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Rap1b is critical for glycoprotein VI-mediated but not ADP receptor-mediated $\alpha_2\beta_1$ activation

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Summary

Background—The platelet $\alpha_2\beta_1$ integrin functions as both an adhesion and signaling receptor upon exposure to collagen. Recent studies have indicated that $\alpha_2\beta_1$ function can be activated via inside-out signaling, similar to the prototypical platelet integrin $\alpha_{IIb}\beta_3$. However, signaling molecules that regulate $\alpha_2\beta_1$ activation in platelets are not well defined. A strong candidate molecule is the small GTPase Rap1b, the dominant platelet isoform of Rap1, which regulates $\alpha_{IIb}\beta_3$ activation.

Objectives—We hypothesized that Rap1b positively regulates $\alpha_2\beta_1$ during agonist-induced platelet activation.

Methods—To test whether Rap1b activates $\alpha_2\beta_1$ downstream of glycoprotein (GP)VI or other platelet receptors, we stimulated platelets purified from *Rap1b*^{-/-} or wild-type mice with diverse agonists and measured $\alpha_2\beta_1$ activation using fluorescein isothiocyanate-labeled monomeric collagen. We also examined the role of Rap1b in outside-in signaling pathways by analyzing adhesion and spreading of *Rap1b*^{-/-} or wild-type platelets on monomeric, immobilized collagen. Finally, we monitored the activation status of related Rap GTPases to detect changes in signaling pathways potentially associated with Rap1b-mediated events.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Doc. S1. Reagents.

Doc. S2. Platelet preparation.

Doc. S3. Rap activation assay.

Doc. S4. Statistics.

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Results—*Rap1b*^{-/-} platelets displayed comparable ADP-induced or thrombin-induced $\alpha_2\beta_1$ activation as wild-type platelets, but reduced convulxin-dependent $\alpha_2\beta_1$ activation. *Rap1b*^{-/-} platelets exhibited increased spreading on immobilized collagen but similar adhesion to immobilized collagen compared to wild-type platelets. *Rap1b*^{-/-} platelets also showed Rap1a and Rap2 activation upon agonist stimulation, possibly revealing functional compensation among Rap family members.

Conclusions—Rap1b is required for maximal GPVI-induced but not ADP-induced activation of $\alpha_2\beta_1$ in murine platelets.

Keywords

$\alpha_2\beta_1$; collagen; GPVI; integrin; platelet and Rap1b

Introduction

Upon blood vessel injury, circulating platelets rapidly adhere to subendothelial collagen, spread and recruit additional platelets to initiate clot formation. Although $\alpha_{IIb}\beta_3$ is critical for these events and is the most abundant integrin on the platelet surface, the less abundant $\alpha_2\beta_1$ provides firm adhesion to type I collagen and assists in the accumulation of platelets at sites of vascular injury [1–3]. Like $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ can be activated in response to physiologic agonists [4,5] and facilitates platelet signaling responses to collagen [6,7], including soluble collagen binding [8] and rapid aggregation [3,7,9,10]. Additionally, individuals with $\alpha_2\beta_1$ polymorphisms that result in increased $\alpha_2\beta_1$ expression have a higher risk of stroke [11], whereas individuals who lack $\alpha_2\beta_1$ exhibit prolonged bleeding times [12].

Glycoprotein (GP)VI is another major collagen receptor on mouse and human platelets. Mouse platelets depleted of GPVI by intraperitoneal injection of an anti-GPVI antibody, JAQ1, show no response to fibrillar collagen [8], and *in vivo* depletion of GPVI protects against arterial thrombosis [13]. GPVI has been proposed to activate $\alpha_2\beta_1$ as part of a redundant collagen signaling system in mouse and human platelets [7]. However, little is known about the intracellular signaling pathways leading to $\alpha_2\beta_1$ activation.

Growing evidence suggests that the small GTPase Rap1b is a key regulator of integrin function in hematopoietic cells. Rap1b was recently identified as the dominant isoform involved in murine B-cell adhesion to vascular cell adhesion molecule-1 (VCAM-1) and chemotaxis [14]. In human and murine platelets, Rap1b is rapidly activated in response to diverse physiologic agonists such as collagen, convulxin (a GPVI agonist), thrombin, ADP, and epinephrine [15–18]. *Rap1b*^{-/-} mice show dramatic defects in $\alpha_{IIb}\beta_3$ -dependent processes such as hemostatic plug and arterial thrombus formation, demonstrating that Rap1b is a crucial regulator of platelet function *in vivo* [19]. CalDAG-GEFI, an upstream guanine nucleotide exchange factor for both Rap1a and Rap1b, is also critical for platelet integrin function [20]. *CalDAG-GEFI*^{-/-} murine platelets show defective $\alpha_{IIb}\beta_3$ -dependent aggregation induced by collagen, defective thrombus formation, defective adhesion to laminin through the $\alpha_6\beta_1$ integrin, and defective adhesion to fibronectin, a process mediated by $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ [20, 21]. In humans, similar integrin defects were observed in leukocyte adhesion deficiency III patients, who lack normal levels of platelet CalDAG-GEFI [22].

Although it is clear that Rap1b signals to $\alpha_{IIb}\beta_3$ in response to fibrillar collagen and convulxin [19], it is unknown whether Rap1b signals to $\alpha_2\beta_1$. We therefore tested the hypothesis that Rap1b positively regulates $\alpha_2\beta_1$ by using soluble monomeric collagen to monitor the activation state of $\alpha_2\beta_1$ on *Rap1b*^{-/-} platelets and by analyzing platelet adherence to immobilized monomeric collagen.

Materials and methods

Reagents

See Supporting Information Doc. S1 for details.

Mice

Rap1b^{-/-} mice were bred on a mixed background of 129 and C57Bl/6 as previously described [19], and used in accordance with guidelines of the IACUC of UNC-CH and the Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI, USA.

Platelet preparation

Washed murine platelets were prepared as previously described, using cardiac puncture or retro-orbital bleeding [23]. See Supporting Information Doc. S2 for more details.

Flow cytometry and data analysis

Platelets were resuspended to 5×10^7 mL⁻¹ in modified Tyrode's buffer. Fluorescein isothiocyanate (FITC)-collagen was added at 5–10 μ g mL⁻¹, followed by the indicated concentrations of ADP, thrombin or convulxin for 30 min in the dark at room temperature. Platelets were then diluted in Tyrode's buffer and analyzed on a FACStar Plus, FACSCanto or LSRII flow cytometer (Becton Dickinson). Data are presented as mean fluorescence intensity from which “no agonist” control values were subtracted.

Adhesion assay

Platelet adhesion was measured as previously described, using a colorimetric phosphatase assay [24]. Briefly, platelets were adjusted to $2\text{--}5 \times 10^7$ mL⁻¹ and preincubated with antibodies at room temperature for 30 min, where indicated. Platelets were attached to microtiter plates coated with 10 μ g mL⁻¹ soluble collagen or 10 mg mL⁻¹ bovine serum albumin (BSA) at room temperature for 20 or 60 min, and non-adherent platelets were removed by aspiration followed by three washes with Tyrode's buffer. Wells not subjected to washing served as a reference for total platelets. To calculate specific adhesion to collagen, raw values of BSA-coated wells were subtracted from values of corresponding collagen-coated wells and divided by values of unwashed total platelets. The values for unstimulated wild-type platelets were normalized to 100 arbitrary units, and all other data points were expressed relative to this value.

Spreading assay

Platelets were adjusted to 1×10^7 mL⁻¹. Coverslips were coated with 10 μ g mL⁻¹ soluble collagen or 10 mg mL⁻¹ BSA in modified Tyrode's buffer at room temperature for 1 h. Platelets were applied to coverslips after treatment with ADP or not as indicated. Spreading was conducted at 37 °C for 1 h. Cells were washed before fixation with 1% paraformaldehyde and permeabilization with 0.1% Triton X-100. Platelets were then incubated for 30 min with Alexa Fluor 546- or Alexa Fluor 488-phalloidin and mounted with FluorSave reagent (Calbiochem, San Diego, CA, USA). Images were obtained on a Nikon TE300 inverted microscope with a 60 \times or 100 \times objective (Tokyo, Japan), and analyzed with Image Pro 5.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

Rap activation assay

Rap-GTP levels were determined essentially as described [18], except that platelets were resuspended to $6\text{--}8 \times 10^8$ mL⁻¹ in 200 μ L and stimulated with thrombin (1 U mL⁻¹). See Supporting Information Doc. S3 for more details.

Results

Rap1b^{-/-} platelets exhibit defective collagen binding to $\alpha_2\beta_1$ upon GPVI but not ADP receptor stimulation

To determine the importance of Rap1b in $\alpha_2\beta_1$ activation, we treated murine *Rap1b^{-/-}* and wild-type platelets with various agonists that activate Rap1b, and used fluorescent soluble collagen (FITC-collagen) as a probe to measure $\alpha_2\beta_1$ activation. Soluble FITC-collagen was used because the other major collagen receptor on platelets, GPVI, preferentially binds fibrillar collagen and because no antibody exists that specifically recognizes activated murine $\alpha_2\beta_1$. ADP dose-dependently stimulated FITC-collagen binding to both *Rap1b^{-/-}* and wild-type platelets to comparable levels (Fig. 1A), even at low ADP concentrations (e.g. 0.1 μ M). Thrombin also stimulated FITC-collagen binding to similar levels in both *Rap1b^{-/-}* and wild-type platelets. However, in agreement with a previous study, ADP-stimulated *Rap1b^{-/-}* platelets displayed impaired fibrinogen binding to $\alpha_{IIb}\beta_3$, showing that Rap1b is required for activation of some integrins but not others downstream of ADP ([19]; data not shown).

As expected, soluble collagen binding is $\alpha_2\beta_1$ -dependent, as an α_2 integrin blocking antibody (HM α 2) completely blocked collagen binding to both wild-type and *Rap1b^{-/-}* platelets in response to ADP (Fig. 1B). ADP-stimulated $\alpha_2\beta_1$ activation did not require outside-in signaling from $\alpha_{IIb}\beta_3$, however, as the $\alpha_{IIb}\beta_3$ blocking antibody 1B5 had no effect on soluble collagen binding to ADP-stimulated platelets (data not shown). Interestingly, a GPVI-specific antibody (JAQ1) blocked collagen binding to wild-type and *Rap1b^{-/-}* ADP-stimulated platelets (Fig. 1B), suggesting that GPVI contributes to soluble collagen binding in a Rap1b-independent manner. JAQ1 did not inhibit FITC-collagen binding to resting platelets, however, indicating that soluble collagen does not detectably bind or activate GPVI in the absence of agonist (data not shown).

As ADP and thrombin receptor signaling did not require Rap1b for $\alpha_2\beta_1$ activation, we next examined whether GPVI requires Rap1b for $\alpha_2\beta_1$ activation. Stimulation of *Rap1b^{-/-}* platelets with the GPVI agonist convulxin resulted in 52% less FITC-collagen binding relative to wild-type platelets (Fig. 1C). Collagen binding to convulxin-stimulated platelets was primarily $\alpha_2\beta_1$ -dependent, as HM α 2 blocked 79% of collagen binding to wild-type platelets and 100% of that to *Rap1b^{-/-}* platelets. Aggregation and dense granule release of *Rap1b^{-/-}* platelets in response to low concentrations of convulxin were also slower and less robust than those of wild-type platelets (data not shown). Similar to the ADP signaling mentioned above, convulxin-stimulated FITC-collagen binding did not require $\alpha_{IIb}\beta_3$ (data not shown). These data indicate that Rap1b is required for optimal signaling downstream of GPVI and that Rap1b may differentially activate $\alpha_2\beta_1$ in response to distinct agonists.

Rap1b is not required for platelet adhesion to monomeric collagen

To investigate the role of Rap1b in outside-in $\alpha_2\beta_1$ signaling, we evaluated platelet adhesion to immobilized monomeric collagen. *Rap1b^{-/-}* platelets exhibited a trend towards increased adhesion vs. wild-type platelets at 20 and 60 min time points, but this was not statistically significant (Fig. 2A,B). ADP did not significantly enhance cell adhesion after 20 min, nor was cell adhesion blocked by the anti-GPVI antibody JAQ1 (Fig. 2A). However, cell adhesion was significantly decreased by HM α 2 at 20 min (Fig. 2A) and at 60 min (Fig. 2B), indicating that *Rap1b^{-/-}* and wild-type platelets adhere normally to monomeric collagen predominantly through $\alpha_2\beta_1$, independently of Rap1b.

Rap1a and Rap2 are expressed and can be activated in *Rap1b^{-/-}* platelets

To investigate whether loss of Rap1b might influence the activity or expression of related GTPases in *Rap1b^{-/-}* platelets, we evaluated Rap1a and Rap2 activation in the presence or

absence of thrombin, using a pulldown assay and specific antibodies. As expected, we detected robust Rap1b activation in response to thrombin in wild-type platelets (Fig. 3, top left panels). Using an antibody that detects both Rap1a and Rap1b, we also detected Rap1a activation in thrombin-stimulated but not resting *Rap1b*^{-/-} platelets (Fig. 3, left 'Rap1a+1b' panel). Rap1 proteins ran as doublets, possibly due to protein phosphorylation [25] or oxidation status [26]. Rap2 is also expressed in both wild-type and *Rap1b*^{-/-} platelets, and can be activated by thrombin (Fig. 3). Rap2 displayed relatively high levels of activation in resting platelets, but even higher levels upon thrombin treatment. These data reveal that Rap1b deficiency does not affect Rap2 activity, and that both Rap1a and Rap2 could potentially compensate for loss of Rap1b in knockout platelets.

Rap1b^{-/-} platelets exhibit increased spreading on monomeric collagen

Platelet spreading on immobilized monomeric collagen was assessed by immunofluorescence microscopy (Fig. 4). Surprisingly, unstimulated *Rap1b*^{-/-} murine platelets showed increased filopodial and lamellipodial extensions on immobilized collagen as compared with wild-type platelets, resulting in increased surface area and a more circular, flattened morphology. Digital image analysis confirmed that the mean polygonal area of the *Rap1b*^{-/-} platelets was significantly greater than that of wild-type platelets (Fig. 4B). However, upon ADP stimulation, wild-type and *Rap1b*^{-/-} platelets exhibited a similar spreading response. JAQ1 did not inhibit spreading of wild-type platelets on immobilized soluble collagen (data not shown), demonstrating that GPVI does not play a critical role in cell attachment or spreading on immobilized monomeric collagen.

Rap1b^{-/-} platelets express more $\alpha_2\beta_1$ and are larger than wild-type platelets

To investigate the mechanism of increased $\alpha_2\beta_1$ -dependent spreading in *Rap1b*^{-/-} platelets, we evaluated $\alpha_2\beta_1$ surface expression. *Rap1b*^{-/-} platelets showed a slight increase in $\alpha_2\beta_1$ expression as compared with wild-type platelets, as indicated by a rightward shift of the flow cytometry histogram (Fig. 5A,B). No difference in surface expression of GPVI was observed between wild-type and *Rap1b*^{-/-} platelets (data not shown). We also found that *Rap1b*^{-/-} platelets display a larger mean forward scatter than wild-type platelets, which may indicate a larger size (Fig. 5C). These data are consistent with a model in which larger platelet size accounts for increased $\alpha_2\beta_1$ expression in *Rap1b*^{-/-} platelets, resulting in enhanced $\alpha_2\beta_1$ -dependent spreading on immobilized monomeric collagen.

Discussion

The small GTPase Rap1b regulates multiple integrins and is strongly activated by agonists that also activate $\alpha_2\beta_1$ in murine platelets. Therefore, we tested the hypothesis that Rap1b regulates platelet $\alpha_2\beta_1$ activation using the recently described *Rap1b*^{-/-} mice [19]. These mice have a severe bleeding phenotype resulting from defective platelets that do not aggregate normally in response to fibrillar collagen or other agonists such as thrombin. Because of this phenotype, we originally predicted that agonist-induced inside-out signals leading to $\alpha_2\beta_1$ activation would also be attenuated in *Rap1b*^{-/-} platelets. Indeed, we found that convulxin-induced but not ADP-induced $\alpha_2\beta_1$ activation is impaired in *Rap1b*^{-/-} platelets. Soluble collagen binding is reduced by more than 50% in Rap1b-deficient platelets in response to convulxin, which mimics the defective convulxin-dependent $\alpha_{IIb}\beta_3$ activation originally observed in *Rap1b*^{-/-} platelets [19]. However, collagen binding to $\alpha_2\beta_1$ is not completely inhibited in convulxin-stimulated *Rap1b*^{-/-} platelets, indicating the existence of Rap1b-independent pathways from GPVI to $\alpha_2\beta_1$. Convulxin-induced collagen binding was completely blocked by an α_2 antibody, revealing the essential role of $\alpha_2\beta_1$ in this process. Collectively, these data demonstrate that Rap1b is critical for GPVI-dependent $\alpha_2\beta_1$ activation, which highlights the sequential nature

of $\alpha_2\beta_1$ activation downstream of GPVI and supports the reciprocal signaling model previously proposed for these two collagen receptors [7,8].

Like convulxin, ADP requires GPVI for $\alpha_2\beta_1$ activation. Soluble collagen binding in response to a low concentration of ADP is significantly blocked by the GPVI antibody JAQ1. JAQ1 may interfere with $\alpha_2\beta_1$ activation by preventing subtle interactions between soluble collagen and GPVI in the presence of ADP, for the following reasons. First, the soluble collagen used here does not activate platelets in the absence of agonist, and therefore does not generate strong GPVI signals. Second, soluble collagen may trigger weak Rap1b-independent signals through GPVI that cooperate with ADP-dependent signals, leading to $\alpha_2\beta_1$ activation. Third, inhibition of $\alpha_2\beta_1$ completely abrogates ADP-induced soluble collagen binding, whereas blockade of GPVI does not. Taken together, these data suggest that weak GPVI signaling and ADP receptor stimulation collaboratively regulate $\alpha_2\beta_1$ via an unknown mechanism that does not involve Rap1b. Another interpretation that is formally possible concerns ADP regulation of GPVI. Although GPVI has not been described as a receptor that undergoes agonist-dependent 'activation', these data may be the first to show that ADP enhances the ability of GPVI to bind certain ligands such as soluble collagen.

In contrast to soluble collagen binding, neither GPVI nor Rap1b signaling is required for platelet adhesion to immobilized monomeric collagen. Rap1b-deficient platelets adhered to immobilized monomeric collagen to the same extent as wild-type platelets in an $\alpha_2\beta_1$ -dependent but GPVI-independent manner, suggesting that Rap1b may not be necessary for adhesion in murine platelets or for solution-phase collagen binding initiated by certain G-protein-coupled receptors.

Previous studies also support our finding that Rap1 is not universally required for integrin activation. For example, $\alpha_4\beta_1$ on human T cells does not require Rap1 activity for ligation to its counter-receptor VCAM-1 [27]. Likewise, sickle red blood cells do not require Rap1 for adhesion to VCAM-1 via $\alpha_4\beta_1$ [28], and adhesion to VCAM-1 is not completely abolished in Rap1b-knockout B cells [14]. These data, combined with our own showing that lack of Rap1b only partially blocks GPVI signaling, strongly suggest the existence of alternative pathways to integrin activation that do not require Rap1.

What are these alternative pathways? We reasoned that ADP-stimulated or thrombin-stimulated collagen binding to *Rap1b*^{-/-} platelets may be catalyzed by other Rap family proteins such as Rap1a and/or Rap2, which are 95% and 65% identical to Rap1b respectively [29]. In a Rap1 pulldown assay, we detected Rap1a activation upon thrombin stimulation in *Rap1b*^{-/-} platelets. We also observed a considerable amount of Rap2 activation in both *Rap1b*^{-/-} and wild-type platelets, indicating that Rap1a and/or Rap2 signaling to $\alpha_2\beta_1$ may occur in the absence of Rap1b. Previous studies have shown that Rap2b is activated by some of the same agonists that activate Rap1b [30] and that Rap1b-independent signaling pathways are operative in response to ADP [19]. For example, Rap1b-deficient platelets show only a partial aggregation response to ADP, but Rap1b-deficient platelets treated with ADP receptor antagonists show a complete loss of aggregation [19], suggesting that Rap1b cannot account for the entire signaling output of ADP receptors and that other GTPases, such as like Rap1a and/or Rap2, may be involved.

Unexpectedly, we found that resting *Rap1b*^{-/-} platelets demonstrated enhanced spreading on collagen relative to wild-type platelets, which may be due to altered cytoskeletal dynamics and organization [31]. To explore whether this increased spreading may be due to enhanced ADP release, we looked for increased dense granule release in knockout platelets. Dense granule secretion was decreased in *Rap1b*^{-/-} platelets as compared with wild-type platelets in response to convulxin. Although dense granule release does not appear to contribute to the enhanced

spreading of *Rap1b*^{-/-} platelets, these data imply that Rap1b may negatively regulate some aspects of $\alpha_2\beta_1$ -dependent signaling, such as cell spreading.

We also found that *Rap1b*^{-/-} platelets have increased cell surface expression of $\alpha_2\beta_1$ and are larger than wild-type platelets. The larger size of Rap1b-deficient platelets may account for their increased $\alpha_2\beta_1$ expression, and other studies have shown that collagen receptor density plays an important role in a cell's ability to bind collagen [32,33]. Increased $\alpha_2\beta_1$ expression did not correlate with a younger, more reticulated population, as *Rap1b*^{-/-} platelets contain slightly less RNA than wild-type platelets (data not shown). Therefore, the larger size and elevated $\alpha_2\beta_1$ surface expression of *Rap1b*^{-/-} platelets may represent a compensatory developmental mechanism that explains the observed adhesion and spreading phenotypes.

In conclusion, we provide evidence for both Rap1b-dependent and Rap1b-independent pathways leading to $\alpha_2\beta_1$ activation. Rap1b is required for efficient $\alpha_2\beta_1$ activation downstream of GPVI, but is not required for ADP-induced or thrombin-induced $\alpha_2\beta_1$ activation or platelet adhesion to immobilized monomeric collagen. Even though these data are the first to reveal Rap1b involvement in GPVI-mediated $\alpha_2\beta_1$ activation, GPVI probably initiates additional Rap1b-independent pathways as well, which impinge upon $\alpha_2\beta_1$. Other small GTPases such as Rap1a and/or Rap2 may function redundantly with Rap1b, especially during ADP signaling or cell adhesion in murine platelets. We have raised important questions about signaling specificity to integrins, and although our study addresses some of these issues, more research is needed to precisely define how signaling pathways are productively coupled to Rap1b activation and what molecular events subsequent to Rap1b activation are critical for $\alpha_2\beta_1$ function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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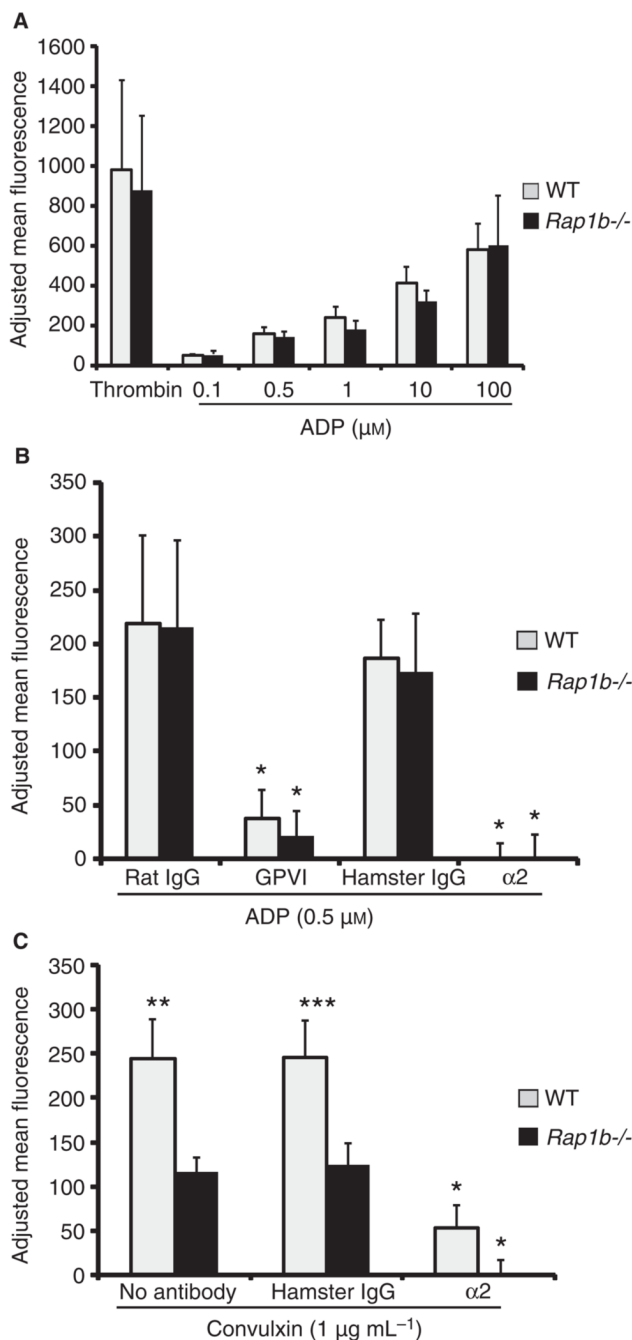


Fig. 1. *Rap1b*^{-/-} platelets display normal ADP signaling but abnormal convulxin signaling to $\alpha_2\beta_1$. (A) Wild-type (WT) (gray bars) or *Rap1b*^{-/-} (black bars) platelets were stimulated by the indicated concentrations of ADP or thrombin (1 U mL⁻¹) followed by the addition of soluble 10 μ g mL⁻¹ fluorescein isothiocyanate (FITC)-collagen. Binding was performed for 30 min at room temperature and detected by flow cytometry. (B) Platelets were pretreated with 20 μ g mL⁻¹ control or blocking antibodies [clone JAQ1 for glycoprotein (GP)VI or HMA2 for $\alpha_2\beta_1$] for 30 min at room temperature, treated with 0.5 μ M ADP for 20 min, and exposed to FITC-collagen as above (**P* < 0.002 for IgG vs. GPVI and IgG vs. α_2). (C) Platelets were pretreated with no antibody, 20 μ g mL⁻¹ control hamster IgG or HMA2 as above before

stimulation with $1 \mu\text{g mL}^{-1}$ convulxin ($*P < 0.005$ for α_2 vs. IgG; $**P < 0.007$ and $***P < 0.02$). For all graphs, the data represent means of FITC–collagen binding adjusted for no agonist background \pm standard error of the mean (SEM), from three to four separate experiments.

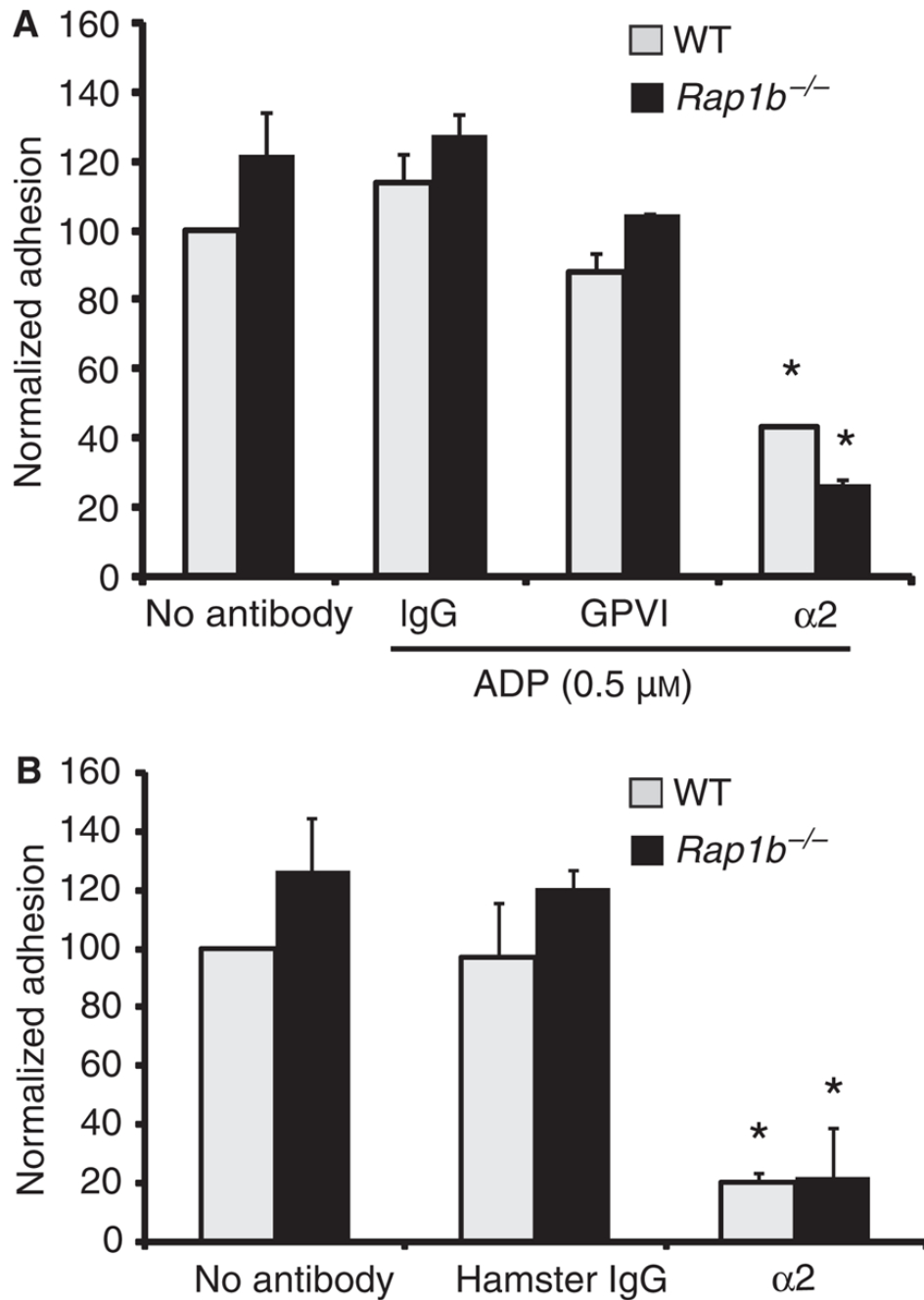


Fig. 2. *Rap1b*^{-/-} and wild-type (WT) platelets adhere to immobilized collagen via $\alpha_2\beta_1$ with similar avidity. (A) Wild-type (gray) and *Rap1b*^{-/-} (black) platelets were pretreated with no antibody, or 20 $\mu\text{g mL}^{-1}$ hamster IgG, JAQ1 (GPVI) or HM α_2 (α_2) as in Fig. 1B. All samples except no-antibody controls were stimulated with 0.5 μM ADP and allowed to adhere to microtiter wells coated with 10 $\mu\text{g mL}^{-1}$ collagen or bovine serum albumin for 20 min at 37 °C before total phosphatase activity was measured using a colorimetric assay (* $P < 0.03$ for α_2 vs. IgG). (B) Platelets were treated with antibodies as in Fig. 1C without agonist and allowed to adhere as above for 60 min at 37 °C for 1 h. Data represent normalized specific adhesion \pm standard

error of the mean (see Materials and methods) from four separate experiments ($*P < 0.0005$ for α_2 vs. IgG). GP, glycoprotein.

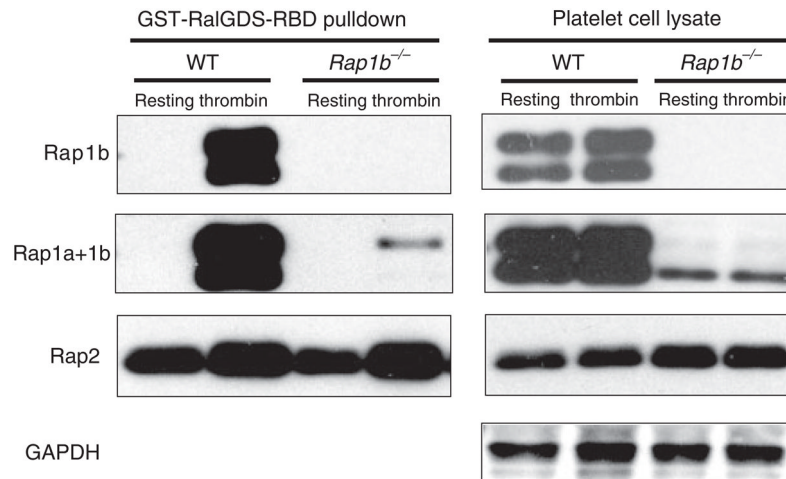


Fig. 3. Thrombin stimulates Rap1a and Rap2 activation in *Rap1b*^{-/-} platelets. Thrombin (1 U mL⁻¹) or buffer was added to $6-8 \times 10^8$ platelets mL⁻¹ in modified Tyrode's buffer for 3 min. Platelets were lysed, and equal amounts of lysates were used in a pulldown assay (left panels) followed by non-reduced sodium dodecylsulfate polyacrylamide gel electrophoresis and western blotting with Rap1b, Rap1a+1b (which recognizes both Rap1a and Rap1b) or Rap2 antibodies. Note that the Rap1b antibody does not crossreact with Rap1a in lysates from *Rap1b*^{-/-} mice (Rap1b panels). Total cell lysates were used as loading controls (right panels). Data are representative of three independent experiments. GST, glutathione-*S*-transferase; RalGDS, Ral guanine nucleotide dissociation stimulator; RBD, Ras binding domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild-type.

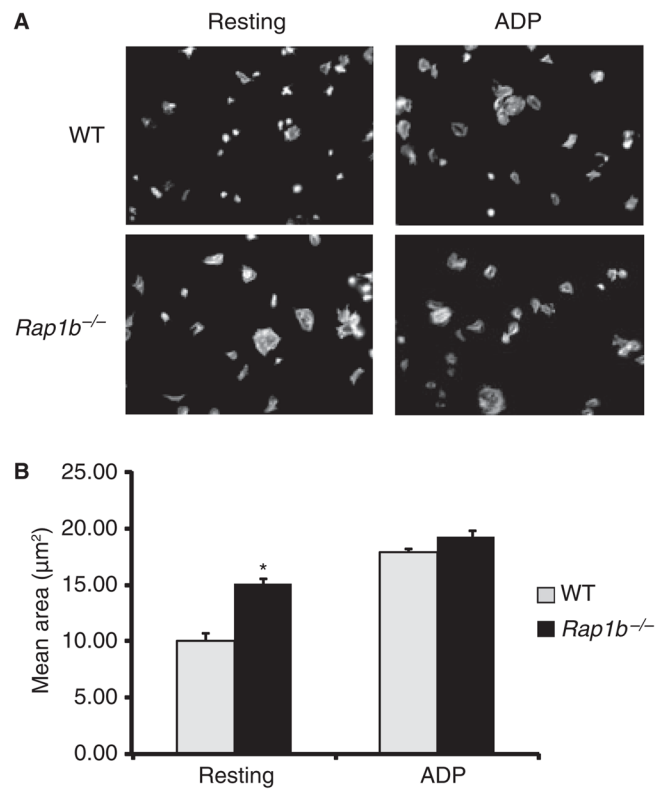


Fig. 4. *Rap1b*^{-/-} platelets show increased spreading on immobilized collagen. (A) Coverslips were coated with 10 µg mL⁻¹ soluble, monomeric collagen. Washed platelets (1 × 10⁷ mL⁻¹) were pretreated with ADP (300 µM) where indicated, and plated for 45–60 min at 37 °C to allow spreading; this was followed by fixation, permeabilization and staining of F-actin with Alexa Fluor 488–phalloidin. Data represent two to six independent experiments. (B) The mean area of resting and ADP-stimulated platelets from two experiments [**P* < 0.0007 for resting wild-type (WT) vs. *Rap1b*^{-/-}]. The area was calculated for 130–291 cells per condition using ImagePro software.

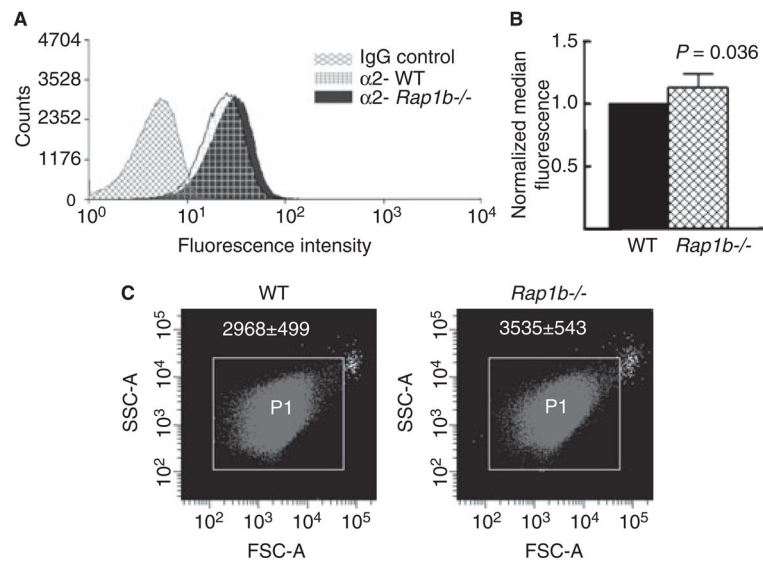


Fig. 5. *Rap1b*^{-/-} platelets display increased $\alpha_2\beta_1$ expression and size. (A) Wild-type (WT) or *Rap1b*^{-/-} platelets were treated with fluorescein isothiocyanate- conjugated IgG control or α_2 antibody (grid for wild type, solid for *Rap1b*^{-/-}), incubated for 30 min on ice in the dark, and analyzed via flow cytometry. IgG control readings for wild-type and *Rap1b*^{-/-} platelets were the same, and are represented as one histogram (IgG control). The histograms shown are representative of three separate experiments. (B) Quantification of data shown in (A) \pm standard deviation. (C) The size of wild-type and *Rap1b*^{-/-} platelets was measured by flow cytometry. Shown is a forward scatter (FSC-A) (x-axis) vs. side scatter (SSC-A) (y-axis) dot plot from one representative experiment gated on P1. *Rap1b*^{-/-} platelets have an increased mean forward scatter (white numbers, $P < 0.05$), but not side scatter, as compared with wild-type platelets.