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The authors inadvertently omitted Daniel E. Conway from the list of authors. The corrected author list appears above. His affiliation is the Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA.

The HTML and PDF versions of this article have been corrected. The error remains only in the print version.
Rac1 functions as a reversible tension modulator to stabilize VE-cadherin trans-interaction

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The role of the RhoGTPase Rac1 in stabilizing mature endothelial adherens junctions (AJs) is not well understood. In this paper, using a photoactivatable probe to control Rac1 activity at AJs, we addressed the relationship between Rac1 and the dynamics of vascular endothelial cadherin (VE-cadherin). We demonstrated that Rac1 activation reduced the rate of VE-cadherin dissociation, leading to increased density of VE-cadherin at AJs. This response was coupled to a reduction in actomyosin-dependent tension across VE-cadherin adhesion sites.

Introduction

The vascular endothelial cadherin (VE-cadherin)–mediated adherens junction (AJ) complex is the primary restrictive barrier of the endothelium to plasma proteins and blood cells (Giannotta et al., 2013). The VE-cadherin extracellular domain undergoes trans-homodimerization after cell–cell contact (Zhang et al., 2009; Brasch et al., 2011). Stabilization of adhesions requires attachment of the cadherin intracellular domain via catenins to the actin cytoskeleton (Baumgartner et al., 2003; Hong et al., 2013). Cadherin “outside-in” signaling orchestrated by the opposing actions of RhoGTPases Rac1 and RhoA is responsible for remodeling of the actin cytoskeleton at the nascent adhesion sites (Noren et al., 2001; Lampugnani et al., 2002; Cain et al., 2010). In epithelial cells, spatiotemporal activity of Rac1 induced polymerization of actin filaments at the cadherin complex (Ehrlich et al., 2002) to initiate cell–cell contact (Yamada and Nelson, 2007), whereas RhoA activity at the contact sites mediated actomyosin-dependent expansion of the adhesion zone (Yamada and Nelson, 2007).

The role of Rac1 at mature cell–cell adhesions in endothelial cells, which mediate the AJ barrier function, is not well understood. Activation of Rac1 can induce a range of responses from stabilization of endothelial AJs on exposure to shear stress or barrier-enhancing mediators, such as sphingosine-1-phosphate (Lee et al., 1999; Mehta et al., 2005; Liu et al., 2013), to disassembly of AJs in response to vascular endothelial growth factor and tumor necrosis factor (van Wetering et al., 2002; Eriksson et al., 2003; Monaghan-Benson and Burridge, 2009; Naikawadi et al., 2012). A likely explanation for this variability is that global Rac1 activation is often accompanied by mobilization of other signaling pathways that modify localized responses at AJs (Komarova and Malik, 2010; Spindler et al., 2010). Most means of Rac1 activation involve activating the GTPase in the cell, and as such, local Rac1-mediated signaling events are not detected or masked by divergent signals. Here, using the photoactivatable (PA) probe bearing the constitutively active Rac1 V12 mutant (Wu et al., 2013), we observed that inhibiting myosin II directly or through photo-release of the caged Rho kinase inhibitor also reduced the rate of VE-cadherin dissociation. Thus, Rac1 functions by stabilizing VE-cadherin trans-dimers in mature AJs by counteracting the actomyosin tension. The results suggest a new model of VE-cadherin adhesive interaction mediated by Rac1-induced reduction of mechanical tension at AJs, resulting in the stabilization of VE-cadherin adhesions.
Cadherin-mediated adhesion is a dynamic event characterized by continuous rearrangements of cadherin adhesive bonds (Hong et al., 2011), lateral movement of cadherin within AJs (Baumgartner et al., 2003), and exchange of cadherin between junctional and intracellular pools (de Beco et al., 2009; Hong et al., 2010). To determine how Rac1 regulated VE-cadherin assembly at AJs, we tagged VE-cadherin to the photoswitchable fluorescent protein Dendra2, which exhibits a shift in emission spectrum from a 488- to 543-nm maximum wavelength after photoconversion (Chudakov et al., 2007). VE-cadherin–Dendra2 was used because irradiation with the 458-nm laser beam required for PA-Rac1 activation did not interfere with photoconversion of Dendra2 (Fig. S2A). Thus, VE-cadherin behavior at AJs as determined from the kinetics of fluorescent recovery at 488 nm defined the association of new VE-cadherin molecules to the AJs, whereas fluorescent decay at 543 nm described the net effect of VE-cadherin lateral movement and dissociation from AJs (Fig. 2A).

We observed that VE-cadherin lateral movement contributed little to the decay kinetics (Fig. S2B). However, VE-cadherin underwent rapid exchange between the junctional and intracellular pools (Fig. 2, E and G). PA-Rac1 activation significantly reduced the rate constant of VE-cadherin dissociation.

Results and discussion

Photoactivation of PA-Rac1 with a 458-nm laser beam occurring with a half-life of ~25 s mimicked the transient endogenous Rac1 activation induced by sphingosine-1-phosphate (Lee et al., 1999; Mehta et al., 2005; Yamada and Nelson, 2007). We observed that activation of PA-Rac1 in different endothelial cell types induced VE-cadherin accumulation within the photoactivation zone (Fig. 1, A–F; and Fig. S1, A–D). The increased VE-cadherin density, however, was not accompanied by expansion of adhesion zone (Fig. S1E), suggesting that any effect of lamellipodia activity was negligible. In contrast, activation of control photoinsensitive (PI) Rac1 (PI-Rac1) or a probe bearing a dominant-negative (DN) Rac1 mutant (PA-Rac1DN; Wu et al., 2009) had no effect on VE-cadherin density at AJs (Fig. 1, C–F). These results showed that spatiotemporal activation of Rac1 at mature AJs functioned by increasing VE-cadherin density of the junctions (Fig. 1G).
and trans-dimers (reaction $d$). After assuming symmetry in VE-cadherin distribution at adherens junctions, the equilibrium between monomer and dimer was described by the equations

\[
\frac{da}{dt} = j - k_a a - 2k_j a^2 + 2k_d d \quad \text{and} \quad \frac{dd}{dt} = k_d a^2 - k_j d,
\]

in which $j$ and $k_a$ are the rates of VE-cadherin monomer–junction association and dissociation; $k_j$ and $k_d$ are the “on” and “off” rate constants between the monomer and trans-dimer. The equilibrium relations between different states of VE-cadherin species were determined from Eqs. 1a and 1b.

The equations in the previous paragraph were solved using COPASI (Complex Pathway Simulator) software (Fig. 3, B–D). COPASI produced an optimal fit to our experimental data (i.e., monomer–junction association [$j$] and dissociation [$k_a$] rate constants [Fig. 2, E and G]), yielding a set of values for in silico on and off rates for trans-interaction (Fig. 3 D). Furthermore, the observed reduction in monomer–junction dissociation...
Rac1 activation occurred at sites of AJs experiencing the highest tension in response to fluid shear stress (Allen et al., 2011; Liu et al., 2013), suggesting that Rac1 functions by opposing the tension developed across VE-cadherin adhesion through counterbalancing RhoA activity (Fig. 4A). To test this concept, we first used a Förster resonance energy transfer (FRET)–based RhoA biosensor (Pertz et al., 2006). Consistent with the antagonistic Rac1 and RhoA relationship (Yamada and Nelson, 2007), we observed spatial reduction of RhoA activity after photoactivation of PA-Rac1 but not of PI-Rac1 (Fig. 4, B–D). Furthermore, activation of PA-Rac1 significantly reduced phosphorylation of myosin-II light chain (MLCII) as compared with controls (Fig. 4, E–G). These findings suggest that Rac1 functioned at AJs by counterbalancing the RhoA-dependent tugging force across the VE-cadherin trans-dimers.

To measure directly actomyosin-mediated tension across VE-cadherin adhesion, we next used the tension sensor for VE-cadherin (VE-t; Fig. 4, F–J; Conway et al., 2013). Endothelial monolayers basally showed low FRET/CFP ratio (Fig. 4, H and I, inset 1), indicative of constitutive intracellular traction forces.
across VE-cadherin adhesions in the confluent endothelium. Photoactivation of Rac1, however, reduced basal tension of ~2.4 nN/molecule to the level of the tailless control (Δtail), a probe that experiences no tension (Fig. 4, H and I, inset 2; Conway et al., 2013). Thus, transient activation of PA-Rac1 at AJs caused spatial relaxation and reduced the tension across VE-cadherin adhesions similar to that seen when applying fluid shear stress to confluent endothelial monolayers (Conway et al., 2013).

We determined whether transient inhibition of RhoA signaling downstream of Rac1 activation and the subsequent reduction in tension at AJs were sufficient to stabilize VE-cadherin trans-interaction. Rho-associated protein kinase (ROCK) is a primary downstream effector of RhoA that inhibits myosin light chain phosphatase and phosphorylates MLCK, leading to actomyosin-dependent contractility (Riento and Ridley, 2003). To establish a causal relationship between RhoA–ROCK signaling and stability of VE-cadherin adhesions, we locally photo-released the caged ROCK inhibitor Rockout (caged Rockout [cRO]; Morckel et al., 2012). Release of cRO at AJs significantly reduced the tension across VE-cadherin adhesion (Fig. 5, A–C) and VE-cadherin dissociation rate from AJs (Fig. 5, D–F). cRO release thus also induced VE-cadherin accumulation at AJs (Fig. S3).

We observed that localized and transient Rac1 activation at AJs mediated VE-cadherin accumulation and stability of VE-cadherin
dynamics. Together, our data indicate that Rac1 activity has a fundamental role in strengthening VE-cadherin adhesion at mature AJs experiencing mechanical tension.

Rac1 also induces formation of lamellipodia involved in annealing of nascent junctions and closure of inter-endothelial gaps after injury (Nola et al., 2011). However, Rac1 stabilization of VE-cadherin adhesive bonds in the present study occurred independently of formation of lamellipodia protrusions. Thus, the role of Rac1 in counterbalancing RhoA signaling at the level of adhesive bonds by modulating actomyosin tension across VE-cadherin adhesions (Fig. 5 G). Interestingly, E-cadherin 1D to A mutation that stabilized trans-swapped dimers also assembled more stable adhesive clusters (Hong et al., 2013). The decrease in VE-cadherin dissociation rate mediated by PA-Rac1 was proportional to the increased VE-cadherin trans-interaction. Destabilization of this interaction with double W2A/W4A point mutations (Brasch et al., 2011) increased VE-cadherin dissociation rate and mitigated the effects of Rac1 activation on VE-cadherin dynamics. Together, our data indicate that Rac1 activity has a fundamental role in strengthening VE-cadherin adhesion at mature AJs experiencing mechanical tension.

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of mature AJs and stabilizing trans-dimers (Fig. 5 G) is distinct from the role of Rac1 in establishing endothelial junctional barrier through lamellipodia formation. The mechanism of Rac1 inhibition of RhoA activity at AJs is unknown. We previously showed that p190RhOGAP-A inhibited RhoA activity at AJs and that the GTPase-activating protein (GAP) activity was regulated by tyrosine nitration at Y1105 (Siddiqui et al., 2011). In epithelial cells, P190RhOGAP-B appears to be the GAP responsible for controlling RhoA activity (Ponik et al., 2013), suggesting that the function of GAPs in regulation RhoA activity at AJs may be cell specific. Importantly, both GAPs required interaction with p120-catenin to inhibit RhoA and protect against permeability increase (Ponik et al., 2013; Zebda et al., 2013). Rac1 and its upstream effectors Tiam1 and Vav2 may be required for localization of P190RhoGAP to the junctions (Wildenberg et al., 2006; Birukova et al., 2011).

In conclusion, we describe here a novel mechanism by which Rac1 relieves myosin-dependent tension across VE-cadherin junctions and stabilizes VE-cadherin–trans-interaction at mature AJs of confluent endothelial cells. This tug-of-war between Rac1 and RhoA ensures prompt restoration of barrier function of AJs after immune and inflammatory insults to regulate tissue fluid balance.

Materials and methods

Plasmids and adenovirus

The human VE-cadherin–GFP adenovirus (pAdG5SV4, the dL327 backbone, cytomegalovirus [CMV] promoter; Shaw et al., 2001) was a gift from F. Luscinskas (Brigham and Women’s Hospital, Boston, MA) and S.K. Shaw (Brown University, Providence, RI). VE-cadherin–Dendra2 (pCDNA3 and CMV promoter) was generated by PCR-based strategy and subcloning VE-cadherin and Dendra2 (a gift from S. Troyanovsky, Northwestern University, Chicago, IL; Hong et al., 2010), into the pCDNA3 vector (Life Technologies) at restriction sites 5’-KpnI and 3’-EcoRI for VE-cadherin and 5’-EcoRI and 3’-XhoI sites for Dendra2. Mutation of tryptophan residues at positions 2 and 4 to alanine (W2A/W4A) on VE-cadherin was generated using a site-directed mutagenesis kit (QuickChange; Agilent Technologies). VE-cadherin tension (VE) and tailless [tail] control FRET-based biosensors were created (pPCX-VE-cadherin; Conway et al., 2013), mCherry-PA-Rac1, mCherry-PA-RAc1-T17N (PA-Rac1TN), mVenus-PA-Rac1-T17N (PA-Rac1DN), and mCherry-PA-Rac1-C450A (PA-Rac1; pTriEx), a hybrid promoter composed of the CMV enhancer fused to the chicken β-actin promoter; Wu et al., 2009) and the FRET-based RhoA biosensor (pTriEx, a hybrid promoter; Pertz et al., 2006) were gifts from K. Hahn (University of North Carolina School of Medicine, Chapel Hill, NC). For CFP-PA-Rac1DN, CFP was amplified using pEFP-C1 (BD) as a DNA template and subcloned into pTriEx-mVenus-PA-Rac1-T17N using 5’-NcoI and 3’-BamHI restriction sites.

Synthesis of cRO

cRO was synthesized according to Morckel et al., 2012 (with some modifications). In brief, starting material NPOCl-6 (6-nitropiperonyloxymethylchloride) was first prepared according to Lusic and Deiters (2006). A mixture of 10.0 mg ROCK inhibitor III (Rockout) and 1 ml dimethylformamide was stirred on ice for 5 min. 1.4 mg sodium hydride (0.058 mmol) was added to the mixture, stirred on ice for an additional hour, and frozen solid using 1 ml ethyl acetate. Next, 0.3 ml dimethylformamide containing 20.5 mg NPOCl-6 (0.079 mmol) was added to the mixture and stirred at room temperature for 12 hours before quenching with 1 ml saturated sodium bicarbonate. The solution was extracted with 10 ml ethyl acetate, dried over sodium sulfate, and concentrated in vacuo. The crude product was purified via silica column chromatography. A mobile phase of 7:3 hexanes/ethyl acetate (vol/vol) removed byproducts and unreacted material, and the product was eluted with a mobile phase of 100% ethyl acetate to yield 5.2 mg (24% yield) of pure cRO as yellow flakes. Purity was assessed via 1H-nuclear magnetic resonance [NMR] spectroscopy. 1H-NMR spectra were acquired on a 360 MHz NMR spectrometer (AVANCE; Bruker) and were obtained in CDCl3 using 0.01% tetramethylsilane as an internal standard. Electrospray mass spectra (positive ion mode) were obtained on a mass spectrometer (ITOF [ion trap time-of-flight]; Shimadzu).

Cell culture, transfection, and treatment

Human dermal microvascular endothelial cells (HMVECs; Ades et al., 1992) were kept in MCD131 medium (Gibco) supplemented with 10% FBS, 0.003 mg/ml human EGF, 0.001 mg/ml hydrocortisone, and -glutamine. Primary human pulmonary arterial endothelial cells were grown in EB2 culture medium (Lonza) supplemented with 15% FBS and EGM-2 bullet kit (Lonza) and were used at passages 2–6. All cell lines were maintained at 37°C and 5% CO2. Endothelial cells were plated on glass-bottom coverslips coated with 0.2% gelatin and transfected at 70–80% confluency using X-tremeGENE HP DNA transfection reagent according to manufacturer’s protocol (Roche). For adenoviral infection, endothelial cells were exposed to the adenoviral particles overnight and were used for live-cell imaging at 24–72 h after infection. The procedure was handled according to National Institutes of Health safety guidelines for materials containing BSL2 organisms. Cells were treated with 20 μM cRO or 10 μM blebbistatin (Toronto Research Chemicals) for 10 min before experiments.

Immunofluorescence staining and analysis of MLCI phosphorylation

Cells expressing mCerulean-PA-Rac1 (Fig. 4, E and F, shown in green) were kept in dark or exposed to white light for 10 min. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.2% Triton X-100. Nonspecific sites were blocked with 4% BSA. Samples were stained for phosphorylated MLCII using the rabbit polyclonal phospho-Thr18/Ser19–specific antibody (3674; Cell Signaling Technology) and TRITC-conjugated donkey anti–rabbit antibody (Jackson Immunoresearch Laboratories, Inc.). In addition, a mouse monoclonal GFP antibody (A11120; Life Technologies) and FITC-conjugated anti–mouse antibody (Jackson Immunoresearch Laboratories, Inc.) were used to stain for mCerulean-PA-Rac1; however, the antibody was found to be nonspecific (staining not depicted in Fig. 4, E and F). Samples were mounted in ProLong Gold reagent (Molecular Probes). Z-stack images were obtained at 100-fold objective using a confocal microscope (LSM 510 Meta; Carl Zeiss) equipped with a 63×, 1.2 NA water immersion objective lenses and Ar ion and dual HeNe lasers and driven by LSM software (Carl Zeiss). Image handling and analysis were performed using MetaMorph software (Molecular Devices), and images were prepared for illustration in Photoshop (Adobe). Cell staining was used to define cell boundaries caused by nonspecificity of the GFP antibody. Transfected cells were chosen using the CFP channel (λ = 458 nm). In these cells, the CFP fluorescence was used to define the cell boundary between dark and light conditions. Relative phosphorylation of MLCII was expressed as mean pixel intensity above background noise. Z-stack images were projected onto a single image. An image with high signal was used to define the threshold for mean pixel intensities and to remove background noise. The threshold value excluded almost all background noise and stress fibers and included only the bright signal from the cortical actin. The same value for the mean pixel intensities was used to threshold all images, thus keeping analysis consistent. The values for mean pixel intensity of threshold images were measured for each cell.

Live-cell imaging

In all experiments, endothelial cells were imaged in phenol red-free EB2 media supplemented with 5% FBS at 37°C using the stage heater (Temp-control 37°; Carl Zeiss). Time-lapse images were acquired using confocal microscope (LSM 710; Carl Zeiss) equipped with a 63×, 1.4 NA oil immersion objectives and Ar ion and dual HeNe lasers and driven by LSM software (Carl Zeiss). Image handling and analysis were performed using MetaMorph software (Molecular Devices), and images were prepared for illustration in Photoshop (Adobe). Cell staining was used to define cell boundaries caused by nonspecificity of the GFP antibody. Transfected cells were chosen using the CFP channel (λ = 458 nm). In these cells, the CFP fluorescence was used to define the cell boundary between dark and light conditions. Relative phosphorylation of MLCII was expressed as mean pixel intensity above background noise. Z-stack images were projected onto a single image. An image with high signal was used to define the threshold for mean pixel intensities and to remove background noise. The threshold value excluded almost all background noise and stress fibers and included only the bright signal from the cortical actin. The same value for the mean pixel intensities was used to threshold all images, thus keeping analysis consistent. The values for mean pixel intensity of threshold images were measured for each cell.

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Image processing

All images were corrected for photobleaching using a modified version of MATLAB software (MathWorks; Hodgson et al., 2006). A photobleaching coefficient was determined from decay kinetics of fluorescent intensity outside of Rac1 photoactivation zone. Each image of time-lapse sequence was corrected for photobleaching, exported to 16-bit format using the LSM program, and analyzed using MetaMorph software.

VE-cadherin–GFP density at AJs was assessed as mean fluorescent intensity of GFP at AJs (above intracellular background) within the photoactivation zone. The increase in VE-cadherin fluorescence after activation of PA-Rac1 or uncaging of cRO above the initial basal values was expressed as relative fluorescent amplitude. The rate constants were calculated by fitting data to a single exponential rise curve.

VE-cadherin–GFP adhesion area was determined by selecting pixels of high intensity values (i.e., VE-cadherin adhesion) using a threshold mapping function in MetaMorph software. The percentage of threshold area within the photoactivation zone was measured before and at 5 min of PA-Rac1 photoactivation.

VE-cadherin kinetics at AJs was assessed with VE-cadherin–Dendra2. The changes in mean fluorescent intensities at 488- and 543-nm maximum emission spectra were measured inside the photoconversion zone. The rate constants for VE-cadherin dissociation (at 543 nm) and association (at 488 nm) were calculated from decay and recovery kinetics, respectively, after VE-cadherin–Dendra2 photoconversion. The rate of VE-cadherin–Dendra2 lateral movement within the cell–cell junction was determined from the rate of fluorescence lateral movement at λ = 543 nm outside of the photoconversion region using kymograph analyses.

For FRET analysis, YFP and CFP channels were corrected for photobleaching. Photobleaching coefficient obtained for YFP was used to correct both YFP and FRET images. The CFP image was corrected using the CFP photobleaching coefficient. The YFP image was used to create a binary mask with a value of 0 outside the cell and a value of 1 inside the cell. To generate a ratio image, the FRET image was first multiplied by a binary mask image and then divided by the CFP image. The ratio images were rescaled to the lower value, and a linear pseudocolor table was applied to generate the color-coded image map. For quantification of FRET data shown in Fig. 4 C and D, a region that included only the thick area (i.e., junctions or membrane overlap) between the two cells within the photoactivation zone was used. The integrated intensity for FRET and CFP within this region was measured for each frame, and numbers were divided to get the FRET/CFP ratio for each frame. Ratios were plotted over time and fitted to an exponential decay. The change in ratio from time 0 to the time at which the curve reaches a plateau was determined. Importantly, quantifications in Fig. 4 C and D were based on the integrated intensities and not from the FRET/CFP ratio images (as shown in Fig. 4 B). The relative activity of RhoA and tension were expressed as mean pixel intensity of FRET/CFP ratio within the irradiation zone at AJs. Changes in RhoA activity or tension across VE-cadherin adhesion were determined before and after PA-Rac1 activation or cRO photo-uncaging.

Computational modeling and parameter estimation

COPASI software was used to fit our experimental data as well as to perform parameter estimation. We assumed that all VE-cadherin–Dendra2 molecules emitted green fluorescence before photoconversion and obeyed rules established by Eqs. 1a and 1b.

The equilibrium relations between different states of VE-cadherin species were determined from Eqs. 1a and 1b as follows:

\[ \frac{da}{dt} = j - k_a a - 2k^\gamma a^2 + 2k^a d - k^-a a - k^a d, \]  
(3a)

\[ \frac{da^*}{dt} = -k_a a^* - k^\gamma a^2 + k^a d, \]  
(3b)

\[ \frac{dd}{dt} = k^\gamma a^2 - k^a d, \]  
(3c)

\[ \frac{dd^*}{dt} = k^\gamma a^2 - k^a d, \]  
(3d)

\[ \frac{dd^**}{dt} = k^\gamma a^2 - k^a d. \]  
(3e)

The equilibrium relations presented in Eqs. 2a and 2b are shown with γ, the fraction of photoconverted molecules (ranging from 0 to 1). The following set of equations allows us to derive the initial equilibrium relations for the model that includes photoconverted and nonphotoconverted VE-cadherin species, under the assumption that the monomers are photoconverted independently of one another even if they form a trans-dimer.

\[ a = (1 - \gamma) a, \]  
(4a)

\[ a^* = \gamma a, \]  
(4b)

\[ d = \frac{a^2}{K_d} + \frac{(1 - \gamma)^2 a}{K_d}, \]  
(4c)


References


