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Rap1-Rac1 Circuits Potentiate Platelet Activation

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Abstract

Objective—The goal of this study was to investigate the potential crosstalk between Rap1 and Rac1, 2 small GTPases central to platelet activation, particularly downstream of the collagen receptor GPVI.

Methods and Results—We compared the activation response of platelets with impaired Rap signaling (double knock-out; deficient in both the guanine nucleotide exchange factor, CalDAG-GEFI, and the Gi-coupled receptor for ADP, P2Y12), to that of wild-type platelets treated with a novel Rac inhibitor, EHT 1864 (wild-type/EHT). We found that Rac1 is sequentially activated downstream of Rap1 on stimulation via GPVI. In return, Rac1 provides important feedback for both CalDAG-GEFI– and P2Y12-dependent activation of Rap1. When analyzing platelet responses controlled by Rac1, we observed (1) impaired lamellipodia formation, clot retraction, and granule release in both double knock-out and wild-type/EHT platelets; and (2) reduced calcium store release in wild-type/EHT but not double knock-out platelets. Consistent with the latter finding, we identified 2 pools of Rac1, 1 activated immediately downstream of GPVI and 1 activated downstream of Rap1.

Conclusion—We demonstrate important crosstalk between Rap1 and Rac1 downstream of GPVI. Whereas Rap1 signaling directly controls sustained Rac1 activation, Rac1 affects CalDAG-GEFI– and P2Y12-dependent Rap1 activation via its role in calcium mobilization and granule/ADP release, respectively.

Keywords

collagen; platelets; signal transduction; CalDAG-GEFI; small GTPases

Platelet activation results in a series of rapid morphological events that depend on actin and are a common feature of all cell-based clotting systems across evolution. Rearrangements of the actin cytoskeleton are crucial for shape change, platelet-substrate (spreading) and platelet-platelet (aggregation) interactions, and granule secretion.[1]

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Disclosure

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The small GTPases of the Ras[2] and Rho[3] families are critical regulators of cellular activation. Small GTPases are molecular switches cycling between a GTP-bound state, able to interact with effector molecules and to initiate downstream responses, and a GDP-bound state. They are tightly regulated by guanine nucleotide exchange factors (GEFs), that accelerate the rate-limiting dissociation of GDP, and guanine nucleotidase-activating proteins, that promote the intrinsic GTPase activity and terminate signal transduction.[4]

RhoA, Cdc42, and Rac1, prototypical members of the Rho family, are the main regulators of actin remodeling in cells.[5] Although RhoA regulates the formation of stress fibers and focal adhesion contacts,[6] Cdc42 promotes the formation of filopodial protrusions.[7] Rac induces the formation of sheet-like lamellipodial protrusions[8] and participates in exocytosis.[9] Independently of actin dynamics, Rac regulates phospholipase Cγ2 (PLCγ2) activation,[10] ROS generation, transcription, MAPK signaling, and cell proliferation.[3] In mammals there are 3 Rac isoforms, but only Rac1 was detected at the protein level in platelets.[11] Recent studies with knockout mouse models and small molecule inhibitors have shown that Rac1 is essential for lamellipodia formation, granule secretion, clot retraction, and PLCγ2 activation in platelets.[11–16] Because of its role in PLCγ2 activation, Rac1 deficient platelets are severely impaired in their response to stimulation via immunoreceptor tyrosine activation motif (ITAM)-coupled receptors, such as the collagen receptor glycoprotein (GP) VI,[16] or the hemITAM receptor C-type lectin-like receptor 2 (CLEC-2).[17]

Within the Ras family, Rap GTPases are most recognized for their role in integrin activation, the regulation of cell-cell contacts, the establishment of cell polarity and the control of exocytosis.[2,18,19] In mammals, the Rap family consists of 2 rap1 genes and 3 rap2 genes encoding proteins that are approximately 65% homologous. Platelets express significant amounts of Rap1B and Rap2B, with Rap1B accounting for approximately 90% of the total Rap protein.[20] Loss of Rap1B inhibits integrin αIIbβ3 activation and platelet spreading on fibrinogen, and it impairs collagen-induced dense granule secretion.[21] In our recent work, we established a 2-pathway model for Rap1-activation in platelets: 1 pathway triggered by calcium and mediated by the guanine nucleotide exchange factor, CalDAG-GEFI, and a CalDAG-GEFI–independent pathway that requires granule release and cosignaling via the Gi-coupled receptor for ADP, P2Y12.[22–24] Blocking of both pathways simultaneously results in inhibition of Rap1 activation, integrin activation, granule secretion[22,25] and thromboxane A_2 generation.[23]

Interestingly, there is growing evidence of cooperation between Rap1 and Rac1 signaling during cellular activation.[26] For example, they are both necessary to form lamellipodial protrusions in migrating fibroblasts,[27] and they regulate the cytoskeleton dynamics required for the exocytotic process in pancreatic acinar cells.[28] In platelets, both Ca^{2+}-dependent and Gαi signaling pathways affect Rac1 activation.[29,30] Thus, the same pathways that regulate Rap1 activation seem to control the activation of Rac1.

In the present study, we investigated the hypothesis that Rac1 in platelets is under the control of Rap1. Using transgenic mice with impaired Rap signaling in combination with a novel inhibitor to Rac1, we found that (1) downstream of the ITAM-coupled receptor, GPVI, Rap1, and Rac1 facilitate optimal platelet activation by providing crucial feedback activation for each other, and (2) there are at least 2 (temporally) distinct pools of Rac1, with only 1 of them controlled by Rap1.

Methods

Reagent and Antibodies

See the Supplemental Material, available online at http://atvb.ahajournals.org, for an exhaustive list of the materials used.

Mice

CalDAG-GEFI–/– × P2Y12–/– mice were obtained from the intercross of CalDAG-GEFI–/–[25] and P2Y12–/–[31] mice. Experimental procedures were approved by the Animal Care and Use Committee of Thomas Jefferson University.

Platelet Preparation

Blood was drawn from the retro-orbital plexus into heparinized tubes. Platelet-rich plasma (PRP) or washed platelets were prepared as described previously.[23] See Supplemental Methods, available online at http://atvb.ahajournals.org, for details.

Small-GTPase Activation Assays

Rap1, Rap2, and Rac1 activation were determined by pull-down assay followed by standard Western blotting procedures as described previously.[23] Rac1-GTP was also quantified with the Rac G-LISA™ (Absorbance Based) Biochem kit (Cytoskeleton, Denver, CO) according to the manufacturer instructions. See Supplemental Methods for details.

Spreading Assay

Spreading of platelets on fibrinogen in the presence or absence of the GPVI agonist convulxin (Cvx) was performed as described in Supplemental Methods.

Platelet Clot Retraction

Washed murine platelets were resuspended in citrated human platelet-poor plasma (4×10^8 platelets/mL) and 150 μmol/L EHT 1864 or DMSO were added. Coagulation was triggered by addition of thrombin (0.4 U/mL). Clots were allowed to retract at 37°C for 1 hour. The extent of clot retraction was determined as the volume of serum extruded from the clot and expressed as percentage of the total reaction volume.

Dense Granule Secretion

The release of ^{3}H-serotonin into platelet supernatants after 10 minutes of stimulation was measured as described previously.[23] See Supplemental Methods for further details.

Flow Cytometry

αIIbβ3 Activation and α-Granule Secretion—Washed platelets diluted in Tyrode's buffer containing 0.35% BSA and 1 mmol/L CaCl2 (10^8 platelets/mL) were stimulated for 10 minutes with increasing doses of Cvx, costained with JON/A-PE[32] (Emfret Analytics, Wuerzburg, Germany) and α-P-selectin-FITC (BD Biosciences, Rockville, MD) for 10 minutes and immediately analyzed by flow cytometry.

Calcium Flux Measurement—Washed platelets were incubated with 5 μmol/L Fluo-4 (Invitrogen, Carlsbad, CA) for 15 minutes, diluted in Tyrode's buffer (10^8 platelets/mL) containing 2.5 mmol/L EDTA, activated with Cvx, and analyzed for fluorescence 1 intensity over a period of 3 minutes.

Aggregometry

Performed as described previously.[23] See Supplemental Methods for details.

Statistics

Results are reported as mean±SD and statistical significance was assessed by unpaired 2-tailed Student *t*-test. A probability value less than 0.05 was considered significant.

Results

The small GTPases Rap1 and Rac1 are critical players in platelet activation, in particular downstream of the collagen receptor GPVI. Studies in other cell systems suggest a crosstalk between Rap1 and Rac1 during cellular activation. In this study, we sought to investigate whether Rap1-Rac1 circuits regulate platelet activation.

In a first step, we evaluated the effect of impaired Rap1 signaling on the activation of Rac1 in platelets stimulated with Cvx, a snake venom toxin that specifically targets the collagen receptor GPVI (Figure 1). In WT platelets, activation of both Rap1 and Rac1 was stronger at t=1 minute than at t=10 minutes after addition of Cvx. Deficiency in CalDAG-GEFI markedly reduced the activation of Rap1 and Rac1 at t=1 minute, while little effect was observed at t=10 minutes. In contrast, inhibition of P2Y12×2-MesAMP reduced Rap1 and Rac1 activation both in the early and the late phase of activation. Virtually complete inhibition of Rap1 and Rac1 activation at t=1 minute and t=10 minutes was observed in 2-MesAMP-treated CalDAG-GEFI–/– platelets, demonstrating a strong correlation between the activation of Rap1 and Rac1 in platelets activated via GPVI (Figure 1A, B). To eliminate any off-target effects of 2-MesAMP,[33] we studied the activation of Rap1 and Rac1 in platelets isolated from mice deficient in CalDAG-GEFI and P2Y12 (double knock-out, DKO) (Figure 1C, D). Consistent with the studies using 2-MesAMP to block P2Y12, we observed virtually complete inhibition of Rap1 and Rac1 activation in Cvx-activated DKO platelets, both at t=1′ and 10′ after addition of the agonist. Importantly, inhibition of αIIbβ3 integrin with a monoclonal antibody during the activation process did not markedly affect Rac1 activation in WT platelets (Supplemental Figure 1, available online at http://atvb.ahajournals.org), demonstrating that the contribution of Rap1 to Rac1 activation is not secondary to Rap1-mediated integrin activation. Furthermore, DKO platelets also failed to activate the small GTPase Rap2 in response to Cvx treatment (Supplemental Figure 2, available online at http://atvb.ahajournals.org), demonstrating that all Rap signaling in platelets is under the control of CalDAG-GEFI and P2Y12.

To confirm the functional association between Rap1 and Rac1 in Cvx-stimulated platelets, we compared the platelet activation response of DKO platelets to that of WT platelets treated with the small molecule inhibitor EHT 1864 (WT/EHT). Previous studies have shown that EHT 1864 specifically blocks Rac, but not RhoA and Cdc42, activation in vitro and in vivo.[34,35] In platelets, EHT 1864 inhibited Rac1 activation downstream of ITAM-coupled receptors[17] at a dosage of 100 μmol/L (Supplemental Figure 3, available online at http://atvb.ahajournals.org). Both WT/EHT and DKO platelets showed impaired spreading on a fibrinogen surface after stimulation with Cvx (Figure 2A and 2B). Shape change and the formation of filopodia and lamellipodia was observed in WT platelets as early as 3 minutes after addition of Cvx. Lamellipodia formation progressed continuously, leading to a more than doubling of the platelet surface area within 30 minutes of activation. In contrast, DKO or WT/EHT platelets underwent shape change and filopodia formation but failed to form lamellipodia. Consequently, their platelet surface area did not markedly increase. Importantly, we did not observe any difference in platelet surface area between DKO, WT/EHT, and DKO/EHT platelets, suggesting that both Rap1 and Rac1 are crucial for

lamellipodia formation and spreading in Cvx-activated platelets. Rac1 has also been implicated in clot retraction, another actin-based mechanism initiated by integrin outside-in signaling. We could confirm this finding in WT/EHT platelets, where clot retraction was significantly delayed when compared to WT controls (Figure 2C, D). Importantly, however, impaired Rap1 signaling (DKO platelets) had a significantly stronger inhibitory effect on clot retraction than inhibition of Rac1, possibly because of the direct role of Rap1 in integrin activation.

Next, we evaluated the ability of DKO and WT/EHT platelets to release their granules (Figure 3). Consistent with recent studies,[16] Cvx-induced release of α granules was markedly impaired in WT/EHT platelets, in particular in response to low and medium doses of the agonist (Figure 3A). At the highest tested concentration of Cvx, however, α granule release in WT/EHT platelets was significantly reduced but not abolished when compared to that of WT platelets. In comparison, α granule release was completely abolished in DKO platelets at all agonist concentrations tested (10-fold the EC50 for Cvx). Pretreatment with EHT 1864 did not affect secretion in Cvx-stimulated DKO platelets. Rap1 and Rac1 signaling contributed in a similar fashion to dense granule release (Figure 3B). Inhibition of Rac1 signaling by EHT 1864 abolished dense granule release at low/medium but not high doses of Cvx, whereas virtually complete inhibition of dense granule release was observed in DKO platelets at all agonist concentrations tested.

In contrast to spreading, clot retraction and granule release, WT/EHT, and DKO platelets did not contribute in a similar way to PLCγ2-mediated calcium (Ca^{2+}) mobilization in Cvx-stimulated platelets (Figure 4A, B). Compared to WT controls, Ca^{2+} store release in WT/EHT platelets was reduced by ≈50%, both at low and high doses of the agonist. Ca^{2+} flux observed in DKO platelets, however, was not significantly different to that in WT cells. Similar results were obtained on stimulation of platelets with an alternative GPVI-specific agonist, collagen-related peptide (CRP) (Supplemental Figure 4, available online at http://atvb.ahajournals.org), suggesting that in GPVI-stimulated platelets both Rap1 and Rac1 signaling contribute to spreading and granule release, but Rac1 alone participates in PLCγ2 activation and subsequent Ca^{2+} mobilization. One potential explanation for this finding is that the activation of Rac1 immediately downstream of the agonist receptor occurs independent of Rap1. Our studies confirm this hypothesis, as we observed that at t=10 seconds after Cvx stimulation Rac1 activation in DKO platelets was comparable to that of WT cells, whereas Rap1 activation was completely abolished. At later time points, however, Rac1-GTP–levels in WT platelets increase and reach a plateau after t=1 minute, while a sharp decrease in Rac1 activation was observed in the DKO platelets (Figure 4C and 4D). Similarly, results were observed using a quantitative ELISA-like approach (Supplemental Figure 5, available online at http://atvb.ahajournals.org).

In our previous work, we demonstrated that Rap1 activation in platelets depends on independent yet synergistic signaling by Ca^{2+}/CalDAG-GEFI and ADP/P2Y12.[22–24] While signaling by Ca^{2+}/CalDAG-GEFI mediates the rapid but reversible activation of Rap1, signaling by ADP/P2Y12 is crucial for the sustained activation of the small GTPase. Consistent with the effect of Rac1 inhibition on calcium mobilization (Figure 4) and granule release (Figure 3), we observed that pretreatment of WT platelets with EHT 1864 impaired both pathways leading to Rap1 activation. Compared to WT controls, Rap1 activation in WT/EHT platelets activated with high-dose Cvx was markedly reduced at both early (t=30″) and late (t=600″) time points (Figure 5A). However, it was not completely abolished and the residual activation was sufficient to facilitate αIIbβ3 activation and platelet aggregation (Figure 5B–5D).

Discussion

Our studies establish a central role for Rap1-Rac1 signaling circuits in platelet activation (Figure 6). Downstream of the collagen receptor, GPVI, 1 pool of Rac1 is activated immediately and contributes to PLCγ2-mediated mobilization of intracellular calcium ([Ca^{2+}]i). Increased [Ca^{2+}]i triggers CalDAG-GEFI–mediated Rap1 activation, which is crucial for the activation of a second pool of Rac1. Sustained Rac1 activation is required for actin-dependent platelet responses such as spreading and granule release. In turn, ADP released from dense granules induces sustained activation of Rap1 via engagement of P2Y12. Thus, a continuous crosstalk between the small GTPases, Rac1 and Rap1, is required for optimal platelet activation downstream of the collagen receptor GPVI.

Using inhibitors to P2Y12 in combination with CalDAG-GEFI–/– platelets, we have recently demonstrated that (1) each pathway independently contributes to Rap1 activation in activated platelets, and (2) Rap1 activation is virtually abolished in P2Y12 inhibitor-treated CalDAG-GEFI–/– platelets.[22–24] To rule out reported off-target effects of P2Y12 inhibitors in our studies,[33] we here used platelets isolated from mice deficient for both CalDAG-GEFI and P2Y12 (DKO). So far, our limited breeding efforts of DKO mice from crosses of mice heterozygous for both CalDAG-GEFI and P2Y12 did not reveal increased embryonic lethality of DKO pups (not shown). This finding is in contrast to the 85% embryonic and perinatal lethality reported in Rap1B–/– mice.[21] However, although Rap1 is widely expressed, both CalDAG-GEFI[36] and P2Y12[37] show a much more restricted expression profile. Thus, it appears likely that the increased lethality observed in Rap1B–/– mice is caused by impaired function of a cell type other than platelets. In addition to viability, peripheral platelet counts and platelet size in DKO mice were comparable to WT controls (not shown). However, Rap1 activation and integrin-mediated aggregation were abolished in the Cvx-stimulated DKO platelets (Figure 1 and 5), confirming our recent studies with P2Y12 inhibitors.[22,23]

We are aware that DKO platelets may have defects that are independent of Rap1 signaling. A prominent consequence of P2Y12 engagement is inhibition of adenylyl cylcase via Gαi.[37] Several studies, however, have documented that there is no causal relationship between adenylyl cyclase inhibition and platelet aggregation.[38,39] P2Y12 also leads to the activation of PI3 kinase signaling. At least part of the PI3 kinase-driven response, however, is mediated via activation of Rap1.[40,41] The serine/threonine protein kinase B/Akt is another effector of PI3 kinase. It is currently not clear if Akt and Rap1 are part of the same signaling pathway or if they operate independent of each other. Although previous works have shown that Rac1 activation is dependent on an increase in [Ca^{2+}]i and potentiated by signaling via P2Y12,[29,30] the mechanism(s) by which Ca^{2+} and P2Y12 modulate this molecule are unknown. Our results suggest that both Ca^{2+}-and P2Y12-dependent Rac signaling is mediated by Rap1, as (1) we observed a strong correlation between the activation kinetics of Rap1 and Rac1 in stimulated platelets (Figure 1); (2) sustained, but not early, Rac1 activation is abolished in DKO platelets (Figure 4C, D); (c) Rap1 and Rac1 mediate similar cellular responses such as spreading, clot retraction, and granule release (Figure 2 and 3), and (4) the GEF activity of CalDAG-GEFI is directed toward Rap but not Rac GTPases.[36] Unfortunately, Rac1 activation has not been examined in platelets from Rap1B–/– mice. A very recent study on platelet function in Rap1B–/– mice, however, shows a role of Rap1B in granule secretion, spreading on fibrinogen and clot retraction, but not in calcium mobilization.[42] Thus, Rap1B–/– platelets show defects in Rac1-dependent platelet responses, supporting our conclusion of a crosstalk between the 2 small GTPases. The defect in secretion of the Rap1B–/– platelets is less severe than that observed in platelets deficient in CalDAG-GEFI and P2Y12. To us the most likely explanation for this difference is provided by back-up signaling in Rap1B–/– platelets via Rap2, which was shown to

associate with granules in human neutrophils.[43] Confirming this hypothesis, we here provide first evidence that CalDAG-GEFI mediates Rap2 activation in Cvx-activated platelets and that simultaneous deficiency of CalDAG-GEFI and P2Y12 prevents the generation of Rap2-GTP (Supplemental Figure 2).

Recent studies in transgenic mice identified a key role for Rac1 in various platelet responses.[11–13,16] In our study we blocked Rac1 activation with EHT 1864, an inhibitor that directly binds Rac, displaces the nucleotide and inhibits further GTP loading.[35] Thus, EHT 1864 inhibits Rac activation independent of which Rac-GEF catalyzes the GDP-GTP exchange, a clear advantage over other Rac inhibitors that target the function of individual Rac-GEFs.[44] Characterization studies in vitro and in cell types other than platelets have shown that EHT 1864 specifically inhibits all known Rac isoforms (Rac1>Rac2≫Rac3), but not other Rho GTPases such as Cdc42 and RhoA.[35] In platelets stimulated via ITAM-coupled receptors, EHT 1864 completely prevents the activation of Rac1,[17] the only Rac isoform expressed at detectable levels in these cells.[11] Moreover, WT platelets treated with EHT 1864 and platelets isolated from Rac1–/– mice[11–13,16] show very similar defects in the platelet activation response, suggesting that neither RhoA nor Cdc42-activation are affected by this compound. Using EHT 1864-treated WT platelets as a control for impaired Rac1 signaling, we here provide the first evidence for Rap1-dependent and -independent pools of Rac1 in platelets (Figure 4C). Although the Rap1-independent pool of Rac1 is critical for Ca^{2+} mobilization in platelets, Rap1-dependent Rac1 signaling controls spreading, clot retraction, and granule release. Rac1 activation independent of Rap1 is consistent with studies in lymphocytes, where the Rac-GEF Vav is activated directly by tyrosine kinases and mediates Rac-dependent PLCγ activation downstream of ITAM-coupled receptors.[45] In platelets, the Vav1 and Vav3 isoforms have been implicated in GPVI-dependent PLCγ2 activation.[46] It is currently not clear how Rap1 controls Rac1 in platelets or other cells, but it has been reported that GTP-bound Rap1 can bind to at least 3 distinct Rho-family GEFs. Studies in fibroblasts showed that Rap1 directly binds the RacGEFs Vav2 and Tiam1, and that Rap1 is important for the translocation of the Rac-GEFs to the plasma membrane but not the GTP-loading of Rac1.[47] However, a more recent study in T cells demonstrates that constitutively active Rap1 directly binds Tiam1 and enhances Rac1 GTP-loading.[48] A separate study further identified that binding of Rap1-GTP to STEF (Tiam2) is necessary for STEF-dependent Rac1 activation in Chinese hamster ovary cells.[49] Our studies show markedly impaired Rac GTP-loading in DKO platelets, suggesting that GEF-mediated activation of the small GTPase rather than the translocation of the GEFs may be responsible for the observed crosstalk in platelets. It is also conceivable that active Rap inhibits a Rac-guanine nucleotidase-activating proteins, which would lead to sustained Rac activity. To the best of our knowledge, however, such an interaction has not been documented. Furthermore, our results do not exclude the possibility that Rap affects Rac indirectly via a different signaling pathway that feeds into Rac1 activation.

Although the spreading defect in DKO and WT/EHT platelets was very similar, we observed interesting differences in granule release and calcium mobilization in these cells. The release of both α- and dense-granules was virtually abolished in DKO platelets, whereas significant granule release was observed in WT/EHT cells stimulated with high-dose Cvx (Figure 3). In contrast, Cvx-induced Ca^{2+} mobilization from internal stores was reduced in WT/EHT but not DKO platelets (Figure 4A and 4B). It is difficult to speculate why granule release in the absence of Rap1 signaling is completely abolished, whereas inhibition of Rac1 or genetic deletion of Rac1[16] only partially blocks this response. One possibility is that integrin outside-in signaling provides important feedback for granule release, and that this process is more profoundly affected in DKO platelets. Supporting this hypothesis, we observed a stronger contribution of Rap1 to clot retraction (Figure 2C and 2D), a cellular response dependent on integrin outside-in signaling.[50] Alternatively, Rap could be directly

involved in granule release as it has been suggested based on its enrichment in granule membranes.[18,43] Further studies are required to address this point.

In conclusion, we show that Rap1-Rac1 circuits potentiate platelet activation downstream of the collagen receptor, GPVI. While signaling via Rac1 affects both the early and the late phase of Rap1 activation, active Rap1 is required for sustained but not immediate activation of Rac1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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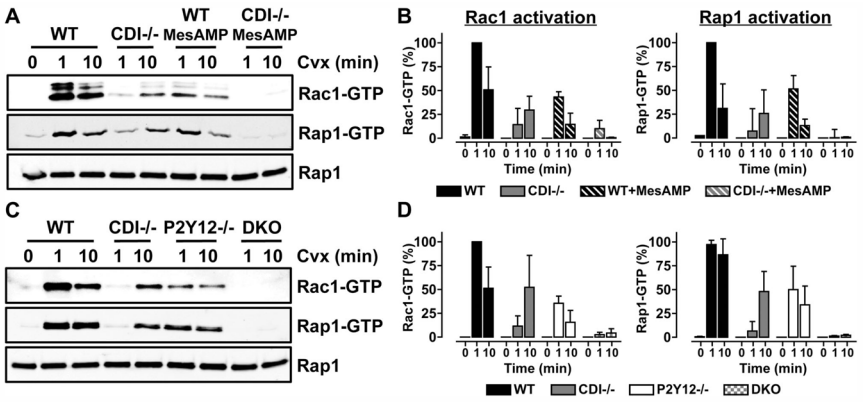

Figure 1.

Rap1 activation correlates with Rac1 activation in platelets. **A**, **B**, Time course of Rac1 (top panel) and Rap1 (central panel) activation on stimulation of wild type (WT, black bars) or CalDAG-GEFI–/– (CDI–/–, gray bars) platelets with 500 ng/mL convulxin (Cvx) in the absence (solid pattern) or presence (striped pattern) of the P2Y12 inhibitor, 2-MesAMP (100 μmol/L, MesAMP). **C**, **D**, Time course of Rac1 and Rap1 activation on stimulation of WT (black bars), CalDAG-GEFI–/– (gray bars), P2Y12–/– (white bars) or CalDAG-GEFI–/– ×P2Y12–/– (double knock-out [DKO], white/gray checkered bars) platelets with 500 ng/mL convulxin. **A**, **C**, Representative images: the bottom panel shows total Rap1 as loading control. **B**, **D**, Densitometric analysis of Rac1-GTP (left) and Rap1-GTP (right) shown as percentage of maximal activation (mean±SD, n=3).

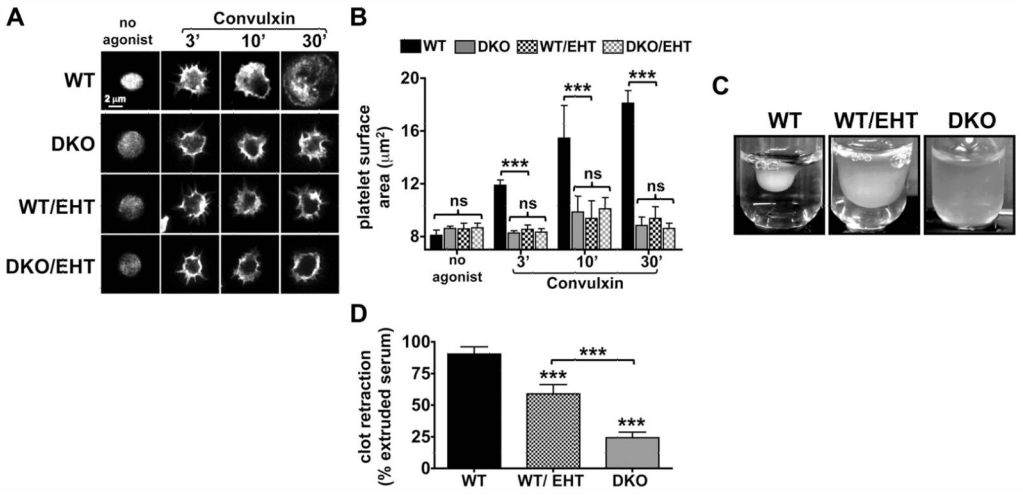

Figure 2.

Rap1 and Rac1 mediate platelet spreading and clot retraction. **A**, **B**, Spreading of convulxin-activated platelets on fibrinogen-coated slides. **A**, Representative images captured with a 100 × oil objective. **B**, Surface area (μm^2) of spreading platelets (mean±SD, n=100/experiment; 3 independent experiments). **C**, **D**, Clot retraction. **C**, Representative images. **D**, The extent of clot retraction expressed as percentage of serum extruded from the clot (mean ±SD, n=5). Platelet preparations studied: wild type (WT, black bar) and CalDAG-GEFI–/– ×P2Y12–/– (double knock-out [DKO], gray bar) in the absence (solid bar) or presence of EHT 1864 (checkered bar). ***P<0.0001.

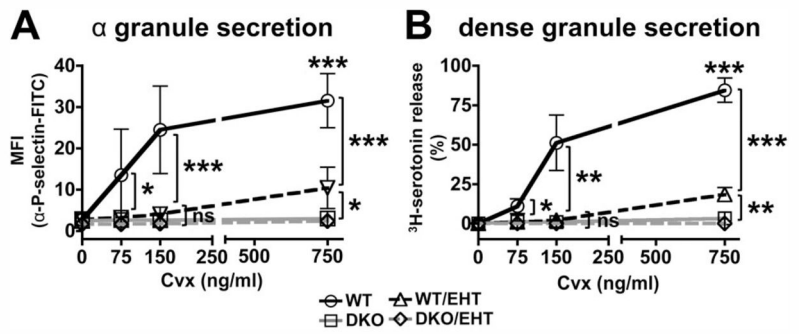

Figure 3.

Rap1 and Rac1 contribute to GPVI-mediated α- and dense-granule secretion. **A**, Dose response of convulxin (Cvx)-induced α-granule secretion determined by flow cytometry (binding of FITC-conjugated α-P-selectin antibody). Data shown are mean fluorescence intensities (MFI)±SD (n=6). **B**, Dose response of Cvx-induced dense granule secretion determined by ^{3}H-serotonin release into platelet supernatants after 10 minutes of stimulation (mean±SD; n=6 in 3 independent experiments), *P<0.05, **P<0.01, ***P<0.001. Platelet preparations studied: wild type (WT, black line) and CalDAG-GEFI–/–×P2Y12–/– (double knock-out [DKO], gray line), in the absence (solid line) or presence (discontinued line) of 100 μmol/L EHT 1864.

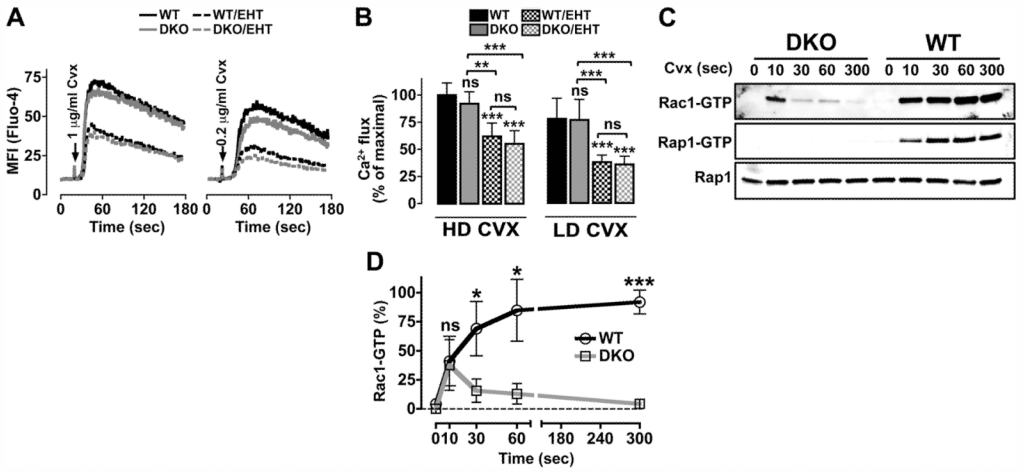

Figure 4.

A distinct pool of Rac1 is activated independently of Rap1 signaling immediately after GPVI stimulation. **A**, Calcium store release of wild type (WT, black) and CalDAG-GEFI–/–×P2Y12–/– (double knock-out [DKO], gray) platelets, in the absence (solid line and bar) or presence (discontinued line, checkered bar) of 100 μmol/L EHT 1864, stimulated with high (left: 1 μg/mL, HD CVX) or low (right: 0.2 μg/mL, LD CVX) dose convulxin (Cvx). **B**, Results expressed as the percentage of maximal Fluo-4 fluorescence intensity±SD measured in the indicated platelet preparations, n=6; ***P*<0.01, ****P*<0.001. **C**, **D**, Time course of Rac1 (top panel) and Rap1 (central panel) activation in WT (black line) and CalDAG-GEFI–/–×P2Y12–/– (DKO, gray line) platelets. **C**, Representative images; the bottom panel shows total Rap1 as loading control. **D**, Densitometric analysis of Rac1-GTP levels shown as percentage of maximal activation (mean±SD, n=3), **P*<0.05.

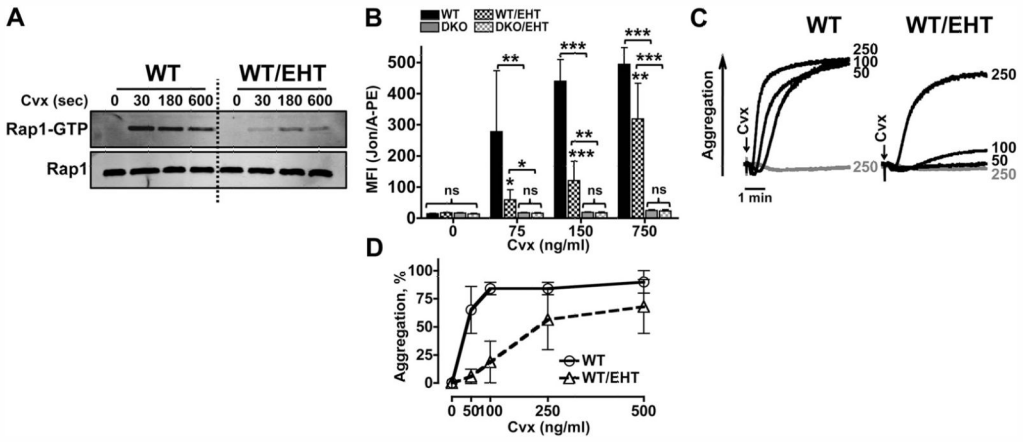

Figure 5.

Rac1 signaling provides feedback for Rap1 and integrin activation in GPVI-stimulated platelets. **A**, Time course of Rap1 activation (upper panel) in platelets stimulated with 250 ng/mL convulxin (Cvx)in the absence (wild type [WT]) or presence of 100 μmol/L EHT 1864 (WT/EHT); representative of 3 independent experiments. The lower panel shows total Rap1 as loading control. **B**, Flow cytometric determination of αIIbβ3 integrin activation (binding of PE-conjugated Jon/A antibody) in Cvx-stimulated platelets (WT: black, double knock-out [DKO]: gray, WT/EHT: checkered black, DKO/EHT: checkered gray). Data shown are mean fluorescence intensities (MFI)±SD, n=6; **P*<0.05, ***P*<0.01, ****P*<0.001. **C**, **D**, Platelet aggregation of WT platelets in the absence (WT, solid line) or presence of 100 μmol/L EHT 1864 (WT/EHT, discontinued line). **C**, Representative aggregation traces (CalDAG-GEFI–/–×P2Y12–/– (DKO) aggregation in response to 250 ng/mL of Cvx is shown for comparison, gray traces). **D**, Percentage of platelet aggregation determined at t=5 minutes after addition of the agonist (mean±SD, n=3).

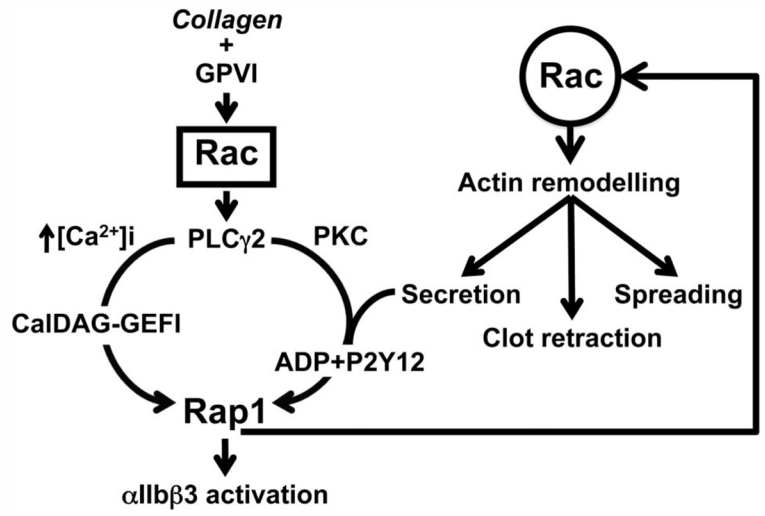

Figure 6.

Schematic representation of the crosstalk between Rac1 and Rap1 in GPVI-stimulated platelets. Two distinct pools of Rac1 control (1) phospholipase Cγ2 (PLCγ2)-dependent calcium (Ca^{2+}) flux (rectangle) and (2) actin dynamics involved in granule secretion, spreading, and clot retraction (circle). Both Rac1 pools potentiate Rap1 activation by amplifying the Ca^{2+}/CalDAG-GEFI– and the secretion/P2Y12-dependent pathways, respectively. In turn, Rap1 positively regulates only Rac1-dependent actin remodelling (circle).