MINIREVIEW

The G Protein α Chaperone Ric-8 as a Potential Therapeutic Target

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ABSTRACT

Resistance to inhibitors of cholinesterase (*Ric-8A* and *Ric-8B*) are essential genes that encode positive regulators of heterotrimeric G protein α subunits. Controversy persists surrounding the precise way(s) that Ric-8 proteins affect G protein biology and signaling. Ric-8 proteins chaperone nucleotide-free $G\alpha$ -subunit states during biosynthetic protein folding prior to G protein heterotrimer assembly. In organisms spanning the evolutionary window of Ric-8 expression, experimental perturbation of *Ric-8* genes results in reduced functional abundances of G proteins because G protein α subunits are misfolded and degraded rapidly. Ric-8 proteins also act as $G\alpha$ -subunit guanine nucleotide exchange factors (GEFs) in vitro. However, Ric-8 GEF activity could strictly be an in vitro phenomenon stemming from the ability of Ric-8 to induce partial $G\alpha$ unfolding, thereby enhancing GDP release. Ric-8 GEF activity

clearly differs from the GEF activity of G protein-coupled receptors (GPCRs). G protein $\beta\gamma$ is inhibitory to Ric-8 action but obligate for receptors. It remains an open question whether Ric-8 has dual functions in cells and regulates G proteins as both a molecular chaperone and GEF. Clearly, Ric-8 has a profound influence on heterotrimeric G protein function. For this reason, we propose that Ric-8 proteins are as yet untested therapeutic targets in which pharmacological inhibition of the Ric-8/ $G\alpha$ protein–protein interface could serve to attenuate the effects of disease-causing G proteins (constitutively active mutants) and/or GPCR signaling. This minireview will chronicle the understanding of Ric-8 function, provide a comparative discussion of the Ric-8 molecular chaperoning and GEF activities, and support the case for why Ric-8 proteins should be considered potential targets for development of new therapies.

Introduction

Resistance to inhibitors of cholinesterase (Ric-8) proteins are ~60-kDa positive regulators of heterotrimeric G protein α subunits found in animals and some fungi. Ric-8 was discovered in the *Caenorhabditis elegans* *ric* genetic screen that was conducted to find mutants with reduced neurotransmitter release. *ric* mutants live by circumventing the neurotoxic effects of cholinesterase inhibitor-induced synaptic acetylcholine accumulation (Miller et al., 1996). Wild-type *RIC* genes positively influence neurotransmission and include components of G protein signaling pathways: *Gaq/egl-30*, *RGS/egl-10*, and *unc-13*, which encodes a protein that regulates synaptic vesicle priming in response to diacylglycerol. *RIC-8* encoded an uncharacterized protein also called synembryn in some databases. *ric-8* mutants were epistatic to *egl-30* (*Gaq*) mutants, indicating that RIC-8 protein action was likely manifested upstream of *Gaq* or in a parallel pathway (Miller et al., 2000). *ric-8* mutants were

later found to be epistatic to *gao-1* (*Gao*), *gsa-1* (*Gas*), and *acy-1* (adenylyl cyclase) mutants in regulation of mitotic centrosome movements and cAMP-regulated synaptic transmission, respectively (Miller and Rand, 2000; Reynolds et al., 2005; Schade et al., 2005). One important conclusion of these works was that *C. elegans* RIC-8 positively regulated signaling pathways headed by three different G proteins (*Gq*, *Gi/o*, *Gs*).

A single *Ric-8* gene copy is present in fungi (excluding *Saccharomyces cerevisiae*), slime mold, worms, and flies (Wilkie and Kinch, 2005). Two distinct genes, *Ric-8A* and *Ric-8B* are present in frogs, fish, and mammals. Demonstration of direct binding between $G\alpha$ subunits and mammalian Ric-8A or Ric-8B proteins was made from yeast two-hybrid (Y2H) screens and protein pull-down experiments (Klattenhoff et al., 2003; Tall et al., 2003). Single-copy ancestral Ric-8 binds all $G\alpha$ subunits present in these organisms, whereas mammalian Ric-8A binds the *Gai/o*, *Ga12/13*, and *Gaq/11* classes, and Ric-8B has exclusive preference for the *Gas/olf*-class (Tall et al., 2003; Matsuzaki, 2005; Von Dannecker et al., 2005; Romo et al., 2008; Chan et al., 2011b). Ric-8 proteins also preferentially bind wild-type or GDP-bound $G\alpha$ subunits over constitutively active mutant (e.g., *Gai1* Q204L) or GTP-bound

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ABBREVIATIONS: ARM, Armadillo (Repeat); GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; HEAT, Huntington, Elongation Factor 3, PR65/A, TOR (Repeat); PPI, protein-protein inhibitors; RNAi, RNA interference.

G α subunits, prompting investigation of Ric-8 influence on G protein guanine nucleotide–cycle kinetics. Purified Ric-8A, Ric-8B, and Ric-8 proteins from multiple organisms exhibited in vitro guanine nucleotide exchange stimulatory activity (GEF) for subsets of G α subunits (Tall et al., 2003; Afshar et al., 2004; Romo et al., 2008; Chan et al., 2011b; Wright et al., 2011; Kataria et al., 2013). The idea that Ric-8 might activate G proteins in cells as a non-PCR GEF was logically consistent with the *C. elegans* genetic studies that showed *ric-8* mutant negative influence of G protein signaling.

The findings that mammalian Ric-8A acted as a GEF and that *C. elegans* RIC-8 was required for regulation of *Gai/o*-dependent mitotic centrosome movements sparked great interest in a hypothesis that Ric-8 activated a GPCR-independent *Gai/o* GTPase cycle to control the microtubule-pulling forces that position the mitotic spindle during cell division. The *C. elegans* works and three concurrent *Drosophila* reports showed that the *Ric-8* and *Gai/o* genes (*C. elegans*, *gpa-16*, *goa-1*; *D. melanogaster*, *G-ia65A* and *G-oa47A*) were required for execution of asymmetric cell division events (Afshar et al., 2004, 2005; Couwenbergs et al., 2004; Hess et al., 2004; David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005). These results were substantiated in a mammalian system, where *Gai* regulation of HeLa cell mitotic spindle positioning in relation to the substratum was *Ric-8A*-dependent (Woodard et al., 2010). A second revealing observation from the *Drosophila* and *C. elegans* studies was that the plasma membrane localization and whole-cell abundances of multiple G protein subunits, including *Gai/o* were reduced when *Ric-8* expression was perturbed through use of null or hypomorphic mutants (i.e., reduction of function mutants), or by using RNA interference (RNAi) techniques (Afshar et al., 2005; David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005).

Ric-8 regulation of G protein cellular abundance is manifested during G α -subunit biosynthesis. G α subunits are synthesized in the cytosol and associate with an endomembrane that serves as the site of G protein heterotrimer assembly (Marrari et al., 2007). Ric-8 proteins act as molecular chaperones to assist in G α -subunit folding prior to G α membrane association. In *Ric-8A*-null cells, newly produced G α subunits were defective in this initial membrane-association event and were prone to rapid degradation (Gabay et al., 2011). In addition, G α subunits were not folded properly when produced in cell-free translation systems lacking Ric-8 (Chan et al., 2013).

Ric-8 GEF-mediated G protein activation and Ric-8 chaperone-mediated folding of G proteins during biosynthesis are both activities that would positively affect G protein signaling. Current Ric-8 studies are directed toward discriminating between these activities or determining if they are one and the same. In this minireview, a case will be made that mammalian Ric-8A and Ric-8B represent untapped pharmacological targets, given Ric-8's dramatic influence over G protein functional abundances and activities. Targeting Ric-8A or Ric-8B may be useful to blunt pathologies driven by constitutively active mutant G proteins and GPCRs, or to augment existing GPCR pharmaceuticals.

Ric-8 Regulation of G Protein Abundance

An essential cellular function of Ric-8 proteins is to maintain proper abundances of G protein subunits. The

observation was first made in three concurrent reports in which genetic disruption of the single *Drosophila Ric-8* gene, or *Ric-8* RNAi treatment of *Drosophila* cells, resulted in reduced levels of plasma membrane-localized G protein subunits (G α subunits and G $\beta\gamma$) (David et al., 2005; Hampoelz et al., 2005; Wang et al., 2005). Corroborating evidence was provided in a *C. elegans ric-8* hypomorph that had reduced cortical/plasma membrane *Gai*-homolog staining during mitosis (Afshar et al., 2005). These studies also concluded that Ric-8 was required for the proper execution of *Gai/o*-directed mitotic spindle pole movements during asymmetric cell divisions. It has not been determined whether *ric-8*-mutant mitotic spindle movement defects are attributable to loss of nonreceptor GEF activation of *Gai/o*, or result from a reduction of functional *Gai/o* levels. Ric-8 regulation of G protein abundances has since been corroborated in multiple model systems spanning filamentous fungi to mice and human cultured cell lines. Table 1 describes most of the major observations in which genetic perturbations to *Ric-8* genes across this entire evolutionary span affected G protein abundances and functional G protein signaling outputs. *Ric-8* genetic perturbations included overexpression experiments and reduction techniques such as transgenic gene disruption, isolation of hypomorphic alleles, or RNA interference.

Ric-8 regulation of G protein abundance is manifested at an early stage of G α -subunit protein biosynthesis. Ric-8 transcriptional control was discounted as a possibility, as both mouse (*Ric-8A* or *Ric-8B*) and *Neurospora* (*Ric-8*) knockouts did little to change G protein–subunit transcript levels (Gabay et al., 2011; Wright et al., 2011). Newly translated G α subunits are folded in the cytosol and targeted to an endomembrane surface thought to be the endoplasmic reticulum or Golgi. G α subunits become palmitoylated and bound to G $\beta\gamma$ at this endomembrane (Rehm and Ploegh, 1997; Michaelson et al., 2002; Marrari et al., 2007). Assembled G protein heterotrimers then traffic to the plasma membrane. Steady-state abundances of *Gai/q/13* and *Gas* were reduced substantially in *Ric-8A*^{−/−} and *Ric-8B*^{−/−} mES cells, respectively (Gabay et al., 2011). The initial endomembrane targeting of newly produced *Gaq* and *Gai* was defective in the *Ric-8A*^{−/−} cells, as a high percentage of these nascent G α subunits remained soluble in the cytosol. It was reasonable to presume that the defect was either with the G proteins, or with a Ric-8A-dependent membrane translocation pathway that prevented G α membrane association. The former situation seemed likely, as the G proteins were degraded ~10 times faster in *Ric-8A*^{−/−} mouse Embryonic Stem cells (mES) cells than in wild type controls (Gabay et al., 2011). Measurements of G protein translation and folding in cell free systems depleted of endogenous Ric-8A (i.e., reticulocyte lysate) or that do not have endogenous Ric-8 (i.e., plants, wheat germ extract) showed that G proteins were in fact misfolded in Ric-8 absence. Reconstitution of the Ric-8A-depleted or Ric-8A-(minus) extracts with recombinant Ric-8A protein restored G protein folding competency (Chan et al., 2013). These results collectively indicate that the observed enhancement of G protein turnover and overall reduced G protein abundances in *Ric-8*-null cells are probably attributable to a normal housekeeping function of cells to degrade a misfolded protein (i.e., misfolded G α subunits). Two independent studies showed that Ric-8A or Ric-8B

TABLE 1

The effects of Ric-8 gene perturbation experiments on G protein functional abundances.

Organism	Ric-8 Homolog	Ric-8 Perturbation	G protein(s) Assessed (Gene Name)	Experimental Findings	References
Mammalian Cultured Cell Lines (HEK293, HeLa, COS, MEF, NIH3T3, mouse embryonic stem cells)	Ric-8A	Overexpression	Gai ₁ (<i>GNAI1</i>)	Transfected Gai ₁ -YFP levels were 5-fold higher in HEK293 cells cotransfected with <i>Ric-8A</i> .	Oner et al. (2013)
		Overexpression	Gai ₂ (<i>GNAI2</i>)	Agonist-stimulated Gai ₂ -dependent inhibition of cAMP levels was enhanced in HEK293 cells cotransfected with <i>Ric-8A</i> .	Fenech et al. (2009)
		siRNA knockdown	Gaq (<i>GNAQ</i>)	HEK293 siRNA knockdown of <i>Ric-8A</i> reduced Gq-coupled GPCR-stimulated ERK activation.	Nishimura et al. (2006)
		siRNA & shRNA knockdown	Gai ₁ (<i>GNAI1</i>)	HeLa cell <i>Ric-8A</i> knockdown decreased Gai ₁ levels and levels of Gai ₁ -dependent mitotic protein complexes at the plasma membrane. Metaphase spindle orientation was impaired.	Woodard et al. (2010)
		shRNA knockdown	Gα13 (<i>GNA13</i>)	<i>Ric-8A</i> -knockdown in mouse embryonic fibroblasts reduced immunostained Gα13 signal at the plasma membrane.	Wang et al. (2011)
		Overexpression	Gai ₂ (<i>GNAI2</i>) Gaq (<i>GNAQ</i>) Gα12 (<i>GNA12</i>)	<i>Ric-8A</i> overexpression in COS cells increased Gai ₂ , Gaq, and Gα12 levels. <i>Ric-8A</i> overexpression mitigated overexpressed Gai ₂ and Gaq ubiquitination and degradation.	Chishiki et al. (2013)
	Ric-8B	shRNA knockdown, overexpression	Gαs (<i>Gnas</i>)	<i>Ric-8B</i> knockdown in NIH3T3 cells reduced Gαs levels. <i>Ric-8B</i> overexpression increased Gαs levels without affecting <i>Gnas</i> transcription. <i>Ric-8B</i> overexpression inhibited Gαs ubiquitination.	Nagai et al. (2010)
		Overexpression	Gαolf (<i>GNAL</i>) Gβ ₁ (<i>GB1</i>) Gγ ₁₃ (<i>GNG13</i>)	<i>Ric-8B</i> overexpression in HEK293 cells increased expressed Gαolf, Gβ ₁ , and Gγ ₁₃ membrane abundance.	Kerr et al. (2008)
		Overexpression	Gαolf (<i>GNAL</i>)	Odorant-stimulated cAMP production in odorant receptor- and Gαolf-transfected HEK293 cells was potentiated by <i>Ric-8B</i> co-overexpression. <i>Ric-8B</i> expression increased Gαolf levels.	Von Dannecker et al. (2006)
		Overexpression	Gαolf (<i>GNAL</i>)	<i>Ric-8B</i> and <i>GNAL</i> cotransfection potentiated agonist-induced cAMP production from two Golf-coupled GPCRs.	Von Dannecker et al. (2005)
		Overexpression	Gαs (<i>GNAS</i>)	Coexpression of <i>Ric-8B</i> with Gαs-coupled odorant receptors in HEK293 cells increased cAMP production.	Yoshikawa and Touhara (2009)
	Ric-8A & Ric-8B	Overexpression	Gai ₁ (<i>GNAI1</i>) Gαs (<i>GNAS</i>)	Ric-8A and Ric-8BFL increased Gai-YFP and Gαs-YFP levels, respectively, in HEK293 cotransfection experiments.	Chan et al. (2011a)
		Transgenic gene deletions <i>Ric-8A</i> ^{-/-} or <i>Ric-8B</i> ^{-/-}	Gai _{1/2} (<i>Gnai1/2</i>) Gαo (<i>Gnao</i>) Gaq (<i>Gnaq</i>) Gα13 (<i>Gna13</i>) Gβ ₁₋₄ (<i>Gnbs 1-4</i>)	Quantitative Western blotting of cultured <i>Ric-8A</i> ^{-/-} and <i>Ric-8B</i> ^{-/-} mouse embryonic stem cell lysates and membranes had substantial reductions in G protein-subunit abundances.	Gabay et al. (2011)

(continued)

TABLE 1—Continued

Organism	Ric-8 Homolog	Ric-8 Perturbation	G protein(s) Assessed (Gene Name)	Experimental Findings	References
Rabbit reticulocyte lysate	Ric-8A	Immunodepletion and Protein Supplementation	Gaq (<i>GNAQ</i>) Gai ₂ (<i>GNAI2</i>) Gai ₁₃ (<i>GNAI3</i>)	Nascent Gai _{1/2} and Gaq were defective in initial membrane association and were degraded 10-fold faster in <i>Ric-8A</i> ^{-/-} versus WT cells. Hormone-stimulated adenylate cyclase and Rho GTPase activities were reduced in <i>Ric-8</i> -null cells. In vitro translated Gα subunits were not folded properly when produced in Ric-8A-immunodepleted RRL. Purified Ric-8A supplementation restored competency of the Ric-8A-depleted RRL to fold functional Gα subunits.	Chan et al. (2013)
<i>Xenopus laevis</i>	Ric-8	Overexpression, siRNA knockdown	Gas (<i>GNAS</i>)	Microinjection of <i>ric-8</i> mRNA in <i>Xenopus</i> oocytes potentiated Gas-inhibition of oocyte maturation. <i>ric-8</i> siRNA knockdown in primed oocytes caused maturation.	Romo et al. (2008)
<i>Caenorhabditis elegans</i>	Ric-8	Hypomorphic <i>ric-8</i> mutation, RNAi knockdown	Gao (<i>goa-1</i>)	Embryos derived from <i>ric-8</i> and single-copy <i>goa-1</i> mutant parents were lethal. Centrosome movements were diminished in <i>ric-8</i> mutants and absent in embryos derived from <i>ric-8/goa-1</i> parents.	Miller and Rand (2000)
		Hypomorphic <i>ric-8</i> mutation	Gaq (<i>egl-30</i>)	<i>ric-8</i> mutants had similar defects as <i>egl-30</i> mutants (e.g., aldicarb resistance, reduced locomotion rates). Defects were rescued by treatment with diacylglycerol analogs.	Miller et al. (2000)
		Hypomorphic <i>ric-8</i> mutations, RNAi knockdown	Gao (<i>goa-1</i>) Gai homolog (<i>gpa-16</i>)	<i>ric-8</i> hypomorphs treated with <i>ric-8</i> RNAi, had decreased mitotic spindle pole movements, resembling the phenotype of <i>goa-1/gpa-16</i> RNAi double knockdown embryos. Less Gao and GPR-1/2 complex was coimmunoprecipitated in <i>ric-8</i> hypomorphs.	Afshar et al. (2004)
		<i>ric-8</i> -null mutant	Gaq (<i>egl-30</i>) Gas (<i>gsa-1</i>)	<i>ric-8</i> -null mutants exhibit a phenotype similar to double <i>gsa-1/egl-30</i> mutants (i.e., paralysis). <i>gsa-1</i> gain-of-function mutation provided only weak rescue of the <i>ric-8</i> -null mutant phenotype.	Reynolds et al. (2005)
		Hypomorphic <i>ric-8</i> mutations	Gai homolog (<i>gpa-16</i>)	<i>ric-8</i> hypomorphs had decreased overall GPA16 protein levels and reduced plasma membrane localization.	Afshar et al. (2005)
		<i>ric-8</i> -null, RNAi knockdown	Gai (<i>G-ia65A</i>) Gβ (<i>Gβ13F</i>)	<i>ric-8</i> mutants had defective Gai and Gβ plasma membrane localization. Gai overexpression in <i>ric-8</i> -null cells did not rescue the Gβ membrane localization defect.	David et al. (2005)
<i>Drosophila melanogaster</i>	Ric-8	<i>ric-8</i> -null (maternal and zygotic Ric-8)	Gai (<i>G-ia65A</i>) Gao (<i>G-oa47A</i>) Gβ (<i>Gβ13F</i>)	Gai and Gβ protein levels were severely reduced in <i>ric-8</i> mutant embryos. Gβ no longer coimmunoprecipitated with	Hampoeiz et al. (2005)

(continued)

TABLE 1—Continued

Organism	Ric-8 Homolog	Ric-8 Perturbation	G protein(s) Assessed (Gene Name)	Experimental Findings	References
				Gai from <i>ric-8</i> mutant cells and embryos. Whole-cell staining showed that <i>Ric-8</i> was required for plasma membrane localization of Gai, Gao, and Gβ.	
		<i>ric-8</i> –null (maternal and zygotic Ric-8)	Gai (<i>G-ia65A</i>) Gβ (<i>Gβ13F</i>)	Localization of Gai and Gβ at the plasma membrane was disrupted in <i>ric-8</i> mutants.	Wang et al. (2005)
Insect (<i>Spodoptera frugiperda</i> , <i>Trichopulsia ni</i>)	Mammalian Ric-8A & Ric-8B	Recombinant mammalian baculoviral overexpression	Gαq (<i>GNAQ</i>) Gαi1 (<i>GNAI1</i>) Gα13 (<i>GNA13</i>) Gas (<i>GNAS</i>) Gαolf (<i>GNAL</i>)	Recombinant Gα coexpression with Ric-8A or B in insect cells boosted Gα levels 25- to 50-fold and permitted an enhanced method to purify recombinant Gα subunits.	Chan et al. (2011a)
<i>Dictyostelium discoideum</i>	Ric-8	<i>ric-8</i> –null mutant gene disruption	Gα2 (<i>gpaB</i>) Gα4 (<i>gpaD</i>)	<i>ric-8</i> is necessary to maintain abundance of the developmental marker and GPCR cAR1. Proper cAR1 abundance was previously shown to require functional Gα2. Gα4-dependent folate chemotaxis was defective in <i>ric-8</i> –null animals.	Kumagai et al. (1991); Kataria et al. (2013)
<i>Neurospora crassa</i>	Ric-8	<i>ric-8</i> –null mutant gene deletion	GNA-1 (<i>gna-1</i>) GNA-2 (<i>gna-2</i>) GNA-3 (<i>gna-3</i>) GNB-1 (<i>gnb-1</i>)	<i>ric-8</i> –null mutants (Δ ric8) had reductions of GNA-1, GNA-2, GNA-3, and GNB-1 whole-cell protein levels. G protein transcript levels were not affected. Δ ric8 mutant and Δ gna-1/ Δ gna-3 double mutant phenotypes were similar (e.g., defects in vegetative growth, absence of female sexual structures, and inappropriate formation of specialized hyphae in submerged cultures).	Wright et al. (2011)
		<i>ric-8</i> –null mutant gene deletion	GNA-1 (<i>gna-1</i>) GNA-2 (<i>gna-2</i>) GNA-3 (<i>gna-3</i>)	Deletion studies show Ric-8 is required for Gα-dependent development of specialized hyphae. Expression of constitutively active Gα mutants within Δ ric-8 background was not sufficient to rescue the Δ ric8 phenotype (e.g., increased proportion of fungal spores).	Eaton et al. (2012)

COS, CV-1 (simian) in Origin, and carrying the SV40 genetic material cells; ERK, extracellular signal-regulated kinase; MEF, Mouse embryonic fibroblasts; RRL, rabbit reticulocyte lysate; WT, wild type; YFP, yellow fluorescent protein;

overexpression “protected” co-overexpressed Gαq or Gas, respectively, from becoming polyubiquitinated (Nagai et al., 2010; Chishiki et al., 2013). Sufficient Ric-8 protein levels are required for efficient Gα-subunit folding, particularly in cases of Gα overexpression, otherwise a portion of the overexpressed G proteins become misfolded and turned over through a default ubiquitin proteasome pathway. This raises a consideration for any G protein overexpression study; should a cognate Ric-8 protein be co-overexpressed to potentiate folded Gα-subunit levels? Studies showing that Ric-8B overexpression was required for efficient odorant receptor coupling to overexpressed Gαolf and Gβγ in HEK293 cells are prime examples (Von Dannecker et al., 2005, 2006; Kerr et al., 2008). Gαolf probably does not fold efficiently in heterologous cells such as human embryonic kidney (HEK)293, and the co-overexpressed Ric-8B allowed production of sufficient functional Gαolf to permit measureable odorant receptor coupling.

Ric-8 as a G Protein α Subunit Guanine Nucleotide Exchange Factor

Ric-8 biochemical action proceeds as expected for a GEF. Ric-8 binds Gα-GDP and enhances the intrinsically slow GDP release rate. A nucleotide-free Gα intermediate state(s) is stabilized, to which GTP binds and induces the active Gα-GTP conformation. Ric-8 has reduced affinity for Gα-GTP and the proteins dissociate. Mammalian Ric-8A acts upon the Gai/q/12/13 classes and Ric-8B has specificity for Gas/olf (Klattenhoff et al., 2003; Tall et al., 2003; Chan et al., 2011b). This same specificity is observed for Ric-8A and Ric-8B regulation of Gα subtype cellular abundances (Gabay et al., 2011). Ric-8 acts catalytically as a GEF with standard Michaelis-Menten behavior to stimulate Gα-GDP release and observed GTPγS binding (Thomas et al., 2008). In steady-state GTP hydrolysis (GTPase) assays, however, the amount of Ric-8 required to

achieve maximal activity ($V_{\max}$) is super-stoichiometric to $G\alpha$ substrate (i.e., excess Ric-8 to $G\alpha$; Fig. 1) (Chan et al., 2011b). High GTP concentrations are required to drive Ric-8-influenced steady-state GTPase reactions in the forward direction because Ric-8 has very high affinity for $G\alpha$ nucleotide-free states. As it is impractical to use GTP concentrations in these assays (i.e., $>10 \mu\text{M}$) that approach the order of cellular levels ($\sim 500 \mu\text{M}$), K_m and $V_{\max}$ calculations can only be considered estimates. Furthermore, Ric-8 has a reduced, albeit measurable affinity for $G\alpha$ -GTP. In $G\alpha$ steady-state GTPase assays with Ric-8, reaction vectors of reverse and futile nucleotide exchange are likely to occur (Chan et al., 2011b). Combined, these parameters account for the kinetic differences observed in the terminal endpoint reactions like GDP release or GTP γ S binding, in which Ric-8 clearly acts catalytically, versus steady-state GTPase assays, where superstoichiometric Ric-8 is required to achieve maximal effect.

The physical mechanism of Ric-8 GEF action is unknown, primarily owing to a lack of Ric-8 structural information. The illuminating work showing the large interdomain movements that $G\alpha$ s underwent when engaged by the agonist-bound β_2 -adrenergic receptor provide the only known example of a $G\alpha$ nucleotide exchange mechanism (Chung et al., 2011; Rasmussen et al., 2011; Westfield et al., 2011). The $G\alpha$ C-terminus is inserted into the seven-transmembrane barrel of the GPCR, and the N-terminus is anchored in parts by the receptor and $G\beta\gamma$. These contacts lead to structural rearrangements within the G protein that reduce nucleotide affinity, allowing the α -helical domain to move liberally as a unit in relation to the Ras homology domain. In a solution-based GEF assay with purified $G\alpha$, Ric-8, and no $G\beta\gamma$, sufficient $G\alpha$ structural alteration must also be made to facilitate GDP release. Like a GPCR, Ric-8A binds the $G\alpha$ Ras homology domain and no interaction has been demonstrated with the $G\alpha$ α -helical domain. Yeast two-hybrid assays with Ric-8A or Ric-8B baits showed positive interactions with prey clones encoding the last 81 and 29 amino acids of $G\alpha_{i1}$ or $G\alpha_{olf}$, respectively (Von Dannecker et al., 2005; Thomas et al., 2011). Furthermore, a $G\alpha_{i1}$ C-terminal peptide blocked Ric-8A-stimulated $G\alpha_{i1}$ nucleotide exchange in vitro (Thomas et al., 2011). $G\alpha$ switch II was implicated as a second Ric-8 contact point because Ric-8 lacks GEF activity for G protein

heterotrimers (Tall et al., 2003). $G\alpha$ switch II binds directly to $G\beta\gamma$, which may occlude interaction with Ric-8 (Wall et al., 1995). The necessity of switch II for Ric-8 binding was further demonstrated by use of a $G\alpha$ s/i chimera in which $G\alpha$ s switch II was swapped for that of $G\alpha_i$. When expressed in HEK293 cells, $G\alpha$ s, but not the chimera, was protected from polyubiquitination by overexpressed Ric-8B (Nagai et al., 2010). These studies collectively indicate that there are at least two critical contact points for $G\alpha$ to bind Ric-8. Both are within the $G\alpha$ Ras homology domain at Switch II and the C-terminus.

We propose that the Ric-8 GEF mechanism fundamentally differs from the GPCR mechanism in that Ric-8 may induce partial unfolding of the $G\alpha$ Ras homology domain. Such unfolding would reduce G protein affinity for GDP, resulting in GDP dissociation, and perhaps permit substantial movement of the $G\alpha$ α -helical domain in relation to the partially unfolded, nucleotide-free Ras homology domain. This mechanism is consistent with the lack of a $G\beta\gamma$ requirement for Ric-8-induced nucleotide exchange. One interesting G protein/GEF pair to consider in regard to this proposed Ric-8 biochemical mechanism is the Rab small GTPase GEF mammalian suppressor of Sec4 (Mss4)/Dss4 combination. Mss4 has weak in vitro guanine nucleotide exchange stimulatory activity toward Rabs. Superstoichiometric Mss4 (20–150-fold excess to Rab) increased Rab GDP release (Nuoffer et al., 1997). However, Mss4 depletion had no influence in a cargo transport assay regulated by the Rab proteins that Mss4 acts upon. Mss4 GEF action might be an in vitro activity attributable to Mss4 induction and chaperoning of partially (un)folded Rab nucleotide-free states. The crystal structure of nucleotide-free Mss4/Rab8 complex revealed the unusual GEF mechanism (Itzen et al., 2006). The Rab8 GTPase P-loop responsible for contacting the α and β phosphates of GDP was “unfolded” when bound to Mss4. In β_2 AR-stabilized nucleotide-free $G\alpha$ s, the P-loop was structured but had undergone rearrangement when compared with the $G\alpha$ s-GTP γ S structure (Coleman et al., 1994; Rasmussen et al., 2011). Alteration of G protein P-loop structure, either by rearrangement, unfolding, or displacement by a GEF element would result in reduced affinity for guanine nucleotide, thereby explaining GEF-mediated GDP release-rate enhancement. NMR spectroscopy and hydrogen/deuterium exchange experiments showed that the Ric-8A- $G\alpha_{i1}$ nucleotide-free complex existed in highly dynamic states predictive of multiple, flexible $G\alpha$ conformations (Thomas et al., 2011). It will be interesting to learn whether the mechanism of Ric-8A GEF action proceeds through induced unfolding of $G\alpha$ nucleotide-binding elements, such as the P-loop, or follows a mechanism similar to that elicited by a GPCR in which the P-element is structured but displaced. Given the findings that Ric-8 proteins chaperone partially folded, nucleotide-free $G\alpha$ subunits during biosynthesis, as may be the case for Mss4 and Rabs, we predict that the mechanism of Ric-8 in vitro GEF action involves partial unfolding of $G\alpha$ -subunit elements responsible for nucleotide binding (Fig. 2).

Fig. 1. Ric-8A enhances $G\alpha_q$ steady-state GTPase activity. Purified Ric-8A or Ric-8B potentiated the rate of $G\alpha_q$ steady-state GTPase activity in a solution-based assay in the presence of $10 \mu\text{M}$ ^{32}P - γ -[GTP]. Steady-state GTPase activity is limited by the rate of $G\alpha_q$ GDP release. Ric-8 proteins in excess of $G\alpha_q$ were required to achieve maximal rates. (This research was originally published in *J. Biol. Chem.* 2011;286:19932–19942. © the American Society for Biochemistry and Molecular Biology.) (Chan et al., 2011b)

How Does Ric-8 Regulate $G\alpha_{i/o}$ Protein-Controlled Mitotic Spindle Positioning?

Many high-profile works have examined a role for Ric-8, $G\alpha_{i/o}$, and accessory proteins in positioning the mitotic spindle prior to cell division [for comprehensive reviews see

Fig. 2. Models comparing the $G\alpha$ -subunit molecular chaperoning and GEF activities of Ric-8 molecular chaperone. Ric-8 participates in biosynthetic folding of $G\alpha$ subunits. The Ras-like (RD) and α -helical domains (AHD) of folded $G\alpha$ exhibited great mobility in relation to each other in the absence of bound nucleotide and when engaged by an agonist-bound GPCR (Chung et al., 2011; Rasmussen et al., 2011; Westfield et al., 2011). During G protein biosynthesis, we propose that these two domains are first folded individually through the aid of cellular folding chaperones including the HSP 70/90 and Chaperonin [TCP-1 ring complex (TRiC) or Chaperonin-containing TCP1 (CCT)] systems (not shown in the model) (Busconi et al., 2000; Farr et al., 1997; Vaiskunaite et al., 2001; Waheed and Jones, 2002). Ric-8 then helps to complete $G\alpha$ RD folding while positioning the RD and AHD to permit development of a nascent guanine nucleotide-binding pocket. GTP binds the newly formed pocket for the first time and Ric-8 becomes dissociated from natively folded $G\alpha$ -GTP. This model presumes the subsequent hydrolysis of one GTP molecule so that $G\alpha$ -GDP can then bind $G\beta\gamma$. In experimental $G\alpha$ -folding situations lacking Ric-8, the $G\alpha$ -Ras domain achieves its proper native fold inefficiently and the protein adopts a misfolded conformation. GEF: Folded $G\alpha$ -GDP is the substrate of in vitro GEF assays. Ric-8 binds $G\alpha$ -GDP and may induce partial unfolding of the $G\alpha$ -RD so that affinity for GDP is reduced. Ric-8 acts to chaperone the nucleotide-free intermediate(s). The open-form G protein binds GTP to induce the active conformation and dissociate Ric-8. The partially folded, nucleotide-free $G\alpha$ state(s) that Ric-8 chaperones during biosynthesis may be similar to the nucleotide-free $G\alpha$ intermediate(s) in in vitro nucleotide exchange assays.

Siderovski and Willard (2005), Knoblich (2008), Siller and Doe (2009), and Tall (2013)]. Movement of the mitotic spindle is mediated by aster microtubules that link the spindle poles to the plasma membrane. A *Gai/o* GTPase cycle regulates “pulling forces” on the aster microtubules to move the spindle into the central position of the cell during the early stages of mitosis. The same G protein system regulates the aster pulling forces during metaphase and anaphase to draw the spindle poles toward the plasma membrane. In some cells that divide asymmetrically, including the *C. elegans* zygote and *Drosophila* neuroblast, the *Gai/o*-directed aster microtubule forces are unequal and the mitotic spindle is pulled closer to one side (pole) of the cell. This positions the metaphase plate asymmetrically, thereby marking a cytokinetic cell-scission site that will result in production of two daughter cells of unequal size and/or content. *C. elegans* and *Drosophila* Ric-8, mammalian Ric-8A, and *Gai/o* are certainly required for these processes, but the mechanism of Ric-8 involvement is unclear (Afshar et al., 2004; David et al., 2005; Hampoelz et al., 2005; Hess et al., 2004; Wang et al., 2005). Ric-8 was postulated to act as the GEF that activated *Gai/o* in a GPCR-independent manner to initiate the required GTPase cycle (Manning, 2003; Hampoelz and Knoblich, 2004; Tall and Gilman, 2005; Siderovski and Willard, 2005; Thomas et al., 2008; Oner et al., 2013). On the other hand, Ric-8 proteins from all of these organisms are required for production of normal, functional *Gai/o* levels. The studies that used Ric-8 genetic ablation to show its necessity in spindle positioning, do not permit discrimination between the affect of Ric-8 on

G protein abundances and the potential nonreceptor GEF activation of *Gai/o*. It remains an open question whether Ric-8 has one or dual function(s) in regulation of mitotic spindle dynamics.

Should Ric-8 Be a Therapeutic Target for Diseases Driven by Mutant $G\alpha$ Subunits or to Attenuate GPCR Signaling?

There is increasing appreciation that constitutively active $G\alpha$ -subunit somatic mutations contribute to a variety of cancers and diseases. Notable examples include the *Gas* Q227L/R/H/K/E or R201C/H/S/L activating mutations found in 4.2% of all analyzed human tumor samples, including 28% of pituitary tumors, 5% of thyroid adenomas, and 6% of colon adenomas or adenocarcinomas (Weinstein et al., 1991; Wood et al., 2007; O’Hayre et al., 2013). Analogous mutations in *Gaq* or *Gα11* were found in 83% of ocular melanomas and 3% of dermal melanoma tumors (Onken et al., 2008; Van Raamsdonk et al., 2009, 2010). Overactive G protein signaling mediated by GPCRs or natural ligands that are inappropriately expressed in non-native tissues, and activating GPCR mutants are well known to contribute to disease including cancers (Schoneberg et al., 2004; Dorsam and Gutkind, 2007; O’Hayre et al., 2013). Any disease mechanism in which heterotrimeric G protein signaling is involved could theoretically be attenuated by inhibition of Ric-8-mediated $G\alpha$ -subunit folding. A Ric-8 inhibition scheme could be particularly beneficial for diseases in which multiple G proteins or GPCRs collectively

contribute to pathogenesis (Johnson and Druey, 2002; Salazar et al., 2007; Lappano and Maggiolini, 2011; Du and Xie, 2012; O'Hayre et al., 2014). In these cases, individual GPCR inhibitors may lack efficacy because redundant, untargeted GPCRs remain active. Ric-8 inhibition would conceivably attenuate signaling from any GPCR, perhaps providing the required inhibitory efficacy depending on the disease context.

Therapeutic Chaperone Inhibition Is an Established Paradigm

Indirect pharmacological targeting of disease-causing proteins by intervention at the level of the molecular chaperones that fold such proteins has clinical utility. Over 20 heat shock protein/cognate 90 or 70 (HSP/C 90/70) inhibitors are being evaluated as standalone or combination therapies against various cancers in later-stage clinical trials (Dhingra et al., 1994; Wadhwa et al., 2000; Schmitt et al., 2003, 2006; Mitsiades et al., 2006; Trepel et al., 2010; Leu et al., 2011; Modi et al., 2011; Jhaveri et al., 2012; Koren et al., 2012; Pacey et al., 2012; Sidera and Patsavoudi, 2014). HSP/C 90/70 are ubiquitous cellular chaperones that work with cochaperones to fold a majority of cellular proteins, including $G\alpha$ subunits (Nathan et al., 1997; Thulasiraman et al., 1999; Busconi et al., 2000; Vaiskunaite et al., 2001; Waheed and Jones, 2002; Echeverria et al., 2011). Cancer cell proliferation depends heavily on stress-induced HSP 90 to fold many oncoproteins (Jonkers and Berns, 2004; Yan et al., 2011; Zuber et al., 2011). Chaperone inhibitors are intended to block oncogenic or disease protein folding and function to sensitize cells to cellular stress generated by conventional chemotherapies (Neckers and Workman, 2012). HSP/C 70/90 are both attractive as therapeutic targets because they contain multiple sites amenable to drug targeting, including ATP cofactor-binding sites, client protein substrate sites, and protein-protein interfaces (PPIs), with cochaperones that facilitate client protein folding activity (Rérole et al., 2011; Assimon et al., 2013; Balaburski et al., 2013; Schlecht et al., 2013). Many HSP/C 90/70 inhibitors are indeed small molecule adenosine analogs that target the nucleotide-binding site (Chiosis et al., 2002; Dymock et al., 2004; Donnelly and Blagg, 2008; Williamson et al., 2009; Day et al., 2010; Budina-Kolomets et al., 2014). While the potential to target active sites in these chaperones is advantageous, limited therapeutic success has been achieved thus far because of the cytotoxic effects of pleiotropic inhibition of protein folding (Banerji et al., 2005; Goetz et al., 2005; Grem et al., 2005).

With these features of therapeutic chaperone inhibition schemes in mind, Ric-8A and Ric-8B are molecular chaperones known to participate in the folding of only twelve (*Gai*_{1,2,3}, *Gao*, *Gaz*, *Gat*, *Gaq*, *Ga11*, *Ga14*, *Ga15*, *Ga12*, *Ga13*) and two (*Gas*/*Gaolf*) protein clients, respectively. Extensive searches have been conducted for additional Ric-8 folding clients or protein-binding partners and very little has been found besides $G\alpha$ subunits. Other reported Ric-8A protein interactions include neural cell adhesion molecule 180, adenylyl cyclase type 5, and protein kinases that may phosphorylate Ric-8A (Wang et al., 2011; Amoureux et al., 2012; Xing et al., 2013; Boularan et al., 2014). Inhibitors of Ric-8 or the Ric-8/ $G\alpha$ PPI could have high target specificity

and profoundly attenuate diseases caused by mutant $G\alpha$ subunits, particularly constitutively active $G\alpha$ subunits that act as oncoproteins. Ric-8A does indeed participate in oncogenic *Gaq*-Q209L protein folding (Chan et al., 2013). The specificity of targeting Ric-8A, and particularly Ric-8B, seems a much better prospect than that of HSP/C 70/90, but there are potential issues of a Ric-8 inhibition scheme, namely potential off-target effects of inhibiting non-disease-causing $G\alpha$ subunits, the current ill-defined nature of the Ric-8/ $G\alpha$ PPI, and whether this interface can be inhibited. Ric-8 probably does not have the same types of active sites as HSP 70/90 chaperones, which are amenable to small molecule targeting.

Defining the Ric-8/ $G\alpha$ PPI and Targeting It for Inhibition

An important first step toward developing an inhibitor strategy to target Ric-8 folding of G proteins is to gain a better understanding of the structural basis of the Ric-8/ $G\alpha$ PPI. Currently, no Ric-8 structure has been resolved. Attempts have been made by multiple groups to crystallize Ric-8 in complex with $G\alpha$ subunits, but the dynamic, multistate nature of the complexes has not yielded suitable crystallization conditions to date. A predicted Ric-8 structure was made with modeling programs using *Xenopus* Ric-8 (Figuroa et al., 2009). This work suggested that Ric-8 contained ten tandem Armadillo (ARM) repeat α -helical elements organized similarly to the way tandem ARM repeats are present in β -catenin and Importin- α (Fig. 3) (Figuroa et al., 2009). ARM repeat-containing proteins do not share amino acid similarity or identity, but are defined by the individual ~42-amino-acid ARM domains, each of which forms a structure composed of three α -helices (Fig. 3A) (Tewari et al., 2010). Structures of many ARM repeat-containing proteins, including β -catenin, Importin- α , and a Ric-8A, prediction comprise an overall superhelical crescent-shaped topology formed by antiparallel arrangement of the ARM repeats (Fig. 3) (Conti et al., 1998; Vetter et al., 1999; Graham et al., 2001; Lee et al., 2005; Zhang, 2008; Kelley and Sternberg, 2009; Roy et al., 2010; Tewari et al., 2010). ARM repeat-containing proteins have diverse functions; interestingly, a subset binds to various small GTPases. The RhoA GTPase regulator smgGDS contains 13–14 tandem ARM repeats and was predicted to form the characteristic crescent shaped structure observed from the Importin- α and β -catenin crystal structures (Hamel et al., 2011; Schuld et al., 2014) (Fig. 3B). Closely related Importin- β contains tandem HEAT (Huntington, Elongation Factor 3, PR65/A, TOR) repeat α -helical elements. HEAT repeats have a common phylogenetic origin and structure that is similar to ARM repeats. The concave side of the Importin- β crescent is the binding site for the small GTPase, Ran, and the concave portion of the smgGDS crescent was predicted to form a similar binding site for RhoA (Vetter et al., 1999; Lee et al., 2005; Hamel et al., 2011). Intriguingly, both of these Armadillo/HEAT repeat crescent-shaped proteins (smgGDS and Importin- β) act as weak guanine nucleotide exchange factors for the small G proteins Rho and Ran, respectively (Lonhienne et al., 2009; Hamel et al., 2011). In both cases, excess GEF to GTPase was required to measure most efficient nucleotide exchange. Furthermore, smgGDS was also shown to chaperone Rho during cycles of regulated enzymatic Rho

Fig. 3. (A) Representative ARM and HEAT domain structures. ARM domain (left) is the second repeat in β -catenin [1JDH in Protein Data Bank (PDB)] (Graham et al., 2001), and HEAT domain (right) is the second repeat in Importin- β [1IBR in PDB (Vetter et al., 1999)]. Helices are identified as H1, H2, and H3 beginning with N-terminal-most helix of each structure. (B) Structures of ARM-repeat proteins. From left to right, ribbon diagrams (top) and electrostatic surface maps (bottom) for human β -catenin [1JDH in (PDB) (Graham et al., 2001)], human smgGDS [predicted structure using Phyre2 Server (Kelley and Sternberg, 2009)], Importin- α (1BK5 in PDB (Conti et al., 1998)], predicted structure of human Ric-8A [I-TASSER Server (Roy et al., 2010; Zhang, 2008)], and Importin- β in complex with Ran (green ribbon representation) [1IBR in PDB (Vetter et al., 1999)]. The N-terminus of each protein is at the top in each representation. Surface area is colored according to local pKa from 0 (blue) to 14 (red). Figure was produced using Discovery Studio 4.0 (Accelrys Software Inc., 2013) to visualize structure coordinates.

lipidation (Schuld et al., 2014). These structure-function relationships between ARM/HEAT-repeat crescent-shaped proteins and their GTPase binding partners may provide insight into the way that Ric-8 interacts with the Ras homology domain of $G\alpha$ subunits. The Importin- β /Ran complex structure revealed a sizeable PPI with two primary points of interaction, between Ran switch II and the Importin- β N-terminus, and between the Ran C-terminus and the mid-to-C-terminal region of Importin- β (Vetter et al., 1999) (Fig. 3B). Switch II and the C-terminus of the $G\alpha$ Ras-homology domain also mediate $G\alpha$ binding to Ric-8 (Coleman et al., 1994; Nagai et al., 2010; Rasmussen et al., 2011; Thomas et al., 2011).

The question of targeting the Ric-8/ $G\alpha$ interaction with small molecules with the ultimate intent of inhibiting Ric-8-mediated $G\alpha$ -subunit folding comes down to evaluating whether this predicted PPI will be amenable to small molecule binding. At least two precedents have been set for blocking the Importin- β /Ran PPI with proven cellular effects (Hintersteiner et al., 2010; Soderholm et al., 2011). A small molecule inhibitor screen of the Importin- β PPI with Ran identified “karyostatin 1A” that blocked regulated nuclear import with micromolar affinity (Hintersteiner et al., 2010; Soderholm et al., 2011). A second Importin- β /Ran small molecule-inhibition screening strategy identified 2,4-diaminoquinazoline, termed “Importazole”, as an inhibitor of Importin- β -mediated nuclear import and mitotic spindle

assembly (Soderholm et al., 2011). Both molecules bind to Importin- β and block Ran binding. If Ric-8 adopts the predicted crescent architecture and uses similar surfaces to bind the Ras-homology domains of $G\alpha$ subunits, then it is reasonable to assume that the Ric-8A PPI will be amenable to small molecule-inhibitor targeting as well. As an alternative to small molecules, peptide inhibitors for Ric-8/ $G\alpha$ PPI could be explored. A peptide comprising the $G\alpha$ C-terminus blocked Ric-8A-mediated $G\alpha_1$ nucleotide exchange (Thomas et al., 2011). A β -catenin peptide inhibitor attenuated growth of cancer cell lines by disrupting β -catenin interaction with the transcription coactivator T-cell factor protein (Grossmann et al., 2012).

Summary

The pair of mammalian Ric-8 proteins (Ric-8A and Ric-8B) act collectively as nonreceptor GEFs and molecular folding chaperones for all classes of heterotrimeric G protein α subunits. Genetic attenuation of *Ric-8* genes in multiple organisms reduced $G\alpha$ -subunit abundances and caused defective G protein signaling. There is controversy whether Ric-8 acts as a GEF to activate GPCR-independent G protein signaling or only serves to fold $G\alpha$ subunits during biosynthesis. Both activities would positively affect G protein signaling, but no current study has clearly distinguished these two activities in vivo. We propose that the mechanism of Ric-8 GEF action

differs significantly from that of GPCRs in that Ric-8 induces partial unfolding of $G\alpha$ Ras-homology-domain elements responsible for GDP binding. This model predicts that Ric-8–induced GDP release from $G\alpha$ in an in vitro GEF reaction is like the reverse reaction vector of Ric-8–assisted folding of nascent $G\alpha$ subunits that have yet to bind nucleotide.

Given the profound influence that Ric-8 proteins have on G protein function, we propose that the proteins represent logical therapeutic targets that could be inhibited to prevent folding of disease-causing or oncogenic $G\alpha$ subunits, or to attenuate pathologic GPCR signaling at the G protein level. Structural predictions suggest that Ric-8 contains tandem armadillo repeats and adopts a superhelical crescent-shaped structure shared by other Armadillo repeat-containing proteins, including some that bind to small GTPases. In the absence of a Ric-8 structure, these examples may provide the best insight into the way that Ric-8 binds to $G\alpha$ -subunit Ras-homology domains. Successful small molecule targeting of an armadillo repeat protein/GTPase interaction was achieved for Importin- β and Ran. This example may provide the paradigm for small molecule targeting of the Ric-8/ $G\alpha$ interface.

Authorship Contributions

Performed data analysis: Tall, Papasergi, Patel.

Wrote or contributed to the writing of the manuscript: Tall, Papasergi, Patel.

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