

Tension on JAM-A activates RhoA via GEF-H1 and p115 RhoGEF

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ABSTRACT Junctional adhesion molecule A (JAM-A) is a broadly expressed adhesion molecule that regulates cell–cell contacts and facilitates leukocyte transendothelial migration. The latter occurs through interactions with the integrin LFA-1. Although we understand much about JAM-A, little is known regarding the protein’s role in mechanotransduction or as a modulator of RhoA signaling. We found that tension imposed on JAM-A activates RhoA, which leads to increased cell stiffness. Activation of RhoA in this system depends on PI3K-mediated activation of GEF-H1 and p115 RhoGEF. These two GEFs are further regulated by FAK/ERK and Src family kinases, respectively. Finally, we show that phosphorylation of JAM-A at Ser-284 is required for RhoA activation in response to tension. These data demonstrate a direct role of JAM-A in mechanosignaling and control of RhoA and implicate Src family kinases in the regulation of p115 RhoGEF.

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INTRODUCTION

Adhesion molecules on the surface of endothelial cells serve as ligands for circulating leukocytes. Interactions between these adhesion molecules and their corresponding receptors facilitate transendothelial migration of the leukocytes to regions of inflammation. As the leukocyte crawls atop the endothelial cell, mechanical forces are imposed on the endothelial cell, resulting in activation of the small GTPase RhoA and an increase in cell stiffness (Liu *et al.*, 2010; Stroka and Aranda-Espinoza, 2011; Heemskerk *et al.*, 2014; Lessey-Morillon *et al.*, 2014; Schaefer and Hordijk, 2015). In a similar manner, homodimerization of adhesion molecules at cell–cell contacts regulates zone-specific contractility through regulation of RhoA (Nelson *et al.*, 2004; Bazellieres *et al.*, 2015; Priya *et al.*, 2015).

RhoA, like other small GTPases, cycles between a GTP-bound, active state and a GDP-bound, inactive state. Addition of GTP is regulated by guanine nucleotide exchange factors (GEFs), whereas hydrolysis of GTP to GDP, which inactivates the protein, is promoted by GTPase-activating proteins (GAPs; Marjoram *et al.*, 2014). When activated, RhoA promotes actomyosin-based contractility, thus regulating cytoskeletal organization (Chrzanowska-Wodnicka and Burridge, 1996). Recent work has demonstrated that mechanical force, in the form of tension, imposed on individual adhesion molecules is sufficient to activate RhoA (Zhao *et al.*, 2007; Guilluy *et al.*, 2011b; Collins *et al.*, 2012; Lessey-Morillon *et al.*, 2014; Schaefer *et al.*, 2014; Barry *et al.*, 2015; Bazellieres *et al.*, 2015). Of interest, the kinetics of RhoA activation and its associated GEFs is unique for individual adhesion molecules, implying pathway specificity and a spatiotemporal response.

Junctional adhesion molecule A (JAM-A) belongs to the immunoglobulin (Ig) superfamily of adhesion molecules. Originally described as a platelet receptor (Naik *et al.*, 1995), the protein is also expressed on endothelial and epithelial cells, as well as in most leukocyte subsets (Martin-Padura *et al.*, 1998). JAM-A participates in a number of cellular functions, including formation and maintenance of cell–cell contacts (Martin-Padura *et al.*, 1998; Aurrand-Lions *et al.*, 2001b), is a reovirus receptor (Barton *et al.*, 2001; Campbell *et al.*, 2005), and is a ligand for the leukocyte-expressed LFA-1 integrin dimer (Ostermann *et al.*, 2002). At cell–cell contacts, JAM-A forms *cis*- and *trans*-homodimers (Severson *et al.*, 2008; Monteiro *et al.*, 2014),

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Abbreviations used: FAK, focal adhesion kinase; GEF, guanine nucleotide exchange factor; HUVEC, human umbilical vein endothelial cell; JAM-A, junctional adhesion molecule-A; PI3K, phosphoinositide 3-kinase; ROCK, Rho-associated protein kinase/Rho-associated, coiled-coil-containing protein kinase; SFK, Src family kinase.

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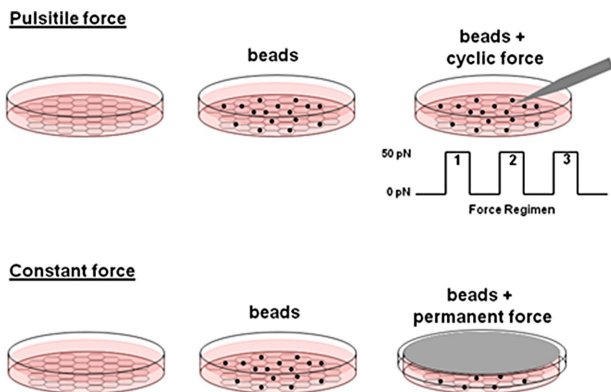


FIGURE 1: Overview of tension models. Cells were grown on fibronectin-coated substratum, and anti-JAM-A-coated paramagnetic beads were added at approximately a 3:1 bead-to-cell ratio. For application of pulsatile forces, the pole tip of a magnetic tweezers was lowered to 25 μm above the bead and force applied using a defined regimen. Bead displacement was imaged at 30 frames/s, and bead movement was tracked using custom software as described in *Materials and Methods*. For application of continuous force, a magnet was suspended parallel to the apical surface of the cells for the determined time. Cells were then lysed and processed for biochemical analysis as needed.

which have been implicated in supporting tension between cells (Bazellieres *et al.*, 2015; Tornavaca *et al.*, 2015). These signaling events require the protein's short C-terminus, which contains a PDZ-binding domain and at least two phosphorylation sites (Severson and Parkos, 2009; Iden *et al.*, 2012; Naik *et al.*, 2014). Through its

interactions with LFA-1, JAM-A is recognized as a critical regulator of leukocyte transendothelial migration, and mice lacking endothelial expression of JAM-A display impaired immune responses (Woodfin *et al.*, 2009; Lakshmi *et al.*, 2012; Schmitt *et al.*, 2014). It is unknown whether tension on JAM-A, such as that found at cell-cell contacts or imposed by a crawling leukocyte, can be mechanically transmitted to support RhoA activation.

The work described here shows that tension on JAM-A activates RhoA, which results in increased cell stiffness. Activation of RhoA is mediated by GEF-H1 and p115 RhoGEF in a phosphoinositide 3-kinase (PI3K)-dependent manner. Tension on JAM-A activates focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) to control GEF-H1, whereas p115 RhoGEF is regulated by Src family kinases (SFKs). Finally, activation of RhoA in response to tension on JAM-A requires phosphorylation of S284 on the protein's C-terminus. These data demonstrate that JAM-A supports tension-induced outside-in signaling to control RhoA and increase cellular stiffness.

RESULTS

Tension imposed on JAM-A activates RhoA via PI3K

Tension imposed on cell surface receptors regulates cellular stiffness through activation of RhoA (Matthews *et al.*, 2006; Guilluy *et al.*, 2011b; Collins *et al.*, 2012; Lessey-Morillon *et al.*, 2014). To determine whether JAM-A could support similar signaling responses, we used models of continuous and pulsatile force as outlined in Figure 1. Paramagnetic beads coated with a monoclonal antibody that recognizes the first Ig-like domain of JAM-A (Mandell *et al.*, 2004) were added to cells, and pulsatile forces were applied using magnetic tweezers. Cell stiffening was determined by measuring

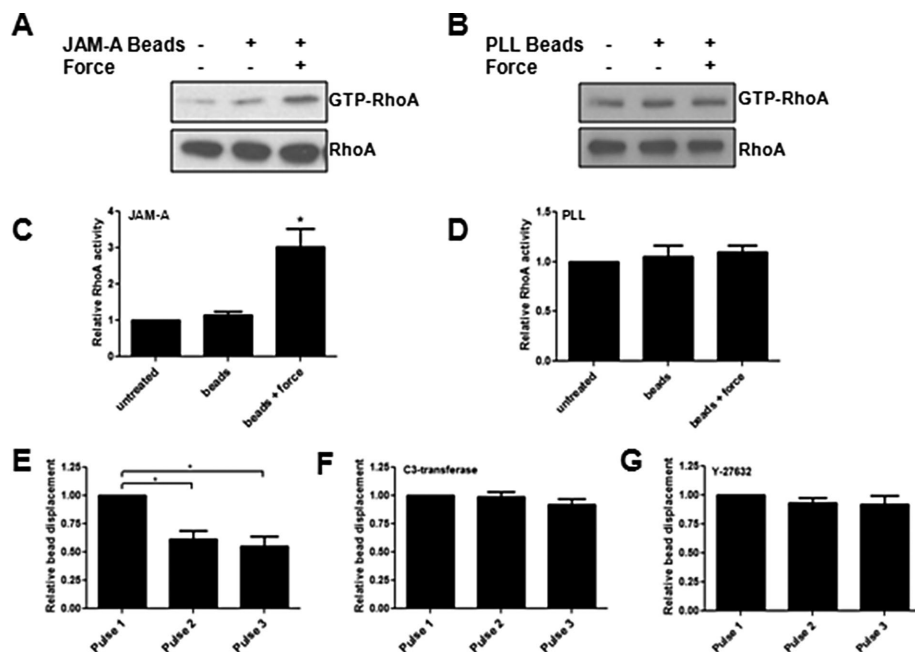


FIGURE 2: Tension on JAM-A activates RhoA to increase cell stiffness. RhoA activity was measured using RBD-pull-down assays on untreated HUVECs, HUVECs treated with anti-JAM-A-coated beads, HUVECs with the same beads plus 3 min of continuous force (A), or the same regimen with PLL-coated beads (B). Data are representative of at least three separate experiments and are quantified as means \pm SEM in C and D. * $p < 0.01$ vs. untreated as determined by *t* test. To determine cell stiffness, HUVECs were untreated (E) or treated with 1 mg/ml C3 transferase for 60 min (F) or 10 μM Y-27632 for 30 min (G) before force application on anti-JAM-A-coated magnetic beads with magnetic tweezers. * $p < 0.01$ vs. pulse 1 as determined by *t* test.

bead displacement using single-particle analysis between successive pulling events. Alternatively, continuous force was generated on JAM-A by suspending a magnet in parallel to the cells and followed by biochemical analysis.

We first wanted to determine whether forces through JAM-A activated RhoA. Tension on anti-JAM-A coated beads increased RhoA activity, whereas addition of beads alone had no effect (Figure 2A). As a control, tension on poly-L-lysine (PLL)-coated beads did not activate RhoA (Figure 2B). Quantification of RhoA activation in response to tension on JAM-A or PLL is shown in Figure 2, C and D, respectively. Previous reports also showed that tension on PLL does not activate RhoA (Collins *et al.*, 2012) or increase cell stiffness in response to force (Collins *et al.*, 2012; Barry *et al.*, 2015). Because tension on JAM-A increased RhoA activity, we determined the effect on cell stiffness. Tension imposed on JAM-A increased cell stiffness, as evidenced by decreased bead displacement between pulse 1 and subsequent pulses (Figure 2E). As seen in Figure 2, F and G, inhibition of RhoA or Rho-associated protein kinase (Rho-associated, coiled-coil-containing protein kinase [ROCK]) prevented the decrease in bead displacement. These data indicate that tension on JAM-A activates RhoA to regulate cell stiffness.

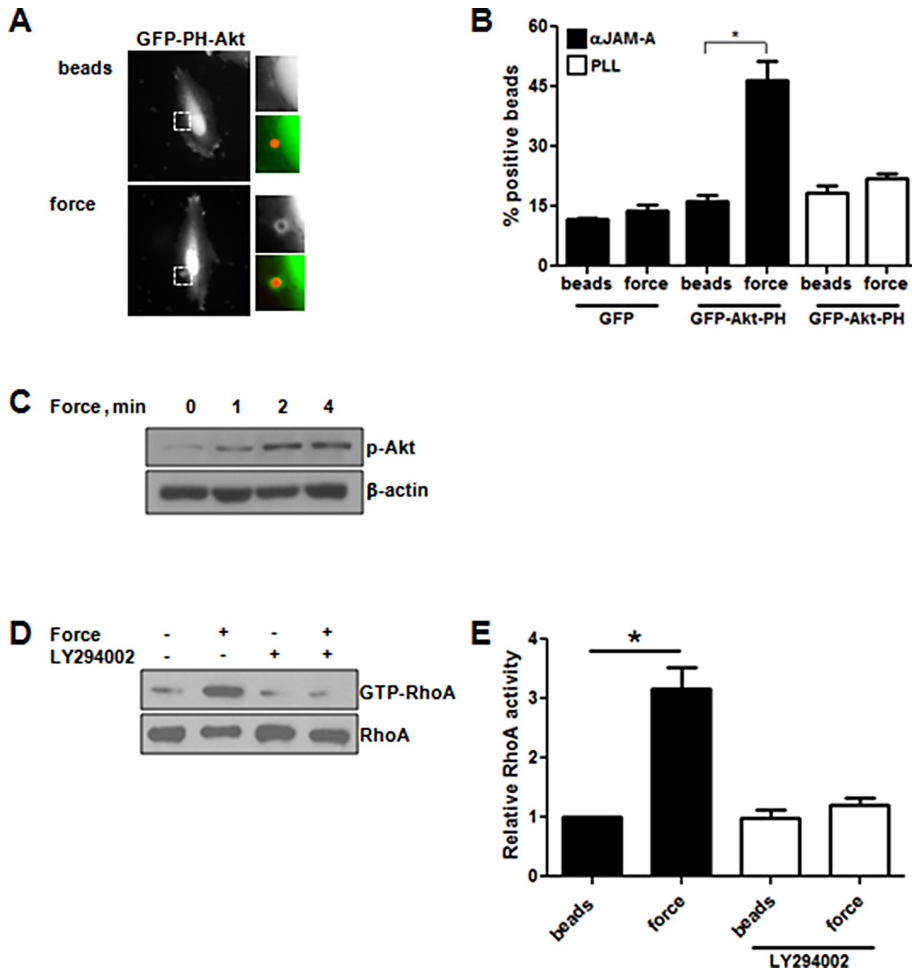


FIGURE 3: JAM-A activates PI3K upstream of RhoA. HUVECs were transfected with GFP or GFP-Akt-PH and incubated with anti-JAM-A- or PLL-coated magnetic beads in the presence or absence of 1 min of continuous force. Cells were fixed with paraformaldehyde, and enrichment of GFP to the area around the bead was determined. (A) Representative images. (B) Quantification. Data are mean \pm SEM of >25 cells/experiment from three independent experiments. * $p < 0.05$ vs. control by *t* test. HUVECs were incubated with anti-JAM-A beads, and force was applied for 0–10 min. Akt phosphorylation, used as a marker of PI3K activation, was determined by Western blot (C). RhoA activity in response to force on JAM-A-coated beads was measured in HUVECs with or without incubation with the PI3K inhibitor LY294002 (10 μ M, 30 min). (D) Representative blots. (E) Means \pm SEM from four experiments. * $p < 0.05$ vs. control by *t* test.

Previous studies showed that tension imposed on some adhesion molecules activates RhoA via phosphoinositide 3 kinase (PI3K; Collins *et al.*, 2012), and recent reports indicate that JAM-A regulates PI3K signaling (Nava *et al.*, 2011; Tuncay *et al.*, 2015). To determine whether PI3K participated in signaling in response to tension on JAM-A, we assessed colocalization between beads and the PI3K sensor green fluorescent protein (GFP)–Akt-PH in the presence or absence of force. Tension imposed on JAM-A recruited GFP-Akt-PH but not GFP alone to the beads, indicating activation of PI3K (Figure 3, A and B). No recruitment of GFP-Akt-PH occurred around PLL beads in the presence or absence of force. To confirm that PI3K was activated in response to force on JAM-A, we examined phosphorylation of Akt. As seen in Figure 3C, tension imposed on JAM-A rapidly increased phosphorylation of Akt. We next wanted to see whether PI3K signaling was required for activation of RhoA. As seen in Figure 3, D and E, the PI3K inhibitor LY294002 prevented RhoA activation downstream of force on JAM-A. Together these

data demonstrate that force on JAM-A activates RhoA in a PI3K-dependent manner.

Tension imposed on JAM-A activates GEF-H1 and p115 RhoGEF to regulate RhoA activity

Rho family GTPases are regulated by the activity of GEFs and GAPs (Schmidt and Hall, 2002; Lessey *et al.*, 2012). To determine which GEFs were activated in response to tension imposed on JAM-A, we used a nucleotide-free RhoA pull-down assay (Garcia-Mata *et al.*, 2006; Guilluy *et al.*, 2011a). As seen in Figure 4, A and B, tension imposed on JAM-A increased the activity of GEF-H1 (ARHGEF2) and p115 RhoGEF (ARHGEF1) but not that of LARG, p190 RhoGEF, or PDZ RhoGEF. To determine whether GEF-H1 and p115 RhoGEF were responsible for RhoA activation in response to tension on JAM-A, we knocked down the expression of these GEFs individually or together. As seen in Figure 5, A and B, small interfering RNA (siRNA)-mediated knockdown of GEF-H1 and p115 RhoGEF individually did not prevent RhoA activation in response to tension on JAM-A. However, knockdown of both GEFs concomitantly prevented RhoA activation in this system. Of importance, knockdown of GEF-H1 and/or p115 RhoGEF did not alter JAM-A expression levels.

Because inhibition of PI3K blocked activation of RhoA downstream of tension on JAM-A, we next investigated the protein's role in the activation of GEF-H1 and p115 RhoGEF. As seen in Figure 5, C and D, inhibition of Akt with the inhibitor LY294002 prevented activation of the two GEFs. These data demonstrate that tension on JAM-A activates GEF-H1 and p115 RhoGEF, both of which are required for RhoA activation, all of which occur downstream of PI3K activation.

Tension imposed on JAM-A activates GEF-H1 through FAK/ERK and p115 RhoGEF through Src family kinases

We next wanted to determine what pathways were leading to GEF-H1 and p115 RhoGEF activation downstream of tension on JAM-A. Previous reports showed that GEF-H1 can be activated downstream of FAK/ERK signaling in response to mechanical forces (Fujishiro *et al.*, 2008; Guilluy *et al.*, 2011b; Collins *et al.*, 2012). To determine whether a similar pathway was operating in our system, we examined phosphorylation of FAK and ERK in response to tension on JAM-A and found increased phosphorylation of both proteins (Figure 6A). As shown in Figure 6B, the upstream inhibitor of ERK, U0126 (mitogen-activated protein kinase kinase [MEK] inhibitor), and FAK inhibitor 14 both inhibited GEF-H1 but had no effect on p115 RhoGEF activation. These results are quantified in Figure 6, C (GEF-H1) and D (p115 RhoGEF).

It was reported that p115 RhoGEF can be activated downstream of JAK2 and protein kinase $C\alpha$ (PKC α ; Guilluy *et al.*, 2010; Peng *et al.*, 2011), and so we examined the involvement of these

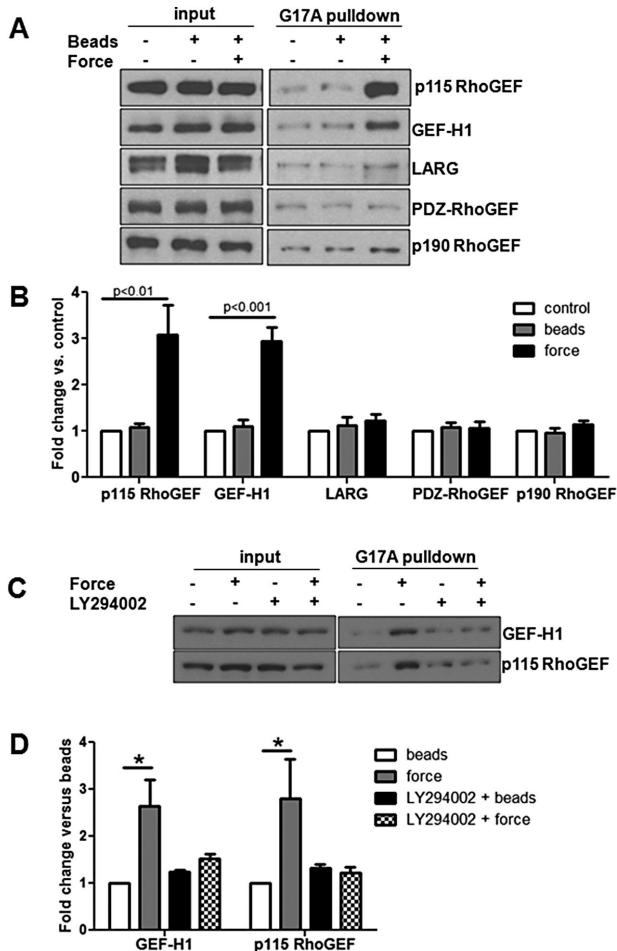


FIGURE 4: Tension on JAM-A activates GEF-H1 and p115 RhoGEF via PI3K. HUVECs were exposed to anti-JAM-A-coated beads, and tension was imposed with a permanent magnet for 3 min. Activation of RhoA GEFs was determined using the GST-RhoA^{G17A} pull-down assay. (A) Representative Western blots. (B) Means \pm SEM from at least three experiments. White bars are untreated control, gray bars are bead only, and black bars are beads plus 3 min of tension. To test for a role of PI3K in activation of GEFs, some cells were treated with LY294002 (10 μ M, 30 min) before addition of beads. GEF activity was assessed using GST-RhoA^{G17A} pull-down assay as described as methods (C). (D) White bars are bead only, gray bars are beads plus 3 min of tension, black bars are LY294002 plus beads, and checkered bars are LY29004 plus beads with tension for 3 min. Statistical analysis was conducted by t test.

kinases in the activation of p115 RhoGEF in response to tension on JAM-A. As seen in Figure 7A, neither inhibition of JAK2 with AG 490 nor inhibition of PKC α with Gö6976 prevented p115 RhoGEF activation in response to tension on JAM-A. To further confirm that PKC α was not required for p115 RhoGEF activation in response to tension on JAM-A, we used siRNA to knock down expression of the protein. As seen in Supplemental Figure S1, knockdown of PKC α did not prevent p115 RhoGEF activation in response to tension on JAM-A. Other candidates for activating p115 RhoGEF are the SFKs, which are known to be activated by mechanical tension (Kostic and Sheetz, 2006; Guilluy *et al.*, 2011b). Inhibition of SFKs with Su6656 caused a significant reduction in p115 RhoGEF activation in response to tension on JAM-A (Figure 7, A and B). These data demonstrate that tension on JAM-A regulates GEF-H1 via FAK/ERK and p115 RhoGEF via SFKs.

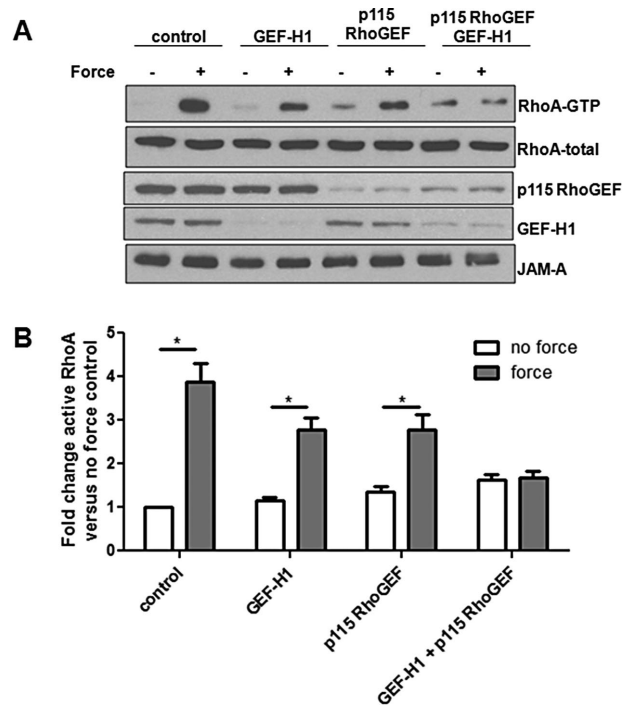


FIGURE 5: JAM-A-mediated RhoA activation requires both GEF-H1 and p115 RhoGEF. HUVECs were transfected with control siRNA or oligos designed against GEF-H1 or p115 Rho GEF. Transfected cells were incubated with anti-JAM-A-coated magnetic beads, and some cells were exposed to force for 3 min. RhoA activity was determined by GST-RBD pull down. Representative Western blots (A) and quantifications (B) from six separate experiments. * $p < 0.05$ vs. no force sample for each condition as determined by t test.

JAM-A phosphorylation is regulated by direct and global mechanical forces

JAM-A is phosphorylated at S285 in mice (S284 in humans) when the protein is localized to tight junctions (Iden *et al.*, 2012). Because tight junctions are regions of high RhoA activity (Priya *et al.*, 2015), we hypothesized that increased tension on JAM-A homodimers within these zones could regulate this phosphorylation event. To test this hypothesis, we examined JAM-A phosphorylation in response to tension on anti-JAM-A beads. Phosphorylation of JAM-A S284 increases rapidly in response to tension on JAM-A (Figure 8A). We next tested to see whether this response was specific to tension on JAM-A or was a general response to mechanical forces. With fluid shear stress as a model, JAM-A S284 phosphorylation rapidly increased before returning to levels at or below baseline within 30 min of shear stress onset (Figure 8B). These data demonstrate that JAM-A phosphorylation is stimulated by mechanical forces.

JAM-A phosphorylation controls RhoA activation in response to tension

To determine whether S284 phosphorylation is required for RhoA activation in response to force on JAM-A, we generated a phosphodeficient S284A mutant and expressed it along with empty vector and wild-type JAM-A in CHO cells that lack endogenous JAM-A. Wild-type human JAM-A expressed in CHO-K1 cells is phosphorylated at S284 (Figure 9A), indicating that the necessary molecular components to control this modification are present. Further, expression of wild-type but not S284A JAM-A increased

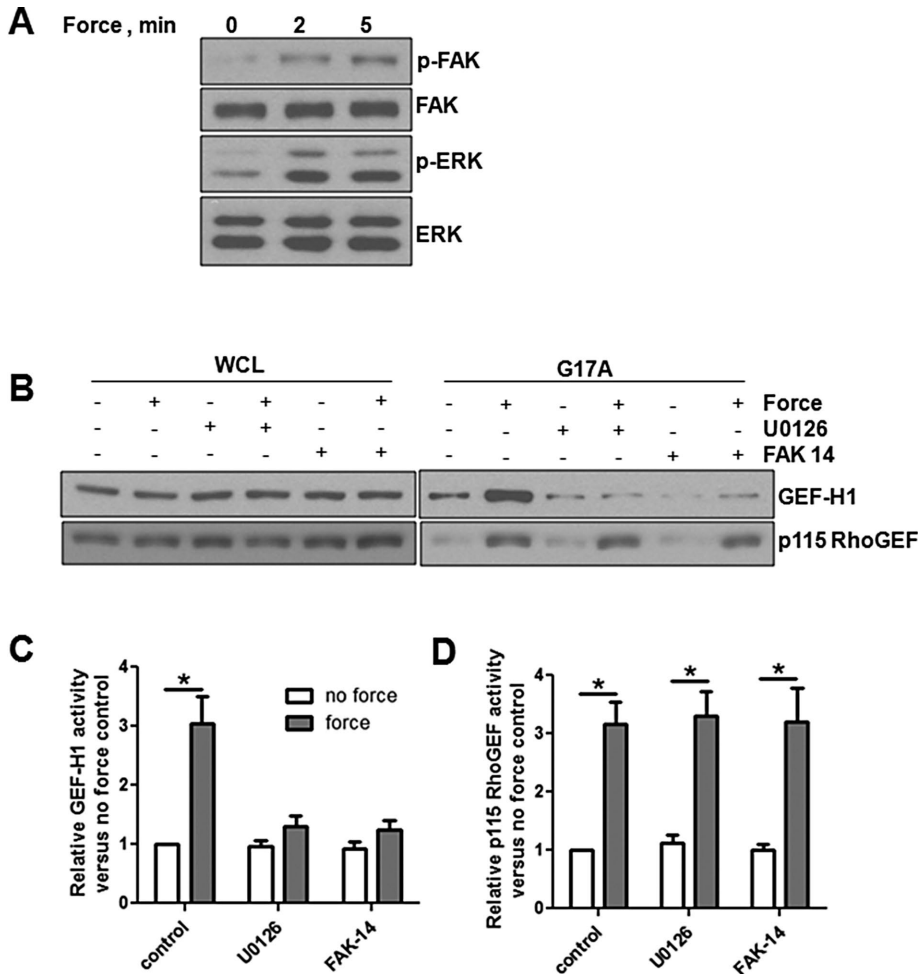


FIGURE 6: GEF-H1 is activated downstream of FAK/ERK in response to tension on JAM-A. (A) HUVECs were incubated with anti-JAM-A-coated magnetic beads, and tension was applied for 0–5 min. Cells were lysed and analyzed for ERK and FAK phosphorylation by Western blot analysis. (B) HUVECs were incubated with anti-JAM-A-coated magnetic beads, and some cells were pretreated with the MEK inhibitor U0126 (25 μ M) or FAK inhibitor 14 (2 μ M) for 30 min before addition of beads, with some samples experiencing 3 min of force. RhoA GEF activity was assessed by GST-RhoA^{G17A} pull-down assay. (C, D) Activation of GEF-H1 and p115 RhoGEF, respectively. Data are mean \pm SEM from at least three experiments. * p < 0.05 vs. no-force control for each condition by *t* test.

barrier function in CHO cells (Figure 9B), as previously reported (Iden *et al.*, 2012), indicating functional differences between the mutant and wild-type protein. We first tested to see whether RhoA activation occurred in the JAM-A S284A mutant. Tension imposed on wild-type JAM-A but not on JAM-A S284A resulted in increased RhoA activity (Figure 9, C and D). As a control, anti-JAM-A beads were added to empty vector-transfected CHO cells, with no RhoA activation observed. Force imposed on wild-type JAM-A resulted in cell stiffening, whereas force imposed on cells expressing the S284A mutant did not (Figure 9, E and F). Attempts were made to measure bead displacement in empty vector-transfected cells, but this measurement was not possible because the beads did not attach to the cell surface and were drawn to the magnetic tweezers instantly. Similar to what was observed in human umbilical vein endothelial cells (HUVECs), cell stiffening in CHO cells could be inhibited by C3-transferase and Y-27632 (Figure 9, F and G, respectively), indicating that RhoA was responsible. These data demonstrate that JAM-A S284 phosphorylation is required for RhoA activation in response to tension on the protein.

JAM-A is phosphorylated at S284 through the actions of PKC ζ (Iden *et al.*, 2012). Previous reports showed that PKC ζ is regulated by mechanical forces (Disatnik *et al.*, 2002; Suzuma *et al.*, 2002; Heo *et al.*, 2011), is regulated via PI3K (Mas *et al.*, 2003; Sarkar *et al.*, 2006), and controls RhoA activity in some systems (Dovas *et al.*, 2006). Therefore we investigated a role for PKC ζ in activation of RhoA downstream of tension on JAM-A. As seen in Figure 10A, tension imposed on JAM-A increased phosphorylation of PKC ζ . To test for a role in RhoA activation, we used a short-peptide inhibitor of PKC ζ to block downstream signaling. As seen in Figure 10B, inhibition of PKC ζ prevented RhoA activation in response to force on JAM-A. Similarly, inhibition of PKC ζ prevents cell stiffening in response to force on JAM-A.

DISCUSSION

Before this study, a role for JAM-A in mechanosignaling was unknown. As diagrammed in Figure 11, we have shown that tension on JAM-A activates RhoA to control cell stiffness (Figure 2). Activation of RhoA in this system requires PI3K (Figure 3) and the combined activities of GEF-H1 and p115 RhoGEF (Figures 4 and 5). Activation of GEF-H1 depends on FAK/ERK (Figure 6), whereas activation of p115 RhoGEF depends on SFKs (Figure 7). Tension imposed on JAM-A or exposure to shear stress increases phosphorylation of JAM-A at S284 (Figure 8). Phosphorylation of JAM-A at S284 is required for activation of RhoA and increased cell stiffness in response to tension on the protein (Figure 9). Finally, PKC ζ is required for activation of RhoA in response to force on JAM-A (Figure 10). Together these results identify JAM-A as a direct transducer of mechanical force, which activates RhoA to regulate cell stiffness.

JAM-A exists in cells as monomers and *cis*- and *trans*-homodimers. At cell–cell junctions, JAM-A homodimers control barrier dynamics (Aurrand-Lions *et al.*, 2001a; Severson *et al.*, 2008; Monteiro *et al.*, 2014). Regulation of barrier function requires interactions between JAM-A's extracellular domain as well as binding partner interactions mediated by the protein's C-terminal PDZ-binding domain (Monteiro and Parkos, 2012). As a monomer, JAM-A has been shown to inhibit integrin signaling (Peddibhotla *et al.*, 2013; Naik *et al.*, 2014), a function that is lost when the protein dimerizes. A possible explanation for the observations described here is that a monomeric form of JAM-A binds to the anti-JAM-A magnetic beads. Once placed under tension, the protein behaves as a homodimer similar to those found at cell–cell junctions. When localized within tight junctions, which are under high levels of tension (Priya *et al.*, 2015), JAM-A is phosphorylated (Iden *et al.*, 2012). Similarly, in our system, JAM-A S284 phosphorylation is rapidly increased by force imposed on the protein.

Monomeric JAM-A inhibits integrin signaling through interaction with CD9. Peddibhotla *et al.* (2013) reported that induction of

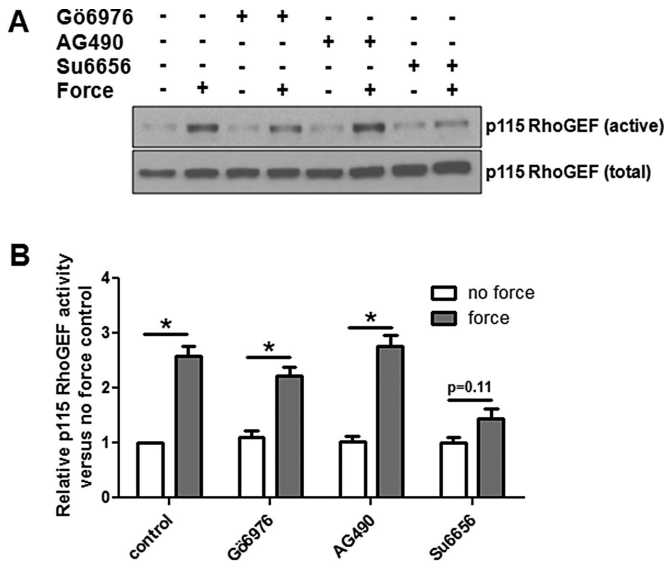


FIGURE 7: Src family kinases activate p115 RhoGEF in response to tension on JAM-A. (A) HUVECs were incubated with inhibitors against JAK2 (AG 490, 25 μ M), PKC α (Gö6976, 10 μ M), or Src family kinases (su6656, 5 μ M) for 30 min, followed by addition of anti-JAM-A-coated magnetic beads. Some cells also experienced force for 3 min. p115 RhoGEF activity was determined by RhoA^{G17A} pull-down assay. (B) Means \pm SEM of at least three separate experiments * $p < 0.05$ vs. no-force control for each condition by t test.

JAM-A dimers results in a disruption of a JAM-A/CD9/ $\alpha_v\beta_3$ complex, leading to increased cell migration. Previous work also showed that activation of platelets results in increased JAM-A dimerization and phosphorylation, as well as decreased interaction with CD9 and $\alpha_{IIb}\beta_3$ (Sobocka *et al.*, 2004). Recently Naik *et al.* (2014) found that JAM-A inhibits $\alpha_{IIb}\beta_3$ by suppressing SFK signaling. In this model, monomeric JAM-A forms a complex with c Src-kinase (Csk) to inhibit integrin-Src complexes. Csk negatively regulates SFK family members via phosphorylation of a conserved tyrosine residue in the protein's C-terminus (Chong *et al.*, 2005). In the context of the present work, tension on JAM-A would increase SFK signaling through dissociation of Csk and integrins. From another perspective, decreasing JAM-A expression should therefore increase similar signaling

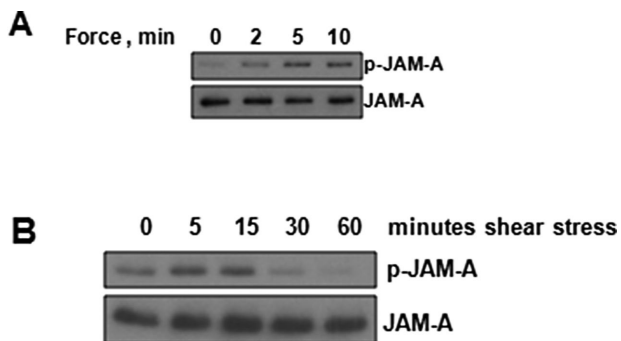


FIGURE 8: JAM-A S284 phosphorylation is regulated by mechanical forces. (A) HUVECs were incubated with anti-JAM-A-coated magnetic beads and exposed to force for 0–10 min. (B) HUVECs were exposed to shear stress for 0–60 min. For both experiments, cells were lysed, and Western blot analysis of total and phosphorylated JAM-A was conducted. Blots are representative of at least three independent experiments

networks due to loss of monomer-associated signaling inhibition. Indeed, Tornavaca *et al.* (2015) showed that focal adhesions are more abundant in cells in which JAM-A has been knocked down. We observed a decrease in phosphorylation of Src at Y527, the site regulated by Csk, and an increase in FAK phosphorylation (Y397) in HUVECs in which JAM-A had been knocked down (Supplemental Figure S2). Increases in FAK phosphorylation in response to JAM-A knockdown could indicate increased RhoA activity or a disruption in focal adhesion turnover by regulating integrin recycling. With regard to this possibility, knockdown of JAM-A could lead to activation of RhoA through activation of p115 RhoGEF. This raises the possibility that distinct pools of JAM-A exist to control spatiotemporal control of cellular contractility. Thus loss of JAM-A-mediated suppression of SFKs would result in increased RhoA activity or at least redistribution of active RhoA. Knockdown of JAM-A could also lead to increased FAK phosphorylation through decreased integrin recycling. Indeed, previous work showed a deficiency in β_1 integrin recycling in JAM-A-null neutrophils, resulting in impaired chemotaxis (Cera *et al.*, 2009).

Previous reports demonstrated that *cis*- (Severson *et al.*, 2008; Peddibhotla *et al.*, 2013) and *trans*-dimerization (Monteiro *et al.*, 2014) mutants of JAM-A control the protein's function. In our model, JAM-A was engaged using an antibody that recognizes the first Ig-like domain, the region involved in dimerization. Alternatively, LFA-1 binds JAM-A in the protein's second Ig-like domain. Binding of LFA-1 to JAM-A has been shown to destabilize homophilic interactions, possibly due to the fact that LFA-1/JAM-A binding can support more tension than JAM-A dimers (Wojcikiewicz *et al.*, 2009). Future studies using *cis*- and *trans*-dimerization mutants engaged with JAM-A/fc-coupled beads, anti-JAM-A-coupled beads, and LFA-1 I-domain beads engaged to the same proteins would provide further insight into the modes of mechanical forces supported by JAM-A.

The data in Figure 6 demonstrate that SFKs control p115 RhoGEF activity. This is not surprising, because SFKs are known to be regulated by mechanical forces. SFK family members Fyn (Kostic and Sheetz, 2006; Chiu *et al.*, 2008; Guilluy *et al.*, 2011b; Fiore *et al.*, 2015), Src (Chaturvedi *et al.*, 2007; Wijetunge and Hughes, 2007), Yes (Niediek *et al.*, 2012), and Lyn (Alessandri-Haber *et al.*, 2008; Hughan *et al.*, 2014) are all activated in response to mechanical forces. Because SFKs are activated by force and Su6656 inhibits multiple SFKs (Blake *et al.*, 2000), it is difficult to determine which kinase(s) are involved without extensive investigation. Fyn has been associated with activation of the RhoGEF LARG in response to force on integrins (Guilluy *et al.*, 2011b). In our system, LARG was not activated in response to tension on JAM-A, but this does not necessarily rule out a role for Fyn in the activation of p115 RhoGEF, as the ligand and cell type used were different between these studies. Previous work showed that p115 RhoGEF is activated in response to integrin engagement to fibronectin (Dubash *et al.*, 2007), a process that likely involves a force component. Although this earlier study demonstrated that p115 RhoGEF was required for cell spreading onto fibronectin, it did not elucidate the mechanism of activation. Determining whether SFKs are responsible for p115 RhoGEF activation after integrin engagement will be pursued in future work.

The present findings may be relevant for several physiological processes. When expressed on endothelial cells, JAM-A participates in leukocyte transendothelial migration by binding to LFA-1. It is known that crawling leukocytes impose forces on endothelial cells, resulting in regional stiffening responses (Schaefer and Hordijk, 2015). The present work demonstrates that forces imposed on JAM-A activate RhoA to elevate cell stiffness. This suggests that

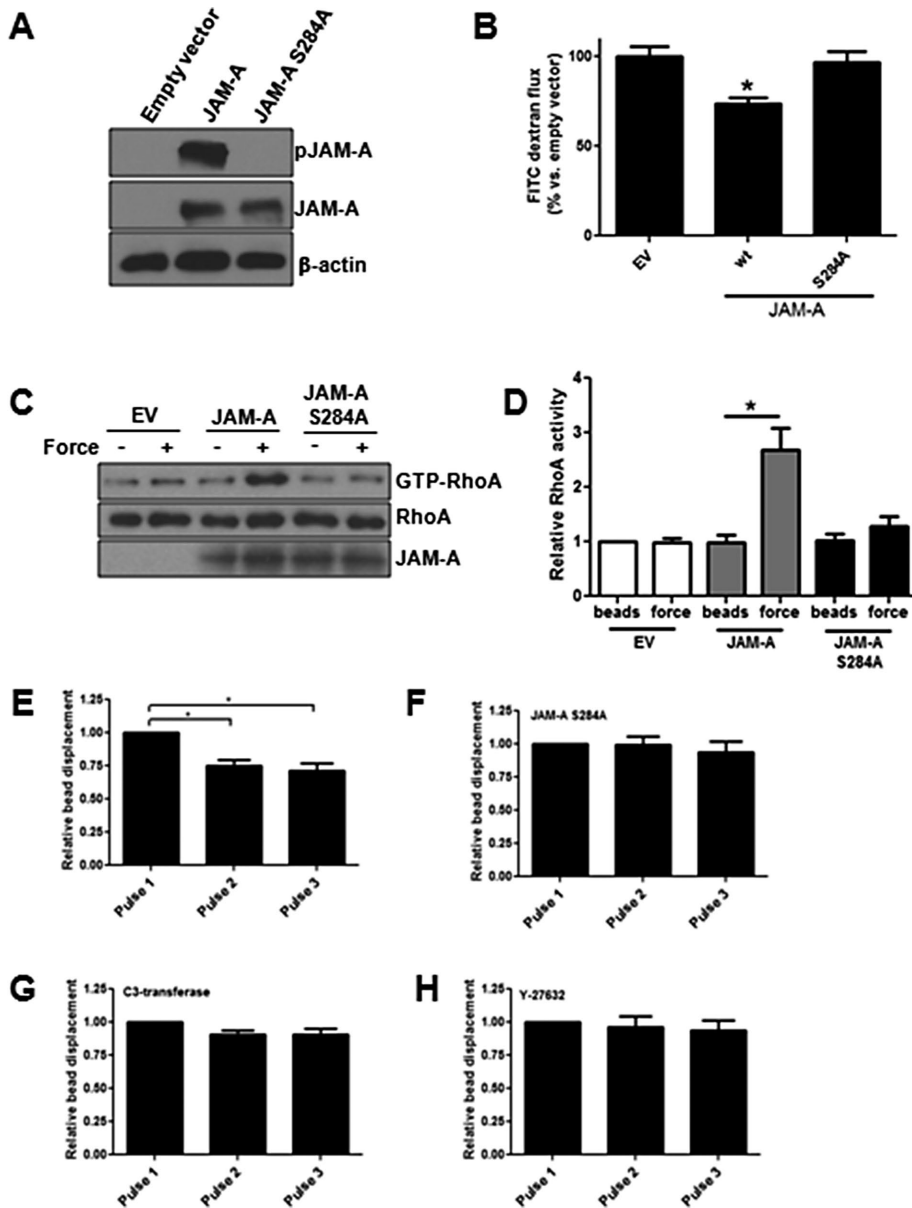


FIGURE 9: JAM-A S284 phosphorylation is required for RhoA activation and cell stiffening in response to force on JAM-A. (A) CHO cells were transfected with empty vector, JAM-A, or JAM-A S284A, and expression of JAM-A and phosphorylated JAM-A was determined by Western blot. (B) Barrier function of CHO cells expressing empty vector (EV), JAM-A, or JAM-A S284A was determined by FITC-dextran flux. Data are representative of three experiments run in triplicate. (C) CHO cells expressing EV, JAM-A, or JAM-A S284A were incubated with anti-JAM-A-coated magnetic beads, and some cells were exposed to force for 5 min. RhoA activity was determined by the RBD pull-down assay. (D) Means \pm SEM of four independent experiments, with $*p < 0.05$ vs. no-force control by *t* test. Cell stiffness was determined in cells expressing (E) JAM-A, (F) JAM-A S284A, (G) JAM-A and incubated with 10 μ M Y-27632, or (H) expressing JAM-A and incubated with 1 mg/ml C3-transferase as described in *Materials and Methods*.

LFA-1/JAM-A interactions would elicit RhoA activation and promote leukocyte migration. A similar pathway was demonstrated for ICAM-1, which also increases RhoA activity and cell stiffening in response to tension (Lessey *et al.*, 2012). It is therefore not surprising that endothelial JAM-A is required for normal leukocyte trafficking to regions of inflammation (Woodfin *et al.*, 2009; Lakshmi *et al.*, 2012; Schmitt *et al.*, 2014). Thus JAM-A and its downstream signaling network could represent a novel therapeutic target for controlling inflammatory responses.

JAM-A also plays a critical role when expressed at cell–cell junctions, as absence of the protein affects the mechanical properties of the cell (Hughan *et al.*, 2014; Tornavaca *et al.*, 2015). Breast cancer provides an intriguing model for the present work. There are conflicting reports on a role for JAM-A in breast cancer. Whereas several studies demonstrated that elevated JAM-A expression is a predictive marker for invasive cellular behavior and poor clinical outcomes, other studies reached opposite conclusions (Naik *et al.*, 2008; McSherry *et al.*, 2009; Murakami *et al.*, 2011; Huang *et al.*, 2014). These studies focused exclusively on total JAM-A expression and did not examine posttranslational modifications such as S284 phosphorylation. Ebnet's group discovered that JAM-A S284 phosphorylation is regulated by PKC ζ and PP2A (Iden *et al.*, 2012), which are often dysregulated in cancer (Switzer *et al.*, 2011; Seshacharyulu *et al.*, 2013; Yin *et al.*, 2014). Thus cells expressing normal levels of JAM-A but that cannot regulate phosphorylation of the protein would behave similarly to cells lacking JAM-A expression. Investigating the levels of JAM-A phosphorylation and correlating these with cell behavior and clinical outcomes are further warranted.

This article has demonstrated that tension on JAM-A activates RhoA to regulate cell stiffness. Activation of RhoA requires GEF-H1 and p115 RhoGEF, as well as phosphorylation of JAM-A at S284. SFKs have been identified as novel regulators of p115 RhoGEF activation. It will be interesting to determine whether changes in JAM-A phosphorylation occur during diseases *in vivo*, such as in cancers of epithelial origin and in vascular disease. Further studies are needed to identify the SFKs required for p115 RhoGEF activation and to determine whether SFKs activate this GEF in other situations in which it is responsible for activating RhoA.

MATERIALS AND METHODS

Cell lines and reagents

HUVECs were cultured in EBM2+ BulletKit (cells and media from Lonza, Rockville, MD). CHO-K1 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in high-glucose DMEM supplemented with 10% fetal bovine serum and antibiotic–antimycotic solution (all from Life Technologies/ThermoFisher Scientific, Grand Island, NY). Y-27632 (ROCK inhibitor) was purchased from Millipore (Billerica, MA). Cell-permeable C3 (RhoA inhibitor) transferase was purchased from Cytoskeleton (Denver, CO). U0126 (MEK inhibitor), LY294002 (PI3K inhibitor), Su6656 (Src family kinase inhibitor), AG 490 (JAK2 inhibitor), Gö6976 (PKC α inhibitor), FAK inhibitor 14, and PKC ζ pseudosubstrate were from Tocris (Minneapolis, MN). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

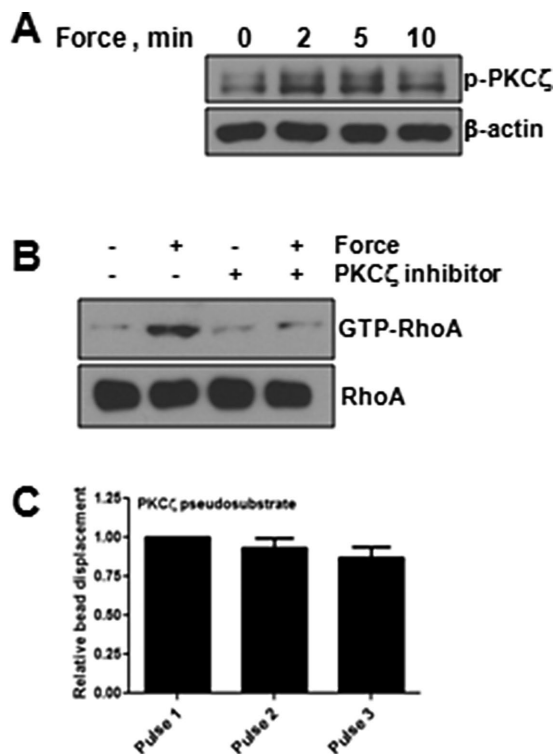


FIGURE 10: Force on JAM-A regulates PKC ζ to activate RhoA. HUVECs were incubated with anti-JAM-A beads in the presence or absence of forces, and PKC ζ phosphorylation was determined by Western blot (A). HUVECs were incubated with anti-JAM-A-coated magnetic beads, and some cells were exposed to force for 3 min. Some cells had been pretreated with PKC ζ inhibitory peptide. RhoA activity was determined by the RBD pull-down assay (B). Cell stiffness was determined in HUVECs after tension was imposed on anti-JAM-A-coated beads in the presence of PKC ζ inhibitor as described in *Materials and Methods* (C).

siRNA, DNA constructs, and transfections

All cells were transfected using Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's protocol. Human JAM-A expressed in pCDNA3.1 was previously described (Naik *et al.*, 2001), and GFP-PH-Akt was a gift from Tamas Balla (Addgene plasmid 51465). Point mutagenesis to generate S284A used the following primers: forward, 5'-AGAAGGTGATTTACAGCCAGCCTGCTGCCCGAAGTGA-3', and reverse, 5'-TCACCTCGGGCAGCAGGCTGGCTGTAAATCACCTTCT-3'. siRNA target sequences are as follows: GEF-H1 duplex 1 sense, 5'-UUUAGAGAUUCGUAGGCAA-3'; GEF-H1 duplex 2 sense, 5'-AGACAGAGGAUGAGGCUUA-3'; p115 RhoGEF duplex 1 sense, 5'-GGGCUGAGGAUGAGGAUUU-3'; p115 RhoGEF duplex 2 sense, 5'-CCACAGAACGGGAGAAAAGU-3'; JAM-A duplex 1 sense, 5'-CGAGUAAGAAGGUGAUUUA-3'; JAM-A duplex 2 sense, 5'-AGGCGCAAGUCGAGAGGAA-3'; PKC α duplex, 5'-AAGAAG-AAGGUGAGUACUA-3'; and control sense, 5'-UAAGGCUAUGAAGAGAUAC-3'.

Antibodies

The following antibodies were used for Western blot analysis: anti-GEF-H1 (4076), anti-p115RhoGEF (3669), anti-RhoA (2117), anti-phospho-FAK (3283), anti-phospho-Erk1/2 (4370), phospho-Akt (2965), FAK (3285), and ERK (9102) were purchased from Cell Signaling (Danvers, MA), anti-JAM-A (612120) was from BD Transduction Laboratories (Forest Lakes, NJ), and anti-phospho-JAM-A

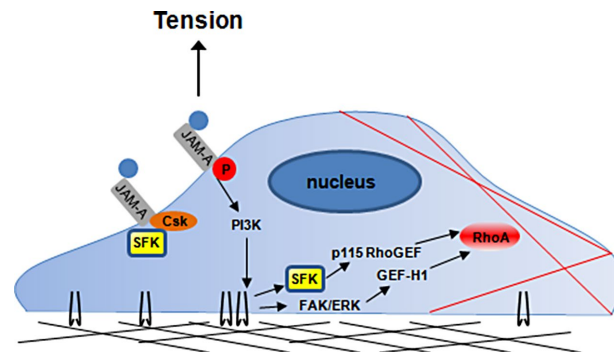


FIGURE 11: Working model of RhoA activation in response to force on JAM-A. Anti-JAM-A-coated magnetic beads engage JAM-A on the cell surface. In response to force, SFKs dissociate from the protein's C-terminus. In short order, PI3K likely signals for the activation of PKC ζ , as well as of GEF-H1 and p115 RhoGEF. Activation of GEF-H1 and p115 RhoGEF also requires FAK/ERK- and SFK-mediated pathways, respectively. Finally, RhoA is activated by either GEF to regulate actomyosin-based cellular contractility and cell stiffness.

(sc-17430-R) was from Santa Cruz Biotechnology (Dallas, TX). Antibodies against LARG and PDZ-RhoGEF were custom produced by Pocono Rabbit Farms and Laboratories (Canadensis, PA) and were previously described (Guilluy *et al.*, 2011b). Anti-p190 RhoGEF was a generous gift of David Schlaepfer (University of California at San Diego, La Jolla, CA).

Application of continuous force

Magnetic beads were prepared as previously described (Lessey-Morillon *et al.*, 2014). Briefly, anti-JAM-A clone J10.4 (sc-53623; Santa Cruz Biotechnology) was conjugated to 4.5- μ m tosyl-activated Dynabeads (ThermoFisher Scientific) in 0.1 M borate buffer, pH 9.5, according to the manufacturer's protocol. After overnight incubation at 37°C with rotation, free sites were quenched by incubation with 0.1% fatty acid-free bovine serum albumin (BSA) for 1 h. For biochemical experiments, a continuous force calculated at ~10 pN was applied to beads using a permanent ceramic magnetic (K&J Magnetics, Jamison, PA) as previously described (Guilluy *et al.*, 2011b).

Detection of cellular stiffening

The same beads used for the application of continuous force were added to cells 10 min before being engaged using the University of North Carolina three-dimensional force microscope. The magnetic tweezers pole tip was positioned 25 μ m above the monolayer, and a force regimen of 3 s of 50-pN force followed by 4 s of no force was applied for repeated cycles. Bead movement was captured using a 40 \times objective (Olympus UplanLN 40 \times /0.75) on an Olympus IX81-ZDC2 inverted microscope (Olympus, Waltham, MA) equipped with a high-speed Rolera EM-C2 camera (QImaging, Surrey, BC, Canada), using MetaMorph software at 30 frames/s. Bead movements were tracked by Video Spot Tracker (<http://cismm.cs.unc.edu>). From 11 to 27 beads per condition were tracked from at least two separate experiments. Data are presented as mean \pm SEM relative to the first pull for each condition tested.

Shear stress

HUVECs were subjected to shear stress as previously described (Dardik *et al.*, 2005). Briefly, cells were grown in six-well plates to

confluence, switched to 3 ml of serum-free medium for 2 h, and then rotated at 210 rpm for the indicated times. Lysates were collected for Western blot analysis.

RhoA activity assay

Cells were lysed for analysis of RhoA (10 mM MgCl₂, 500 mM NaCl, 50 mM Tris, pH 7.6, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 10 µg/ml aprotinin and leupeptin) and cleared at 14,000 × g for 5 min. Lysates were incubated with 50 µg of glutathione-Sepharose-bound glutathione S-transferase (GST)-RBD (Rhotekin-binding domain) for 30 min at 4°C with gentle rocking. Beads were then washed three times in 50 mM Tris, pH 7.6, 10 mM MgCl₂, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 10 µg/ml aprotinin and leupeptin. Released proteins and reserved input control were subjected to Western blot analysis as described later.

GEF activity assay

Active RhoA GEFs were assayed using GST-RhoA G17A as described previously (Guilluy *et al.*, 2011a). Cells were lysed in 150 mM NaCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6, 10 mM MgCl₂, 1% Triton X-100, 1 mM PMSF, and 10 µg/ml aprotinin and leupeptin and incubated with 50 µg/ml glutathione-Sepharose-bound GST-RhoA^{G17A} for 60 min at 4°C and washed in the lysis buffer. Samples were then analyzed by Western blotting as described later.

Barrier function analysis

Cells were seeded (5 × 10⁴ cells/well) onto fibronectin-coated (10 µg/ml) 0.4-µm polycarbonate Transwell membranes (Corning). Forty-eight hours after plating, fluorescein isothiocyanate (FITC)-dextran (10 kDa; Sigma-Aldrich) at a final concentration of 1 mg/ml was added to the upper chamber. After 2 h of incubation, medium from the bottom chamber was collected. Medium was transferred to a black-walled 96-well microtiter plate (Corning), and fluorescence intensity was analyzed using a plate reader (excitation 485 nm, emission 520 nm; Tecan).

Western blotting

Samples were resolved on 12% (RhoA) or 8% (all other proteins) polyacrylamide gels (Sambrook and Russell, 2001) in the presence of SDS. Resolved gels were transferred onto nitrocellulose membranes, blocked with 5% BSA in Tris-buffered saline (25 mM Tris, pH 7.6, 150 mM NaCl) plus 0.1% Tween-20 (TBST) and incubated with primary antibody overnight at 4°C with gentle rocking. Blots were washed extensively in TBST before being incubated with species-appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories) for 1 h at room temperature. Blots were again washed in TBST, and fluorescence was detected using enhanced chemiluminescent reagent (ThermoFisher Scientific) and x-ray film.

PI3K activation

Two days after transfection, cells were plated on fibronectin-coated (10 µg/ml) glass coverslips for 6 h before being incubated with beads. Some cells experienced force for 1 min. Cells were fixed in 4% paraformaldehyde, and images were captured on a Zeiss Axiovert 200 M microscope equipped with a Hamamatsu ORCA-ERAG digital camera.

Statistical analysis

For analysis of cell stiffness, Student's *t* test between pulse 1 and each subsequent pulse was calculated. For GFP-PH localization,

Student's *t* test between beads only and beads plus force was calculated for each construct or ligand. In all instances, *p* < 0.05 was considered significant, and all calculations were conducted in GraphPad Prism 5.1.

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