Tetraspanin CD9 links junctional adhesion molecule-A to αvβ3 integrin to mediate basic fibroblast growth factor–specific angiogenic signaling

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ABSTRACT Junctional adhesion molecule-A (JAM-A) is a member of the immunoglobulin family with diverse functions in epithelial cells, including cell migration, cell contact maturation, and tight junction formation. In endothelial cells, JAM-A has been implicated in basic fibroblast growth factor (bFGF)-regulated angiogenesis through incompletely understood mechanisms. In this paper, we identify tetraspanin CD9 as novel binding partner for JAM-A in endothelial cells. CD9 acts as scaffold and assembles a ternary JAM-A-CD9-αvβ3 integrin complex from which JAM-A is released upon bFGF stimulation. CD9 interacts predominantly with monomeric JAM-A, which suggests that bFGF induces signaling by triggering JAM-A dimerization. Among the two vitronectin receptors, αvβ3 and αvβ5 integrin, which have been shown to cooperate during angiogenic signaling with bFGF and vascular endothelial growth factor (VEGF), respectively, CD9 links JAM-A specifically to αvβ3 integrin. In line with this, knockdown of CD9 blocks bFGF- but not VEGF-induced ERK1/2 activation. JAM-A or CD9 knockdown impairs endothelial cell migration and tube formation. Our findings indicate that CD9 incorporates monomeric JAM-A into a complex with αvβ3 integrin, which responds to bFGF stimulation by JAM-A release to regulate mitogen-activated protein kinase (MAPK) activation, endothelial cell migration, and angiogenesis. The data also provide new mechanistic insights into the cooperativity between bFGF and αvβ3 integrin during angiogenic signaling.

INTRODUCTION

Junctional adhesion molecule-A (JAM-A) is the founding member of the JAM family of immunoglobulin (Ig)-like proteins (Bazzoni, 2003; Ebnet et al., 2004). Originally identified on the surface of human platelets, JAM-A is expressed by many different cell types, including epithelial and endothelial cells, leukocytes, Sertoli cells and spermatozoa, macroglia cells in the brain, and smooth muscle cells. In epithelial cells, JAM-A regulates various processes, including cell migration, cell proliferation, the maturation of intercellular junctions, and the formation of barrier-forming tight junctions (Liu et al., 2000; Rehder et al., 2006; Severson et al., 2009; Nava et al., 2011; Iden et al., 2012). Some of these functions of JAM-A depend on its ability to associate with cytoplasmic proteins through its C-terminal PDZ domain–binding motif. Among those proteins are the scaffolding proteins ZO-1, MUPP1, PAR-3, and AF-6/afadin (Bazzoni et al., 2000;...
CD9 interacts with JAM-A and αvβ3 integrin in endothelial cells to form a ternary JAM-A–CD9–αvβ3 integrin complex

Integrins and IgSF members are the most common binding partners for tetraspanins (Boucheix and Rubinstein, 2001; Levy and Shoham, 2005a). Because JAM-A had been found in a complex with αvβ3 integrin in endothelial cells (Naik and Naik, 2006), we hypothesized that CD9 might form a link between JAM-A and αvβ3 integrin. Immunoprecipitation experiments from various endothelial cell lines and from primary human umbilical vein endothelial cell (HUVEC) lysates indicated that CD9 and JAM-A exist in the same protein complex (Figure 2A and Supplemental Figure S1). Immunofluorescence analysis indicated that both CD9 and αvβ3 integrin colocalize with JAM-A at cell–cell contacts of cultured endothelial cells (Figure 2B). In addition, whole-mount isoelectin B4 labelings of the retinal vasculature revealed that JAM-A and CD9 are coexpressed at the angiogenic front in areas of sprouting angiogenesis of the mouse retina at postnatal day 6 (Figure 2C), suggesting that the association of CD9 and JAM-A is relevant for angiogenic processes in vivo. We next analyzed the presence of αvβ3 integrin in the JAM-A–CD9 complex, and we found that αvβ3 integrin is associated with both JAM-A and CD9 (Figure 2D). The interaction of CD9 with both JAM-A and αvβ3 integrin suggested the existence of a ternary JAM-A–CD9–αvβ3 integrin complex but did not rule out the possibility of independent binary interactions among the three proteins. To address this question, we analyzed the association between JAM-A and CD9.
Figure 1: JAM-A interacts with CD9. (A) JAM-A interacts with CD9 by way of a primary tetraranspin interaction. Top and middle panels, HeLa cells were lysed in either Triton X-100- or Brij79-containing lysis buffer as indicated. JAM-A immunoprecipitates were immunoblotted for CD9 (90% of input) and for JAM-A (10% of input). Bottom panel, Immunoprecipitation was performed in the reverse order: CD9 was immunoprecipitated, and immunoprecipitates were immunoblotted for JAM-A (90% of input) or CD9 (10% of input). Note that JAM-A efficiently interacts with CD9 under both lysis conditions. (B) JAM-A strongly interacts with CD9 in HEK293T cells. CD9 immunoprecipitates obtained from Flag-JAM-A-transfected HEK293T cells were immunoblotted with anti-Flag antibodies (top, 90% of input) or anti-CD9 antibodies (middle, 10% of input). Postnuclear supernatants (PNS) were immunoblotted with anti-Flag antibodies (bottom, 2.5% of total lysate). The asterisk denotes signals resulting from IgG heavy chains. (C) CD9 interacts with the cytoplasmic tail of JAM-A. GST precipitates obtained from HEK293T cells with GST fusion proteins containing the full-length cytoplasmic tail of JAM-A (GST-JAM-A/f.l.) or deletion mutants lacking 3 or 9 C-terminal amino acid residues (GST-JAM-A/Δ3, GST-JAM-A/Δ9) were immunoblotted for CD9 (top panel). Equal loading of GST fusion proteins was verified by immunoblotting aliquots with anti-GST antibodies (bottom panel). Arrowheads indicate GST-JAM-A/Δ3 constructs resulting from proteolytic cleavage. (D) The interaction between JAM-A and CD9 requires the PDZ domain-binding motif of JAM-A. CD9 immunoprecipitates obtained from HEK293T cells transfected with full-length JAM-A (Flag-JAM-A), C-terminal truncation mutants (Flag-JAM-A/Δ3, -Δ6, -Δ9), or triple alanine substitutions (Flag-JAM-A/Δ3A1, Flag-JAM-A/Δ3A2) were immunoblotted with anti-Flag antibodies (top, 90% of input), or with anti-CD9 antibodies (middle, 10% of input). Expression levels of Flag constructs were analyzed by immunoblotting the PNS with anti-Flag antibodies (bottom, 2.5% of total lysate). In addition, all Flag constructs localize to the cell surface as analyzed by flow cytometry (Supplemental Figure S5). Experiments shown in this figure are representative of at least three independent experiments. IP, immunoprecipitation; IB, immunoblotting.
JAM-A could be actively involved in MAPK activation after its dissociation from the complex, possibly by assembling a cytoplasmic signaling complex (Severson et al., 2009). Studies in epithelial cells indicate that many functions of JAM-A, including its role in tight junction barrier formation and β1 integrin–mediated cell adhesion and migration, depend on JAM-A dimerization (Mandell et al., 2004; Rehder et al., 2006; Severson et al., 2008). To test whether the ternary JAM-A–CD9–αvβ3 complex contains monomeric or dimeric JAM-A, we analyzed the association of two dimerization-deficient JAM-A mutants (ΔV-JAM-A, which lacks the V-type Ig domain, and JAM-A/ΔE60RK62E, which contains mutations at two residues in the V-type Ig domain that mediate cis-dimerization [Prota et al., 2003]) with CD9. Both mutants interacted much more strongly with CD9 than with wild-type JAM-A (Figure 4), indicating that the ternary JAM-A–CD9–αvβ3 complex contains predominantly monomeric JAM-A.

CD9 links JAM-A to αvβ3 integrin to assemble a protein complex that specifically mediates bFGF-induced MAPK activation

To test whether the JAM-A–CD9–αvβ3 integrin complex is required for bFGF to stimulate MAPK signaling, we analyzed bFGF-induced ERK1/2 activation in the absence of CD9. To distinguish between contributions of several integrins from those mediated by the two vitronectin receptors αvβ3 and αvβ5 integrin, we grew cells either on plastic or on vitronectin. In control cells, bFGF induced a strong ERK1/2 phosphorylation irrespective of whether cells were grown on plastic or on vitronectin. However, when grown on vitronectin, CD9 knockdown cells failed to respond to bFGF (Figure 5A). CD9 knockdown cells showed a similarly strong bFGF response when grown on plastic. However, when grown on vitronectin, CD9 knockdown cells failed to respond to bFGF (Figure 5A). These observations indicate that CD9 is required for bFGF-induced ERK1/2 activation specifically when cells are costimulated by the two vitronectin receptors αvβ3 and αvβ5 integrin.

Because previous observations indicated a functional and selective cooperation between bFGF and αvβ3 integrin and between vascular endothelial growth factor (VEGF) and αvβ3 integrin during angiogenesis (Friedlander et al., 1995; Hood et al., 2004; Yan et al., 2008), we next addressed the question of whether CD9 is specific for the bFGF–αvβ3 integrin pathway of angiogenesis. For testing this, CD9 knockdown cells were plated either on plastic or on vitronectin and stimulated with either bFGF or VEGF. Control cells responded to both bFGF and VEGF with increased ERK1/2 phosphorylation (Figure 5B, top panel) under both growth

CD9 recruits predominantly monomeric JAM-A into the ternary complex

The bFGF-triggered release of JAM-A opened up the possibility that JAM-A linked to αvβ3 integrin serves to inhibit αvβ3 integrin–mediated MAPK activation under steady-state conditions that would be abrogated after bFGF-induced release of JAM-A. Alternatively, 2003; Yan et al., 2008), we next addressed the question of whether CD9 is specific for the bFGF–αvβ3 integrin pathway of angiogenesis. For testing this, CD9 knockdown cells were plated either on plastic or on vitronectin and stimulated with either bFGF or VEGF. Control cells responded to both bFGF and VEGF with increased ERK1/2 phosphorylation (Figure 5B, top panel) under both growth
conditions. CD9 knockdown cells showed a strong response to both bFGF and VEGF when grown on plastic. As observed before (Figure 5A), CD9 knockdown cells failed to respond to bFGF when grown on vitronectin. However, their ability to respond to VEGF was unchanged (Figure 5B). These observations indicate that CD9 selectively participates in the bFGF–αvβ3 integrin–regulated pathway of MAPK activation. We then performed the same experiment in JAM-A knockdown cells. Control small interfering RNA (siRNA)- and JAM-A siRNA-treated cells grown on either plastic or vitronectin were stimulated with bFGF or VEGF (Figure 5C). Control siRNA-treated cells responded to both bFGF and VEGF with increased ERK1/2 phosphorylation irrespective of whether they were grown on plastic or on vitronectin (Figure 5C, top panel). JAM-A knockdown cells were able to respond to both growth factors with increased ERK1/2 phosphorylation when grown on plastic (Figure 5C, bottom panel). However, as observed for CD9 knockdown cells, when grown on vitronectin, JAM-A knockdown cells failed to respond to bFGF, whereas they responded normally to VEGF. Finally, knockdown of CD9 or JAM-A did not affect the ability of endothelial cells to respond to bFGF when grown on fibronectin or collagen (Supplemental Figure S4). These observations indicate that both CD9 and JAM-A are selectively involved in the bFGF–αvβ3 integrin pathway of angiogenesis (Friedlander et al., 1995; Hood et al., 2003).

Down-regulation of CD9 or JAM-A impairs endothelial cell migration and tube formation

To address the relevance of the JAM-A–CD9–αvβ3 complex during processes linked to angiogenesis, we analyzed the ability of endothelial cells to migrate and to form tube-like structures in a three-dimensional matrix. Knockdown of either JAM-A or CD9 significantly reduced the ability of endothelial cells to migrate through a Matrigel-coated filter in response to bFGF (Figure 6A). In addition, knockdown of either

CoIPs; y-axis: relative signal intensity. Densitometric values obtained from unstimulated cells (no RGDS) were arbitrarily set as 1. Error bars denote the mean ± SE from four separate experiments. Statistical significance was evaluated using one-sample t tests; *, p < 0.05. (B) bFGF dissociates JAM-A from the ternary complex. HUVECs were stimulated with bFGF (10 ng/ml, 10 min). After lysis, JAM-A IPs were analyzed for the presence of CD9 (top, left panel), CD9 IPs were analyzed for the presence of β3 integrin (top, right panel), and β3 integrin IPs were analyzed for JAM-A (bottom, left panel). In all cases, equal and specific IP was verified by immunoblotting 10% of the precipitated material with antibodies against the precipitated protein. The asterisks denote unspecific bands derived from Ig light chains. Bottom, right panel, densitometric analysis of the binary interactions; y-axis: relative signal intensity. Densitometric values obtained from unstimulated cells (no bFGF) were arbitrarily set as 1. Error bars denote the mean ± SE from three separate experiments. Statistical significance was evaluated using one-sample t tests; *, p < 0.05.

FIGURE 3: Ternary complex formation is dependent on integrin activation and negatively regulated by bFGF. (A) Integrin activation promotes ternary complex formation. HUVECs were stimulated with RGDS peptide (100 μg/ml, 20 min). After lysis, JAM-A IPs were analyzed for the presence of CD9 (top, left panel), CD9 IPs were analyzed for the presence of β3 integrin (top, right panel), and β3 integrin IPs were analyzed for JAM-A (bottom, left panel). In all cases, equal and specific IP was verified by immunoblotting 10% of the precipitated material with antibodies against the precipitated protein. The asterisks denote unspecific bands derived from Ig light chains. Bottom, right panel, densitometric analysis of the binary interactions; y-axis: relative signal intensity. Densitometric values obtained from unstimulated cells (no RGDS) were arbitrarily set as 1. Error bars denote the mean ± SE from four separate experiments. Statistical significance was evaluated using one-sample t tests; *, p < 0.05. (B) bFGF dissociates JAM-A from the ternary complex. HUVECs were stimulated with bFGF (10 ng/ml, 10 min). After lysis, JAM-A IPs were analyzed for CD9 (top, left panel), CD9 IPs were analyzed for the presence of β3 integrin (top, right panel), and β3 integrin IPs were analyzed for JAM-A (bottom, left panel). In all cases, equal and specific IP was verified by immunoblotting 10% of the precipitated material with antibodies against the precipitated protein. The asterisks denote unspecific bands derived from Ig light chains. Bottom, right panel, densitometric analysis of the binary interactions; y-axis: relative signal intensity. Densitometric values obtained from unstimulated cells (no bFGF) were arbitrarily set as 1. Error bars denote the mean ± SE from three separate experiments. Statistical significance was evaluated using one-sample t tests; *, p < 0.05.
JAM-A or CD9 significantly impaired the ability of endothelial cells to develop a branched network of tube-like structures when incubated for 24 h in the presence of bFGF in Matrigel (Figure 6B). Finally, knockdown of CD9 resulted in reduced endothelial cell proliferation (Supplemental Figure S3). These observations indicate that both JAM-A and CD9 regulate angiogenesis-related processes such as endothelial cell migration and tube formation. This function of JAM-A and CD9 is most likely due to their ability to associate with αvβ3 integrin in a ternary protein complex that triggers the membrane-proximal signaling events in response to bFGF.

**DISCUSSION**

In this study, we identify CD9 as a novel binding partner for JAM-A in endothelial cells. In addition, we identify αvβ3 integrin as novel binding partner for CD9. CD9 acts as a scaffolding protein that assembles a ternary JAM-A–CD9–αvβ3 integrin complex (Figure 7). This complex contains predominantly monomeric JAM-A, which is released upon bFGF stimulation. Our observations identify CD9 as a ternary JAM-A–CD9–αvβ3 integrin complex that, however, is inactive in the absence of an angiogenic stimulus. On stimulation with bFGF, monomeric JAM-A is released from CD9 and αvβ3 integrin, dimerizes, and adopts its signaling activity.

The selective incorporation of monomeric versus dimeric JAM molecules into specific protein complexes seems to emerge as a common mechanism by which the activity of JAM molecules is regulated. The JAM family–related protein JAM-like (JAM-L) is expressed by different leukocyte subsets and serves to mediate leukocyte endothelial/epithelial cell interactions by interacting with coxsackievirus and adenovirus receptor (CAR) in a trans-heterophilic manner (Moog-Lutz et al., 2003; Zen et al., 2005; Lussient et al., 2008). In resting monocytes and T-cells, JAM-L associates in cis with α4β1 integrin, and requires the C-terminal PDZ domain-containing tetraspanin CD9 for its activity (Lussient et al., 2008). As one example, JAM-A dimerization regulates the close apposition of the two Rap1 regulatory proteins AF-6/afadin and PDZ-GEF2, which both interact with JAM-A (Ebneth et al., 2000; Severson et al., 2009). We therefore speculate that the association of monomeric JAM-A with CD9 serves to assemble a signaling-competent JAM-A–CD9–αvβ3 integrin complex that, however, is inactive in the absence of an angiogenic stimulus. On stimulation with bFGF, monomeric JAM-A is released from CD9 and αvβ3 integrin, dimerizes, and adopts its signaling activity.

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has been identified in a complex with αvβ3 integrin but not αvβ5 integrin in response to stimulation with bFGF and fibrinogen (Sahni and Francis, 2004), which opens up the possibility that JAM-A becomes physically associated with FGFR1 after bFGF stimulation within a JAM-A–CD9–αvβ3 integrin–FGFR1-containing microdomain. As a result of this close proximity, JAM-A could be phosphorylated by FGFR1. Indirect evidence suggests that Tyr-280 phosphorylation of JAM-A is involved in MAPK activation by JAM-A (Naik et al., 2003), which further supports the requirement of a posttranslational modification of JAM-A for its signaling activity.

Another key finding of this study is the specificity of CD9 and JAM-A for the bFGF/αvβ3–mediated angiogenic signaling pathway. Two distinct cytokine-dependent pathways of angiogenesis have been defined based on the involvement of two distinct α integrins: angiogenesis induced by bFGF or TNF-α is cooperatively regulated by integrin αvβ3, whereas angiogenesis induced by VEGF or TGF-α is cooperatively regulated by integrin αvβ5 (Friedlander et al., 1995). Both pathways result in ERK1/2 activation but differ in the signaling components upstream of Raf activation: the bFGF–αvβ3 pathway activates c-Abl and p21-activated kinase-1, resulting in Raf phosphorylation at Ser-338; the VEGF–αvβ5 pathway depends on PKC and Src kinase and results in Raf phosphorylation at Tyr-340 (Eliceiri et al., 2002; Hood et al., 2003; Yan et al., 2008). Our results identify JAM-A and CD9 as upstream components of the bFGF–αvβ3 pathway. The ability of CD9 to connect JAM-A selectively to αvβ3 integrin but not to αvβ5 integrin may thus be a critical factor contributing to the specificity in the cooperativity of bFGF with αvβ3 integrin.

We propose the following molecular mechanism is involved in the membrane-proximal signaling events regulating the

FIGURE 5: Both CD9 and JAM-A specifically cooperate with bFGF in angiogenic signaling. (A) CD9 is required for ERK1/2 phosphorylation in cells grown on vitronectin. CD9 siRNA-treated HUVECs grown either on plastic or on vitronectin were stimulated with bFGF (10 ng/ml, 20 min) as indicated. Cell lysates were analyzed for total ERK1/2 and phosphorylated ERK1/2. Note that the absence of CD9 blocks bFGF-induced Erk1/2 phosphorylation only when cells are grown on vitronectin. (B) CD9 mediates bFGF- but not VEGF-induced ERK1/2 phosphorylation. CD9 siRNA-treated HUVECs were grown on plastic or on vitronectin and stimulated with bFGF (10 ng/ml, 10 min) or VEGF (20 ng/ml, 10 min) as indicated. Cell lysates were analyzed for total ERK1/2 and phosphorylated ERK1/2. Top, right panel, quantification of ERK1/2 phosphorylation; y-axis: relative signal intensity. Bars represent ERK1/2 phosphorylation in CD9 siRNA-transfected cells relative to ERK1/2 phosphorylation in control siRNA-transfected cells. Bottom, right panel, quantification of ERK1/2 phosphorylation; y-axis: relative signal intensity. Bars represent ERK1/2 phosphorylation in CD9 siRNA-transfected cells relative to ERK1/2 phosphorylation in control siRNA-transfected cells. Phosphorylation levels were quantified as detailed in the Materials and Methods section. Error bars denote the mean ± SE from three independent experiments. Statistical significance was evaluated using one-sample t tests; **, p < 0.01. (C) JAM-A mediates bFGF- but not VEGF-induced ERK1/2 phosphorylation. JAM-A siRNA-transfected HUVECs were grown on plastic or on vitronectin and stimulated with bFGF (10 ng/ml, 10 min) or VEGF (20 ng/ml, 10 min) as indicated. Cell lysates were analyzed for total ERK1/2 and phosphorylated ERK1/2. Top, right panel, quantification of ERK1/2 phosphorylation performed as described in (B). Error bars denote the mean ± SE from three independent experiments. Statistical significance was evaluated using one-sample t tests; *, p < 0.05.
bFGF/αvβ3–dependent activation of the ERK1/2 pathway (Figure 7). JAM-A, CD9, and αvβ3 integrin are associated in a ternary protein complex at cell–cell contacts of endothelial cells, whose formation is enhanced by integrin engagement. In this complex, CD9 serves to link monomeric JAM-A to αvβ3 integrin. A signal mediated by bFGF releases monomeric JAM-A from the complex through an unknown mechanism. Liberated JAM-A monomers probably dimerize to form a signaling-active complex, perhaps by bringing molecules associated with the cytoplasmic tail of JAM-A into close proximity. The next important step to further understand the molecular mechanism downstream of JAM-A dimerization will be the identification of these JAM-A–associated molecules.

MATERIALS AND METHODS

Cell culture and transfections

HEK293T and HeLa cells were maintained in DMEM (Life Technologies, Darmstadt, Germany) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine (Lonza, Basel, Switzerland), and 100 U/ml penicillin/streptomycin (Lonza, Basel). MyEnd cells were cultured in the same medium supplemented with 1 mM Na-pyruvate (Biochrom, Berlin, Germany). Human brain microvascular endothelial cells (kindly provided by the Institute of Infectiology, ZMBE, Münster, Germany) were grown in RPMI supplemented with 10% FCS, 10% NuSerum (BD Biosciences, Heidelberg, Germany), 2 mM glutamine, and 100 U/ml penicillin/streptomycin, 1:100 diluted MEM vitamins (PAA, Cölbe, Germany), 1:100 nonessential amino acids (Biochrom), and 1 mM Na-pyruvate. HUVECs were isolated from umbilical veins by dispase treatment and were maintained in EGM medium (Clonetics, Heidelberg, Germany). Transient transfections of plasmids in HEK293T cells were performed using GeneJammer transfection reagent (Stratagene, Amsterdam, Netherlands). Transfections of siRNA oligonucleotides in HUVECs were performed using the Amaxa HUVEC Nucleofector Kit (Lonza, Cologne, Germany) according to the manufacturer’s instructions.

Antibodies and reagents

The following antibodies were used: mouse monoclonal antibody (mAb) anti-CD9 (Millipore, Billerica, MA), rat anti-CD9 mAb KMC8 (eBioscience, Frankfurt, Germany), mouse mAb anti–JAM-A (BD Biosciences), rat anti–JAM-A mAb 106 (Malergue et al., 1998), rabbit polyclonal antibody (pAb) anti-GST (Santa Cruz Biotechnology, Heidelberg, Germany), mouse mAb anti–β3 integrin (BD Biosciences), mouse mAb anti–human integrin αvβ3 (Millipore), mouse mAb anti–α-tubulin (Sigma-Aldrich, Munich, Germany), rabbit mAb anti-ERK1/2 and rabbit mAb anti–Thr-202/Tyr-204–phosphorylated ERK1/2 (Cell Signaling Technology,
DNA constructs, site-directed mutagenesis, and recombinant protein expression

For transient expression of Flag-tagged JAM-A constructs, the human JAM-A cDNA lacking the leader peptide sequence (Flag-hJAM-A, aa 26–299), C-terminal deletion constructs lacking either three or six or nine C-terminal amino acids (Flag-hJAM-A/Δ3, aa 26–296; Flag-hJAM-A/Δ6, aa 26–293; Flag-hJAM-A/Δ9, aa 26–290), and a human JAM-A construct lacking the membrane-distal, V-type Ig domain (Flag-JAM-A/ΔV) were cloned into the pFlag-CMV-1 vector (Sigma-Aldrich). The two hJAM-A mutants with sets of three amino acids at the C-terminus exchanged with alanines (Flag-JAM-A/3A1, F292Q293K294→A292A293A294; Flag-JAM-A/3A2, T295S296S297→A295A296A297), as well as the dimerization mutant with point mutations within the dimerization interface (Flag-JAM-A/E61R/K63E), were generated by a PCR-based approach using mismatch primer pairs with wild-type Flag-hJAM-A as a template. The mouse JAM-A cDNA cloned into pFLAG-CMV-1 has been described before (Ebnet et al., 2001). For recombinant protein expression in E. coli, the pGEX-4T-1 vector containing the entire cytoplasmic tail of murine JAM-A (aa 261–300) or C-terminal deletion mutants (GST-JAM-A/Δ3, aa 261–297; GST-JAM-A/Δ9, aa 261–291) was used as described before (Ebnet et al., 2000). Recombinant proteins were purified from E. coli BL21 as has been described before (Ebnet et al., 2000).

RNA interference

For depleting JAM-A and CD9 in HUVECs, the following siRNA heteroduplexes were used: 5′-GGACGUACUGAAACUUUCUTT-3′ (CD9), 5′-GAAGUGAGGGGGAAUUCAATT-3′ (JAM-A). For depleting β3 integrin, a pool of four different siRNA oligonucleotides (On-TARGETplus Smart Pool; Thermo Fisher Scientific, Schwerte, Germany) was used. As control siRNA, a nontargeting siRNA (On-TARGETplus Non-targeting siRNA; Thermo Fisher Scientific) was used. HUVECs (2 × 10⁴) were transfected with 200 pmol of siRNAs by electroporation using the Amaza HUVEC Nucleofector Kit (Lonza, Cologne, Germany) according to the manufacturer’s instructions. After 48 h, the cells were harvested and analyzed.
HCl, pH 7.4, 1% [vol/vol] Brij97, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, protease inhibitors [Protease Inhibitor Cocktail tablets; Roche Diagnostics]) for 30 min on ice and then centrifuged at 4°C. The supernatants were preclarified by incubation with 15 µl of protein A or protein G Sepharose beads (GE Healthcare), followed by centrifugation. Postnuclear supernatants were incubated with 3 µg of antibodies coupled to protein A or protein G Sepharose beads overnight at 4°C. Immune complex–captured beads were washed five times with lysis buffer without inhibitors and boiled in SDS sample buffer containing 2.5% β-mercaptoethanol. The proteins were separated by SDS–PAGE and analyzed by Western blotting. Under these conditions, the anti-CD9 mAbs used in the study showed a specific signal that was abolished after CD9 knockdown. All CoIP experiments from endothelial cells were performed using Triton X-100 lysis buffer. The results of the CoIP experiments are representative for at least three independent experiments. Quantification of signal intensities was performed using the Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). For each band, the integrated intensity (in kilocounts) was calculated with Odyssey application software (version 3.0). Mean values and SEs were calculated from three independent experiments. Statistical significance was evaluated using one-sample t tests. p values below 0.05 were considered significant.

**Analysis of ERK1/2 phosphorylation**

HUVECs were transfected with JAM-A–specific or CD9-specific siRNAs and incubated for 48 h on regular or vitronectin-coated tissue culture plates. For 14 h prior to stimulation with growth factors, the cells were grown in medium containing 1% BSA instead of FCS (serum starvation). The serum-starved cells were stimulated with either 10 ng/ml bFGF for 10 min or with 20 ng/ml VEGF for 10 min, then lysed with hot SDS sample buffer. Cell lysates were separated by 12% SDS–PAGE, transferred to nitrocellulose membranes, and probed with antibodies against total ERK1/2 or Thr202/Tyr204–phosphorylated ERK1/2. The results of the ERK1/2 phosphorylation experiments are representative for at least three independent experiments. Quantification of signal intensities was performed using the Odyssey imaging system, as described above. Phosphorylation signals were corrected for differences in total ERK1/2 levels. Values obtained from unstimulated cells (baseline phosphorylation) were subtracted from the values obtained from bFGF- or VEGF-stimulated cells, resulting in normalized phosphorylation levels. Bars in Figure 5, B and C, show the increase or decrease in ERK1/2 phosphorylation levels in CD9 (Figure 5B) or JAM-A (Figure 5C) knockdown cells relative to the levels in wild-type cells, which were arbitrarily set as 1.

**Immunofluorescence microscopy**

Immunofluorescence analyses were performed with HUVECs grown on vitronectin-coated Lab-Tek Chamber Slides (ThermoFisher Scientific, Waltham, MA). Cells were fixed in ice-cold EtOH for 30 min and acetone for 3 min at room temperature (RT); this was followed by rehydration in blocking buffer (phosphate-buffered saline [PBS]/10% FCS). After 1 h of blocking, cells were incubated with primary antibodies in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.01% Tween-20, 0.1% BSA for 1 h at RT or overnight at 4°C. After being washed, cells were incubated with Alexa Fluor 488– or Alexa Fluor 568–conjugated, highly cross-adsorbed secondary antibodies for 1 h at RT. After washing, cells were mounted in fluorescence mounting medium (Dako, Hamburg, Germany) and stored at 4°C. Immunofluorescence microscopy was performed using a confocal microscope (Zeiss LSM 510 Meta, Jena, Germany) equipped with Zeiss Plan-Apochromat lenses (Zeiss Plan-Apochromat DIC, oil, 63× magnification, 1.4 numerical aperture).

**Stainings of mouse retina vasculature**

Retina stainings were performed essentially as previously described (Wang et al., 2010). Briefly, eyes were dissected from neonatal mice (postnatal day 6) and fixed in 4% PFA for 2 h on ice. Retinas were permeabilized and blocked in 1% BSA (#A4378; Sigma-Aldrich) and 0.3% Triton X-100 for 2 h at room temperature with gentle rocking. Next retinas were washed three times in Phosphate buffered saline (1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, and 1% Triton X-100 in PBS) and incubated with biotinylated isocitrate B4 (#B-1205, Griffonia simplicifolia lectin 1, 1:50; Vector Labs, Burlingame, CA), and with antibodies against JAM-A (mAb 106) and CD9 (mAb KMC9) overnight at 4°C with gentle rocking. Retinas were washed five times with 0.5% BSA and 0.15% Triton X-100 and incubated with Alexa Fluor–conjugated streptavidin (1:100; Invitrogen) and with the corresponding Alexa Fluor–conjugated secondary antibody (1:500; Invitrogen) in blocking buffer for 2 h at room temperature. Retinas were flat-mounted using Fluoromount-G (#0100-01; SouthernBiotech, Birmingham, AL).

**Cell proliferation**

Cell proliferation was analyzed using a commercially available cell proliferation kit (Cell Proliferation Kit II [XTT]; Roche Diagnostics, Mannheim, Germany). Briefly, cells were seeded on vitronectin-coated 96-well plates at a density of 5 × 10³ cells per well. After 24 h, XTT reagent was added to each well. At 4 and 24 h after XTT addition, the absorption at 475 nm was measured using an enzyme-linked immunosorbent assay reader. The quantification is based on three independent experiments with quintuplicate samples in each experiment. Statistical significance was analyzed using one-sample t test.

**Flow cytometry**

Cell surface expression of transfected JAM-A constructs was analyzed by flow cytometry. After harvest, cells were resuspended in FACS buffer (PBS/3% FCS) at 2 × 10⁶ cells/ml, incubated with primary antibodies (5 µg/ml) for 1 h at 4°C in FACS buffer, washed, and incubated with Cy2-conjugated secondary antibodies (5 µg/ml, 1 h, 4°C). After three washing steps, cells were analyzed by flow cytometry with excitation at 488 nm (BD FACS Calibur; BD Biosciences). For each sample, 10,000 cells were counted.

**Cell invasion**

Twenty-five thousand endothelial cells harvested 48 h after siRNA transfection were resuspended in 0.5 ml serum-free medium and added to the upper compartment of a BioCoat Matrigel Invasion Chamber (BD Biosciences). After overnight incubation, the medium in the lower compartment was supplemented with 50 ng/ml bFGF, and the cells were allowed to invade the lower compartment. After 16 h, the cells present at the bottom surface of the filters were fixed and stained with Diff-Quik dye (Dade Behring, Duedingen, Switzerland). Filter membranes were excised, mounted, and photographed using a Zeiss Axiovert microscope equipped with Axiosvision software (Zeiss) at 100× magnification. For each membrane, cells in five visual fields were counted. For each condition, triplicates were analyzed. Statistical significance was evaluated using one-way analysis of variance (ANOVA) with Dunnett's post hoc test. Mean values and SDs were calculated from three independent experiments.

**In vitro tube formation assay**

In vitro tube formation assays were performed according to the protocol described by Aniautova and Kleinman (2010). Briefly, Cultrex basement membrane extract (BME) with reduced growth factors (Trevigen, Gaithersburg, MD) was thawed overnight at 4°C. After
thawing, individual wells of a flat-bottom 96-well microtitre plate were coated with 50 μl of BME at 37°C and 5% CO2 for 1 h. siRNA-treated HUVECs were serum-starved overnight, resuspended at 1.5 × 10^5 cells/ml in basal medium (EBM 2; Lonza, Cologne, Germany), reconstituted with 40 ng/ml bFGF, and seeded into the BME-coated 96-well plates (100 μl/well). Phase-contrast microscopy images were taken immediately after seeding to control for the seeding density. After 24 h, images were taken again, and the total length of the tube network was analyzed using Image J software (http://rsbweb.nih.gov/ij/). Each condition was performed in triplicate. Statistical analysis was performed using repeated-measures ANOVA with Dunnett’s post hoc test. Mean values and SDs were calculated from three independent experiments.

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