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Ric-8 Enhances G Protein $\beta\gamma$-Dependent Signaling in Response to $\beta\gamma$-Binding Peptides in Intact Cells

Sundeep Malik, Mousumi Ghosh, Tabetha M. Bonacci, Gregory G. Tall, and Alan V. Smrcka

Departments of Pharmacology and Physiology (S.M., M.G., T.M.B., A.V.S.) and Biochemistry and Biophysics (A.V.S.), University of Rochester School of Medicine and Dentistry, Rochester, New York; and Department of Pharmacology (G.G.T.), University of Texas Southwestern Medical Center, Dallas, Texas

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ABSTRACT

Peptides derived from a random-peptide phage display screen with purified $G\beta_1\gamma_2$ subunits as the target promote the dissociation of G protein heterotrimers in vitro and activate G protein signaling in intact cells. In vitro, one of these peptides (SIRKALNILGYPDYD; SIRK) promotes subunit dissociation by binding directly to $G\beta\gamma$ subunits and accelerating the dissociation of $G\alpha$GDP without catalyzing nucleotide exchange. The experiments described here were designed to test whether the mechanism of SIRK action in vitro is in fact the mechanism of action in intact cells. We created a mutant of $G\beta_1$ subunits (β_1W332A) that does not bind SIRK in vitro. Transfection of $G\beta_1$W332A mutant into Chinese hamster ovary cells blocked peptide-mediated activation of extracellular signal-regulated kinase (ERK), but it did not affect receptor-mediated $G\beta\gamma$ subunit-dependent ERK activation, indicating that $G\beta\gamma$ subunits are in fact the direct target in cells responsible for ERK activation. To determine whether free $G\alpha$ subunits were released from G protein heterotrimers upon peptide treatment, cells were transfected with Ric-8A, a guanine nucleotide exchange factor for free $G\alpha$GDP, but not heterotrimeric G proteins. Ric-8A-transfected cells displayed enhanced myristoyl-SIRKALNILGYPDYD (mSIRK)-dependent inositol phosphate (IP) release and ERK activation. Ric-8A also enhanced ERK activation by the G_i-linked G protein coupled receptor agonist lysophosphatidic acid. Inhibitors of $G\beta\gamma$ subunit function blocked Ric-8-enhanced activation of ERK and IP release. These results suggest that one potential function of Ric-8 in cells is to enhance G protein $G\beta\gamma$ subunit signaling. Overall, these experiments provide further support for the hypothesis that mSIRK promotes G protein subunit dissociation to release free $\beta\gamma$ subunits in intact cells.

G protein-coupled receptors (GPCRs) comprise a large family of proteins that bind a diverse array of molecules and communicate this binding information to alterations of cell physiology (Gilman, 1987; Hamm, 1998). Activated GPCRs interact with heterotrimeric G proteins to catalyze the exchange of bound GDP for GTP. This process requires the presence of both $G\alpha$ and $G\beta\gamma$ subunits, and there is evidence for direct binding of the receptor to both $G\alpha$ and $G\beta\gamma$ subunits (Taylor et al., 1994, 1996). Binding of GTP to the $G\alpha$ subunit activates the G protein and is thought to cause dissociation of $G\alpha$ subunits from $G\beta\gamma$ subunits, liberating free $G\alpha$GTP and $G\beta\gamma$ subunits to interact with downstream target proteins and regulate their activities.

It has become apparent that receptor-independent mechanisms exist for G protein activation. AGS proteins, discovered in a yeast screen for activation of the pheromone pathway, all act to release $\beta\gamma$ subunits from α subunits (Cismowski et al., 2001). GPR or GoLoco peptides derived from AGS proteins promote dissociation of $G\alpha$GDP subunits from $G\beta\gamma$ subunits, causing release of $G\beta\gamma$ (Peterson et al., 2000; Kimple et al., 2002; Ghosh et al., 2003). In addition, a novel protein, Ric-8, has been identified that binds specifically to free $G\alpha$GDP subunits and promotes GDP release

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AGS, activator of G protein signaling; SIGK, SIGKAFKILGYPDYD; SIRK, SIRKALNILGYPDYD peptide; mSIRK, myristoyl-SIRKALNILGYPDYD peptide; GFP, green fluorescent protein; LPA, lysophosphatidic acid; ERK, extracellular signaling-regulated kinase; HA, hemagglutinin; HRP, horseradish peroxidase; $bG\beta_1$, biotinylated $G\beta_1$; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium; IP, inositol phosphate; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; wt, wild-type; PTX, pertussis toxin; GEF, guanine nucleotide exchange factor; βARK, β adrenergic receptor kinase; ct, C terminus; EE, EYMPTE.

(Miller et al., 2000; Tall et al., 2003). Thus, a system potentially exists outside of G protein-coupled receptors for G protein activation that involves sequential action of proteins to release αGDP followed by Ric-8-catalyzed nucleotide exchange. Several recent publications suggest a relationship between AGS proteins and Ric-8 in unconventional G protein signaling during spindle pole positioning in the initial cell division events in *Caenorhabditis elegans* zygotes (Afshar et al., 2004; Couwenbergs et al., 2004; Hess et al., 2004).

We have identified a mechanism by which Gβγ binding peptides can activate G protein signaling by a receptor-independent mechanism. Cell-permeant versions of peptides identified by random-peptide phage display screening against G protein βγ subunits promote activation of G protein βγ subunit-dependent pathways, including mitogen-activated protein kinase and phospholipase C activation, in intact cells (Goubaeva et al., 2003). In vitro these peptides bind directly to G protein $\beta_1\gamma_2$ subunits and accelerate dissociation of $G\alpha_iGDP$ subunits from $G\alpha_{i1}\beta_1\gamma_2$ heterotrimers (Ghosh et al., 2003). The structure of G protein $\beta_1\gamma_2$ subunits bound to one of the peptides (SIGKAFKILGYPDYD; SIGK) has been solved (T. L. Davis, T. M. Bonacci, S. R. Sprang, and A. V. Smrcka, submitted). In the structural model, the peptide is bound to a site on $G\beta_1\gamma_2$ subunits normally occupied by the switch II helix of Gα subunits (Wall et al., 1995; Lambright et al., 1996). These data suggest a molecular mechanism by which these peptides promote G protein subunit dissociation by interfering with Gα subunit interactions with Gβγ subunits.

Although these in vitro data support a model for peptide-mediated dissociation of GαGDP from Gβγ as the mechanism for (myristoyl-SIRKALNILGYPDYD) (mSIRK) activation of signaling pathways in intact cells, they do not directly demonstrate this. In this study, we set out to demonstrate that G protein βγ subunits are the direct target of these cell-permeable peptides in cells and that interaction of these peptides with heterotrimeric G proteins results in release of free GαGDP in intact cells. As part of our analysis, we studied the ability of Ric-8 proteins to affect peptide-mediated responses based on the ability of Ric-8 to selectively activate free GαGDP subunits. We were surprised to find that Ric-8 can enhance G protein βγ subunit-mediated responses, probably by a mechanism that involves sequestration of free Gα subunits.

Materials and Methods

Materials and Plasmids. GFP-$G\beta_1$, GFP-$G\beta_1$W332A, $G\gamma_2$, and Ric-8A-3HA were in pCI-Neo. EE-α_{i1} and -α_t were supplied from Guthrie cDNA Resource Center (Rolla, MO) in pcDNA 3.1+; βARKct, kindly supplied by Dr. Robert Lefkowitz (Duke University, Durham, NC), was in pRK5 and Ric-8A; and Ric-8B was in pCMV5. mSIRK and SIGK were synthesized and purified by Alpha Diagnostics International (San Antonio, TX). *myo*-[^{3}H]Inositol (25 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). Pertussis toxin, lysophosphatidic acid (LPA), and ATP were from Sigma-Aldrich (St. Louis, MO). Rabbit anti-ERK and anti-phospho-ERK antisera were from Cell Signaling Technologies Inc. (Beverly, MA). Anti Ric-8A antiserum was generated in rabbits against holo-purified Ric-8A protein by Caprologics, Inc. (Hardwick, MA). Mouse anti-HA and anti-EE antisera were from Covance (Princeton, NJ). Mouse anti-GFP, goat anti-rabbit IgG-horseradish peroxidase conjugate (HRP) and goat anti-mouse IgG-HRP were from Roche Diagnostics (Indianapolis, IN).

Construction and Purification of Biotinylated Gβγ Subunits. Construction of baculovirus encoding biotinylated $G\beta_1$ ($bG\beta_1$) subunit in the baculovirus transfer vector PDW464 was described previously (Goubaeva et al., 2003). For other experiments, G protein β_1 subunits were tagged at the amino terminus with GFP. We used GFP-tagged β_1 subunits to monitor β subunit transfection efficiency by epifluorescence microscopy and to monitor the level of expression of the transfected protein relative to endogenous β subunits by immunoblotting. We (unpublished data) and others have shown that amino terminal modification of Gβ with GFP does not alter Gβγ subunit functions (Azpiazu and Gautam, 2004). Mutants (βW332A and K337A) were created by overlap extension polymerase chain reaction (PCR) using standard methods, and the entire protein coding region was sequenced to confirm the presence of the mutation and lack of additional mutations.

Phage ELISA. The phage used in this study was from the random-peptide phage display screen described previously (Scott et al., 2001). Phage were propagated and ELISA assays with $bG\beta_1\gamma_2$ subunits were performed as described previously (Smrcka and Scott, 2002).

Measurement of α-βγ Interactions via Flow Cytometry. The fluorescein-labeled α_{i1} used in these experiments was prepared as described previously (Sarvazyan et al., 1998), and competition assays were performed as described in detail in Ghosh et al. (2003). In brief, for competition based assays, 100 to 200 pM fluorescein-labeled α_{i1} and indicated concentrations of peptides were added to 50 pM $bG\beta_1\gamma_2$ immobilized on 10^5 beads per milliliter of buffer and incubated at room temperature for 30 min to reach equilibrium. The bead-associated fluorescence was then recorded in the flow cytometer. The data were corrected for nonspecific binding and fit with a sigmoid dose-response curve using Prism 4 (GraphPad Software Inc., San Diego, CA).

Cell Culture and Transfection. All cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Chinese hamster ovary cells obtained from American Type Culture Collection (Manassas, VA) were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. Cells were grown in six-well dishes (35-mm wells) for ERK activation experiments. For these experiments, 200 ng of Ric-8A or Ric-8B was transfected with or without 800 ng of βARK-ct in pRK5, 800 ng of α_t, or pRK5 empty vector control, using LipofectAMINE Plus (Invitrogen) unless otherwise indicated. For inositol phosphate (IP) release measurements, cells were grown in 12-well plates and 200 ng of Ric-8A was transfected with 200 ng of the appropriate inhibitor with a total of 400 ng of DNA transfected in each well. Transfections were performed 48 h before the final treatment and when multiple plasmids were transfected, appropriate amounts of control cDNAs were added such that the total DNA transfected was constant in each experiment.

Measurement of ERK Activation and General Immunoblotting. For measurement of phospho-ERK, serum was removed from 50 to 80% confluent CHO cells 16 h before treatment. Peptides in dimethyl sulfoxide, dimethyl sulfoxide vehicle, or other agonists were diluted 100- to 400-fold into the medium and incubated at 37°C for the indicated times. For all immunoblotting: after treatment, cells were transferred to ice, and the medium was quickly aspirated and replaced with 100 μl of 2× SDS sample buffer. The resulting suspension was boiled for 5 min, and 5 to 10 μl was loaded onto a 12% SDS-polyacrylamide gel. After SDS-PAGE, the proteins were transferred to nitrocellulose for 16 h at 25 V. The transferred proteins were immunoblotted using standard protocols with 1:1000 dilution of primary antibody (unless otherwise indicated) and 1:1000 dilution of the appropriate IgG-horseradish peroxidase conjugate. The proteins were visualized by incubation with the chemiluminescence reagent "Pico" (Pierce Chemical, Rockford, IL) and exposure to film. Film was quantitated by densitometry. Film was quantitated at different levels of exposure to ensure linearity, and results presented are within the linear range.

Inositol Phosphate Assays. Cells in 12-well plates were labeled by adding 3 to 5 μCi of [^{3}H]inositol for 24 to 48 h in inositol-free DMEM. After labeling, the medium was removed and replaced with 1 ml of HEPES-buffered DMEM containing 10 mM LiCl and equilibrated for 20 min at 37°C. Ligands or peptides were added in a volume of 50 μl for 45 min after which the medium was aspirated and replaced with 1 ml of ice-cold 50 mM formic acid and applied to Dowex AG1-X8 columns (Bio-Rad, Hercules, CA). The columns were washed with 50 and 100 mM ammonium formate, followed by elution of the IP-containing fraction with 1.2 M ammonium formate/0.1 M formic acid. The eluted fraction was mixed with scintillation fluid and analyzed by liquid scintillation counting.

Coimmunoprecipitation. CHO cells were plated on 35-mm dishes and transfected with 250 or 500 ng of each cDNA as indicated. Forty-eight hours after transfection, cells were lysed in 1% Nonidet P-40 lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40). After sonication and centrifugation, the supernatant was incubated overnight with the antibody and protein G plus agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C with rocking. Beads were centrifuged for 1 min at 13,000 rpm, washed twice with 1.0 ml of lysis buffer, once with 1.0 ml of phosphate-buffered saline, boiled in 50 μl of 2× SDS sample buffer, and loaded onto a 12% SDS polyacrylamide gel. After SDS-PAGE, proteins were transferred to nitrocellulose for 16 h at 25 V followed by immunoblotting as described above.

Results

Mutation of βW332 to Alanine Inhibits Interaction of Peptides with Gβγ. These experiments were designed to determine whether G protein βγ subunits were indeed the direct target of mSIRK in intact cells responsible for ERK activation. We hypothesized that a transfected mutant Gβ subunit that could not bind SIRK would not be responsive to mSIRK treatment and thus not promote ERK activation. We made single alanine substitutions in the $G\beta_1$ subunit to identify amino acids important for interaction with SIRK. We chose to mutate βW332 to A because this mutation had been shown previously to inhibit activation of PLCβ and not affect inhibition of adenylyl cyclase (Li et al., 1998), and both of these properties were consistent with the ability of SIRK to inhibit βγ-dependent activation of PLCβ but not βγ-dependent inhibition of adenylyl cyclase (Scott et al., 2001). Single alanine-substituted mutant biotinylated-$G\beta_1$ subunits were expressed with γ_2 and 6his-α_{i1} subunits in *Sf*9 insect cells and partially purified by nickel-agarose chromatography. That the Gβγ subunits bound to the nickel column and eluted with AlF_4^- indicates that these mutants folded and assembled properly with γ and α subunits.

We used a phage ELISA assay to examine peptide binding to the partially purified $bG\beta_1\gamma_2$ mutant. In this assay, we used a peptide closely related to SIRK, SIGK, that gives a greater ELISA signal and has a higher affinity for Gβγ subunits than SIRK. Here, SIGK displayed on the surface of an M13-derived phage (f88) was tested for binding to immobilized wt or mutant $bG\beta_1\gamma_2$ subunits. As previously demonstrated, these phages do not give an appreciable binding signal in the absence of $bG\beta_1\gamma_2$, and phages that do not display peptide (f88 control) also do not bind $bG\beta_1\gamma_2$. SIGK-displaying phages bound strongly to wild-type $bG\beta_1\gamma_2$ and $bG\beta_1K337A\gamma_2$, whereas binding to $bG\beta_1W332A\gamma_2$ was negligible (Fig. 1A).

To more quantitatively evaluate the decrease in apparent affinity of SIGK for $G\beta_1W332A$, the ability of SIGK to compete for Gα-Gβγ interactions was tested in a flow cytometry assay (Fig. 1B). The $G\beta_1W332A$ mutation decreased the apparent affinity of peptide for $bG\beta_1\gamma_2$ by approximately 40-fold. Previous reports indicate that heterotrimers containing GβW332A are still capable of interacting with receptors, G protein α subunits, and some effectors (Ford et al., 1998; Li et al., 1998; Myung and Garrison, 2000). The three-dimensional crystal structure of $G\beta_1\gamma_2$ subunits bound to SIGK has been solved, demonstrating a direct interaction of this peptide with W332 on $G\beta_1$ (T. L. Davis, T. M. Bonacci, S. R. Sprang, and A. V. Smrcka, submitted). Thus, the results showing that $G\beta_1W332A$ binds to α subunits in the flow cytometry assay, yet has a decreased

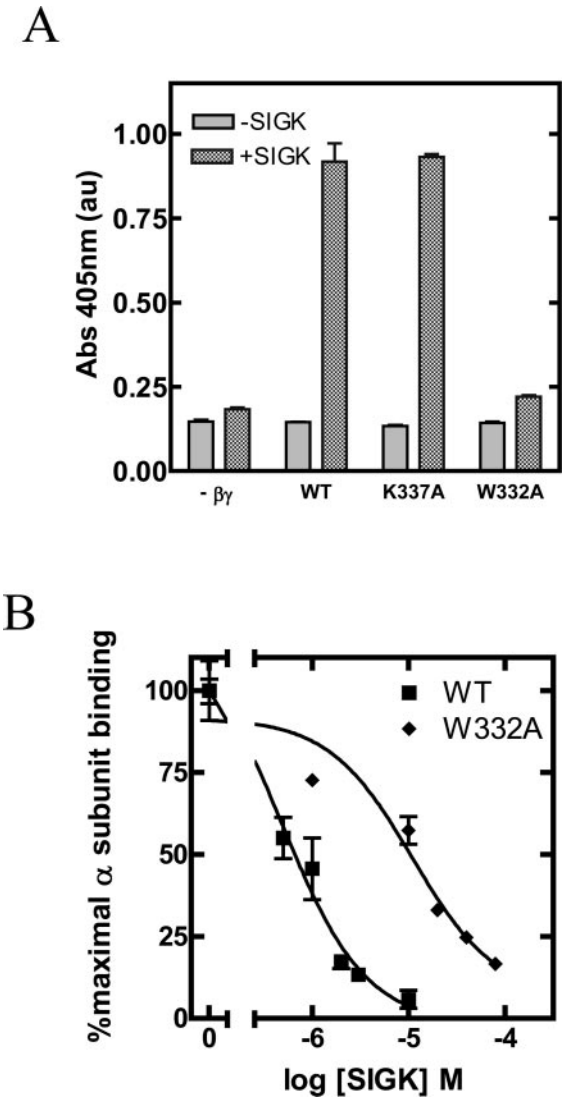

Fig. 1. β_1W332A inhibits SIRK binding. A, binding of control phage or a phage bearing the sequence SIGKAFKILGYPDYD to $bG\beta_1\gamma_2$ in a phage ELISA assay. One hundred nanograms of the indicated wt or mutant $bG\beta_1\gamma_2$ subunits was immobilized and tested for binding of 1×10^{10} phage particles. Bound phages were detected with anti-phage-HRP antibody followed by a colorimetric HRP reaction and optical density measurement at 405 nm as described under *Materials and Methods*. B, synthetic SIGK peptide was tested at the indicated concentrations for its ability to compete for binding of fluorescein isothiocyanate-α_{i1} to either $bG\beta_1\gamma_2$ or $bG\beta_1W332A\gamma_2$ in a flow cytometry assay.

affinity for SIGK, are consistent with previously published data and the crystal structure.

Transfection of βW332A into Intact Cells Inhibits mSIRK-Dependent G Protein Activation. $G\alpha_{i1}$ and $G\gamma_2$ were cotransfected into CHO cells with either GFP-$G\beta_1$ or GFP-$G\beta_1$W332A. We expected that transfection of the wt heterotrimer would enhance mSIRK-mediated ERK phosphorylation, but it did not (Fig. 2A, lanes 1–4). We were surprised to find that transfection of the trimer containing GFP-β_1W332A significantly inhibited the response of these cells to mSIRK (Fig. 2A, compare lanes 1 and 2 with lanes 5 and 6). It is possible that the α subunit transfected with $G\beta_1$W332A was weakened in its interaction with $\beta_1\gamma_2$ containing this mutation and that the excess free $G\alpha$ subunits could sequester endogenous $G\beta\gamma$ subunits released upon peptide addition. To test this, we transfected $G\beta_1$W332A with γ_2 subunits without α subunits. Transfected $G\beta_1$W332A with $G\gamma_2$ also inhibited mSIRK-dependent ERK phosphorylation, whereas transfection of wild-type $G\beta_1$ and $G\gamma_2$ did not (Fig. 2B).

If $G\beta_1$W332A is acting as a dominant negative inhibitor of peptide-mediated ERK activation, then transfection of cells with excess wild-type GFP-β_1 should overcome the inhibition by $G\beta_1$W332A. Cells were transfected with either GFP-$G\beta_1$W332Aγ_2, GFP-$G\beta_1\gamma_2$, or GFP-$G\beta_1$W332Aγ_2 cotransfected with a 2-fold excess of GFP-$G\beta_1\gamma_2$. mSIRK-dependent ERK activation (lanes 1 and 2) was strongly inhibited by transfection of mutant β_1W332Aγ_2 (lanes 3 and 4), and this was largely rescued by the cotransfection of the wild-type $\beta_1\gamma_2$ subunit (lanes 5 and 6).

To determine whether this dominant negative effect was specific to mSIRK-mediated ERK activation, we tested whether transfection of $G\beta_1$W332Aγ_2-affected LPA receptor-dependent ERK activation. In contrast to its effects on mSIRK-mediated ERK activation, transfection of $G\beta_1$W332Aγ_2 had no effect on LPA-mediated ERK activation (Fig. 3A). To confirm that LPA-dependent ERK activation in CHO cells was mediated by $G\beta\gamma$ subunits, we tested the effects of pertussis toxin (PTX) pretreatment. PTX is thought to inhibit GPCR-dependent ERK activation by preventing the release of free $G\beta\gamma$ from Gi heterotrimers (Luttrell et al., 1997). PTX strongly inhibited LPA-mediated responses, indicating that ERK activation by LPA in these cells is through a $G\beta\gamma$ subunit-dependent pathway (Fig. 3B). mSIRK-dependent ERK activation was not inhibited by PTX because mSIRK works through a non–receptor-dependent mechanism. These data indicate that the W332A mutation does not affect the ability of the β subunit to activate ERK through GPCRs and that it is specific for peptide-mediated ERK activation. Thus, a binding site containing W332 on the $G\beta$ subunit is probably the direct target of peptide-mediated ERK activation in intact cells. The exact mechanism for the dominant negative effect of GβW332A on peptide-mediated activation of endogenous ERK pathways is unknown, but we suspect that the overexpressed mutant replaces endogenous $G\beta\gamma$ subunits in endogenous G protein heterotrimers and

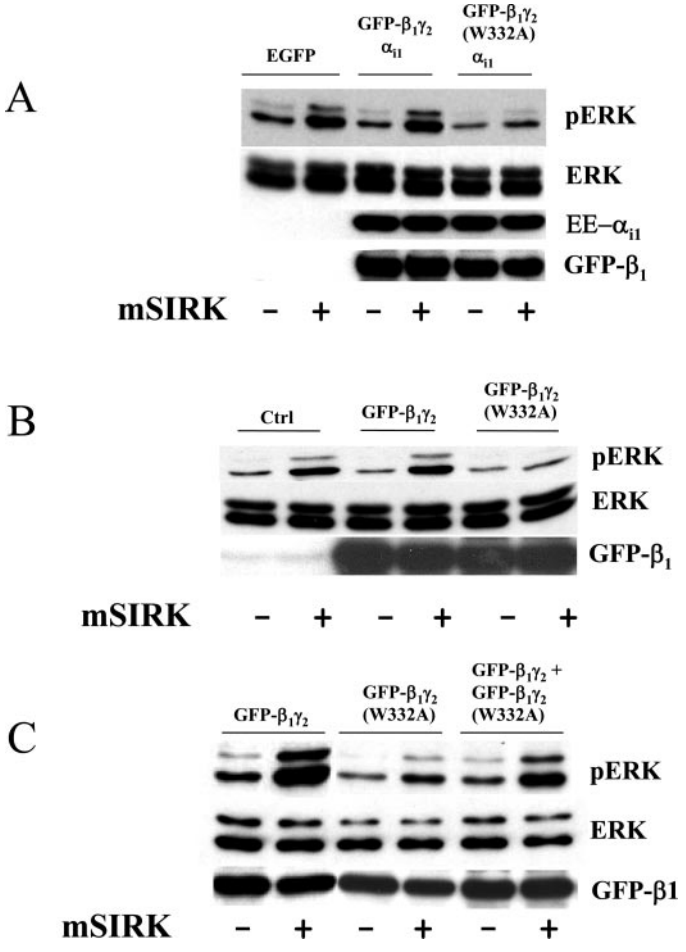

Fig. 2. β_1W332Aγ_2 inhibits peptide-dependent activation of ERK in CHO cells. A, 200 ng of each of the indicated cDNAs was transfected into CHO cells in a six-well plate. Forty-eight hours later, 10 μM mSIRK was added for 5 min. Cells were processed and immunoblotted for phospho-ERK (pERK), total ERK, EE-αi_1 (anti-EE antibody), and GFP-β_1 (anti-GFP antibody) as discussed under *Materials and Methods*. B, either GFP-β_1W332A and γ_2 (200 ng each) or GFP-β_1 and γ_2 (200 ng each) were transfected into CHO cells and treated 48h later with 10 μM mSIRK for 5 min. Cells were processed and immunoblotted for pERK, total ERK, and GFP-β_1 as described in A. In A and B, 1 μg of total DNA was transfected and balanced with pRK5 empty vector. C, CHO cells were transfected with 250 ng of each of the indicated constructs, except for the lanes labeled W332A GFP-$\beta_1\gamma_2$ + GFP-$\beta_1\gamma_2$, where 500 ng of each GFP-β_1 and $G\gamma_2$ were transfected along with W332A GFP-$\beta_1\gamma_2$. In each case, the total DNA transfected in each well was made up to 1.5 μg with pRK5 vector. Treatments were done in the same manner as in A and B. Samples were immunoblotted for pERK, ERK, and GFP-β_1 as described in A.

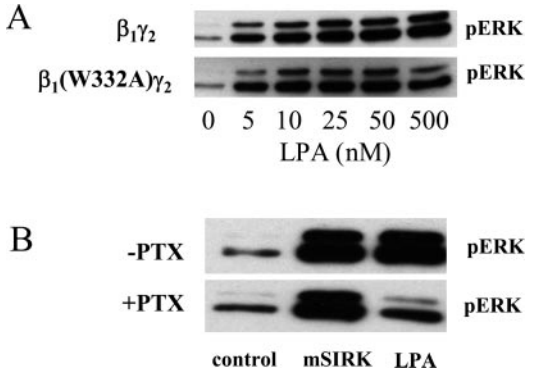

Fig. 3. W332A$\beta_1\gamma_2$ does not inhibit ligand-mediated ERK activation in CHO cells. A, CHO cells transfected with 200 ng of each GFPβ_1 and $G\gamma_2$ or GFP-β_1W332A, and $G\gamma_2$ were treated with the indicated concentrations of LPA for 5 min. Samples were prepared and separated by SDS-PAGE, and proteins were transferred and immunoblotted for pERK as in Fig. 2. B, CHO cells were treated for 16 h with 100 ng/ml PTX and treated with either 10 μM mSIRK or 5 nM LPA for 5 min. Samples were prepared and analyzed as described in A.

these heterotrimers are resistant to mSIRK activation but not to receptor-mediated activation.

Ric-8A Enhances mSIRK-Mediated IP Release. Ric-8A, a recently described G protein guanine nucleotide exchange factor (GEF) for $G\alpha_{q,\ 11,\ i,\ o}$, and $_{12/13}$, exchanges GDP for GTP on free GαGDP but not GαGDPβγ (Tall et al., 2003). We reasoned that if free GαGDP subunits were released from G protein heterotrimers by mSIRK in cells transfected with Ric-8A, the GαGTP subunit-mediated responses to mSIRK would be enhanced. Because $G\alpha_q$GDP is a substrate for Ric-8A in vitro, we predicted that cells expressing Ric-8A would have enhanced mSIRK-dependent IP production because of an increased level of $G\alpha_q$GTP. mSIRK alone causes a small but reproducible increase in IP release in cells transfected with vector control DNA, similar to what we have reported previously (Goubaeva et al., 2003). mSIRK-dependent IP production was enhanced in a dose-dependent manner with transfection of increasing amounts of Ric-8A cDNA (Fig. 4, A and B). On the other hand, Ric-8A had no significant effect on basal IP release (data not shown) or IP release mediated by the GPCR agonists ATP or LPA (Fig. 4, C and D) consistent with previous reports (Tall et al., 2003). Pretreatment with PTX inhibited ATP-dependent IP release by 50% and LPA-dependent IP release by 80% (data not shown), indicating that ATP activates PLCβ through a combination of G_q and $G_i/\beta\gamma$ pathways, whereas LPA is entirely through $G_i/\beta\gamma$ in these CHO cells.

Ric-8A Enhances mSIRK-Dependent IP Production and ERK Activation through a βγ-Dependent Mechanism. To test whether the Ric-8A enhancement of mSIRK-dependent IP production was through α_qGTP or βγ subunits, we determined whether Ric-8A-enhanced IP production could be suppressed by inhibitors of G protein βγ subunit signaling. CHO cells were transfected with Ric-8A or Ric-8A and either the C terminus from βARK (βARK-ct) or the Gα subunit of transducin, α_t. These reagents have been extensively used to sequester free Gβγ subunits without interfering directly with receptor catalyzed G protein activation (Koch et al., 1994). Both transducin and the βARK-ct inhibited responses by mSIRK and mSIRK/Ric-8A to similar levels (Fig. 5, A and B). This indicates that mSIRK-mediated IP release is through free Gβγ subunits and that Ric-8A enhances this βγ-dependent response.

We had previously shown that mSIRK peptides activate ERK in a manner that was blocked by the βARK-ct, strongly suggesting that this response was dependent upon the release of free Gβγ subunits in rat arterial smooth muscle cells (Goubaeva et al., 2003; data not shown). Here, we tested whether Gβγ-dependent ERK activation in CHO cells could be enhanced by transfection of Ric-8A to further explore the

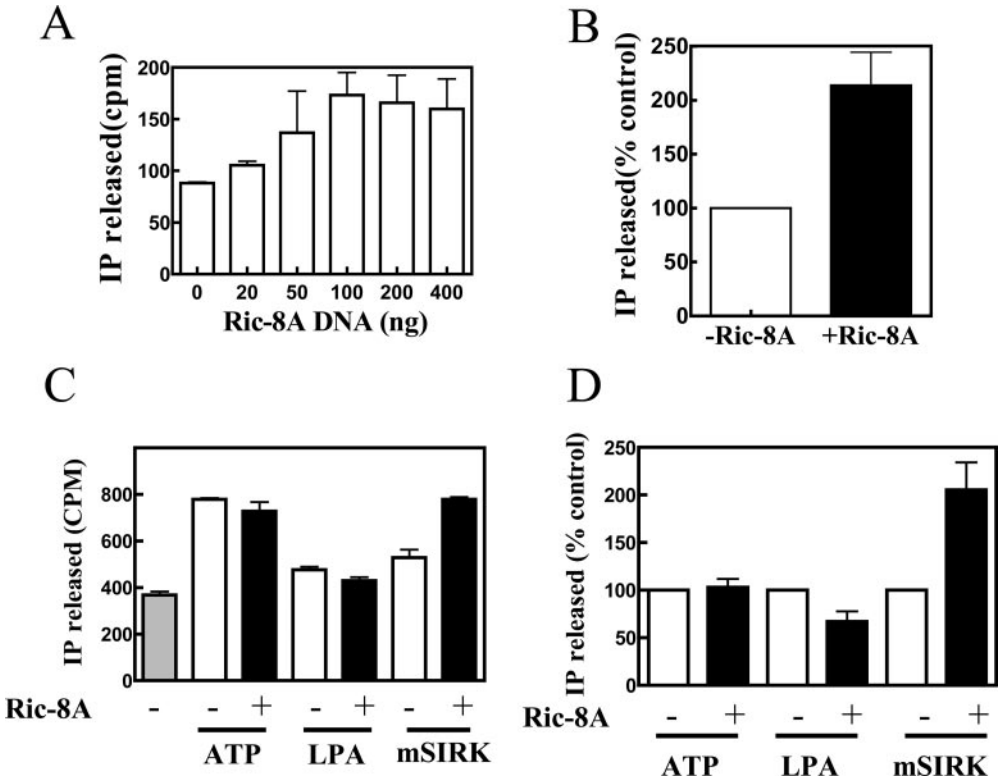

Fig. 4. Ric-8A enhanced mSIRK-dependent IP release in CHO cells. A, CHO cells were transfected with the indicated amounts of Ric-8A pCMV5 [or enhanced green fluorescent protein (pEGFP) control vector, 400 ng total] for 48 h in 12-well dishes. Cells were then treated with mSIRK for 60 min and total IP was measured. IP released is expressed as cpm/well minus basal with no mSIRK treatment. Assays were performed in triplicate, and data are expressed as mean ± S.E.M. The assay was repeated four times. B, pooled data from four experiments with either 200 ng of EGFP or Ric-8A transfected and 10 μM mSIRK was added. mSIRK alone (−Ric-8A) was taken as the 100% control, and data are expressed as mean ± SEM. +Ric-8A is statistically different from −Ric-8A, $P < 0.05$ in a paired t test. C, effect of Ric-8A on hormone-dependent IP release. Cells were transfected with the 200 ng of Ric-8A or pEGFP control DNA and assayed and treated with 0.2 μM ATP, 5 nM LPA, or 10 μM mSIRK for 30 min. D, data from seven experiments as described in C were pooled and analyzed for statistical significance. To facilitate pooling, the data were normalized by subtracting the unstimulated cpm from either ATP-, LPA-, or mSIRK-stimulated cpm in the absence of Ric-8 (100%). Data are expressed as mean ± SEM. ATP alone was not statistically significantly different from ATP + Ric-8A, and LPA alone was not statistically significantly different from LPA + Ric-8A.

idea that Ric-8A can enhance Gβγ-mediated responses. As shown in Fig. 6, A and B, ERK phosphorylation was increased in the presence of mSIRK, and the response was significantly enhanced in cells transfected with Ric-8A or Ric-8B. mSIRK/Ric-8A-dependent ERK activation was significantly attenuated by transducin (Fig. 6, A and B) and βARK-ct expression (data not shown), indicating that Ric-8A enhancement of mSIRK-dependent ERK activation is mediated by Gβγ subunits and not Gα subunits.

We also examined whether Ric-8A or Ric-8B could alter ERK activation in CHO cells in response to the GPCR agonists LPA or ATP. LPA is coupled to ERK activation primarily through Gi/Gβγ, whereas ATP is coupled partially through Gi/βγ and partially through a PTX-insensitive G protein, presumably $G\alpha_q$. Ric-8A and Ric-8B both enhanced ERK activation in response to LPA (Fig. 7, A and B) and ATP (Fig. 8, A and B). LPA-dependent ERK activation was completely blocked by PTX (Fig. 7, A and B), whereas ATP-dependent ERK activation was partially inhibited by PTX (Fig. 8, A and B). These data are consistent with a partial and complete dependence on Gi/βγ pathways for ATP- and LPA-dependent ERK activation, respectively. The enhancement of mSIRK-, LPA-, and ATP-dependent ERK activation by Ric-8A or Ric-8B is modest (a 50–100% increase). For this reason, the results from multiple experiments were quantitated, pooled, and presented in Figs. 6B, 7B, and 8B with

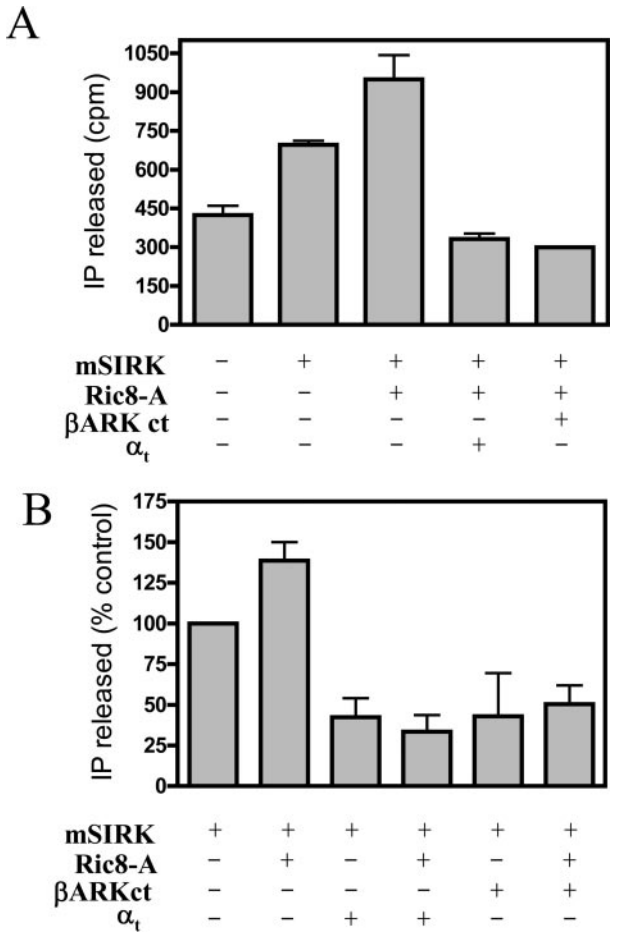

Fig. 5. Inhibitors of βγ subunit signaling inhibit mSIRK and mSIRK/Ric-8A-dependent IP release. A, cells were transfected with pRK5 control DNA (400 ng) or Ric-8A (200 ng) plus pRK5 control vector (200 ng); or Ric-8A (200 ng) plus α_t (200 ng) or βARK-ct (200 ng) followed by treatment with 10 μM mSIRK for 60 min. B, pooled data from seven experiments (α_t) and three experiments (βARKct) expressed as percentage of mSIRK-dependent IP release as in Fig. 4D.

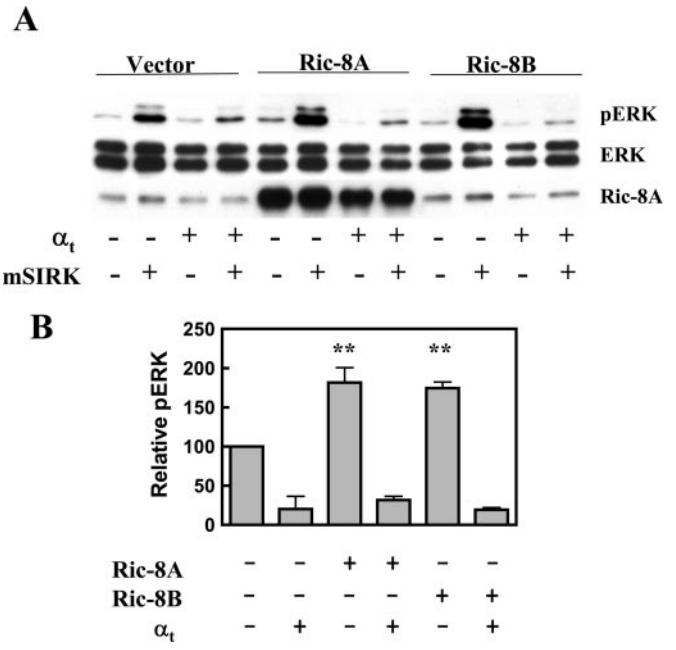

Fig. 6. Ric-8 enhances mSIRK-dependent ERK activation in a βγ subunit-dependent manner. A, cells transfected with 1 μg of vector control or Ric-8A or B DNA (200 ng) and pRK5 (800 ng) or α_t (800 ng) were treated for 5 min with or without 10 μM mSIRK and assayed for phospho-ERK, total ERK, and Ric-8A expression by immunoblotting. B, phosphorylated ERK immunoblots for mSIRK-treated samples were scanned by densitometry, and data were pooled from five experiments and normalized to mSIRK-dependent activation at 100%. Data were analyzed by a one-way analysis of variance followed by Bonferroni's post test; **, $P < 0.01$ compared with mSIRK alone (column 1).

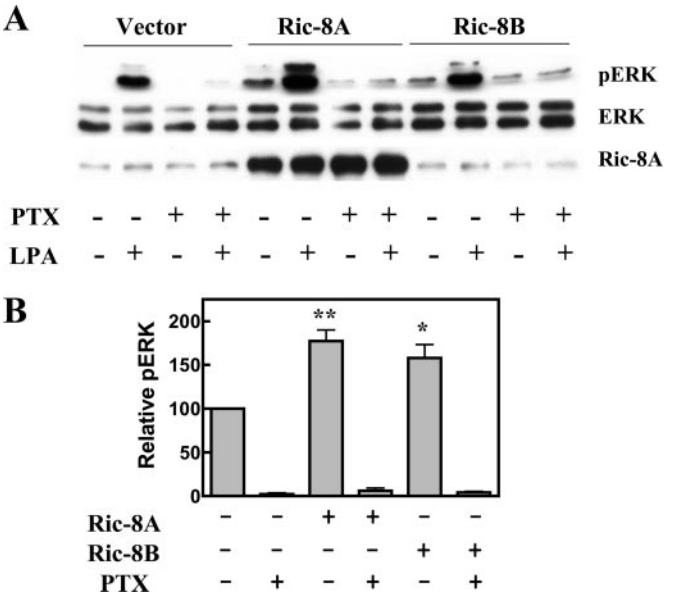

Fig. 7. Ric-8 enhances LPA-dependent ERK activation. A, CHO cells transfected with 200 ng of the indicated constructs were treated with 5 nM LPA for 5 min with or without prior treatment with 100 ng/ml PTX for 16 h. B, phosphorylated ERK immunoblots for LPA-treated samples were scanned by densitometry and data pooled from five experiments and normalized to mSIRK-dependent activation at 100%. Data were analyzed by a one-way analysis of variance followed by Bonferroni's post test; **, $P < 0.01$; *, $P < 0.05$ compared with mSIRK alone (column 1).

analysis for statistical significance. For mSIRK and LPA, the data clearly show a significant enhancement of ERK activation by Ric-8A and Ric-8B. For ATP, there is a trend toward enhancement that it is not statistically significant. This could be because not all of the ATP-dependent ERK activation is mediated by Gβγ subunits. Overall, these data suggest that Ric-8A enhances the responses to these agonists by enhancing G protein βγ-dependent signaling.

Ric-8A Binds α Subunits in Transfected CHO Cells. We were surprised that Ric-8A enhanced βγ-dependent rather than α subunit-dependent responses. To explain this, we hypothesized that excess Ric-8A transfected in cells could bind and sequester the endogenous α subunits, thereby enhancing signaling by βγ subunits. To determine whether Ric-8A stably binds α subunits in CHO cells, we transfected the cells with HA-tagged Ric-8A and either EE-α_{i1} or the empty vector. Cell lysates were prepared, followed by immunoprecipitation with anti-EE antibody. The immunoprecipitate was probed with anti-HA antibody (Fig. 9). Ric-8A-3HA only coimmunoprecipitated from cell lysates containing expressed EE-α_{i1} subunits. Similar results were seen when Ric-8A-3HA was cotransfected with EE-$G\alpha_q$ (data not shown). Together, these results show that in CHO cells Ric-8A can efficiently bind and sequester Gα subunits.

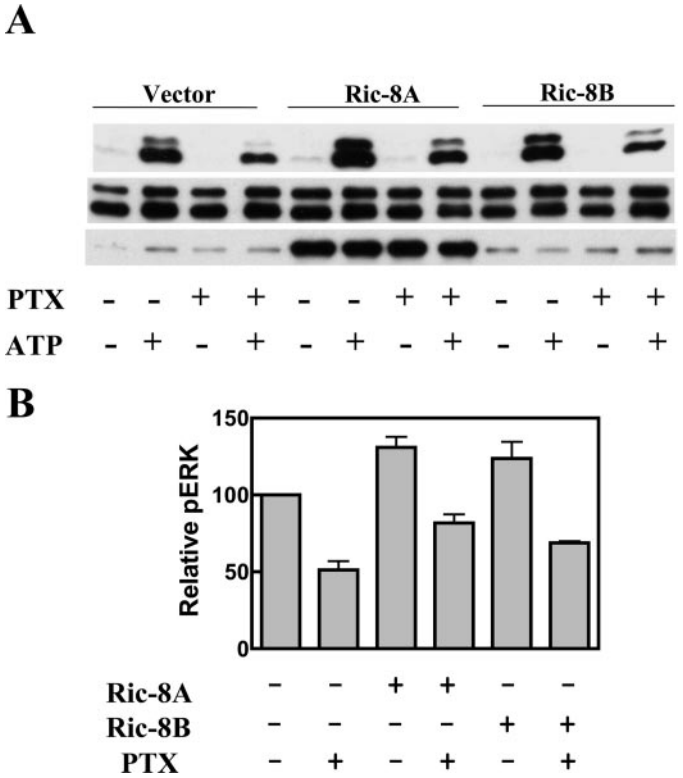

Fig. 8. Ric-8 marginally enhances ATP-dependent ERK activation. A, CHO cells transfected with 200 ng of the indicated constructs were treated with 0.2 μM ATP for 5 min with or without prior treatment with 100 ng/ml PTX for 16 h. B, phosphorylated ERK immunoblots for ATP-treated samples were scanned by densitometry and data pooled from five experiments and normalized to mSIRK-dependent activation at 100%. Data were analyzed by a one-way analysis of variance followed by Bonferroni's post test, and Ric-8A and B enhancements were not found to be statistically significant.

Discussion

We have previously shown that phages display derived peptides that bind to G protein βγ subunits that can activate several signaling pathways in intact cells and promote G protein subunit dissociation in vitro. The cocrystal structure of the peptide bound to G protein βγ subunits was recently solved, with the peptide bound at a position occupied by the switch II helix of $G\alpha_{i1}$ (T. L. Davis, T. M. Bonacci, S. R. Sprang, and A. V. Smrcka, submitted). This provides a plausible explanation at the molecular level of how the peptide causes G protein activation. Here, we present evidence that the peptide binds directly to Gβγ subunits in intact cells and causes α subunits to dissociate from Gβγ subunits to promote Gβγ-dependent signaling.

First, the W332A mutant of $G\beta_1$, but not wt $G\beta_1$, blocked mSIRK-dependent ERK activation in intact cells. $G\beta_1$W332A does not bind to SIRK and should not respond to mSIRK treatment. We expected the GβW332A mutation would alter the behavior of the transfected G protein heterotrimer (both Gα and Gβγ transfected) and were surprised to find that it behaved as a dominant negative inhibitor of peptide-dependent activation of endogenous G protein signaling. We do not fully understand the mechanism of action of this dominant negative inhibition but hypothesize that the overexpressed $G\beta_1$ mutant incorporates into and replaces at least part of the endogenous G protein signaling pool. Regardless of the mechanism, it is clear that transfection of this mutant $G\beta_1$ subunit specifically inhibits ERK activation by mSIRK but not by LPA. The fact that signaling to ERK by endogenous GPCRs remains intact indicates that the ability of $G\beta_1$W332A to activate ERK is not impaired. This is not entirely surprising because this is a binding site for SIRK, and SIRK does not inhibit ERK activation in cells (Goubaeva et al., 2003). In addition, mutation of βW332 to A has previously been shown to selectively inhibit its ability to interact with effectors and does not interfere with its ability to interact with certain receptors (Ford et al., 1998; Li et al., 1998; Myung and Garrison, 2000). This demonstrates that direct binding of mSIRK to Gβγ subunits is required for mSIRK to activate ERK in transfected cells.

Although this result strongly supports the idea that the Gβγ subunits of G protein heterotrimers are the target of these peptides in intact cells, it does not necessarily indicate that binding of the peptide to Gβγ causes subunit dissociation in intact cells. To test this, cells were transfected with Ric-8A, with the idea that it would convert free GαGDP released by mSIRK to GαGTP, which could then activate

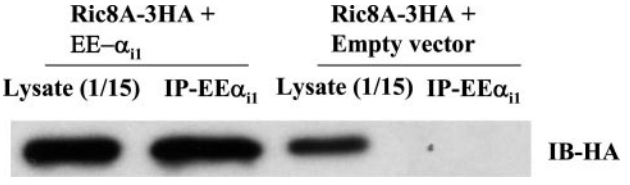

Fig. 9. Ric-8A coimmunoprecipitates with $G\alpha_{i1}$. CHO cells transfected with 500 ng each of Ric-8A-3HA and EE-αi_1 (lanes 1 and 2) or Ric-8A-3HA and pCI-Neo vector (lanes 3 and 4) were immunoprecipitated with anti-EE antibody and probed with anti-HA antibody. Lanes 1 and 3 show expression of Ric-8A-3HA in the transfected cell lysate (1/15 loaded relative to the immunoprecipitate). Lanes 2 and 4 show the Ric8-A 3HA protein that only coimmunoprecipitates when expressed in the presence of EE-αi_1 (lane 2 compared with lane 4).

signal transduction pathways downstream of GαGTP. We had previously shown that mSIRK causes increases in IP production in RASM cells. It was not clear whether this was caused by free Gβγ subunits or by free $G\alpha_q$GDP released that spontaneously exchanged GDP for GTP (Higashijima et al., 1987). If free α_qGDP was released by mSIRK and this was a potential substrate for Ric-8A, then we predicted Ric-8A would enhance mSIRK-mediated IP release. This is in fact what was observed; to our surprise, however, the enhanced IP release seems to be dependent on Gβγ rather than $G\alpha_q$. This is based on the observation that the IP production in response to mSIRK/Ric-8A can be almost completely abrogated by treatment with transducin and the βARK-ct.

The surprising result that Ric-8A can enhance Gβγ-dependent responses is supported by the observation that Ric-8A also enhances mSIRK-dependent ERK activation. We had reported previously, and confirm here in CHO cells, that mSIRK-dependent ERK activation is entirely dependent on Gβγ subunits. Similar results were seen with activation of G protein-coupled receptor agonists where Ric-8A or Ric-8B enhanced the ligand-dependent ERK activation. The enhancement in these cases is modest yet reproducible. For LPA in particular, the entire response was blocked by PTX, indicating that Ric-8A enhanced a Gβγ-dependent pathway.

These data are among the first to show that transfected Ric-8 has a biological effect. Previous work noted that transfected Ric-8A had no effect on $G\alpha_q$-dependent signaling in intact cells (Tall et al., 2003). In those studies, there were multiple possible reasons that transfected Ric-8 was either inactive or unable to access the G protein. The studies presented here show that that Ric-8A binds G protein α subunits in cells and enhances βγ subunit-dependent signaling, yet does not seem to enhance α subunit-mediated responses. If the Ric-8 can access and bind to endogenous G protein α subunits, why is no αGTP subunit-dependent signaling observed? A possibility is that at the high concentrations of Ric-8 expressed in these cells, the excess Ric-8 can bind GαGTP attenuating GαGTP-dependent signaling. Such a possibility is suggested by the observation that Ric-8A stimulates steady-state GTP hydrolysis at low concentrations of Ric-8A, but it inhibits at higher concentrations (G. G. Tall and A. G. Gilman, unpublished observations).

Demonstration that Ric-8 can enhance Gβγ-dependent pathways was unexpected but not entirely inconsistent with its known function. Ric-8A binds to $G\alpha_i$, $G\alpha_o$, $G\alpha_{12/13}$, and $G\alpha_q$ GDP subunits and catalyzes exchange of GDP for GTP. After hydrolysis of GαGTP to GαGDP, the GαGDP might preferentially bind to the expressed Ric-8 over free Gβγ and another round of exchange could occur. Neither free GαGTP, Ric-8:GαGTP, or Ric-8:GαGDP would be expected to rebind to G protein βγ subunits, thus the presence of excess Ric-8 would extend the lifetime of free G protein βγ subunits in the cell. Overall, the data support the notion that free GαGDP subunits are generated in the cell upon treatment with mSIRK because Ric-8 enhances the mSIRK effects.

Several articles have been published suggesting a role for Ric-8 in asymmetric cell division in *C. elegans* (Afshar et al., 2004; Couwenbergs et al., 2004; Hess et al., 2004). Because deletion of Gβ subunits in these animals enhances the G protein-dependent effects on spindle positioning, presumably by raising the level of free Gα subunits in cells, it is unlikely that Gβγ is directly involved in this process. Thus, it is also unlikely that there is a role for Ric-8 in generating free Gβγ subunits in this system. Although it is not entirely clear that release of free Gβγ subunits is a mechanism that occurs with these endogenous Ric-8/G protein signaling systems, our data suggest the possibility that Ric-8 may enhance Gβγ effects through a novel mechanism in more conventional G protein signaling.

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Address correspondence to: Dr. Alan V. Smrcka, Department of Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Box 711, Rochester, NY 14642. E-mail: alan_smrcka@urmc.rochester.edu