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Ric-8 regulation of heterotrimeric G proteins

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Abstract

Resistance to inhibitors of cholinesterase 8 proteins (Ric-8A and Ric-8B) collectively bind the four classes of heterotrimeric G protein α subunits. Ric-8A and Ric-8B act as non-receptor guanine nucleotide exchange factors (GEFs) toward the Gα subunits that each binds *in vitro* and seemingly regulate diverse G protein signaling systems in cells. Combined evidence from worm, fly and mammalian systems has shown that Ric-8 proteins are required to maintain proper cellular abundances of G proteins. Ric-8 proteins support G protein levels by serving as molecular chaperones that promote Gα subunit biosynthesis. In this review, the evidence that Ric-8 proteins act as non-receptor GEF activators of G proteins in signal transduction contexts will be weighed against the evidence supporting the molecular chaperoning function of Ric-8 in promoting G protein abundance. I will conclude by suggesting that Ric-8 proteins may act in either capacity in specific contexts. The field awaits additional experimentation to delineate the putative multi-functionality of Ric-8 towards G proteins in cells.

Keywords

Chaperone; G protein α; guanine nucleotide; exchange factor (GEF)

Resistance to inhibitors of cholinesterase 8 (Ric-8) proteins are unique regulators of heterotrimeric G protein α subunits. The *RIC-8* gene was discovered in *Caenorhabditis elegans* from a mutagenesis screen that sought mutations of genes required for neurotransmission (1). Complementation groups in the *RIC* screen included components of the synaptic vesicle priming process such as the SNARE machinery. Heterotrimeric G protein q signaling components were also identified as *RIC* mutants and included *egl*-30 (Gαq), *egl-10* (RGS) and *unc-13* (encodes a diacylglycerol binding sensor). In subsequent studies, *Ric-8* mutants were shown to be epistatic to mutations in genes that encoded three different classes of G protein α subunits. Genetically, *Ric-8* was predicted to act upstream of, or parallel to, Gαq and Gαs to provide diacylglycerol and cAMP second messenger regulation of synaptic vesicle priming (2–4). *Ric-8* worked with Gαo to control spindle pole movements that regulated mitotic spindle positioning in *C. elegans* embryos (5).

The *C. elegans* genetic studies were suggestive that Ric-8 interacted with G proteins. Demonstration of physical binding was first made with mammalian versions of the proteins. Two mammalian homologs of *C. elegans* Ric-8 were identified in yeast two-hybrid screens conducted to identify novel interactors of Gαo and Gαs baits (6,7). Our 2003 work

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demonstrated the enzymological basis of the interaction; Ric-8A is a non-receptor guanine nucleotide exchange factor (GEF) for Gα subunits. Purified Ric-8A enhanced the rates of GTPγS binding to purified Gαi, Gαq and Gα13 subunits, but not Gαs. This work was followed up by demonstration that mammalian Ric-8B and *Xenopus* Ric-8 acted as GEFs for the Gαs/Gαolf-class (8,9). A key feature of Ric-8-catalyzed Gα subunit *in vitro* nucleotide exchange stimulatory activity that extends to understanding the physiological function of Ric-8 in cells comes from the Michaelis–Menten enzymatic study, in which the GTP substrate dependence of Ric-8-catalyzed nucleotide exchange was examined. Measurement of Gαs steady state GTP hydrolysis showed that Gαs had an intrinsic V_{max} of 0.28 mol GTP hydrolyzed mol $Gαs^{-1}$ min^{-1}, and an intrinsic GTP binding K_m of ~385 nM. The full-length Ric-8B isoform dramatically elevated both these parameters nearly 10- and 100-fold, respectively (Figure 1A). Mechanistically, Ric-8B bound to Gαs-GDP (guanosine diphosphate) and initiated rapid GDP release. Ric-8B stabilized the nucleotide-free Gαs intermediate. The high affinity that Ric-8B has toward nucleotide-free Gαs was reflected by the higher concentrations of GTP (elevated K_m) that were required to dissociate the complex to produce free Gαs-GTP and Ric-8B (Figure 1B). The important point that extends to understand the function of Ric-8 in cells is that Ric-8 proteins have highest affinity for open, nucleotide-free conformations of Gα proteins.

Since the original finding that Ric-8 proteins act as GEFs towards Gα subunits *in vitro*, there have been many investigations of the effects of *Ric-8* gene perturbation on G protein function in cells. The evidence from studies in model organisms and mammalian cells has shown that Ric-8 homologs collectively influence the signaling properties of all four classes of G protein α subunits. Ancestral Ric-8 and mammalian Ric-8A influence Gαi/o-class regulation of spindle pole movements to position the mitotic spindle during symmetric and asymmetric cell division (10–16). *Caenorhabditis elegans* Ric-8 and mammalian Ric-8A affected Gαq-dependent neurotransmission (2,17). Ric-8B expression enabled reconstitution of Golf-dependent odorant receptor signaling in a heterologous system and positively influenced Gαs/Gαolf signaling and protein stability (18–21). Mouse Ric-8A and Ric-8B were required for efficient basal and hormone-stimulated adenylyl cyclase activity in embryonic stem cells (22). Ric-8A was recently purported to act as the GEF that propagated signals derived from the platelet-derived growth factor receptor (PDGFR) to directly activate Gα13 subunits and consequently regulate dorsal ruffling and cell migration (23). In most of these studies, data were also provided to show that Ric-8 proteins positively influenced Gα subunit plasma membrane localization and G protein abundance. We favor the hypothesis that the function of Ric-8 proteins in cells is to promote G protein abundance and that Ric-8 GEF activity *per se* is not necessarily for the purpose of producing activated Gα-GTP to engage downstream effectors. Most observations of Ric-8 pleotropic regulation of varied G protein signaling pathways can be explained because Ric-8 proteins positively regulate G protein abundance in cells.

In support of this idea, we found that co-expression of recombinant GST-tagged Ric-8A or Ric-8B with recombinant Gα subunits in *Sf*9 or High-Five™ (invitrogen) insect cells led to a dramatic up-regulation of the levels of expressed Gα subunits by 25-to-50-fold. The elevated abundances of the recombinant Gα subunits and the finding that much of the over-produced G protein was bound to GST-Ric-8A permitted the development of a new purification procedure for Gα subunits. GST-Ric-8:Gα complexes from insect cell lysates were trapped on a glutathione Sepharose matrix. G proteins were eluted from the matrix with $GDP - AlF_4^-$ buffer that dissociates Ric-8 and Gα. The eluted Gα subunits were highly pure, devoid of contaminating Ric-8 protein and produced in an unprecedented yield. Gαq and Gα13 subunits could formerly be produced only in low quantity (24,25). The new technique using GST-Ric-8 allows the production of ~5–20 mg of Gα subunit per liter of insect cell culture (26). This technique has proven useful to the field in terms of the

increased availability of purified G protein subunit reagents. From the perspective of Ric-8 biology, this study prompted us to investigate potential roles of Ric-8 proteins in the control of G protein biosynthesis and/or resistance to protein turnover.

To this end, we worked with collaborators at Regeneron Pharmaceuticals to produce *Ric-8A* or *Ric-8B* knockout mice. Both strains were embryonic lethal and died early during embryogenesis. Attempts to ascertain the timing and cause of embryonic death were difficult, as no knockout embryos of either *Ric-8* strain could be recovered as early as Day E8.5. An independently derived *Ric-8A* knockout mouse was shown to exhibit embryonic death between Days E6–E8 (27). These *Ric-8A* null embryos had severe gastrulation defects of unknown etiology. To circumvent the inability to use *Ric-8* knockout mice as models, we harvested live blastocysts from *Ric-8A*$^{+/-}$ or *Ric-8B*$^{+/-}$ intercrosses and cultured *Ric-8A*$^{-/-}$ and *Ric-8B*$^{-/-}$ embryonic stem cell lines from them (22). *Ric-8* null mES cells exhibited dramatic G protein signaling defects. Hormone and forskolin-stimulated cAMP production was blunted in both the *Ric-8A* and *Ric-8B*-null cell lines. GPCR-dependent activation of Rho GTP signaling was blunted in the *Ric-8A* knockouts. These signaling defects were attributed to the dramatic reductions in steady state G protein membrane abundances that were observed. *Ric-8A*$^{-/-}$ cells had ~95% reductions in the levels of Gαi/q/13, and *Ric-8B*$^{-/-}$ cells had a dramatic reduction in the level of Gαs. This pattern matches the pattern of Ric-8A or Ric-8B nucleotide exchange stimulatory activity towards Gα subsets. Interestingly, the total Gβ level ($G\beta_{1-4}$) was reduced by ~50% in the *Ric-8A*$^{-/-}$ cells when compared to wild type or R*ic-8B*$^{-/-}$ cells. This was attributed to a bulk reduction in the amount of Gα subunits in the R*ic-8A* null cells, with the Gαi-class contributing the most to the total pool. The extent of the G protein abundance defects in a crude membrane fraction was greater than the overall reductions observed in whole cells, suggesting the probability of a Gα membrane-targeting defect that was manifested by the absence of *Ric-8A*.

Pulse-chase labeling studies were initiated to investigate the timing and extent of nascent Gα subunit membrane association. G protein subunits are produced on soluble ribosomes and assembled as heterotrimers on the outer leaflet of the endoplasmic reticulum membrane prior to trafficking of the intact heterotrimer to the cell surface (28,29). mES cells were briefly pulse labeled with radiolabeled amino acids and then chased with unlabeled amino acids. During the chase, cells were fractionated into crude membranes and cytosol. $G\alpha i_{1/2}$ or Gαq were immunoprecipitated from the fractions and visualized by autoradiography. Virtually all of the nascent Gαq remained soluble in the absence of Ric-8A, whereas most of the Gαq accumulated on a membrane in cells expressing Ric-8A (Figure 2). This defect was observed for Gαi as well, although the extent of the defect was not as great. We reasoned that Gαi is the only class of G protein that becomes lipidated (myristoylation) during translation in the cytosol and this may confer an advantage in membrane association over other Gα subunits that become lipidated only upon achieving membrane association (e.g. Gαq). An extended pulse-chase study was then conducted to measure the rates of G protein turnover in *Ric-8A*$^{-/-}$ and wild type cells. Gαi, Gαq and Gβ were turned over nearly 10-fold faster in *Ric-8A*$^{-/-}$ cells. We reasoned that the inability of Gα subunits to attain membrane residence in the absence of Ric-8A function resulted in enhanced G protein degradation. That is, the primary Gα subunit biosynthetic defect imparted by Ric-8A absence was realized as a secondary consequence of enhanced protein turnover of the mis-targeted, cytosolic G proteins.

The sum of these studies concluded that Ric-8A acted upon Gα subunits at an early point during biosynthesis. We proposed that Ric-8A could work with either the TriC/CCT and/or HSC70/90 chaperone systems to promote Gα subunit folding, or could act after folding upon soluble Gα subunits to escort them to the proper endomembrane for G protein heterotrimer assembly (Figure 3). We have begun investigations using cell-free translation

systems to address these possibilities and have found preliminarily that Ric-8A is required for Gα subunit folding (30).

Perspective

Most research describing roles for Ric-8 in G protein regulation has intimated that Ric-8 acts akin to the action of a GPCR. In this role, Ric-8 is proposed to control Gα subunit activation status in cells as a GEF, producing Gα-GTP and the consequent activation of traditional and non-traditional G protein effector enzyme systems. Most of the observations that contributed to this theory, however, are explained equally as well by Ric-8 controlling the abundance of the signaling G protein subunits. Going forward, the question of how Ric-8 regulates G protein function is probably not limited to a single answer. Ric-8 may have dual function(s) within the cell towards G proteins as a GEF activator and molecular chaperone.

Independent studies have provided some evidence of Ric-8 features that are not intuitively consistent with Ric-8 acting solely as a chaperone during Gα subunit biosynthesis. In asymmetric cell division, biochemical work demonstrated that Ric-8A can act upon Gαi:GPR/GoLoco-domain protein complexes to dissociate them and produce Gαi-GTP (30–32). *Caenorhabditis elegans* Ric-8 and HeLa cell Ric-8A are recruited to mitotic spindles and spindle pole regions during mitosis (14,16). Studies in mammalian cells have shown recruitment of fluorescent protein tagged Ric-8 proteins to the cell periphery in response to chronic GPCR agonist treatments (6). Ric-8A was found to bind to neural cell adhesion molecule (NCAM) and appeared to co-localize at the cell surface with NCAM (33). Recently Blumer and Lanier et al. provided the first experimental evidence that distinguished two separable functions of Ric-8A in cells. Ric-8A regulation of Gαi abundance was insensitive to *pertussis* toxin (PTX), whereas Ric-8A regulation of bioluminescence resonance energy transfer (BRET) interactions between Gαi and AGS3 or AGS4 at the plasma membrane were PTX sensitive (34). ADP-ribosylated Gαi is not a substrate for Ric-8A-catalyzed nucleotide exchange (16). The substrate of PTX is the Gi heterotrimer. Therefore, it is logical that Ric-8 action as a chaperone of nascent Gαi that has not yet bound Gβγ is PTX insensitive, and that Ric-8 action on maturated Gαi at the cell surface (in complex with AGS3 or AGS4) is PTX sensitive. Future work will provide insight into the multiple roles that Ric-8 proteins have in cells including mechanistic detail of Ric-8 chaperoning and/or GEF functions.

Acknowledgments

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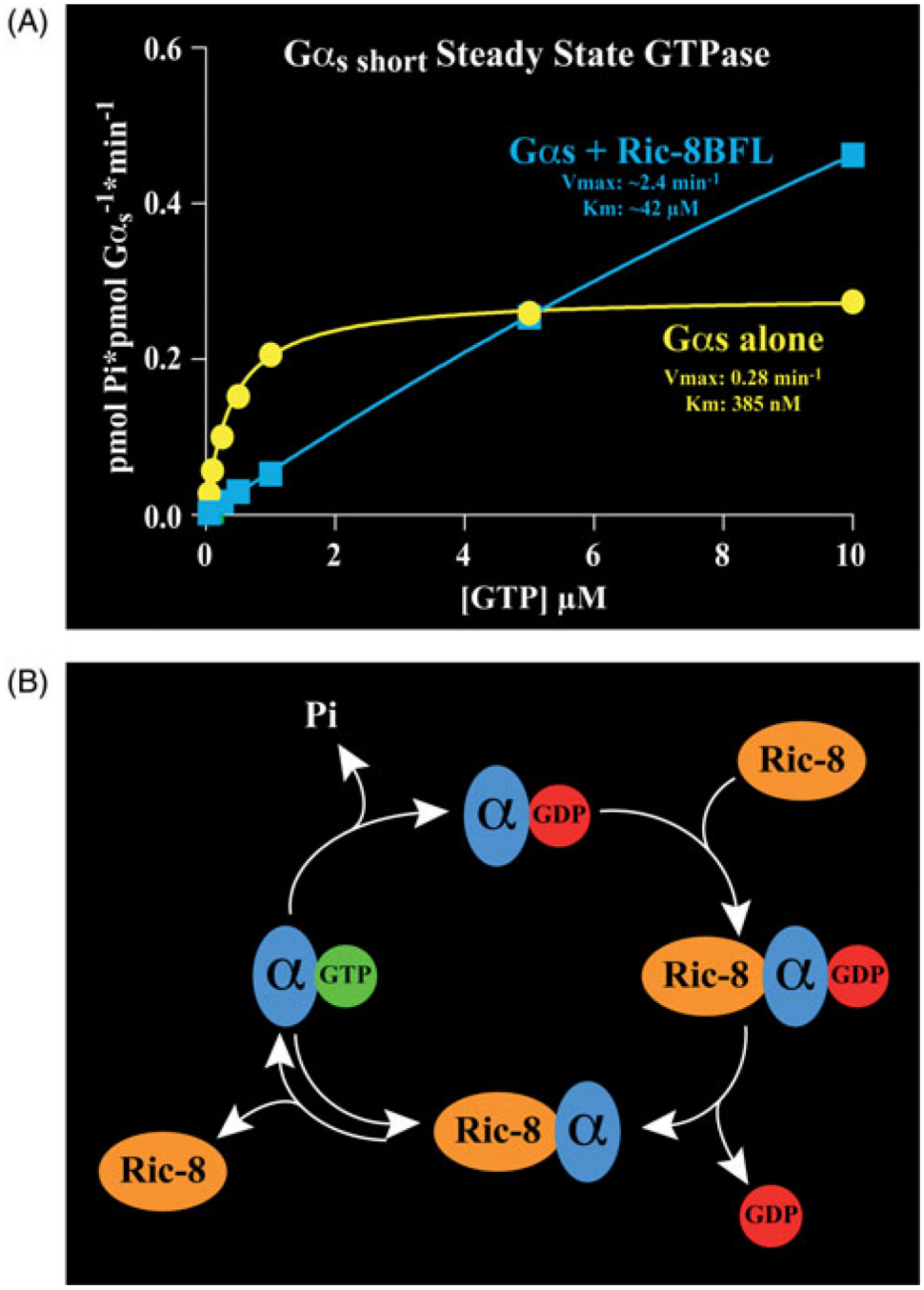

Figure 1.

Ric-8 proteins stabilize the nucleotide-free form of Gα subunits to stimulate GTP-dependent Gα nucleotide exchange. (A) The GTP substrate dependence of Gαs steady state GTP hydrolytic activity was measured in the presence or absence of purified Ric-8B full-length protein. At low GTP concentrations, Ric-8BFL was inhibitory to nucleotide exchange because sufficient GTP is required to dissociate the nucleotide-free Ric-8B:Gα intermediate. At ≥5 μM GTP, Ric-8B stimulated Gαs nucleotide exchange. Physiological GTP concentrations are typically >200 μM. Reproduced from ref. (8). (B) Schematic representation of Ric-8-stimulated Gα nucleotide exchange cycle. Ric-8 binds to Gα-GDP and stimulates rapid GDP release and stabilization of the nucleotide-free Gα. Sufficient GTP

is required to dissociate the Ric-8:Gα nucleotide- free complex to produce Gα-GTP and free Ric-8. Reactions consisting of high affinity Ric-8 and Gα pairs (e.g. Ric-8B:Gαs and Ric-8A:Gαq) can stimulate limited release of GTP(γS) pre-bound to Gα.

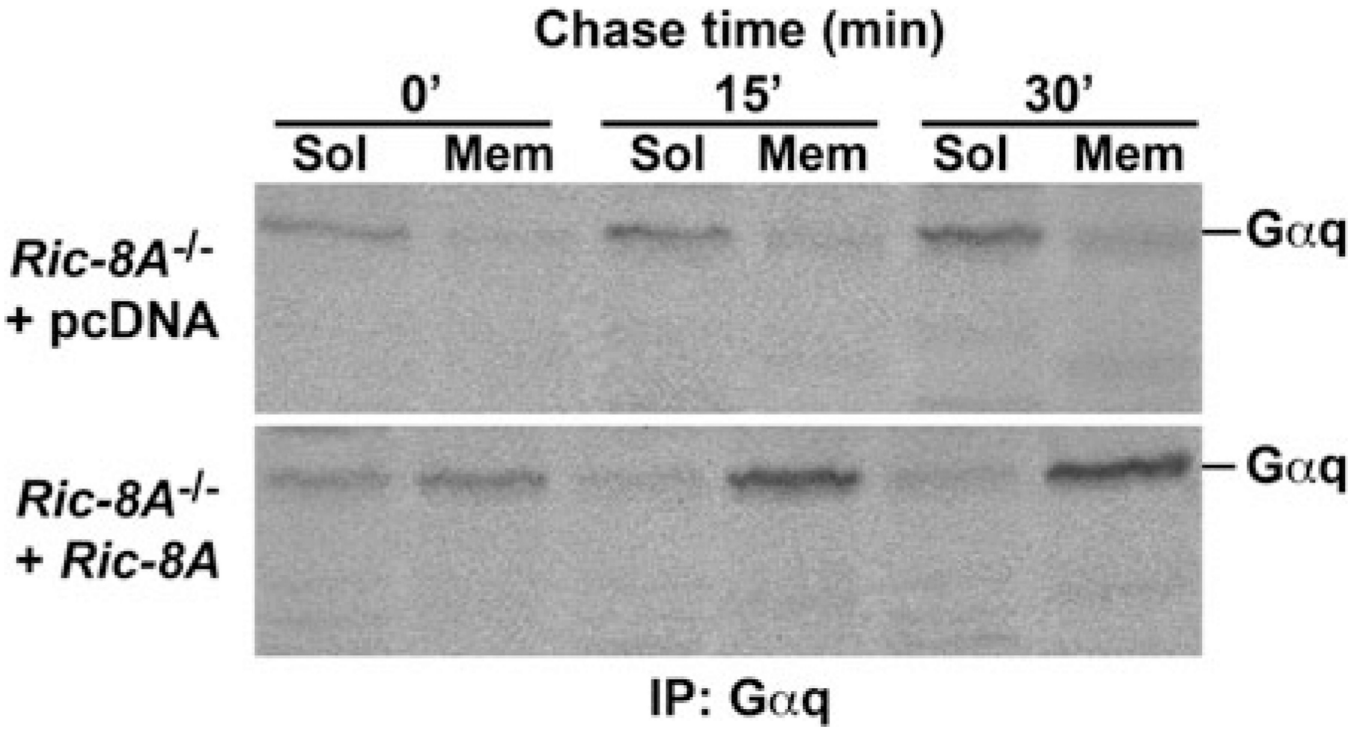

Figure 2.

Nascent Gα q is defective in association with cellular membranes in mES cells lacking Ric-8A. *Ric-8A*$^{-/-}$ mES cells stably expressing *Ric-8A* cDNA or pcDNA were pulse labeled with [^{35}S]-methionine and -cysteine for 10 min and chased with unlabeled amino acids for the indicated times. Cells were fractionated into crude membranes (Mem) and cytosol (Sol) and Gαq was immunoprecipitated from the fractions and visualized by autoradiography. All of the nascent Gαq remained soluble in cells lacking *Ric-8A*, whereas the Gαq accumulated on a membrane in cells that expressed *Ric-8A*. Reproduced from ref. (22).

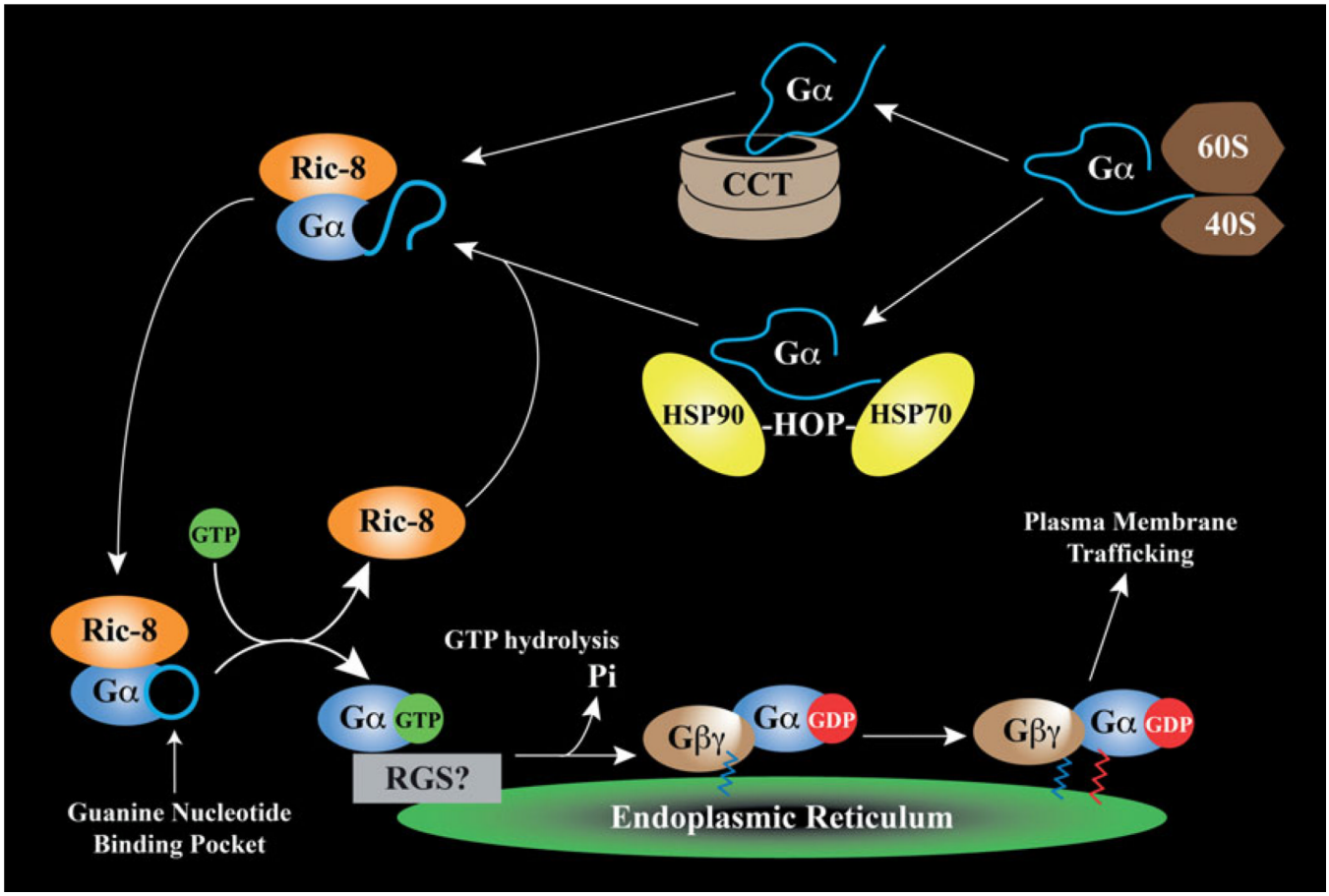

Figure 3.

Model of Ric-8 action during G protein biosynthesis. G protein α subunits are translated by cytosolic ribosomes. The CCT and/or HSC70/HOP/HSC90 chaperone systems were suggested to participate in the folding nascent Gα subunits (35–39). Ric-8A may work in concert with cellular chaperones to promote Gα subunit folding, or work after cellular chaperone-mediated folding in two capacities: (1) Ric-8 could complete the final steps of Gα folding to the native state by promoting GTP binding to the newly folded Gα guanine nucleotide-binding pocket. (2) Ric-8 could act as an escort factor to translocate folded Gα subunits to the outer leaflet of the endoplasmic reticulum. The purely speculative existence of an organelle-specific RGS-like protein has been included in this diagram. Such a factor could serve as a membrane acceptor and/or facilitator of Gα-GTP conversion to the GDP-bound form to enable Gα to bind nascent Gβγ on the membrane. Spontaneous Gα GTP hydrolytic activity could fulfill the same requirement.