Molecular Pharmacology Fast Forward. Published on March 31, 2005 as doi:10.1124/mol.104.010116
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MOL Manuscript# 10116

Ric-8 enhances G protein βγ-dependent signaling in response to βγ-binding peptides in intact cells

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Molecular Pharmacology Fast Forward. Published on March 31, 2005 as DOI: 10.1124/mol.104.010116
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MOL Manuscript# 10116

Running title: Gβγ signaling enhancement by Ric-8

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Non-standard abbreviations: ERK, extracellular signaling regulated kinase; mSIRK, myristoyl-SIRKALNILGYPDYD peptide; IP, inositol phosphate; GEF, guanine nucleotide exchange factor; LPA, lysophosphatidic acid; AGS, activator of G protein signaling; Fα_{i1}, Fluorescein isothiocyanate labeled myristoylated G protein α_{i1} subunit

Number of text pages: 27

Number of Tables: 0

Number of Figures: 9

Number of References: 27

Number of words in Abstract: 249

Number of words in Introduction: 571

Number of words in Discussion: 1049

Molecular Pharmacology Fast Forward. Published on March 31, 2005 as DOI: 10.1124/mol.104.010116
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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 (SIRK) promotes subunit dissociation by binding directly to Gβγ subunits and accelerating the dissociation of GαGDP 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 CHO cells blocked peptide mediated activation of ERK, but did not affect receptor-mediated Gβγ subunit-dependent ERK activation, indicating that Gβγ subunits are in fact the direct target in cells responsible for ERK activation. To determine if free Gα subunits were released from G protein heterotrimers upon peptide treatment, cells were transfected with Ric-8A, a GEF for free GαGDP, but not heterotrimeric G proteins. Ric-8A transfected cells displayed enhanced mSIRK dependent inositol phosphate (IP) release and ERK activation. Ric-8A also enhanced ERK activation by the G_i linked GPCR agonist LPA. Inhibitors of Gβγ 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 Gβγ subunit signaling. Overall these experiments provide further support for the hypothesis that mSIRK promotes G protein subunit dissociation to release free βγ subunits in intact cells.

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Introduction

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 Gα and Gβγ subunits and there is evidence for direct binding of the receptor to both Gα and Gβγ subunits (Taylor *et al.*, 1996;Taylor *et al.*, 1994). Binding of GTP to the Gα subunit activates the G protein and is thought to cause dissociation of Gα subunits from Gβγ subunits, liberating free GαGTP and Gβγ subunits to interact with downstream target proteins and regulate their activities.

Recently 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 βγ subunits from α subunits (Cismowski *et al.*, 2001). GPR or GoLoco peptides derived from AGS proteins promote dissociation of GαGDP subunits from Gβγ subunits causing release of Gβγ (Ghosh *et al.*, 2003;Peterson *et al.*, 2000;Kimple *et al.*, 2002). Additionally, a novel protein, Ric-8, has been identified that binds specifically to free GαGDP subunits and promotes GDP release (Tall *et al.*, 2003;Miller *et al.*, 2000). 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 *C. elegans* zygotes (Afshar *et al.*, 2004;Couwenbergs *et al.*, 2004;Hess *et al.*, 2004)

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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 MAP 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 $G\alpha_i$GDP subunits from $G\alpha_{i1}\beta_1\gamma_2$ heterotrimers (Ghosh *et al.*, 2003). Recently the structure of G protein $\beta_1\gamma_2$ subunits bound to one of the peptides (SIGK) was solved (Davis *et al.*, 2004). 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 (Lambright *et al.*, 1996;Wall *et al.*, 1995). These data suggest a molecular mechanism by which these peptides promote G protein subunit dissociation by interfering with Gα subunit interactions with Gβγ subunits.

While these *in vitro* data support a model for peptide-mediated dissociation of GαGDP from Gβγ as the mechanism for 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 protein 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. Surprisingly we found that Ric-8 can enhance G protein βγ subunit-mediated responses, probably by a mechanism that involves sequestration of free Gα subunits.

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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 in pcDNA 3.1+, βARKct kindly supplied by Dr. Robert Lefkowitz was in pRK5 and Ric-8A and Ric-8B were in pCMV5. mSIRK (myristoyl-SIRKALNILGYPDYD) and SIGK (SIGKAFKILGYPDYD) were synthesized and purified by Alpha Diagnostics International. Myo-[^{3}H]-inositol (25 Ci/mmol) was from PerkinElmer Life Sciences. Pertussis toxin, LPA and ATP were from Sigma. Rabbit anti-ERK and anti-phosphoERK antisera were from Cell Signaling Technologies. Anti Ric-8A antiserum was generated in rabbits against holo purified Ric-8A protein by Caprologics, Inc. Mouse anti HA and anti EE antisera were from Covance. Mouse anti GFP, goat anti Rabbit IgG-horseradish peroxidase conjugate (HRP) and goat anti mouse IgG-HRP were from Roche.

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 previously described (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 PCR using standard methods and the entire protein coding region was sequenced to confirm the presence of the mutation and lack of additional mutations.

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

Measurement of α–$\beta\gamma$ interactions via flow cytometry: The fluorescein labeled α_{i1} ($F\alpha_{i1}$) used in these experiments was prepared as described (Sarvazyan *et al.*, 1998) and competition assays were performed as described in detail (Ghosh *et al.*, 2003). Briefly, for competition based assays, 100-200 pM of $F\alpha_{i1}$ and indicated concentrations of peptides were added to 50 pM of $bG\beta_1\gamma_2$ immobilized on 10^5 beads per mL buffer and incubated at room temperature for 30 minutes to reach equilibrium. The bead-associated fluorescence was then recorded in the flow cytometer. The data was corrected for non-specific binding and fit with a sigmoid dose response curve using Graph Pad Prism 4.

Cell culture and transfection. All cell culture reagents were obtained from Invitrogen, Inc. Chinese Hamster Ovary cells obtained from ATCC were grown in DMEM supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin at 37^{o}C with 5% CO_2. Cells were grown in 6 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 βARK-ct in pRK5, 800 ng α_t, or pRK5 empty vector control, using Lipofectamine Plus (Invitrogen, Inc.) unless otherwise indicated. For IP release measurements, cells were grown in 12 well plates and 200 ng Ric-8A was transfected with 200 ng of the appropriate inhibitor with a total of 400 ng DNA transfected in each well. Transfections were performed 48 h prior to the final treatment and when multiple plasmids were transfected, appropriate amounts of control cDNA's were added such that the total DNA transfected was constant in each experiment.

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Measurement of ERK activation and general immunoblotting. For measurement of phospho-ERK, serum was removed from 50-80% confluent CHO cells 16 h before treatment. Peptides in dimethylsulfoxide (DMSO), DMSO vehicle, or other agonists were diluted 100-400 fold into the medium and incubated at 37^{o}C for the indicated times. For all immunoblotting: after treatment, cells were transferred to ice, the medium quickly aspirated, and replaced with 100 µl of 2x SDS sample buffer. The resulting suspension was boiled for 5 min and 5-10 µl was loaded onto a 12% SDS-polyacrylamide gel. After SDS-PAGE, the proteins were transferred to nitrocellulose for 16 h at 25 volts. 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), 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 3-5 µCi of ^{3}H-inositol for 24-48 h in inositol-free DMEM. After labeling, the medium was removed and replaced with 1ml of HEPES buffered DMEM containing 10 mM LiCl and equilibrated for 20 min at 37^{o}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 formic acid (50 mM) and applied to Dowex AG1-X8 columns (BioRad). The columns were washed with 50 mM 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.

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Co-Immunoprecipitation. CHO cells were plated on 35 mm dishes and transfected with 250 or 500 ng of each cDNA as indicated. 48 hr post transfection, cells were lysed in 1% NP-40 lysis buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 30 mM Sodium pyrophosphate, 50 mM NaF, 100 μM PMSF and 1% NP40). Following sonication and centrifugation, the supernatant was incubated overnight with the antibody and protein G plus agarose beads (Santa Cruz Biotech) 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 PBS, boiled in 50 μL of 2X SDS sample buffer and loaded on to a 12% SDS polyacrylamide gel. After SDS-PAGE proteins were transferred to nitrocellulose for 16 h at 25 volts followed by immunoblotting as described above.

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Results

Mutation of βW332 to alanine inhibits interaction of peptides with G βγ. These experiments were designed to determine if 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 previously been shown to inhibit activation of PLC β 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 PLC β 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 Ni agarose chromatography. That the Gβγ subunits bound to the Ni 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 phage do not give an appreciable binding signal in the absence of $bG\beta_1\gamma_2$ and phage that do not display peptide (f88 control) also do not bind $bG\beta_1\gamma_2$. SIGK displaying phage bound strongly to wild type $bG\beta_1\gamma_2$ and $bG\beta_1K337A\gamma_2$ while binding to $bG\beta_1W332A\gamma_2$ was negligible (Fig 1A).

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To more quantitatively evaluate the decrease in apparent affinity of SIGK for $G\beta_1W332A$, the ability of SIGK to compete for $G\alpha$-$G\beta\gamma$ 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 about 40 fold. Previous reports indicate that heterotrimers containing $G\beta W332A$ are still capable of interacting with receptors, G protein α subunits and some effectors ((Myung and Garrison, 2000;Li *et al.*, 1998;Ford *et al.*, 1998). Recently 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 $G\beta_1$ (Davis *et al.*, 2004). Thus, the results showing that $G\beta_1W332A$ binds to α subunits in the flow cytometry assay, yet has a decreased 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-$G\beta_1W332A$. We expected that transfection of the wt heterotrimer would enhance mSIRK mediated ERK phosphorylation, but it did not (Fig 2A lanes 1-4). Surprisingly, transfection of the trimer containing GFP-β_1W332A significantly inhibited the response of these cells to mSIRK (Fig 2A compare lanes 1 and 2 to lanes 5 and 6). It is possible that the α subunit transfected with $G\beta_1W332A$ was weakened in its interaction with $\beta_1\gamma_2$ containing this mutation and the excess free $G\alpha$ subunits could sequester endogenous $G\beta\gamma$ subunits released upon peptide addition. To test this we transfected $G\beta_1W332A$ with γ_2 subunits without α subunits. Transfected $G\beta_1W332A$ with $G\gamma_2$ also inhibited mSIRK dependent ERK phosphorylation while transfection of wild type $G\beta_1$ and $G\gamma_2$ did not (Fig 2B).

If $G\beta_1W332A$ 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

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inhibition by $G\beta_1W332A$. Cells were transfected with either GFP-$G\beta_1W332A\gamma_2$, GFP-$G\beta_1\gamma_2$, or GFP-$G\beta_1W332A\gamma_2$ cotransfected with a twofold excess of GFP-$G\beta_1\gamma_2$. mSIRK-dependent ERK activation (lanes 1 and 2) was strongly inhibited by transfection of mutant $\beta_1W332A\gamma_2$ (lanes 2 and 4), and this was largely rescued by the co-transfection of the wild type $\beta_1\gamma_2$ subunit (lanes 5 and 6).

To determine if this dominant-negative effect was specific to mSIRK-mediated ERK activation, we tested whether transfection of $G\beta_1W332A\gamma_2$ affected lysophosphatidic acid (LPA) receptor-dependent ERK activation. In contrast to its effects on mSIRK-mediated ERK activation, transfection of $G\beta_1W332A\gamma_2$ had no effect on LPA mediated ERK activation (Fig 3A). To confirm that LPA-dependent ERK activation in CHO cells was mediated by Gβγ 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 Gβγ 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 Gβγ 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 β subunit's ability to activate ERK through GPCRs, and is specific for peptide mediated ERK activation. Thus, a binding site containing W332 on the Gβ subunit is likely 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 Gβγ subunits in endogenous G protein heterotrimers and these heterotrimers are resistant to mSIRK activation but not receptor-mediated activation.

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Ric-8A enhances mSIRK mediated IP release. Ric-8A, a recently described G protein guanine nucleotide exchange factor (GEF) for $G\alpha_{q,\ 11,\ i,\ o\ ,\ 12/13}$, 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, that GαGTP subunit mediated responses to mSIRK would be enhanced. Since $G\alpha_q$GDP is a substrate for Ric-8A *in vitro*, we predicted that cells expressing Ric-8A would have enhanced mSIRK-dependent inositol phosphate (IP) production due to 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 previously reported (Goubaeva *et al.*, 2003). mSIRK-dependent IP production was enhanced in a dose dependent fashion with transfection of increasing amounts of Ric-8A cDNA (Fig. 4A 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. 4C 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 PLC β through a combination of G_q and $G_i/\beta\gamma$ pathways while LPA is entirely through $G_i/\beta\gamma$ (data not shown) 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. 5A 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 idea that Ric-8A can enhance Gβγ mediated responses. As shown in Figures 6A and 6B, 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. 6A and 6B) and βARK-ct expression (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, while 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 7A and B) and ATP (Fig 8A and B). LPA- dependent ERK activation was completely blocked by PTX (Fig 7A and B) while ATP dependent ERK activation was partially inhibited by PTX (Fig 8A 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 figures 6B, 7B, and 8B with analysis for statistical significance. For mSIRK

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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 if Ric-8A stably binds α subunits in CHO cells, we transfected the cells with HA tagged Ric-8A and either, EE-$\alpha_{i1,}$ 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 co-immunoprecipitated from cell lysates containing expressed EE-α_{i1} subunits. Similar results were seen when Ric-8A 3HA was co-transfected with EE-Gα_q (data not shown). Together these results show that in CHO cells Ric-8A can efficiently bind and sequester Gα subunits.

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Discussion

We have previously shown that phage display derived peptides that bind to G protein βγ subunits can activate several signaling pathways in intact cells and promote G protein subunit dissociation *in vitro*. Recently the co-crystal structure of the peptide with G protein βγ subunits was solved with the peptide bound at a position occupied by the switch II helix of G α_{i1} (Davis *et al.*, 2004). 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). Also, 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 (Myung and Garrison, 2000;Li *et al.*, 1998;Ford *et al.*, 1998). This

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demonstrates that direct binding of mSIRK to Gβγ subunits is required for mSIRK to activate ERK in transfected cells.

While 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 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 due to free Gβγ subunits or 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, but surprisingly the enhanced IP release appears 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 previously reported, 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 the ligand dependent ERK activation was enhanced by Ric-8A or Ric-8B. The enhancement in these cases is modest yet reproducible. In the case of LPA in particular, the entire response was blocked by PTX indicating that Ric-8A enhanced a Gβγ-dependent pathway.

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These data are amongst 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 appear 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 inhibits at higher concentrations (Tall and 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 since Ric-8 enhances the mSIRK effects.

Recently a number of papers have been published suggesting a role for Ric-8 in asymmetric cell division in *C. elegans* (Couwenbergs *et al.*, 2004;Afshar *et al.*, 2004;Hess *et al.*, 2004). Since deletion of Gβ subunits in these animals enhances the G

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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. While 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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Footnotes

This work was supported by NIH grants GM60286 (AVS), GM34497 (Tall and Gilman) and an NIH predoctoral Training Grant in Cardiovascular Biology HLT3207949 (TMB)

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Figure Legends

Figure 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. 100 ng of the indicated wt or mutant bG$\beta_1\gamma_2$ subunits were immobilized and tested for binding of 1×10^{10} phage particles. Bound phage were detected with antiphage-HRP antibody followed by a colorimetric HRP reaction, and optical density measurement at 405 nm as described in "Methods". B) Synthetic SIGK peptide was tested at the indicated concentrations for its ability to compete for binding of FITC-α_{i1} to either bG$\beta_1\gamma_2$ or bGβ_1W332Aγ_2 in a flow cytometry assay.

Figure 2. *β_1W332Aγ_2 inhibits peptide dependent activation of ERK in CHO cells.* A) 200 ng of each of the indicated cDNAs were transfected into CHO cells in a 6 well plate. 48h 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 in "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 in A. In A and B, 1μg 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 Gγ_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 2A and 2B. Samples were immunoblotted for pERK, ERK, and GFP-β_1 as described in A.

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Figure 3. *W332A$\beta_1\gamma_2$ does not inhibit ligand mediated ERK activaton in CHO cells.* A) CHO cells transfected with 200 ng of each GFPβ_1 and Gγ_2 or GFP-β_1W332A and Gγ_2 were treated with the indicated concentrations of LPA for 5 min. Samples were prepared, separated by SDS-PAGE, proteins transferred and immunoblotted for pERK as in Figure 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 in A.

Figure 4. *Ric-8A enhanced mSIRK dependent inositol phosphate (IP) release in CHO cells.* A) CHO cells were transfected with the indicated amounts of Ric-8A pCMV5 (or 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 measured. IP released is expressed as CPM/well minus basal with no mSIRK treatment. Assays were performed in triplicate and data expressed as mean +/- SEM. The assay was repeated 4 times. B) Pooled data from 4 experiments with either 200 ng of EGFP or Ric-8A transfected and 10 μM mSIRK 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 7 experiments as 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.

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Figure 5. *Inhibitors of βγsubunit 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 5 min. B) Pooled data from 7 experiments (α_t) and 3 experiments (βARKct) expressed as % of mSIRK dependent IP release as in Fig. 4D.

Figure 6. *Ric-8 enhances mSIRK-dependent ERK activation in a βγsubunit-dependent manner*. A) Cells transfected with 1μg 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 phosphoERK, total ERK and Ric-8A expression by immunoblotting. B) phosphorylated ERK immunoblots for mSIRK treated samples were scanned by densitometry and data pooled from 5 experiments and normalized to mSIRK dependent activation at 100%. Data were analyzed by a one way ANOVA followed by Bonferroni's post test, $^{**}P<0.01$ compared to mSIRK alone (bar 1).

Figure 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 5 experiments and normalized to mSIRK dependent activation at 100%. Data were analyzed by a one way ANOVA followed by Bonferroni's post test, $^{**}P<0.01$; $^{*}P<0.05$ compared to mSIRK alone (bar 1).

Figure 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 5 experiments and normalized to mSIRK dependent activation at 100%. Data were analyzed by a one way ANOVA followed by Bonferroni's post test and Ric-8A and B enhancements were not found to be statistically significant.

Figure 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 co-immunoprecipitates when expressed in the presence of EE-αi_1 (lane 2 compared to lane 4).

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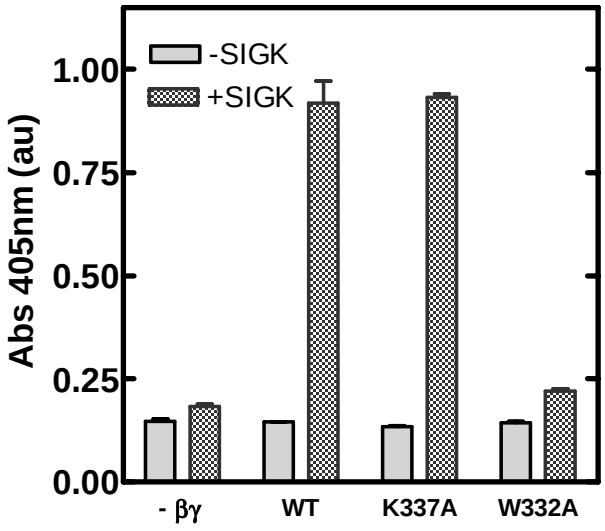

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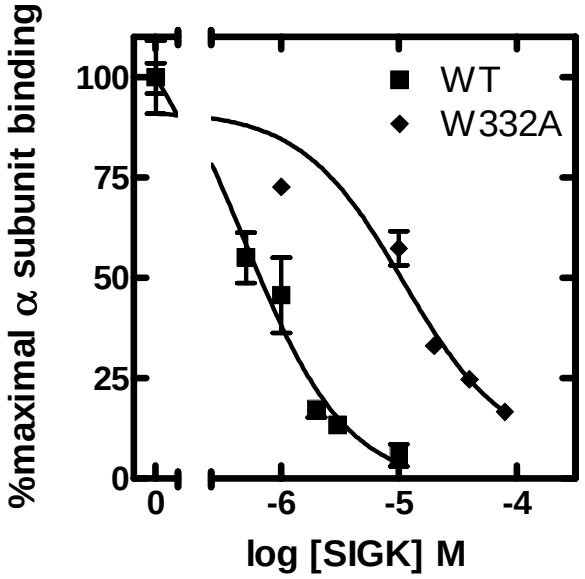

Fig. 1

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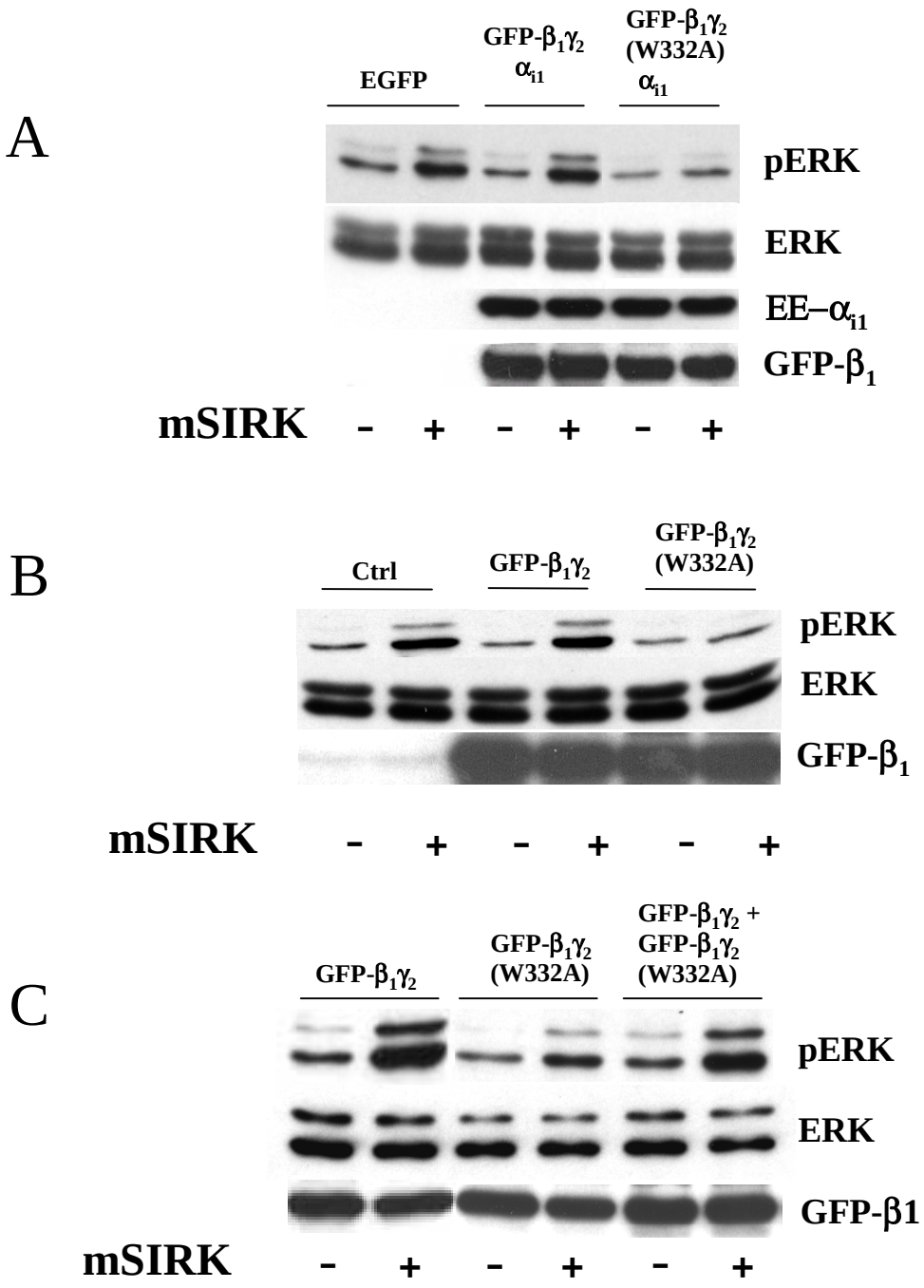

Fig. 2

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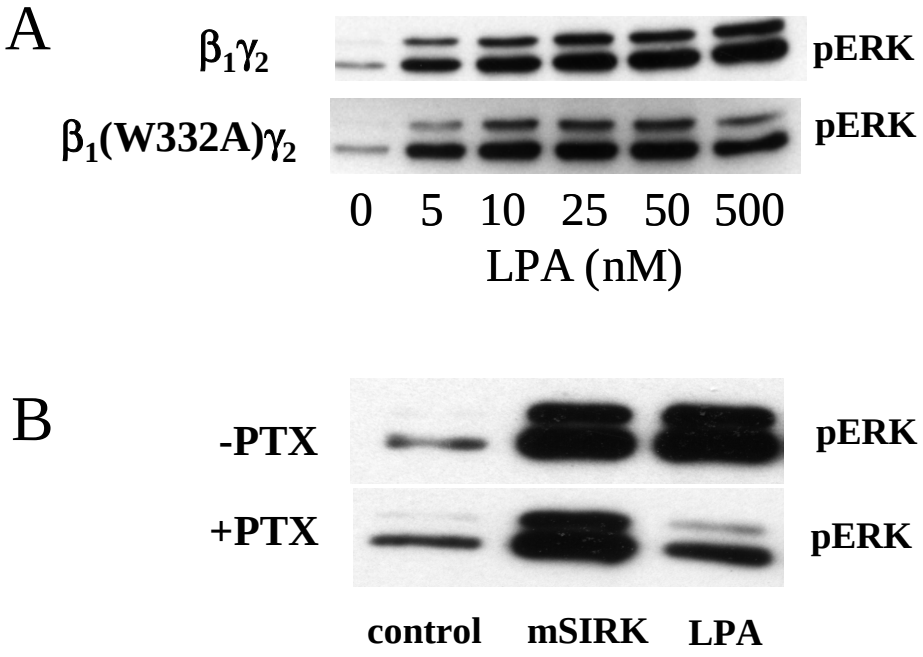

Fig. 3

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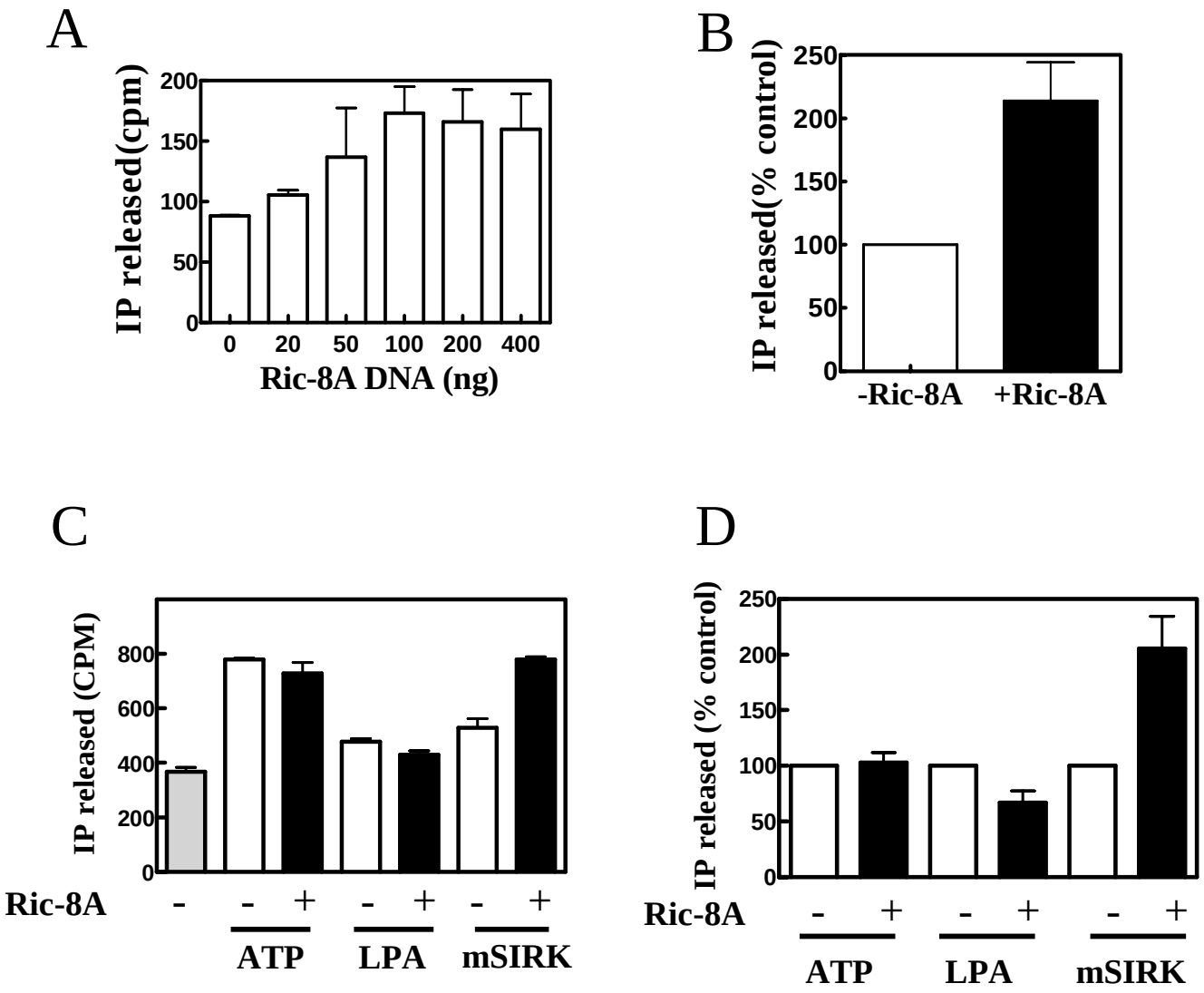

Fig. 4

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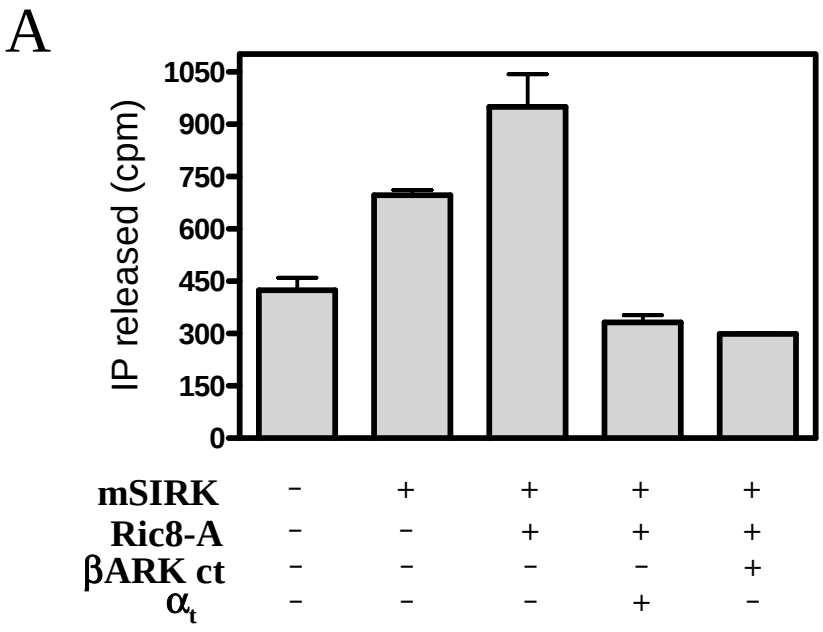

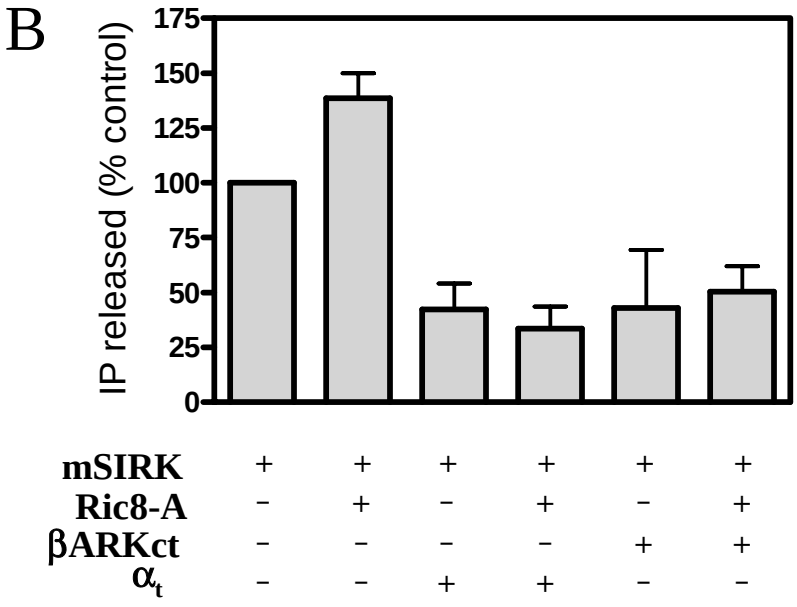

Fig. 5

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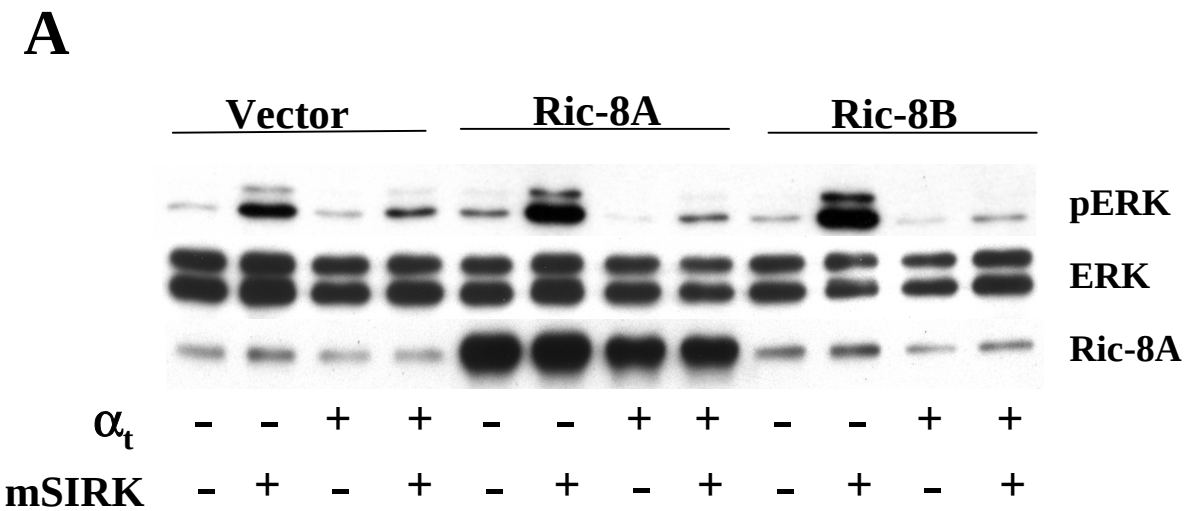

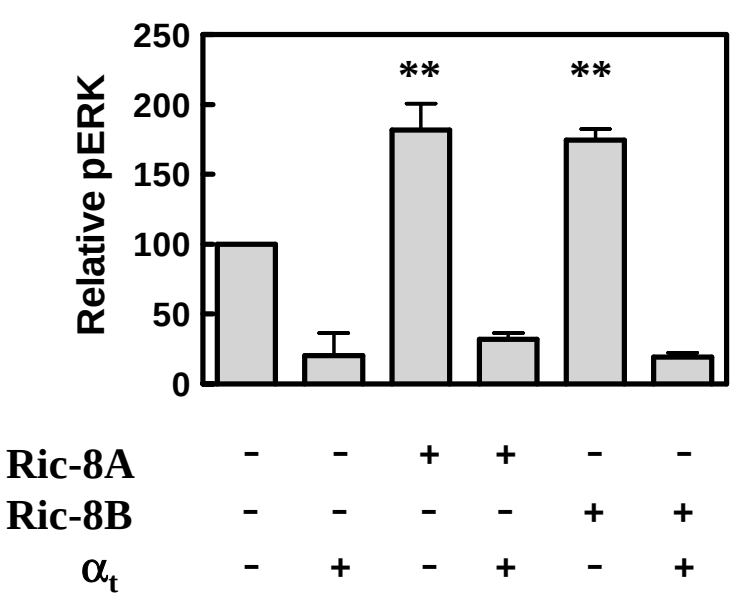

Fig. 6

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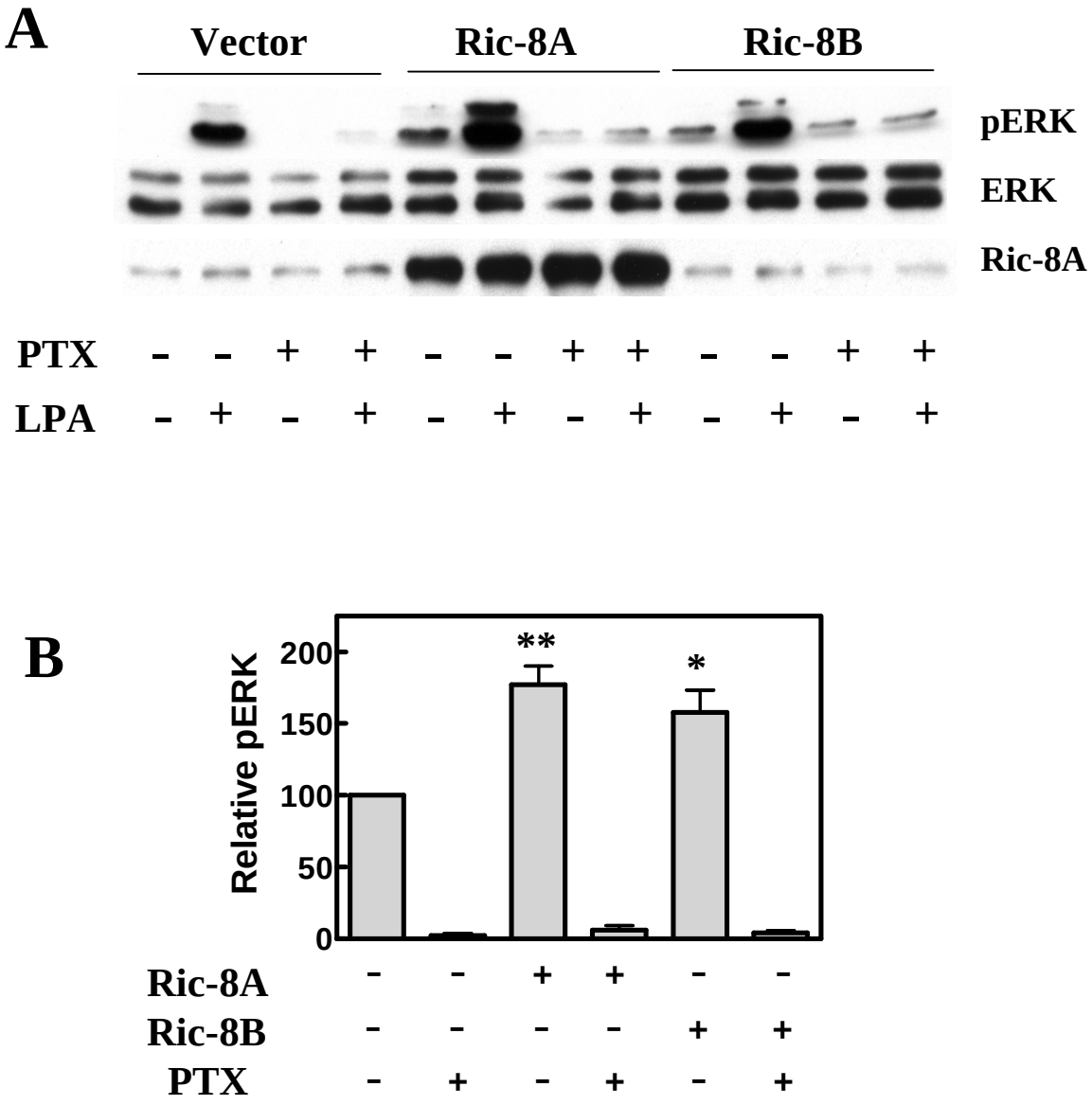

Fig. 7

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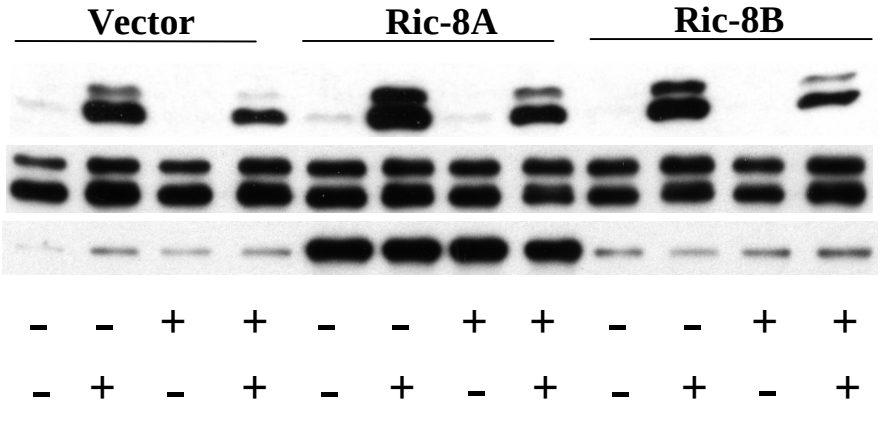

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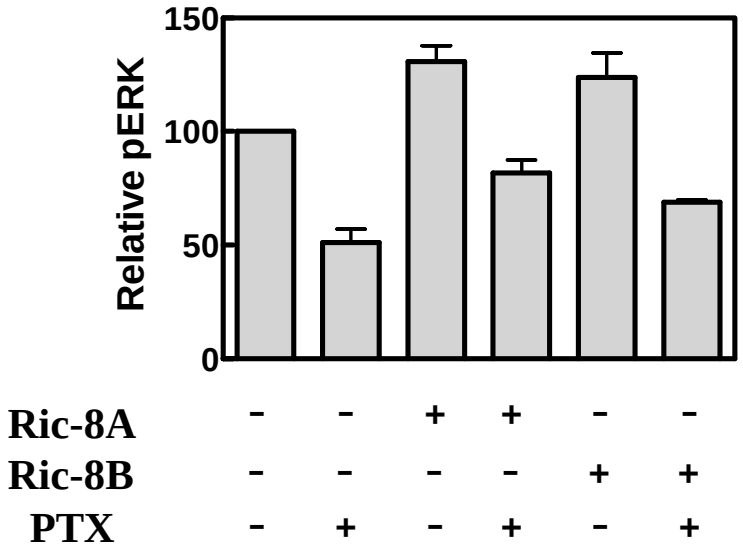

Fig. 8

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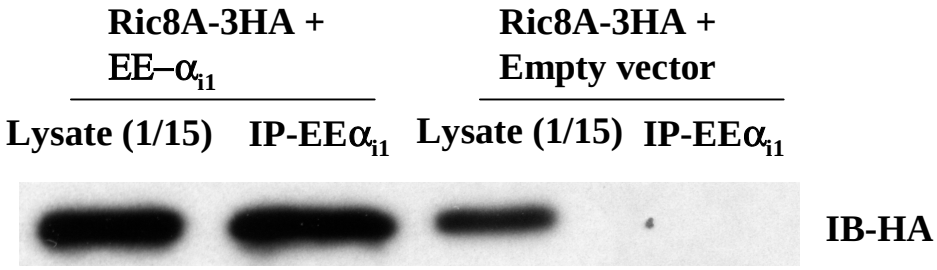

Fig. 9