2 siRNA-mediated decreases observed in Rap1 were each significant (* $p < 0.05$). The graph shown indicates the levels of active Rap1 divided by the levels of total Rap1 and normalized to the mock-transfected sample. The error bars represent SEM. (D and E) Wound closure in PDZ-GEF2 siRNA-treated SK-CO15 monolayers is decreased by 36% (* $p < 0.001$) compared with PDZ-GEF1 or scramble control monolayers. Bar, 200 μm .

Figure 7. Association and colocalization of PDZ-GEF with JAM-A. (A) By IF, JAM-A (red) and PDZ-GEF (green) colocalize in SK-CO15 cells as seen in the merged XY and XZ images. Bar, 20 μ m. (B) Coimmunoprecipitation of JAM-A with PDZ-GEF (200KD) by using JAM-A-specific mAb J10.4. As can be seen, neither JAM-A nor PDZ-GEF immunoprecipitate with the isotype control antibody. (C) Expression of JAM-A is necessary for the association of PDZ-GEF2 with Afadin. FLAG-PDZ-GEF2 was overexpressed in SK-CO15 cells followed by coimmunoprecipitation experiments using anti-Afadin (AF6). The left panel represents immunoblots of FLAG-PDZ-GEF2-expressing cells after treatment with JAM-A or scramble siRNA. As seen in the right panel, JAM-A and FLAG-PDZ-GEF2 coimmunoprecipitate with Afadin (Af6) only in cells treated with scramble control siRNA but not in cells lacking JAM-A expression. (D) A truncated and FLAG-tagged vector containing the PDZ domain of PDZ-GEF2 and expressed in SK-CO15 cells coimmunoprecipitates with JAM-A. The right panel represents immunoblots of the inputs for the immunoprecipitation experiments, and the left panel represents immunoblots of the immunoprecipitated complexes. Both panels were probed for the FLAG epitope tag as well as for JAM-A. As can be seen, JAM-A only immunoprecipitates with the anti-FLAG antibody when the FLAG-tagged PDZ domain of PDZ-GEF2 is present.

noprecipitated with JAM-A and FLAG-PDZ-GEF2 in lysates from control cells that express JAM-A. However, in cells with decreased JAM-A expression, FLAG-PDZ-GEF2 did not coimmunoprecipitate with Afadin. We hypothesized that JAM-A associated with the PDZ domain of PDZ-GEF2 through either direct or indirect interactions with linker scaffolding proteins. To further test this hypothesis, a truncated FLAG-PDZ-GEF construct was made in pCDNA3.0 that contained the putative PDZ binding domain of PDZ-GEF2 (amino acids 530-609). As can be seen in Figure 7D, from lysates of cells transfected with the tagged PDZ binding domain-containing construct, anti-FLAG antibody successfully immunoprecipitated JAM-A. These findings lend further support to a role of the PDZ domain of PDZ-GEF2 in mediating association of PDZ-GEF2 with JAM-A. Whether this association occurs by direct or indirect means awaits further binding studies with purified recombinant proteins. Our combined findings provide evidence that JAM-A dimerization forms the nucleus of a complex containing Afadin and PDZ-GEF2, which in turn activates Rap1A to stabilize β 1 integrin levels and promote cell migration.

DISCUSSION

In this study, we report that intestinal epithelial JAM-A associates with Afadin and PDZ-GEF2 and activates Rap1A. Decreased expression of any of these components results in reduced protein levels of β 1 integrin and decreased cell migration. Because epithelial cell migration is necessary for maintenance of epithelial barrier function and the repair, loss of JAM-A would thus be expected to adversely affect recovery after mucosal injury. Indeed, our results are consistent with a key role of JAM-A in mucosal injury/repair in vivo as JAM-A knockout mice have increased disease severity in a chemically induced mouse model of colitis (Laukoetter *et al.*, 2007; Vetrano *et al.*, 2008). In particular, there was significantly increased colonic mucosal injury and inflammation in JAM-A-deficient animals, compared with wild-type controls. These in vivo data are consistent with our in vitro observations implicating JAM-A as an important regulator of epithelial wound healing.

This study highlights a signaling pathway downstream of JAM-A dimerization that regulates cell migration. We recently reported that in epithelial cells, JAM-A dimerization and the JAM-A PDZ binding motif are necessary for stabilization of β 1 integrin and regulation of cell migration (Severson *et al.*, 2008). These findings are consistent with those observed in endothelial cells where decreased cell migration in JAM-A-deficient endothelial cells was restored after transfection of cells with full-length JAM-A but not expression of JAM-A lacking the PDZ binding motif (Bazzoni *et al.*, 2005). Furthermore, Naik *et al.* (2003b) reported that overexpression of JAM-A increased cell migration in endothelial cells through $\alpha_v\beta_3$ integrin and activation of mitogen-activated protein kinase. Collectively, these observations indicate that JAM-A regulates cell migration through maintenance of cellular β 1 integrin protein levels.

The mechanism(s) by which JAM-A mediates cellular functions are poorly understood. We hypothesized previously that JAM-A regulation of epithelial cell migration was dependent on its *cis*-dimerization, which brings at least two JAM-A PDZ binding motifs into close apposition. It was proposed that such closely apposed PDZ binding motifs would allow for interactions between different scaffolding proteins. The interactions of such scaffolding proteins might then result in signaling that regulates β 1 integrin protein levels (Severson *et al.*, 2008) and cell migration.

To further explore the above-mentioned hypothesis, we tested whether JAM-A associates with candidate PDZ domain-containing scaffolding/signaling molecules in polarized intestinal epithelial cells by using standard biochemical and immunolocalization techniques. We verified a functional linkage of these scaffolding/signaling molecules to JAM-A by comparing the effects of decreased expression of these associated proteins with those observed after decreased JAM-A expression. Using this approach, we observed that decreased expression of JAM-A, Afadin, PDZ-GEF2, or Rap1A, but not ZO-1, PDZ-GEF1, or Rap1B, resulted in reduced levels of $\beta 1$ integrin protein as well as decreased rates of cell migration. Additionally, decreased expression JAM-A, Afadin, and PDZ-GEF2 decreased levels of active Rap1 in a similar manner. The identical biochemical and functional effects observed after decreased expression of these proteins suggest that they are either in the same signaling pathway or separately activate the same effectors. However, because JAM-A, Afadin, and PDZ-GEF2 form part of a complex that is dependent on JAM-A expression, it is likely that these proteins participate in a single signaling pathway.

Of the proteins discussed, only Afadin has been reported to have an effect on cell migration. Lörger and Moelling (2006) reported that down-regulation of Afadin isoform 3 in MCF10A cells led to an increase in cell migration; however, this effect was due to down-regulation of Afadin isoforms 3 and dependant upon interactions with actin (Lörger and Moelling, 2006). We report decreased migration after down-regulation of Afadin in SK-CO15 cells. This difference may be due to variations in the Afadin isoforms expressed, their ability to bind to actin, or the potential absence of an effect of Afadin on $\beta 1$ integrin in MCF10A cells. Additionally, the PDZ binding motif of Afadin has been reported to bind to Rap1GAP, which inactivates Rap1 (Su *et al.*, 2003). It is possible that JAM-A and Rap1GAP compete for binding of Afadin, because they interact with the same protein domain, and such competitive binding could produce cell-type-specific responses. However, these possibilities await elucidation in further studies.

Although decreased expression of PDZ-GEF1 had no effect on $\beta 1$ integrin levels or cell migration, there was a decrease in total Rap1 activity. Furthermore, simultaneous knockdown of PDZ-GEF1 and PDZ-GEF2 resulted in larger decreases in Rap1 activity than that observed with knockdown of either GEF alone. These observations would be explained if PDZ-GEF1 specifically regulates Rap1B; however, further studies are necessary to elucidate the functional consequences of decreased Rap1B activity.

Previously, our laboratory reported a correlation between Rap1B expression and $\beta 1$ integrin levels (Mandell *et al.*, 2005). In that study, we observed that siRNA-mediated down-regulation of Rap1B decreased $\beta 1$ integrin levels. However, those experiments were performed using “smart-pools” of siRNA containing four separate siRNA oligonucleotides. Given that Rap1A and Rap1B are 95% conserved, we determined that the mixture of Rap1B siRNA targets previously used also resulted in partial down-regulation of Rap1A expression and thus decreased $\beta 1$ integrin protein levels. In the current study, we performed real-time RT-PCR to confirm that the siRNA targets used do not cross-react between Rap1A and Rap1B. Taken together, the above-mentioned results suggest that active Rap1A, not active Rap1B, regulates $\beta 1$ integrin protein levels and the rate of cell migration.

Together, the data from this and previous studies support the hypothesis that a complex of dimerized JAM-A, Afadin,

Figure 8. Model for the molecular basis of JAM-A signaling. Dimerized JAM-A interacts with Afadin and PDZ-GEF2, directly or indirectly as a complex through PDZ-mediated binding. Under this circumstance, the activity of Rap1A bound to Afadin is regulated by closely apposed PDZ-GEF2. $\beta 1$ integrin levels and cell migration are regulated by levels of active Rap1A through undefined mechanisms.

and PDZ-GEF2 regulate $\beta 1$ integrin at the protein level by controlling Rap1A activation. Such fine-tuning of $\beta 1$ integrin levels could serve as a regulator of cell migration under a variety of circumstances. As highlighted in Figure 8, our observations support a model of JAM-A-mediated regulation of cell migration through the formation of a PDZ-dependent signaling complex. In this model, JAM-A dimerization leads to the close apposition PDZ-GEF2 and Afadin. Rap1A brought into close proximity to PDZ-GEF2 by Afadin is maintained in an active state to promote cell migration by stabilizing $\beta 1$ integrin levels through, as of yet, unclear mechanisms. The protein interactions depicted in Figure 8 may not be direct but may occur via additional as of yet unidentified scaffolding proteins that may associate with the JAM-A protein complex.

The proposed model of JAM-A signaling (Figure 8) likely applies to the rate of tight and adherens junction formation, because both Rap1 and JAM-A have been implicated in accelerating the rate of formation for adherens and tight junctions (Liu *et al.*, 2000; Glading *et al.*, 2007). In a model of junction reassembly after transient calcium depletion, JAM-A mAbs, such as J10.4, that block dimerization have been shown to slow the formation of tight and adherens junctions (Liu *et al.*, 2000). Similarly, treatment with cAMP analogues that activate Rap1 increase the rate of junction resealing (Kooistra *et al.*, 2005). Thus, the effect of inhibiting JAM-A dimerization of the rate of junctional formation is likely due to inhibition of Rap1 activation through by mechanism(s) similar to that outlined in this current report.

It is important to note that the murine crystal structure of JAM-A predicts the formation of dimers on the same cell (*cis*) that interact between cells (*trans*) (Kostreva *et al.*, 2001). Indeed, the signaling events outlined in Figure 8 would be enhanced through *trans* interactions of JAM-A *cis*-dimers between cells. *Trans* interactions as well as *cis* interactions would result in each JAM-A dimer having two *trans* binding

sites, allowing for localized high density oligomerization of JAM-A between cells. Such localized high concentrations of JAM-A between cell-cell contacts would result in large protein complexes serving to amplify JAM-A-mediated signals. The nature of such predicted *trans* interactions between *cis*-dimers of JAM-A remain to be defined through further studies.

In summary, the findings in this report support a model of JAM-A dimerization-mediated signaling through interactions with Afadin and PDZ-GEF2 that result in activation of Rap1A, to stabilize $\beta 1$ integrin levels and regulate cell migration. Additional studies are necessary to determine the mechanism by which activated Rap1A stabilizes $\beta 1$ integrin levels in epithelial cells. We hypothesize that other reported JAM-A functions may use similar mechanisms for intracellular signaling; however, there are likely to be cell-type-specific signaling intermediates and effector proteins. Further studies are needed to determine whether the general mechanism described in this report is applicable to JAM-A-mediated regulation of other cellular processes.

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