Introduction

Small GTPase RhoA functions as a bi-molecular switch that cycles between active GTP-bound state and inactive GDP-bound state. Interaction of activated RhoA with downstream effectors initiates events critical in regulation of cell migration. Activated RhoA stimulates formation of actin stress fibers, focal adhesion (FA) maturation, and increases contractility of actin cytoskeleton.1,2 Its activation in the rear of a migrating cell has been proposed to be critical for tail retraction.1-3 Interestingly, activated RhoA has also been detected at the front of a migrating cell, where it may participate in the regulation of membrane protrusions and contacts with extracellular matrix.1,3,4 RhoA-mediated contractility of actin cytoskeleton also influences cell–cell adhesions.2,5,6 Thus, spatiotemporal control of RhoA activity determines the location and timing of specific morphological processes occurring in a migrating cell.

RhoA activity is regulated by several distinct families of enzymes. RhoA is negatively regulated by Rho GTPase-activating protein (RhoGAPs) that accelerate the hydrolysis of GTP to GDP, thus inactivating RhoA.7 RhoA bound to GDP is maintained in this inactive state by interacting with Rho guanine nucleotide dissociation inhibitors (RhoGDIs) that sequester inactive RhoA within the cytosol.8 Positive regulation of RhoA is mediated by guanine nucleotide exchange factors (GEFs) that promote dissociation of GDP from inactive RhoA allowing it to bind GTP, which activates RhoA.9 Therefore, activation of RhoA-mediated pathways is primarily achieved through regulation of RhoGEFs.

RhoGEFs belong to a large family of proteins that are characterized by Dbl homology (DH) domain, which is primarily responsible for catalyzing the exchange of GDP for GTP. Members within this family of proteins also contain a C-terminal Pleckstrin homology (PH) domain immediately adjacent to the DH domain. Studies have shown that the DH-PH domains together have greater GEF activity in comparison to only the DH domain.10-12 RhoGEFs employ multiple strategies to regulate activity of DH-PH domains. Growing evidence indicates that in many cases control of GEF activity is achieved by phosphorylation of these RhoGEFs. In some cases phosphorylation functions as a positive regulator of GEF activity, and in others it leads to inactivation of RhoGEFs. Mechanisms of regulation also vary: phosphorylation either directly regulates activity of RhoGEFs or leads to recruitment of proteins that modulate the RhoGEF’s activity. For some RhoGEFs, phosphorylation is the primary regulatory event, whereas in other cases it appears to act in parallel with other regulatory mechanisms. In all of these cases, RhoGEFs serve as critical signaling nodes coupling multiple kinase-mediated signaling cascades to regulation of RhoA.

In the current review, we discuss recent evidence of phosphorylation-mediated regulation of RhoGEF activity and its role in regulation of cell migration.

Phosphorylation of Vav Directly Stimulates GEF Activity

Regulation of Vav by phosphorylation is one of the most well-characterized mechanisms demonstrating direct activation of its GEF activity through phosphorylation-mediated conformational changes. The DH-PH domains of Vav demonstrates higher activity toward Rac1 and Cdc42 GTPases, but can also activate RhoA in vitro.13-15 Furthermore, Vav has also been reported to activate all three Rho GTPase in vivo.15,16 Experimental evidence suggests that Vav-mediated activation of RhoA is required for Vav’s potent

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cell transforming activity. There are three isoforms of Vav: Vav1, Vav2, and Vav3. Vav1 is primarily expressed in hematopoietic cells, whereas Vav2 and Vav3 are more widely expressed. Vav regulates variety of cellular processes such as T-cell activation, phagocytosis, superoxide production in neutrophils, cancer cell proliferation, and cell migration. Current evidence indicates that Vav2-RhoA signaling plays a significant role in regulation of cancer cell migration and invasion. It is interesting to note that Vav2 activation downstream of growth factor receptors, such as transforming growth factor β (TGFβ-R) and epidermal growth factor receptor (EGFR), regulates RhoA activation in variety of tissues, including in different solid tumors. EGFR-mediated activation of Vav2-RhoA signaling has been reported to delay EGFR internalization and degradation in HeLa cells. However, the role Vav2-RhoA signaling in regulation of EGFR internalization and degradation in other cancer cell types and its effect on cancer cell biology is yet to be determined.

Vav has eight well-characterized domains, which include: calponin homology (CH), acidic region (Ac), DH, PH, zinc finger, Src homology 3 (SH3), SH2, and SH3 domains (Fig. 1A). Current evidence demonstrates that the five N-terminal domains play a role in regulation of GEF activity by the DH domain, whereas the C-terminal SH3-SH2-SH3 domains mediate cellular localization of Vav. It is well known that Src and Syk family of kinases play a critical role in regulating Vav’s GEF activity. In its inactive form, Vav assumes an autoinhibited closed conformation where the N-terminal domains interact with DH and PH domains, thus inhibiting its GEF activity (Fig. 1B). Initially, it was described that Src and Syk phosphorylates Vav on Tyr-142 found in the Ac region. Phosphorylation of Tyr-142 in turn resulted in translocation of the N-terminal peptide from its DH domain inhibitory contacts, allowing substrate access to the Vav DH domain. However, as we now know, this is only one part of Vav regulation as the previous study utilized a truncated Vav1 with residues 170–375, which only contained part of the Ac region and the DH domain. A recent study by Yu et al. revealed how CH, Ac, DH, PH, and zinc finger domains (CADPZ) of Vav interact with each other to negatively regulate its basal GEF activity. The study elegantly shows how the CADPZ domains function together to constrain interaction of the inhibitory helix from the C-terminal Ac region with the DH domain. The constraint arising from intramolecular interactions between the CADPZ domains is relieved through multistep phosphorylation of Vav, where Tyr-142 and Tyr-160 in the Ac region are initially phosphorylated, possibly destabilizing the modulatory interactions (Fig. 1B). This results in exposure of the inhibitory helix that can now be readily phosphorylated on Tyr-174, culminating in the opening of the GTPase binding interface on the DH domain (Fig. 1B). The multistep phosphorylation system provides tight control of Vav activity, ensuring that the main regulatory Tyr-174 amino acid can be phosphorylated only when Src activity reaches a certain level sufficient to phosphorylate all three tyrosines. Since Tyr-142 and Tyr-60 are more accessible to phosphorylation, then it is possible that a tyrosine phosphatase keeps basal phosphorylation of these residues low until Src activity is elevated. Activation of a tyrosine phosphatase also could be used to reverse processes initiated by Src and Syk. Tyrosine phosphatases PTP-PEST, PTPN22, and Shp1 have been shown to dephosphorylate Vav. PTP-PEST negatively regulates membrane protrusions by dephosphorylating Vav, and Shp1-mediated dephosphorylation of Vav inhibits cytotoxicity of natural killer cells. Thus, reversible control of Vav phosphorylation provides a mechanism for dynamic regulation of Vav-mediated processes.

![Figure 1. Multi-step phosphorylation-mediated regulation of Vav.](image)

**Regulation of RhoGEFs by Tyrosine Phosphorylation**

Due to its unique domain structure, the mechanism for phosphorylation-mediated regulation of Vav cannot be extrapolated to other RhoGEFs. However, several RhoGEFs have been shown...
to be phosphorylated on tyrosine residues and in most cases it leads to activation of the GEF. However, the mechanisms of activation appear to be different even among close homologs.

RH-RhoGEF subfamily of RhoA-specific GEFs is comprised of p115RhoGEF, PDZ-RhoGEF (PRG), and Leukemia-associated RhoGEF (LARG) (Fig. 2). It is well established that this family of proteins are regulated specifically by G\textsubscript{\alpha12/13} hetero-trimeric G-proteins.\textsuperscript{33} Therefore, RH-RhoGEFs provide a direct functional link from activated G-protein-coupled receptors (GPCRs) coupled to G\textsubscript{\alpha12/13} to RhoA signaling. RH-RhoGEFs share three common domain structures (Fig. 2). They contain the tandem DH-PH domains that catalyze GDP/GTP exchange specifically on RhoA. The distinction of the RH-RhoGEF subfamily comes from the N-terminal regulator of G-protein signaling homology (RH) domain. Structural and biochemical experiments have established that GTP-bound G\textsubscript{\alpha12/13} subunits make direct contacts with the RH-domain, and that this interaction positively regulates RhoGEF activity.\textsuperscript{34-38} It has also been shown that the RH domain of p115 and LARG also can function as negative regulator of G\textsubscript{\alpha12/13} by increasing its intrinsic hydrolysis rate of GTP to GDP.\textsuperscript{33,37,39} While interactions of RH-RhoGEFs with G\textsubscript{\alpha12/13} is believed to be the primary and the most well-characterized mode of regulation, several reports also indicate that additional control could be provided by phosphorylation of tyrosine residues.

The smallest member of RH-RhoGEF subfamily, p115, is ubiquitously expressed with highest expression observed in

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**Figure 2.** Domain structures and phosphorylation sites of RhoA-specific GEFs. Schematic diagram of the domain structure of RhoGEFs, and depiction of phosphorylation sites and kinases that mediate activation (green) and inhibition (red) of RhoGEFs. Kinases that are known to phosphorylate and regulate RhoGEF function where phosphorylation sites have not been identified are listed on the right (see text for detail). CC, coiled-coil; DH, Dbl homology; LR, leucine rich; NLS, nuclear localization signal; PDZ, post-synaptic density 95; disk large, zona occludens-1; PBM, PDZ binding motif; PH, Pleckstrin homology; RH, RGS homology; and ZF, Zinc finger-like binding domain.
hematopoietic cells. Activation of angiotensin II receptor type-1 (AT-1) in vascular smooth muscle cells (VSMCs) leads to phosphorylation of p115 on Tyr-738 by Janus Kinase2 (JAK2). Evidence indicates that phosphorylation Tyr-738 is sufficient for positive regulation of GEF activity of p115, independent from G\textsubscript{\alpha} binding to p115.40 AT-1-mediated phosphorylation of p115 and activation of p115-RhoA signaling was reported to contribute to increased vascular remodeling.40 The mechanism of phosphorylation-mediated activation has not been determined. However, the location of the phosphorylated residue in the PH domain of p115RhoGEF is unusual, suggesting a potentially novel mechanism for regulation of RH-RhoGEFs. Tyr-738 is buried in the hydrophobic pocket of the PH domain (Fig. 3A). Phosphorylation of this tyrosine would dramatically change the environment in the pocket and should result in conformational changes in the PH domain. Another intriguing question is how does JAK-2 access this Tyr-738 residue? Either this particular PH domain has very dynamic structure allowing Tyr-738 to be exposed at least transiently, or there is an alternative conformation that has not yet been determined. Interestingly, Tyr-738 is located within a motif highly conserved among RH-RhoGEFs, but it is still unknown if JAK-2 can phosphorylate LARG and PRG on that residue (Fig. 3B). p115 has also been reported to be phosphorylated by proline-rich tyrosine kinase2 (Pyk2) upon activation of protease-activated receptor-1 (PAR-1) in THP-1 cells.41 However, the phosphorylation site has not been determined and it is not clear if p115 phosphorylation by Pyk2 in this context is sufficient to increase its GEF activity. Non-the-less, evidence indicates that Pyk2 activity and p115 expression is required for thrombin-induced THP-1 cell migration and VSMC migration.41,42 Furthermore, Pyk2 and its close homolog focal adhesion kinase (FAK) can also regulate activity of two other members of RH-RhoGEF family, PRG and LARG.

PRG and LARG are ubiquitously expressed with higher expression of PRG observed in the brain. PRG was the first RH-RhoGEF reported to be regulated by tyrosine phosphorylation. Both PRG and LARG are tyrosine phosphorylated by FAK upon PAR-1 activation in HEK293T cells. The report provides evidence that the tyrosine phosphorylation occurs in the C terminus of PRG and LARG, and that this phosphorylation positively regulates GEF activity.43 Furthermore, existing data demonstrate that PRG and LARG tyrosine phosphorylation is sufficient for positive regulation of its GEF activity independently of G\textsubscript{\alpha} or G\textsubscript{\alpha}\textsubscript{13}.43 Current evidence suggests that C-terminal domains of LARG and PRG mediate their homo- and hetero-dimerization, leading to inhibition of GEF activity.44,45 Thus, it is possible that phosphorylation of the C-terminal portion of LARG and PRG disrupts inhibitory dimerization, and leads to their activation.44,45 Importantly, p115 does not have the similar sequence homology in its C-terminal fragment, suggesting that this mode of regulation is unique for PRG and LARG. In the initial study, the impact of GEF phosphorylation on cell migration was not assessed. However, a study by Iwanicki and colleagues showed that indeed the interaction between FAK and PRG at focal adhesions is critical for trailing-edge retraction in fibroblasts upon LPA stimulation.46 PRG phosphorylation has also been implicated in cancer cell migration. In prostate cancer cells, circumstantial evidence implicated FAK-mediated phosphorylation of PRG downstream of bombesin-2 (BB-2) receptor in regulating prostate cancer cell migration.47 Thus, collective evidence suggests that FAK-mediated regulation of PRG and potentially LARG could represent an alternative mechanism for regulation of cell migration via RH-RhoGEFs downstream of GPCR activation.

Several other tyrosine kinases have been shown to phosphorylate PRG and LARG. Pyk2 phosphorylates PRG providing positive regulation of its GEF activity.48,49 Furthermore, Pyk2-mediated tyrosine phosphorylation of PRG downstream of AT-1 receptor regulates Rho-ROCK cascade in vascular smooth muscle cells leading to increased migration.49 Given the homology between Pyk2 and FAK, it is possible that Pyk2 regulates PRG via the same mechanism as FAK. However, the site(s) of phosphorylation on PRG by Pyk2 have not been mapped. It has
also been shown that LARG is tyrosine phosphorylated by Fyn, a Src family kinase, in response to tensional force resulting in positive regulation of its GEF activity and targeting of LARG to FA. Suzuki et al. have also identified Tec tyrosine kinase to phosphorylate LARG. This study utilizing in vitro reconstitution assays and cell-based assays demonstrated that LARG phosphorylation by Tec makes LARG receptive to activation by Gα12 both in vitro and in vivo. This suggests an interesting mechanism where Tec-mediated phosphorylation of LARG provides an additional mode of control-enhancing GEF activation by GPCR signaling. However, the phosphorylation sites and the details of this potential regulation still remain to be determined.

Another RhoGEF known to be regulated by tyrosine phosphorylation is p190RhoGEF, also known as Rgnef. It is a ubiquitously expressed multi-domain-containing GEF (Fig. 2). p190RhoGEF has been shown to directly interact with FAK, and this interaction helps localize p190RhoGEF to focal adhesions. Biochemical studies have revealed that the interaction of FAK with p190RhoGEF is important for its function. The interaction between p190RhoGEF and FAK has been shown to occur via the coiled-coil domain of p190RhoGEF, which contains the residues 1292–1301, and the focal adhesion targeting (FAT) domain of FAK. Laminin stimulation of Neuro-2a cells promoted FAK-p190RhoGEF complex formation, which enhances p190RhoGEF tyrosine phosphorylation resulting in positive regulation of its GEF activity. Additional studies have also reported the importance of FAK-p190RhoGEF complex in regulation of FA formation and fibronectin-stimulated motility of mouse embryonic fibroblasts. In a pathological condition, evidence also supports the role of FAK-p190RhoGEF complex in regulation of colon cancer cell migration and invasion downstream of gastrin binding to cholecystokinin receptor-2. Furthermore, recent studies have illuminated the mechanisms by which p190RhoGEF may regulate cancer cell migration and invasion through the use of novel Rho biosensors. These studies elegantly demonstrate that p190RhoGEF plays a critical role in formation of leading edge protrusions and invadopodia through spatial regulation of RhoC (a Rho isoform), which leads to regulation of directional cell migration and invasion. These reports suggest that p190RhoGEF interaction with FAK is important for proper localization of p190RhoGEF and regulation of its GEF activity. However, what is unknown is the role of tyrosine phosphorylation of p190RhoGEF by FAK in regulating its activity. Thus, it would be of great interest to identify the tyrosine phosphorylation site(s) of p190RhoGEF and assess if phosphorylation is sufficient to regulate its GEF activity independent of its interactions with FAK.

Regulation of GEFs by Ser/Thr Kinases

While tyrosine kinases appear to predominantly activate RhoGEFs, phosphorylation by Ser/Thr kinases can be both activating and inhibitory. In most cases, phosphorylation on Ser or Thr residues either disrupts or enhances interactions with other proteins that regulates sub-cellular localization of RhoGEFs and influences their GEF activity. Below we discuss several examples of such regulation.

Guanine nucleotide exchange factor-H1 (GEF-H1) is regulated via protein–protein interaction and through protein phosphorylation. It has been reported that GEF-H1 regulates cell motility through spatial control of RhoA activation at the leading edge without having any appreciable effect on overall RhoA activation. This spatial regulation of RhoA activation is turn regulates focal adhesions at the leading edge in HeLa cells. GEF-H1 was originally identified as microtubule-associated nucleotide exchange factor. GEF-H1 directly interacts with either microtubules or microtubule-associated proteins (MAPs) via its N-terminal zinc finger-like domain and through its C-terminal coiled-coil domain (Fig. 2). This interaction has been shown to negatively regulate its GEF activity. Recently, several studies have provided evidence that GEF-H1 is both positively and negatively regulated via phosphorylation. It has been demonstrated that extracellular signal-regulated kinase (ERK) phosphorylates GEF-H1 on Thr-678 in HeLa S3 and HT1080 cells increasing its GEF activity. Similar results were obtained upon stimulation of LLC-PK and MDCK cells with TNFα. An independent study also demonstrated that GEF-H1 is activated by ERK1 downstream of FAK-Raf signaling pathway in rat fibroblasts upon application of force. Intriguingly, a recent study shows that ERK-mediated phosphorylation of GEF-H1 on Ser-959 inhibits its activity and regulates tumor cell migration and invasiveness. It has been reported that PAK-1 phosphorylates GEF-H1 on Ser-885 stimulating interaction of GEF-H1 with 14-3-3 protein. This interaction localizes GEF-H1/14-3-3 complex to microtubules, thus inhibiting its GEF activity. Similarly, Aurora A/B kinase and cyclin B1/cdk1 phosphorylate GEF-H1 on Ser-885 and Ser-959, respectively, thus negatively regulating its activity. Partitioning–defective 1b (PAR1b) kinase can also phosphorylate both Ser-885 and Ser-959 on GEF-H1 in COS-7 cells. Similar to PAK-1-mediated phosphorylation of GEF-H1 on Ser-885, PAR1b-mediated phosphorylation of Ser-885 and Ser-959 increases 14-3-3 binding to GEF-H1, and thus, decreasing its GEF activity. Existing evidence also supports the notion that phosphorylation of GEF-H1 on Ser-885 and S-959 is sufficient to inhibit its GEF activity independent of 14-3-3 interaction. It is worth noting that while phosphorylation in the C-terminal portion (Ser-885 and Ser-959) promotes inhibitory interactions, the activating phosphorylation (Thr-678) occurs between the PH and coiled-coil domain of GEF-H1 (Fig. 2). It still remains to be determined if phosphorylation of Thr-678 affects interaction with 14-3-3 and/or association with microtubules.

Another example of negative regulation by phosphorylation is observed with neuroepithelioma transforming gene 1 (Net1) RhoGEF. Net1 is unusual among the RhoGEFs in that in its N terminus it contains multiple nuclear localization signals (NLS) (Fig. 2). It is thought that the NLS on Net1 plays a critical role in negative regulation of RhoA activation and stress fiber formation. An isofrom of Net1 known as Net1A lacks two NLS, and thus, is less regulated through subcellular localization. It is believed that cytoplasmic redistribution of Net1 is sufficient for its GEF activation. However, there is evidence that supports the existence of additional regulatory elements that control Net1 activity in the cytoplasm. A report by Alberts et al. indicates that
Concluding Remarks

Protein phosphorylation is one of the key post-translational modifications utilized in signal transduction. Thus, it is not surprising that phosphorylation is used to regulate activity of RhoGEFs. Interestingly, unlike for many other enzymes, the direct phosphorylation of the catalytic domain (DH) has not been detected so far and there is only one report demonstrating phosphorylation of PH domain in p15RhoGEF. As evident from current research, regulation of GEF activity is achieved by phosphorylation of residues outside the DH-PH domains affecting either intramolecular interactions or association with binding partners that modulate GEF activity. The diversity of the regulatory mechanisms enables control of different RhoGEFs through independent pathways, and allows use of combination of pathways for regulation of a single RhoGEF. This is particularly important for spatio-temporal regulation of RhoA activation, where phosphorylation and dephosphorylation of different RhoGEFs at specific subcellular locations defines local control of RhoA activity. Better understanding of these processes requires further identification and dissection of phosphorylation-driven mechanisms regulating RhoGEF activity. Recent improvements in phosphoproteomic analysis simplify identification of novel phosphorylation sites in RhoGEFs. Development of novel biosensors for RhoGEFs and application of existing biosensors for RhoA will help to identify local changes in their activity. It is possible to ascertain a more complete picture of kinase-mediated RhoGEF-RhoA activation in “real-time” by employing recently developed techniques that allow for temporal regulation of kinase activity in living cells. Precise control of specific kinases in living cells will be instrumental for dissection of phosphorylation-mediated regulation of RhoGEFs.

Phosphorylation-mediated regulation of RhoA signaling goes beyond modulating RhoGEF activity. Other molecular entities that regulate RhoA activity, such as GDIs and GAPs, are also controlled by phosphorylation. Evidence indicates that RhoA itself is phosphorylated on Ser-188 by cGMP-dependent protein kinase (cGK), protein kinase A (PKA), and by Ste20-related kinase (SLK). It has been shown that RhoA Ser-188 phosphorylation negatively regulates its function by promoting its association with RhoGDIs. Thus, phosphorylation of all components has to be taken into account in order to describe the signaling network controlling RhoA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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