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Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction

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Abstract

The integrin family of cell adhesion receptors mediates bi-directional signaling: “inside-out” signaling activates the ligand binding function of integrins and “outside-in” signaling mediates cellular responses induced by ligand binding to integrins leading to cell spreading, retraction, migration, and proliferation. Integrin signaling requires both heterotrimeric G proteins and monomeric small G proteins. This review focuses on recent development in the roles of G proteins in integrin outside-in signaling. The finding of direct interaction between the heterotrimeric G protein subunit $G_{\alpha 13}$ and integrin β subunits reveals a new mechanism for integrin signaling, and also uncovers a crosstalk between the signaling pathways initiated by G protein-coupled receptors (GPCRs) and integrins. This crosstalk, which may be referred to as “inside-outside-in” signaling, dynamically regulates contractility and greatly promotes integrin outside-in signaling

Introduction

The integrin family of heterodimeric cell adhesion receptors not only plays an important role in mediating cell adhesion to the extracellular matrix (ECM), but also in signal transduction [1]. Integrins transmit signals bidirectionally. Intracellular signals initiate so-called “inside-out signaling” by inducing the binding of talin and kindlin to the cytoplasmic domains of integrin β subunits, which activate the ligand binding function of integrins [2–5]. Conversely, the interaction between integrins and their various ligands induces “outside-in” signals across the membrane, allowing the cell to sense the extracellular environment and react correspondingly [6,7]. Integrin outside-in signaling induces cell spreading, retraction, migration, proliferation, and survival. G proteins (guanine nucleotide-binding proteins) play critical roles in mediating integrin inside-out and outside-in signaling. The roles of G proteins in inside-out signaling have been excellently reviewed elsewhere [5,8]. This review focuses on the molecular mechanisms whereby G proteins regulate outside-in signaling (Fig. 1).

G proteins are GTPases (guanosine triphosphatases) that cycle between a GDP-bound form and a GTP-bound form. The GTP-bound G protein is an active form that interacts with downstream effectors and transmits signals, during which the bound GTP is often hydrolyzed to GDP and the G protein recycles into the inactive GDP-bound form. The activity of G proteins is regulated mainly through three classes of regulatory proteins:

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GTPase-activating proteins (GAPs), guanine nucleotide-exchange factors (GEFs), and guanine nucleotide-dissociation inhibitors (GDIs) [9,10]. GAPs promote the intrinsic GTPase activity of G proteins accelerating their conversion to the inactive GDP-bound form. Activation of G proteins requires exchange of bound GDP with GTP, a process facilitated by GEFs and inhibited by GDIs. According to their structures and molecular masses, there are monomeric small G proteins and heterotrimeric G proteins. Of the monomeric small G proteins, we focus on the roles of the Rho family of small GTPases, such as members of the Rho, Rac, and Cdc42 subfamilies, respectively [9]. Heterotrimeric G proteins are composed of three subunits, α , β and γ . The α subunit binds to guanine nucleotides. Heterotrimeric G proteins are typically activated by G protein-coupled receptors with 7 transmembrane domains (GPCRs). Upon activation, the GTP-bound α subunit dissociates from β/γ subunits, and serves as the major signaling messenger by interacting with its signal acceptors (downstream effectors) [11]. Here we review recent progress and emerging concepts on the roles and regulatory mechanisms of G proteins in integrin signaling leading to cell spreading and retraction.

Heterotrimeric G protein subunit $G\alpha 13$ mediates integrin outside-in signaling

Various isoforms of heterotrimeric G proteins, such as Gq/11 and Gi/o, are important in initiating “inside-out” signaling, activating the ligand binding function of integrins [5,12]. Deletion of the $G\alpha 13$ gene in mice, however, only partially reduces integrin-mediated platelet aggregation in response to low concentrations of GPCR agonists, suggesting that its primary role is in the secondary amplification response to integrin activation [13]. We recently showed that $G\alpha 13$ directly binds to the cytoplasmic domain of integrin β subunits, including $\beta 1$ and $\beta 3$ [14]. The integrin binding site is located within switch region I (SRI) of $G\alpha 13$, which is also the binding site for the $G\alpha 13$ effector p115RhoGEF. Therefore, The competitive inhibition of p115RhoGEF- $G\alpha 13$ interaction is a possible mechanism contributing to integrin-mediated negative regulation of Rho signaling pathway. Although GDP-bound $G\alpha 13$ is able to interact with integrins, the $G\alpha 13$ –integrin interaction is significantly greater when $G\alpha 13$ is activated. Importantly, this interaction is induced by integrin ligation [14]. Thus, $G\alpha 13$ -integrin interaction is dually regulated by integrin ligands and GPCR signaling. The importance of $G\alpha 13$ in integrin outside-in signaling was revealed by the data that suppression of $G\alpha 13$ expression or inhibition of the $G\alpha 13$ SRI binding site results in inhibition of integrin-dependent activation of Src family kinases (SFK) (an established key player in integrin outside-in signaling), and diminished integrin-dependent cell spreading, a phenotype similar to that observed with inhibition of SFKs [14–19]. Thus, it appears that $G\alpha 13$ mediates integrin signaling and cell spreading by stimulating the activation of SFKs. Thus far, this is the earliest identified step of outside-in signaling following integrin ligation and clustering. The short cytoplasmic domain of integrin β subunits interacts with several intracellular molecules, including talin, kindlins and c-Src. It remains to be shown how the binding of $G\alpha 13$ and other signaling molecules to integrin β subunits are coordinated during integrin signaling.

The roles of Rho and Rac in integrin-mediated cell retraction

The binding of ECM ligands to integrins initiates outside-in signaling, leading to complex cellular responses that vary in different cell types. However, integrin-mediated cell spreading and subsequent retraction are common to nearly all cell types. In migratory cells, coordinated spreading in the leading edge and retraction in the rear drive cell migration on the ECM [20]. Cell retraction requires a retractile force generated by actin-myosin interaction and can be defined as the inward movement of cell membranes and the associated cytoskeleton. When cells are imbedded in flexible tissues and gel-like matrices,

cell retraction causes these tissues or gel-like matrices to shrink, as in the cases of wound healing and clot retraction. On rigid surfaces, retraction of actin-myosin complexes results in the formation of stress fibers. In non-muscle cells, actin-myosin-dependent retractile forces are stimulated by phosphorylation of myosin light chain (MLC), which can be mediated by MLC kinase (MLCK) and downregulated by MLC phosphatase (MLCP). GTP-bound Rho activates its downstream effector Rho-kinase (ROCK), which subsequently induces phosphorylation and inhibition of MLCP [21], culminating in the generation of a retractile force. Members of the Rho subfamily of small GTPases also interact with different isoforms of formins, which facilitate actin polymerization and formation of stress fibers [22,23]. Three highly homologous Rho isoforms, RhoA, RhoB and RhoC, activate ROCK and retractile forces. However, these different Rho isoforms may play different roles in cells, as they differ in subcellular localization, regulator and effector specificity [22–24]. Integrin ligation dynamically regulates Rho activity. During the early phase of cell spreading, Rho activity is transiently downregulated probably via Src-dependent phosphorylation of p190RhoGAP [18,25,26], but is later activated [18]. A recent study shows that tensional force applied to ligated integrins induces SFK Fyn-dependent activation of leukemia-associated Rho GEF (LARG) and the mitogen-activated protein kinase (MAPK)- and focal adhesion kinase (FAK)-enhanced activation of GEF-H1 [27], both of which activate RhoA.

Interestingly, Rac, which antagonizes RhoA-dependent MLC phosphorylation in some cell types [28], is important in stimulating integrin-dependent MLC phosphorylation and cell retraction in other cell types [29]. In platelets, Rac1 stimulates the activation of the MAPK pathway, leading to increased phosphorylation of MLC and clot retraction [29]. Platelet-mediated clot retraction is totally abolished only when both Rac1 and RhoA are inhibited [29]. Thus, Rho and Rac play co-operative or synergistic roles in mediating cell retraction in this system.

The roles of Rho family small G proteins in integrin-dependent cell spreading

Integrin-dependent cell spreading is characterized by the outward movement of cell membranes and the underlying cytoskeletal structure (as opposed to cell retraction), where cells adherent on integrin ligands put forth extensions to contact the surface and form new integrin-dependent adhesions. Cell spreading involves visually distinct morphological changes of cell membranes including waves of protrusions, filopodial extensions, and lamellipodia. A pioneer study using microinjection of recombinant proteins demonstrated that Cdc42 mediates filopodia formation, Rac mediates lamellipodia formation, and Rho is important in stress fiber formation [30]. The roles of Rac and Cdc42 in cell spreading have been supported by numerous subsequent studies, although there is a recent controversy with respect to whether blood platelets are able to form filopodia and spread normally on integrin ligands in the absence of Cdc42 [31,32]. GTP-loaded Rho has also been observed at the leading edge of membrane protrusions in migrating cells, about 2 μm in front of where activated Rac and Cdc42 is located [33]. RhoC appears to interact with the formin isoform FMNL3 and restrict the broadening of lamellipodia [23]. RhoA may regulate Rac activity to restrict over-extension of the cell [23]. Interestingly, cycles of membrane protrusion and retraction occur locally in the leading edge of spreading cells. A study suggests that the function of RhoA in regulating clock rhythm of protrusion waves is controlled by PKA phosphorylation of RhoA at Ser188 and consequent increase in its GDI affinity [34]. Nevertheless, even though Rho may play an important role during the normal process of cell spreading and migration, Rho isoforms do not seem to be required for cell spreading. In fact, inhibition of Rho by siRNA knockdown, dominant negative mutants, or pharmacological inhibitors leads to accelerated or increased cell spreading [14,18,23,35]. Also, RhoA is

transiently inhibited during the early phase of cell spreading [14,18,25,35,36]. These studies suggest that Rho-mediated contraction may serve to limit cell spreading.

Cell spreading is associated with small G protein-mediated waves of actin polymerization in filopodia and lamellipodia. It has been presumed that actin polymerization is required for cell spreading; however, there has been evidence indicating that actin polymerization does not directly cause cell spreading, particularly the early phase of cell spreading. A study on the physical properties of cell spreading dynamics suggest that the dynamics of cell spreading follows a universal power-law behavior, and can be modeled as a viscous adhesive cortical shell (membrane and associated cytoskeleton) enclosing a less viscous interior (cytosol) [37]. Inhibition of actin polymerization with cytochalasin D accelerates cell spreading, suggesting that actin polymerization may not be required for cell spreading but may serve as a constraint [37]. Consistently, although Rac and Rho both facilitate actin polymerization, Rac and Rho play opposing roles during integrin-dependent cell spreading. In this respect, Rac induces phosphorylation of p190RhoGAP and inhibition of RhoA, directly or indirectly via a ROS-dependent mechanism, and this function is important in promoting cell spreading [38–40]. Another mechanism whereby Rac suppresses Rho activity is via its effector PAK, which phosphorylates and inactivates p115RhoGEF, a RhoGEF known to be important in stimulating GPCR-mediated RhoA activity [41].

The activity of Rac is stimulated during integrin outside-in signaling. SFK activation has been proposed to be a mechanism linking integrin ligation with downstream activation of small GTPases [16,42,43]. SFKs, particularly c-Src, have been shown to directly or indirectly mediate phosphorylation and activation of various GEFs for Rac, including Vav1 [42], Vav2 [44], and DOCK180 [45]. In platelets Src-dependent activation of Vav1 is mediated through the tyrosine kinase Syk [16,42]. A recent report also suggests that ribosome protein S6 kinase (S6K1) and its upstream regulator mTOR play a role in stimulating Src-dependent activation of Rac1 through association with the Rac1-GEF Tiam1 [43]. In addition, there is also evidence that integrin-mediated activation of other GEFs, such as α - and β -PIX, may be important in Rac activation in cells spreading on integrin ligands [46,47].

Switch between integrin-mediated cell spreading and retraction

Upon cell adhesion to integrin ligands, cells spread and retract. In polarized cell movement, cell spreading mainly occurs in the leading edge, while retraction dominates in the rear. Clearly, some molecular mechanisms must exist to allow a cell to switch its response to integrin outside-in signaling from spreading to retraction. Such a switch mechanism has been characterized for $\beta 3$ integrins (Fig. 2): following the binding of extracellular ligands to integrin $\alpha_{IIb}\beta 3$, $G_{\alpha 13}$ - $\beta 3$ interaction and consequent activation of $\beta 3$ -bound c-Src induces c-Src-dependent inhibition of RhoA [14,18]. This process is required for cell spreading. c-Src also phosphorylates $\beta 3$ at Y747 and Y759 [48], protecting $\beta 3$ from cleavage by calpain [49], a calcium-dependent protease. Following cell spreading, dephosphorylation of Y⁷⁵⁹ and consequent cleavage of $\beta 3$ by calpain at Y⁷⁵⁹ removes the c-Src-binding site thus abolishing c-Src-mediated inhibition of RhoA, allowing subsequent activation of RhoA-dependent contractile signaling and cell retraction [18,50]. Cell spreading mediated by other integrin subtypes, such as $\beta 1$ integrin, also requires transient inhibition of RhoA via SFK-dependent activation of p190RhoGAP [26,35,51]. However, c-Src does not appear to directly bind to the C-terminal sequence of $\beta 1$ [52] but rather can be recruited to the integrin-focal adhesion complex by FAK, which is implicated in $\beta 1$ -dependent activation of Src [53]. Thus, although calpain activity is also important in $\beta 1$ -dependent cell migration on matrix proteins [54], it is possible that the exact molecular mechanisms regulating the “switch” from spreading to retraction are different amongst integrin subtypes.

Crosstalk between GPCR signaling and integrin outside-in signaling

The discovery of an important role for Gα13-integrin interaction in outside-in signaling also reveals that a crosstalk exists linking the GPCR signaling pathway and the integrin outside-in signaling pathway. This crosstalk is distinct from the established role of GPCR pathways in stimulating integrin inside-out signaling. There are two distinct aspects of the crosstalk. First, GPCRs mediate the activation of Gα13, and thus stimulates Gα13 binding to integrin β subunits. This provides a molecular mechanism whereby the GPCR-Gα13 pathway directly enhances integrin outside-in signaling (Fig 3)[14]. Secondly, Gα13 binding to integrin β subunits negatively regulates GPCR-stimulated activation of RhoA. Interestingly, GPCR-stimulated RhoA activation is also mediated by GTP-bound Gα13, which activates p115 RhoGEF [55,56]. However, initial GPCR/Gα13-mediated activation of RhoA is transient [14]. While the mechanism inducing the downregulation of RhoA activity following initial GPCR activation was previously unclear, the discovery of the binding of Gα13 to the cytoplasmic domain of integrin following integrin ligation provides a plausible mechanism for the dynamic regulation of RhoA activity (Fig. 2) [14]. The binding of Gα13 to integrin induces the activation of SFKs, which suppress the activity of RhoA by activating p190RhoGAP [26,35]. In addition, the integrin binding site on Gα13 is located within switch region I, the same site that also interacts with RhoGEFs [14,57]. Thus, integrin binding to Gα13 should competitively block Gα13-p115 RhoGEF interaction. Therefore, the crosstalk between the Gα13-coupled GPCR pathway and the integrin outside-in signaling pathway, together with the above-mentioned calpain-dependent switch mechanism, forms an ideal regulatory system that controls Rho-dependent contractility and coordinates cell spreading and retraction (Fig. 2).

Conclusions

Integrin outside-in signaling requires both the heterotrimeric G protein Gα13 and monomeric small G proteins to mediate cell spreading, retraction, and migration. Gα13 plays a dual role in the process of integrin outside-in signaling. It mediates integrin outside-in signaling and integrin-dependent regulation of small G proteins, particularly Rho. It is also required for the crosstalk between the GPCR signaling pathway and the integrin outside-in signaling pathway. In the latter case, integrin serves as an effector for the GPCR-Gα13 signaling pathway. Thus, it appears that, in addition to the inside-out signaling and outside-in signaling of integrins, there is a third integrin signaling pathway that coordinates cell spreading and retraction: the “inside-outside-in” signaling pathway (Fig. 3). In this pathway, GPCR-induced intracellular (inside) signals greatly enhance integrin outside-in signaling to stimulate cellular responses independent of the inside-out signaling pathway. The mechanism whereby Gα13 stimulates integrin-dependent activation of SFKs remains unclear. Likewise, the complex networking of the small GTPases and their regulators remains to be fully elucidated. Further studies on the roles of G proteins in integrin-dependent cell adhesion, spreading, retraction, and migration will help to shed light on the intriguing and complex signaling networks regulating these fundamentally important cellular processes.

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Figure 2. Dynamic regulation of Rho by GPCRs and integrins

A. GPCR agonists (such as thrombin) stimulate the activation of $G_{\alpha 13}$ and $G_{\alpha 13}$ -dependent activation of RhoGEFs (such as p115 RhoGEF), leading to Rho activation and cell contraction. GPCRs also initiate inside-out signaling to activate integrins. B. Following ligand binding to integrins, GPCR-activated $G_{\alpha 13}$ binds to integrin β subunits, which inhibits Rho activity by Src-dependent activation of p190RhoGAP and by competitive inhibition of $G_{\alpha 13}$ -RhoGEF interaction. Inhibition of Rho facilitates cell spreading probably by relieving the constraint of retractile forces. Cleavage of integrin $\beta 3$ by calpain abolishes $\beta 3$ -Src association and thus Src-dependent inhibition of Rho. Tensional force on integrins also activates RhoGEFs. These events lead to reactivation of Rho and cell retraction [14].

Figure 3. Inside-outside-in signaling

GPCR/ $G\alpha_{13}$ -dependent signals inside a cell converge with integrin ligand-induced outside-in signal to stimulate cellular responses by inducing $G\alpha_{13}$ -integrin interaction.

Table 1**G proteins Involved in Integrin Outside-in Signaling**

G protein	Function
RhoA	Actomyosin contraction and actin polymerization [21,30,58]
RhoB/RhoC	Similar to RhoA but different localization and specificity for formin isoforms [23,24]
RhoG	Potential Rac activator [59,60]
RhoH	Potential Rac inhibitor [61]
Rnd	Rearrangement of actin cytoskeleton [62]
Rif	Filopodia formation through formin [63]
Rac1	Lamellipodia, activation of PAK and MAPK pathways [29,30,64]
Cdc42	filopodia formation and activation of PAK pathway [30,64,65]
Ras	Gene regulation, proliferation, and apoptosis through MAPK pathway [66]
Gα13	Src activation and RhoA regulation [14]