Expression of junctional adhesion molecule-A prevents spontaneous and random motility

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Summary

Junctional adhesion molecule-A (JAM-A) is a cell-surface glycoprotein that localizes to intercellular junctions and associates with intracellular proteins via PSD95-Dlg-ZO1-binding residues. To define the functional consequences of JAM-A expression, we have produced endothelial cells from JAM-A-deficient mice. We report here that the absence of JAM-A enhanced spontaneous and random motility. In turn, the enhanced motility of JAM-A-negative cells was abrogated either on transfection of exogenous JAM-A or on treatment with inhibitors of glycogen synthase kinase-3β (GSK-3β). In addition, in JAM-A-positive cells, motility was enhanced on inactivation of protein kinase Cζ (PKCζ), which is an inhibitor of GSK-3β. Although these findings suggested that JAM-A might inhibit GSK-3β, we found that expression per se of JAM-A did not change the levels of inactive GSK-3β. Thus, JAM-A expression may regulate effectors of motility that are also downstream of the PKCζ/GSK-3β axis. In support of this view, we found that JAM-A absence increased the number of actin-containing protrusions, reduced the stability of microtubules and impaired the formation of focal adhesions. Notably, all the functional consequences of JAM-A absence were reversed either on treatment with GSK-3β inhibitors or on transfection of full-length JAM-A, but not on transfection of a JAM-A deletion mutant devoid of the PSD95-Dlg-ZO1-binding residues. Thus, by regulating cytoskeletal and adhesive structures, JAM-A expression prevents cell motility, probably in a PSD95-Dlg-ZO1-dependent manner.

Key words: Junction adhesion molecule, Motility, Glycogen Synthase kinase 3, PDZ domains, Microtubules, Focal adhesions

Introduction

Junctional adhesion molecule-A (JAM-A), which is also known as JAM, JAM-1 and F11R, belongs to a family of cell adhesion molecules that contain two immunoglobulin-like folds in the extracellular domain (Bazzoni, 2003). JAM-A localizes to intercellular junctions (Martin-Padura et al., 1998) and mediates processes as diverse as junction assembly (Liu et al., 2000; Liang et al., 2000), leukocyte transmigration (Martin-Padura et al., 1998; Del Maschio et al., 1999), platelet activation (Sobocka et al., 2000; Amieva et al., 2003). JAM-A comprises an extracellular domain, a transmembrane segment and a cytoplasmic tail. The extracellular domain forms parallel dimers (Kostrewa et al., 2001) and binds several ligands, such as JAM-A itself (Liu et al., 2000; Bazzoni et al., 2000a), the leukocyte integrin αLβ2 (Ostermann et al., 2002) and the Reovirus protein σ-1 (Barton et al., 2001). The cytoplasmic tail contains the carboxyl terminal residues F298-L299-V300 (FLVCOOH), which represent a recognition motif for type II PSD95-Dlg-ZO1 (PDZ) domains. JAM-A associates directly with at least five different PDZ proteins, namely ZO-1 (Bazzoni et al., 2000b; Ebnent et al., 2000), AF6/Afadin (Ebnent et al., 2000), CASK/Lin-2 (Martinez-Estrada et al., 2001), Par3/ASIP (Ebnent et al., 2001; Itoh et al., 2001) and MUPP-1 (Hamazaki et al., 2002), as evaluated by in vitro binding assays. Importantly, all these interactions are abolished on deletion of the FLVCOOH motif.

Although the junctional localization of most of these PDZ proteins suggests a role in the assembly or stabilization of JAM-A-containing junctions, they may bind other molecules and play additional roles. For example,ZO-1 (Fanning et al., 2002) and AF6/Afadin (Boettner et al., 2000) bind directly F-actin and profilin, respectively. CASK/Lin-2 interacts with protein 4.1, which nucleates actin and spectrin filaments (Cohen et al., 1998). Finally, Par3 associates with Par6 and atypical protein kinase C (PKC), which in turn regulate cell polarity (Ohno, 2001). To date, however, the functional role of JAM-A and its PDZ-dependent associations remains largely unknown.

To further define the function of JAM-A, we have produced and characterized endothelial cells from JAM-A-deficient mice. We report here that JAM-A absence enhanced spontaneous and random motility. In turn, the enhanced motility of JAM-A-defective cells was abolished on transfection of full-length JAM-A (JAM-A FL), but not a deletion mutant (JAM-A delFLV) that lacks the FLVCOOH motif. Thus, JAM-A inhibits cell motility in a PDZ-dependent way. Finally, we report that JAM-A controls cell motility by regulating cytoskeletal and...
Materials and Methods

**Cell lines**

The 164-JAM-A+/+ and 160-JAM-A+/− cells were obtained from floxP-JAM-A-floxP mice and JAM-A−/− mice (the latter derived from mating floxP-JAM-A-floxP with Cre transgenic mice), respectively. The floxP-JAM-A-floxP and JAM-A+/− mice have been generated as described previously (Cera et al., 2004). To prepare the cells, 3-month old mice were killed by cervical dislocation and exsanguination. Then, lungs were explanted and digested with collagenase (Sigma, St Louis, MO). Endothelial cells were immortalized by polyoma middle-T antigen to establish the 164-JAM-A+/+ and 160-JAM-A−/− cells. Finally, the 160-JAM-A FL and 160-JAM-A delFLV cells were produced by transfection of recombinant constructs (Bazzoni et al., 2000b). Cells were kept in culture (with 1:2 splitting every second day) in D-MEM supplemented with 10% (vol:vol) FBS (Gibco Invitrogen, Carlsbad, CA), 10 µg/ml heparin (Sigma) and 5 µg/ml endothelial cell growth supplement.

**Antibodies and reagents**

The following antibodies were used: rat anti-JAM-A mAbs BV12, BV11, and BV19; rat anti-PECAM-1 mAb 12.3 (kindly provided by A. Vecchi, Mario Negri Institute, Milan); mouse anti-vinculin mAb hVIN-1 (Sigma); mouse anti-paxillin mAb 349 (Transduction Laboratories, Belgium); mouse anti-α-tubulin mAb B-5-1-2 and anti-acetylated-α-tubulin mAb 6-11B-1 (Sigma); mouse anti-GSK3β mAb 7 (Transduction). Rabbit GSK-3α/β Ser21-P antiserum (Biosource International, Camarillo, CA); rabbit PKCζ antisemur sc-216 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit ZO-1 and occludin antisera (Zymed Laboratories, San Francisco, CA). TRITC- and FITC-labeled anti-IgG (H+L) antibodies were from Jackson antisera (Zymed Laboratories, San Francisco, CA). FITC-Phalloidin, colloidal gold, and nocodazole were from Sigma. Myristoylated PKCζ pseudo-substrate (PKCζ-PS) was from Biosource International.

**Fluorescence flow cytometric analysis**

Fluorescence flow cytometric analysis was performed with a FACStar Plus apparatus (Becton Dickinson, Mountainview, CA). Cells were detached by incubation with 0.25% Trypsin (Gibco) in phosphate-buffered saline. Aliquots (100 µl) of cell suspensions (10^6 cells/ml) were incubated with 2% (wt:vol) BSA in PBS (for 60 minutes on ice), then washed with PBS plus 0.2% BSA, and incubated with primary antibodies (for 45 minutes on ice) at a concentration of 20 µg/ml. Cells were then washed three times, incubated (for 45 minutes on ice) with secondary fluorescein isothiocyanate-conjugated goat F(ab′)2 anti-rat IgG (CalTag Laboratories, Burlingame, CA) and washed three times before analysis.

**Immunofluorescence microscopy**

Single-cell suspensions were seeded at different densities onto glass coverslips (that had been coated with human plasma fibronectin; 7 µg/ml) and incubated for 120 minutes at 37°C. Cells were then fixed with 3% (wt:vol) paraformaldehyde for 15 minutes, permeabilized with 0.5% (vol:vol) Triton-X-100 for 3 minutes, and stained with primary and TRITC-labeled secondary antibodies, as previously described (Martinez-Estrada et al., 2001). Coverslips were mounted in 488-Mowiol and analyzed with a Zeiss Axioshot microscope (equipped with 100× Plan-Neofluar Ph3 objective lens). Images were recorded with a Zeiss 456070 camera on 3200 ASA Kodak films and processed using Adobe Photoshop.

Phagokinetic track assay

The assay was performed as described (Albrecht-Buhler, 1977). Briefly, cells were plated onto glass coverslips (previously coated with colloidal gold) and incubated for 24 hours at 37°C. Then, cells were fixed with 3% paraformaldehyde. Pictures of individual cells were analyzed with the NIH ImageTM software for measuring particle-free surfaces containing one cell were analyzed, in order to exclude surfaces produced by either intersection of two (or more) cells or cells derived from cell division during the 24 hours.

Microtubule polymerization assay

The assay was performed as described previously (Minotti et al., 1991). Briefly, cells were grown in a 6-well plate. Before reaching confluence, cells were lysed with taxol-containing lysis buffer (20 mM Tris-HCl, pH 6.8; 140 mM NaCl; 0.5% NP-40; 1 mM MgCl2, 2 mM EGTA; 4 µg/ml taxol). Wells were scraped and washed with lysis buffer, while any remaining insoluble residue was solubilized with Laemmli sample buffer. Then, the aliquot of taxol-containing lysis buffer was centrifuged, and the pellet was combined with Laemmli-solubilized material to obtain the NP40-insoluble fraction. Samples were separated by SDS-PAGE. Filters were analyzed by western blotting with anti-tubulin antibody, secondary HRP-conjugated antimurine IgG antibody and chemiluminescence.

Wound assay

Cells were seeded (at a density of 10^5/cm²) onto gelatin-coated 24-well plates and grown to confluence. Then, the culture medium was removed, and scratch wounds were produced using a plastic tip for 1000 µl pipettes. After two washes with D-PBS, cells were incubated with D-MEM medium containing 10% serum. Finally, cells were fixed and stained (5 minutes at room temperature) with a solution of crystal violet in methanol/water (80:20; vol:vol). The migrated distance was obtained by subtracting the width of the wound at 24 hours from the width at time 0 (i.e. immediately after wounding) and by averaging the results obtained at the different 1 mm intervals. At time 0, wounds measured 1118±36 (164-JAM-A+/+), 1110±25 (160-JAM-A−/−), 1204±45 (160-JAM-A FL) and 1108±63 µm (160-JAM-A delFLV; means±s.e.m., n=15).

Results

JAM-A expression in endothelial cells

We have produced JAM-A-positive (164-JAM-A+/+) and JAM-A-negative (160-JAM-A−/−) endothelial cells from wild-type and JAM-A-deficient mice, respectively (Cera et al., 2004). In addition, we have transfected the 160-JAM-A−/− cells with either full-length JAM-A (160-JAM-A FL) or a deletion mutant lacking the FLVCOOH motif (160-JAM-A delFLV). As assessed by fluorescence flow cytometric analysis, endogenous JAM-A was expressed in 164-JAM-A+/+ but not in 160-JAM-A−/− cells. Also, exogenous JAM-A FL and delFLV were expressed at similar levels in 160-JAM-A FL and 160-JAM-A delFLV cells, respectively (Fig. 1). By comparison, the expression levels of PECAM-1 and occludin (two other molecules that localize to intercellular contacts in endothelial cells) did not differ among the four cell lines, as assessed by fluorescence flow cytometric analysis. Specifically, in 164-JAM-A+/+, 160-JAM-A−/−, 160-JAM-A FL and 160-JAM-A delFLV cells, the values of mean fluorescence intensity for PECAM-1 were 43.3, 44.4, 47.6 and 40.5 units, respectively. Also, the values for occludin were 96.3, 106.8, 104.9 and 114.5 units, respectively.
JAM-A regulates cell motility

As assessed by IF microscopy, JAM-A localized to intercellular junctions in all cell lines, with the exception of 160-JAM-A–/–. Nonetheless, all cell lines displayed similar expression of junctional markers and similar levels of paracellular permeability (data not shown), thus indicating that JAM-A is dispensable for junction formation and function in contacting cells. Next, in search for additional functions of JAM-A, we analyzed noncontacting cells.

JAM-A expression reduces the number of membrane protrusions

When plated onto fibronectin, the four cell lines formed actin-containing protrusions of variable length and shape (Fig. 2A). However, 160-JAM-A–/– cells formed a higher number of long protrusions than 164-JAM-A+/+ cells. Also, on transfection of 160-JAM-A–/– cells with JAM-A FL (but not JAM-A delFLV), the number of long protrusions decreased to the level observed in 164-JAM-A+/+ cells. Then, cells were analyzed individually for measuring the distribution of this type of protrusion (Fig. 2B). Specifically, we counted protrusions that were longer than 2 µm. The average number of long protrusions per cell was about 1.5-fold higher in 160-JAM-A–/– (8.3±0.4 protrusions; mean±s.e.m.; n=168 cells) and 160-JAM-A delFLV cells (8.6±0.3; n=123), compared with 164-JAM-A+/+ (5.5±0.3; n=176) and 160-JAM-A FL cells (5.9±0.3; n=129). Thus, expression of JAM-A (but not JAM-A delFLV) reduced the protrusive activity at the membrane. As actin-based protrusions are associated with cell motility (Condeelis, 1993), we next evaluated the effect of JAM-A expression on motility.

JAM-A expression prevents spontaneous and random motility

To study motility, we deployed the phagokinetic track assay (Albrecht-Buehler, 1977). Over a 24-hour interval, most 164-JAM-A+/+ cells produced a small particle-free surface, with no (or very few) tracks (Fig. 3A). By contrast, most 160-JAM-A–/– cells produced a broader particle-free surface, with many more tracks. As above, on transfection of 160-JAM-A–/– cells with JAM-A FL (but not JAM-A delFLV), the phagokinesis pattern became similar to the one observed in 164-JAM-A+/+ cells.

Then, cells were analyzed individually for measuring the distribution of particle-free area (Fig. 3B) and number of tracks (Fig. 3C). The average particle-free area per cell was greater in 160-JAM-A–/– (522±32 µm²; mean±s.e.m.; n=61 cells) and 160-JAM-A delFLV cells (530±34 µm²; n=58), compared with 164-JAM-A+/+ (239±14 µm²; n=72) and 160-JAM-A FL cells (328±19 µm²; n=53). Similarly, the average number of tracks per cell was higher in 160-JAM-A–/– (2.9±0.2; mean±s.e.m.; n=181) and 160-JAM-A delFLV cells (3.3±0.2; n=182), compared with 164-JAM-A+/+ (0.2±0.1; n=180) and 160-JAM-A FL cells (1.4±0.1; n=182).

Inactivation of GSK-3β mimics JAM-A-dependent inhibition of motility

Similarly to the expression of JAM-A in our system, inactivation of GSK-3β was shown to reduce long protrusions in keratinocytes (Koivisto et al., 2003). Thus, we surmised that JAM-A expression might inhibit protrusions (and possibly motility) by inactivating GSK-3β. To address this issue, we first treated cells with chemical inhibitors of GSK-3β. In 160-JAM-A–/– and 160-JAM-A delFLV cells, either lithium (40 mM;
Table 1A) or SB216763 (20 µM; Table 1B) reduced the number of protrusions, the particle-free area and the number of tracks per cell almost to the levels observed in 164-JAM-A+/+ and 160-JAM-A FL cells. Thus, inactivation of GSK-3β mimicked the inhibitory effect of JAM-A expression on protrusions and motility.

PKCζ is one of the endogenous regulators of GSK-3β. Notably, PKCζ-dependent inactivation of GSK-3β (via phosphorylation on Ser9) is instrumental in controlling cell migration (Etienne-Manneville and Hall, 2003). To test the role of PKCζ in our system, we treated cells with a cell-permeable and PKCζ-specific pseudo-substrate (PKCζ-PS). In 160-JAM-A FL cells, PKCζ-PS (20 µM; Table 1C) increased the number of protrusions, the particle-free area and the number of tracks per cell to the levels observed in 160-JAM-A–/– cells. Thus, complementing the GSK-3β inhibitor data, PKCζ-PS prevented the inhibitory effect of JAM-A expression on protrusions and motility. The effect of PKCζ-PS was probably due to GSK-3β activation, as an inhibitor of GSK-3β could prevent it. For instance, in PKCζ-PS-treated 160-JAM-A FL cells, SB216763 (5 µM) strongly reduced the effect of PKCζ-PS (20 µM) on the particle-free area (610±64 µm² and 312±56 µm², in the absence and presence of SB216763, respectively).

JAM-A expression does not influence the activation state of GSK-3β

To examine the direct effect of the inhibitors on GSK-3β, we analyzed inactive GSK-3β (GSK-3β Ser9-P) by western blotting. In 160-JAM-A FL cells, constitutive levels of GSK-3β Ser9-P were detectable even in absence of the inhibitors. Either lithium (40 mM) or SB216763 (20 µM) increased the levels of GSK-3β Ser9-P, while PKCζ-PS (20 µM) reduced them. In all conditions tested, no changes in total GSK-3β were observed (data not shown).

Then, to test whether JAM-A expression per se influenced the activation state of GSK-3β, we compared the constitutive levels of GSK-3β Ser9-P among the cell lines. We found similar levels of GSK-3β Ser9-P in 160-JAM-A+/+, FL and delFLV cells (data not shown). Thus, although JAM-A expression (see Figs 2 and 3) and GSK-3β inhibitors alike (see Table 1) reduced protrusions and motility, JAM-A expression did not affect the activation state of GSK-3β. Rather, we hypothesized that JAM-A expression regulated motility-related responses downstream of the PKCζ-GSK-3β axis.

JAM-A expression stabilizes microtubules

As shown above, one of these responses is the formation of actin-based protrusions, which are reduced in number on either expression of JAM-A (Fig. 2) or treatment with GSK-3β inhibitors (Table 1). Stabilization of microtubules is another effect of GSK-3β inactivation that is important for cell motility (Zumbrunn et al., 2001). To test whether JAM-A affected microtubule dynamics, we first studied microtubule assembly (by IF microscopy) and tubulin polymerization (by western blotting) following nocodazole withdrawal. By IF microscopy (Fig. 4A), microtubules disappeared from all cell lines after 16-hour treatment with 10 µM nocodazole. However, by 2 hours after nocodazole withdrawal, microtubules were more numerous in 164-JAM-A+/+ and 160-JAM-A FL cells than in 160-JAM-A–/– and 160-JAM-A delFLV cells. In addition, by western blotting (Fig. 4B), NP40-insoluble (i.e. polymerized) tubulin was strongly reduced in all cell lines after nocodazole withdrawal. As a loading control, similar amounts of NP40-insoluble ZO-1 were detectable in the four cell lines on nocodazole withdrawal. On the basis of these results, we next investigated whether JAM-A expression might actually stabilize microtubules.

Cold temperature causes microtubule disassembly (Bershadsky et al., 1979), even though diverse cell types differ
JAM-A regulates cell motility in the cold-stability of microtubules (Lieuvin et al., 1994). In addition, it is well established that stable microtubules accumulate acetylated tubulin (Westermann and Weber, 2003). So, to analyze microtubule stability, we examined whether JAM-A expression enhanced the amount of either cold-stable microtubules or acetylated tubulin, or both. To this purpose, we incubated cells at either 4°C or 37°C for 30 minutes. Then we used antibodies against bulk tubulin (Fig. 5A) and acetylated tubulin (Fig. 5B) to examine cold-stability and tubulin acetylation, respectively. At 37°C, similar amounts of microtubules were detectable in both 164-JAM-A+/+ and 160-JAM-A–/– cells (Fig. 5A, left panels). Remarkably, however, more acetylated tubulin was detectable in 164-JAM-A+/- than in 160-JAM-A–/– cells (Fig. 5B, left panels).

### Table 1. PKC-ζ-dependent inactivation of GSK-3β mimics JAM-A-dependent inhibition of protrusions and motility

| Treatment and cell line | Number of protrusions/cell | Particle-free area/cell (µm²) | Number of tracks/cell |
|-------------------------|----------------------------|-------------------------------|-----------------------|
|                         | Control                    | Lithium                       | Control               | Lithium               | Control               | Lithium               |
| 164-JAM-A+/+            | 5.3±0.6                    | 5.7±0.6                       | 324±53                | 332±23                | 1.2±0.3               | 1.5±0.4               |
| 160-JAM-A–/–            | 9.7±0.6                    | 6.7±0.5                       | 818±103               | 354±17                | 4.8±0.4               | 2.3±0.3               |
| 160-JAM-A FL            | 4.7±0.4                    | 4.9±0.5                       | 312±64                | 306±35                | 1.9±0.3               | 1.9±0.3               |
| 160-JAM-A delFLV        | 9.0±0.5                    | 6.2±0.5                       | 1027±128              | 337±33                | 4.1±0.4               | 2.3±0.3               |
|                         | **SB216763**               |                               |                       |                       |                       |                       |
|                         | Number of protrusions/cell |                               |                       |                       |                       |                       |
|                         | Control                    | SB216763                      | Control               | SB216763              | Control               | SB216763              |
| 164-JAM-A+/+            | 8.6±0.8                    | 4.8±1.2                       | 319±20                | 325±22                | 1.4±0.3               | 1.4±0.3               |
| 160-JAM-A–/–            | 4.5±0.6                    | 3.8±0.6                       | 884±94                | 308±18                | 3.8±0.4               | 1.5±0.4               |
| 160-JAM-A FL            | 4.5±0.6                    | 3.8±0.6                       | 324±34                | 338±29                | 1.7±0.5               | 1.6±0.4               |
| 160-JAM-A delFLV        | 8.9±0.8                    | 3.9±0.8                       | 934±111               | 317±21                | 3.7±0.5               | 1.4±0.3               |
|                         | **PKCζ-PS**                |                               |                       |                       |                       |                       |
|                         | Number of protrusions/cell |                               |                       |                       |                       |                       |
|                         | Control                    | PKCζ-PS                       | Control               | PKCζ-PS               | Control               | PKCζ-PS               |
| 164-JAM-A+/+            | n.d.                       | n.d.                          | n.d.                  | n.d.                  | n.d.                  | n.d.                  |
| 160-JAM-A–/–            | 9.3±1.5                    | 9.3±1.3                       | 735±73                | 622±118               | 3.1±0.4               | 2.8±0.3               |
| 160-JAM-A FL            | 3.3±1.0                    | 9.8±2.0                       | 312±28                | 575±14                | 1.2±0.3               | 2.6±0.4               |
| 160-JAM-A delFLV        | n.d.                       | n.d.                          | 731±136               | 715±105               | 3.2±0.5               | 2.9±0.3               |

Cells were incubated in the absence (Control) or presence of lithium (40 mM), SB216763 (20 µM), and PKCζ-PS (20 µM). Cells were then analyzed for number of actin-based protrusions, particle-free area and number of tracks. Data are expressed as mean±s.e.m. (n=30-32 cells; n.d.=not done).
Next we examined the effect of cold temperature. Following exposure to 4°C, the amount of microtubules was only slightly reduced in 164-JAM-A+/+ cells, whereas it was reduced to a greater extent in 160-JAM-A−/− cells (Fig. 5A, middle panels). In addition, in 164-JAM-A+/+ cells, greater amounts of acetylated tubulin were detectable at 37°C than at 4°C. At variance, in 160-JAM-A−/− cells, acetylated tubulin was barely detectable at both 37°C and 4°C (Fig. 5B, left and middle panels). As a control, pretreatment with 10 nM taxol (for 30 minutes before exposure to cold) preserved microtubules and acetylated tubulin in both cell types (right panels). Finally, we found that 160-JAM-A FL cells displayed higher amounts of cold-resistant and acetylated tubulin than 160-JAM-A delFLV cells (not shown). Thus, expression of JAM-A (but not JAM-A delFLV) enhanced microtubule stability.

JAM-A expression increases the number of focal adhesions
As highly dynamic microtubules cause disassembly of focal adhesions (Small and Kaverina, 2003), we examined whether JAM-A expression affected focal adhesions as well. By IF microscopy of vinculin- (Fig. 6A) and paxillin- (Fig. 6B) stained cells, we found that, in 160-JAM-A+/+ and 160-JAM-A delFLV cells, adhesions were fewer in number and more peripheral in location, compared with 164-JAM-A+/+ and 160-JAM-A FL cells. Cells were analyzed individually for measuring the distribution of the number of vinculin-based focal adhesions (Fig. 6C). The average number of focal adhesions per cell was greater in 164-JAM-A+/+ (141±9; mean±s.e.m.; n=15 cells) and 160-JAM-A FL cells (121±6), compared with 160-JAM-A−/− (53±4) and 160-JAM-A delFLV cells (52±4).

In 160-JAM-A−/− cells, 10 nM taxol (a dose that stabilizes microtubules; see above) (Jordan et al., 1993) induced numerous adhesions. Similarly, lithium (40 mM) increased the number of adhesions in 160-JAM-A−/− cells, whereas PKCζ/PS reduced it in 160-JAM-A FL cells (Fig. 6D). Hence, the effect of JAM-A expression on focal adhesions (Fig. 6) was related to the effect on microtubules (Figs 4 and 5), probably downstream of the PKCζ/GSK-3β axis.

JAM-A causes morphological changes in migrating cells at a wound edge
Next, we analyzed cell migration into a wounded monolayer. Using this system, we found that all cell lines migrated with similar efficiency. For instance, at 24 hours after wounding, the migrated distances were 767±43 µm (164-JAM-A+/+), 808±29 µm (160-JAM-A−/−), 807±35 µm (160-JAM-A FL) and 751±35 µm (160-JAM-A delFLV; mean±s.e.m., n=15). Hence, expression of JAM-A (but not JAM-A delFLV) reduced spontaneous motility (see particle-free area) and randomness of motion (see number of tracks), while leaving migration efficiency (see migrated distances in the wound) unaffected.

However, morphological differences among the cell lines were detectable at the wound edge (Fig. 7A). First, compared with 160-JAM-A−/− and 160-JAM-A delFLV cells, 164-JAM-A+/+ and 160-JAM-A FL cells aligned cell body (first row) and actin filaments (second row) in a much more orderly fashion and more in parallel to the wound. Second, vinculin-containing focal adhesions were more numerous and central in 164-JAM-A+/+ and 160-JAM-A FL cells aligned cell body (first row) and actin filaments (second row) in a much more orderly fashion and more in parallel to the wound. Second, vinculin-containing focal adhesions were more numerous and central in 164-JAM-A+/+ and 160-JAM-A FL cells than in 160-JAM-A−/− and 160-JAM-A delFLV cells (third row). Third, the percentage of cells orienting flat protrusions in parallel to the major axis of the wound was about 2.6-fold greater in 164-JAM-A+/+ cells (41.2±1.4%) than in 160-JAM-A−/− cells (15.7±1.0%; mean±s.e.m.; n=900 cells). Thus, JAM-A absence induced morphological changes in cell motility not only in noncontacting cells (Figs 2 and 6), but also in cohesive cell sheets (Fig. 7).

JAM-A effect is independent of cell-cell contact and expression levels
To evaluate this issue in further detail, we examined membrane protrusions and focal adhesions in small groups of contacting cells (two or three cells). We found that, similarly to single noncontacting cells (Figs 2 and 6), contacting 164-JAM-A+/+ cells formed fewer actin-based protrusions at the membrane (Fig. 7B, left panels) and more vinculin-based focal adhesions (right panels) compared with 160-JAM-A−/− cells. Thus, the
JAM-A regulates cell motility and morphological changes associated with JAM-A expression were not influenced by cell-cell contact.

Finally, we examined whether the functional consequences of JAM-A expression were influenced by the levels of JAM-A expression at the cell surface. To this purpose, we sorted the 160-JAM-A FL cells and obtained the 160-JAM-A FL1 and 160-JAM-A FL2 cells, which express JAM-A at medium and high levels, respectively (Table 2). Using these cells (in parallel with 164-JAM-A+/+ and 160-JAM-A–/– cells for comparison), we found that expression of JAM-A at either medium (FL1) or high (FL2) levels similarly decreased the migrated area, reduced the number of membrane protrusions and increased the number of focal adhesions. Thus, JAM-A reversed the functional consequences of JAM-A absence independently of its levels of expression.

**Discussion**

The major findings of this manuscript are as follows. First, the absence of JAM-A enhances cell motility, increases membrane

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**Table 2. JAM-A effect is not dependent on the expression levels at the cell surface**

| Cell line        | JAM-A expression (MFI) | Number of protrusions/cell | Particle-free area/cell (µm²) | Number of adhesions/cell |
|------------------|------------------------|---------------------------|-------------------------------|--------------------------|
| 164-JAM-A+/+     | 54                     | 4.5±0.2                   | 258±24                        | 116±5                    |
| 160-JAM-A+/+     | 0                      | 9.5±0.3                   | 1021±162                      | 63±4                     |
| 160-JAM-A FL1    | 70                     | 4.3±0.2                   | 269±36                        | 120±4                    |
| 160-JAM-A FL2    | 850                    | 4.0±0.2                   | 403±60                        | 118±7                    |

The 160-JAM-A FL cells were sorted by fluorescence flow cytometry to obtain the 160-JAM-A FL1 and FL2 cells, which express JAM-A at medium and high levels, respectively. Cells were then analyzed for JAM-A expression (by flow cytometry), number of actin-based membrane protrusions, gold particle-free area and number of vinculin-based focal adhesions. Data are expressed as mean±s.e.m. (n=15,000 for JAM-A expression; n=30 for protrusion number; n=9 for particle-free area and adhesion number).
protrusions, affects microtubule stability, and reduces focal adhesions. Second, the consequences of JAM-A absence are reversed either on transfection of JAM-A (in a way that critically requires the PDZ-binding motif) or on treatment with GSK-3β inhibitors.

JAM-A and motility

Although JAM-A expression modulates pleotropic targets as diverse as protrusions, adhesions and microtubules, the final outcomes are all consistent with a coordinated inhibition of spontaneous and random motility. First, the formation of a single lamellipodial protrusion at the cell front is one of the earliest steps of cell migration that ensures regular and directional motility (Lauffenburger and Horwitz, 1996). At variance, the formation of numerous protrusions (such as those that appear around the periphery of JAM-A-defective cells) is predictive of irregular patterns of motility (as observed in these cells). In this context, transfection of JAM-A not only restricts overall motility (as reflected in the smaller particle-free area) but also prevents frequent changes in direction (as reflected in the lower number of tracks). Second, focal adhesions are another key factor of motility (Webb et al., 2002). As shown in other systems, numerous adhesions strengthen the restraint to free motion that the substrate exerts on the cells (Ilic et al., 1995). Thus, the higher number of focal adhesions in JAM-A-expressing cells is consistent with the lower motility of these cells. In partial agreement with this view, other authors have reported that JAM-A blockade reduced adhesion to vitronectin (Naik et al., 2003). Third, microtubules play a role in cell motility. Notably, focal adhesions are among the key effectors of motility that are also potential targets for microtubules. In particular, microtubule instability (with microtubules that repeatedly direct to and retract from the adhesions) has been associated with adhesion disassembly (Kaverina et al., 1999; Small and Kaverina, 2003). Conversely, taxol, besides stabilizing microtubules, reduces cell motility (Liao et al., 1995). Hence, the ability of JAM-A to stabilize microtubules provides a probable explanation for the higher number of adhesions and the lower motility of JAM-A-expressing cells (even though we cannot exclude a direct effect of JAM-A on the adhesions). The observation that taxol fully reverses the defective formation of focal adhesions in JAM-A+/− cells further supports the notion of a JAM-A-dependent and coordinated regulation of microtubules and adhesions in the inhibition of cell motility.

Finally, JAM-A expression (besides favoring microtubule assembly on nocodazole withdrawal) was associated with enhanced amounts of acetylated tubulin, which is a known marker of microtubule stability (Westermann and Weber, 2003). To further strengthen the link between microtubule stability and cell motility, it is worth mentioning that reduced tubulin acetylation (in cells that overexpress histone deacetylase-6) was associated with enhanced cell motility (Hubbert et al., 2002), even though the precise molecular mechanism remains unclear (Palazzo et al., 2003).

JAM-A and the PKCζ/GSK-3β axis

In addition to its key role in metabolism and proliferation (Manoukian and Woodgett, 2002), GSK-3β regulates protrusions, motility and microtubules. First, GSK-3β inhibitors prevent the formation of long lamellipodia in keratinocytes (Koivisto et al., 2003) and filopodia in neurons (Owen and Gordon-Weeks, 1999). To further strengthen the link between microtubule stability and cell motility, it is worth mentioning that reduced tubulin acetylation (in cells that overexpress histone deacetylase-6) was associated with enhanced cell motility (Hubbert et al., 2002), even though the precise molecular mechanism remains unclear (Palazzo et al., 2003).
stabilizes microtubules by preventing the phosphorylation of adenomatous polyposis coli (Zumbrunn et al., 1999) and microtubule-associated protein 1B (Goold et al., 1999). Building on this evidence, our data (with chemical inhibitors) further show that GSK-3β inhibition reverses the protrusive and motile activity of JAM-A-negative cells, thus establishing a link between JAM-A, the PKCζ/GSK-3β axis and motility.

All the herein-described effects of JAM-A expression required the PDZ-binding residues FYLCOOH, thus suggesting the involvement of PDZ partner(s) of JAM-A. Among these molecules, it is known that Par3 associates with the Par6–PKCζ complex (Lin et al., 2000; Joberty et al., 2000), thus representing a putative molecular linkage between JAM-A and GSK-3β. In addition, a JAM-A deletion mutant lacking the PDZ-binding residues redistributes Par3 from cell contacts in the cytosol (Ebnét et al., 2001). Finally, Par3 overexpression causes the loss of contact-dependent inhibition of cell motility (Mishima et al., 2002), and soluble Par3 inhibits PKCζ activity (Lin et al., 2000). On the basis of this evidence, we surmised that, in the absence of JAM-A, increased availability of Par3 might result in PKCζ inactivation and, consequently, in GSK-3β activation. However, JAM-A expression did not increase the absolute levels of inactive GSK-3β Ser9-P (at least within the sensitivity limits of western blotting), thus ruling out a general effect of JAM-A (and Par3) on GSK-3β activation. Interestingly, it has been recently shown that JAM-C (another member of the JAM family) regulates the polarized distribution of a PKCζ-containing complex in differentiating spermatids (Gliki et al., 2004). Thus, it remains possible that, by analogy, JAM-A might regulate the polarized distribution of PKCζ and/or inactive GSK-3β in our cells (without changing the absolute levels of inactive GSK-3β).

At first sight, the observation that JAM-A expression did not decrease the levels of GSK-3β activation is at odds with the finding that JAM-A expression and GSK-3β inhibitors both inhibited motility. As a plausible explanation for this apparent paradox, we propose that JAM-A controls a regulator of motility, which is also downstream of GSK-3β. Also, the ability of PKCζ-PS to induce motility in JAM-A-expressing cells strengthens the idea that such a putative regulator of motility lies at the crossroads of signals that originate from both JAM-A and the PKCζ/GSK-3β axis. However, given the existence of several mechanisms of microtubule stabilization, we cannot rule out at present that JAM-A and GSK-3β have no other target in common, apart from the microtubules.

We also report that JAM-A expression affected protrusions and focal adhesions in both isolated and cohesive cells (at the wound edge). Interestingly, however, JAM-A expression limited the extent of motility (in isolated cells) but not the efficiency of migration (in cohesive cells). Migration efficiency is dependent on instantaneous velocity and directional persistence (Laufenburger and Horwitz, 1996). Thus, we speculate that intercellular cohesion may enhance either the instantaneous velocity of JAM-A-positive cells or the directional persistence of JAM-A-negative cells, even though real-time measurements are essential to test this hypothesis. Finally, regardless of the cause, the discrepancy between isolated and cohesive cells also suggests that different mechanisms may underlie different types of motility. In this respect, it should be stressed that some of the aforementioned reports analyzed wound edge migration (e.g. Etienne-Manneville and Hall, 2003) and thus the relevant mechanisms (e.g. localization of inactive GSK-3β) may not necessarily apply to our experimental system, which uses isolated cells.

With these caveats, however, our findings point to a novel link between JAM-A expression and cell motility. On the basis of these findings, we propose the following model. JAM-A expression favors microtubule stability, probably in a PDZ-dependent manner. As a consequence, more focal adhesions form. In turn, more adhesions may restrain spontaneous cell motility, possibly by providing stronger anchorage to the matrix. Although JAM-A expression does not directly affect GSK-3β activation, inhibitors of GSK3β mimic the effect of JAM-A expression, thus suggesting the existence of an as-yet-unidentified mediator at a crossroad of signals that originate from both JAM-A/PDZ and PKCζ/GSK3β axis.

It is also noteworthy that the effect of JAM-A on motility was contact independent, as it was detectable in noncontacting cells. In this respect, the inhibitory mechanism reported here is novel and unrelated to the well established mechanism of contact-dependent inhibition of motility (Abercrombie and Heaysman, 1953).

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