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Rho GTPases in Platelet Function

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Summary

The Rho family of GTP binding proteins – also commonly referred to as the Rho GTPases – are master regulators of the platelet cytoskeleton and platelet function. These low molecular weight or "small" GTPases act as signaling switches in the spatial and temporal transduction and amplification of signals from platelet cell surface receptors to the intracellular signaling pathways that drive platelet function. The Rho GTPase family members RhoA, Cdc42 and Rac1 have emerged as key regulators in the dynamics of the actin cytoskeleton in platelets and play key roles in platelet aggregation, secretion, spreading and thrombus formation. Rho GTPase regulators, including GEFs and GAPs and downstream effectors, such as the WASPs, formins and PAKs, may also regulate platelet activation and function. In this review, we provide an overview of Rho GTPase signaling in platelet physiology. Studies of Rho GTPases and platelets have had a shared history, as platelets have served as an ideal, non-transformed cellular model to characterize Rho function. Likewise, recent studies of the cell biology of Rho GTPase family members have helped to build an understanding of the molecular regulation of platelet function and will continue to do so through the further characterization of Rho GTPases as well as Rho GAPs, GEFs, RhoGDIs and Rho effectors in actin reorganization and other Rho-driven cellular processes.

Keywords

actin; cytoskeleton; platelet; Rac; Rho; Cdc42

Introduction

Platelets function as the primary cellular mediators of hemostasis and thrombosis [1]. As the guardians of vascular integrity, platelets patrol the circulation for vessel leakage in a quiescent, bidiscoid shape. Upon detecting molecular cues of vessel damage, platelets undergo a dramatic change in shape, bind to adhesive protein substrates and aggregate to form vascular plugs to ultimately halt bleeding. As platelets encounter exposed extracellular matrix proteins such as collagen and laminin, receptors on platelets trigger intracellular signaling events that rapidly result in platelet activation and a complex rearrangement of platelet morphology to form filopodia and lamellipodia (Fig. 1). Finger-like platelet filopodial extensions and actin-rich sheets of lamellipodia dramatically increase platelet surface area and stabilize platelet aggregates to form thrombotic plugs (Fig. 1). The molecular events that drive platelet surface adhesion, aggregation and thrombus formation are critical to platelet function in physiology as well as pathology, as platelet activation is a contributing factor to atherothrombosis, sepsis, and cancer as well as other disease states [2–4]. Cellular biological paradigms of signal transduction have refined the understanding of the molecular basis of platelet regulation as a crosstalk between phosphorylation-, cyclic

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nucleotide-, calcium-, lipid-based signaling pathways that commit platelets to an activated state through rapid changes in the platelet cytoskeleton [5]. These systems that control platelet function are interdependently coordinated by a number of molecular mediators that integrate, amplify and regulate intracellular signals, including GTP-binding proteins, or small GTPases.

Small GTPase proteins serve as signaling mediators in the control of diverse cellular processes [6, 7]. These intracellular mediators of signal transduction are homologous to the Gα subunits of heterotrimeric G proteins that couple to G protein coupled receptors (GPCRs) and similarly bind GTP to promote an interaction with specific effectors, and in turn activate downstream signaling events (Fig. 2). Like Gαs, the intrinsic GTPase activity of small GTPases leads to the hydrolysis of GTP to GDP, serving as a molecular and temporal "on-off" switch in the control of effector activation and function. Small GTPases differ in part from Gαs in that they lack intrinsic GTPase activating domains, which accounts for their "small" molecular weights in the range of 21 kD, and their slower GTP hydrolysis rates. A fortunate convergence of findings in the cell biology, virology and oncology fields described the rat sarcoma virus gene product, Ras, as the first small GTPase protein, linking retrovirus gene expression to aberrant GTPase signaling, cellular proliferation and oncogenesis [8, 9]. A quest to identify other Ras-like genes led to the discovery of a set of Ras homolog, or Rho, GTPase proteins first in marine snails [10, 11] and subsequently in yeast [12], where the Rho homologous Cdc42 GTPase was found to be required for budding and cell polarity [13–15]. Unlike the Ras GTPase, the Rho proteins were found to be targets of the *Clostridium botulinum* C3 transferase enzyme, which modifies and inactivates Rho GTPases by ADP ribosylation at a specific, conserved asparagine residue to promote microfilament disassembly [16, 17]. The roles of the Rho proteins in actin cytoskeletal dynamics became further evident in early experiments showing that botulinum C3 enzyme induced a loss of actin stress fibers in epithelial cells through Rho modifications [16]. Similar experiments led to the discovery of Rac, a Ras-related C3 botulinum toxin substrate and small GTP binding protein that was noted to be abundant in platelets [18, 19]. While over 20 Rho family members have been identified in mammalian cells, RhoA, Cdc42 and Rac have held the spotlight as central players in cellular functions including cell division, exocytosis and actin cytoskeletal dynamics [20, 21].

Initial studies of Rho GTPases in platelet function alluded to their important functional roles in platelet cytoskeletal rearrangements [22, 23]. Accordingly, platelets have served as an important model for studies of Rho GTPase function owing to their conserved actin pathways [24, 25]. Studies of the Rho GTPases have fueled discoveries central to the functional biology of platelets and hemostasis (Table 1). In platelets, RhoA plays a role in actin contractility and contributes to platelet shape change upon activation as well as thrombus stability [26–29]. Rac1 controls the formation of platelet lamellipodia [30–33]. The specific roles of Cdc42 in platelet function are controversial [34, 35]; however, Cdc42 activation is associated with platelet filopodia formation and granule secretion [25, 36]. Despite these generalized functions of the Rho GTPases in specific steps of platelet activation, an interdependent model of Rho function in platelet physiology is emerging that integrates a crosstalk amongst integrins, GPCRs and Rho proteins in platelet cytoskeletal regulation, aggregation, secretion, thrombus formation and other cellular processes.

RhoA, platelet contractility and thrombus stability

The highly homologous Rho GTPase proteins RhoA, RhoB and RhoC are best known for their roles in focal adhesion formation and actomyosin contractions, as microinjection or overexpression of these Rho isoforms in fibroblasts promotes actin stress fiber formation [11, 37]. Experiments using the clostridial enzyme C3 transferase, which inhibits these three

Rho isoforms through ADP ribosylation, has helped to identify Rho protein family members and define their functions in a number of cell types, including platelets [22, 23, 38]. RhoA is likely the most highly expressed Rho isoform in human platelets [23, 39, 40] and is most noted for its general role in actomyosin contractility to drive changes in shape from a quiescent discoid shape to small spheres upon activation [5, 27, 28]. In platelets, RhoA works downstream of a number of G-protein coupled receptor pathways to control platelet shape, as RhoA maintains platelet sphericity and promotes contractility, contributing to platelet shape change upon activation (Fig. 3).

Over the past decade, multidisciplinary studies in the GPCR, integrin and platelet biology fields have synergistically established a model of how RhoA mediates platelet function from the levels of protein structure to thrombus formation *in vivo* [5, 41]. The Rho proteins were first suspected as regulators of platelet function after studies demonstrated that C3 enzyme blocked platelet function and that RhoA was a major C3 target in platelets [22, 23]. An investigation of GPCR signaling pathways downstream of thromboxane A2 (TxA2) receptors first found that upon stimulation with the prostaglandin PGH2 analog and thromboxane mimetic, U46619, platelet $G\alpha_q$ activates PLC to drive lipid and calcium signaling. While $G\alpha_q$-deficient platelets fail to aggregate or release granules upon agonist stimulation, they still undergo a characteristic shape change upon stimulation [42]. However, Rho inhibition with C3 enzyme or the ROCK inhibitor, Y-27632, blocks this shape change and actin polymerization in $G\alpha_q$-deficient systems in which $G\alpha_{12}/G\alpha_{13}$ are active [27]. These classic studies were the first to show that stimulation of platelets with U46619 in $G\alpha_q$-deficient systems leads to the activation of $G\alpha_{12}/G\alpha_{13}$ and MLC phosphorylation and shape change in a Rho and ROCK dependent manner. In its active form, RhoA-GTP binds to and activates ROCK to drive actin remodeling events [43]. Once active, ROCK phosphorylates and inactivates the myosin light chain (MLC) phosphatase to result in a net increase in MLC phosphorylation [44, 45] and the subsequent actomyosin contractions that promote a change in platelet shape from discoid to spheres [27, 46]. In addition to the $G\alpha_{13}$-Rho-ROCK pathway, MLC phosphorylation is also promoted through $G\alpha_q$ signaling, which stimulates MLC kinase activation and MLC phosphorylation directly [47].

Platelets express both $G\alpha_{12}$ and $G\alpha_{13}$; however, $G\alpha_{12}$-null platelets have limited phenotypic alterations and thus $G\alpha_{13}$ has emerged as a dominant player in platelet RhoA activation downstream of GPCR stimulation [48, 49]. In its GTP-bound form, $G\alpha_{13}$ directly activates a specific guanine nucleotide exchange factor (GEF), p115RhoGEF, which promotes the activation of RhoA through its $G\alpha_{13}$ switch region 1 (SRI). This switch region of $G\alpha_{13}$ interacts with p115RhoGEF to mediate Rho activation [50] and is critical for protease-activated receptor (PAR) mediated platelet shape changes and aggregation [50]. The RhoA-ROCK-MLC axis is cyclically targeted through a number of steps in the platelet activation pathway to temporally mediate cell spreading, thrombus stability and clot retraction (Fig. 3). In the initial stages of platelet activation, the coordinated inhibition of the MLC phosphatase and activation of MLC kinase is thought to promote platelet secretion and rounding (Fig. 3A). After this first wave of platelet RhoA activation, $G\alpha_{13}$-GPCR-integrin crosstalk, in which $G\alpha_{13}$ binds to the cytosolic domain of integrin β_3, mediates Src family kinase (SFK) activation and platelet spreading through RhoA inhibition (Fig. 3B) [5, 51]. In this step, $G\alpha_{13}$ associates with the cytosolic domain of integrin β_3 to promote the activation of c-Src. c-Src then phosphorylates and activates p190RhoGAP, a GTPase activating protein that stimulates Rho-GTP to GDP hydrolysis and RhoA inactivation as platelets spread upon a surface [52]. The cytosolic domain of integrin β_3 that activates c-Src is targeted by the calcium-dependent calpain protease [26, 31]. After platelets mobilize calcium from intracellular stores during the early stages of platelet activation, calpains eventually cleave integrin β_3 to effectively inhibit c-Src activation to again promote contractility and clot

retraction (Fig. 3C) [26]. In accordance with this model, compared to wild type platelets, platelets from *calpain*$^{-/-}$ mice show temporal alterations in RhoA and Rac1 activation as they spread on extracellular matrix proteins [53]. In addition to shape changes, spreading and clot retraction, RhoA-mediated platelet contraction may also have a role in thrombus stability under shear flow conditions, as RhoA and ROCK stabilize and maintain platelet-matrix and platelet-platelet interactions [29, 54].

Studies of megakaryocyte/platelet-specific RhoA-deficient mice have confirmed that RhoA has roles in the platelet shape change, $G\alpha_{13}$-mediated GPCR crosstalk activation of integrin $\alpha_{IIb}\beta_3$, granule secretion, clot retraction and thrombus formation and stability [28]. Platelets from RhoA-null mice have impaired shape changes and mild aggregation deficits downstream of $G\alpha_{13}$ as well as $G\alpha_q$ activation. While RhoA is not required for platelets to spread upon a surface of the integrin $\alpha_{IIb}\beta_3$ substrate fibrinogen, RhoA is required for full integrin activation and granule secretion in response to thrombin, or combined ADP and U46619 stimulation, as well as for clot retraction [28]. These *in vitro* observations translate into *in vivo* functions as RhoA-deficient mice show reduced thrombus formation, prolonged tail-bleeding times and partial protection from ischemic stroke [28].

Cdc42 in platelet filopodia formation and granule secretion

Since its discovery as a Rho-related GTP-binding protein and mediator of budding and cell polarity in yeast [14, 15, 55], Cdc42 has become established as a generalized regulator of filopodia formation [21, 56, 57] as well as exocytosis and secretion [58, 59]. While Cdc42 activation is commonly associated with microspike and filopodia formation at the periphery of a number of cell types [56, 57, 60], including platelets [34, 36], the requirement of Cdc42 in filopodial formation is not clear. Some studies have reported filopodia formation in Cdc42-free or Cdc42-inhibited conditions [61–63], including studies of platelets from Cdc42 megakaryocyte/platelet deficient mice [35]. In contrast, other studies have shown that Cdc42 has very specific roles in platelet filopodia formation and plays a critical part in granule secretion [34].

While Cdc42 activation is associated with platelet spreading and secretion, the specific roles of Cdc42 in platelet biology remain vague. Early experimental observations supported the hypothesis that Cdc42 would play critical roles in platelet filopodia formation, as Cdc42 was found to be abundant in platelets [15]. Upon stimulation with thrombin receptor activating peptide (TRAP) or ADP, Cdc42 translocates to the platelet cytoskeleton in an integrin-dependent manner, also requiring actin polymerization and tyrosine kinase activation [25]. Cdc42 is activated by PAR stimulation in solution [64, 65] and also as platelets spread on collagen [66]. Treatment of platelets with the Cdc42-specific inhibitor, secramine A, blocks platelet adhesion on collagen, and collagen-induced aggregation [36].

To date, two separate approaches to characterizing the roles of platelet Cdc42 in knockout systems of mouse megakaryocyte/platelet or hematopoietic cells have provided varied results regarding the roles of Cdc42 in platelet function [34, 35]. The reasons for these inconsistent conclusions are not clear but may be due to the different knockout strategies employed to eliminate Cdc42 expression by gene excision in hematopoietic cells after the administration of multiple doses of polyinosinic acid-polycytidylic acid [34, 67] versus a Cre-recombinase based model that targets Cdc42 in megakaryocytes and platelets directly through the *platelet factor 4* (*Pf4*) promoter during megakaryocyte maturation [35, 68].

A study of mice with conditional excision of the *Cdc42* gene under the *Pf4* promoter in megakaryocyte lineage specific cells suggests that the role of Cdc42 in platelet biology is complex [35]. Consistent with a role for GPIb upstream of Cdc42 activation in the formation

of filopodia, Cdc42-null platelets from these mice show a reduced ability to form filopodia on a surface of the GPIb ligand, von Willebrand Factor (vWF), but not on surfaces of collagen-related peptide (CRP) or fibrinogen, suggesting that Cdc42 has a specific role in platelet filopodia formation downstream of GPIb but not GPVI or integrin $\alpha_{IIb}\beta_3$. Unlike the requisite role of Cdc42 in secretory processes in nucleated cells, Cdc42 deletion was found to increase granule secretion in response to low doses of U46619, CRP or collagen, but showed no differences in aggregation in response to thrombin. Consistent with this increased secretion, *Cdc42*$^{-/-}$ platelets formed larger aggregates when flowed over collagen and increased thrombus formation *in vivo*. However, although erratic, tail bleeding times were generally increased in these *Cdc42*$^{-/-}$ mice.

Studies of conditional knockout mice lacking Cdc42 in hematopoietic cells, including platelets, have shown results more consistent with the hypothesized functions of Cdc42 in filopodia formation [34]. Platelets from these mice showed reduced phosphorylation of the Cdc42 effector PAK in response to stimulation with thrombin. Such Cdc42-deficient platelets demonstrated reduced filopodia formation and spreading on surfaces of fibrinogen and CRP as well as reduced secretion, Akt phosphorylation, and aggregation. These Cdc42-null mice also displayed prolonged tail-bleeding times. Despite the phenotypic disparities between the two described knockout models of platelet Cdc42 function, both studies demonstrate that Cdc42 deletion leads to reduced platelet counts, suggesting that Cdc42 plays a key role in platelet production.

Rac regulates platelet lamellipodia formation

Since their initial discovery as substrates of botulinum C3 enzyme and regulators of actin dynamics [18], the Rac proteins have been an active topic of investigation in platelet biology. In nucleated cells, the Rac proteins are known for roles in lamellipodia formation, as dominant-negative Rac mutants inhibit lamellipodia extension, membrane ruffling and cell migration in a variety of cell types [21]. Of the Rac1, Rac2 and Rac3 isoforms, only Rac1 is expressed in platelets [32]. Early studies of Rac in platelet function found that Rac is activated in platelets upon stimulation with a number of agonists, including collagen and thrombin [69], and surfaces such as fibrinogen [66]. Similar to Cdc42, Rac translocates to the platelet cytoskeleton upon activation [25, 64]. Inhibition of Rho GTPase signaling with clostridial toxin B blocks the activation of the Rac/Cdc42 effector PAK and platelet lamellipodia formation [65], while inhibition of RhoA signaling with the ROCK inhibitor Y-27632 reduced platelet stress fiber formation but not the degree of platelet spreading [54], providing the first suggestion that Rac or Cdc42 would play critical roles in platelet lamellipodia formation [66].

A specific role for Rac in platelet lamellipodia formation and function was established through studies of mice with hematopoietic cell deletion of Rac1. Rac1 is required for lamellipodia formation as platelet spread upon surfaces of fibrinogen, vWF, laminin and collagen [32, 70]. Rac is also required to maintain the stability of platelet aggregates formed under physiological conditions of shear as well as for thrombus stability at sites of injury *in vivo*. Experiments taking advantage of the Rac-specific inhibitor, NSC23766, as well as genetic models of Rac activation show that Rac has a role in granule secretion and PAK activation downstream of thrombin or U46619, as well as in aggregation in response to ADP, collagen, thrombin and U46619 [30]. Pharmacological inhibition of Rac with a separate Rac-specific inhibitor, EHT 1864, also provides evidence for Rac function downstream of platelet integrin $\alpha_{IIb}\beta_3$ activation as well as platelet aggregation in response to collagen and platelet aggregate stability under flow *in vitro* [71]. Rac, but not ROCK, activation is also required for platelet integrin-mediated p38 and ERK phosphorylation, suggesting that in addition to roles in cell spreading, Rac may also have a role in clot

retraction via MLC phosphorylation through p38 and ERK, independent of the Rho and ROCK pathway [31].

The mechanism by which platelet agonists activate Rac is not well defined, but may be similar to Cdc42 activation in that it is similarly regulated by the release of 14-3-3ζ from the glycoprotein GPIb-IX subunit of the adhesion receptor GPIb (Fig. 4) [72]. The exact manner by which 14-3-3ζ mediates Rac activation is not yet understood, but may involve recruitment of the Rac GEF and T-cell Lymphoma Invasion and Metastasis protein TIAM1 to membranes [73] or 14-3-3 dimerization [74]. Like RhoA, Rac1 activation by thromboxane A2 (TxA2) occurs in an $\alpha_{IIb}\beta_3$-independent manner [75]. Unlike RhoA, which is activated by $G\alpha_{13}$, Rac is activated by $G\alpha_q$ and is not required for platelet shape change [75]. In nucleated cells, Rac can also be activated through G_i as well as through $G\beta\gamma$ activation of PI3K [76, 77]. In platelets, however, activation of G_i alone is not sufficient for Rac1 activation, as thrombin, ADP, or combined ADP and U46619 treatments do not activate Rac in $G\alpha_q$-deficient platelets. However, full activation of Rac requires direct $G\alpha_q$ signaling as well as a $G\alpha_q$-mediated release of G_i coupled receptor agonists (Fig. 4) [75, 78].

While Rac has a role in platelet PI3K activation, PI3K activation is also required for Rac to become completely active, supporting a model of platelet activation in which Rho GTPases regulate multidirectional, feed-forward and feed-back signaling between receptors, phospholipase enzymes, calcium signaling systems and PI3Ks to control platelet lamellipodia formation, secretion and thrombus stability (Fig. 4) [79]. In agreement with such a multi-modal model of platelet Rac1 function, platelet Rac1 activation is calcium dependent [69, 75]. Intracellular calcium signaling plays a key role in platelet activation, adhesion, secretion, and thrombus formation [5, 80, 81]. Platelets use two major pathways to elevate intracellular calcium. Soluble agonists (ADP, thrombin, TxA2) stimulate GPCRs coupled to $G\alpha_q$ to stimulate phospholipase Cβ (PLCβ) [42]. Alternatively, PLCγ2 is activated downstream of immunoreceptor tyrosine activation motif (ITAM) receptors GPVI or CLEC-2, which are receptors for collagen or rhodocytin, respectively [82]. PLCs generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers calcium mobilization from internal stores; DAG activates PKC which may also contribute to calcium entry from non-internal stores. Rac may play a role in the activation of PLCγ2 through a mechanism independent of tyrosine phosphorylation [83]. In platelets, Rac1 is required for PLCγ2 activation downstream of GPVI/ITAM, but not for PLCβ activation downstream of PARs (Fig. 4) [84, 85]. Accordingly, Rac1 is required for GPVI-mediated platelet aggregation, $\alpha_{IIb}\beta_3$ activation, granule secretion, and ADP secretion downstream of calcium mobilization but plays only a minor role in thrombin responses. Rac1 is also required for vWF-induced activation of $\alpha_{IIb}\beta_3$ upstream of PI3K/Akt activation to drive platelet responses to GPIb-IX engagement [86]. Many of the observed defects in thrombus formation in Rac-deficient platelets can be rescued with exogenous ADP and U46619 [84], supporting a role for Rac1 in exocytosis and secretion [87, 88]. Consistent with platelet Rac signaling upstream and downstream of both calcium and PI3K signaling, a separate pool of Rac1 has recently been proposed to work immediately downstream of GPVI receptor activation to regulate CalDAG-GEF1- and P2Y12-dependent activation of the non-Rho, Ras-related small GTPase, Rap1, leading to integrin $\alpha_{IIb}\beta_3$ activation [33]. A second pool of Rac1 downstream of PLCγ and calcium signaling regulates platelet secretion, aggregation and spreading events through the activation of Rac effectors such as the WAVE and Arp2/3 system or the PAK kinases (Fig. 4) [33].

Actin reorganization downstream of Rho GTPase activation

The current paradigms of Rho GTPase action in the mediation of actin remodeling, filopodia extension and lamellipodia formation rely on the activation of the actin related protein

Arp2/3 complex [21] and the formin proteins. Through this model, Cdc42 mediates filopodia formation through the Wiskott–Aldrich syndrome proteins WASP and neuronal N-WASP to induce actin branching via Arp2/3 [89–91]; Rac activates Arp2/3 to form lamellipodial structures through the WASP-family verprolin-homologous WAVE complex proteins [92, 93]. Rho GTPases also activate formin proteins, including mDia and Daam, to nucleate, elongate and bundle actin fibers in the control of cell spreading, motility and division [94]. In this regard, RhoA mediates actin assembly through the formins mDia1, Daam [95] and FHOD1 [96] while Rac works through mDia2 [11].

Early studies of platelet Rac1 implicated Rho GTPases in processes of platelet actin elongation, as exogenous Rac protein stimulated phosphoinositide synthesis and actin filament uncapping in permeabilized platelets [97]. In platelets, Rac1 interacts with the phosphatidylinositol kinase, PIP5K, which synthesizes phosphatidylinositol-4,5-bisphosphate, or PI(4,5)P(2), a lipid that dissociates capping proteins such as gelsolin from actin filament barbed ends [98, 99]. This uncapping process has a role in platelet Arp2/3 complex activity at the barbed ends of actin filaments [100, 101]. Platelets express WAVE-1, WAVE-2 and low levels of WAVE-3 [102]. Like Rac1-deficient platelets, lamellipodia formation and aggregation in response to GPVI agonists including CRP and laminin are disrupted in Scar/WAVE-1-null platelets [103]. These findings suggest that platelet spreading may be driven by a Rac-WAVE-Arp2/3 axis rather than Cdc42-WASP, as platelets deficient in WASP activate Arp2/3 normally and have no deficits in actin cytoskeletal function, spreading, lamellipodia formation and aggregation [102, 104].

A serial analysis of gene expression (SAGE) library study of formin RNA expression in megakaryocytes found that six of the 15 mammalian formins (mDia1, mDia2, Daam1, Fmnl1, Fmnl3 and FHOD1) are present in mouse megakaryocytes, as well as human megakaryocytes, which also contain mDia3 [96]. Notably, FHOD1 was found to be the most highly expressed formin in megakaryocytes. FHOD1 is activated by ROCK phosphorylation in platelets in a RhoA-dependent and Rac1-independent manner by thrombin, CRP, or a combined ADP and U49919 treatment, but not in platelets on a surface of fibrinogen [96]. FHOD1 has a role in actin stress fiber formation following thrombin treatment of endothelial cells [105]; however, a similar function of FHOD1 in platelets remains to be confirmed.

A number of downstream effectors of Cdc42 have been identified in non-platelet systems with roles in filopodia formation. For instance, Cdc42 activates IRSp53, an insulin receptor substrate protein known to mediate actin bundling and filopodia formation [106, 107]. The role of IRSp53 in platelet physiology has not yet been determined. Given the lack of a requirement for WASP in platelet Arp2/3 activation [108] and the minimal role of N-WASP in platelet physiology due to its low levels of expression [108, 109], platelet filopodia formation may be regulated by other mechanisms, perhaps involving Rif [110], a newly characterized Rho GTPase in filopodia that works independently of Rac, Cdc42 and Arp2/3 to drive filopodia formation through mDia1 [62] and mDia2 [63]. In cultured cells, Cdc42 and Rac can also mediate actin microspike formation downstream of thrombospondin stimulation through the actin bundling protein fascin [111]. Interestingly, Rac plays a role in controlling the interaction of PKC with fascin in cell migration [112]. The role of Rho GTPases in fascin-mediated actin processes in platelets has not yet been defined.

The PAKs as Rho GTPase effectors

The ~21 kD Rho GTPases Cdc42 and Rac support the autocatalytic activation of the p21 activated kinases, or PAKs, to mediate actin reorganization processes in focal adhesion formation and cell migration [113]. The PAKs represent perhaps the best characterized effectors of Rac and Cdc42 [114]. Upon activation by GTP-bound Rac or Cdc42, the PAKs

phosphorylate a number of substrates to coordinate adhesion complex formation and actin dynamics [114]. Interestingly, PAK2, the most abundant and ubiquitously expressed PAK isoform [114], was originally described as a thrombin-activated kinase in platelets [115]. Platelets express a number of PAK isoforms [116], and like Rac, PAK is activated as platelets spread on collagen in a Src- and PI3K-dependent manner [65, 66]. In platelets, PAK acts downstream of Rho GTPases to regulate the intracellular distribution of cortactin [65], an established regulator of actin polymerization that associates with the Arp2/3 complex and has role in actin polymerization in lamellipodial structures [117, 118]. The adaptor protein SLP-76 has also been proposed to potentiate PAK activity downstream of Rac activation to mediate platelet lamellipodia formation [119]. However, the exact roles of PAKs in platelet function have not yet been characterized.

A notable PAK effector linked to platelet function is LIM domain kinase, LIMK1, which phosphorylates and inactivates the actin binding protein cofilin to regulate actin assembly and disassembly [120]. Stimulation of platelets with thrombin causes a rapid dephosphorylation of cofilin [121] that is associated with platelet secretion [116] but not with the platelet shape change [122]. However, platelet cofilin regulation is complex, as cofilin is rephosphorylated minutes following its dephosphorylation [121]. LIMK1 activity is regulated by PAK phosphorylation of LIMK1 in a Rac- and calcium-dependent manner that does not require PKC or PI3K activation [116]. Platelet LIMK1 activity is also regulated by RhoA [122]. It has been proposed that RhoA activation of LIMK has a role in the platelet shape change independent of cofilin phosphorylation, while calcium-dependent LIMK signaling regulates platelet secretion and aggregation [123]. Hence, the LIMK-cofilin system may represent an important coordinator of RhoA as well as Cdc42/Rac1-PAK signaling in multiple stages of the platelet activation process.

Other PAK substrates of potential significance to platelet function include the G protein-coupled receptor kinase interactor GIT and the PAK interacting exchange factor PIX proteins, which lie both upstream and downstream of Rac and Cdc42 activation in the regulation of actin processes [114, 124]. Upon integrin engagement, platelet GIT1, is phosphorylated in a Src-dependent manner and may play a role at recruiting βPIX to areas of active actin remodeling [125]. The functions of GIT and PIX in platelet actin dynamics have not yet been defined; however, a computational analysis of proteomics data suggests a role for GIT proteins in platelet function [126]. Moreover, in endothelial cells, GIT1 regulates focal adhesion assembly and cell contractility in response to thrombin stimulation [127]. PIX and GIT proteins are PAK effectors downstream of Rac activation but also serve as GAPs, respectively, in the regulation of Rac activation. Accordingly, GIT and PIX may cyclically regulate feed-back and feed-forward waves of Rac and PAK activation at sites of active actin remodeling in platelets and other cells [128].

Rho GEFs, GAPs and GDIs in platelet function

A number of intracellular regulatory mechanisms control Rho GTPase function. Guanine nucleotide exchange factors, or GEFs, interact with Rho GTPases to catalyze the exchange of GDP for GTP to activate GTP binding proteins (Fig. 2 and reviewed in [129]). While over 60 different GEFs for Rho family and other GTPases have been identified in mammalian cells, few have been examined for expression or function in platelets. Notably, as described above, upon platelet activation, the RhoA GEF p115RhoGEF is activated by $G\alpha_{13}$ directly to mediate RhoA and subsequently ROCK activation to promote platelet shape changes (Fig. 3).

To date, no Cdc42-specific GEFs have been described with roles in platelet function. The Vav proteins represent perhaps the best-characterized Rac GEFs in platelet function. Vav

was originally identified as a protooncogene from hematopoietic cells [130]. The three Vav isoforms (Vav1, Vav2 and Vav3) have GEF activities towards Rac, Cdc42 and Rho proteins, with preference for Rac. Vav proteins have been shown to mediate several cytoskeletal-associated cellular processes downstream of receptor activation and tyrosine kinase signal transduction. Thrombin-, collagen- and integrin-mediated platelet activation, but not ADP or thromboxanes, activate Vav in platelets [131]. Vav1, unlike Vav2, has a role in Rac activation in response to GPVI agonists but not thrombin [132]. Double knockout mouse studies have shown that Vav1 and Vav3 together have redundant roles in platelet activation, as platelets from *Vav1*$^{-/-}$/*Vav3*$^{-/-}$ knockout mice show defects in platelet spreading on fibrinogen [133] and aggregation in response to GPVI agonists such as collagen [134].

In addition to Vav, platelets also express the Rac GEF TIAM1, which localizes to the leading edge of platelets spread on fibrinogen surfaces [71]. Platelets from mice deficient for expression of the phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 (P-Rex1) show that the Rac GEF P-Rex1 is not required for platelet spreading on surfaces of fibrinogen, collagen or thrombin [135]. However, P-Rex1 has a role in secretion and aggregation, suggesting that the roles of Rho GEFs in platelet physiology are complex [136]. Notably, P-Rex1 links the mammalian target of rapamycin, or mTOR, signaling network to Rac regulation and the control of cell motility [137]. Traditionally, mTOR serves as a master orchestrator of a number of cellular signaling systems, linking cellular homeostasis to protein translation and cellular growth [138]. Platelet express mTOR, which has a role in platelet function upstream of Rac [71, 139] and protein translation events associated with clot retraction [140]. A number of studies show that mTOR activation is under the control of small GTPases, including Rac [141, 142]. Other studies show a role for mTOR in the control of cell motility, specifically through the regulation of Rho GTPase activity [143, 144]. Given the emerging co-dependent functions of mTOR and small GTPases in the regulation of diverse cellular activities, the mTOR system may emerge as centralized nexus of Rho GTPase regulation in platelets [139]. In addition, the mTOR-Rac axis has roles in platelet function, as inhibition of mTOR prevents platelet Rac activation downstream of $\alpha_{IIb}\beta_3$, blocks platelet aggregation in response to collagen, and has a role in platelet aggregate stability [71]. These effects may be specific to integrin and glycoprotein receptor signaling, as mTOR inhibitors have no effect on platelet aggregation in response to PAR agonists [145] but inhibit $\alpha_{IIb}\beta_3$-mediated clot retraction [140]. In addition to mTOR, other proteins with more traditional roles in gene expression and disease are also emerging as Rho GTPase regulators in platelets. For instance, Wnt-3a was recently shown to play a role in Rho GEF activation through Dvl and Daam [146, 147].

While Rho GEFs promote Rho-GTP binding and activation, Rho GTPases are inactivated by specific GTPase activating proteins, or GAPs, that accelerate the hydrolysis of GTP to GDP to inactivate GTPase signaling (Fig. 2) [148]. Platelets have served important roles in the history of GAP studies, as the first RhoGAP for Cdc42 was originally discovered using platelet as a model cellular system [149]. While over 70 RhoGAPs have been identified, the roles of RhoGAPs in platelet function remain mostly uncharacterized [148]. The Rho GAP p190RhoGAP has a role in contractility in the control of clot retraction (Fig. 3). Emerging evidence suggests that other RhoGAPs may have roles in platelet function. Thrombin, but not collagen treatment, leads to the translocation of the Rac/Cdc42 IQ-domain containing GAP IQGAP2 to the cytoskeleton of platelet filopodia [150]. Oligophrenin1 (OPHN1) as well as Nadrin - Rho GAPs that activate Rac, Cdc42 and Rho GTPase activity - have also been proposed to regulate platelet function [151, 152].

In addition to GEFs and GAPs, Rho GTPases are also held in check by specific Rho-guanine nucleotide dissociation inhibitor RhoGDI proteins that act as an “invisible hand” in the regulation of total cellular Rho GTPase activity (Fig. 2) [153]. As Rho GTPase binding

proteins, the RhoGDIs regulate Rho GTPase function by sequestering Rho GTPases to specific intracellular locations, regulating GTPase enzymatic activity and also controlling Rho GTPase expression. Like Rho GAPs, the first RhoGDI discovered for Cdc42 was also first found using platelets [154, 155]. The roles of RhoGDIs in platelet function remain unexplored.

Future perspectives

Platelets have served as an ideal non-transformed system in the studies of signaling processes independent of nuclear events, and will continue to do so as novel paradigms of Rho GTPase regulation develop. For instance, recent studies have shown that Rho GTPases and many Rho regulators are targets of micro RNAs (miRNAs) [156]. miRNAs have been increasingly recognized as important regulators of platelet function and hemostasis [157]. Given the abundance of Rho-related RNA transcripts in platelets [40] and the roles of RNA translation in platelet function [158], real-time miRNA regulation of Rho GTPase expression and activity may emerge as an important mechanism of platelet physiological function, and platelets may serve as a model cell for the study of miRNA regulation of cytoskeletal dynamics.

The roles of the Rho GTPases in regulating non-actin based platelet cytoskeletal structures is less known. For instance, as platelets activate, a ring-like marginal band of tubulin at the platelet periphery that holds platelet shape disappears to allow for the platelet shape change [24]. Studies from nucleated cells suggest a close relationship between Rho GTPases and tubulin dynamics in cell motility [159, 160], but the relation of Rho signaling to microtubule dynamics in platelets remains unclear. Interestingly, *RhoA*$^{-/-}$ platelets show altered tubulin structures [28]. Actin and tubulin dynamics play a critical role in platelet formation by megakaryocytes [161, 162]. Accordingly, Rho GTPases have been and will continue to be a topic of investigation in platelet biogenesis and may reveal insights into roles of cytoskeletal dynamics in hematopoesis and hematological disease [163, 164].

Conclusions

Since their discovery as mediators of intracellular signaling and cytoskeletal dynamics, the Rho GTPases have continually been demonstrated to play critical roles in actin mediated processes in a number of cellular systems, including platelets [11, 21, 82, 165]. Cell biological studies of the Rho GTPases have linked specific Rho GTPase signaling axes to the dynamics of platelet function over the course of platelet activation, thrombus formation, thrombus stability and clot retraction. Newly emerging methods for studies of Rho GTPase function that take advantage of single-molecule and single-cell systems combined with computational and “omics” tools will serve to define the link between platelet Rho signaling and platelet cellular mechanics [166–168]. These studies may lead to the development of Rho GTPases-based pharmacologic therapies for platelet-centric disease states [169].

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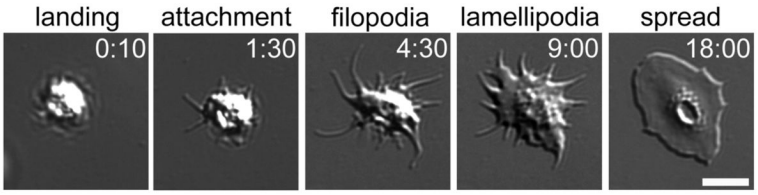

Figure 1. Platelet filopodia and lamellipodia formation

As platelets land upon and attach to surfaces of adhesive proteins, such as fibrinogen, they spread upon surfaces by first forming actin-rich, finger-like filopodia (examples indicated with white arrow) which are then filled in by actin-rich sheets of lamellipodia (example indicated by white arc). Rho GTPase proteins RhoA, Cdc42 and Rac1 have specific roles in these actin-driven processes. Visualized by DIC microscopy [170]. Times shown in [min:sec]. Scale bar = 5 μm.

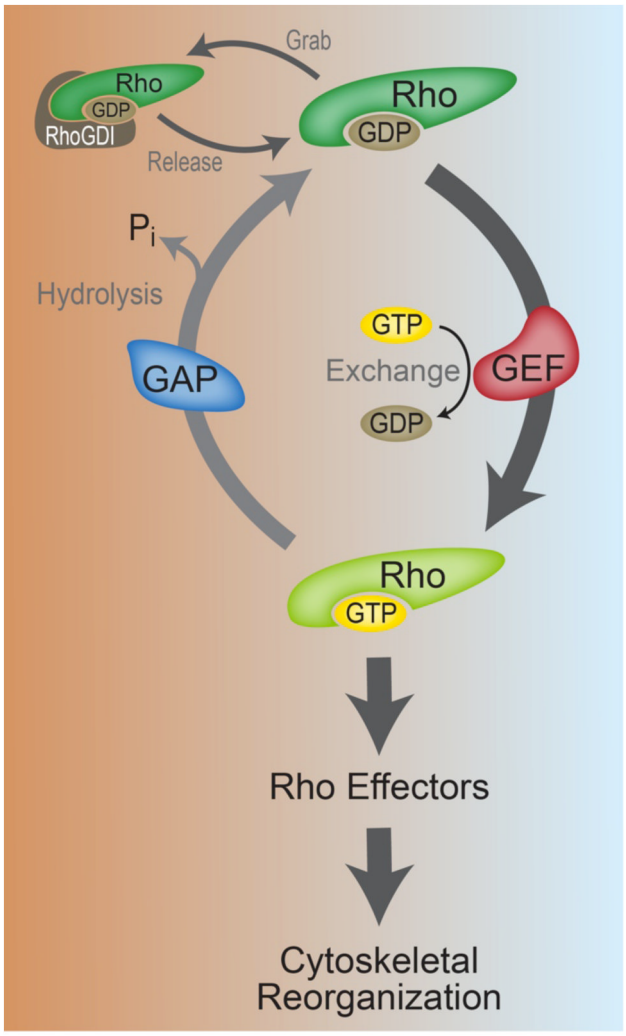

Figure 2. Rho GTPase activation and regulation

In their GTP-bound states, the Rho GTPases, including RhoA, Cdc42 and Rac1, associate with specific downstream effectors to regulate cytoskeletal remodeling events and platelet function. RhoA, Cdc42 and Rac1 are cyclically regulated by specific GEF and GAP proteins. GEFs promote GDP to GTP exchange to activate Rho GTPases. GAP proteins accelerate the hydrolysis of Rho-GTP to GDP and effectively inhibit Rho GTPase activation. Rho proteins may also be regulated by RhoGDI proteins, which “grab” and “release” Rho GTPases to sequester their activities [153].

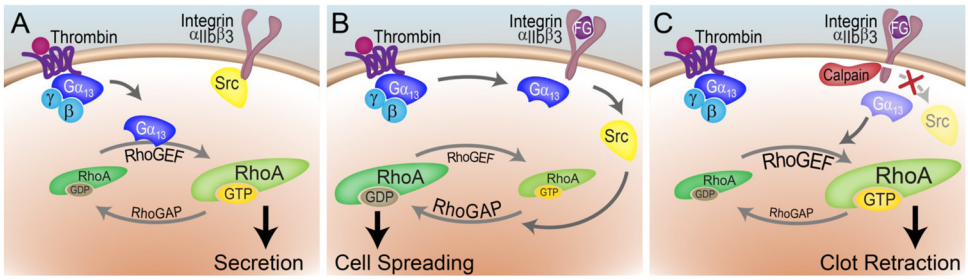

Figure 3. RhoA in platelet shape change, spreading and clot retraction

RhoA activation and inactivation drives separate steps of platelet activation. **(A)** Upon activation with thrombin, $G\alpha_q$ activates p115RhoGEF to promote RhoA-GTP formation, ROCK activation and MLC phosphorylation to drive platelet shape change and secretion events. **(B)** $G\alpha_q$ later supports the activation of c-Src through an association with the cytosolic domain of integrin $\alpha_{IIb}\beta_3$. c-Src activates p190RhoGAP to stimulate RhoA-GTP to GDP hydrolysis to effectively shut down RhoA contractile activity and facilitate platelet spreading. **(C)** Later, calcium signaling activates the calpain protease which cleaves integrin β_3, resulting in the loss of c-Src activity, driving platelet contraction and clot retraction [5, 29, 51, 54].

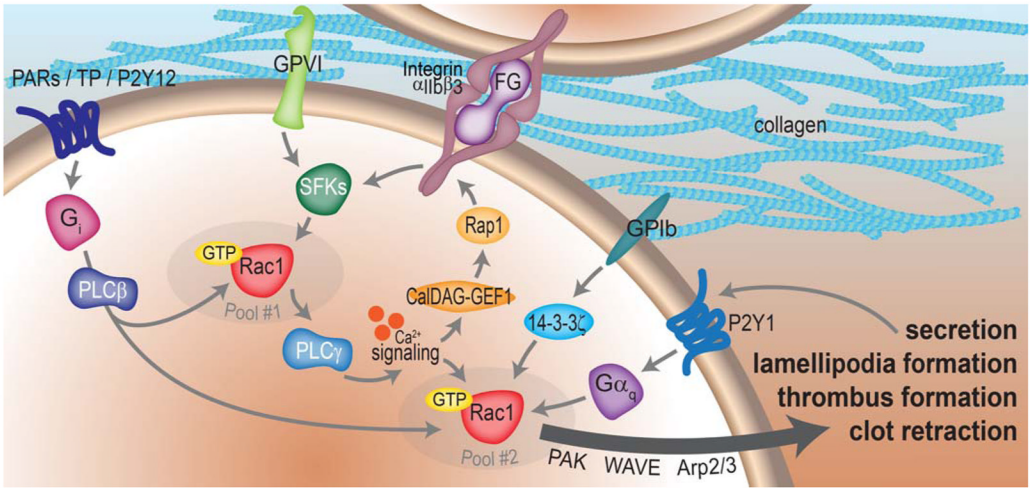

Figure 4. Regulation of platelet Rac1 activation and lamellipodia formation

Upon stimulation of platelet GPCRs such as the PARs, TPs or P2Y12, $G\alpha_q$ mediates the activation of Rac1 through PLCβ [42]. Integrin- and GPVI-coupled Src family kinase (SFK) activation also supports Rac1 activation [30, 32, 71]. GPIb release of 14-3-3ζ also has a role in platelet Rac1 activation [72]. G_i coupled to P2Y1 receptors is also required for full platelet Rac1 activation [78]. Separate pools of Rac1 may work in distinct steps of the platelet activation process. A first pool proximal to SFK-coupled receptor activation drives PLCγ activation and calcium signaling to activate CalDEG-GEF1 and the Rap1 GTPase to support integrin $\alpha_{IIb}\beta_3$ activation [33, 84]. A second pool of Rac1 downstream of PLCγ and calcium signaling then supports secretion, aggregation and spreading events through the activation of Rac effectors, such as the WAVE and Arp2/3 system or the PAK kinases.

Table 1

Summary of physiological functions reported for Rho GTPases in platelets from selected mouse model and inhibitor studies.

Rho GTPase	Function	Reference
RhoA	Platelet production	[28]
	Platelet shape change (U46619 stimulation)	[27, 28]
	Platelet aggregation (U46619 or PAR-4 stimulation)	[28]
	Platelet integrin $\alpha_{IIb}\beta_3$ activation (U46619+ADP or thrombin stimulation)	[28]
	Platelet granule secretion (U46619+ADP or thrombin stimulation)	[28]
	Regulates platelet rolling velocity on vWF	[28]
	Maintains strength of $\alpha_{IIb}\beta_3$ – vWF contacts under shear	[29]
	Clot retraction	[28]
	Thrombus formation *in vivo*	[28]
	Hemostasis *in vivo*	[28]
	Pathology of cerebral ischemia *in vivo*	[28]
Cdc42	Platelet production	[34, 35]
	Regulates platelet life span	[35]
	Platelet filopodia formation (on vWF surface)	[35]
	Platelet spreading (on collagen or fibrinogen)	[34]
	Platelet granule secretion	[34]
	Platelet ATP secretion	[34]
	Regulates secretion output	[35]
	Platelet aggregation (collagen or thrombin stimulation)	[34]
	Controls platelet aggregate size on collagen	[35]
	Hemostasis *in vivo*	[34]
	Regulates rate of thrombus formation	[35]
Rac1	Platelet aggregation (collagen or collagen peptide stimulation)	[30, 32, 71, 84]
	Platelet aggregation (thrombin stimulation)	[30, 116]
	Platelet aggregation (ADP or U46619 stimulation)	[30]
	Platelet aggregation (vWF stimulation)	[86]
	Platelet spreading on FG, collagen or laminin	[32]
	Platelet $\alpha_{IIb}\beta_3$ activation (collagen stimulation)	[84]
	Platelet $\alpha_{IIb}\beta_3$ activation (vWF stimulation)	[86]
	Platelet ATP secretion (thrombin stimulation)	[30]
	Platelet granule secretion (collagen peptide, convulxin or ristocetin stimulation)	[84]
	Platelet granule secretion (thrombin stimulation)	[30, 116]
	Calcium mobilization (collagen peptide stimulation)	[84]
	PLCγ2 activation (convulxin stimulation)	[84]
	Thromboxane synthesis (vWF stimulation)	[86]
	Aggregation on collagen surface under flow	[32, 84]

Rho GTPase	Function	Reference
	Aggregation on vWF surface under flow	[32, 86]
	Clot Retraction	[31]
	Thrombus formation *in vivo*	[32, 84]
	Hemostasis *in vivo*	[30]