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Production of Phosphorylated Ric-8A Proteins using Protein Kinase CK2.

Wenxi Yu, Maiya Yu, Makaia M. Papasergi-Scott, and Gregory G. Tall*

Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

Abstract

Resistance to Inhibitors of Cholinesterase-8 (Ric-8) proteins are molecular chaperones that fold heterotrimeric G protein α subunits shortly after biosynthesis. Ric-8 proteins also act as test tube guanine nucleotide exchange factors (GEF) that promote G α subunit GDP for GTP exchange. The GEF and chaperoning activities of Ric-8A are regulated by phosphorylation of five serine and threonine residues within protein kinase CK2 consensus sites. The traditional way that Ric-8A proteins have been purified is from *Spodoptera frugiperda* (Sf9) or *Trichoplusia ni* (Tni) insect cells. Endogenous insect cell kinases do phosphorylate the critical regulatory sites of recombinant Ric-8A reasonably well, but there is batch-to-batch variability among recombinant Ric-8A preparations. Additionally, insect cell-production of some Ric-8 proteins with phosphosite alanine substitution mutations is proscribed as there seems to be interdependency of multi-site phosphorylation for functional protein production. Here, we present a method to produce wild type and phosphosite mutant Ric-8A proteins that are fully occupied with bound phosphate at each of the regulatory positions. Ric-8A proteins were expressed and purified from *E. coli*. Purified Ric-8A was phosphorylated *in vitro* with protein kinase CK2 and then reisolated to remove kinase. The phosphorylated Ric-8A proteins were ~99% pure and the completeness of phosphorylation was verified by chromatography, phos-tag SDS-PAGE mobility shifts, immunoblotting using phospho-site specific antibodies, and mass spectrometry analysis. *E. coli*-produced Ric-8A that was phosphorylated using this method promoted a faster rate of G α subunit guanine nucleotide exchange than Ric-8A that was variably phosphorylated during production in insect cells.

Keywords

Ric-8A; phosphorylation; GEF; Protein kinase CK2; Casein Kinase 2; Phos-tag gel

*To whom correspondence should be addressed. gregtall@med.umich.edu.

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Introduction

Resistance to Inhibitors of Cholinesterase-8 (Ric-8) proteins are molecular chaperones that participate in the biosynthetic protein folding process of heterotrimeric G protein α subunits [1, 2]. Ric-8 proteins also act as guanine nucleotide exchange factors (GEF) that promote G α subunit GDP for GTP exchange [3-9]. Ric-8A substrates include G α_i -, G α_q -, and G α_{13} -class subunits, whereas G α_s /G α_{olf} are substrates for Ric-8B [3, 4, 10, 11]. We proposed that Ric-8 binds to nascent G α subunits and renders a conformation that is capable of productively binding GTP for the first time [12]. G α subunits that are produced in systems lacking Ric-8 protein(s) may be folded improperly and adopt a bottleneck conformation(s) that lacks guanine nucleotide. Mis-folded proteins are often degraded rapidly in cells, which is consistent with observations that newly produced G α subunits are turned over nearly ten-times faster in *Ric-8A*-null cells versus wild type cells [1]. This may account for the dramatic reductions (by ~95%) of functional G α_i /q/13 or G α_s subunit abundances in *Ric-8A* or *Ric-8B* knock-out cells, respectively [1, 2, 13-15]. Purified Ric-8 proteins are also effective catalysts for the preparation of purified G α proteins that are bound stoichiometrically to GTP γ S for use in G protein effector enzyme studies [16].

Ric-8A is post-translationally modified by phosphorylation at various residues, which may affect its subcellular localization and ubiquitination [17-20]. We recently demonstrated that phosphorylation of (Rat) Ric-8A residues S435 and T440 (Human Ric-8A S436 and T441) dramatically potentiated G α subunit GEF and chaperoning activities [21]. Three additional C-terminal phosphosites, S522, S523, and S527 were identified and postulated to work in concert with S435-PO₄ and T440-PO₄ to affect Ric-8A activities and to promote high affinity G α subunit binding. All five of these Ric-8A phosphosites are protein kinase CK2 consensus sites, four of which are functionally conserved in all Ric-8 homologs, including mammalian Ric-8B. The protein kinase CK2 phosphosites of Ric-8A and Ric-8B appear to be efficiently phosphorylated in normal cells and tissues, and when produced as recombinant proteins in *S9* insect cells. These observations are consistent with the consideration that protein kinase CK2 is a constitutively active kinase [22].

Attempts to parse out the individual contributions of the five Ric-8A phosphosites by creating single and combinatorial alanine substitution mutant Ric-8A proteins produced in insect cells was met with mixed results. Some combinations of Ric-8A alanine substitution mutants were proscribed because they resulted in poor yields or completely dysfunctional recombinant proteins. This was not the case when the same mutant Ric-8A proteins were produced in *E. coli*, which lacks protein kinase CK2 and produces unmodified, recombinant Ric-8A. We interpret this to mean that there may be critical interactions among the different protein kinase CK2 phosphorylated residues that are important for Ric-8 structure and function. Additionally, the CK2 consensus sites of insect cell-produced Ric-8A or Ric-8B are only partially phosphorylated. This confounds the interpretation of Ric-8-regulated G protein guanine nucleotide exchange and protein folding measurements.

Here we present a detailed method for the preparation of *E. coli*-produced Ric-8A proteins that are stoichiometrically-phosphorylated using recombinant protein kinase CK2 holoenzyme. The method entails a re-purification step to isolate phosphorylated Ric-8

proteins away from kinase. The phosphorylated Ric-8 proteins exhibited dramatically enhanced ability to stimulate G α subunit guanine nucleotide exchange. This method will find utility for the production of homogenous preparations of phosphorylated Ric-8 proteins for structural studies, G protein enzymatic analyses, and as catalytic tools for the production of purified G α -GTP γ S.

Results and Discussion

Production and Purification of Ric-8A from *E. coli*.

Bacterial expression plasmids for Ric-8A and Ric-8B were created by sub-cloning the amino-terminally tagged glutathione-S-transferase (GST)-tobacco etch virus protease (TEV)-Ric-8A cassette from the pFastBac-1 vector into the T7 bacterial promoter vector, pET21a [3, 23]. The GST tag was chosen over a His6 tag for improved solubility of the fusion protein and because amino-terminally tagged His6-Ric-8 proteins do not readily adsorb to metal chelate resins. Ric-8A can be readily changed to encode phospho-site point mutant Ric-8A proteins via standard site-directed mutagenesis procedures as required. Competent BL21 (DE3) *E. coli* were transformed with pET21a-GST-TEV-Ric-8A and the cells were expanded and cooled to 16 °C for induction. Slow induction at lower temperature is essential to mitigate recombinant Ric-8A protein aggregation.

E. coli cell lysates are prepared in two steps. Collected cell pastes are frozen, thawed, resuspended in lysis buffer and treated with lysozyme. Viscosity is reduced in this “first lysis” using DNase I and the lysate is clarified by differential centrifugation. The pellet contains many unbroken cells and is subjected to a “second lysis” by rapid freezing with liquid nitrogen, followed by shearing in additional lysis buffer with a hand-held kitchen blender. The secondary lysis step is very important, as it contains much more total protein than the first lysis. The combined lysates are clarified and loaded onto the gravity-driven glutathione Sepharose 4B column.

After loading, the column is washed and drained (Fig. 1, lanes 2-5). His6-TEV protease is triturated directly into the column matrix to cleave the recombinant GST-TEV-Ric-8A fusion protein. TEV-cleaved Ric-8 proteins eluted from the column are typically found to be ~90% pure (Fig. 1, lane 5 and Table I). Resin-bound GST and uncleaved GST-Ric-8 proteins are subsequently eluted with reduced glutathione to evaluate TEV protease cleavage efficiency (Fig. 1, lane 6). TEV-eluted Ric-8A is diluted to ~1 mg/ml and loaded onto a Hi-trap Q HP column using an FPLC. This anion exchange polishing purification step achieves ~99% pure Ric-8 proteins and typical recoveries exceed ~80% (Fig. 1 lane 7-15). Purification Table 1 presents a quantitative analysis of the enrichment and purity attained during each of the individual steps of the Ric-8A purification procedure. Typical yield of recombinant Ric-8A prior to the elective phosphorylation procedure is ~5 mg per liter of culture.

In vitro protein kinase CK2 phosphorylation of Ric-8A

E. coli-produced Ric-8 proteins are unmodified. Protein kinase CK2 holoenzyme is used to fully phosphorylate recombinant rat Ric-8A at its five protein kinase CK2 consensus sites: S435, T440, S522, S523, and S527. These sites were identified by tryptic and chymotryptic

peptide-mapping mass spectrometry analysis [21]. Concentrated stock solutions of ATP (100 mM) that are required for the kinase reaction were found to be low pH, perhaps due to laboratory water, but nonetheless caused Ric-8A protein aggregation if mixed directly into a kinase reaction. Therefore, freshly prepared, concentrated ATP stock solutions are buffered with 50 mM Hepes pH 8.0 and testing with pH strips is employed to ensure near neutral pH. The kinase reaction is then assembled, which typically results in 95% of the Ric-8 protein preparation becoming fully phosphorylated.

Fully phosphorylated Ric-8A proteins (e.g. Ric-8A-5PO₄) are polished and separated from residual kinase by high-resolution Mono Q 10/100 GL anion exchange chromatography (GE Healthcare). Hi-Trap Q HP columns do not provide sufficient resolution to discriminate fully phosphorylated from lesser phosphorylated Ric-8A species. Figure 2A shows the resultant chromatographs of a kinase and mock kinase reaction with Ric-8A. Unmodified Ric-8A eluted at lower ionic strength (220 mM NaCl) than phosphorylated Ric-8A (270 mM NaCl). Leftover ATP was also adsorbed to the column and separated from Ric-8A during NaCl gradient development. The diminution of the ATP peak represents the amount consumed by kinase during Ric-8A phosphorylation.

To evaluate phosphorylation of the key regulatory sites of purified Ric-8A and a Ric-8A truncation mutant protein consisting of 1-452 amino acids, phosphosite-specific antibodies that recognize Ric-8A phosphorylated at positions S435 or T440 were used to immunoblot the Ric-8A proteins that had been treated with or without protein kinase CK2 and re-purified [21]. Kinase-specific phosphorylation of S435 and T440 was observed for both proteins (Fig 2B). Phosphorylated and unphosphorylated Ric-8A and Ric-8A 1-452 were then resolved by Phos-tag SDS-PAGE and visualized by Coomassie-staining (Fig. 2C). The Phos-tag reagent binds selectively to phosphate groups and reduces the gel mobility of phosphorylated proteins in a manner that is relative to the number of occupied phosphosites [24]. Ric-8A contains five phosphosites and the Ric-8A 1-452 protein lacks the three C-terminal sites (S522, S523, S527). Ric-8A-5PO₄ exhibited a relative phostag gel mobility shift of 9-10% compared to unphosphorylated Ric-8A, whereas Ric-8A 1-452 (2PO₄) exhibited a smaller mobility shift of 6% compared to unphosphorylated Ric-8A 1-452 (Fig. 2C).

We attempted to express and purify a full-length Ric-8A protein with the S522, S523, and S527 phosphosites mutated to encode alanines ("Ric-8A triple A") using the *Sf9* or *Tni* expression systems. Very low yields of inactive Ric-8A triple A protein were attained from the insect cells. We speculate that there is an interdependent structural requirement of phosphorylation of one or more of the C-terminal phosphosites and S435 and/or T440 phosphorylation for functional Ric-8A protein production. Despite being distant in the linear amino acid sequence, all five phosphosites were predicted to reside in very close proximity in a three-dimensional Ric-8A structural prediction model [21]. S435 and T440 of Ric-8A triple A could be phosphorylated by endogenous insect cell kinases. However, the presence of unnatural alanines in place of S522, S523, and/or S527 might create a structural conflict when S435 or T440 are phosphorylated. Therefore, we reasoned that if we produced Ric-8A triple A protein in *E. coli*, where the protein could be folded in the absence of S435 and T440 phosphorylation, the protein might be active and could then be phosphorylated *ex vivo* with protein kinase CK2. This hypothesis was validated by the production of highly pure,

phosphorylated Ric-8A triple A protein at a yield only ~2/3 that of wild-type Ric-8A (Table 1). After *in vitro* protein kinase CK2-mediated phosphorylation, the phosphorylation status of Ric-8A triple A was compared to wild type Ric-8A by phostag gel mobility shift assay. Phosphorylated and unphosphorylated Ric-8A and Ric-8A triple A had gel mobility shifts of 10% and 4%, respectively (Fig. 2D), which is consistent with the known quintuple and predicted dual phosphorylation status of the two proteins.

Functional Testing of Phosphorylated Ric-8 proteins – G protein GTP γ S binding assays

Ric-8A is a guanine nucleotide exchange factor (GEF) that stimulates the rate of G α subunit guanine nucleotide exchange [3-9]. Dual phosphorylation of Ric-8A at positions T440 and S435 dramatically accelerated the rates of Ric-8A-stimulated G α_q , G α_{i1} , and G α_{13} guanine nucleotide exchange [21]. We previously found that insect cell-produced Ric-8A preparations were heterogeneously phosphorylated [21]. *In vitro* kinase-treated *E. coli*-produced Ric-8A-5PO $_4$ appeared by phostag gel and chromatography analyses to be homogeneously phosphorylated (Fig. 2). We sought to directly measure the completeness of phosphorylation of recombinant Ric-8A produced in insect cells or in *E. coli* and subjected to *in vitro* protein kinase CK2 phosphorylation. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis demonstrated that insect cell-produced Ric-8A was variably modified ranging from one to five phosphorylated species (Fig 3A). In contrast, *in vitro* protein kinase CK2-treated Ric-8A was phosphorylated efficiently, as a major five-phosphorylated protein was observed (Fig. 3B). Likewise, the protein kinase CK2-treated Ric-8A triple A preparation was found to predominantly consist of a dual phosphorylated protein (Fig. 3C).

We then examined the activity of the two wild type Ric-8A protein preparations. Protein kinase CK2-phosphorylated Ric-8A exhibited dramatically higher GEF activity compared to unmodified Ric-8A (Fig. 3D). Interestingly, near-homogeneously phosphorylated Ric-8A-5PO $_4$ that was produced using the *in vitro* protein kinase CK2 phosphorylation method stimulated G α_q guanine nucleotide exchange significantly faster than Ric-8A produced from insect cells (rates of $0.015 \pm 0.001 \text{ min}^{-1}$ and $0.08 \pm 0.01 \text{ min}^{-1}$, respectively) (Fig. 3D). This activity difference was examined in closer detail by measuring the initial rates of G α_q GTP γ S binding in response to two concentrations of each Ric-8A protein preparation. The initial G α_q guanine nucleotide exchange rates stimulated by Ric-8A-5PO $_4$ produced in *E. coli* were ~50% faster than the rates stimulated by the equivalent protein produced in insect cells (Fig. 3E).

Conclusions

We have developed a method to produce wildtype and mutant Ric-8A proteins from *E. coli* that can be phosphorylated at the regulatory protein kinase CK2 phosphosites to near homogeneity. The phosphorylation status and activity of the purified Ric-8A proteins was verified by Phos-tag SDS-PAGE mobility shift assay, immunoblotting blotting with phosphosite-specific antibodies, mass spectrometry, and by G α GTP γ S binding activity assay. Due to the high purity and high degree of phosphorylation of Ric-8A proteins produced by this method, they may be ideal for protein structural studies. They may also

find utility for the study of interactions between Ric-8A and G proteins, screening for Ric-8A modulators, and additional Ric-8A binding partners. Ric-8A proteins are also used traditionally as catalysts for the production of activated G α -GTP γ S for use in G protein effector assays [16]. The dramatically enhanced activity of phosphorylated Ric-8A proteins produced by this method should prove advantageous for this use.

Materials and Methods

Bacterial Culture, Induction, and Cell Lysate Preparation:

BL21 (DE3) competent *E. coli* (NEB) were transformed with plasmids encoding rat Ric-8A amino-terminally tagged with Glutathione S-transferase and a tobacco etch virus (TEV) protease cleavage site (GST-TEV) and selected on LB agar containing carbenicillin (50 μ g/mL). GST-TEV-Ric-8A cassette was produced by restriction digestion of pFastBac-1 vector harboring the corresponding gene using BamHI and SalI, and was subcloned into the T7 bacterial promoter vector, pET21a (Millipore). Transformants were pre-cultured in 80 ml LB-carb medium overnight. The next morning, cells were centrifuged to remove pre-culture medium, resuspended in fresh LB-carb medium, and distributed evenly into five 1 L LB-carb cultures. Cells were grown to an O.D.₆₀₀ of 0.6 and then cooled to 16 °C and induced with 30 μ M IPTG and allowed to grow for 8 hours at 150 rpm to induce protein production. The cells from 5 L of culture were collected by centrifugation at 10,000 x g for 10 min. The cell paste was frozen with liquid nitrogen and then thawed into 700 ml of lysis buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM DTT) and protease inhibitors (23 μ g/mL phenylmethylsulfonyl fluoride, 21 μ g/mL Na-*p*-tosyl-L-Lysine-chloromethyl ketone, 21 μ g/mL L-1-*p*-tosylamino-2-phenylethyl-chloromethyl ketone, 3.3 μ g/mL leupeptin, and 3.3 μ g/mL soy bean trypsin inhibitor). 750 mg of chicken egg white lysozyme (Sigma) was added and the mixture was stirred for 45 min at 4 °C. DNase I (7 mg) and 750 μ l of 1M MgCl₂ were added and the mixture was stirred for an additional 5 min at 4 °C. The lysate was centrifuged at 5000 x g for 10 min and the supernatant was collected. The 5000 x g pellet was rapidly frozen with liquid nitrogen and thawed into 700 ml of fresh lysis buffer. DNase I (7 mg) and 750 μ l of 1M MgCl₂ were added and a hand-held blender was used to thoroughly homogenize the biomass and to facilitate further cell lysis. The secondary lysate was then centrifuged at 5000 x g. The combined 5000 x g supernatants were centrifuged at 100,000 x g for 45 min.

Glutathione Sepharose 4B Chromatography:

The 100,000 x g supernatant was loaded by gravity onto a 3 ml bed volume glutathione Sepharose 4B column (GE Healthcare) at ~1 ml / min. The column was washed with 200 ml of lysis buffer containing 80 mM NaCl. 2 ml of TEV protease (2 mg/ml) was added for 18 h at 4 °C to cleave the Ric-8A from the GST tag. The sample was collected and the column was eluted further with 10 ml of Anion Exchange Buffer A (20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT) and protease inhibitors. The combined eluate was reappplied to the column and collected. Additional Anion Exchange Buffer A was then used to elute the column until no more protein could be detected by Bradford Assay. The Ric-8 protein samples are ~90% pure at this point.

HiTrap Q HP Chromatography:

The Ric-8A protein sample was purified further using a 5 ml bed volume HiTrap Q HP anion exchange column (GE Healthcare). The TEV-eluted Ric-8A sample was passed through a 0.22 μ m polyethersulfone syringe filter and loaded onto the HiTrap Q HP column in Anion Exchange Buffer A using an AktA FPLC. The column was washed with 25 ml Anion Exchange Buffer A and eluted with a 100 ml linear gradient to 550 mM NaCl in Anion Exchange Buffer A while collecting 2 ml fractions. The protein-containing fractions were analyzed by Coomassie-stained SDS-PAGE. The Ric-8A-containing fractions were identified, pooled, and concentrated using an Amicon Ultra 30 KDa NMWL ultra centrifugal device (Millipore). The purified Ric-8A samples were rapidly frozen in liquid nitrogen and stored at -80°C .

Protein Kinase CK2 holoenzyme phosphorylation of Ric-8A:

A stock solution of 100 mM buffered ATP was prepared by dissolving ATP powder in 50 mM HEPES pH 8.0. The pH of the solution was verified to be near neutral by testing with pH colorimetric indicator strips (GE Healthcare). Purified Ric-8A (25 mg) was incubated in a 10 ml kinase reaction containing 25 mM HEPES pH 8.0, 10 mM MgCl_2 , 1 mM EGTA, 1 mM DTT, protease inhibitor cocktails, 100 mM NaCl, 30 μ l of Protein Kinase CK2 (*i.e.* Casein Kinase 2, CK2) holoenzyme (NEB), and 2.5 mM of buffered ATP. The kinase reaction was incubated for 1.5 h at 30°C and then at 22°C for 1 h. The reaction was cooled to 4°C , diluted to 25 ml with Anion Exchange Buffer A containing 50 mM NaCl and protease inhibitor cocktails, and then passed through a 0.22 μ m sterile syringe filter. The filtered reaction was loaded onto a Mono Q 10/100 GL anion exchange chromatography column (GE Healthcare) at 1.5 ml / min with Anion Exchange Buffer A. The column was washed with 40 ml Anion Exchange Buffer A and eluted with a 160 ml linear gradient to 525 mM NaCl in Anion Exchange Buffer A while collecting 2 ml fractions. Ric-8A-5PO₄ typically elutes from the column at ~ 310 mM NaCl. Purified, phosphorylated Ric-8 proteins were concentrated using an Amicon Ultra 30 KDa NMWL ultra centrifugal device and stored at -80°C .

Phos-tag SDS-PAGE

In order to qualitatively assess Ric-8A phosphorylation, the proteins were resolved on 8% SDS-acrylamide gels containing Phos-tag-reagent (Wako Chemical Industries) that retards the electrophoretic mobility of phosphorylated proteins. Phos-tag PAGE was performed using an established method with additional modifications [25]. Phos-tag acrylamide resolving gels were comprised of 8% 29:1 acrylamide/bis-acrylamide (Bio-Rad), 350 mM bis-tris, pH 6.8 (Fisher Scientific), 75 μ M Phos-tag reagent (Wako Chemical Industries), 150 μ M $\text{Zn}(\text{NO}_3)_2$, 1% tetramethylethylenediamine (TEMED), and 0.05% ammonium persulfate (APS). The resolving gel was overlaid with isopropanol and allowed to polymerize. The stacking gel was comprised of 4% 29:1 acrylamide/bis-acrylamide, 350 mM bis-tris, pH 6.8, 0.05% APS, and 0.1% TEMED.

Purified Ric-8A proteins were buffer exchanged into 20 mM HEPES, pH 8.0, 50 mM NaCl by passage through PD Spintrap G-25 columns (GE Healthcare) to remove all EDTA, EGTA, and free phosphates, which interfere with phostag acrylamide gel resolution.

Phosphorylated or unphosphorylated Ric-8A were mixed 1:1 with 2X Phos-tag sample buffer (0.1% bromophenol blue, 2% SDS, 20% glycerol, 0.5 M Tris-HCl pH 8.5 and 0.2 M DTT) and heated at 95 °C for 5 min. The samples were cooled to room temperature and $\text{Zn}(\text{NO}_3)_2$ was added to 4 mM prior to gel loading. Protein samples corresponding to 5 μg protein were loaded onto 8% Phos-tag gels. Gels were run at a constant 100 V for ~3.5 h in gel running buffer (50 mM Tris-HCl, pH 7.8, 50 mM 3-N-morpholino-propanesulfonic acid (MOPS), 0.1% SDS, and freshly made 5 mM sodium bisulfite). Proteins were detected by Coomassie staining. Relative phostag gel mobilities were calculated as the ratio of distance traveled by each Ric-8A protein from the origin of the gel to the 20 kDa molecular mass standard.

Western blotting

Protein samples corresponding to 40 ng protein were resolved by 10% SDS-PAGE, and transferred to BA85 nitrocellulose (GE Healthcare). Immunoblotting was performed using 1/2000 dilution of rabbit monoclonal phospho-Casein Kinase II motif (pS/pT)-D-X-E antibody (Cell Signaling Technologies) that specifically recognizes the T440 phosphosite of rat Ric-8A. A 1/3000 dilution of IgG purified from rabbit antiserum 6383 was used to detect the S435 phosphosite of Ric-8A [21]. A 1/6000 dilution of IgG purified from rabbit polyclonal antiserum 1184 was used to detect purified Ric-8A [21].

Whole protein Mass Spectrometry analysis of Ric-8A phosphorylation.

Purified Ric-8A proteins were buffer exchanged into 5 mM HEPES pH 8.0, 100 mM NaCl and 100 μM DTT by passage through PD Spintrap G-25 desalting columns (GE Healthcare). Proteins were diluted to 30 μM and 50 μL each were injected into a Thermo-Fisher LTQ-Velos Orbitrap Elite mass spectrometer to determine intact protein masses via electrospray ionization tandem mass spectrometry (ESI-MS/MS). The mass spectrometry and analysis was performed in conjunction with the University of Michigan Medical School Biomedical Research Core Proteomics and Peptide Synthesis Facility.

Gα_q GTPγS Binding Assays

Gα_q [^{35}S]-GTPγS binding or guanine nucleotide exchange extended progress curve assays were performed as described with minor modifications [4]. In brief, Gα_q (100 nM) and Ric-8A proteins (100 nM each) were mixed and incubated at 25 °C in GTPγS binding buffer (20 mM Hepes, pH 8.0, 100 mM NaCl, 4 mM DTT, 1 mM EDTA, 10 mM MgCl_2 , 0.05% Genapol C-100 and 10 μM [^{35}S]-GTPγS (~15,000 – 20,000 cpm /pmol). Triplicate aliquots were taken from the reactions at 0, 2, 4, 7, 10, 15 and 30 min and quenched in GTP quench buffer (20 mM Tris, pH 7.7, 100 mM NaCl, 10 mM MgCl_2 , 1 mM GTP, and 0.08% (m/v) Genapol C-100), and filtered onto BA85 nitrocellulose filters to capture the [^{35}S]-GTPγS-bound Gα. The filters were washed with 20 mM Tris, pH 7.7, 100 mM NaCl, 2 mM MgCl_2 , dried and subjected to scintillation counting to determine the fraction of Gα_q bound to GTPγS. For the initial rate determination assays, the experiments were conducted as described above except that 50 nM or 25 nM of Ric-8A proteins were included in each reaction. Triplicate aliquots were taken from the reactions at 0, 0.5, 2 and 4 min and processed as described above.

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Abbreviations

GEF	guanine nucleotide exchange factor
CK2	Casein Kinase 2

References:

1. Gabay M, et al., Ric-8 proteins are molecular chaperones that direct nascent G protein alpha subunit membrane association. *Sci Signal*, 2011 4(200): p. ra79. [PubMed: 22114146]
2. Chan P, et al., Molecular chaperoning function of Ric-8 is to fold nascent heterotrimeric G protein alpha subunits. *Proc Natl Acad Sci U S A*, 2013 110(10): p. 3794–9. [PubMed: 23431197]
3. Tall GG, Krumins AM, and Gilman AG, Mammalian Ric-8A (synembryn) is a heterotrimeric Galpha protein guanine nucleotide exchange factor. *J Biol Chem*, 2003 278(10): p. 8356–62. [PubMed: 12509430]
4. Chan P, et al., Ric-8B is a GTP-dependent G protein alphas guanine nucleotide exchange factor. *J Biol Chem*, 2011 286(22): p. 19932–42. [PubMed: 21467038]
5. Wright SJ, et al., RIC8 is a guanine-nucleotide exchange factor for Galpha subunits that regulates growth and development in *Neurospora crassa*. *Genetics*, 2011 189(1): p. 165–76. [PubMed: 21750256]
6. Thomas CJ, et al., Ric-8A catalyzes guanine nucleotide exchange on G alpha1 bound to the GPR/GoLoco exchange inhibitor AGS3. *J Biol Chem*, 2008 283(34): p. 23150–60. [PubMed: 18541531]
7. Hess HA, et al., RGS-7 completes a receptor-independent heterotrimeric G protein cycle to asymmetrically regulate mitotic spindle positioning in *C. elegans*. *Cell*, 2004 119(2): p. 209–18. [PubMed: 15479638]
8. Romo X, et al., xRic-8 is a GEF for Galpha and participates in maintaining meiotic arrest in *Xenopus laevis* oocytes. *J Cell Physiol*, 2008 214(3): p. 673–80. [PubMed: 17960561]
9. Tall GG and Gilman AG, Resistance to inhibitors of cholinesterase 8A catalyzes release of Galphai-GTP and nuclear mitotic apparatus protein (NuMA) from NuMA/LGN/Galphai-GDP complexes. *Proc Natl Acad Sci U S A*, 2005 102(46): p. 16584–9. [PubMed: 16275912]
10. Matsuzaki F, Drosophila G-protein signalling: intricate roles for Ric-8? *Nat Cell Biol*, 2005 7(11): p. 1047–9. [PubMed: 16385732]
11. Von Dannecker LE, Mercadante AF, and Malnic B, Ric-8B, an olfactory putative GTP exchange factor, amplifies signal transduction through the olfactory-specific G-protein Galphao1f. *J Neurosci*, 2005 25(15): p. 3793–800. [PubMed: 15829631]
12. Papasergi MM, Patel BR, and Tall GG, The G protein alpha chaperone Ric-8 as a potential therapeutic target. *Mol Pharmacol*, 2015 87(1): p. 52–63. [PubMed: 25319541]
13. Hampoelz B, et al., Drosophila Ric-8 is essential for plasma-membrane localization of heterotrimeric G proteins. *Nat Cell Biol*, 2005 7(11): p. 1099–105. [PubMed: 16228011]
14. Tall GG, Ric-8 regulation of heterotrimeric G proteins. *J Recept Signal Transduct Res*, 2013 33(3): p. 139–43. [PubMed: 23384070]
15. David NB, et al., Drosophila Ric-8 regulates Galphai cortical localization to promote Galphai-dependent planar orientation of the mitotic spindle during asymmetric cell division. *Nat Cell Biol*, 2005 7(11): p. 1083–90. [PubMed: 16228010]
16. Tall GG and Gilman AG, Purification and functional analysis of Ric-8A: a guanine nucleotide exchange factor for G-protein alpha subunits. *Methods Enzymol*, 2004 390: p. 377–88. [PubMed: 15488189]
17. Yan M, Ha JH, and Dhanasekaran DN, Galphai3 Stimulates the Tyrosine Phosphorylation of Ric-8A. *J Mol Signal*, 2015 10: p. 3. [PubMed: 27096001]

18. Lundby A, et al., Quantitative maps of protein phosphorylation sites across 14 different rat organs and tissues. *Nat Commun*, 2012 3: p. 876. [PubMed: 22673903]
19. Boularan C, et al., Resistance to inhibitors of cholinesterase (Ric)-8A and Galphai contribute to cytokinesis abscission by controlling vacuolar protein-sorting (Vps)34 activity. *PLoS One*, 2014 9(1): p. e86680. [PubMed: 24466196]
20. Xing B, et al., Atypical protein kinase Clambda is critical for growth factor receptor-induced dorsal ruffle turnover and cell migration. *J Biol Chem*, 2013 288(46): p. 32827–36. [PubMed: 24092753]
21. Papasergi-Scott MM, et al., Dual phosphorylation of Ric-8A enhances its ability to mediate G protein alpha subunit folding and to stimulate guanine nucleotide exchange. *Sci Signal*, 2018 11(532).
22. Turowec JP, et al., Protein kinase CK2 is a constitutively active enzyme that promotes cell survival: strategies to identify CK2 substrates and manipulate its activity in mammalian cells. *Methods Enzymol*, 2010 484: p. 471–93. [PubMed: 21036246]
23. Chan P, et al., Purification of heterotrimeric G protein alpha subunits by GST-Ric-8 association: primary characterization of purified G alpha(olf). *J Biol Chem*, 2011 286(4): p. 2625–35. [PubMed: 21115479]
24. Kinoshita E, et al., Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics*, 2006 5(4): p. 749–57. [PubMed: 16340016]
25. English JG, et al., MAPK feedback encodes a switch and timer for tunable stress adaptation in yeast. *Sci Signal*, 2015 8(359): p. ra5. [PubMed: 25587192]

Optimized production of phosphosite mutant Ric-8A proteins with high purity.

Produce homogenous and fully phosphorylated Ric-8A proteins.

Phosphorylated Ric-8A produced by this method has higher Gα guanine nucleotide exchange activity than insect cell-produced Ric-8A.

Figure 1. Purification of Ric-8A from *E. coli*.

GST-Ric-8A was produced in BL21 (DE3) *E. coli*. Cell pellets were lysed, clarified by centrifugation, loaded onto a glutathione Sepharose 4B column and washed with lysis buffer (Lane 2, column load, Ld; Lane 3, column flow through, FT; Lane 4, column wash, W). TEV protease was triturerated into the column resin to cleave the GST tag from Ric-8A and release Ric-8A from the column matrix (Lane 5, TEV). The column was then eluted with Anion Exchange Buffer A containing 20 mM reduced glutathione to visualize the amount of uncleaved Ric-8A and cleaved GST (Lane 6, Glut). The TEV-eluted Ric-8A was then loaded onto a HiTrap Q HP column using an AkTA FPLC. The column was washed and Ric-8A was eluted with a linear NaCl gradient of 100 mM to 550 mM. Lanes 7-15, fractions were collected during gradient development and the peak fractions were resolved by SDS-PAGE and proteins were visualized by staining the gel with Coomassie Brilliant Blue.

Figure 2. Phosphorylation of Ric-8A proteins using Protein Kinase CK2.

(A) Ric-8A (1 mg) was treated ± protein kinase CK2 and purified by Mono Q 10/100 GL anion exchange chromatography. The chromatographs demonstrate resolution of ATP, unmodified and fully phosphorylated Ric-8A-5PO₄. (B) Protein Kinase CK2-treated and mock-treated Ric-8A and Ric-8A 1-452 were immunoblotted with phosphosite-specific antibodies and a pan-Ric-8A antibody as indicated. (C) Purified Ric-8A and Ric-8A 1-452 were treated ± protein kinase CK2, re-purified by Mono Q 10/100 GL chromatography and resolved by phos-tag SDS-PAGE. The proteins were detected by staining the gels with Coomassie Brilliant Blue in order to visualize the phosphorylation-dependent gel mobility shifts. (D) Purified full length Ric-8A and Ric-8A triple A were treated ± protein kinase CK2, re-purified by Mono Q 10/100 GL chromatography and resolved by phos-tag SDS-PAGE. The proteins were detected with Coomassie Brilliant Blue staining to visualize the phosphorylation dependent gel mobility shifts.

Figure 3. Functional Testing of Protein Kinase CK2 phosphorylated Ric-8A Proteins. Mass spectra of (A) insect cell-produced Ric-8A, (B) *in vitro* phosphorylated Ric-8A and (C) *in vitro* phosphorylated Ric-8A triple A were obtained through intact protein ESI/MS analysis. (D) Ric-8A purified from insect cells, or Ric-8A purified from *E. coli* were treated ± CK2 and tested for stimulation of Gαq GTPγS binding activity. (E) Initial Gαq GTPγS binding rates stimulated by Ric-8A produced by *in vitro* CK2 phosphorylation, or from insect cells. Error bars (n = 3) are the mean ± S.E.M. The size of the error bars was sometimes smaller than the height of the plotted symbols. Two-way ANOVA analysis was performed. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Table 1Purification of Ric-8A-(5)PO₄ and Ric-8A triple A-(2)PO₄ from 5L of *E. coli*

Step	Volume (mL)	Total Protein (mg)	Ric-8A (mg)	Recovery (%)	Purity (%)
Clarrified Cell Lysate	1400	1300	169	100	13
Absorbed to Glutathione Sepharose 4B	-	-	45.6	27	-
TEV Protease Elution	25	35	31	68	89
Hi-Trap Q	5	25	24.8	81	99
CK2/Mono Q 10/100	4	19	19	77	>99
CK2/Mono Q 10/100 Ric-8A triple A protein	-	12.6	12.6	60	>99