Different Raf Protein Kinases Mediate Different Signaling Pathways to Stimulate E3 Ligase RFFL Gene Expression in Cell Migration Regulation*

Received for publication, April 18, 2013, and in revised form, September 13, 2013 Published, JBC Papers in Press, October 10, 2013 DOI 10.1074/jbc.M113.477406

Xiaoqing Gan†, Chen Wang‡, Maulik Patel§, Barry Kreutz*, Maggie Zhou†, Tohru Kozasa‡, and Dianqing Wu††

From the ††Department of Pharmacology and Program in Vascular Biology and Therapeutics, Yale School of Medicine, New Haven, Connecticut 06520, the †Department of Pharmacology, University of Illinois at Chicago, Chicago, Illinois 60612, and the †Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153, Japan

Background: Cell migration requires persistent PKC phosphorylation, which can be achieved through the RFFL-mTORC2 pathway.

Results: We show how Ga12 specifically activates ARAF to stimulate RFFL expression, which can also be stimulated by EGF, via CRAF, and activated BRAF.

Conclusion: Different signaling pathways, through different Raf proteins, stimulate RFFL expression to support cell migration.

Significance: The RFFL-PKC pathway has a broad significance in cell migration regulation.

We previously characterized a Ga12-specific signaling pathway that stimulates the transcription of the E3 ligase RFFL via the protein kinase ARAF and ERK. This pathway leads to persistent PKC activation and is important for sustaining fibroblast migration. However, questions remain regarding how Ga12 specifically activates ARAF, which transcription factor is involved in Ga12-mediated RFFL expression, and whether RFFL is important for cell migration stimulated by other signaling mechanisms that can activate ERK. In this study, we show that replacement of the Ga12 residue Arg-264 with Gln, which is the corresponding Ga13 residue, abrogates the ability of Ga12 to interact with or activate ARAF. We also show that Ga12 can no longer interact with and activate an ARAF mutant with its C-terminal sequence downstream of the kinase domain being replaced with the corresponding CRAF sequence. These results explain why Ga12, but not Ga13, specifically activates ARAF but not CRAF. Together with our finding that recombinant Ga12 is sufficient for stimulating the kinase activity of ARAF, this study reveals an ARAF activation mechanism that is different from that of CRAF. In addition, we show that this Ga12-ARAFAK pathway stimulates RFFL transcription through the transcription factor c-Myc. We further demonstrate that EGF, which signals through CRAF, and an activated BRAF mutant also activate PKC and stimulate cell migration through up-regulating RFFL expression. Thus, RFFL-mediated PKC activation has a broad significance in cell migration regulation.

Heterotrimeric G proteins mediate signal transduction of a diverse range of biologically active molecules. Among the four families of Ga subunits, the Ga12/13 family, comprised of Ga12 and Ga13, is ubiquitously expressed. Although Ga12 and Ga13 share less than 70% amino acid sequence identity, mouse genetic studies have shown that these two Ga proteins are functionally redundant in regulating vascular smooth muscles, neuron migration, cardiac morphogenesis, T and B cell migration, and trafficking (1, 2). The redundant functions may be mediated by a group of Rho guanine nucleotide exchange factors that both Ga proteins activate (2). Nevertheless, evidence has emerged to suggest that these two proteins may also have additional functions that differ between the isoforms. Ga13 appears to regulate platelet activation (3, 4), PDGF-induced cell migration (5), and angiogenesis (6). On the other hand, Ga12 was shown to be involved in LPA-induced mitogenic activity (7), S1P-induced COX2 expression (8), and TCR-mediated IL-2 expression (9). Some of the specific in vivo functions of Ga13 may be mediated by its interaction with integrin αIIbβ3 and GEF115 (3, 10, 11).

We recently characterized a Ga12-specific signaling mechanism activated by lysophosphatidic acid (LPA)2 (12). LPA induces diverse cellular responses, including proliferation, adhesion, migration, morphogenesis, differentiation, and survival (13), and has an important role in pulmonary fibrosis (14). We found that LPA induced two phases of PKC hydrophobic motif (HM) phosphorylation. The late, sustained phase is mediated by Gi12 but not other G proteins, including its close homolog Gi13 (12). We also found that Ga12, but not Ga13, specifically interacted with ARAF, but not CRAF, to stimulate transcription of the E3 ubiquitin ligase RFFL via MEK and ERK. RFFL can poly-ubiquitinate and destabilize PRR5L, an mTORC2-associated protein that inhibits mTORC2-mediated HM phosphorylation of PKCζ but not AKT. Its elimination by RFFL resulted in PKCζ HM phosphorylation and activation. We further showed that this Ga12-mediated pathway is critically important for fibroblast migration and the development of pulmonary fibrosis (12).

2 The abbreviations used are: LPA, lysophosphatidic acid; HM, hydrophobic motif; MEF, mouse embryonic fibroblast; MARCKS, myristoylated alanine-rich C kinase substrate; CR, conserved region.
The Raf family of protein kinases, consisting of ARAF, BRAF, and CRAF, plays important roles in signal transduction by regulating the MEK and ERK cascade. ARAF is the least studied among the three RAF isoforms (15, 16). It is poorly activated by growth factors, overexpressed tyrosine kinases, or activated Ras. Ras is considered the primary Raf upstream activator (17, 18). However, our recent study demonstrates that ARAF is primarily regulated by G protein-coupled receptors (GPCRs) via Go12 independently of Ras (12). Thus, it would be important to understand why Go12, but not Go13, specifically interacts with and activates ARAF but not CRAF. Ras activates CRAF by binding to its N-terminal Ras-binding domain to relieve N terminal-mediated autoinhibition, so the question is whether Go12 activates ARAF via a similar mechanism. Although the LPA-Go12 pathway acted specifically through CRAF to activate ERK, leading to RFFL transcription activation, ERK can also be activated by CRAF and BRAF, which are activated by growth factors. Moreover, many cancer cells, particularly melanoma cancer cells, contain activated BRAF mutations, and activation of CRAF and BRAF by either growth factors or activated mutations is expected to activate ERK. The questions are whether these CRAF and BRAF activating mechanisms lead to RFFL expression and persistent PKC activation and whether RFFL-mediated persistent PKC activation is important in cell migration stimulated by growth factors and activated BRAF mutants.

In this report, we identified an important Go12 amino acid required for its interaction with ARAF and the ARAF sequence required for its interaction with and activation by Go12. We also demonstrated that EGF acted specifically through CRAF in mouse embryonic fibroblast (MEF) cells to up-regulate RFFL expression and stimulate cell migration. In addition, in two tumor cell lines that harbor the BRAF-activating V600E mutation, RFFL is involved in elevated PKC activation and plays an important role in cell migration.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were obtained from the following sources: protein A/G-PLUS-Sepharose from Santa Cruz Biotechnology, Inc.; antibodies to PKC\(\beta\), p44/42 MAPK, c-Myc, histone H3, phospho-p44/42 MAPK, phospho-PKC\(\beta\)/\(\theta\)(Ser-643/676), phospho-MARCKS(Ser-152/156), phospho-CRAF(Ser-338), phospho-ARAF(Ser-299), and phospho-MEK1/2(Ser-217/221) from Cell Signaling Technology, Inc.; antibodies to Go12, Go13, and Elk1 (H-160) from Santa Cruz Biotechnology, Inc.; Lipofectamine/Plus reagent and Lipofectamine RNAiMax from Invitrogen; L-\(\alpha\)-lysophosphatidic acid from Sigma; a chromatin immunoprecipitation assay kit from Millipore; and His-MEK1(K97A) from SignalChem.

**Cell Culture and Stimulation**—HEK293T cells and MEFs were maintained in DMEM with 4.5 g/liter glucose supplemented with 10% FBS. W2N266-4 cells were maintained in minimum Eagle’s medium with 10% FBS. NCI-460 cells were maintained in RPMI 1640 with 10% FBS. Transient transfection was carried out using Lipofectamine and Plus (Invitrogen), and samples were collected 24 h after transfection. Synthetic siRNA oligos, which were obtained from Dharmacon (the sequences are listed in supplemental Table S1), were transfected using Lipofectamine RNAiMax, and samples were collected 48 h after transfection. Cells were starved overnight in DMEM without serum before being stimulated with LPA or EGF.

**Protein Purification**—GST-ARAF, ARAF mutants, or CRAF was expressed in *Escherichia coli* BL21 (DE3). After isopropyl-1-thio-\(\beta\)-D-galactopyranoside (100 \(\mu\)M) induction at 22 °C for 24 h, proteins were extracted with 0.5 mg/ml lysozyme in buffer A (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). The high-speed supernatant of the extract was loaded on glutathione-agarose and eluted with buffer A with 5 mM reduced glutathione. The recombinant protein was dialyzed into 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5% glycerol.

**Immunoprecipitation**—Cells were lysed with cell lysis buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.7% Triton X-100, 1 mM EGTA, 5 mM MgCl\(_2\), protease inhibitor mixture, and phosphatase inhibitor mixture (Roche)) and AMF (50 \(\mu\)M AlCl\(_3\), 10 mM MgCl\(_2\), and 5 mM NaF). Immunoprecipitation was carried out using anti-Go12 antibody.

**In Vitro Binding Assay and Kinase Assay**—Approximately 60 pmol of purified Go12 or Go13 was incubated with either 10 \(\mu\)M GTP\(\gamma\)S for 2 h at 30 °C in buffer (50 mM HEPES (pH 8.0), 1 mM EDTA, 3 mM DTT, and 0.05% polyoxyethylene 10 lauryl ether containing 10 mM MgSO\(_4\)). Reactions were divided and then incubated with glutathione-agarose-bound GST fusion proteins for 2 h at 4 °C. For the kinase assay, the above mixtures were incubated with 200 ng of His-MEK1 and 500 \(\mu\)M ATP for 30 min at 30 °C and stopped by SDS sample buffer. For the binding assay, the glutathione-agarose was pelleted, washed with buffer, and analyzed by Western blotting.

**ChIP Assay**—The ChIP assays were performed using the ChIP immunoprecipitation assay kit (Millipore, MA) and 10\(^7\) HEK293T cells by following the instructions of the manufacturer.

**Cell Migration Assay**—For the scratch migration assay, cells were grown to confluency in a monolayer in six-well plates. A linear gap was generated by scratching the bottoms of the wells using a sterile 200-\(\mu\)l pipette tip. Phase-contrast microscopy images were acquired 0 and 16 h after the gaps were created. The distance migrated in 16 h by the control cells are taken as 1. The transwell assay was done using transwells coated with Matrigel (1 mg/ml). Cells were harvested by trypsin/EDTA, washed, and resuspended in medium containing 0.1% BSA at a density of 10\(^6\) cells/ml. Cell suspensions (100 \(\mu\)l) were loaded onto the transwells and incubated at 37 °C for 6 h. The cells from the upper portion of the transwell filters were removed by cotton swab. Cells at the bottom of the filters were stained with crystal violet and counted under a light microscope.

**RESULTS AND DISCUSSION**

To understand how Go12, but not its close homolog Go13, specifically regulates ARAF, we generated a Go12 chimeric mutant in which the Go12 amino acid sequence from residues Val-225 to Leu-294 was substituted with the corresponding Go13 sequence as depicted in Fig. 1A. The reason for selecting this stretch of amino acid sequence is that these regions of Go subunits have been recognized for effector interactions (19–22). This Go12 chimeric mutant also contains the constitutively active Q229L mutation and was designated as Go12QL-
Regulation of RFFL-mediated Signaling by Raf Kinases

A. We have shown previously that Gα12Q, but not Gα13Q, stimulated ERK phosphorylation in HEK293 cells and that Gα12Q-mediated ERK phosphorylation exclusively depends on ARAF in these cells (12). Thus, ERK phosphorylation was used as a surrogate marker to test Gα12 for its ability to activate ARAF. We found that expression of Gα12Q-Ch, unlike Gα12Q, failed to stimulate ERK phosphorylation in HEK293T cells (Fig. 1B), suggesting that the residues from Val-225 to Leu-294 may contain amino acids critical for ARAF activation.

Comparison of these Gα12Q residues (Val-226 to Leu-293) with Gα13 revealed only a few residues that are not conserved, including Gln-232, Gln-234, and Arg-264 (Fig. 1A). We replaced Gln-232 and Gln-234 with corresponding Gα13 residues (Glu and Lys) to create Gα12Q-QRQ and replaced Arg-264 with Gln to create Gα12Q-R (Fig. 1A). Although Gα12Q-QRQ still retained its ability to stimulate ERK phosphorylation, Gα12Q-R showed a markedly reduced ability (Fig. 1B). These results suggest that the Gα12 residue Arg-264 is critically important for Gα12 to stimulate ERK phosphorylation. Consistent with the fact that Gα12 stimulates ERK phosphorylation via MEK1 (12), we also observed that expression of Gα12Q-R only weakly increased MEK-1 phosphorylation compared with that of Gα12Q (supplemental Fig. S1). To confirm that Gα12 Arg-264 is also important for Gα12 to bind to ARAF, we carried out the coimmunoprecipitation assay as we did previously (12) and found that the interaction of Gα12Q-R with ARAF was markedly reduced (Fig. 1C).

To determine whether the Gα12 residues Val-226 to Leu-293 are sufficient for activating ARAF and stimulating ERK phosphorylation, we generated a Gα13 chimera, designated as Gα13Q-Ch, by substituting these Gα12 residues for the corresponding Gα13 residues (Fig. 1A). Expression of Gα13Q-Ch had a marginal effect on ERK phosphorylation in HEK293T cells (Fig. 1D), suggesting that these Gα12 residues are largely insufficient to activate ARAF and stimulate ERK phosphorylation.

Next we investigated the basis for specific activation of ARAF, but not CRAF, by Gα12. There are three highly conserved regions (CR1–3) in the Raf family of protein kinases (Fig. 2A). However, sequences in regions other than these CRs are less conserved. We first generated two ARAF chimeric mutants with substitution of the CRAF sequences between CR1 and CR2 and between CR2 and CR3, respectively, for those in ARAF. When these chimeric mutants were tested in a cotransfection assay with Gα12, they showed similar synergistic effects with Gα12 compared with the wild-type ARAF, suggesting that these regions may not be important for Gα12 activation (data not shown). We then generated an ARAF chimera with substitution of the CRAF C terminus (starting at residues His-611) for ARAF C-terminal residues (starting at Arg-572), which was designated ARAF-C (Fig. 2A). In HEK293 cells, expression of ARAF alone induced little ERK phosphorylation. However, ERK phosphorylation was markedly greater in HEK293 cells coexpressing ARAF and Gα12Q than that in cells expressing Gα12Q alone. We used this synergistic effect of ARAF with Gα12Q to examine whether the ARAF mutants were able to be activated by Gα12Q. Unlike ARAF, ARAF-C and CRAF failed to show any synergistic effect with Gα12Q (Fig. 2B). In addition, ARAF-C appeared to reduce Gα12Q-induced ERK phosphorylation (Fig. 2B, compare lane 6 with lane 2), suggesting that it may act as a dominant negative mutant. These results together suggest that the C-terminal sequence downstream of the ARAF kinase domain is required for its activation by Gα12. To further confirm this conclusion, we examined the interaction of Gα12 with ARAF-C using a coimmunoprecipitation assay and found that ARAF-C acted like CRAF rather than ARAF and showed little interaction with Gα12 (Fig. 2C). Consistent with these coimmunoprecipitation results, recombinant GTPγS-bound Gα12 protein strongly interacted with the ARAF protein but not with ARAF-C or the CRAF protein (Fig. 2D). These results collectively indicate that the C-terminal...
region of ARAF is required for its interaction with and activation by Ga12.

To determine whether the C-terminal ARAF sequence is sufficient for activation by Ga12, we generated a CRAF chimeric mutant with substitution of the ARAF C-terminal sequence for the CRAF C-terminal sequence, designated as CRAF-C (Fig. 2A). CRAF-C showed no synergistic effect with Ga12 (Fig. 2E), thus suggesting that the ARAF C-terminal sequence is not sufficient for activation by Ga12. ARAF sequences beyond this C-terminal region might also be involved in the interaction with Ga12. This conclusion is consistent with the observation that ARAF-C acts as a dominant negative mutant (Fig. 2B).

Although we have shown that Ga12 directly interacts with and activates ARAF in transfected cells, it is important to also know whether Ga12 can activate ARAF kinase activity independently of other factors. This is particularly crucial because Ga12 binds to a region on ARAF that is different from the one to which the Ras proteins, the best characterized Raf activators, bind. Thus, we performed an in vitro kinase assay using recombinant Ga12 and Ga13 proteins that were loaded with GTPγS and recombinant ARAF, ARAF-C, or CRAF protein in the presence of ATP and kinase-dead MEK1 as substrates. The Raf kinase activity was detected by using an anti-phospho-MEK1 antibody. Ga12, but not Ga13, stimulated the phosphorylation of MEK1 only when ARAF, but not CRAF or ARAF-C, was present (Fig. 3). These results clearly demonstrate that Ga12 is sufficient for stimulating ARAF kinase activity.

Because Ga12 and ARAF stimulate RFFL expression via ERK (12), we also tested if Ga12QL-R and ARAF-C could stimulate RFFL expression. Neither Ga12QL-R nor ARAF-C, when expressed in HEK293T cells, stimulated RFFL expression, whereas their wild-type proteins could (Fig. 4A). These results are consistent with their inability to stimulate ERK phosphorylation. We also investigated which transcription factors are involved in RFFL transcription regulation by this pathway. Elk and c-Myc are transcription factors known to be regulated by ERK. We examined their involvement by using a ChIP assay and found that LPA, which is upstream of Ga12 (12), stimulated the binding of c-Myc, but not Elk, to the RFFL promoter regions in a MEK-dependent manner (Fig. 4, B and C). In addition, expression of Ga12QL or ARAF, but not Ga12QL-R or ARAF-C, stimulated c-Myc binding to the RFFL promoter elements (Fig. 4D). To further characterize the importance of c-Myc in RFFL transcription regulation, we transfected the cells with c-Myc siRNAs, which silenced c-Myc expression (Fig. 4E), and inhibited LPA-stimulated expression of RFFL (F). Together, these data demonstrate that the LPA-Ga12-ARAF pathway up-regulates RFFL gene expression via c-Myc.

Not only can ARAF activate the MEK-ERK pathway, but the ARAF homolog CRAF and BRAF are also known to activate the

---

**FIGURE 2. The ARAF C-terminal sequence is required for ARAF interaction with and activation by Ga12.** A, schematic of the Raf family of kinases and the C-terminal amino acid sequences of ARAF and CRAF. The amino acid sequence of CRAF is underlined. B, the effect of ARAF C-terminal substitution on ERK phosphorylation stimulated by Ga12QL. HEK293T cells were transfected with Ga12QL and Ras, and Western blotting was carried out the next day. C and D, the effect of ARAF C-terminal substitution on its interaction with Ga12. The interaction between Ga12 and Ras was assessed by coimmunoprecipitation (IP) using HEK293 cells transfected with Ga12QL and Ras (C) or by pull-down of recombinant GTPγS-loaded Ga12 or Ga13 and Ras proteins (D). E, the effect of CRAF C-terminal substitution on ERK phosphorylation stimulated by Ga12QL. HEK293 cells were transfected with Ga12QL and/or ARAF or CRAF-C, and Western blot analysis was performed 24 h after transfection.
pathway, and they should up-regulate RFFL as well. In fact, expression of WT CRAF or mutant BRAF carrying an activating
mutation, V600E, also led to up-regulation of RFFL expression in HEK293T cells (Fig. 5A). Interestingly, in MEFs, EGF-upregulated
RFFL primarily depended on CRAF, but not BRAF or ARAF, because knockdown of CRAF, but not BRAF or ARAF, inhibited EGF-induced RFFL expression (Fig. 5B, C, and supplemental Fig. S2A, A and B). Consistent with
this observation, knockdown of CRAF, but not ARAF or BRAF, inhibited EGF-induced phosphorylation of PKCδ HM and MARCKS (Fig. 5D and supplemental Fig. S2C). MARCKS is a PKC substrate, and its phosphorylation indicates PKC activation (12). We have shown previously that RFFL mediated long-term PKCδ HM phosphorylation in MEFs, which is important for MEF migration (12). In agreement with these findings, RFFL knockdown primarily inhibited EGF-induced phosphorylation of PKCδ and MARCKS at 120 min rather than at 30 min (Fig. 5E and supplemental Fig. S2D) and impaired EGF-induced migration (Fig. 5F and supplemental Fig. S2E). We also investigated the role of RFFL in the migration of two tumor cell lines harboring the V600E BRAF mutation. They are WM266-4 (a melanoma cell line) and NCI-H460 (a non-small cell lung carcinoma cell line). Knockdown of either BRAF or RFFL inhibited migration of these cells (Fig. 5G and supplemental Fig. S2A and F). Consistent with the effects on cell migration, BRAF or RFFL knockdown also decreased the phosphorylation of PKCδ and MARCKS (Fig. 5H and supplemental Fig. S2G).
In this study, we provided insights into the molecular basis for the specific interactions between Ga12 and ARAF. In addition, we showed that although Ga12, unlike Ras, binds to the C terminus of ARAF, it may achieve a similar outcome in activation of ARAF, probably by relieving the autoinhibition by the N terminus. The fact that Ga12 alone is sufficient to activate ARAF kinase activity further confirms the above conclusion, although other cofactors, including KSR (kinase suppressor of Ras), Raf phosphorylation, and dimerization, may help to stabilize or further activate ARAF. Thus, the results in this study further solidify the conclusion that ARAF is a specific, direct effector of Ga12. In this study, we also demonstrated that EGF, acting through CRAF, and activated Braf could also up-regulate RFFL expression to regulate PKC activity and cell migration. Thus, different signaling pathways, either activated by a GPCR ligand (such as LPA) via ARAF, a growth factor (such as EGF) via CRAF, or activated Braf, mutations, as depicted in Fig. 6, can achieve persistent PKC activation through the activation of ERK and RFFL transcription to support cell migration. This RFFL-mediated signaling pathway may have a much broader significance in cell migration regulation than illustrated in this and our previous study (12) because many tumor cells have amplified EGF signaling activity or mutated Ras and Raf genes that can also lead to RFFL expression and persistent PKC activation. Therefore, RFFL, mTORC2, and PKC may be potential targets for blocking tumor migration and, hence, metastasis, in addition to treating pulmonary fibrosis, as we indicated previously (12). This hypothesis needs to be tested with in vivo models, particularly the spontaneous genetic tumor models.

Questions also remain on how RFFL activates PKC. We have shown previously that RFFL induces PKC HM phosphorylation by polyubiquitinating and destabilizing PRR5L, a protein that inhibits mTORC2-mediated PKC HM phosphorylation. It is not known whether the phosphorylation at the HM site of PKC8 is sufficient for its activation. We have substituted a glutamine residue for the HM site serine residue but did not observe any change in the kinase activity (data not shown). Given the known caveat of phosphomimic mutations, additional studies are needed to resolve this question.

ACKNOWLEDGMENTS—We thank Michelle Orsulak for technical assistance.

REFERENCES

1. Herroeder, S., Reichardt, P., Sassmann, A., Zimmermann, B., Jaeneke, D., Hoeckner, J., Hofmann, M. W., Fischer, K. D., Vogt, S., Grosse, R., Hogg, N., Gunzer, M., Offermanns, S., and Wettscuehck, N. (2009) Guanine nucleotide-binding proteins of the G12 family shape immune functions by controlling CD4+ T cell adhesiveness and motility. *Immunology* 30, 708–720

2. Worzfeld, T., Wettscuehck, N., and Offermanns, S. (2008) G(12)/G(13)-mediated signalling in mammalian physiology and disease. *Trends Pharmacol. Sci.* 29, 582–589

3. Gong, H., Shen, B., Flevaris, P., Chow, C., Lam, S. C., Vojno-Yasenetskaya, T. A., Kozasa, T., and Du, X. (2010) G protein subunit Ga13 binds to integrin αIIbβ3 and mediates integrin ‘outside-in’ signaling. *Science* 327, 340–344

4. Moers, A., Niewandt, B., Massberg, S., Wettscuehck, N., Grüner, S., Konrad, I., Schulte, V., Aktas, B., Gratacap, M. P., Simon, M. I., Gawaz, M., and Offermanns, S. (2003) G13 is an essential mediator of platelet activation in hemostasis and thrombosis. *Nat. Med.* 9, 1418–1422

5. Shan, D., Chen, L., Wang, D., Tan, Y. C., Gu, J. L., and Huang, X. Y. (2006) The G protein Gα(13) is required for growth factor-induced cell migration. *Dev. Cell* 10, 707–718

6. Sivaraj, K. K., Takefuji, M., Schmidt, I., Adams, R. H., Offermanns, S., and Wettscuehck, N. (2013) G13 controls angiogenesis through regulation of VEGFR-2 expression. *Dev. Cell* 25, 427–434

7. Radhiya, V., Hee Ha, J., Jayaraman, M., Tsim, S. T., and Dhanasekaran, N. (2005) Mitogenic signaling by lysophosphatidic acid (LPA) involves Ga12. *Oncogene* 24, 4597–4603

8. Ki, S. H., Choi, M. J., Lee, C. H., and Kim, S. G. (2007) Ga12 specifically regulates COX-2 induction by phosphanising 1-phosphate. Role for INK-dependent ubiquitination and degradation of iNOS*. *J. Biol. Chem.* 282, 1938–1947

9. Won, H. Y., Min, H. J., Lee, W. H., Kim, S. G., and Hwang, E. S. (2010) Ga12 is critical for TCR-induced IL-2 production and differentiation of T helper 2 and T helper 17 cells. *Biochem. Biophys. Res. Commun.* 394, 811–816

10. Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W. J., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Direct stimulation of the guanine nucleotide exchange factor of p115 RhoGEF by Ga13. *Science* 280, 2112–2114

11. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) p115 RhoGEF, a GTPase activating protein for Ga12 and Ga13. *Science* 280, 2109–2111

12. Gan, X., Wang, J., Wang, C., Sommer, E., Kozasa, T., Srinivasula, S., Alessi, D., Offermanns, S., Simon, M. I., and Wu, D. (2012) PRR5L degradation promotes mTORC2-mediated PKC-δ phosphorylation and cell migration downstream of Ga12. *Nat. Cell Biol.* 14, 686–696

13. Anliker, B., and Chun, J. (2004) Cell surface receptors in lysophospholipid signaling. *Semin. Cell Dev. Biol.* 15, 457–465

14. Tager, A. M., LaCerrera, P., Sheu, B. S., Campanella, G. S., Selman, M., Zhao, Z., Polosukhin, V., Wain, J., Karimi-Shah, B. A., Hart, W. K., Pardo, A., Blackwell, T., Xu, Y., Chun, J., and Luster, A. D. (2008) The lysophosphatidic acid receptor LPA1 links pulmonary fibrosis to lung injury by mediating fibroblast recruitment and vascular leak. *Nat. Med.* 14, 45–54

15. Roskoski, R., Jr. (2010) RAF protein-serine/threonine kinases. Structure and regulation. *Biochem. Biophys. Res. Commun.* 399, 313–317

16. Udell, C. M., Rajakulendran, T.,icher, F., and Therrien, M. (2011) Mechanistic principles of RAF kinase signaling. *Cell. Mol. Life Sci.* 68, 553–565

17. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997) Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. *J. Biol. Chem.* 272, 4378–4383

18. Bajlul, A., Mueller, T., Dreuxler, H. C., Hekman, M., and Rapp, U. R. (2007) Unique N-region determines low basal activity and limited inducibility of...
Regulation of RFFL-mediated Signaling by Raf Kinases

19. Chen, Z., Singer, W. D., Sternweis, P. C., and Sprang, S. R. (2005) Structure of the p115RhoGEF rgRGS domain-Gα13/i1 chimera complex suggests convergent evolution of a GTPase activator. Nat. Struct. Mol. Biol. 12, 191–197

20. Tesmer, J. I., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) Crystal structure of the catalytic domains of adenyl cyclase in a complex with Gαs·GTPγS. Science 278, 1907–1916

21. Slep, K. C., Kercher, M. A., He, W., Cowan, C. W., Wensel, T. G., and Sigler, P. B. (2001) Structural determinants for regulation of phosphodiesterase by a G protein at 2.0 A. Nature 409, 1071–1077

22. Kreutz, B., Yau, D. M., Nance, M. R., Tanabe, S., Tesmer, J. I., and Kozasa, T. (2006) A new approach to producing functional Gα subunits yields the activated and deactivated structures of Gα(12/13) proteins. Biochem. 45, 167–174