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Critical Role of Lysine 204 in Switch I Region of $G\alpha_{13}$ for Regulation of p115RhoGEF and Leukemia-Associated RhoGEF

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ABSTRACT

Heterotrimeric G proteins of the G12 family regulate the Rho GTPase through RhoGEFs that contain an amino-terminal regulator of G protein signaling (RGS) domain (RGS-RhoGEFs). Direct regulation of the activity of RGS-RhoGEFs p115 or leukemia-associated RhoGEF (LARG) by $G\alpha_{13}$ has previously been demonstrated. However, the precise biochemical mechanism by which $G\alpha_{13}$ stimulates the RhoGEF activity of these proteins has not yet been well understood. Based on the crystal structure of $G\alpha_{i1}$ in complex with RGS4, we mutated the $G\alpha_{13}$ residue lysine 204 to alanine ($G\alpha_{13}$K204A) and characterized the effect of this mutation in its regulation of RGS-RhoGEFs p115 or LARG. Compared with wild-type $G\alpha_{13}$, $G\alpha_{13}$K204A induced much less serum-response factor activation when expressed in HeLa cells. Recombinant $G\alpha_{13}$K204A exhibits normal function in terms of nucleotide binding, basal GTP hydrolysis, and formation of heterotrimer with $\beta\gamma$. We found that lysine 204 of $G\alpha_{13}$ is important for interaction with the RGS domain of p115 or LARG and for the GTPase-activating protein activity of these proteins. In addition, the K204A mutation of $G\alpha_{13}$ impaired its regulation of the RhoGEF activity of p115 or LARG. We conclude that lysine 204 of $G\alpha_{13}$ is important for interaction with RGS-RhoGEFs and is critically involved in the regulation of their activity.

Heterotrimeric G proteins are composed of α, β, and γ subunits and are activated by members of the seven-transmembrane helix family of receptors (G protein-coupled receptors) (Kaziro et al., 1991; Hepler and Gilman, 1992). Upon agonist binding, activated receptor catalyzes GDP-GTP exchange on the α subunit. Nucleotide exchange induces conformational changes at three switch regions of the Gα subunit and facilitates the dissociation of GTP-bound Gα from $\beta\gamma$ subunits. Both GTP-bound Gα subunit and free $\beta\gamma$ subunits have the capacity to regulate various downstream effectors. The Gα subunit hydrolyzes bound GTP to GDP by its intrinsic GTPase activity, and this rate is accelerated by the presence of GTPase-activating proteins (GAPs), such as regulators of G protein signaling (RGS) proteins (Hollinger and Hepler, 2002). The signaling event is terminated when the GDP-bound Gα subunit reassociates with the $\beta\gamma$ subunit to form the inactive heterotrimer. In this signaling system, the strength and duration of the signal is determined by the precise control of the amount of GTP-bound Gα subunit.

α Subunits of G12 and G13 have been shown to transduce signals from G protein-coupled receptors to Rho activation (Aragay et al., 1995; Gohla et al., 1998; Kranenburg et al., 1999). It is well established that Rho family monomeric GTPases are involved in various cellular functions through regulation of the actin cytoskeleton and gene expression (Hall, 1998; Schmidt and Hall, 2002). We have identified that RhoGEFs that contain an RGS domain within their amino-terminal region (RGS-RhoGEFs) constitute direct links between heterotrimeric G12/13 and the Rho GTPase (Hart et al., 1998; Kozasa et al., 1998; Suzuki et al., 2003). Currently, three mammalian RhoGEFs, p115RhoGEF, PDZ-RhoGEF/GTRAP48, and LARG, have been isolated in this RGS-RhoGEF subfamily. The RGS domain of each of these RhoGEFs specifically interacts with $G\alpha_{12}$ and $G\alpha_{13}$ (Kozasa et al., 1998; Fukuhara et al., 1999; Booden et al., 2002). GAP activ-

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ABBREVIATIONS: GAP, GTPase-activating protein; RGS, regulator of G protein signaling; GEF, guanine nucleotide exchange factor; PDZ, PSD-95/Dlg/ZO-1 homology; PDE, phosphodiesterase; LARG, leukemia-associated RhoGEF; SRE, serum-response element; DMEM, Dulbecco's modified Eagle's medium; IP, immunoprecipitation; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; NTA, nitrilotriacetic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; SRF, serum-response factor; DH, Dbl homology; PH, pleckstrin homology.

ity of the RGS domain of p115RhoGEF or LARG for $G\alpha_{12}$ and $G\alpha_{13}$ has been demonstrated (Kozasa et al., 1998; Suzuki et al., 2003). In addition, these RhoGEFs serve as direct effectors of these $G\alpha$ subunits. In vitro reconstitution experiments using purified components demonstrated that the active form of $G\alpha_{13}$ stimulates Rho activation through p115RhoGEF or LARG (Hart et al., 1998; Suzuki et al., 2003).

Members of the RGS family share a homologous domain (RGS domain) of about 120 residues. It has been demonstrated biochemically that the RGS domain of several family members possesses GAP activity for $G\alpha$ subunits, most of them for α subunits of the Gi/o or Gq subfamilies (Hollinger and Hepler, 2002). The crystal structure of the complex of $G\alpha_{i1}$ with the RGS domain of RGS4 suggested that the RGS domain functions as a GAP by stabilizing the transition state of GTP hydrolysis of the $G\alpha$ subunit (Tesmer et al., 1997). In this structure, the RGS domain makes extensive direct contacts with the switch regions of $G\alpha_{i1}$. In particular, threonine 182 of $G\alpha_{i1}$ forms critical contacts with several amino acid residues of the RGS domain of RGS4. This threonine residue is conserved at the corresponding region of all $G\alpha$ subunits except for $G\alpha_s$ and $G\alpha_{12/13}$, with $G\alpha_{12}$ and $G\alpha_{13}$ each containing a lysine residue at this position. In the $G\alpha_{i1}$-RGS4 complex, the side chain of a lysine residue cannot be accomodated in the position of threonine 182, supporting biochemical evidence that RGS4 does not act as a GAP for $G\alpha_{12}$ (Berman et al., 1996).

Although the amino acid sequence homology of the RGS domains of RGS-RhoGEFs with the RGS domain of RGS4 is low, the recently solved crystal structures of the RGS domain of p115RhoGEF or PDZ-RhoGEF demonstrated that the overall three-dimensional structure of the RGS domain is well conserved in the RGS domains of these RhoGEFs (Chen et al., 2001; Longenecker et al., 2001). We thus tested the hypothesis that lysine 204 of $G\alpha_{13}$, which corresponds to threonine 182 of $G\alpha_{i1}$, is important for interaction with the RGS domain of RGS-RhoGEFs. In the present study, we have investigated this possibility using biochemical reconstitution assays. Although lysine 204 of $G\alpha_{13}$ is not required for nucleotide binding or heterotrimer formation, we found that it is important for interaction with p115RhoGEF or LARG and is essential for the regulation of their activity.

Materials and Methods

Expression Constructs. The $G\alpha_{13}$K204A point-mutant was created by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), according to manufacturer's instructions. Primers used to generate the K204A mutation were the following (mutated bases underlined): 5′-GCTTGCCAGAAGGCCC ACTGCAGGCATCCATGAGTACG-3′ and 5′-CGTACTCATGGATGCCTGCAGTGGGC CTTCTGGCAAGC-3′. pCMV5-$G\alpha_{13}$ (wild-type), pCMV5-$G\alpha_{13}$K204A, pCMV5-$G\alpha_{13}$Q226L, pcDNAmyc-RGSp115 (amino acids 1–252), and pcDNAmyc-ΔPDZ-LARG (amino acids 307-1543; Suzuki et al., 2003) constructs were used for the expression of respective proteins in serum-response element (SRE)-luciferase or coimmunoprecipitation studies. The pGL3-SRE.L reporter construct used for SRE-luciferase assays was kindly provided by Dr. Paul Sternweis (University of Texas Southwestern Medical Center, Dallas, TX). The glutathione *S*-transferase-rhotekin RBD construct was kindly provided by Dr. Gary Bokoch (Scripps Research Institute, La Jolla, CA). Generation of the baculovirus transfer vector for expression of His_6-LARG has been described previously (Suzuki et al., 2003). $G\alpha_{13}$K204A was subcloned into pFastBac1 for preparation of its baculovirus. Each construct was confirmed by DNA sequencing.

SRE-Luciferase and Rho GTP Pulldown Assays. HeLa cells were maintained in DMEM/10% fetal bovine serum and were passaged to 24-well plates at a density of 7×10^4 cells per well, 1 day before transfection. Transfections were performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). For all conditions, cells were transfected with pGL3-SRE.L reporter (0.1 μg) and pCMV5-β-galactosidase (0.1 μg). To indicated wells, cells were additionally transfected with pCMV5-$G\alpha_{13}$ wild-type, pCMV5-$G\alpha_{13}$K204A, or pCMV5-$G\alpha_{13}$Q226L (0.01 μg). After 6 h, media were changed to fresh serum-free DMEM, and cells were harvested 24 h post-transfection. Luciferase activity of cell extracts was quantified according to manufacturer's instructions (Promega, Madison, WI). Total amount of plasmids transfected per well was balanced by addition of empty vector. β-Galactosidase activity in cell extracts was used to normalize for transfection efficiency. Rho GTP pulldown assays using GST-rhotekin RBD were performed as described previously (Ren and Schwartz, 2000). HeLa cells (6×10^6/condition) were cultured in 100-mm plates and transfected with 10 μg of indicated expression plasmids. After 24 h, media were changed to fresh serum-free DMEM, and cells were harvested 48 h post-transfection. Immunoblot analyses were performed using either anti-RhoA (26C4; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-$G\alpha_{13}$ (A-20; Santa Cruz Biotechnology, Inc.) antibodies.

Coimmunoprecipitation Studies. COS-1 cells were cultured in 100-mm plates to a density of 6×10^6 cells per plate. Cells were transfected with either 5 μg of pcDNA3.1-myc-ΔPDZ-LARG or pCMV5-myc-RGSp115 (residues 1–252), in combination with either 0.5 μg of pCMV5-$G\alpha_{13}$ wild type, pCMV5-$G\alpha_{13}$Q226L, pCMV5-$G\alpha_{13}$K204A, or pCMV5-$G\alpha_{13}$K204A/Q226L. Cells were harvested 24 h after transfection and lysed in 500 μl of ice-cold IP buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 5 μM GDP, 10 mM Na_3VO_4, 10 mM β-glycerophosphate, 0.7% Triton X-100, 16 μg/ml phenylmethylsulfonyl fluoride, 16 μg/ml *N*-tosyl-L-phenylalanine-chloromethyl ketone, 16 μg/ml *N*-tosyl-L-lysine-chloromethyl ketone, 3.2 μg/ml leupeptin, and 3.2 μg/ml lima bean trypsin inhibitor). To indicated samples, 30 μM $AlCl_3$ and 5 mM NaF (AlF_4^-) were added. Soluble lysates (100,000*g*, 20 min, 4°C) were first precleared by incubation with protein G-Sepharose for 30 min at 4°C, and then incubated for 1 h at 4°C with protein G-Sepharose coupled to monoclonal anti-myc antibody (9E10; Covance, Richmond, CA). Beads were pelleted (10,000*g*, 5 min, 4°C) and washed three times with IP buffer (with or without AlF_4^-). Finally, beads were boiled in SDS-PAGE sample buffer. Protein samples either bound to beads or in total lysates were resolved by SDS-PAGE. Immunoblot analyses were performed using specific antibodies raised against either the myc epitope tag (9E10) or $G\alpha_{13}$ (B-859; Singer et al., 1994).

Expression and Purification of Recombinant Proteins. Recombinant baculoviruses were prepared and amplified using the Bac-to-Bac system (Invitrogen). Mutant $G\alpha_{13}$K204A was purified from the membranes of Sf9 cells coinfected with baculoviruses encoding $G\alpha_{13}$K204A, β_1, and His_6-γ_2, using methods described previously for purification of wild-type $G\alpha_{13}$ (Kozasa, 1999). Glu-Glu-tagged p115RhoGEF or His_6-LARG were expressed and purified from Sf9 cells using either anti-Glu-Glu (Covance) immunoaffinity or nickel-NTA (QIAGEN, Valencia, CA) immobilized metal affinity chromatography, respectively (Suzuki et al., 2003). Recombinant His_6-RhoA used in RhoGEF assays was purified from Sf9 cells infected with baculovirus encoding His_6-RhoA. Cell pellets from 1 liter of culture were resuspended in 200 ml of lysis buffer (20 mM Na-HEPES, pH 7.4, 10 mM 2-mercaptoethanol, 50 mM NaCl, 1 mM $MgCl_2$, 10 μM GDP, and proteinase inhibitors) and lysed by nitrogen cavitation for 30 min at 4°C. Lysates were centrifuged (1000*g*, 15 min, 4°C), and the supernatant was extracted with 1% cholate for 1 h on ice. After ultracentrifugation (100,000*g*, 30 min, 4°C), the supernatant was loaded onto a 1-ml nickel-NTA column equilibrated with 10 volumes of buffer A (lysis buffer supplemented with 1% cholate).

The column was washed with 30 volumes of buffer B (lysis buffer supplemented with 400 mM NaCl, 10 mM imidazole, and 0.5% cholate). Recombinant His_6-RhoA was eluted in 5 fractions of 1 volume of buffer C (lysis buffer supplemented with 100 mM NaCl, 100 mM imidazole, and 1% cholate). Peak elution fractions containing His_6-RhoA were pooled and exchanged to buffer D (20 mM Na-HEPES, pH 7.4, 1 mM DTT, 100 mM NaCl, 1 mM $MgCl_2$, 1 μM GDP, and 1% cholate) using a Centricon YM-10 unit (Millipore Corporation, Billerica, MA). Finally, octyl-β-D-glucopyranoside was added to a final concentration of 1% (Calbiochem, San Diego, CA).

GTPγS Binding Assays. GTPγS binding to $G\alpha_{13}$ or $G\alpha_{13}$K204A (1 pmol) was measured at 30°C in binding buffer (50 mM Na-HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 0.05% $C_{12}E_{10}$, 10 μM GTPγS, and 2000 cpm/pmol [^{35}S]GTPγS). Fifty-microliter aliquots were withdrawn at the indicated time points and mixed with wash buffer containing 10 mM $MgSO_4$ to terminate reactions. Samples were applied to BA-85 filters (Schleicher & Schuell, Keene, NH) and filtered under vacuum. After washing three times, radioactivity remaining on filters was measured by liquid scintillation counting. The inhibitory effect of G$\beta\gamma$ on GTPγS binding to $G\alpha_{13}$ wild-type or $G\alpha_{13}$K204A (2.5 pmol) was evaluated in the same buffer described above. Samples were incubated for 90 min at 30°C, either in the absence or presence of purified $\beta_1\gamma_2$ (7.5 pmol).

GTPase Assays. Single-turnover GTP hydrolysis activity of $G\alpha_{13}$ wild-type or $G\alpha_{13}$K204A mutant was assessed essentially as described previously (Kozasa et al., 1998). Thirty picomoles of recombinant, purified $G\alpha_{13}$ or $G\alpha_{13}$K204A protein was loaded with γ-[^{32}P]GTP (50–100 cpm/fmol) for 40 min at 30°C in the presence of 5 mM EDTA and 5 μM GTP. Samples were rapidly gel filtered through Sephadex G-50 (Amersham Biosciences Inc., Piscataway, NJ) to remove unbound nucleotide and free ^{32}P-labeled phosphate, and GTP hydrolysis at 15°C was monitored after the addition of γ-[^{32}P]GTP-labeled Gα protein to the reaction mixture (50 mM Na-HEPES, pH 8.0, 1 mM DTT, 5 mM EDTA, 8 mM $MgSO_4$, 1 mM GTP, 0.05% $C_{12}E_{10}$, and 100 nM either EE-p115-RhoGEF or His_6-LARG). Fifty-microliter aliquots were taken at the indicated time points and mixed with 750 μl of 5% (w/v) NoritA in 50 mM NaH_2PO_4. Radioactivity in the supernatants after centrifugation (1200*g*, 10 min, 4°C) was measured by liquid scintillation counting.

RhoGEF Assays. $G\alpha_{13}$ or $G\alpha_{13}$K204A (1.5 pmol) was first incubated in the presence of 60 μM $AlCl_3$, 5 mM $MgCl_2$, and 20 mM NaF for 15 min at 0°C, and then incubated with His_6-RhoA (25 pmol) in the presence of indicated proteins at 30°C in binding buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.5 mM EDTA, 50 mM NaCl, 5 mM $MgCl_2$, 0.05% $C_{12}E_{10}$, 10 μM GTPγS, and ~500 cpm/pmol [^{35}S]GTPγS), in a final reaction volume of 50 μl. Binding reactions were terminated by addition of wash buffer containing 10 mM $MgSO_4$, followed by filtration through BA-85 nitrocellulose filters. After washing three times, radioactivity remaining on filters was measured by liquid scintillation counting.

Miscellaneous Procedures. Statistical significance was assigned based on results of *t* test analyses of data.

Results

$G\alpha_{13}$K204A Shows Impaired Serum-Response Factor (SRF) Activation in Cells. To analyze the functional role of lysine 204 of $G\alpha_{13}$ in the regulation of RGS-RhoGEF activity, we first examined whether $G\alpha_{13}$K204A could stimulate Rho activity in cells similar to wild-type $G\alpha_{13}$. Transcription from a SRE-luciferase reporter gene in HeLa cells was measured as an index of Rho activation (Fromm et al., 1997). When wild-type $G\alpha_{13}$ or the $G\alpha_{13}$K204A mutant was examined in this assay, $G\alpha_{13}$K204A activated SRF to less than one-half of the extent of wild-type $G\alpha_{13}$, although these proteins were expressed at similar levels in transfected cells (Fig. 1, A and B). We directly assessed the activity of Rho in cells expressing forms of $G\alpha_{13}$ by Rho-GTP pulldown assays (Ren and Schwartz, 2000). Compared with HeLa cells expressing wild-type $G\alpha_{13}$, Rho activity was reduced in the lysate of cells expressing $G\alpha_{13}$K204A (Fig. 1C). These data suggest that $G\alpha_{13}$K204A exhibits a defect in stimulating Rho activation through effector RGS-RhoGEFs in cells. Lysine 204 of $G\alpha_{13}$ may therefore be involved in the mechanism of RhoGEF activation in cells.

$G\alpha_{13}$K204A Is Defective for RGS-RhoGEF Interaction. It is well established that $G\alpha_{13}$ interacts with the RGS domains of p115RhoGEF or LARG in an activation-dependent manner (Kozasa et al., 1998; Booden et al., 2002). Thus, we next examined whether KA mutation of $G\alpha_{13}$ affects its physical interaction with these RGS domains. Either wild-type $G\alpha_{13}$ or $G\alpha_{13}$K204A was coexpressed in COS-1 cells together with myc-tagged RGS domain of p115RhoGEF or ΔPDZ-LARG, a construct of LARG that lacks the amino-terminal PDZ domain but includes the RGS, DH, and PH domain. The binding of wild-type $G\alpha_{13}$ or $G\alpha_{13}$K204A to the RGS domain of RGS-RhoGEFs was examined by coimmunoprecipitation either in the absence or presence of AlF_4^-. Compared to wild-type $G\alpha_{13}$, AlF_4^--activated $G\alpha_{13}$K204A interacts poorly with the RGS domain of either p115RhoGEF or LARG (Fig. 2). Thus, lysine 204 of $G\alpha_{13}$ seems to be important for its activation-dependent interaction with RGS-RhoGEFs through the RGS domain. In contrast to previous reports, we could not detect the interaction of $G\alpha_{13}$Q226L with RGS-RhoGEFs under the experimental conditions described

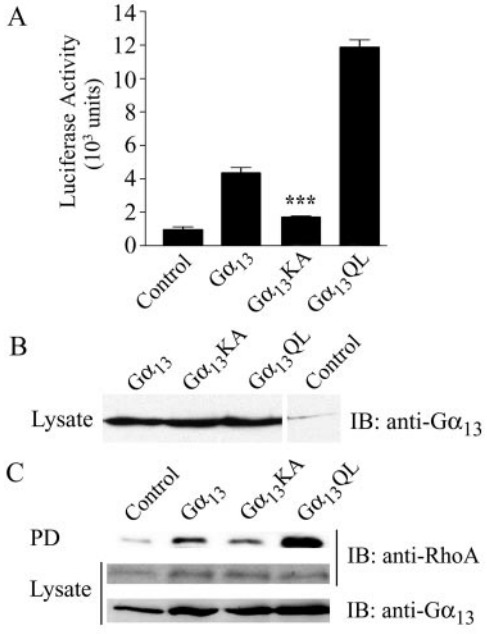

Fig. 1. SRF activation by $G\alpha_{13}$ or $G\alpha_{13}$K204A in cells. A, HeLa cells were transfected with plasmid constructs directing the expression of indicated proteins. Luciferase activity of lysates is represented as the mean (±S.E.) of triplicate samples, which was normalized for protein expression. Statistical comparison of $G\alpha_{13}$ and $G\alpha_{13}$K204A data indicate significant difference (***, $p < 0.001$). Data shown are from a single experiment representative of three independent experiments having similar results. B, lysates of HeLa cells transfected with empty vector (Control) or vector directing the expression of the indicated form of $G\alpha_{13}$ were immunoblotted with anti-$G\alpha_{13}$ antibody. C, Rho-GTP pulldown (PD) from HeLa cells expressing indicated proteins. HeLa cells were transfected with either pCMV5 vector (Control) or with pCMV5-$G\alpha_{13}$, $G\alpha_{13}$K204A, or $G\alpha_{13}$Q226L. Data shown are from a single experiment representative of three independent experiments having similar results.

above (Fukuhara et al., 2000). This suggests that the transition state of $G\alpha_{13}$ exhibits a higher affinity for these RGS domains than does the activated state, as was reported for RGS4-$G\alpha_{i1}$ interaction (Berman et al., 1996).

$G\alpha_{13}$K204A Is Active as a Gα Subunit. To further characterize the functional role of lysine 204 of $G\alpha_{13}$ in $G\alpha_{13}$-RhoGEF interaction, recombinant $G\alpha_{13}$K204A was purified from Sf9 cells coinfected with baculoviruses encoding $G\alpha_{13}$K204A, $G\beta_1$, and His_6-$G\gamma_2$, following the purification methods described previously (Kozasa, 1999). $G\alpha_{13}$K204A apparently interacts with $\beta_1\gamma_2$ in membrane extracts of Sf9 cells, because $G\alpha_{13}$K204A was retained on the nickel-NTA column with His_6-$\beta_1\gamma_2$, and could be eluted in an AlF_4^--dependent manner (Fig. 3A). Further biochemical assays using purified, recombinant $G\alpha_{13}$K204A confirmed these observations. In the G protein heterotrimer, G$\beta\gamma$ inhibits GDP dissociation from Gα and thus inhibits its subsequent binding to GTP. To confirm whether purified $G\alpha_{13}$K204A could interact with $\beta\gamma$, we examined whether $\beta\gamma$ inhibits GTPγS binding to $G\alpha_{13}$K204A. Addition of purified $\beta_1\gamma_2$ inhibited GTPγS binding to either $G\alpha_{13}$K204A or wild-type $G\alpha_{13}$ to a similar extent (Fig. 3B). $G\alpha_{13}$K204A bound GTPγS similar to wild-type $G\alpha_{13}$ over the time course analyzed (Fig. 3C). Furthermore, the intrinsic GTPase activity of the $G\alpha_{13}$K204A mutant was similar to wild-type $G\alpha_{13}$ (Fig. 5). Although K204A mutation of $G\alpha_{13}$ disrupts its interaction with the RGS domains of p115 or LARG, these data suggest that the basic properties of $G\alpha_{13}$, such as guanine nucleotide binding, GTP hydrolysis, and interaction with G$\beta\gamma$ subunits, are not affected by K204A mutation.

$G\alpha_{13}$K204A Has an Impaired Response to the GAP Activity of RGS-RhoGEFs. The RGS-RhoGEF proteins p115RhoGEF and LARG are GAPs specific for $G\alpha_{13}$ and $G\alpha_{12}$ (Kozasa et al., 1998; Suzuki et al., 2003). Because we detected the defective binding of $G\alpha_{13}$K204A to the RGS domain by coimmunoprecipitation studies, we next analyzed whether these RGS-RhoGEF proteins could function as GAPs for the $G\alpha_{13}$K204A mutant. Purified proteins used in these and subsequent reconstitution experiments are shown (Fig. 4). In single-turnover GTPase assays of $G\alpha_{13}$, GAP activity of each of these RGS domains for $G\alpha_{13}$ was severely impaired with K204A mutation, even at the high concentration of RGS domain (100 nM) used in these assays (Fig. 5). These results indicate that lysine 204 of $G\alpha_{13}$ is critically involved in the GAP reaction with the RGS domain of p115RhoGEF or LARG.

$G\alpha_{13}$K204A Fails to Stimulate the RhoGEF Activity of p115 or LARG. We also examined whether $G\alpha_{13}$K204A could stimulate the RhoGEF activity of p115 or LARG. RhoGEF activity was measured as the activity to facilitate GTPγS binding to Rho after GDP release in reconstitution assays. In these assays, the binding of GTPγS to $G\alpha_{13}$ was negligible in the presence of AlF_4^- (data not shown).

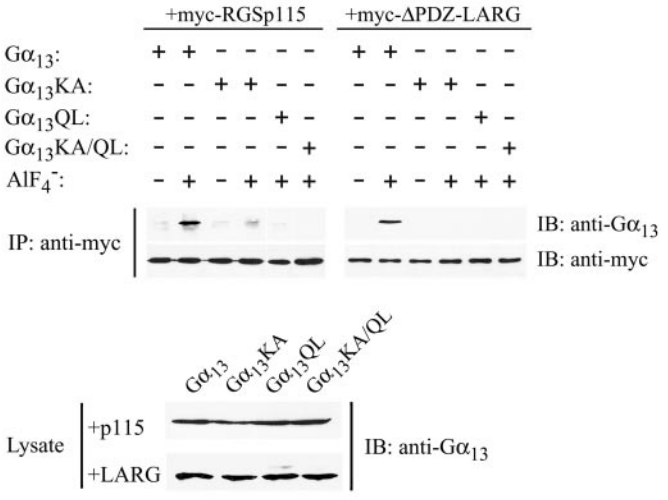

Fig. 2. $G\alpha_{13}$K204A is defective for interaction with the RGS domain of p115RhoGEF or LARG. Top, COS-1 cells were transfected with expression constructs of $G\alpha_{13}$, $G\alpha_{13}$Q226L, $G\alpha_{13}$K204A, or $G\alpha_{13}$K204A/Q226L together with either myc-tagged RGS domain of p115RhoGEF (RGSp115) (left) or ΔPDZ-LARG (right). RhoGEFs were immunoprecipitated (IP) from lysates using anti-myc antibody, either in the presence or absence of AlF_4^-. Immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting (IB) with either anti-$G\alpha_{13}$ antibody or anti-myc antibody. Bottom, COS-1 cell lysates used in coimmunoprecipitation experiments were immunoblotted with anti-$G\alpha_{13}$ antibody.

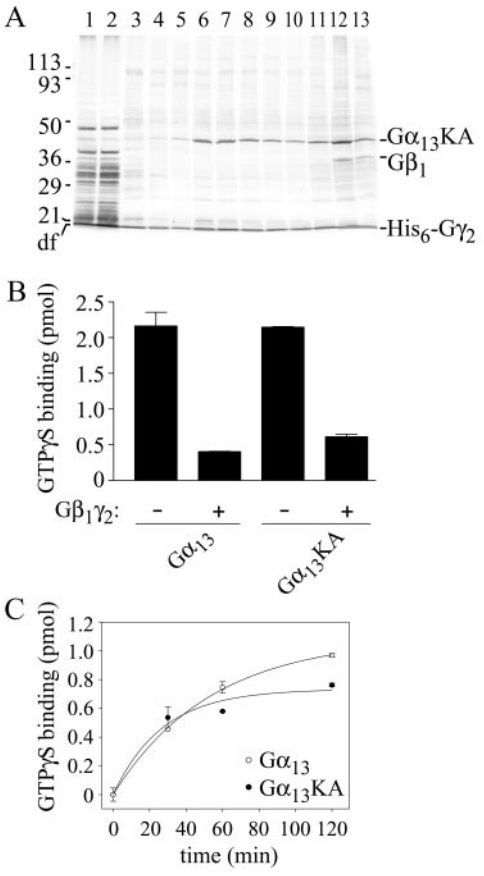

Fig. 3. Purification of $G\alpha_{13}$K204A and characterization as a G protein α subunit. A, purification of $G\alpha_{13}$K204A was performed using His_6-tagged $\beta_1\gamma_2$ immobilized on Ni-NTA resin. Lane 1, load; lane 2, flow-through; lane 3, salt wash; lane 4, wash with octylglucoside; lanes 5–10, elution with AlF_4^-; and lanes 11–13, elution with 150 mM imidazole. Ten-microliter fractions were resolved by SDS-PAGE followed by staining with silver nitrate. $G\beta_1$ is negatively stained by silver nitrate and His_6-γ_2 runs at the dye front (df). The apparent molecular weight of standard proteins is in kilodaltons. B, GTPγS binding of $G\alpha_{13}$ or $G\alpha_{13}$K204A (2.5 pmol) was measured in the absence or presence of purified $\beta_1\gamma_2$ (7.5 pmol). Reactions were incubated for 90 min at 30°C. Data are represented as the mean (±S.E.) of triplicate samples. C, time course of GTPγS binding to $G\alpha_{13}$ or $G\alpha_{13}$K204A. $G\alpha_{13}$ (○) or $G\alpha_{13}$K204A (●) was mixed with binding buffer and incubated at 30°C, and duplicate samples were taken at the indicated time points. Data are represented as the mean (±S.E.) of duplicate samples.

$G\alpha_{13}$K204A did not demonstrate RhoGEF activation of either p115RhoGEF or LARG (Fig. 6). The dose-response curve of the $G\alpha$ subunit for p115RhoGEF activation indicated that even at higher concentrations (100 nM), the $G\alpha_{13}$K204A mutant could not stimulate the RhoGEF activity of p115 (data not shown).

Discussion

In the present study, we have identified an amino acid residue of $G\alpha_{13}$ that is critically involved in the regulation of RGS-RhoGEFs. Mutating lysine 204 in the switch I region of $G\alpha_{13}$ to alanine severely affected its binding to p115RhoGEF or LARG when coexpressed in cells. The $G\alpha_{13}$K204A mutant also demonstrated a defective response to the GAP activity of the RGS domain of p115 or LARG. In addition, $G\alpha_{13}$K204A could not stimulate the RhoGEF activity of p115 or LARG.

In the crystal structure of the complex of $G\alpha_{i1}$-RGS4, threonine 182 of $G\alpha_{i1}$ makes contacts with multiple residues of RGS4 and is considered to play a critical role during GTP hydrolysis (Tesmer et al., 1997). This threonine residue in the switch I region is highly conserved among different $G\alpha$ subunits except in the case of $G\alpha_{12}$ or $G\alpha_{13}$, where it corresponds to lysine 204 in $G\alpha_{13}$ or lysine 207 in $G\alpha_{12}$. In the complex of $G\alpha_{i1}$-RGS4, a lysine residue cannot be accommodated in the position of threonine 182, supporting evidence that $G\alpha_{12}$ or $G\alpha_{13}$ is not a target for the GAP activity of RGS4 (Berman et al., 1996). Recent crystal structures of the RGS domain of p115RhoGEF or PDZ-RhoGEF demonstrated that their three-dimensional structures are largely similar to the RGS domain of RGS4, despite the low amino acid sequence homology between the RGS domains of RGS-RhoGEFs and RGS4 (Chen et al., 2001; Longenecker et al., 2001). Mutagenesis studies of the RGS domain of p115 also demonstrated the global similarity of these interaction surfaces (Chen et al., 2003). These results, together with the results of the present study, suggest that K204 of $G\alpha_{13}$ will be the critical residue of $G\alpha_{13}$ to interact with the RGS domain of p115 or LARG and that it will have a functional role similar to threonine 182 of $G\alpha_{i1}$ in the GTP hydrolysis reaction of $G\alpha_{13}$.

Even though the RGS domain of RGS-RhoGEFs is clearly involved in the interaction with $G\alpha_{13}$, recent evidence suggests that the region containing DH-PH domain may also interact with $G\alpha_{13}$. In the case of p115RhoGEF, coimmunoprecipitation experiments demonstrated that $G\alpha_{13}$ could bind to a truncated protein consisting of the DH-PH domain but lacking the RGS domain (Wells et al., 2002). Similar interaction was also detected for $G\alpha_{13}$ with a LARG construct lacking the amino-terminal RGS domain (N. Suzuki and T. Kozasa, unpublished observations). Thus, although the major interaction is mediated through the RGS domain, $G\alpha_{13}$ likely interacts through the DH-PH domain of RGS-RhoGEF as well. In the present study, disruption of the interaction of

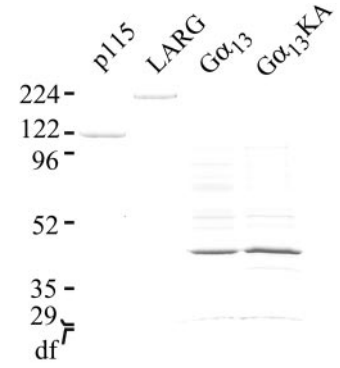

Fig. 4. Purified proteins used in reconstitution assays. Purified p115RhoGEF, LARG, $G\alpha_{13}$, and $G\alpha_{13}$K204A (10 pmol each) were resolved by SDS-PAGE and stained by Coomassie Brilliant Blue. The apparent molecular weight of standard proteins is in kilodaltons.

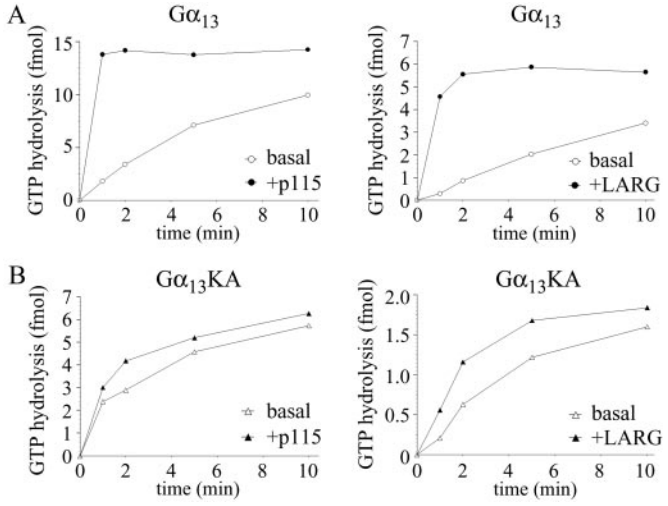

Fig. 5. Impaired GAP activity of p115RhoGEF or LARG toward $G\alpha_{13}$K204A. Single-turnover hydrolysis of GTP bound to $G\alpha_{13}$ (A) or $G\alpha_{13}$K204A (B) was measured over a 10-min time course at 15°C, in the absence (open symbols) or presence (filled symbols) of 100 nM p115RhoGEF or LARG, as indicated.

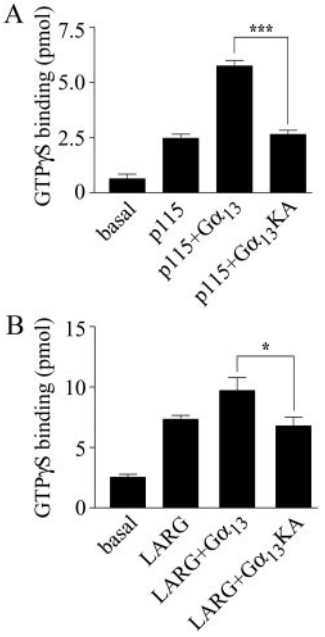

Fig. 6. Defective stimulation of the RhoGEF activity of p115RhoGEF or LARG by $G\alpha_{13}$K204A. GTPγS binding to Rho (25 pmol) was measured after incubation at 30°C for 10 min under the following conditions: basal, in the presence of the indicated RhoGEF, or in the presence of the indicated RhoGEF and AlF_4^--activated $G\alpha_{13}$ or $G\alpha_{13}$K204A. Final concentration in reaction: 500 nM Rho, 30 nM $G\alpha_{13}$ or $G\alpha_{13}$K204A, 5 nM p115RhoGEF, and 2 nM ΔPDZ-LARG. Data are represented as the mean (±S.E.). Statistical comparisons: ***, $p < 0.001$ calculated from data obtained from four independent experiments having similar results; *, $p < 0.05$ calculated from data obtained from four independent experiments having similar results.

$G\alpha_{13}$ with the RGS domain of RhoGEFs severely affected the regulation of GEF activity by $G\alpha_{13}$. It is possible that interaction of $G\alpha_{13}$ with the RGS domain may positively regulate the interaction of $G\alpha_{13}$ with the DH-PH domain-containing region. The structure of $G\alpha_t$ complexed with its effector, PDEγ, and its GAP, RGS9-1, demonstrated that $G\alpha_t$ interacts with RGS9-1 and PDEγ using separate surfaces of its switch regions (Slep et al., 2001). It was also recently demonstrated that the affinity of RGS9-1 for $G\alpha_t$ is enhanced in the presence of its effector PDEγ (Skiba et al., 2000). Furthermore, studies using mice that lack the RGS9–1 gene indicate that RGS9-1 is required for the precise temporal regulation of PDEγ activity by $G\alpha_t$ (Chen et al., 2000). Thus, it is likely that some Gα subunits maintain separate interaction surfaces for the RGS domain and the effector domain and that both interactions are necessary for the proper regulation of effector activity. Further investigation will be necessary to examine the functional role of the interaction of RhoGEFs with $G\alpha_{13}$ through the DH-PH domain-containing region.

Although the K204A mutant of $G\alpha_{13}$ is defective for interaction with its effector molecules, it can still interact with the $\beta\gamma$ subunit and form the G protein heterotrimer. Since the K204A mutation is not localized within the possible receptor-interacting region, it is likely that the heterotrimer containing $G\alpha_{13}$K204A would interact with receptor similar to the wild-type G13 heterotrimer. Therefore, this mutant may function as a dominant-negative mutant of $G\alpha_{13}$ when expressed in cells. Overexpressed $G\alpha_{13}$K204A is expected to form heterotrimers with endogenous $\beta\gamma$ subunits and will likely attenuate the receptor-G13 mediated response without affecting the G12-mediated pathway. Thus, $G\alpha_{13}$K204A and possibly the similar mutant of $G\alpha_{12}$ may become useful reagents to analyze G13- or G12-specific cellular responses. This possibility will be examined using the $G\alpha_{13}$-mediated cellular pathway, such as lysophosphatidic acid-induced Rho activation (Gohla et al., 1999; Kranenburg et al., 1999).

In this study, we have presented evidence to support a critical role of lysine 204 of $G\alpha_{13}$ for RGS-RhoGEF interaction. To further understand the interaction between $G\alpha_{13}$ and RGS-RhoGEFs in detail, determination of the crystal structure of the complex of $G\alpha_{13}$ with an RGS-RhoGEF will be the next important step. The structure of this complex will not only elucidate the molecular mechanism for Rho activation by the heterotrimeric G protein G13 but also provide further insight into the regulation of G protein-mediated signaling by RGS proteins.

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