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G-protein $G_{\alpha_{13}}$ Functions with Abl Kinase to Regulate Actin Cytoskeletal Reorganization

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Abstract

Heterotrimeric G-proteins are essential cellular signal transducers. One of the G-proteins, $G_{\alpha_{13}}$, is critical for actin cytoskeletal reorganization, cell migration, cell proliferation and apoptosis. Previously we have shown that $G_{\alpha_{13}}$ is essential for both G protein-coupled receptor (GPCR) and receptor tyrosine kinase (RTK)-induced actin cytoskeletal reorganization such as dynamic dorsal ruffle turnover and cell migration. However, the mechanism by which $G_{\alpha_{13}}$ signals to actin cytoskeletal reorganization is not completely understood. Here we show that $G_{\alpha_{13}}$ directly interacts with Abl tyrosine kinase which is a critical regulator of actin cytoskeleton. This interaction is critical for $G_{\alpha_{13}}$ -induced dorsal ruffle turnover, endothelial cell remodeling, and cell migration. Our data uncover a new molecular signaling pathway by which $G_{\alpha_{13}}$ controls actin cytoskeletal reorganization.

Graphical abstract

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Actin cytoskeletal reorganization
Cell-shape change
Cell migration

Keywords

cell signaling; G-protein; Abl; tyrosine kinases; actin cytoskeleton

INTRODUCTION

Heterotrimeric G proteins ($G\alpha$ and $G\beta\gamma$ trimer) are essential for the transmembrane signaling by G protein-coupled receptors (GPCRs). A structurally diverse repertoire of ligands activates GPCRs to elicit their physiological functions. Ligand-bound GPCRs, in turn, function as guanine nucleotide-exchange factors (GEFs) to catalyze the exchange of GDP bound on the $G\alpha$ subunit with GTP, causing the dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ dimer to form two functional units ($G\alpha$ and $G\beta\gamma$) [1]. Both $G\alpha$ and $G\beta\gamma$ subunits signal to various cellular signaling pathways. Based on the sequence and functional homologies, $G\alpha$ proteins are grouped into four families: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ [2]. Among the four families of $G\alpha$ proteins, the physiological functions and signaling mechanisms of $G\alpha_{12/13}$ family is the least understood. In this family, there are two members, $G\alpha_{12}$ and $G\alpha_{13}$. $G\alpha_{12}$ knockout mice appeared normal [3]. $G\alpha_{13}$ knockout mice displayed embryonic lethality [4].

$G\alpha_{13}^{-/-}$ mouse embryos died at E9.5 [4]. The yolk sac of $G\alpha_{13}^{-/-}$ mouse embryos (at E9.5) did not show any blood vessels. Endothelial cell (EC)-specific $G\alpha_{13}$ knockout mouse embryos also died at E9.5 with the same phenotypes [5]. Re-expression of $G\alpha_{13}$ in ECs rescued the development of EC-specific $G\alpha_{13}$ knockout mouse embryos, and postponed the embryonic death from E9.5 to E13.5 in global $G\alpha_{13}^{-/-}$ embryos (eventually embryos died due to defects in other types of cells) [5]. These mouse genetic studies demonstrate that $G\alpha_{13}$ is a master regulator of vascular functions. Furthermore, $G\alpha_{13}$ also plays critical roles in adult angiogenesis such as tumor angiogenesis (pathological conditions), and ovarian as well as postnatal retinal angiogenesis (physiological conditions) [6, 7]. One cellular effect of $G\alpha_{13}$ in ECs is cell remodeling (such as cell shape changes). In the absence of $G\alpha_{13}$, serum-

induced capillary-tube formation, an in vitro model of EC remodeling, was impaired [5–8]. This defective actin cytoskeletal reorganization in the absence of $G\alpha_{13}$ was also observed in fibroblasts [8–11]. Although $G\alpha_{13}$ could act through a family of Rho-GEFs to activate RhoA linking to actin cytoskeleton [12], RhoA activation seemed not responsible for EC remodeling [5]. In $G\alpha_{13}$ -deleted ECs, RhoA activation was normal [5].

We previously made a surprising discovery that $G\alpha_{13}$ is also required for receptor tyrosine kinases (RTK)-induced actin cytoskeletal reorganization and cell migration [8]. Hence, $G\alpha_{13}$ plays a critical role in actin cytoskeletal reorganization and cell migration induced by both GPCRs and RTKs [8, 13]. The earliest ultra-structural changes of cells treated with growth factors are the intensive bursts of ruffling of the dorsal surface plasma membranes as seen under the phase-contrast microscope [14–16]. This cell remodeling event involves the formation and breakdown of dorsal ruffles (the dorsal ruffle turnover process). The physiological functions of dorsal ruffles, including macropinocytosis, cell migration and invasion, are continually expanding [17–20]. It has been suggested that one major function of dorsal ruffles is to reorganize the actin cytoskeleton to prepare a static cell for motility [21]. Growth factors (such as platelet-derived growth factor (PDGF)) initiate rapid formation of dorsal ruffles, and these ruffles move along the dorsal side of the cells, contract, close and eventually disassemble. We previously reported that, in mouse embryonic fibroblast (MEF) cells, dorsal ruffles formed within ~5 min after PDGF treatment [9]. These dorsal ruffles started to disassemble ~10 min after PDGF treatment. Dorsal ruffles formed only one time after PDGF stimulation. After the disassembly of these dorsal ruffles, protrusion of large peripheral membrane ruffles was observed [9]. In $G\alpha_{13}$ -deleted MEF cells, the formation of dorsal ruffles was normal after PDGF stimulation. However, the disassembly of dorsal ruffles was disrupted (~10-times slower), identifying $G\alpha_{13}$ as the first controller of dorsal ruffle disassembly [9]. Furthermore, we have shown that Ric-8A, a non-GPCR guanine nucleotide exchange factor for some $G\alpha$ proteins, is critical in relaying RTK signals to $G\alpha_{13}$ [10]. However, the signaling pathways from $G\alpha_{13}$ to dorsal ruffle turnover and cell migration are not well defined.

The mammalian Abelson nonreceptor tyrosine kinase family has two members, Abl1 (also named Abl) and Abl2 (also called Arg, Abl-related kinase) [22]. Abl is a multi-functional kinase, and has been implicated in the regulation of cell proliferation, differentiation, survival, death, retraction, adhesion, migration and morphology, depending on the cell types and physiological and pathological conditions [22]. Unlike most signaling protein kinases which are mainly involved in specific pathways, Abl kinases have been shown to be regulated by diverse signals and to function through various downstream substrates and/or binding proteins [23]. Most of the substrates are involved in actin polymerization or in transcription and chromatin [22]. It is believed that every signal only activates a pool of Abl kinases in a specific subcellular location leading to a selective biological response [22]. Furthermore, Abl shuttles between the nucleus and the cytoplasm, and can interact with DNA and actin polymers. Abl^{-/-} mice died at embryonic and neonatal stages with variable penetrance depending on the mouse strain background [24, 25]. Arg^{-/-} mice appeared normal [26]. Abl^{-/-}Arg^{-/-} mice led to embryonic lethality at E8 to E9 [26]. Mutations in Abl could lead to cancers; for example, the oncogenic BCR-Abl fusion leads to a constitutively activated kinase in human chronic myelogenous leukemia [27]. Abl kinase

inhibitors, such as imatinib, dasatinib and nilotinib, have been successfully used in treating chronic myelogenous leukemia [27]. In the growth factor-induced dorsal ruffle formation pathway, Abl signals to activate Rac [28]. Genetic screens in *Drosophila* have identified several gastrulation regulators including $G\alpha_{13}$ -like G protein *Concertina* and Abl [29, 30], all involved in controlling the actin cytoskeletal reorganization. Here we show that $G\alpha_{13}$ directly interacts with Abl in vitro, and they form a complex in cells. Their interaction is critical for the regulation of actin cytoskeletal reorganization.

RESULTS

Direct interaction between $G\alpha_{13}$ and Abl

To understand the molecular signaling mechanisms by which $G\alpha_{13}$ plays its physiological functions, we performed co-immunoprecipitation assays to identify possible $G\alpha_{13}$ -interacting proteins that are known to be involved in actin cytoskeletal reorganization. One of the identified proteins was Abl tyrosine kinase. Given the known role of Abl in actin cytoskeletal reorganization, here we focused on the $G\alpha_{13}$ and Abl interaction. From co-immunoprecipitation assays, we observed that endogenous $G\alpha_{13}$ could form a complex with endogenous Abl tyrosine kinase in cells (Fig. 1A). An anti- $G\alpha_{13}$ antibody co-immunoprecipitated endogenous Abl from cell extracts (Fig. 1A). In control experiments with $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ MEF cells and $Abl^{-/-}Arg^{-/-}$ MEF cells, the bands representing Abl were not observed (Fig. 1 A and B). $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ MEF cells were derived from $G\alpha_{12}$ and $G\alpha_{13}$ double knockout mouse embryos [8]. $Abl^{-/-}Arg^{-/-}$ MEF cells were from Abl and Arg double knockout mouse embryos [26]. A control antibody (anti-GFP antibody) did not co-immunoprecipitate Abl (Fig. 1C). The specificity of the anti- $G\alpha_{13}$ and anti-Abl antibodies was verified by the absence of corresponding bands in $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells and $Abl^{-/-}Arg^{-/-}$ cells, respectively (Fig. 1 A–C). These data demonstrate that $G\alpha_{13}$ and Abl form a specific complex in cells.

To study whether $G\alpha_{13}$ and Abl interact directly, we purified recombinant $G\alpha_{13}$ and Abl proteins. Abl contains an N-terminal half (including the SH3, SH2 and kinase domains) and a C-terminal half (including binding sites for DNA, G-actin and F-actin) (Fig. 1D). Recombinant $G\alpha_{13}$ was purified as His₆-tagged proteins from insect cells and the N-terminal half of Abl (GST-NT-Abl) was purified as GST-fusion proteins from *E. coli*. While $G\alpha_{13}$ did not pull-down GST control protein, it pulled-down the N-terminal half of Abl (Fig. 1E). We then produced GST-fusion proteins of the SH3SH2 domains of Abl (SH3SH2-Abl) and the kinase domain of Abl (Kin-Abl), and showed that $G\alpha_{13}$ directly interacted with the kinase domain of Abl (Fig. 1E). The other member of the $G_{12/13}$ -family of G-proteins, $G\alpha_{12}$ (but not other G-protein $G\alpha_i$, $G\alpha_s$, or $G\alpha_q$ families), also directly interacted with Abl, and $G\alpha_{13}$ could also interact with the other member of the Abl-family nonreceptor tyrosine kinases Arg (our unpublished data). G-proteins could exist in either GDP-bound or GTP-bound states. To investigate whether $G\alpha_{13}$ states have any effect on its interaction with Abl, we expressed the GTPase-deficient $G\alpha_{13}(Q226L)$ mutant (mainly in GTP-bound state in cells) and the mainly GDP-bound $G\alpha_{13}(G225A)$ mutant (Fig. 1F). The N-terminal half of Abl interacted with both GDP-bound $G\alpha_{13}$ and GTP-bound $G\alpha_{13}$ (Fig. 1F). Taking advantage that GDP-bound $G\alpha_{13}$ could interact with Abl, we examined whether the same

binding site on $G\alpha_{13}$ was occupied by Abl and $G\beta\gamma$. Since $G\beta\gamma$ subunits preferentially bind to GDP-bound $G\alpha_{13}$, we performed a competition experiment between $G\beta\gamma$ subunits and Abl for binding to GDP-bound $G\alpha_{13}$ (Fig. 1G). In the presence of $G\beta\gamma$, less GDP-bound $G\alpha_{13}$ proteins were pulled-down by GST-NT-Abl, implying that $G\beta\gamma$ competed with Abl for GDP-bound $G\alpha_{13}$. Hence Abl likely interacts with the same site on $G\alpha_{13}$ that also binds $G\beta\gamma$ (Fig. 1G).

Moreover, we used the isothermal titration calorimetry (ITC) technique and measured the direct interaction of purified $G\alpha_{13}$ and Abl proteins (Fig. 1 H and I). ITC is a direct measurement of the heat generated or absorbed when molecules interact. It is used to determine the thermodynamic parameters of interactions of molecules in solution. The experimental raw data consists of a series of spikes of heat flow (with every spike corresponding to one injection of Abl protein) (Fig. 1I). These spikes are then integrated with respect of time (Fig. 1I). The pattern of these heat effects as a function of the molar ratio $[Abl]/[G\alpha_{13}]$ could then be analyzed to give the thermodynamic parameters of the interaction (see Methods). ITC assay revealed that $G\alpha_{13}$ could directly interact with Abl (Fig. 1I). The K_a was $\sim 1.5 \times 10^5$, giving a K_d of $\sim 6.7 \mu M$ (Fig. 1I). The stoichiometry was ~ 1 (Fig. 1I). Together, these data demonstrate that $G\alpha_{13}$ directly interacts with Abl, and thus Abl is a novel direct signaling effector of $G\alpha_{13}$.

Role of Abl in EC remodeling

We then investigated the biological relevance of this newly discovered interaction between $G\alpha_{13}$ and Abl. As described before, $G\alpha_{13}$ is involved in EC remodeling. To examine whether Abl-family kinases are also involved in EC remodeling, we used two different cellular models of EC remodeling: three-dimensional spheroid spouting and capillary-tube formation [6, 31]. In the EC spheroid sprouting assay, ECs grew in hanging drops, aggregated as multicellular “spheroids” and remodeled to form sprouts. In the presence of serum, wild-type SVEC mouse endothelial cells formed rounded spheroids with multiple long sprouts (Fig. 2A). Treatment with Abl-family kinase inhibitor GNF2 reduced the number of sprouts and the length of the sprouts (Fig. 2 A–C). Similarly, down-regulation of $G\alpha_{13}$ in SVEC cells by siRNA decreased the number of sprouts and the length of the sprouts (Fig. 2 A–C). In the capillary-tube formation assay, ECs remodel and form capillary-like structures (tubes) in three-dimensional environment with the surrounding extracellular matrix. In the presence of serum, wild-type SVECs formed networked tubes with multiple branches (Fig. 2D). Treatment of Abl-family kinase inhibitor GNF2 or $G\alpha_{13}$ siRNA decreased the number, length and area of the capillary-like structures (Fig. 2 D–F). Together, these data demonstrate a role for Abl-family kinases in EC remodeling.

Control of actin cytoskeletal reorganization by $G\alpha_{13}$ and Abl

Underlying EC remodeling is the cytoskeletal reorganization. Previously we showed that $G\alpha_{13}$ controls actin cytoskeletal reorganization, but has no effect on microtubule cytoskeletal reorganization [9–11]. To take advantage of the availability of $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ and $Abl^{-/-}Arg^{-/-}$ mouse MEFs, we next explored the regulatory mechanisms of actin cytoskeletal reorganization by $G\alpha_3$ and Abl in these cells. We have shown that G-protein $G\alpha_{13}$ is essential for RTKs-induced dorsal ruffle turnover and cell migration [8–10]. The

signals from these RTKs (including PDGFRs) are relayed to $G\alpha_{13}$ via a non-GPCR guanine nucleotide exchange factor Ric-8A [10]. To visualize directly the effect of $G\alpha_{13}$ and Abl on actin cytoskeletal reorganization, we utilized live-cell imaging techniques to compare the dynamics of actin cytoskeletal reorganization in wild-type, $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ and $Abl^{-/-}Arg^{-/-}$ MEF cells. We injected plasmid DNA encoding actin-GFP into MEF cells, $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ cells and $Abl^{-/-}Arg^{-/-}$ cells. After expression for 1~2 hours, we treated the cells with PDGF-BB (20 ng/ml) and monitored the subcellular distribution of actin polymers. In MEF cells, actin-based dorsal ruffles formed within 3 min (3.07 ± 0.07 minutes, $n = 28$) after PDGF treatment (Fig. 3 A and D). These dorsal ruffles lasted for ~8 min (8.29 ± 0.57 minutes, $n = 28$) and disassembled after PDGF treatment (Fig. 3 A and E). Without PDGF treatment, actin-GFP was uniformly distributed in MEF cells [10]. On the other hand, in $Abl^{-/-}Arg^{-/-}$ cells, formation of actin-based dorsal ruffles was slightly delayed (5 ± 0.22 minutes, $n = 45$; comparing with MEF cells, $p < 0.0001$) (Fig. 3 B and D). Also, dorsal ruffles took a longer time to disassemble (17.6 ± 0.81 minutes, $n = 45$) (Fig. 3 B and E). In $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells, dorsal ruffles formed within 3 min (3.2 ± 0.21 minutes, $n = 20$) after PDGF-BB treatment (Fig. 3 C and D). However, these dorsal ruffles then stayed much longer (64.2 ± 2.08 minutes, $n = 20$) before disassembling (Fig. 3 C and E). In addition to these single-cell analyses, we also investigated this actin reorganization process in populations of cells. Wild-type MEFs, $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$, and $Abl^{-/-}Arg^{-/-}$ cells were stimulated with PDGF-BB for various time periods. The cells were then stained with phalloidin to visualize the actin polymers. Consistent with the single-cell analysis, after 20 min treatment, dorsal ruffles could not be observed in wild-type MEFs, while ~40% of $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells and ~20% of $Abl^{-/-}Arg^{-/-}$ cells still had dorsal ruffles, indicating dorsal ruffle turnover in $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ and $Abl^{-/-}Arg^{-/-}$ cells was slower than MEF cells (Fig. 3F). We noticed that, after 5 min stimulation with PDGF-BB, while 20~30% of MEF and $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells had dorsal ruffles, ~60% of $Abl^{-/-}Arg^{-/-}$ cells had dorsal ruffles (Fig. 3F). All together, our data demonstrate that, while $G\alpha_{13}$ controls the rate of the breakdown of dorsal ruffles, Abl-family kinases participate in both dorsal ruffle formation and disassembly. Hence both $G\alpha_{13}$ and Abl participate in this actin cytoskeletal reorganization process.

Role of the interaction between $G\alpha_{13}$ and Abl in actin cytoskeletal reorganization

To further investigate whether the $G\alpha_{13}$ -Abl interaction is critical for the actin cytoskeletal reorganization, we identified $G\alpha_{13}$ mutants with impaired interaction with Abl and studied their rescue ability in $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ cells. G-proteins use their switch regions to contact $G\beta\gamma$ and their downstream effectors [32]. Since $G\alpha_{12}$ and $G\alpha_{13}$, but not $G\alpha_i$, interact with Abl, we mutated residues in the three switch regions of $G\alpha_{13}$ that were the same in $G\alpha_{12}$, but different in $G\alpha_i$ (Fig. 4A). These residues in $G\alpha_{13}$ were mutated to the corresponding residues in $G\alpha_i$ (Fig. 4A). We purified these recombinant $G\alpha_{13}$ proteins and examined their interaction with purified Abl proteins by GST pull-down assays. We found that two adjacent mutants were with impaired ability to interact with Abl: while Glu235 to His had decreased binding to Abl, the mutant Phe234 to Iso had increased binding to Abl (Fig. 4B). We then tested whether these two $G\alpha_{13}$ mutants could rescue the dorsal ruffle disassembly in $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ cells. While wild-type $G\alpha_{13}$ accelerated the disassembly of dorsal ruffles in single-cell imaging studies, the two $G\alpha_{13}$ mutants with impaired interaction with Abl did

not (Fig. 4C). Furthermore, we tested these two $G\alpha_{13}$ mutants in rescuing the EC remodeling defect in $G\alpha_{13}$ knockdown SVEC cells. In SVEC cells stably expressing $G\alpha_{13}$ shRNA, we expressed the two $G\alpha_{13}$ mutants (in a human $G\alpha_{13}$ background and were resistant to the $G\alpha_{13}$ shRNA). As shown in Fig. 4D, while wild-type $G\alpha_{13}$ rescued the capillary-tube formation defect in $G\alpha_{13}$ knockdown SVEC cells, $G\alpha_{13}$ (Phe234Iso) and $G\alpha_{13}$ (Glu235His) mutants did not.

Moreover, we investigated the role of the interaction between $G\alpha_{13}$ and Abl in cell migration. We used the Boyden chamber assay to quantitatively measure the migration of $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ cells and $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ cells stably transfected with wild-type $G\alpha_{13}$, and the two $G\alpha_{13}$ mutants. In this chamber assay, MEF cells were loaded onto the top of the Boyden chamber. After about six hours, cells migrated into the bottom of the chamber filter were counted. As shown in Fig. 4E, wild-type $G\alpha_{13}$ rescued the PDGF-induced migration of $G\alpha_{13}^{-/-}G\alpha_{12}^{-/-}$ cells. $G\alpha_{13}$ (Phe234Iso) and $G\alpha_{13}$ (Glu235His) mutants did not. These results were confirmed by a second, complementary cell migration assay, the wound-healing assay. Although these two $G\alpha_{13}$ mutants might also affect the interaction of $G\alpha_{13}$ with other proteins, these data are consistent with the notion that the interaction of $G\alpha_{13}$ and Abl is critical for the regulatory function of $G\alpha_{13}$ in actin cytoskeletal reorganization.

DISCUSSION

We have shown here that Abl is a new direct signaling effector of $G\alpha_{13}$. We did not observe a significant effect of $G\alpha_{13}$ on the catalytic kinase activity of Abl. Rather, the interaction of $G\alpha_{13}$ and Abl is critical for actin cytoskeletal reorganization. This is reminiscent of the interaction of $G\alpha_i$ and the NuMA/LGN complex during asymmetric cell division [33]. Both $G\alpha_{13}$ and Abl have been shown to be critical for EC functions. Deletion of $G\alpha_{13}$ gene leads to impaired EC remodeling, defective vascular development and mouse embryonic lethality. $Abl^{-/-}Arg^{-/-}$ mice die at E10.5 with hemorrhage and pericardial edema [26]. EC-specific deletion of Abl and Arg results in embryonic lethality [34, 35][36]. Based on our data, we propose that the direct interaction of $G\alpha_{13}$ and Abl controls actin cytoskeletal reorganization, remodeling and migration of ECs, thus contributing to blood vessel formation.

Previously we showed that $G\alpha_{13}$ controls actin cytoskeletal reorganization by regulating the disassembly of dorsal ruffles [9]. In the absence of $G\alpha_{13}$, the activated state of Rac stayed much longer than in normal cells [9], indicating that $G\alpha_{13}$ controls the duration of Rac activation. It is not clear how $G\alpha_{13}$ is linked to Rac. Currently there are quite a few proteins have been identified to interact with the $G\alpha_{12/13}$ family of G-proteins. $G\alpha_{13}$ (but not $G\alpha_{12}$) is able to directly increase the activity of p115RhoGEF and related RhoGEF proteins [PDZ-RhoGEF and leukemia-associated RhoGEF (LARG)] by membrane recruitment and direct interaction [37]. Other G-proteins, such as $G\alpha_q$, could also increase the activity of RhoGEFs, such as p63RhoGEF, linked to RhoA activation [38, 39]. In endothelial cells, RhoA activation is normal in $G\alpha_{13}$ -knockout cells, but is impaired in $G\alpha_q$ -knockout cells [40]. Several proteins were reported to interact with $G\alpha_{12}$. These proteins include Btk-family tyrosine kinases, Gap1, rasGAP, cadherins, α -SNAP, and p120-catenin [41–44]. Similarly, several proteins (such as Btk-family tyrosine kinase, cadherins, radixin, Hax-1,

and Integrin $\alpha_{\text{iii}}\beta_3$) were reported to interact with $G\alpha_{13}$ [42, 45–49]. While RhoGEF provides a direct link of $G\alpha_{13}$ to Rho (Fig. 4F), it is not clear how $G\alpha_{13}$ is linked to Rac.

Rac mainly functions via the WAVE complex which activates the Arp2/3 complex, leading to actin polymerization (Fig. 4F) [50]. Genetic and biochemical studies have demonstrated that Abl can regulate the dynamic actin cytoskeletal reorganization at multiple levels (Fig. 4F). These regulations are complex. In *C. elegans*, during the actin cytoskeletal reorganization processes of the engulfment of apoptotic cells and of the migration of distal tip cells, genetic and biochemical data indicate that Abl inhibits the function of its binding partner Abi to regulate the activity of the WAVE complex, in a pathway parallel to the Rac pathway, leading to the inhibition of engulfment and migration [51] (Fig. 4F). In these processes, Rac was activated by the complex of DOCK180/ELMO, which is regulated by the adaptor Crk [51] (Fig. 4F). DOCK180 (also known as DOCK1) is an atypical Rac GEF. In *Drosophila*, during the actin cytoskeletal reorganization process of axon patterning, genetic and biochemical data suggest that Abl suppresses the activity of *Enabled* (*Ena*) to limit extension of actin filaments, and activates Trio (a GEF for Rac) to promote formation of branched actin networks [52] (Fig. 4F). In PDGF-induced dorsal ruffle formation pathway in mammalian cells, Abl signals to activate Rac [28, 35, 53–56]. This activation might involve DOCK180 since the complex of Crk/DOCK180 has been shown to control Rac activation during dorsal ruffle formation [57] (Fig. 4F). DOCK180 binds to phosphatidic acid through the C-terminal polybasic amino acid cluster and accumulates at the dorsal ruffle membrane in response to PDGF-stimulation. Therefore Abl can positively and negatively module the actin network. These might underline our observation that Abl regulates both the dorsal ruffle assembly and disassembly. Future investigation will be needed to understand the dynamic and cyclic regulation of Rac activity by $G\alpha_{13}$ and Abl. Nevertheless, given that $G\alpha_{13}$ is essential for blood vessel formation, our data should open up new avenues using agents that inhibit the specific interactions between $G\alpha_{13}$ and particular interacting proteins such as Abl for therapeutic intervention for human diseases such as atherosclerosis, tumor angiogenesis, and macular degeneration.

MATERIALS AND METHODS

Cell Culture and Co-Immunoprecipitation

MEF cells, $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells and $Abl^{-/-}Arg^{-/-}$ cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen). Co-immunoprecipitation and western-blots were performed as previously described [58, 59]. Cells in 10-cm plates were washed with ice-cold phosphate buffered saline (PBS, pH7) and detached from the plate by incubating with 3 ml PBS containing 5 mM EDTA. After centrifugation, the cell pellet was resuspended in 1 ml of RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 1% Triton X-100, and protease inhibitors). The cells were homogenized with a Dounce homogenizer and centrifuged at $900 \times g$ for 10 min. The supernatant was transferred to a fresh tube and pre-cleared by incubating with 20 l of 50% protein A-agarose slurry for 30 min at 4°C. After centrifugation, the supernatant was transferred to a fresh tube and incubated with 2 μ g of antiserum for 2 hr at 4°C. Forty μ l of protein A-agarose were then added and the mixture was incubated overnight at 4°C. The

supernatant was removed after centrifugation. The pellet was washed three times with RIPA buffer. The proteins were eluted with SDS sample buffer and subjected to SDS-PAGE and western-blot analyses. Anti-G α_{13} , anti-Abl, and anti-GFP antibody were from Santa Cruz Biotechnology.

Protein Purification

GST-tagged various domains of Abl were purified from *E. coli*. Two liters of BL21(DE3) with these plasmids were grown at 37°C until the turbidity reached OD₆₀₀ = 1.2. Protein expression was induced with 10 μ M IPTG (Sigma) for 4 hr. Cells were harvested by centrifugation and the pellet was resuspended in ice-cold 1 $\times$ PBS containing 1 mg/ml lysozyme, 1 mM DTT, 100 μ g/ml DNase I, 0.5 μ g/ml leupeptin, 1 μ g/ml pepstatin, 2 μ g/ml aprotinin and 1 mM PMSF. After sonication, Triton X-100 (1%) was added to the lysate and the mixture was stirred for 30 min at 4°C. The lysate was then cleared of debris by centrifugation and applied to glutathione agarose beads (Sigma). After washing, GST-tagged protein was eluted with glutathione.

Histidine-tagged G α_{13} protein was purified from High Five insect cells (Invitrogen). Baculovirus containing His₆-G α_{13} was generated and amplified in Sf9 cells using Invitrogen's Bac-to-Bac system. Two liters of High-Five cells were infected with the baculovirus and incubated at 27°C for 48 hr. After harvesting the infected cells by centrifugation, the cell pellet was resuspended in 100 ml of 1 $\times$ PBS containing 2% Triton X-100, 5 mM β -mercaptoethanol and protease inhibitors. The lysis of the cells was facilitated by sonication. The lysate was then centrifuged at 20,000 rpm for 30 min. The supernatant was discarded. The pellet was resuspended and incubated at 4°C for one hour in 20 ml of 1 $\times$ PBS containing 1% Triton X-100 and protease inhibitors. The mixture was then centrifuged at 20,000 rpm for 30 min. The supernatant was collected. Purification of the protein was monitored by western-blot with an anti-G α_{13} antibody. The protein was further purified by Hi-trap Q-sepharose HP column (Pharmacia).

GST Pull-Down Assay

GST pull-down assays were done with purified proteins [38, 41, 59–61]. In a 100 μ l solution, components such as GST-NT-Abl or GST were mixed with His-tagged G α_{13} in the absence or presence of purified G $\beta\gamma$ proteins. Ten μ g of each protein were used in a reaction mixture. After incubating the samples for 30 min at 4°C, 40 μ l of 50% slurry of glutathione beads were added. The mixtures were incubated for at least 1 hr at 4°C. The beads were collected by centrifugation and washed three times with 1 $\times$ PBS. Proteins were eluted with SDS sample buffer and subjected to SDS-PAGE and western-blot.

Isothermal Titration Calorimetry

Calorimetric measurements were carried out using a Low Volume Nano ITC instrument from TA Instruments (New Castle, DE). In each experiment, GST-NT-Abl was titrated to G α_{13} solution present in the 500 μ L sample cell. All measurements were carried out at 25 °C. G α_{13} was dissolved in 20 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl to a concentration of 60 μ M. GST-NT-Abl solution (0.7 mM) in the same buffer as the protein was used. After a stable baseline had been achieved, the titration was initiated. The injection

sequence composed 20 injections of 2.4 μL in time intervals of 180 s until complete saturation was obtained. Heat changes caused by each injection were obtained from the integral of the calorimetric signal. Data were analyzed using the NanoAnalyze software (TA Instruments) for fitting points to the independent binding model. Experimental heats of the Abl- $\text{G}\alpha_{13}$ titration were corrected for heats of dilution by subtracting the average heat of the last three measurements after saturation of the protein binding sites has occurred. All measurements were carried out at least in triplicate. Given energy values, binding constants and standard deviations were derived from data fitting and subsequent averaging of the corresponding measurements. Standard Gibbs free energy values were calculated using the equation $G_0 = -RT \ln K_b$ (where $R = 8.3144 \text{ J mol}^{-1} \text{ K}^{-1}$, K_b binding constant).

RNA Interference

RNAi of $\text{G}\alpha_{13}$ was performed in SVEC cells with Qiagen's HiPerformance 2-for-Silencing siRNA duplexes. Transfections of siRNAs were done using Oligofectamine (Invitrogen). Briefly, siRNA and Oligofectamine were diluted and mixed in OptiMEM I (Invitrogen) to allow the formation of siRNA-liposome complexes. After a 20-min incubation at room temperature, the mixture was overlayed onto SVEC cells (50% confluent) cultured in medium without antibiotics. The transfected cells were analyzed 72–96 hr after transfection. shRNAs were in pSuper vector, and individual cell clones were selected and the expression of $\text{G}\alpha_{13}$ was examined by western blots.

Endothelial Spheroid Formation

Endothelial cell spheroids were generated as described [31, 62]. SVEC cells were suspended in culture medium containing 0.25% (w/v) carboxymethylcellulose and seeded in nonadherent round-bottom 96-well plates. Under these conditions all suspended cells contribute to the formation of a single EC spheroid. These spheroids were harvested within 24 hours and embedded into collagen gels. A collagen stock solution was prepared prior to use by mixing acidic collagen extract of rat tails (equilibrated to 2 mg/ml, 4°C; 8 vol.) with 10 $\times$ PBS (1 vol.) and 0.1 M NaOH (approx. 1 vol.) to adjust the pH to 7.4. This stock solution (0.5 ml) was mixed with 0.5 ml of room temperature medium with 40% FBS containing 0.5% (w/v) carboxymethylcellulose to prevent sedimentation of spheroids prior to polymerization of the collagen gel, 50–100 SVEC cell spheroids, and the corresponding test substance. The spheroid-containing gel was rapidly transferred into pre-warmed 24-well plates and allowed to polymerize (for 1 minute), after which 0.15 ml basal medium was pipetted on top of the gel. The gels were incubated at 37°C in 5% CO_2 at 100% humidity.

Tube Formation Assay

Matrigel growth factor reduced (500 μL , BD Biosciences) was applied to each well of a 24-well plate and incubated at 37°C for one hour. Mouse SVEC4-10 cells were added in serum-containing medium. Tube formation was examined beginning 6 hours later. Photographs of representative 10 $\times$ fields were taken, and endothelial tubes were quantified by counting length and branches using the NIH Image J software.

Microinjection

Cells were maintained in DMEM supplemented with 10% fetal bovine serum in a 37°C incubator humidified with 5% CO₂. Cells were seeded onto heat sterilized 25 mm round coverslips and grown for 36 hr prior to microinjection. Cell nuclei were pressure microinjected with plasmid DNAs prepared in HKCl microinjection buffer (10 mM HEPES, 140 mM KCl, pH 7.4). GFP- β -actin with or without other plasmids were injected at a concentration of 20 μ g/ml using back-loaded glass capillaries and a Narishige micromanipulator (Narishige, Greenvale, NY). For experiments in which multiple plasmid DNAs were injected, the final concentration of each plasmid DNA in the injection needle was kept at 20 μ g/ml. After injection, cells were maintained at 37°C in a humidified CO₂ environment for 90 min to allow for expression of injected plasmid DNAs. Cells were transferred to Recording Medium (Hanks Balanced Salt Solution, without phenol-red, supplemented with 20 mM HEPES, 1% dextran-charcoal stripped FBS (Atlanta Biologicals), 4.5 g/L glucose, essential and non-essential amino acids) and the formation of dorsal ruffles in the presence of added PDGF (20 ng/ml) was monitored by time-lapse fluorescence microscopy of actin-GFP at 37°C.

Time-Lapse Fluorescence Microscopy

After microinjection cells were transferred to a Sykes-Moore chamber (Bellco Glass Inc, Vineland, NJ) and placed in a thermal-controlled microincubator (Harvard Apparatus, Holliston, MA) mounted on a Nikon TiE inverted microscope equipped with Perfect Focus™ (Nikon Inc., Melville, NY). The cells and recording medium were allowed to equilibrate at 37°C for 5 min prior to addition of PDGF and beginning time-lapse recordings. GFP-actin fluorescence was imaged directly with a fluorescein/GFP filter cube (Chroma Technology Corp, Bellows Falls, VT). Images were collected using an Andor Neo, CMOS camera (Andor Technology, Belfast, Ireland) and were transferred to a computer workstation running the Nikon Elements™ software. Formation of dorsal ruffles in the presence of added PDGF (20 ng/ml) was monitored using a 20 $\times$ objective (plan apo λ , NA 0.75) and images were collected at one min intervals for ~60 minutes after addition of PDGF. Post-acquisition analysis and processing of images was performed using Elements™.

Fluorescence microscopy

Staining and observation of dorsal ruffles were performed as previously described [9]. Cells were starved, treated with PDGF-BB (20 ng/ml) for indicated time points, and plated onto coverslips coated with gelatin. Cells were then fixed with 3.7% formaldehyde. The fixed cells were then permeabilized in 0.1% Triton X-100 for 5 min. After washing in PBS, phalloidin conjugated to Alexa Fluor 488 (Molecular Probes) in a solution containing PBS and 1% BSA was added to stain actin filaments. After incubation for 30 min at room temperature, the cells were washed extensively to reduce nonspecific interactions. The coverslips were then fixed onto slides and imaged using a Zeiss AXIOVERT 200 fluorescence microscope. Post-acquisition analysis and processing of images were performed using MetaMorph.

Boyden Chamber Cell Migration Assay

Cell migration was assayed using Boyden chambers [8.0 μ m pore size polyethylene terephthalate membrane, FALCON cell culture insert (Becton-Dickinson)] [9, 63–65]. Cells were starved for 15 hours, trypsinized and counted. 200 μ l of $5\text{--}10 \times 10^4$ cells in serum free medium were added to the upper chamber and 500 μ l of appropriate medium with 20 ng/ml PDGF-BB were added to the lower chamber. Transwells were incubated for 6 hours at 37°C. Cells on the upper surface of the membrane were removed with a cotton swab, and cells on the under-surface of the membrane were fixed with 3.7% formaldehyde and stained with crystal violet. Photographs of three random regions (10 $\times$ objective) were taken and the number of cells counted to calculate the average number of cells that had transmigrated per field.

Wound-healing Cell Migration Assay

Cells in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) containing 10% FBS were seeded into wells of 24-multiwell plates (Becton-Dickinson). After starvation for 15 hours, wounds were made with sterile pipet tips. Cells were washed with Phosphate Buffered Saline (PBS) and refreshed with medium containing 20 ng/ml platelet-derived growth factor (PDGF-BB). After ~16-hours incubation at 37°C, cells were fixed and photographed (with 100 $\times$ magnification).

Statistical Analysis

Data are expressed as mean $\pm$ SEM and analyzed by the Student *t*-test with significance defined as $p < 0.05$.

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Highlights

- Signaling mechanism by which G-protein $G\alpha_{13}$ regulates actin cytoskeletal reorganization was investigated.
- $G\alpha_{13}$ and Abl tyrosine kinase directly interact.
- $G\alpha_{13}$ and Abl regulate the dynamic dorsal ruffle turnover.
- This interaction between $G\alpha_{13}$ and Abl is critical for actin cytoskeletal reorganization.

Figure 1.

Direct interaction between $G\alpha_{13}$ and Abl. **A–C**, Co-immunoprecipitation of $G\alpha_{13}$ and Abl in cells. Whole-cell lysates were used for immunoprecipitation. $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ MEF cells and $Abl^{-/-}Arg^{-/-}$ MEF cells, as well as anti-GFP antibody were used as controls. IgG: immunoglobulin heavy chain. **D**, Diagram of the functional domains of Abl and its related Arg kinase. NT-Abl: N-terminal fragment of Abl. **E**, Pull-down assays to map the binding domain on Abl. Purified His₆-tagged $G\alpha_{13}$ proteins were mixed with purified GST-tagged NT-Abl, GST-SH3SH2-Abl (the SH3 and SH2 domains of Abl), or GST-Kin-Abl (the kinase domain of Abl). Ni-beads were used for pull-down. Anti-GST antibody was used for

western blot. **F** Both $G\alpha_{13}(GDP)$ and $G\alpha_{13}(GTP)$ could interact with Abl. $G\alpha_{13}(G225A)$ (mainly in GDP-bound state in cells) and $G\alpha_{13}(Q226L)$ (mainly in GTP-bound state in cells) mutants were transfected into HEK293 cells. GST-NT-Abl fusion protein bound on glutathione beads were used for pull-down and the interacting $G\alpha_{13}$ protein was shown with anti- $G\alpha_{13}$ antibody. The right panel was the result from western blot of the whole-cell lysates from these transiently transfected cells. **G**, Competition binding for $G\alpha_{13}$ between $G\beta\gamma$ and Abl. Purified GST or GST-NT-Abl proteins were used to pull-down His₆-tagged $G\alpha_{13}(GDP)$ in the absence (lanes 1 and 4) or presence (lanes 3 and 6) of purified $G\beta\gamma$ proteins. **H** and **I**, Isothermal titration calorimetry (ITC) assay shows the direct binding of $G\alpha_{13}$ to Abl. **H**, Coomassie blue staining of purified $G\alpha_{13}$ and GST-NT-Abl proteins used in the ITC assays. **I**, ITC experimental data. Data are representative of three similar experiments.

Figure 2.

Role of Abl in EC remodeling. **A – C**, SVEC cells were cultured and let to form spheroids in the presence of serum, serum + Abl inhibitor (GNF2), or pre-treated with Gα₁₃ siRNAs. Images of representative spheroids are shown in **A**. The cumulative sprout length per spheroid is quantified in **B**. The number of sprouts per spheroid is presented in **C**. **D – F**, SVEC cells were cultured in the presence of matrix gel with serum, serum + Abl inhibitor (GNF2), or pre-treated with Gα₁₃ siRNAs. Images of representative fields are shown in **D**. The average branch length per field is quantified in **E**. The number of branches per field is

presented in **F**. Control: under starvation condition (without serum). Data are shown as mean $\pm$ SEM of three to five experiments. *, $p < 0.05$ by t test.

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Figure 3.

Control of actin cytoskeletal reorganization by $G\alpha_{13}$ and Abl. **A – C**, Actin-GFP plasmids were microinjected into individual MEF cells (**A**), $Abl^{-/-}Arg^{-/-}$ cells (**B**), or $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells (**C**). After the addition of PDGF-BB, the actin cytoskeletal reorganization (here the formation and disassembly of dorsal ruffles, marked by arrowheads) was imaged in live-cells. Representative cells are shown. **D**, The time from the addition of PDGF-BB to the first appearance of dorsal ruffles is quantified. **E**, The duration time from the first appearance of dorsal ruffles to the disappearance of dorsal ruffles is quantified. Data are shown as mean $\pm$ SEM of 20 to 45 cells. **F**, Cells were stimulated with PDGF-BB. The

percentage of cells with dorsal ruffles among the total number of cells in a given field is quantified at different time points after PDGF-BB stimulation. Data are shown as mean $\pm$ SEM of three similar experiments.

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Figure 4.

Role of the interaction between Gα₁₃ and Abl in actin cytoskeletal reorganization, EC remodeling and cell migration. **A**, Amino acid sequences of Gα₁₃, Gα₁₂ and Gα₁₁ in their three switch regions which have different configurations in the GDP and GTP bound forms. Residues same in Gα₁₃ and Gα₁₂, but different in Gα₁₁, are labeled in red or green. **B**, Interaction between Gα₁₃ mutants and Abl. Purified GST-NT-Abl proteins were used to pull-down various purified recombinant Gα₁₃ mutant proteins. Anti-Gα₁₃ antibody was used for western blot. The binding of wild-type Gα₁₃ with Abl was used as control. **C**, Wild-type Gα₁₃, Gα₁₃(F234I), or Gα₁₃(E235H) plasmids were co-microinjected with actin-

GFP into $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells. The dorsal ruffle formation and disappearance were recorded in live-cells. The duration of dorsal ruffles is shown. **D**, SVEC cells with wild-type human $G\alpha_{13}$, $G\alpha_{13}(F234I)$, or $G\alpha_{13}(E235H)$ were used for tube forming assay in matrigel. The average branch number is quantified. **E**, Boyden chamber cell migration assay. $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ control cells and $G\alpha_{12}^{-/-}G\alpha_{13}^{-/-}$ cells stably expressing wild-type $G\alpha_{13}$ or $G\alpha_{13}$ mutants were used for cell migration assay in response to PDGF-BB. **F**, An outline of the possible mechanisms of action for the interaction between $G\alpha_{13}$ and Abl on the cytoskeletal reorganization, cell-shape change, and morphogenesis. We should note that not all actions of $G\alpha_{13}$ are through Abl. Not all listed functions of Abl are affected by $G\alpha_{13}$. Also, only some of the possible functions of $G\alpha_{13}$ and Abl are listed here to illustrate their regulatory roles in cytoskeletal reorganization. Data are shown as mean $\pm$ SEM of three independent experiments. *, $p < 0.05$ vs. control.