Activated $G\alpha_{13}$ Impairs Cell Invasiveness through p190RhoGAP-Mediated Inhibition of RhoA Activity

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Abstract

The GTPase RhoA is a downstream target of heterotrimeric G_{13} proteins and plays key roles in cell migration and invasion. Here, we show that expression in human melanoma cells of a constitutively active, GTPase-deficient $G\alpha_{13}$ form ($G\alpha_{13}$QL) or lysophosphatidylcholine (LPC)-promoted signaling through $G\alpha_{13}$-coupled receptors led to a blockade of chemokine-stimulated RhoA activation and cell invasion that was rescued by active RhoA. Melanoma cells expressing $G\alpha_{13}$QL or cells stimulated with LPC displayed an increase in p190RhoGAP activation, and defects in RhoA activation and invasion were recovered by knocking down p190RhoGAP expression, thus identifying this GTPase-activating protein (GAP) protein as a downstream $G\alpha_{13}$ target that is responsible for these inhibitory responses. In addition, defective stress fiber assembly and reduced migration speed underlay inefficient invasion of $G\alpha_{13}$QL melanoma cells. Importantly, $G\alpha_{13}$QL expression in melanoma cells led to impairment in lung metastasis associated with prolonged survival in SCID mice. The data indicate that $G\alpha_{13}$-dependent downstream effects on RhoA activation and invasion tightly depend on cell type–specific GAP activities and that $G\alpha_{13}$-p190RhoGAP signaling might represent a potential target for intervention in melanoma metastasis. [Cancer Res 2008;68(20):8221–30]

Introduction

Rho GTPases control the dynamics of the actin cytoskeleton during cell migration, contributing to the development of cell protrusions and adhesion at the leading edge and to retraction at the cell rear (1, 2). Their activity is tightly regulated by guanine-nucleotide exchange factors (GEF), which stimulate exchange of bound GDP by GTP, and inhibited by GTPase-activating proteins (GAP), which promote GTP hydrolysis (3, 4). Therefore, cell migration is finely regulated by the balance between GEF and GAP activities on Rho GTPases.

Activation of cell migration machinery is required at different steps of metastasis, such as in invasion across basement membranes and interstitial tissues, intravasation into blood or lymphatic circulation, and extravasation and invasion through subendothelial basement membranes for colonization of distant organs (5, 6). Involvement of Rho GTPases in cancer is well documented (reviewed in ref. 7), providing control of both cell migration and growth. RhoA and RhoC are highly expressed in colon, breast, and lung carcinomas (8, 9), whereas overexpression of RhoC in melanoma leads to enhancement of cell metastasis (10).

Chemokines are chemotactic cytokines which stimulate cell migration and activation and exert their functions upon binding to heterotrimeric guanine nucleotide-binding (G) protein-coupled receptors (GPCR; refs. 11, 12). Activation of Rho GTPases represents a target of chemokine signaling, providing cell adhesion and directionality during migration. Most solid cancer cells express CXCR4, a receptor for the chemokine CXCL12 (also called SDF-1), which is expressed in lungs, bone marrow, and liver (13). Similarly to the key role of chemokines for the homing of immune cells during immune surveillance, chemokines contribute to tumor cell trafficking and colonization of organs during metastasis (14, 15).

CXCR4 is expressed on melanoma, a highly aggressive cancer when metastasis starts (16), providing activation of the cell invasive machinery upon interaction with CXCL12 (13, 17–19). CXCL12 stimulates RhoA activation on melanoma cells, and expression of a mutant dominant-negative (DN) form of Rho abolishes chemokine-promoted invasion (18). In addition, it was shown that the GEF Vav2 is an upstream molecule regulating CXCL12-mediated RhoA activation in these cells (20).

Interaction of chemokine receptor with their agonists elicits different cellular responses, which depend on initial receptor-heterotrimeric G-protein binding (11). Heterotrimeric G proteins consist of a α subunit and a complex formed by β and γ subunits (21, 22). Basally, $G\beta\gamma$ and GDP-bound $G\alpha$ are associated, and upon interaction with an activated receptor, GTP replaces GDP and GTP-$G\alpha$ dissociates from the $G\beta\gamma$-dimer. These two elements interact with effector proteins leading to the activation of distinct signaling pathways. The GTPase activity inherent to $G\alpha$ limits G-protein activation, as GTP hydrolysis causes reassociation of GDP-$G\alpha$ and $G\beta\gamma$. G proteins are classified into four subfamilies, G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$, according to the $G\alpha$ protein present in the complex (21, 22).

In addition to its well-known role on cell growth and transformation, $G_{12/13}$ proteins also regulate RhoA activation and cell migration (23). Thus, expression of constitutively active (CA) mutant forms of $G\alpha_{12}$ and $G\alpha_{13}$, which lack GTPase activity due to substitution of a glutamine (Q231 or Q226) to a leucine residue, stimulates Rho-dependent stress fiber and focal adhesion assembly (24, 25). In prostate and breast cancer, a key role for $G\alpha_{12/13}$ activation during tumor cell motility has been proposed (26, 27), as it was shown that G_{12} signaling promotes Rho-mediated cell invasion *in vitro*.

In the present work, we studied how $G\alpha_{13}$ activation controls chemokine-stimulated human melanoma cell invasion and metastasis. Interestingly, we found that expression of a GTPase-deficient $G\alpha_{13}$ form ($G\alpha_{13}$QL) impaired melanoma cell invasion and *in vivo*

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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metastasis that was associated with inhibition of RhoA activation. We provide here mechanistic characterization of the molecular components involved in these unexpected effects. The data suggest that cancer cell–type specific differences might underlie distinct $G\alpha_{13}$-dependent activation of signaling pathways leading to different biological responses.

Materials and Methods

Cells, antibodies, and reagents. The human melanoma cell lines BLM (28) and MeWo were cultured as reported (18). MDA-MB-231 human breast cancer and mouse fibroblastic Swiss 3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (BioWhittaker). Anti-$G\alpha_{13}$, anti-$G\alpha_{12}$, anti-RhoA, and anti-phosphotyrosine antibodies were purchased from Santa Cruz Biotechnology, anti-green fluorescent protein (GFP) from Molecular Probes, antibodies to paxillin from BD Biosciences, anti–β-actin from Sigma-Aldrich, and anti-p190RhoGAP from Upstate. Control P3X63 monoclonal antibody (mAb) was from Dr. Francisco Sánchez-Madrid (Hospital de la Princesa). Anti-CXCR4 mAb and CXCL12 were obtained from R&D Systems. Lysophosphatidylcholine (LPC) was from Sigma-Aldrich, U46619 from Alexis Biochemicals, and PP2 and PP3 from Calbiochem-Novabiochem Co.

Vectors, RNA interference, and transfections. Vectors coding for GFP-fused forms of wild-type $G\alpha_{13}$ ($G\alpha_{13}$wt) or $G\alpha_{13}$QL were generated by subcloning $G\alpha_{13}$ cDNAs (gifts from Dr. Piero Crespo, Universidad de Cantabria, and Dr. Silvio Gutkind, NIH) into pEGFP-C1 (Clontech). Vectors coding for $G\alpha_{13}$wt or $G\alpha_{12}$QL were purchased from UMR cDNA. Vectors coding for GFP-fused forms of wild-type RhoA and Rac1, DN N19-RhoA, and activated V14-RhoA and V12-Rac1 were gifts from Dr. Francisco Sánchez-Madrid. For small interfering RNA (siRNA), we used a control siRNA duplex (29) and designed three target-specific siRNA duplexes against $G\alpha_{13}$ mRNA. Sense strands were $G\alpha_{13}$ (1) 5′-GGCAUCCAUGAAUACGACUdTdT-3′, targets bases 610-630; $G\alpha_{13}$ (2) 5′-CUUUAUGGUGACCUCCUAUdTdT-3′, targets bases 1575-1595; and $G\alpha_{13}$ (3) 5′-GUUCAUGGUAUCGUGCAUGdTdT-3′, targets bases 2894-2914. In addition, we used two siRNAs against p190RhoGAP, p190A (1) and p190A (2), siGENOME duplex D-004158-03 and D-004158-04, respectively (Dharmacon). Cells were transiently transfected with expression vectors or siRNA (100 nmol/L) using Lipofectamine (Invitrogen Corp.) or X-tremeGENE (Roche Diagnostics), respectively, according to manufacturer's instructions. Transfectants were tested in the different assays 48 h posttransfection.

Invasion assays. Invasions were done as earlier reported (18). Briefly, cells were loaded on the upper compartments of invasion chambers coated with Matrigel (BD Biosciences). The lower compartments were filled with invasion medium with or without CXCL12. Invasive cells were fixed, stained, and counted under a microscope.

Immunoprecipitation, Western blotting, and GTPase assays. For immunoprecipitation, melanoma cells were lysed (30) and extracts were incubated with antibodies followed by specific coupling to protein A-Sepharose beads (Amersham Pharmacia Biotech). Proteins were eluted in Laemmli buffer, resolved by SDS-PAGE, and subjected to Western blotting with primary antibodies, followed by incubation with horseradish peroxidase–conjugated secondary antibodies and detection with Super-Signal chemiluminiscent substrate (Pierce). GTPase activity assays were performed, as previously reported (18). In brief, cells were incubated with or without CXCL12, and upon cell lysis, aliquots from extracts were kept for total lysate controls and the remaining volume incubated with GST-C21 (for RhoA) or GST-PAK-CD (for Rac1) fusion proteins (31) in the presence of glutathione-agarose beads. After elution of bound proteins, they were subjected to Western blotting with anti-RhoA or anti-Rac1 antibodies.

Retroviral gene transfer and animal studies. $G\alpha_{13}$wt or $G\alpha_{13}$QL cDNAs were cloned into pRETRO-bsd vector (gift from Dr. Reuven Agami, National Cancer Institute, Amsterdam, the Netherlands). Vectors were cotransfected by Lipofectamine with pNGVL-VSV-G and pNGVL-gag-pol vectors (gifts from Dr. Rafael Delgado, Hospital 12 de Octubre) into 293FT packaging cells. After 48 h, conditioned medium containing viral particles was used to infect BLM cells, which were selected with blasticidin (Invitrogen Corp.) for 3 wk. For xenografting studies, 6-wk to 9-wk sex-matched BALB/c SCID mice (Harlan), bred and maintained under specific pathogen-free conditions at the Centro de Investigaciones Biológicas Animal Facility, were injected s.c. in the lateral thoracic wall or i.v. into the tail vein with 1×10^6 cells in 0.2 mL PBS. The Consejo Superior de Investigaciones Científicas Ethics Committee approved the protocols used for experiments with mice. Mice were inspected on a daily basis for local tumor growth and general condition and were killed when signs of respiratory stress were noted or when s.c. tumors reached a volume of 2.5 cm^3.

Statistical analyses. Data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons. In both analyses, the minimum acceptable level of significance was $P < 0.05$.

Results

Expression of $G\alpha_{13}$QL in melanoma leads to inhibition of chemokine-promoted cell invasion and RhoA activation. The chemokine CXCL12 activates RhoA in the highly aggressive BLM human melanoma cell line, and expression of DN Rho results in inhibition of invasion toward the chemokine (18). As Rho constitutes a well-known downstream effector of $G\alpha_{13}$ (23), we expressed in BLM cells the $G\alpha_{13}$ Q226L ($G\alpha_{13}$QL) mutant, which leads to a CA GTPase-deficient form of $G\alpha_{13}$ in fibroblasts (24), and tested cell invasion and RhoA activation in response to CXCL12. $G\alpha_{13}$QL BLM transfectants displayed a large inhibition of Matrigel invasion toward the chemokine compared with $G\alpha_{13}$wt or mock cell invasion (Fig. 1*A*). Expression of endogenous, as well as GFP-fused forms of $G\alpha_{13}$, was monitored by Western blotting (Supplementary Fig. S1*A, left*), showing similar expression levels of $G\alpha_{13}$wt and $G\alpha_{13}$QL species, and control experiments revealed that their expression did not affect cell viability (Supplementary Fig. S1*B*). Notably, whereas RhoA was activated by CXCL12 in mock and $G\alpha_{13}$wt cells (7-fold to 8-fold), activation was deficient in $G\alpha_{13}$QL melanoma transfectants (Fig. 1*B*). Instead, all transfectants showed similar levels of chemokine-promoted activation of Rac1 (Supplementary Fig. S1*C*), another RhoGTPase that is involved in ruffle and lamelipodia formation and which controls cell migration. Moreover, expression in BLM cells of $G\alpha_{12}$QL similarly caused defective RhoA activation compared with mock and $G\alpha_{13}$wt transfectants (Fig. 1*C*). We also found that impairment in RhoA activation on $G\alpha_{13}$QL transfectants was associated with inhibition in chemokine-promoted spreading on fibronectin (Supplementary Fig. S1*D*).

Consistent with previous results (24, 27), $G\alpha_{13}$QL expression in mouse fibroblastic Swiss 3T3 and human MDA-MB-231 breast carcinoma cells (Supplementary Fig. S1*A, right*) led to a robust induction in basal RhoA activation compared with $G\alpha_{13}$wt transfectants (4-fold to 6-fold; Fig. 1*D*), and activation was further increased by CXCL12 in MDA-MB-231 cells. In contrast, no RhoA activation was detected on MeWo melanoma cells expressing $G\alpha_{13}$QL.

To confirm that inhibition of melanoma cell invasion and RhoA activation could be directly attributed to $G\alpha_{13}$QL, we transfected $G\alpha_{13}$ siRNA to silence $G\alpha_{13}$ expression. $G\alpha_{13}$ siRNA (3) inhibited by >80% the expression of endogenous $G\alpha_{13}$, whereas $G\alpha_{13}$ siRNA (1) and (2) did not affect its expression (Fig. 2*A, top*). Importantly, $G\alpha_{13}$ (3) siRNA knocked down the expression of $G\alpha_{13}$QL in addition of the endogenous counterpart (Fig. 2*A, bottom*), and it reverted $G\alpha_{13}$QL-dependent inhibition of invasion in cotransfection experiments (Fig. 2*B*). Moreover, $G\alpha_{13}$ silencing rescued CXCL12-triggered RhoA activation that is impaired in $G\alpha_{13}$QL transfectants,

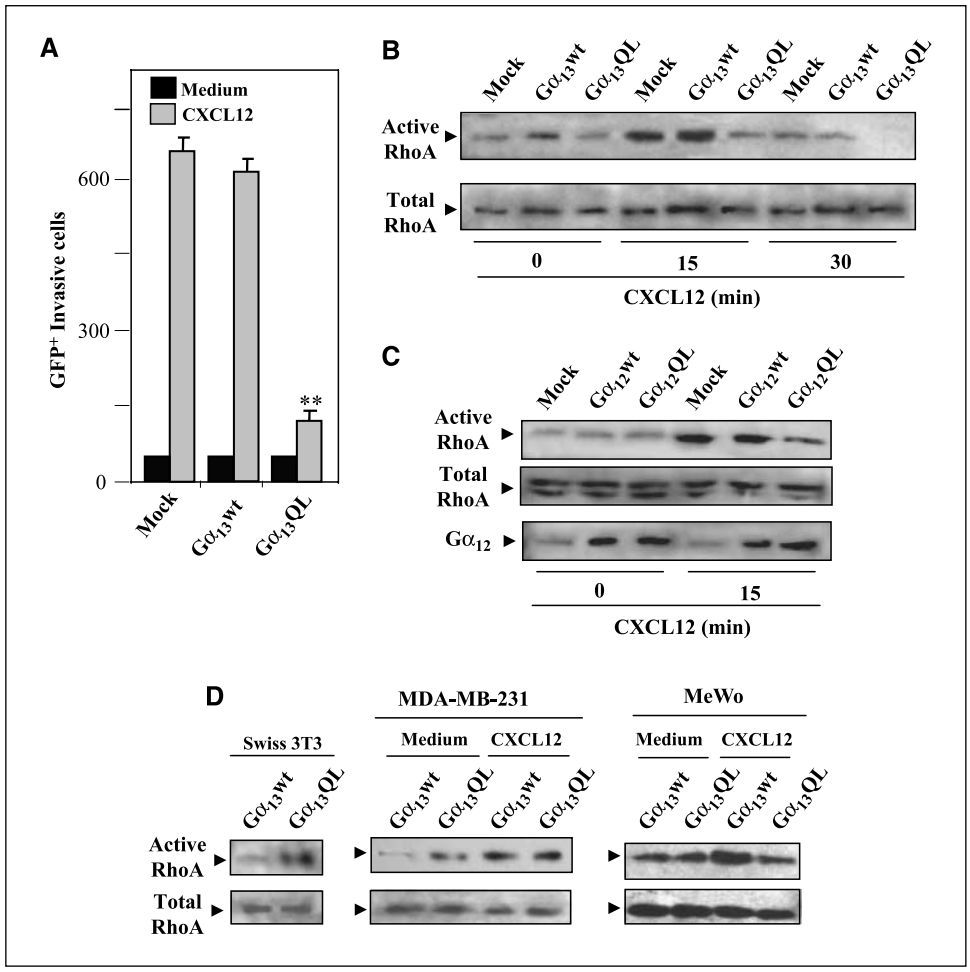

Figure 1. Expression of $G\alpha_{13}QL$ in melanoma leads to defective CXCL12-promoted cell invasion and impaired RhoA activation. *A,* BLM cells were transfected with GFP-fused forms of $G\alpha_{13}wt$, $G\alpha_{13}QL$, or GFP vector alone (mock), and transfectants were subjected to Matrigel invasion assays toward CXCL12 or medium alone. Invasion data represent the mean ± SD of three independent experiments done in duplicate. **, invasion was significantly inhibited, $P < 0.01$. *B* and *C,* mock, $G\alpha_{13}$, or $G\alpha_{12}$ BLM transfectants incubated for the indicated times with CXCL12 (150 ng/mL) were subjected to GTPase assays to detect active RhoA. $G\alpha_{12}$ expression was analyzed by immunobloting with anti-$G\alpha_{12}$ antibodies. *D,* cells were transfected as in *A* and incubated with medium alone (Swiss 3T3) or with medium in the absence or presence of CXCL12 (MDA-MB-231 and MeWo). Transfectants were then subjected to GTPase assays to detect active RhoA.

without affecting Rac activation (Fig. 2*C*), suggesting that RhoA-dependent invasion is interfered by $G\alpha_{13}QL$ expression. To prove this hypothesis, we cotransfected $G\alpha_{13}QL$, $G\alpha_{13}wt$, or empty vector with wild-type, DN, or CA forms of RhoA and checked transfectant invasion to CXCL12. Results showed that $G\alpha_{13}QL$-dependent defective invasion was recovered when Rho CA, but not Rac CA, forms were cotransfected (Fig. 2*D* and Supplementary Fig. S2*A* and *B*). In addition, inhibition of melanoma cell invasion by Rho DN expression in $G\alpha_{13}wt$ and mock transfectants reached levels similar to those attained by $G\alpha_{13}QL$ counterparts, again pointing to the inhibitory action of $G\alpha_{13}QL$ on RhoA activation. These data indicate that inefficient $G\alpha_{13}QL$ transfectant invasion is a direct consequence of inhibition of RhoA activation.

The results from Fig. 2*B* already suggested that chemokine-promoted melanoma cell invasion might be independent of endogenous $G\alpha_{13}$ function. Both $G\alpha_i$ and $G\alpha_{13}$ associated to CXCR4 in BLM cells, and exposure to CXCL12 transiently increased this association (Supplementary Fig. S3*A*). However, CXCL12-stimulated cell invasion was not affected by silencing the endogenous $G\alpha_{13}$ expression (Supplementary Fig. S3*B*). Activation of heterotrimeric G proteins leads to separation of Gα subunits from Gβγ dimers, which are both capable of downstream signaling (21, 22). Pertussis toxin (PTx) prevents this dissociation, inhibiting $G\alpha_i$-triggered and Gβγ-triggered signaling. Abolishment by PTx of BLM cell invasion induced by CXCL12 correlated with a large inhibition of RhoA activation in PTx-treated cells (Supplementary Fig. S3*C*). In addition, BLM transfection, either with a vector encoding human transducin $G\alpha_t$ subunit, a protein that associates with Gβγdimers (32), or with a vector coding for the carboxy terminus of GPCR kinase (GRK2-CT; ref. 33), both treatments causing suppression of Gβγ-dependent responses, did not alter cell invasion toward CXCL12 (Supplementary Fig. S3*D*). These data show that chemokine-stimulated melanoma cell invasion involving RhoA activation predominantly depends on $G\alpha_i$-mediated responses and that endogenous $G\alpha_{13}$ or Gβγ do not play relevant roles in this process. Furthermore, they indicate that $G\alpha_{13}QL$ actions functionally oppose to CXCL12-promoted, $G\alpha_i$-dependent signaling.

p190RhoGAP mediates $G\alpha_{13}QL$-dependent inhibition of RhoA activation and melanoma cell invasion. GAP proteins stimulate the slow intrinsic rate of GTP hydrolysis of Rho GTPases, leading to Rho GTPase inactivation. A candidate molecule mediating $G\alpha_{13}QL$-dependent inhibition of RhoA activation is p190RhoGAP, a protein containing a GAP domain on its COOH terminal region (34, 35), and shows preferential activity on RhoA after its phosphorylation on Y1105 (36). We found that p190RhoGAP is expressed in BLM melanoma cells and that CXCL12 stimulates its tyrosine phosphorylation (Fig. 3*A, left*). Furthermore, $G\alpha_{13}QL$ BLM transfectants displayed higher p190RhoGAP phosphorylation and increased binding to RhoA than $G\alpha_{13}wt$ and mock counterparts, both in the absence or presence of CXCL12 (Fig. 3*A, right*). Importantly, p190RhoGAP silencing restored the defective

$G\alpha_{13}$QL cell invasion toward CXCL12, without affecting invasion of $G\alpha_{13}$wt and mock transfectants, and this effect was associated with recovery of RhoA activation by CXCL12 in $G\alpha_{13}$QL cells (Fig. 3*B, top* and *bottom,* and 3*C, top* and *bottom*). Therefore, these results identify p190RhoGAP activation as a mechanism responsible for impaired RhoA activation and invasion promoted by CXCL12 in $G\alpha_{13}$QL transfectants. As Src proteins are involved in p190RhoGAP tyrosine phosphorylation (37, 38), we addressed whether they might mediate $G\alpha_{13}$QL-dependent phosphorylation of the GAP protein by using the PP2 Src inhibitor. Treatment of $G\alpha_{13}$QL transfectants with PP2 abolished p190RhoGAP tyrosine phosphorylation in comparison with cells treated with the control nonblocking PP3 reagent (Fig. 3*D*), suggesting that Src proteins are highly candidates to mediate $G\alpha_{13}$QL-promoted p190RhoGAP phosphorylation in melanoma cells.

The Vav proteins are GEF members whose phosphorylation is required for their GEF activity toward Rho GTPases (39). As Vav2 is needed for chemokine-stimulated RhoA activation and BLM cell invasion (20), we tested whether its activation was altered in $G\alpha_{13}$QL cells. We found that CXCL12-stimulated Vav2 phosphorylation was comparable in the three transfectants and that similar amounts of RhoA were recovered in anti-Vav2 immunoprecipitates from cell lysates after exposure to CXCL12 (Supplementary Fig. S4). These results indicate that chemokine-promoted Vav2 activation and association with RhoA is not altered in $G\alpha_{13}$QL melanoma transfectants.

LPC inhibits chemokine-stimulated melanoma cell invasion and RhoA activation through $G\alpha_{13}$-p190RhoGAP activation. We next investigated whether extracellular ligands that activate $G\alpha_{13}$ upon interaction with their receptors could mimic the

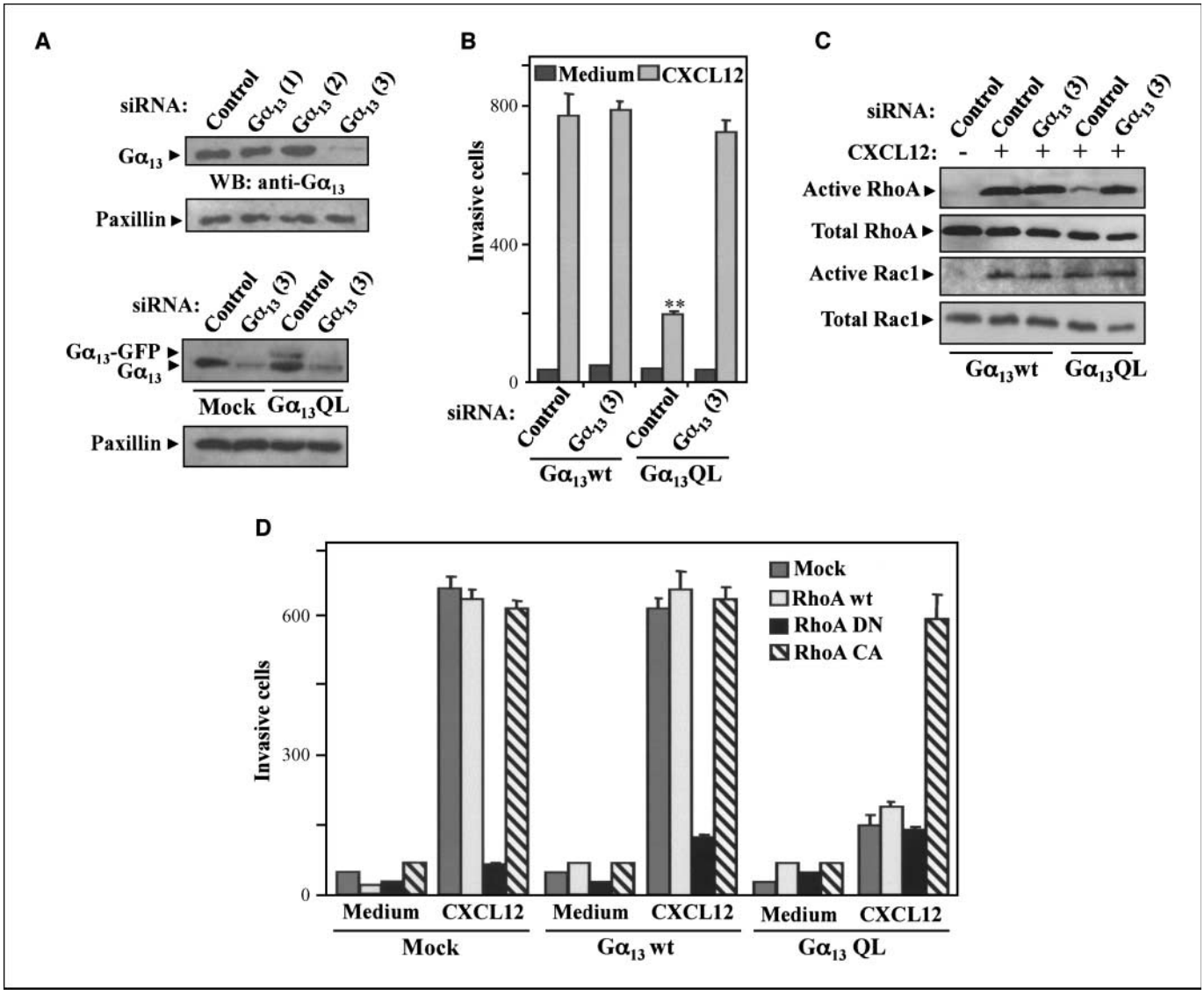

Figure 2. $G\alpha_{13}$QL-dependent inhibition of melanoma cell invasion and RhoA activation is rescued by silencing $G\alpha_{13}$QL or by expression of CA RhoA. *A, top,* immunoblotting of BLM cells transfected with control or the indicated siRNA for $G\alpha_{13}$. Control loading was assessed with anti-paxillin antibodies (*bottom*). Empty GFP vector (mock) or $G\alpha_{13}$QL were cotransfected together with control or $G\alpha_{13}$ siRNA, and cell lysates were subjected to Western blotting with anti-$G\alpha_{13}$ antibodies. *B,* BLM cells cotransfected with $G\alpha_{13}$wt or $G\alpha_{13}$QL vectors and control or $G\alpha_{13}$ siRNA were analyzed in Matrigel invasion. **, invasion was significantly inhibited, $P < 0.01$ ($n = 3$). *C,* same transfectants were tested in GTPase assays to detect RhoA or Rac1 activation. *D,* BLM cells were transfected with empty GFP (mock), $G\alpha_{13}$wt, or $G\alpha_{13}$QL vectors, together with wild-type, DN, or CA forms of GFP-fused RhoA, and transfectants were subjected to invasion assays. Invasion data represent the mean ± SD of two independent experiments done in duplicate.

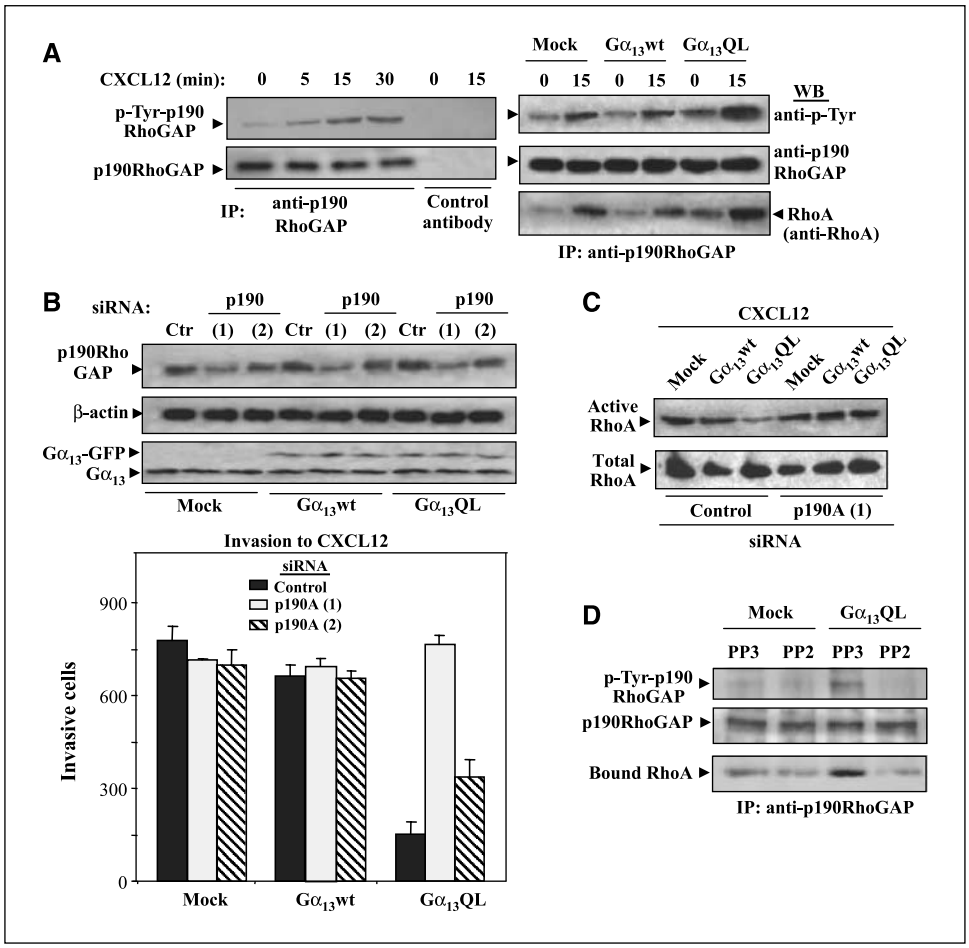

Figure 3. $G\alpha_{13}$QL-dependent inhibition of melanoma cell invasion and RhoA activation is mediated by p190RhoGAP. *A,* BLM cells incubated for the indicated times with CXCL12 were subjected to immunoprecipitation with control or anti-p190RhoGAP antibodies, followed by Western blotting with the antibodies shown. *B,* cells were transfected with GFP, $G\alpha_{13}$wt, or $G\alpha_{13}$QL vectors together with control (*Ctr*) or with the indicated siRNA for p190RhoGAP [p190A (1) and (2)]. Transfectant lysates were subjected to immunoblotting with anti-p190RhoGAP or anti-$G\alpha_{13}$ antibodies or with anti–β-actin antibodies for loading controls (*top*) or cells tested in invasion assays toward CXCL12 (*bottom*). *C,* cells cotransfected with the indicated vectors and siRNA were tested in GTPase assays for detection of RhoA activation in response to CXCL12 (20 min). *D,* transfectants were incubated with PP2 or PP3 (2 h, 3 μmol/L), and p190RhoGAP tyrosine phosphorylation and bound RhoA were measured as in *A*.

biological actions of $G\alpha_{13}$QL on melanoma cells. LPC and thromboxane A2 (TXA_2) are bioactive lipids that exert their functions involving G_{13}-mediated, as well as $G_{q/11}$-mediated, signaling (40). G2A and GPR4 are postulated to function as receptors mediating LPC actions (41), whereas TPα and TPβ are receptors for TXA_2 (42, 43). PCR analyses revealed that BLM cells express GPR4, G2A, and TPα, but we did not detect TPβ (Supplementary Fig. S5). Invasion of BLM cells to CXCL12 was significantly inhibited by LPC and the TXA_2 analogue U46619 (Fig. 4*A, left*). LPC-mediated impairment in invasion was rescued when $G\alpha_{13}$ expression was silenced (Fig. 4*A, right*). Instead, $G\alpha_{13}$ knocking down did not recover invasion of U46619-incubated cells, indicating that LPC actions were dependent on $G\alpha_{13}$-mediated signaling, whereas U46619 blocking of cell invasion was $G\alpha_{13}$-independent. Notably, CXCL12-promoted RhoA activation was inhibited by LPC, which was rescued by $G\alpha_{13}$ silencing (Fig. 4*B* and *C*). Control experiments showed that Rac1 activation by CXCL12 was not altered in LPC-treated cells (Fig. 4*C*), and incubation with LPC did not affect BLM viability (Supplementary Table S1). Moreover, LPC stimulated p190RhoGAP tyrosine phosphorylation and its binding to RhoA and cooperated with CXCL12 in further augmenting this phosphorylation (Fig. 4*D*). LPC-dependent phosphorylation of p190RhoGAP was mediated by $G\alpha_{13}$, as it was absent in $G\alpha_{13}$-silenced cells (Fig. 4*D*). Together, these data indicate that blockade by LPC of chemokine-promoted melanoma cell invasion involves $G\alpha_{13}$-dependent activation of p190RhoGAP and subsequent inhibition of RhoA activation.

Expression of $G\alpha_{13}$QL in melanoma inhibits lung metastasis. We next used retroviral gene transfer to generate BLM transfectants stably expressing $G\alpha_{13}$QL to investigate its influence on *in vivo* metastasis. Total $G\alpha_{13}$ protein was overexpressed in $G\alpha_{13}$wt and $G\alpha_{13}$QL stable transfectants compared with mock counterparts (Supplementary Fig. S6*A*), which was associated with impaired $G\alpha_{13}$QL transfectant invasion toward CXCL12 and decreased two-dimensional migration (Fig. 5*A* and Supplementary Fig. S6*B*). Furthermore, these transfectants displayed defective RhoA activation in response to the chemokine (Fig. 5*B*), which correlated with inefficient actin stress fiber formation on Matrigel, as well as with inhibition in the generation of focal contacts, as detected with anti-vinculin antibodies (Supplementary Fig. S6*C*). No significant differences in the cell cycle of 5′-bromodeoxyuridine–labeled transfectants were found, with doubling times of ~22 hours. In addition, expression levels of CXCR4 and integrin β1 were similar among these transfectants (Supplementary Fig. S6*D* and *E*).

S.c. inoculation of melanoma $G\alpha_{13}$QL, $G\alpha_{13}$wt, or mock stable transfectants into SCID mice gave rise to primary tumors with similar growth rates (Fig. 5*C, left*). However, two of seven $G\alpha_{13}$wt and mock-injected mice developed several lung metastatic nodes, whereas there was no lung colonization in mice injected with $G\alpha_{13}$QL transfectants (Fig. 5*C, right*). Melanoma cells derived from s.c. tumors retained overexpression of $G\alpha_{13}$ and displayed invasiveness across Matrigel similarly to their original transfectants (Supplementary Fig. S7*A*), indicating that they conserved the invasive properties during tumor growth.

Upon 4 weeks of i.v. inoculation with $G\alpha_{13}$wt or mock transfectants, all mice progressively developed breathing difficulties, which was associated with formation of lung metastatic nodes, with no signs of additional metastases in other organs. After 7 to 8 weeks, there were no surviving $G\alpha_{13}$wt or mock mice (Fig. 5*D, left*). Instead, $G\alpha_{13}$QL mice had a longer disease-free period (6–7 weeks), as well as a significantly prolonged survival, with the last mice of this group dying after 13 weeks. Appearance and abundance of metastatic nodes in lungs from these mice were similar to $G\alpha_{13}$wt or mock counterparts (Fig. 5*D, right*). The human origin of the tumors was confirmed by their expression of $G\alpha_{13}$ and glyceraldehyde-3-phosphate dehydrogenase mRNA detected by PCR with specific primers (Supplementary Fig. S7*B*). These data indicate that expression of $G\alpha_{13}$QL on melamoma cells impairs their *in vivo* lung metastasis.

Discussion

Dynamic reorganization of the actin cytoskeleton by Rho GTPases drives cells in motion (1, 2). Rho GTPase activation is finely regulated by the functional interplay between GEFs and GAPs, and thus migratory responses closely reflect their balance of activities. Heterotrimeric G_{12} proteins constitute upstream molecules, which control Rho activation after interaction of GPCR with their agonists (23). Here, we show that expression in melanoma cells of $G\alpha_{13}$QL, a GTPase-deficient form of $G\alpha_{13}$, led to inhibition of chemokine-promoted RhoA activation and cell invasion, which was dependent on p190RhoGAP function (Fig. 6). Defective RhoA activation in melanoma cells was also detected after expression of $G\alpha_{12}$QL, indicating that activated $G\alpha_{13}$ and $G\alpha_{12}$ trigger similar functional responses. Contrary to melanoma, we found that expression of $G\alpha_{13}$QL in Swiss 3T3 fibroblasts or in MDA-MB231 breast carcinoma cells recapitulated the reported Rho activation (24, 27), indicating that cell type–specific differences might exist in the molecular signals regulating RhoA activation. Whereas RhoA activation by CXCL12 was deficient in $G\alpha_{13}$QL melanoma cells, activation of Rac was unaltered, suggesting that stimulation of these GTPases either occurs independently of each other or that Rac activation is an upstream event of Rho activation.

Defective RhoA activation and invasion was a direct effect of $G\alpha_{13}$QL rather than from endogenous $G\alpha_{13}$ function, as both responses were blocked when $G\alpha_{13}$ siRNA was transfected together with $G\alpha_{13}$QL, but not in single $G\alpha_{13}$ siRNA transfectants. Although CXCL12 stimulated $G\alpha_{13}$ and $G\alpha_i$ association with CXCR4,

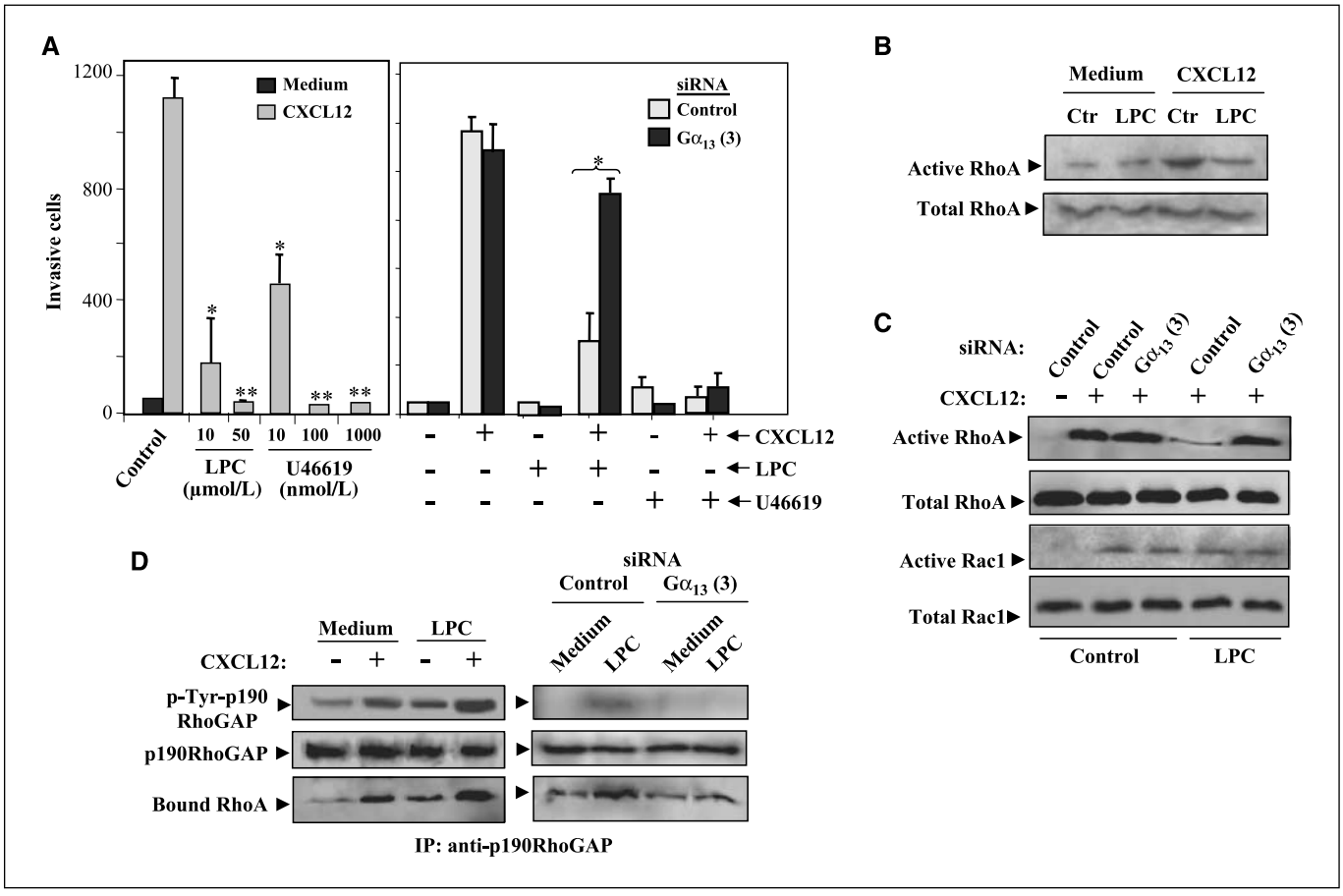

Figure 4. Lysophosphatidylcholine inhibits CXCL12-promoted melanoma cell invasion and RhoA activation and stimulates p190RhoGAP activation through $G\alpha_{13}$. *A, left,* BLM cells were tested in invasion assays toward CXCL12 in the absence (control) or presence of the indicated concentrations of LPC or U46619. **, invasion was significantly inhibited, $P < 0.01$; *, $P < 0.05$. *Right,* cells were transfected with control or $G\alpha_{13}$ siRNA and subjected to invasion assays toward CXCL12 in the absence or presence of LPC (10 μmol/L) or U46619 (100 nmol/L), as indicated. *, invasion was significantly restored, $P < 0.05$. *B* and *C,* nontransfected or control or $G\alpha_{13}$ siRNA-transfected BLM cells were tested in GTPase assays for RhoA or Rac activation by CXCL12 (20 min) after preincubation without (control, *Ctr*) or with LPC (10 μmol/L, 2 h). *D, left,* BLM cells were preincubated as in *B*, and upon cell lysis extracts were immunoprecipitated with anti-p190RhoGAP antibodies, followed by immunoblotting with anti-phosphotyrosine, anti-p190RhoGAP, or anti-RhoA antibodies. *Right,* control or $G\alpha_{13}$ siRNA transfectants were incubated with or without LPC and subjected to immunoprecipitation and immunoblotting as in left panel.

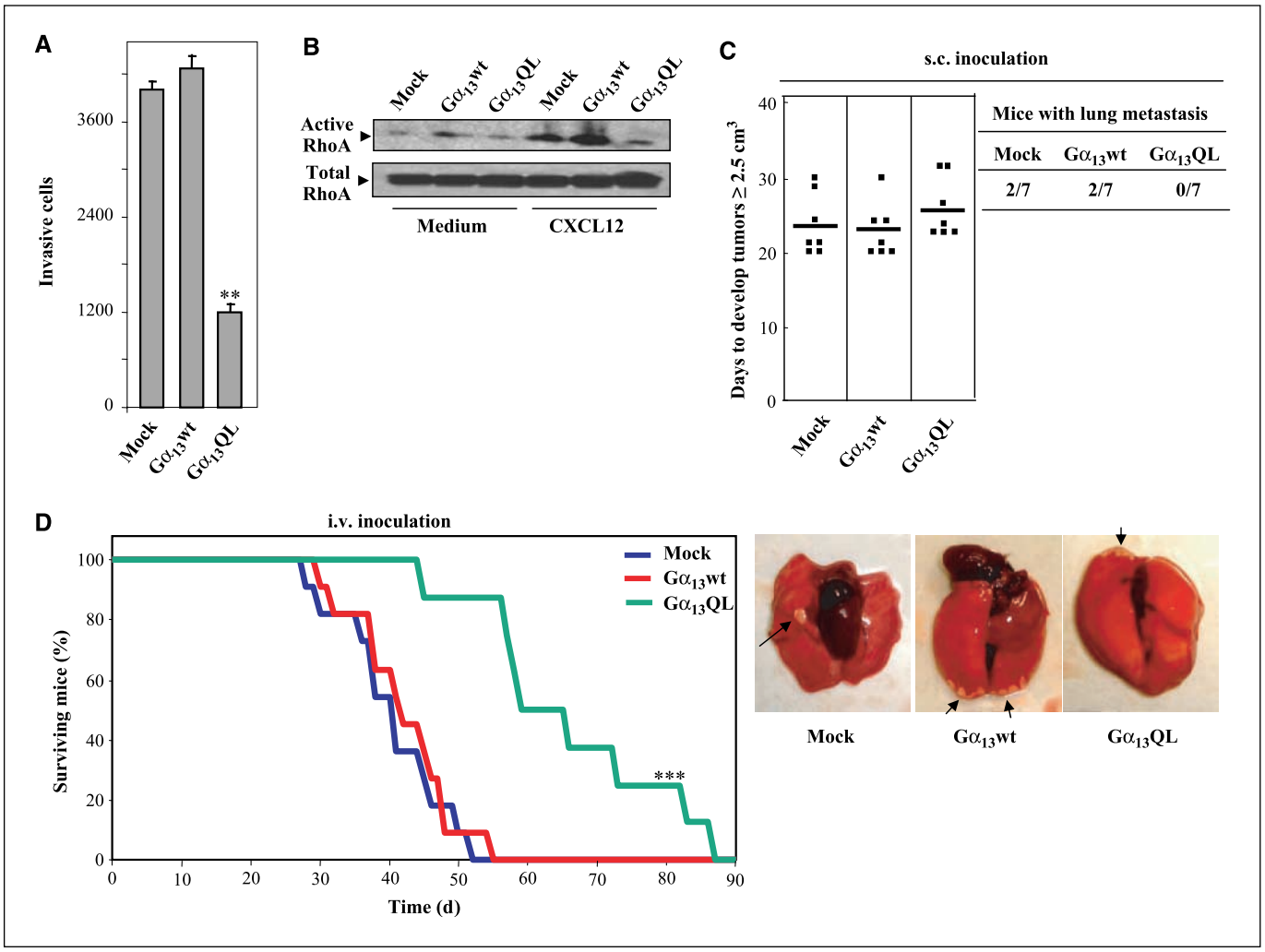

Figure 5. Expression of $G\alpha_{13}QL$ on melanoma cells inhibits lung metastasis. *A,* transfectants were subjected to Matrigel invasion toward CXCL12. **, invasion was significantly inhibited, $P < 0.01$. *B,* transfectants incubated for 20 min in the absence or presence of CXCL12 were tested in RhoA GTPase assays. *C,* SCID mice were s.c. inoculated with the indicated stable melanoma BLM transfectants ($n = 7$). Data show the time that tumors reached volumes of ≥ 2.5 cm^3 (*left*) and number of mice having lung metastases (*right*). *D, left,* survival curves of SCID mice i.v. inoculated with the indicated melanoma transfectants (mock and $G\alpha_{13}$wt, $n = 11$; $G\alpha_{13}QL$, $n = 8$; ***, $P < 0.001$); *right,* lung metastases from mice inoculated with mock (49 d), $G\alpha_{13}$wt (46 d), or $G\alpha_{13}QL$ (66 d) melanoma transfectants. *Arrows,* metastatic nodes.

chemokine-promoted RhoA activation was largely inhibited by pertussis toxin, indicating a predominant $G\alpha_i$ involvement. Importantly, expression of a CA Rho form rescued $G\alpha_{13}QL$-dependent inhibition on invasion, confirming that impairment in Rho activation underlay the defective $G\alpha_{13}QL$ melanoma cell invasion.

Potential mechanisms mediating $G\alpha_{13}QL$-dependent defective RhoA activation in melanoma include activation of RhoGAP function or inhibition of RhoGEF activity. p190RhoGAP has GAP activity preferentially on Rho, this activity being stimulated by tyrosine phosphorylation (34, 36, 44). Stimulation by CXCL12 or $G\alpha_{13}QL$ expression enhanced tyrosine phosphorylation of p190RhoGAP in melanoma cells. Furthermore, we found higher amounts of RhoA associated with p190RhoGAP in $G\alpha_{13}QL$ transfectants than in mock or $G\alpha_{13}$wt counterparts, and silencing p190RhoGAP expression in $G\alpha_{13}QL$ cells led to rescue of RhoA activation and invasion in response to CXCL12, therefore identifying p190RhoGAP as responsible for the inhibitory effects due to $G\alpha_{13}QL$ (Fig. 6). Thus, higher GAP activity of p190RhoGAP toward RhoA in $G\alpha_{13}QL$ melanoma transfectants might constitute one of the mechanisms accounting for decreased RhoA activation compared with Swiss 3T3 and MDA-MB-231 $G\alpha_{13}QL$ counterparts. As mentioned in a recent review (23), although it is assumed that G_{12}-mediated signaling causes Rho activation and *in vitro* invasion of breast and prostate cancer cells (26, 27), this might not be always the rule. Thus, glioblastoma cells stimulated with sphingosine-1-phosphate display G_{12}-mediated inhibition of cell migration (45), suggesting that tumor type–specific differences in RhoA activation might be responsible for the distinct invasion responses.

Silencing the GEF Vav2 in BLM cells blocks CXCL12-promoted RhoA activation (20). However, Vav2 activation and association with RhoA were similar in $G\alpha_{13}QL$, $G\alpha_{13}$wt, and mock transfectants, indicating that $G\alpha_{13}QL$-mediated blockade in RhoA activation was not due to alterations in Vav2 function. It is surprising that RGS domain-containing RhoGEFs, such as p115RhoGEF, PDZ-RhoGEF, and LARG, known to interact with $G\alpha_{12/13}$ proteins and stimulate Rho activation (23), apparently do

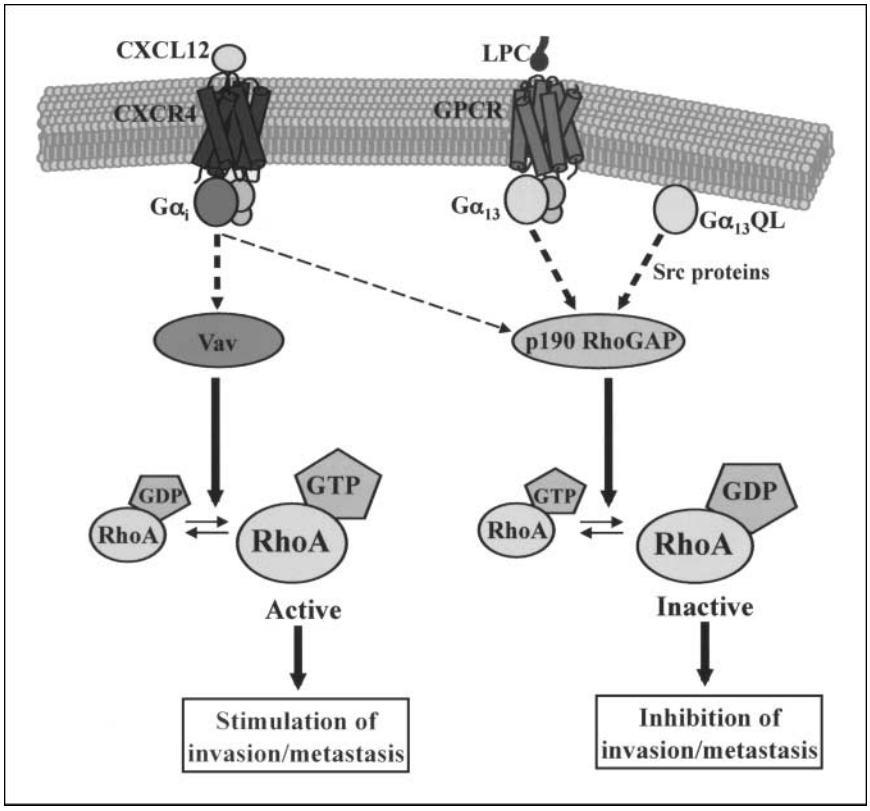

Figure 6. Model for Rho-dependent regulation of melanoma cell invasion in response to GPCR stimulation. Stimulation or inhibition of cell invasion is dependent on the levels of Rho activation due to GPCR-mediated signaling. See text for details.

not importantly contribute to Rho activation of melanoma cells in response to chemokines. Should any of these RhoGEFs mediate $G\alpha_{13}QL$ actions in BLM cells, we would theoretically expect increased RhoA activation and invasion as a result of cooperation with Vav proteins, which is not what we observe. Our previous work with Vav-silenced cells indicated that Vav proteins are main GEFs involved in chemokine-promoted RhoA activation and invasion of melanoma cells (20), and the present data suggest that RGS-RhoGEF role in $G\alpha_{13}QL$ transfectants might be obscured by the potent p190RhoGAP activity. Another potential mechanism which could contribute to $G\alpha_{13}QL$-dependent, p190RhoGAP-mediated inhibition of Rho activation could arise from the fact that active $G\alpha_{13}$ binds to E-cadherin (46) and that p120ctn links p190RhoGAP to the E-cadherin complex in Rac-activated cells, causing Rho inactivation (47). However, BLM cells or $G\alpha_{13}$ transfectants express very low amounts of cell membrane E-cadherin (not shown), suggesting the unlikelihood of defective RhoA activation by E-cadherin–associated p190RhoGAP in $G\alpha_{13}QL$ cells.

The above data raised the interesting possibility that stimuli that activate $G\alpha_{13}$ after interaction with their GPCR could limit chemokine-promoted RhoA activation and invasion of melanoma cells. We found that stimulation by CXCL12 of BLM cell invasion and Rho activation was opposed by LPC and the TXA_2 analogue U46619, which are lipids that bind to $G\alpha_{13}$-coupled receptors (40). Candidate LPC receptors GPR4 and G2A (41) were found to be expressed on BLM melanoma cells, and knocking down $G\alpha_{13}$ led to rescue of both invasion and Rho activation in response to CXCL12, indicating that LPC-dependent inhibition was mediated by $G\alpha_{13}$, potentially involving GPR4 and/or G2A. Although BLM cells also express the TPα receptor for TXA_2, $G\alpha_{13}$ silencing did not result in recovering invasion that was inhibited by U46619, suggesting that this TXA_2 analogue might be exerting its inhibition through G_q, an additional G protein mediating TXA_2 actions (48). Importantly, interference by LPC of chemokine-promoted melanoma cell invasion correlated with LPC-dependent, $G\alpha_{13}$-mediated stimulation of p190RhoGAP tyrosine phosphorylation and blockade of RhoA activation. These results show that activation in melanoma cells of the $G\alpha_{13}$-p190RhoGAP route by extracellular stimuli opposes chemokine-triggered RhoA activation and cell invasion *in vitro* and indicate that LPC-dependent and $G\alpha_{13}QL$-dependent inhibition of RhoA activation and invasion share a common control point that is p190RhoGAP.

Therefore, these data suggest that a tight balance of RhoA activation due to GEF and GAP actions controls melanoma cell invasion (Fig. 6). On one hand, CXCL12 stimulates $G\alpha_i$-dependent Vav2-RhoA activation, as well as tyrosine phosphorylation of p190RhoGAP (ref. 20, and this work), overall causing RhoA activation and stimulation of cell invasion, indicating that, under these conditions, GEF-promoted activation prevails over GAP activity toward Rho. When $G\alpha_{13}$ is activated, either through $G\alpha_{13}QL$ expression or by LPC, p190RhoGAP-mediated RhoA inactivation overcomes CXCL12-dependent stimulation, causing defective RhoA activation and impaired invasion. Initial characterization of the molecular components involved in p190RhoGAP tyrosine phosphorylation in melanoma cells led to the identification of Src proteins as likely candidates to mediate this phosphorylation, as suggested from the results obtained with Src inhibitors. p190RhoGAP is a well-known Src substrate (37, 38), and thus, Src-dependent phosphorylation represents a potential mechanism regulating the GAP activity on Rho and, hence, the invasion of melanoma cells.

To investigate if $G\alpha_{13}$QL-dependent impairment in *in vitro* melanoma cell invasion could influence *in vivo* metastasis, we generated transfectants stably overexpressing $G\alpha_{13}$QL or $G\alpha_{13}$wt to be used in xenograft studies. $G\alpha_{13}$QL stable transfectants retained the inhibition of invasion and RhoA activation in response to CXCL12, which was associated to a decrease in transfectant migration speed.

BLM human melanoma cells were originally selected by their high potential to disseminate into lungs of immunodeficient mice (28). CXCR4 strongly contributes to BLM cell metastasis into lungs, as its silencing leads to inhibition of melanoma lung colonization.[1] Although there were no significant differences in primary tumor growth between s.c. inoculated $G\alpha_{13}$ BLM transfectants, confirming the *in vitro* results, near a third of mice injected with $G\alpha_{13}$wt or mock transfectants developed melanoma lung metastatic nodes, whereas no lung metastases were observed in mice inoculated with $G\alpha_{13}$QL melanoma cells. When $G\alpha_{13}$QL transfectants were i.v. inoculated, we found a significantly prolonged disease-free and survival period, compared with $G\alpha_{13}$wt or mock counterparts. These results suggest that activated $G\alpha_{13}$ might represent a molecular brake for initiation of invasive steps leading to melanoma metastasis. If melanoma cells expressing activated $G\alpha_{13}$ reach blood circulation, deficient homing into lungs occurs, suggesting that extravasation and/or tissue invasion capabilities might be affected. Based on the *in vitro* results, it can be proposed that p190RhoGAP-mediated inhibition of RhoA activation in $G\alpha_{13}$QL melanoma cells could impair stress fiber assembly, hindering cell motility and leading to inefficient invasion and metastasis *in vivo*. Although our results strongly suggest that $G\alpha_{13}$QL expression mainly affects cell motility, we cannot, however, exclude that $G\alpha_{13}$ activation might impair, to some extent, melanoma cell survival in the lungs.

Little is known on the expression pattern of G_{12} and p190RhoGAP proteins in human tumor samples. Histopathologic analyses using human tissue specimen revealed higher $G\alpha_{13}$ expression in breast and prostate cancer (26, 27), whereas p190RhoGAP has been proposed to play tumor suppressor roles in glioma (35, 49). Further analyses are required to better define the function of these proteins in tumor cell growth and metastasis, and it will be relevant to functionally correlate their roles in *in vivo* models of melanoma metastasis.

From the above data, it is tempting to speculate that $G\alpha_{13}$-coupled receptors might represent a cell entrance way to inhibit RhoA activation via stimulation of p190RhoGAP. GPCRs represent important regulators of tumor cell growth and metastasis, and as recently proposed, they might provide powerful opportunities for cancer prevention and treatment (50). The present results should contribute to widen our knowledge of key signaling components activated through these receptors whose function is needed for efficient tumor cell invasion.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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[1] Bartolomé et al., submitted for publication.

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