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Ric-8B Stabilizes the α Subunit of Stimulatory G Protein by Inhibiting Its Ubiquitination*[S]

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The α subunit of stimulatory G protein ($G\alpha_s$) activates adenylyl cyclase, which catalyzes cAMP production, and regulates many physiological aspects, such as cardiac regulation and endocrine systems. Ric-8B (resistance to inhibitors of cholinesterase 8B) has been identified as the $G\alpha_s$-binding protein; however, its role in G_s signaling remains obscure. In this study, we present evidence that Ric-8B specifically and positively regulates G_s signaling by stabilizing the $G\alpha_s$ protein. An *in vitro* biochemical study suggested that Ric-8B does not possess guanine nucleotide exchange factor activity. However, knockdown of Ric-8B attenuated β-adrenergic agonist-induced cAMP accumulation, indicating that Ric-8B positively regulates G_s signaling. Interestingly, overexpression and knockdown of Ric-8B resulted in an increase and a decrease in the $G\alpha_s$ protein, respectively, without affecting the $G\alpha_s$ mRNA level. We found that the $G\alpha_s$ protein is ubiquitinated and that this ubiquitination is inhibited by Ric-8B. This Ric-8B-mediated inhibition of $G\alpha_s$ ubiquitination requires interaction between Ric-8B and $G\alpha_s$ because Ric-8B splicing variants, which are defective for $G\alpha_s$ binding, failed to inhibit the ubiquitination. Taken together, these results suggest that Ric-8B plays a critical and specific role in the control of $G\alpha_s$ protein levels by modulating $G\alpha_s$ ubiquitination and positively regulates G_s signaling.

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) transmit extracellular signals from the G protein-coupled receptor to effector proteins, controlling a wide variety of cellular processes. The G protein consists of α, β, and γ subunits and undergoes an activation-inactivation cycle dependent on bound guanine nucleotides. In the basal state, GDP-bound α subunit ($G\alpha$) and $\beta\gamma$ subunits ($G\beta\gamma$) are associated. Once the G protein-coupled receptor is stimulated by its specific ligand, the exchange reaction of GDP to GTP on $G\alpha$ is promoted. The GTP-bound $G\alpha$ dissociates from $G\beta\gamma$, and both $G\alpha$ and $G\beta\gamma$ independently or cooperatively modulate the activity of specific effectors. G protein signaling is terminated by GTP hydrolysis, returning the protein to the GDP-bound state and allowing reformation of the inactive heterotrimer (1, 2). Although G proteins are primarily regulated by G protein-coupled receptors, growing evidence demonstrates that non-receptor types of regulators, including RGS (regulators of G protein signaling) and AGS (activators of G protein signaling) proteins, also modulate G protein signaling (3, 4).

Ric-8 is a novel non-receptor type of the G protein regulator that was originally identified by a genetic screening of *Caenorhabditis elegans* mutants, which are resistant to inhibitors of acetylcholinesterase (5). Ric-8 functions as a guanine nucleotide exchange factor (GEF)[2] for $G\alpha$ *in vitro* (6). Genetic studies indicate that Ric-8 is involved in asymmetric cell division in *C. elegans* embryos (6–8) and *Drosophila melanogaster* neuroblasts (9–11). In contrast to invertebrates, which have one Ric-8, in mammals, there are two homologues of *C. elegans* Ric-8, named Ric-8A and Ric-8B (12). Previous studies have indicated that Ric-8A also functions as a GEF for $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, and $G\alpha_{13}$ *in vitro* (12) and potentiates G_q signaling (13). On the other hand, Ric-8B was shown to interact with $G\alpha_s$ and $G\alpha_q$, and some evidence suggests that Ric-8B potentiates olfactory-specific G protein (G_{olf})-mediated signaling (14, 15). Recently, a small pigment phenotype caused by a defect of the zebrafish synembryn-like protein, which is a homologue of mammalian Ric-8B, was rescued by treatment with forskolin, an activator of adenylyl cyclase (16). These findings collectively suggest that Ric-8B is involved in G_s signaling; however, whether and how Ric-8B regulates G_s signaling remain to be clarified.

In this study, we found a novel regulatory mechanism for G_s signaling by Ric-8B. The GEF activity of Ric-8B could not be observed *in vitro*; however, the knockdown of Ric-8B in NIH3T3 cells suppressed cellular cAMP accumulation in response to a β-adrenergic agonist. Surprisingly, knockdown of Ric-8B resulted in the reduction of the $G\alpha_s$ protein but not of other $G\alpha$ and $G\beta$ proteins. In contrast, overexpression of Ric-8B increased the $G\alpha_s$ protein without affecting $G\alpha_s$ mRNA levels. These results suggest that Ric-8B specifically regulates $G\alpha_s$ protein levels. Furthermore, we found that the $G\alpha_s$ protein was covalently modified with ubiquitin and degraded by the proteasome. The ubiquitination of $G\alpha_s$ was suppressed by the overexpression of Ric-8B. These results suggest that Ric-8B specifically regulates $G\alpha_s$ protein levels by suppressing $G\alpha_s$ ubiquitination and positively regulates G_s signaling.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Materials and Methods" and Figs. S1–S7.

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[2] The abbreviations used are: GEF, guanine nucleotide exchange factor; shRNA, short hairpin RNA; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CHX, cycloheximide.

EXPERIMENTAL PROCEDURES

Molecular Cloning—The open reading frames of mouse full-length Ric-8B, Ric-8BΔ9, and Ric-8BΔ3Δ9 were amplified by PCR using forward primer 5′-GGCGGATCCATGGATGAAGAGCGCGCCCT-3′, reverse primer 5′-GGCGGATCCTCAGTCTGTGTCCGAGCTGG-3′, and cDNAs prepared from mouse brain (full-length Ric-8B) or heart (Ric-8BΔ9 and Ric-8BΔ3Δ9). The PCR products were cloned into the BamHI site of the pCMV5 expression vector. The cDNA encoding full-length Ric-8B was subcloned into the BglII site of pCMV5-FLAG or into the BamHI site of pCMV-Myc, pFASTBac-GST, and pGEX-6P-1 (GE Healthcare). The Ric-8BΔ9 and Ric-8BΔ3Δ9 cDNAs were subcloned into the BglII site of pCMV5-FLAG. pcDNA3.1-human $G\alpha_{olf}$ was obtained from the Missouri S&T cDNA Resource Center and digested with KpnI and XhoI. The fragment was ligated into the KpnI and SalI sites of pCMV-FLAG. Short hairpin RNAs (shRNAs) directed against mouse Ric-8B (two different sequences) and Ric-8A were generated from the following annealed primers: Ric-8B2, 5′-GATCCCCACAGTTGGAAGGTGCATAATTCAAGAGATTATGCACCTTCCAACTGTTTTTTA-3′ (sense) and 5′-AGCTTAAAAAACAGTTGGAAGGTGCATAATCTCTTGAATTATGCACCTTCCAACTGTGGG-3′ (antisense); and Ric-8B3, 5′-GATCCCCGGCAGCAACTCTAGATGAATTCAAGAGATTCATCTAGAGTTGCTGCCTTTTTA-3′ (sense) and 5′-AGCTTAAAAAGGCAGCAACTCTAGATGAATCTCTTGAATTCATCTAGAGTTGCTGCCGGG-3′ (antisense). These annealed primers were inserted into the BglII and HindIII sites of pSUPER-retro-puro (Oligoengine, Seattle, WA). The $G\alpha_{s/i}$ chimera constructs were generated by overlapping PCR ($G\alpha_{s/i}$-SWI_i, residues 1–185 of $G\alpha_{s\text{-}short}$, residues 177–195 of $G\alpha_i$, and residues 205–380 of $G\alpha_{s\text{-}short}$; $G\alpha_{s/i}$-$SWII_i$, residues 1–214 of $G\alpha_{s\text{-}short}$, residues 206–217 of $G\alpha_i$, and residues 227–380 of $G\alpha_{s\text{-}short}$; and $G\alpha_{s/i}$-$SWIII_i$, residues 1–232 of $G\alpha_{s\text{-}short}$, residues 224–240 of $G\alpha_i$, and residues 250–380 of $G\alpha_{s\text{-}short}$).

Cell Culture and Transfection—HEK293T and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C with 5% CO_2. Plasmid DNAs were transfected into HEK293T cells using the calcium phosphate method or Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Retroviral Production and Infection—HEK293T cells were transfected with ecotropic helper retroviral plasmid together with pSUPER-retro-puro vectors encoding shRNA directed against Ric-8B. Viruses harvested 24–60 h post-transfection were pooled. NIH3T3 cells (1×10^5 cells/60-mm dish) were infected twice with 1.5 ml of retrovirus-containing supernatant supplemented with 8 μg/ml Polybrene at 2-h intervals. Twenty-four hours after infection, cells were selected in 7.5 μg/ml puromycin for 48 h.

Protein Purification—Escherichia coli Rosetta(DE3) pLysS strain (Novagen) cells harboring pGEX6P-1-mouse Ric-8B were incubated in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. When A_{600} was between 0.5 and 0.6, isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM. Cells were incubated for 8 h at 20 °C and collected by centrifugation. Pelleted cells were suspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 20% glycerol, and 0.5% sodium cholate) including protease inhibitors (16 μg/ml phenylmethylsulfonyl fluoride, 16 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 16 μg/ml N^{α}-tosyl-L-lysine chloromethyl ketone, 3.2 μg/ml leupeptin, and 3.2 μg/ml lima bean trypsin inhibitor) and disrupted by sonication. After the lysate was clarified by centrifugation, glutathione-Sepharose 4B (1-ml bed volume/liter of culture) was added to the lysate and gently agitated for 1 h at 4 °C. The resin was washed with extraction buffer containing 300 mM NaCl and subsequently wash buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, and 0.7% CHAPS). Glutathione *S*-transferase (GST)-Ric-8B was eluted with 20 mM glutathione in wash buffer. To separate the aggregated proteins, the eluate was loaded onto HiLoad 16/60 Superdex 200 pg (GE Healthcare) pre-equilibrated in 50 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl, and 10% glycerol at 0.5 ml/min. Fractions containing non-aggregated GST-Ric-8B were pooled and concentrated in a 30,000 molecular weight cut-off Amicon Ultra filter unit (Millipore). Purification of GST-Ric-8A was performed as described previously (13) with some modification. Briefly, the proteins eluted from glutathione-Sepharose were loaded onto HiLoad 16/60 Superdex 200 pg pre-equilibrated in 20 mM HEPES-NaOH, pH 8.0, containing 100 mM NaCl and 1 mM DTT at 0.5 ml/min. Peak fractions containing non-aggregate GST-Ric-8A were pooled and concentrated in a 30,000 molecular weight cut-off Amicon Ultra filter unit. Baculoviruses encoding $G\alpha_q$, $G\beta_1$, and His-$G\gamma_2$ were kindly provided by Dr. Tohru Kozasa (University of Illinois at Chicago). Purification of $G\alpha_q$ was performed as previously described (17). To prepare recombinant $G\alpha_{s\text{-}short}$, the cDNA encoding bovine $G\alpha_{s\text{-}short}$ (spliced variant 4) was subcloned into the XbaI and HindIII sites of pQE60 (Qiagen). The expression and purification of $G\alpha_s$ proteins were performed as described previously (18, 19).

In Vitro Binding Assay—The *in vitro* binding assay of GST-Ric-8B and $G\alpha_s$ or $G\alpha_q$ was performed as described previously (12). Briefly, 100 nM $G\alpha_s$ or $G\alpha_q$ was incubated with 100 nM GST or GST-Ric-8B in binding buffer (20 mM HEPES-NaOH, pH 8.0, 100 mM NaCl, 10 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, and 0.05% Lubrol PX) for 1 h at 25 °C. Glutathione-Sepharose 4B (GE Healthcare) was added to the reaction mixture and gently agitated for 1 h at 4 °C. The resins were washed three times with binding buffer and treated with SDS-PAGE sample buffer. The eluted proteins were resolved by SDS-PAGE, stained with Coomassie Blue, and immunoblotted with anti-$G\alpha_s$ or anti-$G\alpha_q$ antibodies.

GTPγS Binding Assays—GTPγS binding reactions were initiated by the addition of 5 pmol (50 nM) of $G\alpha_s$ or $G\alpha_q$ to reaction buffer (20 mM HEPES-NaOH, pH 8.0, 100 mM NaCl, 10 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, and 0.05% $C_{12}E_{10}$) containing 20 pmol (200 nM) of GST, GST-Ric-8A, or GST-Ric-8B and 10 μM [^{35}S]GTPγS (10,000 cpm/pmol) in a total volume of 100 μl at 20 °C. The reaction buffer for $G\alpha_q$ was identical to that for $G\alpha_s$ except that 0.05% Genapol C-100 detergent was used instead of $C_{12}E_{10}$. Aliquots (20 μl) were removed at the indi-

cated times, and ice-cold buffer containing 20 mM Tris-HCl, pH 7.7, 100 mM NaCl, 2 mM $MgSO_4$, 0.05% $C_{12}E_{10}$, and 1 mM GTP was added before filtration through BA85 nitrocellulose membranes. The membranes were washed twice with an ice-cold wash buffer (20 mM Tris-HCl, pH 7.7, 100 mM NaCl, and 2 mM $MgSO_4$) and dried. The radioactivity of each membrane was measured using an LS6500 liquid scintillation counter (Beckman Coulter).

Intracellular cAMP Accumulation—NIH3T3 cells infected with retroviruses that express shRNA directed against mouse Ric-8B or HEK293T cells were pretreated with 0.5 mM 3-isobutyl-1-methylxanthine for 1 h and subsequently stimulated with 10 μM isoproterenol or 10 nM pituitary adenylate cyclase-activating polypeptide for the indicated times, respectively. Cyclic AMP was measured using the AlphaScreen cAMP assay kit (PerkinElmer Life Sciences) according to the manufacturer's protocol.

Degradation of Gα$_s$ after Inhibition of Protein Synthesis (Cycloheximide (CHX) Chase)—HEK293T cells transfected with an empty vector or FLAG-Ric-8B were grown to 80% confluence in 60-mm dishes and treated with 100 μg/ml CHX for the indicated times. Cells were harvested and subsequently lysed with lysis buffer (20 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were analyzed by immunoblotting using anti-Gα$_s$, anti-FLAG, and anti-actin antibodies.

In Vivo Ubiquitination Assay—An *in vivo* ubiquitination assay was performed as described previously (20). HEK293T cells were transfected with pMT107-6xHis-ubiquitin (21) and other expression plasmids. Twenty-four hours post-transfection, cells were treated with 10 μM MG132 for 12 h, and cells were harvested by centrifugation. Cells were lysed with urea lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaH_2PO_4, 8 M urea, 10% glycerol, 0.1% Triton X-100, 0.5 M NaCl, 10 mM imidazole, and 10 mM 2-mercaptoethanol) and disrupted by sonication. Lysates were centrifuged at 15,000 × *g* for 5 min at room temperature, and supernatants were collected. For purification of His_6-tagged ubiquitinated proteins, nickel-nitrilotriacetic acid-agarose (Qiagen) was added to the supernatant and gently agitated for 4 h at room temperature. The resins were washed five times with 20 mM imidazole in urea lysis buffer and treated with 150 mM Tris-HCl, pH 6.8, containing 200 mM imidazole, 5% SDS, 30% glycerol, and 0.72 M 2-mercaptoethanol. Ubiquitinated Gα$_s$, FLAG-Gα$_s$, or FLAG-Gα$_{olf}$ proteins were detected by immunoblotting using anti-Gα$_s$ or anti-FLAG antibodies.

Reverse Transcription-PCR—Total RNAs were prepared using TRIzol reagent (Invitrogen) from HEK293T cells transfected with FLAG-Ric-8B. First-strand cDNAs were synthesized from 2 μg of total RNA with SuperScript II (Invitrogen). PCR mixtures (50 μl) containing 0.5 μl of cDNA and 0.5 μM each forward and reverse primers were heated at 94 °C for 2 min, followed by 25 (Gα$_s$) or 20 (glyceraldehyde-3-phosphate dehydrogenase) cycles of 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 30 s. PCR products were analyzed in 2% agarose gels stained with ethidium bromide. The following primers were used: Gα$_s$, 5′-GCACCATTGTGAAGCAGATG-3′ (forward) and 5′-TCATCCTCCCACAGAGCCTT-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse).

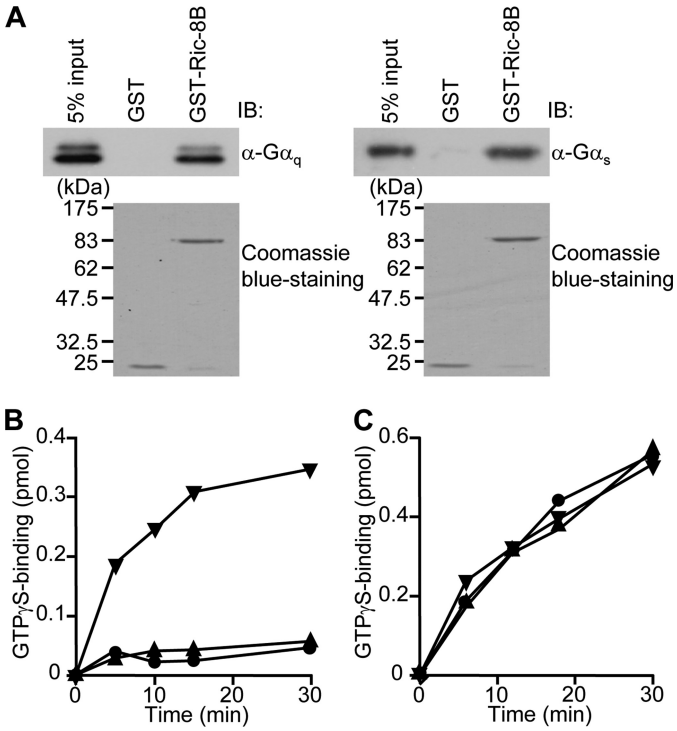

FIGURE 1. **Effect of Ric-8B on guanine nucleotide exchange.** *A*, GST or GST-Ric-8B (100 nM each) was incubated with Gα$_s$ or Gα$_q$ (100 nM each) for 1 h at 25 °C. These mixtures were bound to glutathione-Sepharose and washed extensively with a buffer. The proteins were eluted with an SDS-PAGE sample buffer. Co-precipitation of Gα proteins was detected by immunoblotting (*IB*). *B* and *C*, Gα$_q$ (*B*) and Gα$_s$ (*C*) (5 pmol each) were incubated with [^{35}S]GTPγS in reaction mixture (100 μl) containing GST (●), GST-Ric-8B (▲), or GST-Ric-8A (▼) (20 pmol each). Aliquots (20 μl) of these reaction mixtures were taken at the indicated time points and filtered to absorb nucleotide-bound protein. The amount of G protein-bound [^{35}S]GTPγS was determined by scintillation counting.

RESULTS

Effect of Ric-8B on GTPγS Binding to Gα$_s$ and Gα$_q$ in Vitro—Previously, it has been demonstrated that *C. elegans* Ric-8 (6) and mammalian Ric-8A (12) possess GEF activity for Gα *in vitro*. In addition, it has been reported that Ric-8B potentiates Gα$_{olf}$-mediated signaling in HEK293 cells (14, 15). These findings suggest that Ric-8B might have GEF activity for Gα; however, no data for the GEF activity of Ric-8B have been reported so far. First, we confirmed the binding of Ric-8B to Gα$_s$ and Gα$_q$. GST-Ric-8B, which was prepared from *E. coli* in the presence of glycerol using gel filtration chromatography as non-aggregate, was incubated with Gα$_s$ or Gα$_q$, and the protein complexes were then precipitated with glutathione-Sepharose. Gα$_q$ and Gα$_s$ were co-precipitated with GST-Ric-8B, indicating that Ric-8B directly binds to Gα$_q$ and Gα$_s$ (Fig. 1*A*). Next, the kinetics of GTPγS binding to Gα$_s$ or Gα$_q$ proteins was investigated in the presence of GST-Ric-8B or GST-Ric-8A. GST-Ric-8A dramatically increased the rate of GTPγS binding to Gα$_q$ but not Gα$_s$, as reported previously (12). In contrast, GST-Ric-8B did not affect the GTPγS-binding activity of Gα$_q$ and Gα$_s$ (Fig. 1, *B* and *C*). In these experiments, we used recombi-

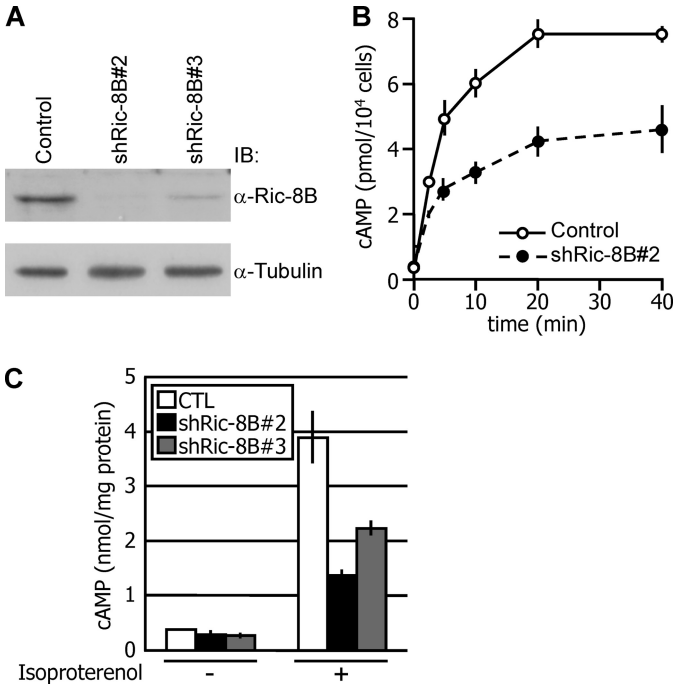

FIGURE 2. **Knockdown of Ric-8B decreases isoproterenol-induced cAMP accumulation.** *A*, cell lysates from NIH3T3 cells infected with retroviruses encoding control shRNA or two different sequences of shRNAs directed against Ric-8B were analyzed by immunoblotting (*IB*) using anti-Ric-8B and anti-tubulin antibodies. *B*, shown is the time course of cAMP accumulation. cAMP accumulation of NIH3T3 cells infected with retroviruses encoding control (○) or Ric-8B2 (*shRic-8B#2*; ●) shRNA was measured following exposure to 10 μM isoproterenol for the indicated times. *C*, NIH3T3 cells infected with retroviruses were exposed to 10 μM isoproterenol for 20 min, and cAMP accumulation was measured. The data are expressed as the mean ± S.D. from three independent experiments. *CTL*, control.

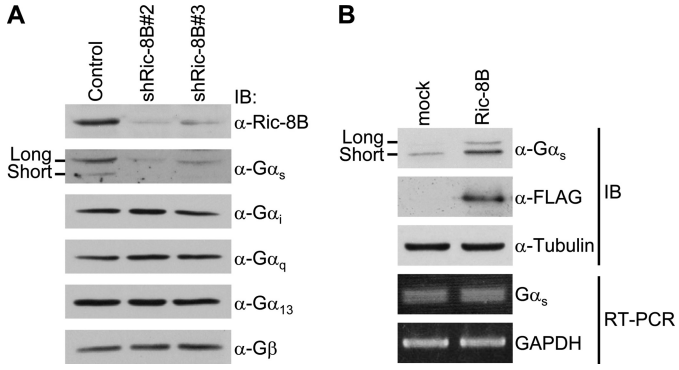

FIGURE 3. **Ric-8B positively regulates Gα$_s$ protein levels.** *A*, cell lysates were prepared from NIH3T3 cells infected with retroviruses encoding control shRNA or two different sequences of shRNAs directed against Ric-8B (*shRic-8B*) and were analyzed by immunoblotting (*IB*) using the indicated antibodies. The Gα$_s$ protein exists as two spliced forms, short and long. The ratio of the spliced forms varies in different cell types. The positions of the long and short Gα$_s$ variants are indicated by *lines*. *B*, HEK293T cells were transfected with FLAG-Ric-8B and harvested for RNA and protein preparations. Semi-quantitative reverse transcription-PCR (*RT-PCR*) and immunoblotting were performed to assess the effect of Ric-8B on the Gα$_s$ mRNA and protein levels, respectively. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

nant Gα$_s$ and Gα$_q$ proteins that were expressed in *E. coli* and Sf9 cells, respectively. Because N-terminal myristoylation of Gα$_{i1}$ greatly improves the ability of the protein to serve as a substrate for Ric-8A-stimulated guanine nucleotide exchange (22), we examined the effect of Ric-8B on palmitoylated Gα$_s$. Palmitoylated Gα$_s$ was purified from a membrane fraction of Sf9 cells that were infected with baculoviruses encoding Gα$_{s\text{-short}}$, Gβ$_1$, and His-Gγ$_2$. However, we could not observe the effect of GST-Ric-8B on the guanine nucleotide exchange reaction for palmitoylated Gα$_s$ (data not shown). These results suggest that Ric-8B alone does not possess GEF activity for Gα$_s$ and Gα$_q$ *in vitro*.

Knockdown of Ric-8B Reduces Isoproterenol-induced cAMP Accumulation—Next, to test the involvement of Ric-8B in G$_s$ signaling, we prepared two retroviral constructs expressing the shRNA directed against mouse Ric-8B and infected NIH3T3 cells with them. As shown in Fig. 2*A*, both shRNAs showed effective reduction of the Ric-8B protein, and their efficiency of knockdown was above 80%. In control cells, cAMP accumulation was promoted in response to isoproterenol. In cells expressing shRNA directed against Ric-8B, isoproterenol-induced cAMP accumulation was greatly reduced (Fig. 2*B*). The extent of the inhibition of cAMP accumulation was correlated with the protein reduction of Ric-8B (Fig. 2, *A* and *C*). These results indicate that Ric-8B positively regulates G$_s$ signaling and are consistent with previous observations reporting the functional requirement of Ric-8B in Gα$_{olf}$-mediated signaling in mammals (14) and pigment dispersion in zebrafish (16). In addition, Ric-8B seems to be specifically involved in G$_s$ signaling because we did not observe any significant effect of knockdown of Ric-8B on UTP-stimulated (Gα$_q$-coupled) intracellular calcium mobilization and platelet-derived growth factor-induced extracellular signal-regulated kinase (ERK) activation (supplemental Fig. S1, *A* and *B*).

Ric-8B Positively Regulates Gα$_s$ Protein Levels—To investigate how Ric-8B is involved in G$_s$ signaling, we first examined the effect of knockdown of Ric-8B on the protein levels of α and β subunits of G proteins. Surprisingly, knockdown of Ric-8B dramatically reduced Gα$_s$ protein levels (Fig. 3*A*). This effect was not observed in other Gα proteins or the Gβ subunit. In contrast, overexpression of Ric-8B greatly increased the Gα$_s$ protein level (Fig. 3*B*). These results indicate that Ric-8B specifically and positively regulates Gα$_s$ protein levels. Next, we performed a reverse transcription-PCR analysis using HEK293T cells transfected with Ric-8B. Overexpression of Ric-8B increased the Gα$_s$ protein but had little effect on its transcriptional level (Fig. 3*B*), suggesting that Ric-8B affects Gα$_s$ protein stability.

To examine whether Ric-8B knockdown affects the expression and function of other G$_s$ signaling components, G$_s$-coupled receptor, and adenylyl cyclase, we evaluated the β-adrenergic receptor expression and adenylyl cyclase activity. Ligand binding assays using a radiolabeled β-adrenergic receptor antagonist, [^{125}I]iodocyanopindolol, were carried out. The numbers of endogenous β-adrenergic receptors were determined by Scatchard analysis of [^{125}I]iodocyanopindolol saturation binding. The level of β-adrenergic receptor expression was not affected by Ric-8B knockdown (supplemental Fig. S2*A*). Intrinsic adenylyl cyclase activity was evaluated by Gα$_s$ reconstitution assay. Membranes of NIH3T3 cells expressing shRNA directed against Ric-8B were incubated with GTPγS-preloaded Gα$_s$, and then cAMP production was measured. No effect of Ric-8B knockdown on intrinsic adenylyl cyclase activity was observed (supplemental Fig. S2*B*).

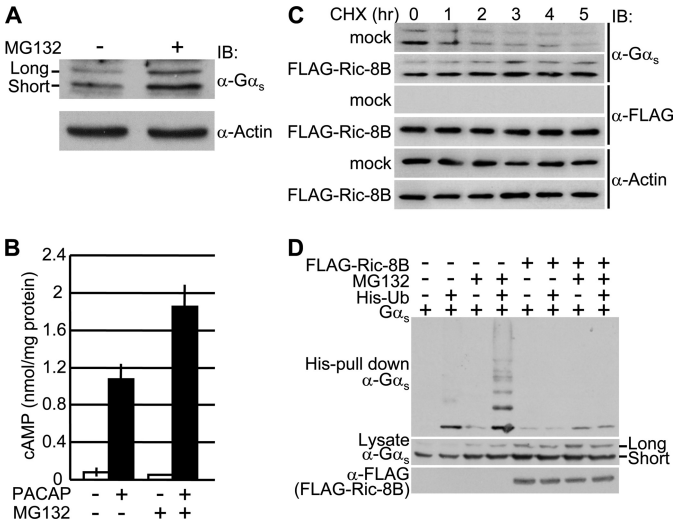

FIGURE 4. **Ric-8B inhibits Gα$_s$ degradation and ubiquitination.** *A*, HEK293T cells were treated with 10 μM MG132 for 12 h, and cell lysates were analyzed by immunoblotting (*IB*) using anti-Gα$_s$ and anti-actin antibodies. *B*, HEK293T cells were pre-incubated with 10 μM MG132 for 8 h, and cAMP accumulation was then measured following exposure to 10 nM pituitary adenylate cyclase-activating polypeptide (*PACAP*) for 15 min. *C*, HEK293T cells transfected with an empty vector or FLAG-Ric-8B were treated with 100 μg/ml CHX for the indicated times. Cell lysates were analyzed by immunoblotting using anti-Gα$_s$, anti-FLAG, and anti-actin antibodies. *D*, HEK293T cells were transfected with Gα$_{s\text{-short}}$, His-ubiquitin (*His-Ub*), and FLAG-Ric-8B. Twenty-four hours after transfection, cells were treated with 10 μM MG132 for 12 h and subsequently lysed with an 8 M urea-containing buffer, and His-tagged ubiquitinated proteins were precipitated with nickel-agarose resin. Ubiquitinated Gα$_s$ was detected by immunoblotting using the anti-Gα$_s$ antibody.

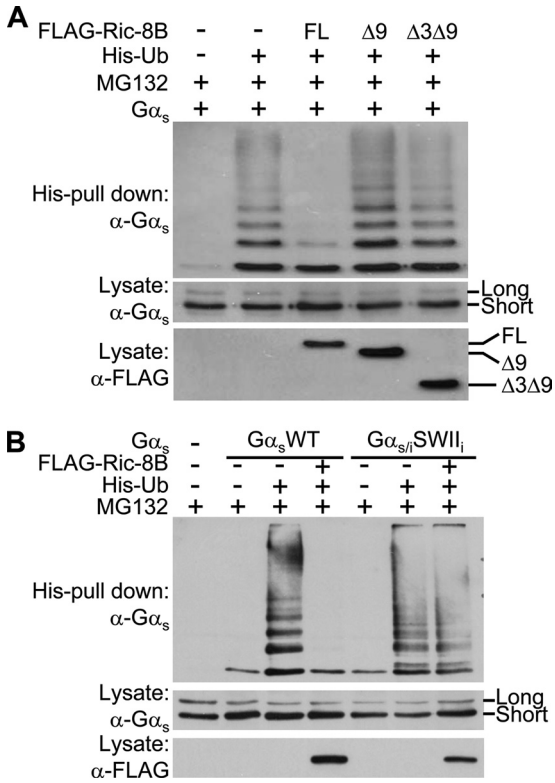

FIGURE 5. **Interaction of Ric-8B with Gα$_s$ is important for inhibiting Gα$_s$ ubiquitination.** HEK293T cells were transfected with Gα$_{s\text{-short}}$, His-ubiquitin (*His-Ub*), and FLAG-tagged full-length Ric-8B (*FL*) or spliced variants (*A*) or with His-ubiquitin, FLAG-Ric-8B, and wild-type Gα$_{s\text{-short}}$ (*Gα$_s$WT*) or Gα$_{s/i}$-SWII$_i$ (*B*). The ubiquitinated Gα$_s$ was detected as described in the legend to Fig. 4*D*.

Ric-8B Inhibits Gα$_s$ Degradation and Ubiquitination—We focused on the ubiquitin-proteasome pathway, which is involved in protein stability (23). We first investigated whether the Gα$_s$ protein is controlled by the ubiquitin-proteasome pathway. HEK293T cells were treated with a potent proteasome inhibitor, MG132. Interestingly, treatment with MG132 increased the Gα$_s$ protein and potentiated the pituitary adenylate cyclase-activating polypeptide-induced cAMP accumulation (Fig. 4, *A* and *B*). These results suggest that the Gα$_s$ protein is controlled by proteasomal degradation. Next, we monitored the endogenous Gα$_s$ protein level after treatment with a protein synthesis inhibitor, CHX. HEK293T cells transfected with FLAG-Ric-8B or an empty vector were treated with CHX for 0–5 h. In control cells, the levels of the Gα$_s$ protein decreased after exposure to CHX. In cells transfected with FLAG-Ric-8B, the degradation of the Gα$_s$ protein was inhibited (Fig. 4*C*), indicating that Ric-8B enhances Gα$_s$ stability. These results suggested that Ric-8B modulates the post-translational modification, such as ubiquitination, of Gα$_s$. Therefore, we performed an *in vivo* ubiquitination assay. HEK293T cells were transfected with Gα$_s$ together with His-tagged ubiquitin and treated with MG132 for 12 h. These cells were subsequently lysed under denaturing conditions. His-tagged ubiquitinated proteins were collected with nickel-agarose resin, and the precipitated Gα$_s$ was detected by immunoblotting. We detected a ladder of ubiquitinated Gα$_s$ proteins whose intensity increased with MG132 treatment (Fig. 4*D*). To examine the effect of Ric-8B on Gα$_s$ ubiquitination, we expressed Ric-8B in HEK293T cells together with Gα$_s$ and His-tagged ubiquitin. Overexpression of Ric-8B increased the Gα$_s$ protein, as shown in Fig. 3*B*; however, the amount of ubiquitinated Gα$_s$ proteins was reduced. These findings strongly suggest that Gα$_s$ is a novel substrate for ubiquitination and that Ric-8B regulates Gα$_s$ protein stability by suppression of the ubiquitination and degradation of Gα$_s$ in mammalian cells.

Interaction of Ric-8B with Gα$_s$ Is Important for Inhibiting Gα$_s$ Ubiquitination—To understand in detail the mechanism whereby Ric-8B inhibits Gα$_s$ ubiquitination, we hypothesized that interaction of Ric-8B with Gα$_s$ is required for Ric-8B-mediated inhibition of Gα$_s$ ubiquitination. To examine this hypothesis, we used two spliced variants of Ric-8B (supplemental Fig. S3*A*). Ric-8BΔ9, which lacks exon 9, was previously reported (14). In addition, we identified another novel spliced variant, Ric-8BΔ3Δ9, which lacks both exons 3 and 9. HEK293T cells were transfected with FLAG-tagged full-length Ric-8B and its variants. Endogenous Gα$_s$ protein was co-immunoprecipitated with FLAG-tagged full-length Ric-8B but not with Ric-8BΔ9 or Ric-8BΔ3Δ9 (supplemental Fig. S3*B*). As expected, these spliced variants failed to inhibit Gα$_s$ ubiquitination (Fig. 5*A*), suggesting that the suppressive effect of Ric-8B on the ubiquitination of Gα$_s$ requires the interaction between Gα$_s$ and Ric-8B.

Next, we generated a series of the Gα$_{s/i}$ chimeric proteins because the Gα$_i$ protein does not interact with Ric-8B in cells (supplemental Fig. S4) (12). Gα contains three switch regions (SWI, SWII, and SWIII), and each switch region of Gα$_s$ was replaced with that of Gα$_i$ (supplemental Fig. S5*A*). These constructs retain proper conformation because chimeric α sub-

units were dissociated from the Gβγ complex in the presence of AlF_4^- (supplemental Fig. S5*B*). Among these chimeric proteins, only Gα$_{s/i}$-SWII$_i$ dramatically reduced the affinity for FLAG-Ric-8B in HEK293T cells (supplemental Fig. S5*C*), suggesting that the switch II region of Gα$_s$ is required for interaction with Ric-8B. Similarly to the wild-type Gα$_s$ protein, the Gα$_{s/i}$-SWII$_i$ chimeric protein was also ubiquitinated; however, the ubiquitination of Gα$_{s/i}$-SWII$_i$ was not inhibited by the overexpression of Ric-8B (Fig. 5*B*). Taken together, these results strongly support our hypothesis that the interaction of Ric-8B with Gα$_s$ is important for inhibiting Gα$_s$ ubiquitination.

DISCUSSION

Previously, Tall *et al.* (12) and our group (13) reported that Ric-8A, another mammalian homologue of Ric-8, exhibits GEF activity for Gα$_q$ and contributes to the G$_q$ signaling pathway. According to the analogy to Ric-8A, it was speculated that Ric-8B would also harbor GEF activity for Gα$_s$ because Ric-8B showed potent ability to interact with Gα$_s$ (12). However, we could not observe the GEF activity of Ric-8B *in vitro* (Fig. 1). On the other hand, our analysis utilizing shRNA against Ric-8B clearly demonstrated the functional involvement of Ric-8B in ligand-induced cAMP accumulation (Fig. 2). Our observation suggested that Ric-8B plays an essential role in G$_s$ signaling without its GEF activity. Furthermore, we found that the expression level of Ric-8B apparently affected the expression level of Gα$_s$ without any changes in the amount of Gα$_s$ mRNA (Fig. 3).

We demonstrated that Gα$_s$ is ubiquitinated and that both its ubiquitination and degradation are suppressed by Ric-8B (Fig. 4). Combining all of our current data, we propose a new mode of regulatory mechanism whereby Ric-8B stabilizes G$_s$ signaling through suppressing the ubiquitination and degradation of Gα$_s$. According to a previous report, Gα$_{olf}$-mediated cAMP accumulation is also potentiated by Ric-8B (14). However, the mechanism whereby Ric-8B emphasizes olfactory signaling remained obscure. In this study, we demonstrated that overexpression of Ric-8B increased the protein amount of Gα$_{olf}$ and inhibited its ubiquitination similarly to Gα$_s$ (supplemental Fig. S6). These findings raise the possibility that Ric-8B may also amplify Gα$_{olf}$ signaling by the stabilization of Gα$_{olf}$. However, it is still possible that Ric-8B harbors Gα$_s$/Gα$_{olf}$-specific GEF activity with the additional factors in the cells. Detailed biochemical analysis for the Ric-8B-interacting proteins that may be critical for GEF activity would prove this possibility.

Several observations in this study suggest that the interaction between Ric-8B and Gα$_s$ is important for the suppression of ubiquitination of Gα$_s$ (Fig. 5). The Gα$_s$ protein preferentially locates in the plasma membrane, whereas Ric-8B mostly localizes in the cytoplasm in quiescent cells. Klattenhoff *et al.* (24) reported that isoproterenol induces the translocation of Ric-8B into the plasma membrane and the co-localization of Ric-8B with Gα$_s$. Consistent with these findings, another group reported that the overexpression of Ric-8B increases the amount of Gα$_{olf}$ protein on the plasma membrane (15, 25). In addition, we observed that the polyubiquitinated Gα$_s$ protein seemed to localize in the plasma membrane and that MG132 treatment resulted in the accumulation of Gα$_s$ in the plasma membrane (supplemental Fig. S7). Although the manner in which a ligand induces membrane localization of Ric-8B is unclear, it allows us to provide a novel regulatory mechanism whereby Ric-8B may interact with Gα$_s$ in response to G$_s$ activation and to stabilize Gα$_s$ through the suppression of the ubiquitination of Gα$_s$. Interestingly, several reports suggest that the activation of Gα$_s$ shortens the half-life of Gα$_s$ (26, 27).

Our observation provides a model of how Ric-8B stabilizes the Gα$_s$ protein and enhances its signals; Ric-8B may mask the ubiquitination site of Gα$_s$ and then perturb the accessibility of Gα$_s$-specific E3 ubiquitin ligase to the Gα$_s$ protein. Although E3 ubiquitin ligase for Gα$_s$ has not been identified yet, several observations provide some clues to explore the Gα$_s$-specific E3 ubiquitin ligase. A recent study described an RGS-GAIP-interacting protein, GIPN, which possesses E3 ubiquitin ligase activity and promotes the proteasome-dependent degradation of Gα$_{i3}$ (28). There are two main classes of E3 ubiquitin ligases: RING finger and HECT E3 ubiquitin ligases (29). RING finger E3 ubiquitin ligases bind to a specific E2 ubiquitin-conjugating enzyme through their RING finger domain, which is prerequisite for ubiquitination of the substrate protein. It has been reported that GIPN harbors the RING finger-like motif, suggesting that GIPN may exhibit E3 ubiquitin ligase activity through this non-canonical RING finger domain. Similarly, Gα$_s$-specific E3 ubiquitin ligase may be an interacting protein of RGS-PX, which has been reported as a sole RGS molecule for Gα$_s$ so far (30). A recent genetic and biochemical study indicated that Rsp5 is an E3 ubiquitin ligase for yeast Gα Gpa1 (31). Although Gpa1 is ubiquitinated in a region that is absent in mammalian Gα (32), NEDD4 proteins, which are mammalian homologues of yeast Rsp5, might have the E3 ubiquitin ligase activity for mammalian G protein α subunits. More recently, it was reported that MGRN1 (Mahogunin ring finger-1) attenuates melanocortin receptor-mediated cAMP accumulation by competing the interaction between Gα$_s$ and the melanocortin receptor (33). MGRN1 contains a RING finger domain and has been shown to display ubiquitin ligase activity for some proteins other than Gα$_s$ (34, 35). MGRN1 might also function as E3 ubiquitin ligase for Gα$_s$ and might attenuate G$_s$ signaling. The determination of E3 ubiquitin ligase for Gα$_s$ and analysis of the mechanism whereby E3 ubiquitin ligase regulates G$_s$ signaling will be the focus of a future study.

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