Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G_{12} family to Rho

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Abstract A putative guanine nucleotide exchange factor (GEF), termed leukemia-associated RhoGEF (LARG), was recently identified upon fusion to the coding sequence of the *MLL* gene in acute myeloid leukemia. Although the function of LARG is still unknown, it exhibits a number of structural domains suggestive of a role in signal transduction, including a PDZ domain, a LH/RGS domain, and a Dbl homology/pleckstrin homology domain. Here, we show that LARG can activate Rho in vivo. Furthermore, we present evidence that LARG is an integral component of a novel biochemical route whereby G protein-coupled receptors (GPCRs) and heterotrimeric G proteins of the $G\alpha_{12}$ family stimulate Rho-dependent signaling pathways.

Key words: Rho; G protein; Ras; Signal transduction; G protein-coupled receptor

1. Introduction

The Rho family of GTP-binding proteins plays an important role in the regulation of cell morphology, cell aggregation, tissue polarity, cytokinesis, cell motility and also in smooth muscle contraction [1–3]. Recent evidence suggests that Rho proteins are also integral components of signaling pathways regulating gene expression. For example, activated alleles of Rho induce expression from the serum responsive element (SRE) through the activation of the serum response factor [4], and two members of the Rho family of GTPases, Rac and Cdc42, regulate the activity of the c-Jun amino-terminal kinase (JNK) [5], which, in turn, phosphorylates transcription factors such as c-Jun and ATF2 thereby stimulating their transcriptional activity [6].

Rho proteins are members of the Ras superfamily of GTPases, which cycle between an inactive GDP-bound and an active GTP-bound form under the tight regulation of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Whereas GEFs promote the exchange of GDP for GTP thus activating Ras-like proteins [7], GAPs enhance the low intrinsic rate of GTP hydrolysis of small GTPases and are negative modulators [8]. Many GEFs for GTP-binding proteins of the Rho family, including *dbl*, *ost*, *lfc*, *lbc*, *vav*, *ect2*, *tim*, and *net* were discovered by virtue of their ability to transform murine fibroblasts upon overexpression or when activated by truncations [9]. These proteins share a highly related structural domain of about 250 amino acids, termed Dbl homology (DH) domain, adjacent to a pleckstrin homology (PH) domain [9]. The DH domain is responsible for the nucleotide exchange activity towards GTPases of the Rho family [10,11], and often the overexpression of the DH/PH domain alone is sufficient to stimulate guanine nucleotide exchange on Rho proteins in vivo [10]. However, how cell surface receptors regulate the activity of these GEFs thereby stimulating Rho proteins is still largely unknown.

Of interest, in a recent study Kourlas et al. have identified a novel putative RhoGEF, termed leukemia-associated RhoGEF (LARG), whose coding sequence was fused to that of the *MLL* gene upon chromosomal rearrangement in a patient with acute myeloid leukemia [12]. The predicted protein product of the *LARG* gene includes a number of structural domains suggestive of a function in signal transduction, including a PDZ domain, a LH/RGS domain, and a tandem DH/PH domain highly related to those of RhoGEFs [12]. However, the biological and/or biochemical activities of LARG are still unknown. In this study, we present evidence that LARG links G protein-coupled receptors (GPCRs) and heterotrimeric G proteins of the $G\alpha_{12}$ family to Rho, thus providing a mechanism by which this large family of cell surface receptors activates Rho-dependent signaling pathways.

2. Materials and methods

2.1. Cloning of full length LARG cDNA and expression plasmids

A partial LARG cDNA (KIAA0382) encoding amino acids 795–1544 was kindly provided by Dr. T. Nagase, Kazusa DNA Research Institute, Japan. The upstream sequence of LARG was amplified by the reverse transcription (RT)-PCR technique using human brain total RNA, and was confirmed by sequencing. Sequence of the oligonucleotides used for RT-PCR will be made available upon request. The full coding sequence of LARG was subcloned in frame with that of an AU1-tag in the pCEFL vector (pCEFL AU1 LARG) [13]. cDNAs encoding LARG mutants were generated by restriction enzyme digestion and polymerase chain reaction amplification using pCEFL AU1 LARG as a template.

Plasmids expressing AU1-tagged PDZ-RhoGEF, an AU5-tagged form of Rho, activated forms of $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_q$, and β-galactosidase as well as m1 and PAR1 GPCRs were described previously [13,14]. Reporter plasmid encoding the chloramphenicol acetyltransferase (CAT) gene under the control of mutant serum response element from the c-fos promoter, which lacks the ternary complex factor (TCF)-binding site (SREmutL), was kindly provided by Dr. R. Treisman [4].

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2.2. Cell lines and transfection

Human kidney 293T and murine NIH 3T3 cells were maintained in Dulbecco's modified Eagles's medium (DMEM) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and calf serum, respectively. Transfection of DNA plasmids into these cells was carried out using LipofectAMINE Plus, according to manufacturer's protocol. For the transfection into 293T cells, tissue culture plates were coated with poly-D-lysine to prevent cell detachment from the plates, as described before [13].

2.3. In vivo Rho activation assay

In vivo Rho activity was assessed by a modified method described elsewhere [15]. Briefly, 293T cells were transfected with the DNA encoding AU5-tagged wild type of Rho together with the indicated plasmids. After serum starvation for 1 day, cells were lysed at 4°C in a buffer containing 20 mM HEPES, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 10 mM EGTA, 40 mM β-glycerophosphate, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated with a GST fusion protein including the Rho-binding domain (RBD) of rhotekin previously bound to glutathione-Sepharose beads, followed by four washes with lysis buffer. GTP-bound forms of Rho associated with GST-rhotekin-RBD were released from beads by an addition of protein loading buffer, and quantified by Western blot analysis using AU5 antibodies.

2.4. Co-immunoprecipitation and Western blot analysis

293T cells were transfected with vectors expressing AU1-tagged wild type and mutant forms of LARG together with plasmids for the activated forms of $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_q$ or for green fluorescent protein (GFP) as a control. After culture for 48 h, cells were washed twice with phosphate-buffered saline and lysed at 4°C in the lysis buffer used for Rho activation assays. AU1-tagged wild type and mutant forms of LARG were immunoprecipitated from the cleared lysate with the specific antibodies against AU1 (BABCO), and immunocomplexes were recovered with the aid of Gamma-bind Sepharose beads (Pharmacia). Lysates and anti-AU1 immunoprecipitates were analyzed by Western blotting after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to Immobilon-P Transfer Membranes (Amersham Life Science) and immunoblotted with rabbit polyclonal anti-$G\alpha_q$ (Santa Cruz Laboratories) or anti-$G\alpha_{12/13}$ and mouse monoclonal AU1 antibody as indicated in the corresponding figure. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Life Science) using goat anti-rabbit or goat anti-mouse IgGs coupled to horseradish peroxidase as a secondary antibody (Cappel).

2.5. Reporter gene assay

The SRE activity was determined as described previously [13]. Briefly, NIH 3T3 cells were transfected with the indicated plasmids together with pSREmutL, the reporter plasmid expressing a CAT gene under the control of the mutant SRE lacking a TCF-binding site, together with pcDNA3-β-galactosidase, a plasmid encoding the enzyme β-galactosidase. After transfection, cells were cultured for ~24 h in serum free DMEM, stimulated with the indicated agonists for an additional 6 h, when required, then lysed using reporter lysis buffer (Promega). CAT and β-galactosidase activities were measured as described previously [13].

3. Results and discussion

3.1. LARG induces the accumulation of Rho-GTP in vivo

The structure of the predicted LARG protein is highly related to that of PDZ-RhoGEF and a *Drosophila* RhoGEF, DRhoGEF2 [12]. Indeed, these molecules share the presence of a PDZ and a LH/RGS domain in addition to a tandem DH/PH domain (Fig. 1). The PDZ domain is a protein–protein interacting domain, which is thought to participate in the formation of macromolecular complexes [16]. The LH/RGS domain exhibits sequence similarity to GAPs for heterotrimeric G proteins [13,17]. LARG is also related to p115-RhoGEF, which exhibits a LH/RGS domain but not a PDZ domain [18].

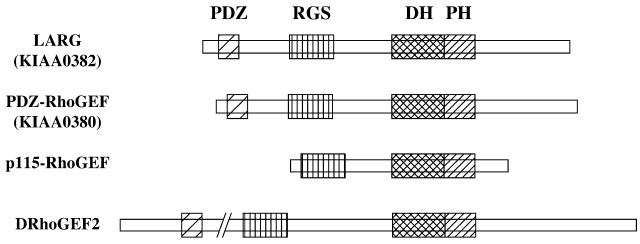

Fig. 1. Structures of RGS domain-containing RhoGEFs. Accession numbers: LARG (KIAA0382), AF180681; PDZ-RhoGEF (KIAA0380), AB002378; p115-RhoGEF, U64105; DRhoGEF2, AF032870.

We first confirmed that the open reading frame of the *LARG* gene expresses a protein of the predicted molecular weight, 173 kDa. As shown in Fig. 2, the epitope-tagged form of LARG was detectably expressed in transiently transfected 293T cells, albeit to a much lower level than that of PDZ-RhoGEF that exhibits a similar molecular weight and was used as a control. Next, we investigated whether LARG can act as a Rho exchange factor by examining its ability to stimulate the exchange of GDP for GTP on Rho using a recently developed in vivo Rho guanine nucleotide exchange assay [15]. For these experiments, 293T cells were transfected with a plasmid encoding AU5-tagged Rho together with expression vectors for the AU1-tagged LARG and AU1-tagged PDZ-RhoGEF or for GFP as a control. Lysates from transfected cells were incubated with a GST fusion protein including the RBD of rhotekin, previously bound to glutathione-Sepharose beads. The levels of GTP-bound form of Rho associated with GST-rhotekin-RBD were quantified by Western blot analysis using anti-AU5 antibodies. As shown in Fig. 2, overexpression of LARG as well as PDZ-RhoGEF effectively increased the amount of GTP-bound form of Rho without causing any changes in the expression levels of AU5-tagged

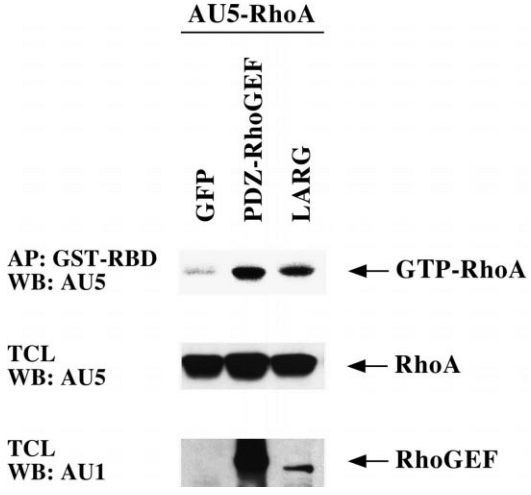

Fig. 2. In vivo activation of Rho by LARG and PDZ-RhoGEF. 293T cells were transfected with a DNA encoding AU5-tagged RhoA together with plasmids for AU1-tagged LARG and PDZ-RhoGEF or for GFP as a control. The level of GTP-bound form of Rho in vivo was determined as described under Section 2.

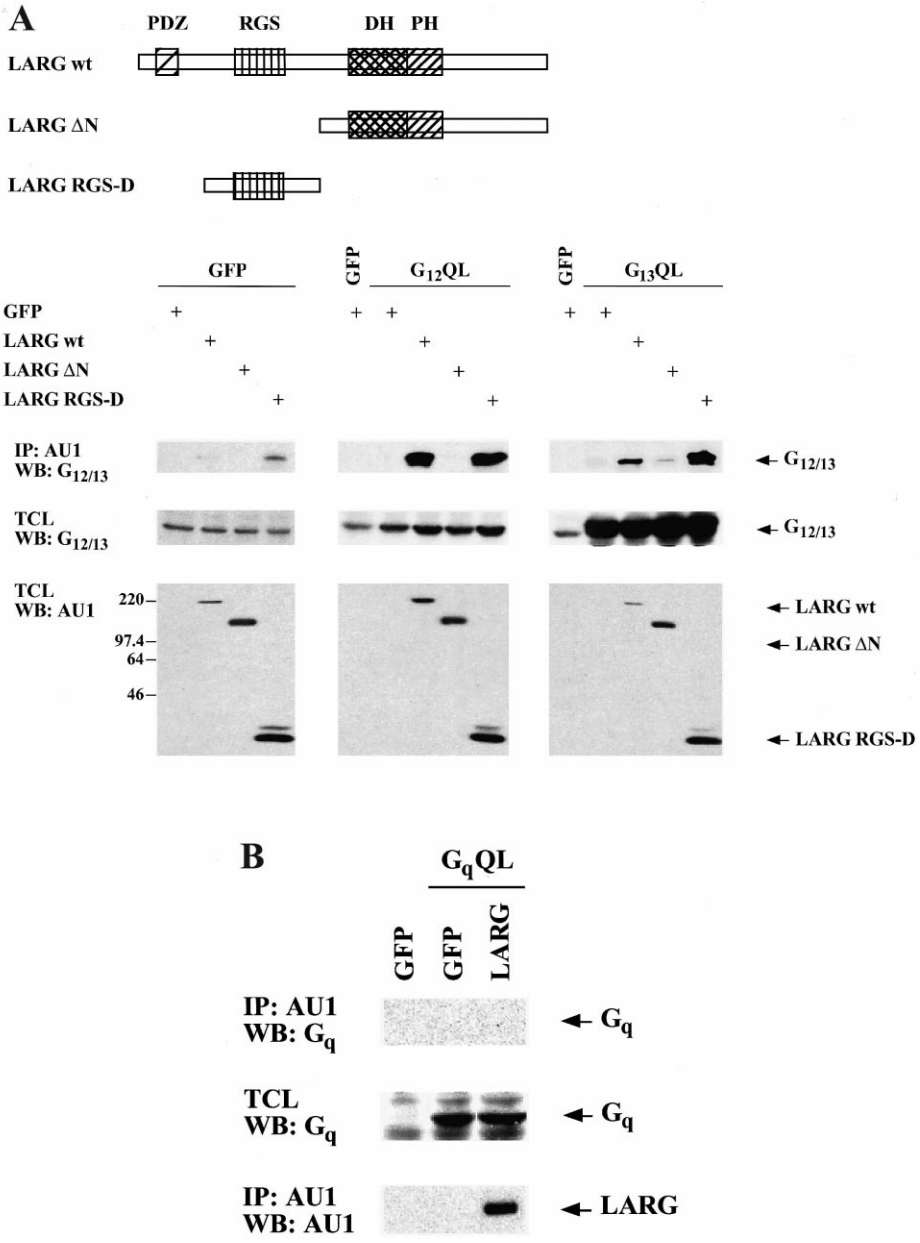

Fig. 3. Interaction of LARG through its LH/RGS domain with heterotrimeric G protein α subunit of the G_{12} family. 293T cells were transfected with expression vector for AU1-tagged wild type and mutant forms of LARG, whose structures are depicted in the upper panel, together with plasmids carrying cDNAs for the constitutively activated mutants of (A) $G\alpha_{12}$, $G\alpha_{13}$ and (B) $G\alpha_q$ as indicated. Lysates, prepared as described under Section 2, were immunoprecipitated (IP) with anti-AU1 antibody and subjected to Western blot (WB) analysis using rabbit polyclonal antibodies against $G\alpha_{12/13}$ or $G\alpha_q$ (A and B, respectively). In addition, total cellular lysate (TCL) was subjected to Western blot (WB) analysis with G protein-specific antisera and anti-AU1 antibodies as indicated.

Rho, thus indicating that LARG and PDZ-RhoGEF are able to act as RhoGEFs in vivo.

3.2. *LARG associates with $G\alpha_{12}$ and $G\alpha_{13}$ heterotrimeric G protein α subunits*

GPCRs are key upstream regulators of Rho GTPases [19]. Furthermore, work from several laboratories indicates that heterotrimeric G proteins of the G_{12} family, G_{12} and G_{13}, utilize Rho to induce the formation of actin-based stress fibers [20], gene expression driven by serum response element [21,22], and cellular transformation [21]. However, there is still limited information on how G proteins and their coupled receptors stimulate Rho. In this regard, it has recently been shown that PDZ-RhoGEF and p115-RhoGEF can provide a direct link between the $G\alpha_{12}$ family of heterotrimeric G protein α subunits and Rho. In addition, the $G\alpha_q$ family of G proteins has also been reported to activate Rho-dependent signaling pathways [21,22]. However, how $G\alpha_q$ communicates to Rho is still completely unknown. Thus, the observation that LARG exhibits a LH/RGS domain raised the possibility that this RhoGEF may link these heterotrimeric G proteins to Rho. To address this possibility, we investigated the ability of LARG to associate with $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_q$ upon transfection of 293T cells with a DNA vector encoding AU1-tagged wild type LARG together with plasmids for GFP (as a control) and activated forms of $G\alpha_{12}$ ($G\alpha_{12}$QL), $G\alpha_{13}$

($G\alpha_{13}$QL) and $G\alpha_q$ ($G\alpha_q$QL). As shown in Fig. 3A,B, LARG co-immunoprecipitated with $G\alpha_{12}$QL and $G\alpha_{13}$QL, but not $G\alpha_q$QL. Similarly, we did not detect any specific association of LARG to $G\alpha_s$, $G\alpha_i$, or βγ subunits (not shown). Furthermore, as depicted in Fig. 3A (upper panel), we prepared DNA constructs encoding an N-terminal deletion mutant of LARG, lacking its RGS domain, and an epitope-tagged RGS domain alone. Each protein was detectably expressed, as judged by Western blot analysis using anti-epitope antibodies (Fig. 3A, lower panel). As shown in Fig. 3A, the protein encoding the RGS domain alone associated with $G\alpha_{12}$QL and $G\alpha_{13}$QL efficiently, whereas the LARG N-terminal deletion mutant, lacking its LH/RGS domain, could not. These results indicate that LARG associates physically with $G\alpha_{12}$ and $G\alpha_{13}$, and that the LH/RGS domain is the molecular determinant of its ability to form stable complexes with these G protein α subunits.

3.3. The RGS/LH domain of LARG diminishes the SRE response elicited by $G\alpha_{12}$ and $G\alpha_{13}$ but not $G\alpha_q$

These results and recent published reports suggest that $G\alpha_{12}$ proteins may signal to Rho through LH/RGS domain-containing RhoGEFs [13,23]. To explore this possibility, we examined the effect of expression of LH/RGS domains derived from LARG and PDZ-RhoGEF on SRE activation induced by the constitutively active mutants of $G\alpha_{12}$, $G\alpha_{13}$ and $G\alpha_q$, as these RGS domains might act as a dominant negative molecules. As shown in Fig. 4, the induction of SRE by $G\alpha_q$QL was unaffected by the expression of the LH/RGS domains of PDZ-RhoGEF and LARG. In contrast, these RGS domains significantly inhibited the SRE activation induced by the expression of $G\alpha_{12}$QL and $G\alpha_{13}$QL.

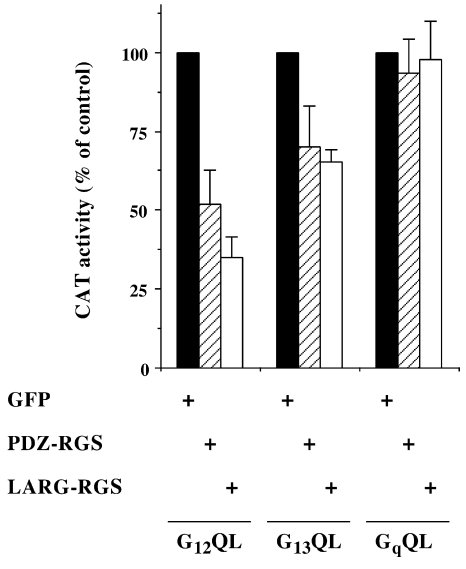

Fig. 4. Inhibition of $G\alpha_{12}$- and $G\alpha_{13}$-mediated SRE activation by the RGS domain of LARG and PDZ-RhoGEF. NIH 3T3 cells were co-transfected with pSREmutL and pCDNA3-β-gal together with the indicated expression vectors. Cells were processed as described under Section 2. The data represent CAT activity normalized by the β-galactosidase activity present in each cellular lysate, expressed as a percentage relative to that observed in control, and are the mean ± S.E.M. of triplicate samples from a typical experiment. Similar results were obtained in three independent experiments.

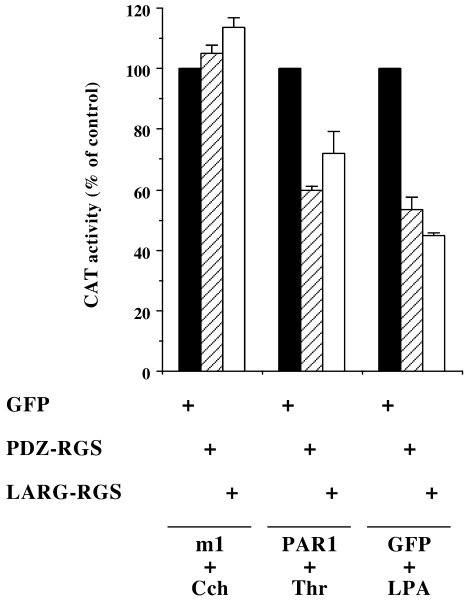

Fig. 5. Inhibition of GPCR-mediated SRE activation by the RGS domain of LARG and PDZ-RhoGEF. NIH 3T3 cells were co-transfected with pSREmutL and pCDNA3-β-gal together with the expression vectors for m1 and PAR1 receptors or GFP (for LPA stimulation) as well as plasmids expressing RGS domains of LARG and PDZ-RhoGEF. Cells were stimulated with 100 μM carbachol (Cch) for m1-transfected cells, 10 U/ml thrombin (Thr) for PAR1-transfected cells, and 5 μM LPA for GFP-transfected cells for 6 h, and were processed as described under Section 2. The data represent CAT activity normalized by the β-galactosidase activity present in each cellular lysate, expressed as a percentage relative to that observed in control, and are the mean ± S.E.M. of triplicate samples from a typical experiment. Similar results were obtained in three independent experiments.

3.4. Evidence for a role of LARG downstream from LPA and thrombin receptors

Next, we asked whether LARG might play a role in signaling from GPCRs to Rho. In this regard, thrombin and LPA receptors are able to couple to the $G\alpha_{12}$ family of heterotrimeric G proteins [19], whereas m1 muscarinic acetylcholine receptors represent typical $G\alpha_q$-coupled receptors. Stimulation of both classes of GPCRs is known to induce Rho-dependent signaling pathways [21,22]. Thus, we investigated the ability of the RGS domains of LARG and PDZ-RhoGEF to interfere with the activation of the SRE when induced by these GPCRs. As shown in Fig. 5, the expression of the RGS domains of LARG and PDZ-RhoGEF inhibited the activation of SRE induced by thrombin and LPA receptors. In contrast, the transcriptional response elicited by m1 receptors was unaffected. Taken together, these results suggest that LARG and PDZ-RhoGEF can mediate in signaling from G_{12}- and G_{13}-coupled receptors to Rho, whereas additional molecular mechanisms may link G_q-coupled receptors to Rho GTPases (Fig. 6).

Whether the MLL–LARG fusion protein exhibits also RhoGEF activity is at present unknown. However, this fusion protein includes the majority of the LARG coding sequence but its PDZ domain [12]. Thus, it would be expected to retain its ability to stimulate Rho proteins. In this regard, LARG is likely to function as a Rho-specific GEF, as it failed to induce MAPK and JNK, typical responses elicited by Ras and Rac/Cdc42, respectively, when overexpressed in a number of cel-

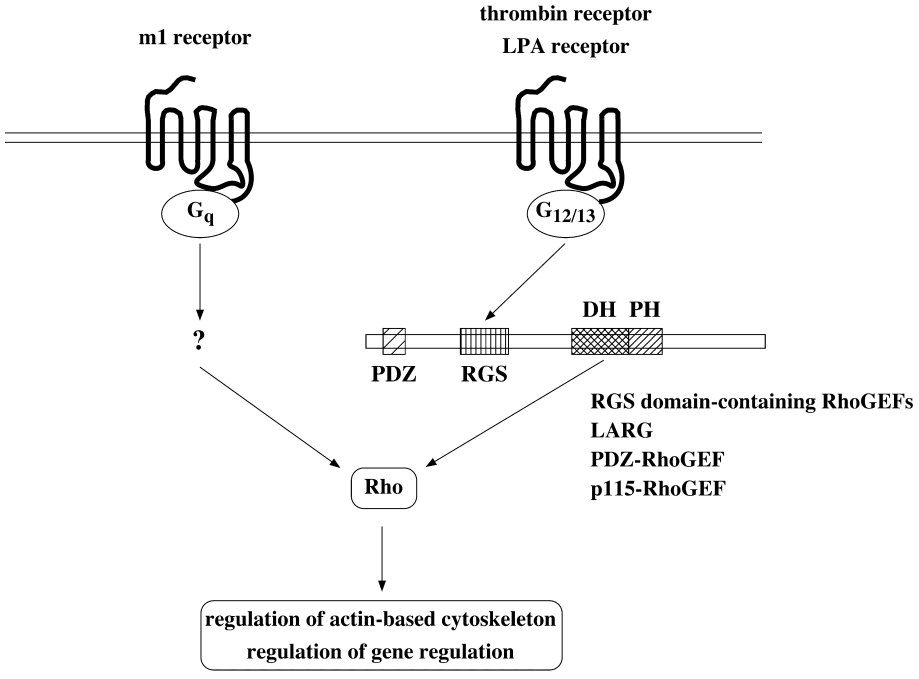

Fig. 6. Proposed mechanism whereby GPCRs stimulate Rho-dependent signaling pathways. The stimulation of certain GPCRs results in the activation of $G\alpha_{12}$ and $G\alpha_{13}$ which, in turn, interact with LH/RGS domain-containing RhoGEFs such as LARG, PDZ-RhoGEF and p115-RhoGEF through their LH/RGS domains, thereby causing their activation. Subsequently, the activated RGS domain-containing RhoGEFs catalyze the exchange of GDP for GTP on Rho through their DH/PH domain, which leads to the activation of Rho-dependent signaling pathways. G_q-coupled receptors also stimulate Rho-mediated signaling pathways, albeit by a still poorly understood mechanism.

lular systems (data not shown). Furthermore, Rho and molecules acting upstream from Rho, such as $G\alpha_{12}/G\alpha_{13}$ and certain GPCRs, can harbor transforming potential [24,25]. Thus, these observations raise the possibility that aberrant expression of the MLL–LARG protein or signaling molecules acting on Rho pathways may contribute to the development of acute myelogenous leukemia, a possibility that warrants further investigation.

The striking structural similarity to PDZ-RhoGEF and to the *Drosophila* DRhoGEF2 suggests that LARG and PDZ-RhoGEF represent the mammalian homologs of DRhoGEF2. Of interest, genetic analysis suggests that DRhoGEF2 acts downstream of the *concertina* gene, a *Drosophila* $G\alpha_{12/13}$ homolog involved in gastrulation during early embryogenesis [26,27]. Thus, the GPCR-$G\alpha_{12/13}$-LARG/PDZ-RhoGEF-Rho pathway may represent a signaling route highly conserved throughout evolution. On the other hand, whether LARG and PDZ-RhoGEF perform similar or distinct functions is at the present unclear. Both RhoGEFs are ubiquitously expressed and exhibit similar structural features. However, their C-terminal domain is highly divergent [12], which may result in distinct biological activities. We expect that ongoing efforts aimed to disrupt the PDZ-RhOGEF and LARG genes will soon provide a wealth of information on their unique or mutually redundant functions.

Taken together, we can conclude that LARG represents a novel RhoGEF whose aberrant expression as a fusion protein to MLL may play a role in human acute myeloid leukemia. At the molecular level, LARG can associate physically with $G\alpha_{12}$ and $G\alpha_{13}$ through its LH/RGS domain and stimulate the accumulation of GTP-bound Rho, likely through its DH/PH domain. Furthermore, available evidence suggests that LARG is an integral component of a novel signaling route by which the large family of G protein-linked receptors coupled to G_{12}/G_{13} can activate Rho, thereby initiating the activity of multiple intracellular signaling pathways dependent on Rho GTPases.

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