Low-affinity binding in cis to P2Y$_2$R mediates force-dependent integrin activation during hantavirus infection

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ABSTRACT Pathogenic hantaviruses bind to the plexin-semaphorin-integrin (PSI) domain of inactive, $\beta_3$ integrins. Previous studies have implicated a cognate cis interaction between the bent conformation $\beta_3/\beta_3$ integrins and an arginine-glycine-aspartic acid (RGD) sequence in the first extracellular loop of P2Y$_2$R. With single-molecule atomic force microscopy, we show a specific interaction between an atomic force microscopy tip decorated with recombinant $\alpha_{IIb}\beta_3$ integrins and (RGD)P2Y$_2$R expressed on cell membranes. Mutation of the RGD sequence to RGE in the P2Y$_2$R removes this interaction. Binding of inactivated and fluorescently labeled Sin Nombre virus (SNV) to the integrin PSI domain stimulates higher affinity for (RGD)P2Y$_2$R on cells, as measured by an increase in the unbinding force. In CHO cells, stably expressing $\alpha_{IIb}\beta_3$ integrins, virus engagement at the integrin PSI domain, recapitulates physiological activation of the integrin as indicated by staining with the activation-specific mAb PAC1. The data also show that blocking of the Go$_{13}$ protein from binding to the cytoplasmic domain of the $\beta_3$ integrin prevents outside-in signaling and infection. We propose that the cis interaction with P2Y$_2$R provides allosteric resistance to the membrane-normal motion associated with the switchblade model of integrin activation, where the development of tensile force yields physiological integrin activation.

INTRODUCTION Pathogenic hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Sin Nombre virus (SNV) is a Category A pathogen that causes the most severe form of HCPS with case fatality ratios of 30–50% (Vaheri et al., 2013; Hjelle, 2014). Inhalation of excreta from hantavirus rodent hosts is the primary route of infection. From the lung parenchyma, they are taken up in phagocytes and transported to draining lymph nodes, where they establish infection and then disseminate to various organs, where they replicate principally in vascular endothelial cells (Hjelle, 2014). Tissue tropism involves the vascular endothelium of the heart, kidney, lung, and lymphoid organs. HCPS causes dysregulation of the endothelial barrier function edema and focal hyaline membranes (Zaki et al., 1995; Koster and Mackow, 2012; Mackow et al., 2014). Death results from low-output cardiogenic shock and multi-organ failure (Jonsson et al., 2008, 2010; Vaheri et al., 2013).
Currently there is no approved, effective therapy. Treatment of severe cases is supportive and typically includes the use of extracorporeal membrane oxygenation (ECMO) (Wernery et al., 2011).

Previous studies have identified β3 and β1 integrins as the primary receptors for pathogenic and nonpathogenic hantaviruses, respectively (Gavrilochkaya et al., 1998; Mackow and Gavrilochkaya, 2009). Pathogenic hantaviruses bind to the PSI domain of the bent/ inactive form (Figure 1A) of the human β3 integrin subunit of αvβ3 and αvβ6 integrins (Raymond et al., 2005). However, the mechanism of integrin activation postoccupancy of the PSI domain is unknown.

Integrins are heterodimers of noncovalently associated α- and β-subunits that regulate cell-surface adhesion through changes in the conformation in their ectodomains (Luo and Springer, 2006). Inactive, low-affinity integrins assume a bent structure. Activated integrins adopt a higher affinity for extracellular ligands and convert to an extended or open conformation (Calderwood, 2004). Integrins bind to signal by causing the binding of adaptor molecules such as talins, kindlins, 14-3-3 proteins, or filamins to the integrin β-subunit (Gahmberg et al., 2009). The engagement of adaptor proteins is necessary for coupling to the underlying actin cytoskeleton, which enables the induction of full integrin activation (Gahmberg et al., 2009). High-affinity integrin binding to the ECM initiates outside-in signaling, which leads to cell spreading, retraction, migration, and proliferation (Takagi et al., 2002; Chen et al., 2006; Zhu et al., 2007; Shen et al., 2012). More recent studies have shown that outside-in signaling requires the binding of a G protein subunit, Gα13, to the cytoplasmic domain of the β3 integrin, which overlaps with the talin binding domain (Gong et al., 2010; Shen et al., 2013).

The linkage between the cytoskeleton and integrins facilitates transduction of force across the membrane (Hu et al., 2007; Kanchanawong et al., 2010). In this way, integrins guide forces generated by the cytoskeleton onto the ECM to produce the traction force necessary for cell adhesion and migration (Volk et al., 1990; Welch et al., 1990; Vogel and Sheetz, 2006; Yu et al., 2015). Recent studies have shown that integrin conformational state is correlated with force exertion (Zhu et al., 2008; Nordenfelt et al., 2016). Force application is optimal when integrins engage immobilized ligands that resist traction force applied to the ligands through the integrin, which results in increased signaling output (Schurpf and Springer, 2011; Nordenfelt et al., 2016). Conversely, soluble ligands cannot transduce tensile force, which is based on allosteric resistance to translation (Schurpf and Springer, 2011; Nordenfelt et al., 2016) (Figure 1).

The PSI domain is believed to be a locus of integrin activation, based either on activating amino acid substitutions (Zang and Springer, 2001; Arnaout, 2002; Sun et al., 2002; Xiong et al., 2004) or epitope location of activation-sensitive monoclonal antibodies such as AP5. AP5 antibodies recognize the hexapeptide sequence GPNICT (residues 1–6) at the amino terminus of β3 integrins of extended conformation (Honda et al., 1995; Smaghe et al., 2010). Nevertheless, studies have shown that polarized epithelial and endothelial cells are susceptible to hantavirus infection exclusively from the apical surface but not the basolateral side (Krautkramer and Zeier, 2008; Buranda et al., 2014), despite integrins being sorted to the latter domain (Drubin and Nelson, 1996). A glycosylphosphatidylinositol-linked protein, CD55/decay-accelerating factor (DAF), which is sorted to the apical surface of polarized cells (Brown et al., 1999), was identified as a cognate cell entry coreceptor for pathogenic hantaviruses (Krautkramer and Zeier, 2008). However, studies of the functional mechanisms of integrin activation associated with these proteins are not yet available. It is known that pathogenic hantaviruses bind to DAF with high-affinity (Buranda et al., 2014) and that H319 anti-DAF antibodies can be used to block infection in polarized cells but not in isotropic cells (Krautkramer and Zeier, 2008; Buranda et al., 2014).

In this study, we sought to investigate an alternative effector for integrin activation during hantavirus infection. We targeted the P2Y2...
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Materials and Methods

RT–PCR was performed in triplicate for each well by Taqman assay as described under Materials and Methods. (B) Plot of P2ry2 mRNA knocked down in CHO-K1 stably transfected with αβ3 integrin measured 24 h after siRNA transfection, **p < 0.05.

RESULTS

AFM detection of RGD-specific interactions in astrocytoma cells expressing exogenous P2Y2R

To explore the binding specificity between β3 integrin and P2Y2R we used single-molecule AFM to probe for changes in adhesive interactions between an AFM cantilever functionalized with recombinant αβ3β3 integrins and astrocytoma 1321N1 cells and CHO-K1 cells. The 1321N1 astrocytoma cells that were transfected with hemagglutinin- (HA) tagged human P2Y2R or HA-tagged low-affinity RGE mutant P2Y2R were tested for functional P2Y2 receptor activity and for their ability to be immunoprecipitated with HA antibody, as demonstrated in Liao et al. (2007). Figure 2A shows the expression levels of P2ry2 mRNA in the cell lines that we used in this study. Figure 2B indicates that small interfering RNA (siRNA) transfection of CHO-K1 cells achieved 60% knockdown efficiency of P2ry2 mRNA in CHO-K1.

The experimental setup for single-molecule interactions between an AFM tip decorated with recombinant αβ3β3 integrins and cells expressing P2Y2R is shown in Figure 3. To enable measurement of single-molecule interactions between the AFM tip and cellular membranes, we optimized compression force and contact duration to attain an adhesion frequency of ∼33% in the measurements. Figure 3B shows a typical force displacement scan recording the interaction between an AFM tip and substrate in our experimental setting. Single-molecule adhesion between the tip and cell membrane appears as a hysteresis between the engagement and retraction events of the trace. The force jump that accompanies the unbinding of the adhesion is indicated as FU in Figure 3B.

During a force-displacement scan, pulling an integrin across the cell surface is expected to encounter nonspecific adhesions with cell-surface proteins. With 1321N1 cells, we first sought to determine whether expression of exogenous RGD-P2Y2R would lead to specific adhesions connected to P2Y2R expression. Substitution in the RGD sequence to RGE reduces affinity by three orders of magnitude (Hautanen et al., 1989). Thus, by comparing AFM measurements using cells transfected with RGD-P2Y2R to RGE-P2Y2R, we expected to demonstrate specificity for (RGD)–P2Y2R. To assess the strength of single-molecule interactions, we limited the contact between the AFM cantilever and 1321N1 cells expressing RGD-P2Y2R by minimizing contact force (150 pN) and contact time (0.08 s). Under these conditions, an adhesion frequency of ∼33% in the force measurements emerged for RGD-P2Y2R cells. In this way, the probabilities of forming single, double, and triple adhesion bonds between AFM tip and surface were 81%, 16%, and 2%, respectively (Chesla et al., 1998). These settings were used to record adhesion events between the AFM tip and the three cell types (P2Y2R-null WT 1321N1, RGE-P2Y2R, and RGD-P2Y2R expressing cells). For each cell type, a minimum of 400 repeated force scans was conducted using multiple cell-probe pairs. A large number of scans ensured robust statistics. As shown in Figure 4A under the same contact force, duration, and scan rates, the adhesion frequencies associated with WT and RGE-P2Y2R were comparable (∼10%), whereas the AFM probe adhered to the RGD-P2Y2R cells with higher frequency (∼33%). We plotted samples of 10 consecutive force-distance scans for the three cell types. The WT (Figure 4B) and RGE-P2Y2R (Figure 4C) cells displayed adhesions of comparable magnitudes appearing as hysteresis on their respective fourth and sixth traces. Among the 10 consecutive traces associated with RGD-P2Y2R expressing cells, four adhesion events were apparent in traces 2, 4, 6, and 9 (Figure 4D). The magnitude of the unbinding force in the fourth trace was comparable to the WT adhesion events.
Measurements of $\alpha_{\text{IIb}}\beta_3/\text{RGD}$ interactions in siRNA P2Y$_2$R knockdown and Mn$^{2+}$- and SNV-treated CHO-K1 cells

The preceding experiments demonstrated that the $\alpha_{\text{IIb}}\beta_3$-functionalized AFM specifically recognizes exogenous RGD$^{\text{P2Y}_2}$R and not RGE$^{\text{P2Y}_2}$R. (A) Adhesion frequency of $\alpha_{\text{IIb}}\beta_3$-functionalized AFM cell membranes of P2Y$_2$R-null WT, RGE$^{\text{P2Y}_2}$R, and RGD$^{\text{P2Y}_2}$R 1321N1 cells under similar AFM scan conditions. Values are means ± SD of force scans of greater than or equal to five different cell measurements. Statistical significance was determined by Student’s t test **** $p < 0.0001$. (B–D) Representative single force distance (retraction) curves between $\alpha_{\text{IIb}}\beta_3$-functionalized AFM tip and the surface of parent WT 1321N1 cells devoid of P2Y$_2$R, and 1321N1 cells expressing exogenous RGE$^{\text{P2Y}_2}$R and RGD$^{\text{P2Y}_2}$R. The fractions shown represent the number of positive force scans in the numerator and the denominator, the total number of scans for each cell type.

Measurements of $\alpha_{\text{IIb}}\beta_3$/RGD interactions in siRNA P2Y$_2$R knockdown and Mn$^{2+}$- and SNV-treated CHO-K1 cells

The preceding experiments demonstrated that the $\alpha_{\text{IIb}}\beta_3$-functionalized AFM specifically recognizes exogenous RGD$^{\text{P2Y}_2}$R, expressed in astrocytoma cells. To further explore the specificity of the integrin/RGD$^{\text{P2Y}_2}$R interaction, we turned to CHO-K1 cells that express endogenous RGD$^{\text{P2Y}_2}$R but are devoid of $\beta_3$ integrins. We optimized contact force (200 pN), contact time (0.15 s), and retraction speed (3.7 µm/s) to achieve an adhesion frequency of $\sim 33\%$ between the AFM tip and CHO-K1 cells. Figure 6A shows a series of 10 consecutive force–distance curves indicating the interaction of the integrin functionalized tip with WT CHO-K1 cells. The three of 10 unbinding events found in the fourth, sixth, and ninth traces correspond to specific interactions between the AFM tip and RGD$^{\text{P2Y}_2}$R. A much weaker unbinding event in the seventh trace was attributed...
and siRNA P2Y2R knockdown CHO-K1. We found a >50% drop in adhesion frequency events on the surfaces of P2Y2R knockdown cells compared with the WT CHO-K1 cells (Integrin vs. siRNA vs. CHO-K1 in Figure 6B). This result was consistent with the knockdown efficiency of P2Y2R shown in Figure 2B. We also performed single-molecule force displacement scans in the presence of integrin-activating cation Mn2+ (Dransfield et al., 1992) (Integrin w/Mn2+ vs. CHO-K1 in Figure 6B). In this experiment, 2 mM Mn2+ was added to the cell sample chamber before AFM engagement. In a separate set of experiments, the AFM probe was incubated with UV inactivated and fluorescently labeled SNV (Buranda et al., 2010, 2014) to allow the virus to attach to the immobilized integrins. The probe was washed once and used in force retraction measurements (Integrin w/SNV vs. CHO-K1 in Figure 6B). A peptide derived from the β3 integrin PSI domain is a competitive inhibitor of SNV binding to β3 integrins (Buranda et al., 2010). We inhibited fluorescently labeled SNV from binding to the integrin-decorated cantilever by preincubating the SNV with 6 µM PSI peptide (Integrin w/SNV & PSI vs. CHO-K1 in Figure 6B). The force-distance scans were performed under comparable conditions for probing unitary adhesion events. CHO-K1 cells are devoid of αv and β3 integrins and known to express α5β1 endogenously. We tested whether SNV interacted with CHO-K1 membranes. Therefore SNV particles were directly conjugated to the AFM tip using the same method for immobilizing the integrin. Pulling an SNV functionalized cantilever on the surface of CHO-K1 found weak nonspecific adhesions (SNV vs. CHO-K1 in Figure 6B). This result was expected because SNV does not interact with endogenous β3 integrins (Gavrilovskaya et al., 1999). We therefore used CHO-A24 cells, which are stably transfected with recombinant αIIbβ3 (Zhu et al., 2007) (SNV vs. CHO-A24 in Figure 6B). Sample histograms of unbinding forces measured under the conditions described above are shown in Figure 7 and discussed below.

Binding of SNV to integrin PSI induces integrin activation

Using force histogram data, we next explored the effect of SNV binding to the PSI domain of αIIbβ3 on the αIIbβ3/α5β1 interaction. The most frequent unbinding force of the low-affinity control (Figure 7A) was 55.0 pN. The mode of the nonspecific adhesions is similar to WT and RGE.
Using cloned CHO cell lines, CHO-A24 cells that stably express functionalized cantilever had negligible adhesive interactions pN and high-affinity, 65.0 pN (Figure 7F). As noted above, SNV-domain peptide partially blocked SNV binding to the AFM tip and yielded a bimodal force histogram with two peaks implying two bound states representing low affinity, 55.0 pN (Figure 7E) and 65.0 pN (Figure 7H), which was significantly higher than the integrin-specific measure shown in Figure 7D. This last result confirmed that SNV binds to the PSI domain with high-affinity and provides a basis for a future study in which we will characterize the affinity of SNV-PSI interactions during infection.

SNV-activated αβ3-RGD-P2Y2R bonds are more resistant to tensile force compared with Mn2+ activation

The preceding data show that the presence of Mn2+ and SNV caused the formation of higher affinity αβ3-RGD-P2Y2R bonds that are devoid of β3 integrins. Measurements of SNV-integrin (PSI) interaction measured in CHO-A24 cells stably expressing αβ3 are included. SNV-activated αβ3-RGD-P2Y2R bonds are more resistant to tensile force compared with Mn2+ activation. The preceding data show that the presence of Mn2+ and SNV caused the formation of higher affinity αβ3-RGD-P2Y2R bonds that are devoid of β3 integrins.
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Forces for breaking single integrin RGDp2Y\(_2\)R over a wide range of loading rates (300–7000 pN/s). The resulting dynamic force spectra (DFS), represented by a plot of most frequent unbinding forces of \(\alpha_{\text{IIb}}\beta_3\)-integrin/RGD interactions versus the logarithm of loading rate is shown in Figure 8. As predicted by the Bell-Evans model, the most frequent unbinding force, \(f^*\) in Eq. 2) is linearly dependent on the logarithm of the loading rate \(r\) (in Eq. 2) (Bell, 1978; Evans and Ritchie, 1997). The two-dimensional dissociation rates in the absence of force \(k^0\) (in Eq. 2), as well as the barrier widths \(\gamma\) in Eq. 2, were determined from a linear regression fit to Eq. 2 (Figure 8A). The best-fit parameters for \(k^0\) were 0.39 s\(^{-1}\), 0.097 s\(^{-1}\), and 0.13 s\(^{-1}\) for resting, Mn\(^{2+}\) and SNV-treated integrins, respectively. These force-free dissociation rates indicated that the activation of \(\alpha_{\text{IIb}}\beta_3\)-integrin by Mn\(^{2+}\) and SNV resulted in comparable increases (four- and threefold, respectively) in strengths of the MD tension (in the range of 0–100 pN), on the lifetimes of \(\alpha_{\text{IIb}}\beta_3\)-integrin interactions in the presence of SNV and Mn\(^{2+}\). The semi-logarithmic plot of bond lifetimes versus applied tensile force shows an exponential decrease of bond lifetimes with increasing tensile force (Figure 8C). The graph shows that the force-dependent decreases in the bond lifetimes of SNV and Mn\(^{2+}\)-activated integrins diverge with increasing tensile force. The SNV activated bond appears to be more resistant to force compared with the resting and Mn\(^{2+}\)-activated bonds. This behavior is rationalized in Figure 8D. According to the Bell-Evans model, a pulling force \(f\) (in Eq. 1) distorts the intermolecular potential of a ligand-receptor complex, leading to a lowering of the activation energy and a shortening of the bond lifetime. As illustrated in Figure 8D, the net effect of tensile force on bond dissociation is attenuated by \(f\). In this way, the applied tensile force is less attenuated during SNV activation compared Mn\(^{2+}\) activation because of the smaller value of \(\gamma\) associated with the former. In summary, the DFS data suggest that, in a nonphysiological setting, SNV and Mn\(^{2+}\) induce stabilization of the \(\alpha_{\text{IIb}}\beta_3\cdot\text{RGDp2Y}_2\)R bond to a comparable extent in the absence of a tensile force (i.e., \(k^0\) (SNV) \(\sim k^0\) (Mn\(^{2+}\))). For our purposes, this finding is important because it allows us to make estimates on the scale of SNV-induced activity based on what is known about the effect of Mn\(^{2+}\) on induced integrin activity (reviewed in Ye et al., 2012).

Occupancy of the PSI domain induces physiological integrin activation in living cells

The canonical switch-blade model for integrin activation postulates that integrins undergo conformational changes involving integrin extension and an opening of the headpiece leading to high affinity (Shimaoka et al., 2002; Schurpf and Springer, 2011; Zhu et al., 2013). We tested whether SNV binding to the PSI domain caused conformational changes in \(\alpha_{\text{IIb}}\beta_3\) similar to physiologically activated \(\alpha_{\text{IIb}}\beta_3\) (cf. Figure 1). The IgM mAb PAC1 with an RGD integrin-targeting site (Abrams et al., 1992) selectively binds to the open conformation fibronogen-binding site of physiologically active \(\alpha_{\text{IIb}}\beta_3\) integrins, with \(~1\) nM affinity (Hantgan and Stahle, 2009). It is used as a surrogate marker for physiologic integrin activation (O’Toole et al., 1990; Du et al., 1991; Ye et al., 2012). We used CHO-A24 cells stably expressing wild-type \(\alpha_{\text{IIb}}\beta_3\) integrin and CHO cells expressing the \(\alpha_{\text{IIb}}\cdot\text{W968C/B3I693C mutant} \) (C3) for this assay (Zhu et al., 2007). In the C3 cells, the \(\alpha_{\text{IIb}}\) 968 tryptophan (W) and the 693 isoleucine (I) residues were replaced with cysteines to prevent the separation of the cytoplasmic domains through constitutive disulfide bond formation (Luo et al., 2004) (Figure 9). Exposure of CHO-A24 and CHO-C3 cells to SNV for 5 min produced a threefold increase in PAC1 binding to A24 cells compared with the resting state (Figure 9A), with no significant change in PAC1 staining of C3 cells in the same setting (Figure 9B). These results indicated that transmembrane domain separation is required for the high-affinity integrin that is induced by SNV binding.

Integrin activation to full extension is dependent on the transmission of mechanical force through the integrin \(\beta\) subunit (Figure 1) (Gahnberg et al., 2009; Nordenfelt et al., 2016). To test the idea that integrin tension was required for stabilizing the fully active state (Zhu et al., 2008), in our experiments, we used the soluble fibronectin hexapeptide GRGDS to disrupt the presumed cis interaction of \(\alpha_{\text{IIb}}\beta_3\) with RGDp2Y\(_2\)R in suspension cells. The affinity constant, \(K_d\), for GRGDS binding to a low-affinity purified \(\alpha_{\text{IIb}}\beta_3\) headgroup is \(-0.4\pm 0.26\) \(\mu\)M (Lin et al., 2016). Elsewhere GRGDS was shown to inhibit fibrinogen binding to purified \(\alpha_{\text{IIb}}\beta_3\) with an IC\(_{50}\) of 2.0 \(\mu\)M (Suehiro et al., 1996). PAC1 (1:10 dilution) and 1 \(\mu\)M GRGDS were coadministered to 20,000 CHO-A24 cells in suspension, before exposure of the cells to SNV. Staining of CHO-A24 cells with PAC1 in the presence of 1 \(\mu\)M GRGDS did not rise above background in SNV-stimulated cells compared with mock-treated cells (Figure 9C).
Western blot analysis of SNV N-protein expression as described in A24 cells were infected in the presence of various titrations (0.3–10 μM) remained obstructed. In a second set of experiments, CHO-A24 cells were infected in suspension with 0.1 moi SNV for 30 min. Cells were then suspended in 20 µl media containing 1:10 dilution PAC-1 antibody, which abrogates cis-interaction of the integrin and RGDp2Y2R, inhibits infection of suspension CHO-24 cells. CHO-A24 cells were infected in suspension with 0.1 moi SNV for 30 min. Cells were then washed in low pH media three times and then incubated in normal media for 24 h and then assayed for viral N-protein.

This result suggests that competitive inhibition of the relatively low-affinity α9β3-RGDp2Y2R interaction by GRGDSP prevented the integrin from achieving full activation post SNV binding. In this way, the cognate binding site for PAC1 (Kd = 1 nM) (Hantgan and Stahle, 2009) remained obstructed. In a second set of experiments, CHO-A24 cells were infected in the presence of various titrations (0.3–10 μM) of GRGDSP. Cell entry and replication by SNV was analyzed by Western blot analysis of SNV N-protein expression as described in the methods. Loading controls of the gels were established by using a typical standard housekeeping gene, calnexin, on the same membrane. Quantitative analysis of the gel was performed using a Bio-Rad Molecular Imager. The data indicate that GRGDSP inhibited infected infection (Figure 9D). GRGDSP was also shown to inhibit infection in telomerase-immortalized human microvascular endothelial cell line (TIME) cells (Supplemental Figure S1B). Our results therefore demonstrate that SNV-induced global conformational changes in integrin conformation that are necessary for PAC1 binding and infection require the integrin to engage an immobilized ligand (Schurpf and Springer, 2011).

Gα13 binding to β3 integrin mediates infection

Integrin binding to immobilized ligands mediates outside-in signaling. Recent studies have shown that the heterotrimeric Gα13 protein binds to a fully activated ligand-occupied integrin (Gong et al., 2010). We therefore explored whether a myristoylated peptide, mP6, (MVR-SEEER-OH) (Shen et al., 2013), which blocks the binding of Gα13 to the cytoplasmic domain of β3 integrin, prevents outside-in signaling and infection of cells by SNV. Outside-in signaling stimulates cytoskeletal remodeling, receptor clustering (Qin et al., 2004), internalization, and trafficking. GTP loading of several GTPases was used as a surrogate measure of SNV-induced integrin activation as previously shown (Buranda et al., 2013). We used a multiplex G-trap effector binding assay which employs a quantitative flow cytometric read-out (Buranda et al., 2013) (Figure 10). We measured GTP loading of (Rap1) associated integrin activation (Bos, 2005), cytoskeletal remodeling (RhoA, Rac1) (Burridge et al., 2004) and cargo trafficking (Rab7) (Feng et al., 1995) within 5 min after exposure to SNV to capture early events of integrin activity. mP6 blocked the GTP loading of Rac1, Rap1, and Rab7 (Figure 10, B–D). GTP loading of RhoA was minimal, which was consistent with an expected down-regulation of Rho signaling during the initial wave of Gα13-mediated outside-in signaling (Figure 4F in rGong et al. [2010] and Figure 3G in Shen et al. [2013]).

mP6 is known to inhibit Gα13–β3 interaction without disrupting talin-dependent integrin functions (Shen et al., 2013), and therefore we investigated how mP6 affects chemically induced inside-out signaling under our experimental conditions. Intracellular free Ca²⁺ ([Ca²⁺]i) and Rap1GTP have been implicated in the activation of inside-out signaling for integrins (Crittenden et al., 2004). We therefore exploited the fact that thapsigargin (TG) (Thasstrup et al., 1990) stimulates the discharge of intracellular Ca²⁺ stores to gain the [Ca²⁺]i-mediated inside-out signaling function in CHO-A24 cells (Figure 10D). We tested the effect of mP6 in an infection assay measuring the production of viral N-protein. mP6 inhibited infection of CHO-A24 cells (Figure 10E). Thus the results confirmed that SNV-induced integrin outside-in signaling and infectivity required Gα13 binding to the β3 cytoplasmic domain. The infection of Tg-treated cells, replete with a sustained bolus of elevated [Ca²⁺], was enhanced by a factor of 2 (Figure 10E), and mP6 did not block SNV infection of Tg-treated cells. This result suggested that inside-out signaling not associated with SNV engagement dispenses the requirement of Gα13 binding to the β3 integrin tail for infection (Figure 10E).

We explored in several assays the functional significance of RGDp2Y2R-mediated integrin activation post-SNV binding. First, we measured SNV infectivity in P2Y2R-knockdown CHO-A24 cells and in mP6-treated and GRGDSP-treated TIME endothelial cells. siRNA knockdown of P2Y2R expression in CHO-A24 cells decreased SNV infectivity by 60% (Supplemental Figure S1A). mP6 and GRGDSP equally inhibited infection of TIME cells (Supplemental Figure S1B). These results further suggested cis binding to P2Y2R and binding of Gα13 to β3 were both required for SNV infection. Second, the P2Y2R...
is a Gαi coupled receptor. We speculated that inside-out signaling initiated by P2Y2R agonists, ATP, UTP, and ATPβS, a nonhydrolyzable form of ATP, could enhance SNV infectivity in CHO-A24, TIME, and Vero E6 cells. We initially compared SNV infectivity of CHO-A24 cells in regular media and media containing P2Y2R agonists or apyrase, a nuclease that acts extracellularly to hydrolyze ATP and ADP (Corriden et al., 2010). The exogenous P2Y2R agonists and nuclease had no significant effect on SNV infectivity of CHO-A24 cells (Supplemental Figure S1C). This result was not surprising as αiβ3 integrins expressed in CHO cells are known to be refractory to agonist stimulation (Watanabe et al., 2008). However, the CHO-A24 data suggested that binding of Gs13 to the RGD/P2Y2R-occupied integrin was independent of inside-out signaling as expected (Shen et al., 2013). On the contrary, UTP-stimulated a nominal two-fold increase in viral N-protein expressed in TIME cells (Supplemental Figure S1D). To obtain a more quantitative measure of this effect, we turned to Vero E6 cells, a modified kidney epithelial cell line from the African green monkey that lacks the interferon gene, which suppresses viral replication in normal Vero cells (Emeny and Morgan, 1979). SNV focus forming units (FFU) were assayed using serial supernatant samples of infected Vero E6 cells cultured in ATPβS replete media. The results show that at 7 d postinfection (pi), cells infected in ATPβS-replete media yielded a 10-fold increase in progeny SNV compared with cells infected without ATPβS (Supplemental Figure S1E). Thus, collectively, the data suggest that integrin bidirectional signaling synergistically stimulates SNV infectivity even if SNV induced outside-in integrin activation is sufficient for productive infection.

Cell polarity disrupts the cis interaction between P2Y2R and β3 integrin and infectivity

Adhesion of cells to the extracellular matrix and neighboring cells provides external cues that establish structurally and functionally distinct apical and basolateral membrane domains (Drubin and Nelson, 1996). As mediators of cell adhesion, integrins are sorted to the basolateral surface, (Drubin and Nelson, 1996) while the P2Y2R is sorted to the apical surface (Qi et al., 2005) (Figure 11A). We hypothesized that SNV infectivity of isotropic suspension cells was more efficient than polarized cells because nonpolarized cells allow for optimal cis interactions, where polarization segregated β3 integrins and RGD/P2Y2R. To test this idea, we plated

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**FIGURE 10:** Gs13 is an effector of SNV-induced GTPase activation and infection of CHO-A24 cells. (A) Histograms of a mixture of five bead populations with graded levels of red fluorescence to form a bead array. Each bead is functionalized with an effector molecule for Rac1 (Pak1), H-Ras (Raf), Rho A (Rhotekin), Rap1(Ral), and Rab 7 (Rilp). The beads act as sensors for GTP-GTPases in a cell lysis solution. Gates were used to select beads associated with known effector proteins labeled with green fluorescent antibodies. (B) Top panels show typical histograms of beads incubated in CHO-A24 cell lysates derived from cells treated with SNV and vehicle (0.1 DMSO), 250 µM DMSO solubilized mP6 and SNV, and resting cells. Bottom panels represent beads incubated in cell lysis buffer to determine nonspecific binding of multiplex antibodies. In 48-well plates 50,000 CHO-A24 cells in each well were exposed to purified and fluorescently labeled UV inactivated virus for 5 min. The reaction was quenched on ice, after which cells were lysed with radio immunoprecipitation assay buffer (RIPA) buffer. Subsequent incubations with effector beads and antibodies were performed at 4°C to limit GTP hydrolysis. The assay took 4 h to complete. (C) Respective plots of site occupancy/bead of active GTPases, determined from median channel fluorescence (MCF) of CHO-A24 cell-lysate histograms after correction for nonspecific binding. The error bars represent triplicate measurements for each target. (D) CHO-A24 cells were plated at confluence into a 96-well plate and then loaded with Fura 2AM and 10 µM BAPTA-AM following standard protocols and analyzed on a Biotek H2 Synergy Plate Reader. Thapsigargin (Tg, 10 µM) was used to stimulate the discharge intracellular Ca²⁺ stores in the Fura 2 AM-loaded CHO-A24 cells. The traces show fluorescence intensity of the Ca²⁺-sensitive Fura 2 AM in the presence of Ca²⁺ (Tg) or absence (Tg + BAPTA) after [Ca²⁺]. Each trace was background-corrected using fluorescence of nonstimulated cells. The traces are an average of three separate wells. (E) Cells were treated with 250 µM mP6 for 30 min. The 10 µM Tg was added to cells 5 min before infection with 0.1 moi SNV inocula and incubated for 30 min and washed in triplicate at low pH to remove unbound SNV and resuspended in fresh media and analyzed after 24 h. Data show comparison of SNV N-protein in CHO-A24 cells infected with SNV inocula among samples treated with mock treated (Veh.), mP6 (mP6), 10 µM Tg (Tg), and mP6 and 10 µM Tg (mP6+Tg). Error bars are SEs for the four data points shown in the graph, *p < 0.05.
Cells as DAF/CD55 provides an alternate pathway for integrin activation of integrins and P2Y\(_{\text{R}}\) (250,000 DAF/CD55 per cell) (Buranda et al., 2008), is highly expressed on Vero E6 cells. DAF/CD55, a cognate apical coreceptor for pathogenic hantaviruses, the infection rates for plated and suspension Vero E6 were similar.

CHO-A24, TIME, and Vero cells in a 48-well plate, at confluence in eight-well Nunc Lab-Tek chambers and allowed to propagate for 1–2 d. At room temperature, cells were fixed with 3% paraformaldehyde for 20 min and subsequently permeabilized with 0.2% Triton X-100 (in PBS) for 15 min. Cells were blocked for nonspecific staining with 1% BSA in PBS for 30 min and then incubated with mouse monoclonal anti-integrin \(\alpha_v\beta_3\) (1:40, Millipore MxH1, MAB81976) and rabbit polyclonal anti-P2Y\(_{\text{R}}\) (1:100, Abcam, ab10270) were used with Alexa 488 and Alexa 580, respectively.

(A) Confocal image of polarized Vero E6 cells expressing antibody-stained basolateral \(\alpha_v\beta_3\) and apical P2Y\(_{\text{R}}\). The top panel is a confocal image in which the focus plane was parallel to the monolayer (XY scan), whereas the bottom panel shows the focus plane as a vertical cross section of the monolayer (XZ scan). The white line indicated by the arrow in the XY scan indicates the path of the XZ scan. To enable visualization, Vero E6 cells were transiently transfected with P2Y\(_{\text{R}}\) and plated at confluence in eight-well Nunc Lab-Tek chambers and allowed to propagate for 1–2 d. At room temperature, cells were fixed with 3% paraformaldehyde for 20 min and subsequently permeabilized with 0.2% Triton X-100 (in PBS) for 15 min. Cells were blocked for nonspecific staining with 1% BSA in PBS for 30 min and then incubated with mouse monoclonal anti-integrin \(\alpha_v\beta_3\) (1:40, Millipore MxH1, MAB81976) and rabbit polyclonal anti-P2Y\(_{\text{R}}\) (1:100, Abcam, ab10270) were used with Alexa 488 and Alexa 580, respectively.

(B) Plot of SNV FFU/ml vs. cells in suspension and on plates. CHO-A24, TIME, and Vero E6 cells were plated at confluence in vitronectin treated 48-well plates for 24 h. Confluent cells in triplicate wells were inoculated with 0.1 moi SNV for 1 h, washed in low pH media, and then incubated in normal media for 24 h. Media from infected cells was collected after 24 h diluted and subjected to a 7-day focus assay in Vero cells (see Supplemental Figure S1E for typical SNV FFU). **p < 0.001.

**FIGURE 11:** Cell polarity disrupts the cis interaction between P2Y\(_{\text{R}}\) and \(\beta_3\) integrin and infectivity. (A) Confocal image of polarized Vero E6 cells expressing antibody-stained basolateral \(\alpha_v\beta_3\) and apical P2Y\(_{\text{R}}\). Conversely, CHO cells are devoid of DAF, while TIME cells (derived from microvascular cells of the foreskin) express low levels of DAF. These data provide additional evidence that cis interaction between \(\text{RGDP}P2Y_{\text{R}}\) and \(\beta_3\) integrin is necessary for SNV induced integrin activation, which leads to infection. Given the above result on receptor distribution in polarized cells, it is important to note that under normal physiological conditions integrins are expressed on the apical surface of vascular endothelial cells (Conforti et al., 1991, 1992). The luminal expression of integrins in vivo allows the potential for cis interaction between \(\beta_3\) integrins and P2Y\(_{\text{R}}\) in vivo to be exploited as a mechanism for cell entry by pathogenic hantaviruses.

**DISCUSSION**

In the present study, we investigate the role of P2Y\(_{\text{R}}\)-\(\beta_3\) integrin cis interaction in integrin activation during the first step of viral infection, the binding of SNV to low-affinity \(\beta_3\) integrins. We draw the following conclusions from the data. SNV binds to the PSI domain of the integrin and activates the integrin by stimulating an increase in its ligand binding affinity coupled with changes in conformation. Unbending of the integrin generates a tensile force between the integrin and the purinergic receptor in the plane of the membrane, which induces full integrin activation. For these studies, we used single-molecule force spectroscopy and multiple cell lines that were chosen because of targeted expression of receptor proteins relevant to the study. Wild-type astrocytoma cells are devoid of P2Y\(_{\text{R}}\) expression (Parr et al., 1994). Therefore, using wild-type 1321N1 cells and well-characterized stable transfectants of wild-type \(\text{RGDP}P2Y_{\text{R}}\) (positive control) and a nonfunctional \(\text{RGDP}P2Y_{\text{R}}\) (negative control) enabled us to demonstrate the specificity of the cognate interaction between \(\text{RGDP}P2Y_{\text{R}}\) and \(\beta_3\) integrin (Figures 4 and 5). The astrocytoma cells have been well characterized previously in biochemical experiments showing the possible cis integrin, \(\text{RGDP}P2Y_{\text{R}}\) interactions mediated G\(_{\alpha_12}\)- and Go-dependent signaling in \(\alpha_v\beta_3\) integrin, \(\text{RGDP}P2Y_{\text{R}}\) expressing cells but not \(\alpha_v\beta_3\) integrin, \(\text{RGDP}P2Y_{\text{R}}\) cells (Erb et al., 2001; Bagchi et al., 2005; Liao et al., 2007; Erb and Weisman, 2012). 1321N1 cells are devoid of \(\beta_3\) integrin subunits and are not permissive to productive SNV infection in our hands. We therefore used CHO-K1 cells that are devoid of DAF/CD55 (a coreceptor for SNV cell entry) to establish, by AFM, that interaction of SNV with the integrin PSI domain causes integrin activation. The AFM provides a unique capability of measuring the cell metabolism independent activation of integrins on the binding of SNV to the integrin PSI domain (Figure 7, C and D).

Under conditions established for unitary adhesive interactions, the frequency of adhesion events is governed by Poisson statistics (Chesla et al., 1998). A large number of scans ensured robust statistics. The frequency of adhesive interactions between the AFM tip and cell membranes depends on several factors. Surface decoration of the AFM tip with \(\omega_{\alpha_v\beta_3}\) was minimized to make sure that the preponderance of the observed rupture events was the result of single \(\alpha_v\beta_3\)-\(\text{RGDP}P2Y_{\text{R}}\) interactions. The topography of the cell membrane is also a contributing factor. Unlike the relatively smooth surface of CHO cells, the surface of astrocytes is known to have many projected structures such as dendrites. During the course of our studies, we found that differential design of the AFM cantilever was a critical determinant for establishing unitary binding events in astrocytes and CHO cells. Because of projections located on the surface of CHO cells, the surface of astrocytes is known to have many projected structures such as dendrites.
effective at limiting the probability of nonspecific interactions. Thus the frequency of binding events comprising \( \leq 33\% \) of total force displacement scans and \( \geq 95\% \) of unbinding events occurring in a single step (cf. step 4 in Figure 3B) indicated unitary adhesive interactions (Zhang et al., 2002; Rankl et al., 2008). At our established set-point for unitary adhesive interactions, the probability for weaker nonspecific interactions was \( \approx 10\% \) in 1321N1 cells (Figure 5) and \( \approx 6\% \) in CHO-K1 cells (Figure 6). The nonspecific events were solely present in WT and RGE 1321N1 cells, whereas both nonspecific and RGD-specific interactions were present in \( \text{ROP2Y}^2 \text{R} \) expressing 1321N1 and CHO-K1 cells. We found that the presence of Mn\(^{2+} \) and SNV induced an increase in the population of higher affinity interactions represented in the force histograms relative to quiescent samples (Figure 7). In the resting integrins, the histogram data show that only 35% of unbinding interactions are defined by Fu values \( >55 \) pN (the most frequent unbinding force of quiescent samples measured in CHO-K1 cells) (Figure 7A). For stimulated integrins, the population of adhesions above this threshold increases to 67% in the presence of Mn\(^{2+} \) (Figure 7B) and 70% in the presence of SNV (Figure 7D).

Mn\(^{2+} \) is a widely used positive control for integrin activation. However, the extent to which Mn\(^{2+} \) induces integrin activation is variable and depends on context and isotype of the target integrin (reviewed in Ye et al., 2012). While stimulation of \( \alpha_R \beta_3 \) integrin by Mn\(^{2+} \) is known to induce both integrin extension and change in affinity, Mn\(^{2+} \) stimulation produces only a change in affinity without the extension in \( \alpha_{IIb} \beta_3 \) integrin (Kamata et al., 2005). Elsewhere Cluzel et al. (2005) observed Mn\(^{2+} \)-induced clustering of \( \alpha_{IIb} \beta_3 \) integrins in focal complexes when attached to immobilized ligands. To extend the comparison between SNV- and Mn\(^{2+} \)-induced states of integrin activation, we analyzed the dynamic force spectrum (DFS) of resting Mn\(^{2+} \)- and SNV-treated samples. From the DFS analysis, we showed that Mn\(^{2+} \) and SNV exposure confers a \( \approx 3.5\)-fold decrease in the two-dimensional dissociation rate of the \( \alpha_{IIb} \beta_3 \text{-ROP2Y}^2 \text{R} \) bond in the absence of force. Using the Bell-Evans formalism, we found that the bond lifetimes of resting Mn\(^{2+} \)- and SNV-treated samples decreased monotonically with increasing tensile force, suggesting slip-bond dissociation (Dembo et al., 1988; Marshall et al., 2003) in all cases. However, the difference in the potential energy barrier widths for the \( \alpha_{IIb} \beta_3 \text{-ROP2Y}^2 \text{R} \) interaction, under SNV (0.35 nm) or Mn\(^{2+} \) (0.40 nm) activation, are small but consequential in that the narrower barrier width associated with SNV activity confers a nominally longer bond lifetime under tensile force (Figure 8C). In recent studies, the threshold limit of tensile force required to activate outside-in signaling has been set to 56 pN (Wang and Ha, 2013; Wang et al., 2015). Because SNV-activated \( \alpha_{IIb} \beta_3 \text{-ROP2Y}^2 \text{R} \) bonds are shown here to be more resistant to tensile force compared with Mn\(^{2+} \), we suggest that SNV activated \( \alpha_{IIb} \beta_3 \text{-ROP2Y}^2 \text{R} \) bonds are sufficiently stable to allow outside-in mediated adhesion signaling, like Mn\(^{2+} \) (Ye et al., 2012). In summary, these single molecule two-dimensional events could be extended to biologically relevant bulk experiments induced by other stimuli.

Biochemical evidence for cis interaction between integrins and P2Y2R is extensive. Studies to establish this association used human 1321N1 astrocytoma cells that are devoid of P2Y2R (Parr et al., 1994; Erb et al., 2001). A set of cells were stably transfected with the wild-type \( \text{ROP2Y}^2 \text{R} \) designed to test whether integrin interaction with the P2Y2R was an essential noncanonical effector for the \( G_\lambda \)PCR to couple to \( \alpha_{IIb} \beta_3 \) and G\( \lambda \) proteins. Nonfunctional RGE mutant P2Y2R served as negative controls. Evidence for cis interaction between \( \alpha_R \) integrins and the RGD domain of the P2Y2R was demonstrated in a series of papers that showed that the \( \alpha_R \) integrin coimmunoprecipitates with the \( \text{ROP2Y}^2 \text{R} \) but not with the nonfunctional \( \text{RGD} \)P2Y2R in 1321N1 astrocytoma cells (Erb et al., 2001; Bagchi et al., 2005; Liao et al., 2007; Erb and Weisman, 2012). The authors then showed that the presumptive \( \text{ROP2Y}^2 \text{R} \) integrin engagement conferred to the \( G_\lambda \)PCR the ability to activate the heterotrimeric G-protein \( \text{G}\lambda_{12} \), which stimulated RhoA signaling (Liao et al., 2007). Under these circumstances \( \text{G}\lambda_{12} \) associated with the wild-type P2Y2R and with \( \alpha_R \beta_3 \) integrins. Also, function-blocking antibodies directed against \( \alpha_R \beta_3 \) inhibited UTP-induced Rho activation and stress fiber formation. Conversely, UTP treatment of the RGE mutant neither caused \( \text{G}\lambda_{12} \) activation nor conferred RhoA activity (Liao et al., 2007).

SNV-permissive CHO-A24 cells stably expressing \( \alpha_{IIb} \beta_3 \) were used to establish the requirement of cis interaction for infectivity as follows. We first showed that SNV binding to the PSI domain recapitulates physiological integrin activation. SNV binding to the PSI domain in CHO-A24 cells induces full extension of the integrin ectodomain and opening of the headpiece as indicated by binding of the fibrinogen-mimetic PAC-1 mAb. We then showed that blocking of the \( \alpha_{IIb} \beta_3 \text{-ROP2Y}^2 \text{R} \) interaction, with a soluble GRGDSP peptide, inhibits full integrin activation and infection (Figure 9). siRNA knockdown of P2Y2R in CHO-A24 cells inhibits infectivity (Supplemental Figure S1). Cell polarization limits infection of CHO-A24 and physiologically relevant TIME cells by segregating \( \beta_3 \) integrins from the P2Y2R by differential sorting of the two receptors to the basolateral and apical domains, respectively (Figure 11 and Supplemental Figure S1). Integrin activation to an extended open structure is associated with a \( \approx 100 \) Å vertical shift of the integrin's ligand recognition domain away from the plasma membrane (Takagi et al., 2002; Chigaev et al., 2003, 2015). Thus extension displaces the head of the integrin above the \( \text{ROP2Y}^2 \text{R} \) (Figure 1). In this setting, one might imagine the development of tensile force from the integrin head pulling away from a membrane-anchored P2Y2R. Zhu et al. have described a mechano-mechanical model for bidirectional integrin activation that involves engagement to immobilized extracellular matrix ligands (Zhu et al., 2008). Thus, for this study, one might expect a combination of vertical and lateral (cis) tensile forces to be exerted during the change in conformation while being resisted by the membrane fixed \( \text{ROP2Y}^2 \text{R} \) (Figure 1B [5 and 6]) (Schurpf and Springer, 2011; Nordenfelt et al., 2016). Importantly, our study shows that SNV infectivity is inhibited when soluble GRGDSP abrogates possible cis interaction of the integrin with P2Y2R.

Integrin signaling is bidirectional and involves, inside-out and outside-in signaling processes. This process involves dynamic interactions of the integrins with cyto- and ectoplasmonic proteins (Shattil et al., 2010). External forces transmitted through integrins’ connection to the cellular cytoskeleton contribute to outside-in signaling (Iskratsch et al., 2014). Prototypical inside-out signaling, is initiated by receptor signaling (commonly a GPCR), where a downstream intracellular integrin activator, such as talin, binds to the cytoplasmic \( \beta \)-integrin domain (Calderwood et al., 1999). This leads to conformational changes that result in increased affinity for extracellular ligands. Integrin binding to extracellular ligands and subsequent changes in conformation initiates outside-in signaling, which in turn can generate signals that cause inside-out signaling. Recent studies have shown that inside-out and outside-in signaling phases are regulated by a “timeshare occupancy” of a common domain on the \( \beta \)-integrin cytoplasmic domain by talin and \( \text{G}\lambda_{12} \). These proteins are principle effectors for inside-out and outside-in signaling, respectively (Gong et al., 2010; Shen et al., 2013).

Our results indicate that the binding of \( \text{G}\lambda_{12} \) to the integrin \( \beta_3 \) subunit is a critical determinant of SNV infectivity. We demonstrate
that mP6, a membrane permeable peptide inhibitor of integrin–Gα13 binding inhibits cell signaling and infection. Of importance, our results indicate that the implicit binding of Gα13 to β3 integrin does not require upstream activity by a GPCR. In previous studies, Gα13 activation by a ligand-activated GPCR was viewed as an important upstream signaling component for Gα13–integrin binding (Gong et al., 2010; Shen et al., 2013). Ultimately, physiologic integrin activation in the presence of macromolecular ligands such as fibrinogen (but not small soluble peptides) are indispensable for Gα13 coupling to the integrin (Shen et al., 2013). Our data recapitulate the model shown in Figure 1, where the ligand-binding pocket of bent integrins faces toward the plasma membrane, which favors physical interaction with membrane-proximal RGD/P2Y2R. After SNV binds to the integrin PSI domain, activation of the integrin initiates switchblade-like conformational change and development of tensile force. The increase in PAC1 staining of SNV-activated cells indicates that the rupture of P2Y2R-integrin interactions occurs after the integrin is fully activated, which favors Gα13 binding to β3 integrin with higher affinity (Shen et al., 2013). It is now established that outside-in signaling is localized at focal adhesions in a process that includes the recruitment of adhesion molecules such as talin and vinculin (Askari et al., 2010; Yu et al., 2015). Termination of the tensile force results in integrin turnover with the exchange of adhesion related molecules by endocytic adaptor proteins (Puklin-Faucher and Sheetz, 2009; Yu et al., 2015; Nordenfelt et al., 2016). It is therefore likely that the rupture of the integrin–P2Y2R junction results in endocytosis. Studies of the signaling steps that lead to internalization are ongoing. In summary, our study reports a new mechanism for integrin activation post binding of SNV to the PSI domain of the integrin. The findings have potential impact for the discovery of novel therapeutic targets of hantavirus infection, due to a high case mortality ratio.

MATERIALS AND METHODS

Materials
Octadecyl rhodamine B chloride (R18), 5-octadecanoyl amino fluorescein (F18), BAPTA-AM, amine-reactive Alexa Fluor 488 carboxylic acid, and succinimidyl ester probe were purchased from Life Technologies (Carlsbad, CA). Recombinant DAF was purchased from R&D Systems. Rabbit polyclonal H319 anti-DAF antibody was purchased from Santa Cruz Biotechnology. Primary antibodies used for immunostaining were mouse monoclonal anti-integrin αβ3 (1:40, Millipore MsxHu, #MAB 1976), rabbit polyclonal anti-P2Y2 (1:100, Abcam, ab10270), and fluorescein isothiocyanate mouse anti-human PAC-1 (BD Biosciences). Secondary antibodies used were anti-mouse immunoglobulin G (IgG) Alexa488, anti-mouse IgG Alexa647, anti-mouse IgG Cy5, and anti-rabbit Alexa647, all from Molecular Probes. Phosphate-buffered saline (PBS) was purchased from Life Technologies. Tyrode’s buffer, dimethyl sulfoxide (DMSO), and Sephadex G-50 were purchased from Sigma. TRIS (10 mM or 25 mM Tris, 150 mM NaCl, pH 7.5) and HHB (30 mM HEPES, 110 mM NaCl, 10 mM KCl, 1 mM MgCl₂,6H₂O, and 10 mM glucose, pH 7.4) buffer, and Hanks balanced saline solution (HBSS) (0.35 g NaH₂CO₃, 0.049 g MgSO₄, and 1 mM CaCl₂ or 1 mM MnCl₂) were prepared under sterile conditions and stored in 50-ml tubes at -20°C. Chemical reagents sources and conditions of use are summarized in Table 1.

Effector proteins
The GST-effector chimeras consisting of the minimal GTPase binding domains (RBD) used for the studies were as follows. PAK-1 RBD, a Rac1 effector, and Raf-1 RBD, a RAS effector protein, were obtained from Millipore. Rhotekin-RBD, a Rho effector protein, was purchased from Cytoskeleton. RalGDS-RBD, a RAP1 effector protein, was expressed and purified from a plasmid kindly provided by Keith Burridge (UNC Chapel Hill) (Witchen and Burridge, 2008). GST-RILP RBD was purified as described in Buranda et al. (2013).

Cell culture
Vero E6 were purchased from the American Type Culture Collection and maintained in DMEM (Life Technologies) at 37°C in a water-jacketed 5% CO₂ incubator. The medium contains 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 2 mM l-glutamine.

CHO cells lines: Zhu et al. (2007) have generated stable CHO-K1 cell lines expressing wild-type recombinant αIIbβ3 integrins (A24) or disulfide mutant αIIbW968C/B3I693C (C3). In the C3, the αIIb 968 tryptophan (W) and the 693 isoleucine (I) residues were replaced with cysteines to prevent the separation of the cytoplasmic domains through constitutive disulfide bond formation (Luo et al., 2004). They showed that the mutant integrin was defective in adhesion-induced outside-in signaling (Zhu et al., 2007). CHO A24 were grown in DMEM/F12 HAM mixture (Life Technologies) with 2 mM l-glutamine and 1 mg/ml G418 (Sigma). CHO C3 cells were cultured in F-12K media (Cellgro, Mediatech), 100 U/ml penicillin/streptomycin, and 2 mM l-glutamine.

Human lung microvascular endothelial cell (HLMEVEC) and TIME were maintained in EBAM-2 Basal Medium with EGM-2 SingleQuot Kit supplements and growth factors (Lonza, Walkersville, MD).

Human 1321N1 astrocytoma cells were transfected with HA-tagged human P2Y2R, or HA-tagged low-affinity RGE mutant P2Y2R. The cells were tested for functional P2Y2 receptor activity and for their ability to be immunoprecipitated with HA antibody, as demonstrated in Liao et al. (2007). Wild-type (WT) human 1321N1 astrocytoma cells (lacking detectable expression of any subtype of P2Y receptor), human 1321N1 astrocytoma cells stably transfected with the human P2Y2 receptor (pLXSN vector with an mmo-terminal HA tag for detection with HA antibodies), and human 1321N1 astrocytoma cells stably transfected with the RGE mutant human P2Y2 receptor (pLXSN vector with an amino-terminal HA tag) were cultured in DMEM containing 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂, and 95% air (Bagchi et al., 2005). The amount of 500 µg/ml Genetec (G418, Life Technologies) was added to maintain the expression of pLXSN-P2Y2 receptor constructs in the transfected cells.

| Reagent (source) | Target; concentration | Incubation time | Solvent |
|------------------|-----------------------|----------------|---------|
| ATPγS, ATP, UTP (Sigma) | P2Y2R, Gaq; 100 µM | 0 | H₂O |
| BAPTA-AM (Life Technologies) | [Ca²⁺]; 10 µM | 30 min | DMSO |
| H319 (Santa Cruz) | DAF/CD55; 40 µg/ml | 1 h | H₂O |
| mP6 (New England Peptide) | G13; 250 µM | 30 min | DMSO |
| Tg (Sigma) | [Ca²⁺], stores; 10 µM | ±2 min | DMSO |

A24 cells were incubated with inhibitors or activators for the indicated time and concentration. Control samples were incubated with the solvent used to solubilize specific drugs.

TABLE 1: Signaling modulation assays.

Molecular Biology of the Cell
All cell lines are tested for the presence of mycoplasma at every cell passage using commercial kits such as the MycoAlert mycoplasma detection kit (www.lonza.com).

Cell imaging
Tight junction markers occludin or ZO1 were imaged in cell monolayers with Zeiss META or LSM 510 systems using 63 x 1.4 oil immersion objectives as previously described. One hundred fifty thousand cells were plated at confluency in eight-well Nunc Lab-Tek chambers (thermoscientific.com) or in inverted transwell filter supports and allowed to propagate for 2 d. At room temperature, cells were fixed with 3% paraformaldehyde for 20 min and subsequently permeabilized with 0.2% Triton X-100 (in PBS) for 15 min. Cells were blocked for nonspecific staining with 1% bovine serum albumin (BSA) in PBS for 30 min and then incubated with either anti-ZO1 (rabbit polyclonal from Abcam; 1:100 dilution in blocking buffer) or occludin (mouse mAb from Invitrogen; 1:200 dilution in blocking buffer) overnight at 4°C. Cells were then incubated with a secondary Alexa488 tagged secondary antibody (Millipore; 1:100 dilution in blocking buffer for 1.5 h at room temperature). Samples were washed and imaged on the confocal microscope. For receptor imaging, mouse monoclonal anti-integrin αvβ3 (1:40, Millipore MxShu, MAB#1976), and rabbit polyclonal anti-P2Y2 (1:100, Abcam, ab10270) were used with Alexa 488 and Alexa 580, respectively.

Knockdown of P2Y2R expression by siRNA
RNAi knockdown of P2Y2R in TIME was performed using P2Y2R siRNA (h): sc-42579 purchased from Santa Cruz Biotechnologies. siRNA for CHO cells was custom designed from a partial sequence of Cricetulus griseus and synthesized by Dharmacon. P2Y2R siRNA ribonucleotides (40 nM) were pooled from four target-specific duplex components (sense: 5′ U.C.U.G.C.A.A.C.U.G.U.G.G.G.C.A.U.U.U.U 3′ and antisense: 5′ A.A.U.C.G.C.A.C.C.A.G.U.U.G.C.A.G.A.U.U.U.U 3′; sense: 5′ C.A.G.C.U.G.G.G.A.G.C.G.A.-G.A.C.U.A.U.U.U.U 3′ and antisense: 5′ U.U.A.G.U.C.U.-C.G.U.C.C.C.A.G.C.G.U.U.U.U 3′; sense: 5′ G.G.G.A.U.-G.A.A.C.U.G.G.G.C.C.U.A.A.C.A.U.U.U.U 3′ and antisense: 5′ U.U.G.U.A.G.C.C.C.A.G.U.U.A.C.U.C.C.U.U.U.U 3′; sense: 5′ C.A.G.U.C.A.U.C.U.G.U.G.U.C.G.U.U.U.A.U.U.U.U 3′ and antisense: 5′ U.A.A.C.A.G.A.C.G.G.-A.U.G.A.C.G.U.G.U.U.U.U 3′) and was used to knock down P2Y2R in CHO-A24 cells expressing wild-type integrin (Zhu et al., 2007). Transfection was performed with Lipofectamine 2000 (Thermofisher) following the manufacturer’s protocol. Knockdown efficiency was determined by quantitative PCR.

Quantitative PCR
Total RNA was isolated from CHO A24 and TIME cells, and TaqMan gene expression assays were performed to determine the expression of P2Y2R in wild-type and siRNA knockdown cells. RNA was extracted using a Qiagen RNeasy kit (cat. no. 74104), quantified with a Thermo Fisher Scientific Nanodrop. PCR and TaqMan reactions were carried out using GeneAmp PCR System 9700 from ABI and Bio-Rad CFX96 Real-Time PCR Systems, respectively. Quantitative reverse transcription PCR (RT-PCR) was performed using primers for P2Y2R (Hs04176264_s1 FG from Life Technologies) and a standard curve derived from serial dilutions of a WT P2Y2R plasmid.

Production of Sin Nombre virus
We propagated and titered SNV in Vero E6 cells inside biosafety level 3 (BSL3) facilities and practices (CDC registration number C20041018-0267) (Bharadwaj et al., 1999). To inactivate SNV with UV radiation, we placed 100 µl of virus stock (isolate SN77734, typically (1.5–2) x 10⁶ FFU/ml). In each well of a 96-well plate we subjected the virus to UV irradiation at 254 nm for various time intervals (~5 mW/cm²) as described elsewhere (Buranda et al., 2010).

Fluorescence labeling of SNV
The envelope membrane of hantavirus particles was stained with the lipophilic lipid probe octadecyl rhodamine (R18) and purified as previously described (Buranda et al., 2010). The typical yield of viral preparation was 1 ± 0.5 x 10⁶ particles/µl in 300 µl tagged with ~10,000 R18 probes/particle. Small aliquots for single use were stored at ~80°C.

Infection assays
Virus inocula (0.1 moi) were added to cells in a serum-free medium and incubated for 30 min at 37°C. Various cell signal-inhibiting or -activating drugs were used at the concentrations shown in Table 1. Unbound virions were removed by triple washing, and cells were incubated for 24 h at 37°C. The infection was analyzed by a standard Western blot analysis of N-protein expression where cell lysates were boiled in SDS buffer and separated by 8% SDS–PAGE and transferred to a nitrocellulose membrane. SNV N protein was detected with αSNV/N (hyperimmunum rabbit anti-SN virus N protein) (Elgh et al., 1997) used at a 1:1000 dilution, with overnight incubation, washed, and then stained for an hour incubation with a secondary antibody (Peroxidase AffiniPure Goat Anti-Rabbit IgG; used at 1:1000 dilution) from Jackson Immuno Research Laboratories (cat. no. 111-035-003, lot no. 104668). The nitrocellulose membrane was then treated with an HRP substrate from Pierce, SuperSignal West Pico (product no. 0034077) for 5 min before imaging. Quantitative analysis of the gel was performed using a BioRad Molecular Imager, ChemiDoc XRS+ equipped with Image Lab Software 4.1. Equal loading was verified by the detection of β-actin or calnexin on the same membrane with the anti-β-actin (clone AC-74 used at 1:2000 from Sigma) or anti-calnexin (clone C20041018-0267; Bharadwaj et al., 1999). To inactivate SNV with UV radiation, we placed 100 µl of virus stock (isolate SN77734, typically (1.5–2) x 10⁶ FFU/ml). In each well of a 96-well plate we subjected the virus to UV irradiation at 254 nm for various time intervals (~5 mW/cm²) as described elsewhere (Buranda et al., 2010).

Focus assay
These experiments were performed under a protocol that was approved by the University of New Mexico Biosafety Committee. This protocol is typically applied to grow the virus in our lab (Buranda et al., 2010, 2013, 2014) The medium for propagating virus is typically limited in nutrients (2% FBS) to minimize cell proliferation. We inoculated Vero E6 monkey kidney cells with a low multiplicity of infection (moi) of virus (<0.01) and then incubated infected cells, serially collecting supernatants of the culture into aliquots for 1–9 d post-inoculation (pi) and freezing those supernatants down at ~80°C as 1-ml aliquots. The virus titers were determined via a Focus Reduction Neutralization Test (FRNT) (Bharadwaj et al., 1999). A 7-d FRNT assay was carried out to quantify the viral progeny in each serial sample using 1:10 and 1:1000 dilutions for samples collected on day 1 and day 7 samples, respectively. Viral antigen was visualized by addition of rabbit anti-SN virus nucleocapsid protein serum followed by peroxidase-conjugated goat anti-rabbit IgG and DAB/metal concentrate as substrate.

GTPase effector trap flow cytometry assay (G-trap)
This has been previously described (Buranda et al., 2013). CHO A24 were plated in 48-well plates and starved overnight in serum-free medium followed by stimulation with activator or...
inhibitor at concentrations and times shown in Table 1. After incubating cells with various inhibitors, activators, and SNV$^{18}$ for desired times, cells were lysed with 100 µl ice-cold RIPA buffer. For standardization, a fraction of the supernatant was collected to measure protein concentrations. Lysates were kept cold on ice at all times to limit hydrolysis of active GTPases. For each effector assay, 10,000 beads were added to 50 µg of protein in 100 µl of cell lysate in RIPA buffer. Optimizing the assay for each new lot of antibody is critical.

**Cytometry experiments**

Equilibrium binding interactions of fluorescent antibodies with cells or beads were analyzed by flow cytometry as described previously (Buranda et al., 2010, 2011, 2014).

**Atomic force microscopy measurements of individual integrin–P2Y$_2$R interactions**

Recombinant human α$_{IIb}$β$_3$ integrin (R&D Systems, Minneapolis, MN) was attached to an AFM cantilever (MLCT or MLCT-BIO-DC, Bruker Nano, Camarillo, CA) by covalently crosslinking the two using a heterobifunctional acetal-PEG27-NHS linker (Ebner et al., 2007; Wildling et al., 2011, MN). Recombinant heterodimeric soluble human α$_{IIb}$β$_3$ integrin was from R&D Systems (Minneapolis, MN). The protein contains the ectodomains of α$_{IIb}$ (Leu32-Arg993) and β$_3$ (Gly27Asp718) followed by an ACID peptide and a His(6) tag at the carboxyl terminus of α$_{IIb}$ and by a BASE peptide at β$_3$. The ACID/BASE coiled-coil peptides can stabilize the heterodimer and have been widely used in expressing soluble integrins (Takagi et al., 2002, 2003; Xiao et al., 2004; Bennett, 2005; Jeong et al., 2013; Ozawa et al., 2016). All single-molecule force measurements were conducted using a custom-designed apparatus designed for operation in the force spectroscopy mode (Zhang et al., 2002, 2004). The cantilever (320-µm-long by 22-µm-wide triangle) was calibrated with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°. Whereas the regular MLCT has a relatively sharp tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°, MLCT-BIO-DC offers a dull tip with an open angle of 35–40°. The cantilever (320-µm-long by 22-µm-wide triangle) was calibrated before modification with PEG. The cantilever showed very low nonspecific interactions further. With the MLCT-BIO-DC and additional 5% FBS, we were able to lower the nonspecific adhesions on astrocotoma to ~10%. We next determined whether SNV binding to the PSI domain causes a change in affinity at the RGD binding site in CHO-K1 cells. The positive control for integrin activation was 2 mM Mn$^{2+}$ added to the media (Zhang et al., 2002; Li et al., 2003; Trache et al., 2010; Ligezowska et al., 2011). For SNV assays, the cantilever was preincubated with fluorescently labeled neat SNV$^{18}$, or a mixture of SNV$^{18}$ and 25 µM PSI domain polypeptide (Buranda et al., 2010) to competitively block the interaction between the integrin functionalized cantilever and SNV$^{18}$. Association of SNV and the integrin functionalized AFM tip was confirmed by imaging SNV fluorescence on the AFM tip. The binding interaction between the AFM tip and binding sites was then determined from the deflection of the cantilever via a position-sensitive two-segment photodiode (Figure 3C). Experimental conditions allowing for measurement of single-molecule unbinding forces ($f_u$) of the α$_{IIb}$β$_3$-RGD site complex were established following a method described by Evans (2001).

Typically, 5–10 separate cells were probed for each setting. For each pulling speed, 200–600 force curves were recorded, which yielded ≥50 to ≥150 unbinding forces. Curve fitting was performed using IGOR Pro or Origin software by minimizing the chi-square statistic for the optimal fit. The unbinding force ($f_u$) of the α$_{IIb}$β$_3$-integrin/P2Y$_2$R complex was derived from the force jump that accompanies the unbinding of the complex (Figure 3B) (Hinterdorfer and Dufrene, 2006; Rankl et al., 2008).

**Dynamic force spectroscopy**

The Bell-Evans model, a theory to determine energy landscape properties, describes the influence of an external force on the rate of bond dissociation (Bell, 1978; Evans and Ritchie, 1997). According to this model, a pulling force, $f$, distorts the intermolecular potential of a ligand-receptor complex, which leads to a lowering of the activation energy and an increase in the dissociation rate $k_f$ as follows:

$$k_f = k_0 \exp\left(\frac{f \gamma}{k_B T}\right).$$

(1)

where $k^0$ is the dissociation rate constant in the absence of a pulling force, $\gamma$ is the linear distance (nm) of the transition state from the bottom of the potential well, $T$ is the absolute temperature, and $k_B$ is the Boltzmann constant. For a constant loading rate $r$, the most probable, or most frequent unbinding force $f^*$ is given by

$$f^* = \frac{k_B T}{\gamma} \ln\left(\frac{\gamma}{k^0 k_B \beta}\right) + \frac{k_B T}{\gamma} \ln(r) + \frac{k_B T}{\gamma} \ln(r).$$

(2)

Hence the Bell model predicts that $f^*$ is a linear function of the logarithm of the loading rate. Experimentally, $f^*$ was determined from the mode of the unbinding force histograms. The Bell model parameters were determined by fitting Eq. 2 to the plot of $f^*$ versus ln($r$).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism. Differences between the data were tested for statistical significance using Dunnett’s multiple comparisons or Student’s t test; $p < 0.05$ was considered statistically significant.

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