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A Role for Rac1 in Glycoprotein Ib-IX-mediated Signal Transduction and Integrin Activation

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Abstract

Objective—The platelet receptor for von Willebrand factor (VWF), the glycoprotein Ib-IX (GPIb-IX) complex, mediates platelet adhesion at sites of vascular injury and transmits signals leading to platelet activation. VWF/GPIb-IX interaction sequentially activates the Src Family Kinase (SFK) Lyn, phosphoinositide 3-kinase (PI3K) and Akt, leading to activation of integrin $\alpha_{IIb}\beta_3$, and integrin-dependent stable platelet adhesion and aggregation. It remains unclear how Lyn activates the PI3K/Akt pathway following ligand binding to GPIb-IX.

Methods and Results—Using platelet-specific $Rac1^{-/-}$ mice and the Rac1 inhibitor NSC23766, we examined the role of Rac1 in GPIb-IX-dependent platelet activation. $Rac1^{-/-}$ mouse platelets and NSC23766-treated human platelets were defective in GPIb-dependent stable adhesion to VWF under shear stress, integrin activation, thromboxane A_2 (TXA_2) synthesis and platelet aggregation. Interestingly, GPIb-induced activation of Rac1 and the guanine nucleotide exchange factor (GEF) for Rac1,Vav, was abolished in both $Lyn^{-/-}$ and PP2-treated platelets but was unaffected by the PI3K inhibitor LY-294002, indicating that Lyn mediates activation of Vav and Rac1 independently of PI3K. Furthermore, GPIb-induced activation of Akt was abolished in Rac1-deficient platelets, suggesting that Rac1 is upstream of the PI3K/Akt pathway.

Conclusions—A Lyn/Vav/Rac1/PI3K/Akt pathway mediates VWF-induced activation of integrin $\alpha_{IIb}\beta_3$ to promote GPIb-IX-dependent platelet activation.

Keywords

platelet; glycoprotein Ib-IX; platelet adhesion; von Willebrand factor; Rac1

INTRODUCTION

Under the high shear rate flow conditions present in arteries and capillaries, platelet adhesion to the site of vascular injury is mediated by the interaction between subendothelial-bound VWF and its platelet receptor, the GPIb-IX complex.[1–3] The interaction between VWF and GPIb-IX not only mediates transient platelet adhesion to the injured vessel wall but also initiates a signal transduction cascade culminating in the activation of integrin

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DISCLOSURES
None.

$\alpha_{IIb}\beta_3$, leading to stable platelet adhesion, spreading, and aggregation.[2, 4–6] This process is essential for thrombosis under high shear conditions. GPIb-IX-mediated platelet activation signaling is known to involve several intracellular signaling molecules and pathways, including the SFK Lyn, the PI3K/Akt pathway, and the cGMP and mitogen-activated protein kinase (MAPK) pathways.[2, 7–16] GPIb-IX signaling is also greatly amplified by the activation of the immunoreceptor tyrosine-based activation motif (ITAM), TXA_2, and adenosine diphosphate (ADP) signaling pathways [2, 8, 17, 18]. Although the importance of GPIb-IX-mediated platelet activation to arterial thrombosis is well understood, the signaling pathway that regulates GPIb-IX-dependent platelet activation is poorly characterized. Thus far, the identified most proximal step to GPIb-IX that propagates platelet activation signals is the activation of Lyn and Lyn-dependent activation of the PI3K/Akt pathway. Interestingly, although the PI3K/Akt pathway is activated downstream of Lyn and is critical for promoting GPIb-IX-mediated platelet activation, the exact molecular mechanisms governing this process are unclear.

The Rho family small GTPase (guanosine triphosphatase), Rac1, has been shown to be important for arteriole thrombosis *in vivo*.[19, 20] Rac1 is activated by binding to guanosine triphosphate (GTP) and plays roles in multiple cellular processes, including actin polymerization[21], lamellipodia formation[19, 22–24] and cell retraction[25]. However, the role of Rac1 in GPIb-IX-mediated signal transduction has not been explored.

Here we show that Rac1 is a critical mediator of GIb-IX-dependent early signaling leading to integrin activation, stable platelet adhesion under shear stress, TXA_2 production, and platelet aggregation. Most importantly, we demonstrate that Rac1 mediates Lyn-dependent activation of the PI3K/Akt signaling pathway during GPIb-IX-dependent platelet activation. Thus, our study reveals an important new link in the GPIb-IX signaling pathway and a novel mechanism of Rac1-dependent PI3K/Akt activation.

MATERIALS AND METHODS

Generation of Mice with Rac1$^{-/-}$ Platelets

Mice containing the Rac1 conditional allele ($Rac1^{loxP/loxP}$) [26] were crossed with mice carrying the Pf4-Cre transgene ($Pf4\text{-}Cre^{+}$).[27] $Pf4\text{-}Cre^{+}/Rac1^{loxP/loxP}$ mice are notated as $Rac1^{-/-}$ and $Pf4\text{-}Cre^{-}/Rac1^{loxP/loxP}$ mice as wild type (WT). Mice were maintained on a mixed SV/129/C57/Bl-6 background and littermates used as control. Animal usage and protocol were approved by the institutional animal care committee of the University of Illinois at Chicago.

Platelet Preparation and Adhesion Under Flow

Human and murine platelets were prepared as previously described and used at 3×10^8/mL. [28, 29] Analysis of platelet adhesion under flow was performed as described previously. [11, 12] Data are representative of ≥ 3 experiments and statistical significance was determined via ANOVA and post-test.

Fibrinogen Binding Assay

Buffers contained 1% BSA and 2 mM $MgCl_2$. Washed human (1×10^8/mL) and mouse (2×10^8/mL) platelets were stimulated with ristocetin/botrocetin ± VWF in the presence of 5 or 2 μg/mL Oregon Green-labeled fibrinogen (Oregon Green-Fg: Molecular Probes) for 20 or 10 min., respectively. Integrilin and RGDS were used to determine the level of non-specific binding of Oregon Green-Fg to the platelet surface. Platelets were diluted 1:20 in Modified-Tyrode's buffer and analyzed with a BD Accuri C6 flow cytometer. Specific fibrinogen binding was determined by subtracting the geometric means of fluorescence intensity of the

nonspecific binding (integrin antagonist present) from the total binding (integrin antagonist absent).

VWF Binding Assay

2 mM $MgCl_2$, 1% BSA, and 10 mM EDTA were added to all buffers and 1×10^8 platelets/mL were used. Washed human and mouse platelets were incubated 5 or 10 min. with VWF ± ristocetin/botrocetin, respectively. Platelets were fixed with a final concentration of 1% paraformaldehyde (PFA) in HEPES-saline buffer for 20 min., centrifuged at 100 g for 1 min., and the pellet stained for 30 min. in 0.1 mL Modified-Tyrode's buffer containing 4 μg/mL SZ-29-FITC. Samples were diluted 1:20 with Modified-Tyrode's buffer and analyzed using a BD Accuri C6 flow cytometer. Specific VWF binding was determined by subtracting the geometric means of fluorescence intensity of VWF binding in the presence of botrocetin/ristocetin alone from the VWF binding in the absence of botrocetin/ristocetin.

Platelet Aggregation and TXB_2 Generation Assay

Platelet aggregation [30] and measurement of the TXA_2 metabolite, TXB_2 [15, 17] was performed as described previously. Briefly, following platelet aggregation for 8 minutes, the reaction was stopped with EDTA and aspirin, the platelets briefly spun down in a microfuge, and the resultant supernatant used in a TXB_2 ELISA (Assay Designs). TXB_2 is used to indicate levels of TXA_2. Data are from ≥ 3 experiments and expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using the Student's *t*-test.

Immunoblot Detection of SFKs, Vav, Akt, and P38 MAPK

SDS-PAGE analysis of phosphorylated signaling proteins was performed as described previously.[12] Anti-pan phospho SFK (#2101: Tyr416), which reacts with phosphorylated Lyn, anti-phospho P38 (#9211: Thr180/Tyr182), anti-phospho Akt (#4058: Ser473), and anti-pan Akt (#2920) antibodies are from Cell Signaling Technology. Anti-pan phospho Vav (sc-16408-R), anti-pan Vav (sc-55482), anti-Lyn (sc-7274) and anti-P38 (sc-535) antibodies are from Santa Cruz Biotechnology.

Immunoblot Detection of Small GTPases

GST-PAK pull-down assays were performed as described previously to detect levels of GTP-bound Rac1.[26, 31] Anti-Rac1 antibody (#61051: BD Biosciences) was used for Western Blot.

RESULTS

Generation of Megakaryocyte- and Platelet-Specific Conditional Rac1 Knockout mice

In order to study the role of Rac1 in GPIb-IX-induced platelet activation, we generated mice that lack expression of Rac1 exclusively in megakaryocytes and platelets and used a small molecule inhibitor of Rac1, NSC23766. To obtain megakaryocyte- and platelet-specific conditional Rac1 knockout mice, Pf4-Cre transgenic mice[27] were cross-bred with mice containing the $Rac1^{loxP/loxP}$ allele.[26] Deletion of Rac1 in $Rac1^{-/-}$ mouse platelets was verified via Western Blot analysis of platelet lysates (Figure 1A). $Rac1^{-/-}$ mouse platelets did not show any noticeable difference in routine hematological parameters including morphology and counts compared to WT platelets. This is similar to previous reports, where $Rac1^{-/-}$ mouse platelets were generated using the Mx1-Cre transgene.[19, 20]

The Role of Rac1 in the Early GPIb-IX-Mediated Integrin Activation Signaling Pathway Leading to Stable Platelet Adhesion to VWF Under Flow Conditions

We initially evaluated the effect of Rac1 deficiency on GPIb-IX-induced, integrin-dependent stable platelet adhesion to VWF under shear stress, which was created using a cone-plate rheometer. As shown in Figure 1, stable adhesion of human and mouse platelets to VWF was completely inhibited by the integrin antagonist RGDS (Figure 1B–D). This is consistent with our previous results that stable platelet adhesion to VWF under shear flow conditions requires not only GPIb-IX but also GPIb-IX-dependent activation of integrin $\alpha_{IIb}\beta_3$.[11, 12] Stable platelet adhesion to VWF under flow is partially inhibited by the cyclooxygenase (COX) inhibitor aspirin, which is also consistent with our previous data that the COX pathway plays an amplifying role in promoting platelet adhesion under shear stress.[11, 12] Interestingly, stable platelet adhesion to VWF was dramatically impaired in $Rac1^{-/-}$ mouse platelets or NSC23766-treated human platelets (Figure 1B–D). These data indicate that Rac1 plays a critical role in GPIb-IX-mediated, integrin-dependent platelet adhesion to VWF under shear stress. Importantly, deficiency of Rac1 led to significantly greater inhibition of stable platelet adhesion than aspirin (Figure 1B–D), indicating that the role of Rac1 in GPIb-IX-mediated platelet activation exceeds the COX-dependent amplification signaling pathway.

Rac1 is Important for GPIb-IX-Induced Integrin Activation

We next investigated whether Rac1 is important for GPIb-IX-induced integrin activation. WT and $Rac1^{-/-}$ mouse platelets were stimulated with VWF in the presence of botrocetin, and analyzed for the binding of Oregon Green-Fg using flow cytometry. VWF/botrocetin-induced fibrinogen binding was diminished in $Rac1^{-/-}$ mouse platelets compared to WT platelets, indicating that $Rac1^{-/-}$ mouse platelets were defective in GPIb-IX-induced activation of $\alpha_{IIb}\beta_3$ (Figure 2A, B). These results were recapitulated in human platelets, where VWF/ristocetin-induced fibrinogen binding was abolished by Rac1 inhibitor-treatment, as compared to the control solvent DMSO (Figure 2C, D). These results demonstrate that Rac1 plays an important role in the signaling pathway of GPIb-IX-dependent integrin activation.

Platelet Deficiency in Rac1 Does Not Affect the VWF Binding Function of GPIb-IX

To exclude the possibility that the functional defects observed in Rac1-deficient platelets derive from a defect in the VWF-binding function of GPIb-IX, washed WT and $Rac1^{-/-}$ mouse platelets or washed human platelets treated with DMSO or NSC23766 were incubated with human VWF in the presence of botrocetin or ristocetin, respectively, to induce the binding of VWF to GPIb-IX. The platelets were then fixed, stained with a FITC-labeled anti-VWF antibody, and analyzed via flow cytometry. As expected, botrocetin or ristocetin induced the binding of soluble VWF to platelets. There was no difference in botrocetin-induced binding of VWF to WT and $Rac1^{-/-}$mouse platelets (Figure 3A, B) or ristocetin-induced binding of VWF to DMSO- and NSC23766-treated human platelets (Figure 3C, D). Therefore, Rac1 is not involved in regulating the VWF binding function of GPIb-IX, but rather is important in GPIb-IX-dependent signal transduction.

Rac1 is Required for GPIb/VWF-mediated Platelet Aggregation and TXA_2 Production

In order to further confirm the role of Rac1 in GPIb-IX-mediated platelet activation, we also evaluated the effect of Rac1 deficiency on VWF-induced platelet aggregation. VWF-induced platelet aggregation is characterized by two distinct waves.[2, 8] The first wave mainly comprises VWF/GPIb-IX-dependent platelet agglutination and an often minor component of GPIb-IX-induced integrin-dependent platelet aggregation.[9, 32] The second wave represents TXA_2-, secretion- and integrin-dependent platelet aggregation. It requires

GPIb-IX-induced activation of integrin $\alpha_{IIb}\beta_3$ and subsequent TXA_2 synthesis, which initiates integrin- and TXA_2-dependent granule secretion and secretion-dependent amplification of platelet activation. As expected, stimulation of WT mouse platelets with VWF/botrocetin or stimulation of human platelets with VWF/ristocetin lead to two waves of platelet aggregation (Figure 4A, B). However, $Rac1^{-/-}$ platelets and WT mouse platelets treated with NSC23766 both had a defect in the second-wave of VWF-induced aggregation (Figure 4A). Similarly, NSC23766-treated human platelets were also defective in the second wave of VWF-induced platelet aggregation (Figure 4B). Thus, these results further verify that Rac1 is required for VWF/GPIb-IX-mediated platelet activation in both human and mouse platelets. In addition, $Rac1^{-/-}$ mouse platelets (Figure 4C) or NSC23766-treated human platelets (Figure 4D) were defective in GPIb-IX-mediated TXA_2 synthesis, indicating that Rac1 is important in the early GPIb-IX-mediated signaling pathway that is upstream of TXA_2 synthesis.

Rac1 is required for GPIb-IX-mediated activation of the PI3K/Akt pathway

Previous studies from our laboratory and from others have shown that GPIb-IX-mediated early signaling leading to integrin activation involves GPIb-IX-dependent activation of the SFK Lyn, and Lyn-dependent activation of the PI3K/Akt pathway, which subsequently stimulates the cGMP and MAPK pathways.[9–12, 32, 33] To determine how Rac1 is involved in GPIb-IX-mediated platelet activation, we determined whether and how loss of function of Rac1 affected this signaling pathway by evaluating VWF/GPIb-IX-mediated phosphorylation of SFK, Akt and p38 MAPK during the early agglutination phase of VWF-induced platelet aggregation (2 minute time point) prior to the second wave of platelet aggregation.[2, 8] VWF/GPIb-IX-induced phosphorylation of Akt was abolished in $Rac1^{-/-}$ mouse platelets (Figure 5A) or NSC23766-treated human platelets (Figure 5B), indicating that Rac1 is upstream of Akt in the GPIb-IX signaling pathway. Furthermore, platelet deficiency in Rac1 also abolished GPIb-IX-mediated phosphorylation of P38 MAPK (Figure 5A, B). These data suggest that Rac1 is required for activation of the PI3K/Akt pathway as well as the downstream p38 MAPK pathway. Consistent with the observation that Rac1 functions upstream of the TXA_2 synthesis pathway (Fig 4), aspirin had no effect on GPIb-IX-mediated phosphorylation of Akt and P38, which was fully inhibited by NSC23766 in the presence of aspirin. In contrast, VWF/GPIb-IX-induced phosphorylation of SFKs was not negatively affected by deficiency of Rac1 (Figure 5A, B). Thus, Rac1 is not required for GPIb-IX-dependent activation of SFKs.

Activation of Vav and Rac1 during GPIb-IX signaling requires the SFK Lyn

To assess whether Lyn and/or PI3K were involved in GPIb-IX-mediated activation of Rac1, the levels of GTP-bound Rac1 in human platelets treated with the SFK inhibitor PP2 or PI3K inhibitor LY294002, or in $Lyn^{-/-}$ mouse platelets, were assayed following ligation of GPIb-IX. Rac1 was activated within 1 minute and remained active throughout VWF-induced platelet aggregation in WT platelets (Figure 6A). However, VWF-induced GTP loading of Rac1 was abolished in $Lyn^{-/-}$ (Figure 6A) and PP2-treated platelets (Figure 6B), indicating that Rac1 is activated downstream of Lyn in the GPIb-IX signaling pathway. Interestingly, VWF-induced GTP-loading of Rac1 remained unaffected by LY294002 (Figure 6B, C), indicating that Rac1 activation occurs independent of PI3K. In fact, under the same conditions, LY294002- and NSC23766-treated platelets were both defective in GPIb-IX-mediated activation of Akt and P38 MAPK but not SFKs (Figure 5B). Taken together, these results indicate not only that Rac1 becomes activated during GPIb-IX-dependent platelet activation, but that Rac1 is activated downstream of Lyn and functions upstream of the PI3K/Akt/MAPK pathway.

Rac1 can be activated by multiple GEFs, including Vav. Recent studies suggest that Vav can be activated by Lyn-dependent phosphorylation at Tyr^{174}.[34] To evaluate whether Lyn may mediate GPIb-IX-dependent activation of Rac1 via phosphorylation of Vav, we determined whether ligation of GPIb-IX induces Vav phosphorylation and whether GPIb-IX-dependent Vav phosphorylation is affected in $Lyn^{-/-}$ mouse platelets or PP2-treated platelets during the early phase of VWF-induced platelet agglutination/aggregation prior to the second wave of platelet aggregation. Indeed, ligation of GPIb-IX induced Vav activation, which was abolished in $Lyn^{-/-}$ mouse platelets (Figure 6D) or PP2-treated human platelets (Figure 6E). In contrast, activation of Vav was unaffected by either LY294002 or NSC23766 (Figure 6E). Thus, Lyn mediates GPIb-IX-induced activation of Vav and Rac1 independent of the PI3K pathway. Collectively, we have demonstrated that a novel Lyn-Vav-Rac1-PI3K-Akt signaling pathway is important in the early phase of GPIb-IX-mediated signal transduction leading to platelet activation.

DISCUSSION

In this study, we demonstrate that Rac1 plays a critical role in stimulating GPIb-IX dependent platelet activation. We further show that Rac1 is important in the early GPIb-IX signaling pathway leading to activation of integrin $\alpha_{IIb}\beta_3$ and stable platelet adhesion under flow. Importantly, we have discovered that Rac1 and its GEF, Vav, are activated downstream from Lyn, and that Rac1 is required for activating the PI3K/Akt pathway. Thus, our study not only reveals a novel role for Rac1 in platelet activation but also as an important mediator of GPIb-IX-induced, Lyn-dependent activation of the PI3K/Akt signaling pathway leading to integrin activation.

We conclude that Rac1 is required for GPIb-IX-mediated platelet activation. This conclusion is supported by the data that $Rac1^{-/-}$ mouse platelets and NSC23766-treated human platelets were defective in GPIb-IX-dependent integrin activation and stable platelet adhesion to VWF under flow. Moreover, the TXA_2-, -secretion-, and integrin-dependent second wave of platelet aggregation was abolished in Rac1-deficient platelets without affecting VWF binding, further supporting an important role for Rac1 in GPIb-IX-mediated platelet activation signaling. The platelet activation process induced by GPIb-IX can be divided into early phase GPIb-IX-specific signaling events and late phase amplification signaling pathways shared by all other platelet agonists.[2, 8] Several signaling molecules and events have been shown to be important in the early GPIb-IX signaling pathway leading to integrin activation and stable platelet adhesion, including the SFKs c-Src and Lyn, the PI3K/Akt pathway, intracellular calcium oscillation, cGMP-dependent protein kinase (still controversial), and p38/ERK MAPKs.[2, 8, 10, 13, 14, 18, 35–37] Some other signaling molecules, such as components of the ITAM signaling pathway, have been shown to be important in the late signal amplification phase of GPIb-IX-induced platelet activation, which facilitates aggregation and the recruitment of additional platelets to the growing thrombus.[2] However, the distinct role of many other signaling molecules in either the early or late phase of GPIb-IX-induced platelet activation remains poorly characterized. This is because the aggregation response of platelets to GPIb-IX-specific early phase receptor signaling is often masked by VWF-mediated platelet agglutination and the second phase of VWF-induced platelet aggregation is complicated by the platelet response to amplification signals induced via the integrin, ITAM, TXA_2, and ADP signaling pathways. It is thus difficult to use the routine platelet aggregation assay to effectively specify the role of a particular signaling molecule in GPIb-IX-specific platelet responses. We thus determined the specific role of Rac1 in early GPIb-IX signaling by analyzing the effect of Rac1 deficiency in platelet adhesion to VWF under flow conditions. Under shear stress, stable platelet adhesion to immobilized VWF requires the “early” GPIb-IX mediated signaling pathway that stimulates the inside-out activation of integrin, leading to integrin-dependent stable platelet adhesion. Stable platelet

adhesion under shear stress does not require molecules important in the secondary signaling pathways such as Syk and ITAM [11, 12], although it is amplified by TXA_2. Our data demonstrate that stable platelet adhesion to VWF under shear stress was dramatically impaired in $Rac1^{-/-}$ mouse platelets and NSC23766-treated human platelets. Furthermore, the impairment in $Rac1^{-/-}$ mouse platelets and NSC23766-treated platelets is significantly greater than the inhibitory effects of saturable concentrations of aspirin. These data indicate that the role of Rac1 in stable platelet adhesion is not limited to its role in TXA_2 synthesis and signal amplification, but rather that Rac1 plays an important role in the early, TXA_2-independent GPIb-IX-dependent signaling pathway that activates $\alpha_{IIb}\beta$ This conclusion is further supported by the data showing that Rac1 stimulates activation of Akt and P38 MAPK independent of the TXA_2 pathway (Figure 5B) and that Rac1 is also upstream of the TXA_2 synthesis pathway (Figure 4C, D). Importantly, we have provided direct evidence that Rac1 is critical for GPIb-IX-dependent integrin activation (Figure 2). These results, for the first time, indicate a new small GTPase-dependent signaling mechanism in the early GPIb-IX signaling pathway, leading to integrin activation and stable platelet adhesion under shear stress. These results, however, do not exclude an important role for Rac1 also in the secondary platelet amplification pathways such as granule secretion and integrin outside-in signaling, which have been demonstrated previously.[26, 38]

It is established that the SFK Lyn and the PI3K/Akt pathway are important in early GPIb-IX signaling.[11–13, 15, 16] It remains unclear, however, as to how GPIb-IX activates these molecules and how these molecules are linked into a signaling pathway or network that mediates GPIb-IX signaling leading to integrin activation. Previous studies have shown the co-immunoprecipitation of GPIb-IX with PI3K[39] and Lyn[40], and it was postulated that the GPIb-associated p85 subunit of PI3K functions as a scaffold that recruits SFKs to GPIb, thereby facilitating the activation of SFKs.[40] Here we show that GPIb-IX-mediated activation of the PI3K/Akt signaling pathway requires Rac1. We also show that GPIb-IX-mediated activation of Rac1 requires Lyn, and likely involves Lyn-dependent phosphorylation of Vav. Furthermore, the catalytic function of PI3K is not required for GPIb-IX-mediated activation of Rac1, as LY294002 had no effect on GPIb-IX-dependent Rac1 activation. Thus, although we do not exclude the role of PI3K as a scaffold, our study reveals a novel signaling mechanism in which ligand binding to GPIb-IX induces the sequential activation of the SFK Lyn, the GEF Vav, and Rac1. Rac1 then mediates activation of the PI3K/Akt signaling pathway. Thus, this study represents a significant advance by identifying an important molecule in the early GPIb-IX signaling pathway leading to integrin activation.

The identification of Rac1 as a downstream effector of Lyn that stimulates the PI3K/Akt pathway is a novel finding not only to the GPIb-IX signaling pathway in platelets, but also may have general implications to the mechanisms regulating PI3K activation. The SFKs Lyn/Fyn and Rac1 have both been implicated in stimulating the activity of PI3K by binding to the p85 subunit of PI3K.[41–43] Our data, however, indicate that Lyn activates the Rac1 GEF Vav and thus Rac1, and requires Rac1 to mediate activation of PI3K. It will be interesting to further investigate whether this new model of Lyn- and Rac1-dependent PI3K activation is a common mechanism in other cell types.

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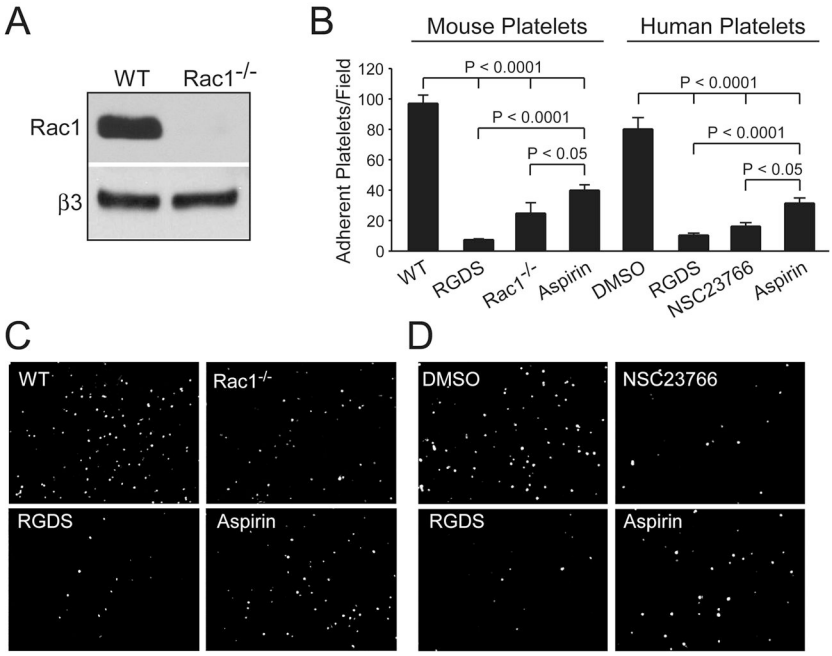

Figure 1.

The role of Rac1 in integrin-dependent stable platelet adhesion to VWF under flow. **A**, Washed WT and Rac1$^{-/-}$ mouse platelets were solubilized in SDS-buffer and protein expression of Rac1 and β_3 was analyzed via SDS-PAGE and western blot. **B**, Washed WT and Rac1$^{-/-}$ mouse platelets, pretreated with 2 mM RGDS or 1 mM Aspirin for 5 minutes, or washed human platelets pretreated with 0.1% DMSO, 100 μM NSC23766, 2 mM RGDS or 1 mM aspirin for 5 minutes, were loaded onto VWF-coated glass slides in the presence of 10 μM mepacrine. Platelets were subjected to a constant shear rate for 5 minutes. After washing, adherent platelets were viewed and photographed. The number of adherent platelets per field was quantified and shown as mean ± SEM. **C**, Representative images of adherent WT and Rac1$^{-/-}$ mouse platelets from **B. D**, Representative images of adherent human platelets from **B**.

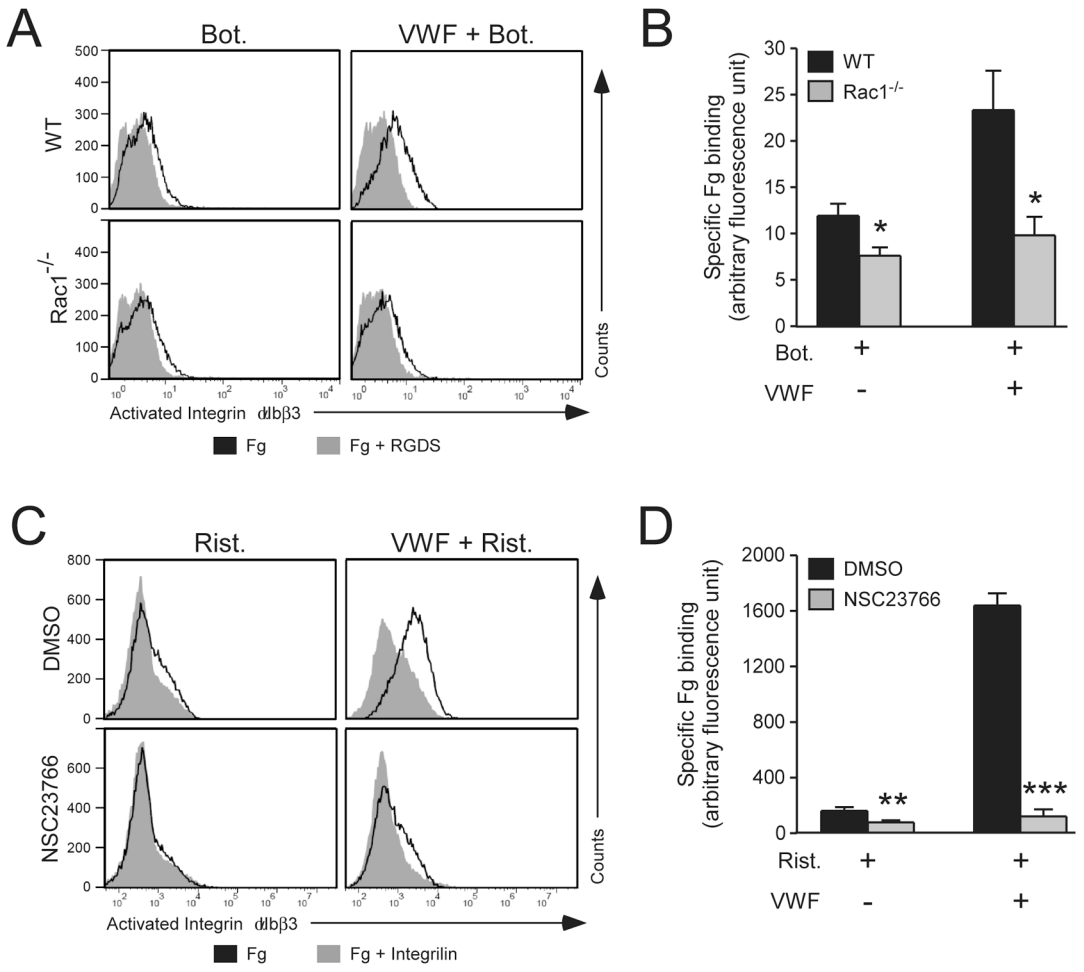

Figure 2.

Rac1 is important for GPIb-IX-induced integrin activation. **A**, Flow cytometric analysis of the binding of Oregon Green-conjugated fibrinogen (Oregon Green-Fg) to washed WT and $Rac1^{-/-}$ mouse platelets stimulated with 2 μg/mL botrocetin (Bot.) ± 10 μg/mL VWF in the presence (grey filled) or absence (black line) of 2 mM of the integrin receptor antagonist RGDS. **B**, The quantitative results from 3 independent experiments as described in **A. C**, Flow cytometric analysis of the binding of Oregon Green-Fg to washed human platelets treated with 0.1% DMSO or 100 μM NSC23766 for 5 minutes and subsequently stimulated with 0.6 mg/mL ristocetin (Rist.) and 10 μg/mL VWF in the presence (grey filled) or absence (black line) of 40μg/mL of the integrin receptor antagonist integrilin. **D**, The quantitative results from 3 independent experiments as described in **C**. ***, **, and * represents statistical significance (P<0.0001, P<0.001, and P<0.05, respectively) as determined by Student's *t*-test. Data shown as mean ± SEM.

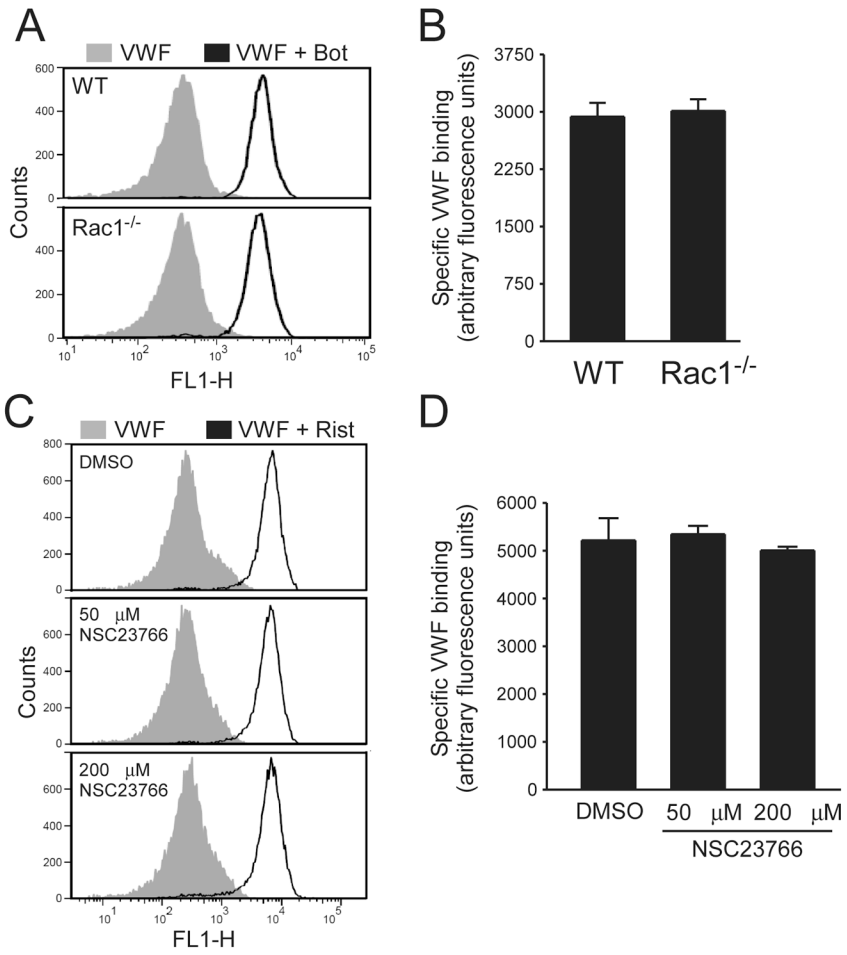

Figure 3.

Platelet deficiency in Rac1 does not affect the VWF binding function of GPIb-IX. **A**, Flow cytometric analysis of VWF binding, as determined by the binding of FITC-labeled anti-VWF antibody, to washed WT and Rac1$^{-/-}$ mouse platelets after addition of 20 μg/mL VWF in the presence (black line) or absence (grey filled) of 1.25 μg/mL botrocetin for 10 minutes. **B**, Quantification of specific VWF binding from **A. C**, Flow cytometric analysis of VWF binding to washed human platelets that were pretreated with either 0.1% DMSO, 50 μM or 200 μM NSC23766 and stimulated with 20 μg/mL VWF in the presence (black line) or absence (grey filled) of 0.5 mg/mL ristocetin for 5 minutes. Data are shown as mean ± SEM and are from 3 experiments.

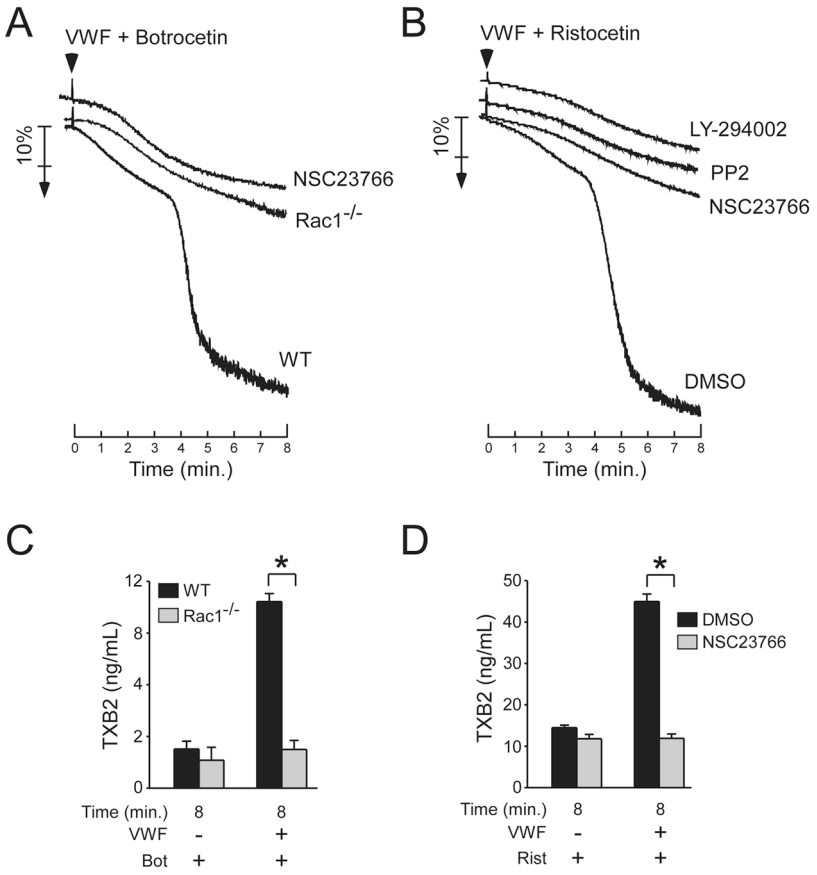

Figure 4.

Rac1 is required for VWF-induced platelet aggregation and TXA_2 synthesis. **A**,Washed WT platelets, treated with 100 μM NSC23766 or $Rac1^{-/-}$ platelets were stimulated with 5 μg/mL and 1 μg/mL botrocetin to induce aggregation in a lumi-aggregometer. **B**, Washed human platelets, preincubated with 0.1% DMSO, 100 μM NSC23766, 10 μMPP2, or 20μMLY294002 were stimulated with 5 μg/mL VWF and 0.3 mg/mL ristocetin to induce aggregation in a lumi-aggregometer. **C** to **D**, the amount of TXB_2 was determined in WT and $Rac1^{-/-}$ mouse platelets, or human platelets treated with 0.1% DMSO or 100 μMNSC23766, following platelet aggregation stimulated by 1 μg/mL botrocetin or 0.3 mg/mL ristocetin, respectively, ± 5 μg/mL VWF.

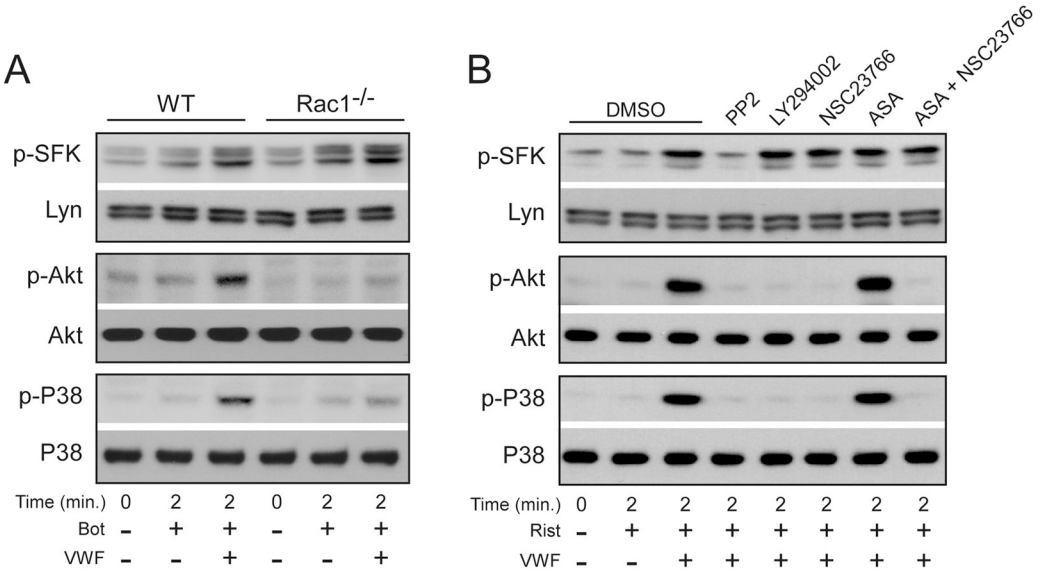

Figure 5.

Rac1 is required for GPIb-IX-mediated activation of the PI3K/Akt pathway. **A**, Washed WT and $Lyn^{-/-}$ mouse platelets or **B,** washed human platelets treated with 0.1% DMSO, 10μM PP2, 20 μM LY294002, 100 μM NSC23766, 1 mM Aspirin (ASA), or 100 μM NSC23766 plus 1 mM ASA were stimulated with 1 μg/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist), respectively, with or without 5 μg/mL VWF in a lumi-aggregometer. **A** and **B**, The amount of phosphorylated SFK (Tyr^{416}), Akt (Ser^{473}), and P38 MAPK (Thr^{180}/Tyr^{182}) was determined using SDS-PAGE and Western blot with appropriate antibodies. Total Lyn, Akt, and P38 are shown as loading controls.

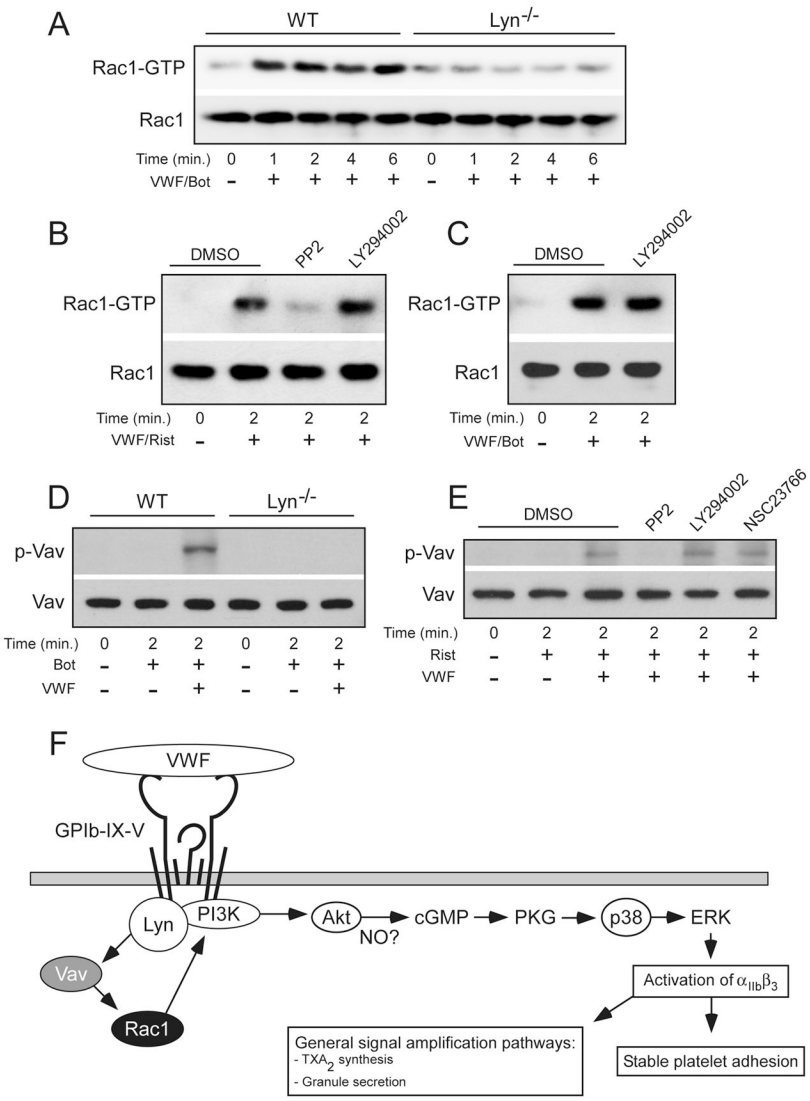

Figure 6.

Activation of Vav and Rac1 during GPIb-IX signaling requires the SFK Lyn. **A**, Washed WT and Lyn$^{-/-}$ mouse platelets, **B,** washed human platelets treated with 0.1% DMSO, 10 μM PP2 or 20 μM LY294002, and **C,** washed WT mouse platelets treated with 0.1% DMSO or 20μM LY294002 were stimulated with 5 μg/mL VWF together with 1 μg/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist) in a lumi-aggregometer. The amount of GTP-bound Rac1 was determined using the GST-PAK pull-down assay, SDS-PAGE, and Western blot. Total Rac1 is shown as loading control. **D,** Washed WT and Lyn$^{-/-}$ mouse platelets or **E,** human platelets treated with 0.1% DMSO, 10 μM PP2, 20 μM LY294002 or 100 μM NSC23766 were stimulated with 1 μg/mL botrocetin (Bot) or 0.3mg/mL ristocetin (Rist), respectively, with or without 5μg/mL VWF. The level of phosphorylated Vav (Tyr^{174}) was determined using SDS-PAGE and Western blot. Total Vav is shown as loading control. **E**, A schematic of the GPIb-IX signaling pathway. VWF binding to GPIb-IX sequentially activates Lyn, Vav, Rac1, PI3K, and Akt. Akt stimulates cGMP-dependent activation of MAPKs (there are still controversies), leading to integrin activation and stable platelet adhesion. This further leads to activation of signal amplification pathways, in which Rac1 may also play a role. Encircled signaling molecules were investigated in this study.