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Ric-8: Different Cellular Roles for a Heterotrimeric G-Protein GEF

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ABSTRACT

Signaling via heterotrimeric G-proteins is evoked by agonist-mediated stimulation of seven transmembrane spanning receptors (GPCRs). During the last decade it has become apparent that Gα subunits can be activated by receptor-independent mechanisms. Ric-8 belongs to a highly conserved protein family that regulates heterotrimeric G-protein function, acting as a non-canonical guanine nucleotide exchange factors (GEF) over a subset of Gα subunits. In this review we discuss the roles of Ric-8 in the regulation of diverse cell functions, emphasizing the contribution of its multiple domain protein structure in these diverse functions. J. Cell. Biochem. 113: 2797–2805, 2012.

KEY WORDS: Ric-8; GEF; G-PROTEIN; SIGNAL TRANSDUCTION

Signal transduction systems, in particular those mediated by heterotrimeric G-proteins, interpret and propagate external signals through plasma membrane-associated protein complexes, that are composed of a receptor, a G-protein transductor and an effector system amplifier [Birnbaumer, 2007]. This coupling of receptors to G-proteins provides plasticity in the response as it allows regulation of different intracellular effector systems [Goldsmith and Dhanasekaran, 2007]. The majority of the proteins that contribute to this type of signaling exist as different isoforms that exhibit different regulatory properties. For example, in mammals, 20 Gα, 5 Gβ, and 12 different Gγ subunits have been described, which can associate in different combinations within the cells to generate an assorted population of heterotrimeric G-proteins [Birnbaumer, 2007]. Even though this diversity generates high complexity during signal integration, it allows the cells to respond in a very specific manner to a particular external stimulus. Furthermore, many signaling cascades share molecular components to produce highly specific cell responses. This raises the important question of, how are cells capable of integrating highly diverse external stimuli and reply to each of them in an also highly specific manner? During recent years, several groups have intensively addressed this question by searching for determinants that support the specificity of the signaling response. Among these determinants, we find those that: (a) Define the regulatory mechanisms that control gene expression patterns during embryonic development, (b) establish the estequiometry of regulatory protein–protein interactions within cells, (c) define the precise subcellular distribution and compartmentalization of regulatory components, (d) determine post-translational modifications at regulatory components, and (e) define the contribution of accessory proteins.

Among the relevant components in establishing regulatory signaling complexes, are multi-domain proteins that function as scaffolds. These proteins have concentrated significant interest in the last few years, mainly because of their ability to associate with several different components of a given signaling pathway which may contribute to a tighter regulation of the signal transduction process and a high level of specificity [Good et al., 2011]. Moreover, scaffold proteins can determine the localization of different signaling components in an adequate cellular microenvironment, hence facilitating formation of a well-organized signal transduction protein complex. In this review, we will discuss recent research progress on the function of the Ric-8 proteins. We will address the

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contribution of these proteins in key cellular events, including, but not limited to, G-protein-mediated signaling pathways and control of mitotic cell division.

G-PROTEIN SIGNALING COMPONENTS

Heterotrimeric G-proteins are composed of three different subunits, Gα, Gβ, and Gγ, that are considered biological switches that oscillate between ON and OFF states [Birnbaumer, 2007]. The OFF state is represented by the Gα-GDP/Gβγ heterotrimer whereas the ON state by the dissociated Gα-GTP complex and the Gβγ dimer. In un-stimulated conditions, the inactive GαGDP/Gβγ complex predominates in the cell membrane and the signaling pathway remains OFF. In contrast, binding of a specific ligand to the membrane receptor, results in a conformational change in its intracellular domain, which in turn triggers the exchange of GDP by GTP in the Gα subunit with the concomitant dissociation of the Gα-GDP/Gβγ heterotrimer. This dissociation generates the two active species Gα-GTP and Gβγ, which can act as downstream regulators by interacting with different effector systems, including adenylyl cyclase, phospholipase C, ionic channels, and kinases, among others [Goldsmith and Dhanasekaran, 2007]. The signaling impulse is then terminated by the hydrolysis of GTP to GDP catalyzed by the intrinsic GTPase activity of the Gα subunit. Gα-GDP then re-associates with Gβγ thus reconstituting the inactive heterotrimer. In the last decade, novel families of accessory proteins have been identified which can modulate the ON-OFF state of G-proteins, therefore raising the level of complexity of the regulatory mechanisms that control these signaling processes [Sato et al., 2006].

REGULATORS OF THE HETEROTRIMER G-PROTEIN ON-OFF STATE

Among several regulators of heterotrimeric G-proteins, we find the Regulators of G-protein signaling (RGS), which function as GAPs (GTPase activating proteins) by stimulating the intrinsic Gα subunit GTPase activity. Therefore, these GAPs function as negative regulators of G protein signaling. RGS protein family comprises more than 20 different members that are involved in diverse cellular responses, including cell migration, growth, and differentiation [Hollinger and Hepler, 2002]. Alternatively, there are proteins that can inhibit the dissociation of GDP from Gα subunits, called guanine nucleotide dissociation inhibitors (GDIs), and which then function by stabilizing the GDP-bound state in the Gα subunit, decreasing the nucleotide exchange and consequently its activation. In addition, there are also accessory proteins called guanine nucleotide exchange factors (GEFs), that can induce the nucleotide exchange at Gα subunits, thus promoting G-protein activation.

Ligand-GPCR complexes have been considered for a long time the traditional GEFs for heterotrimeric G proteins [Pierce et al., 2002]. However, in the past few years, several novel non-receptor G-protein activators have been discovered. Studies by Cismowski et al. [2001], using a yeast-based screen for activators of the pheromone pathway, identified potential receptor-independent activators of heterotrimeric G-protein signaling, which were named AGS proteins (for activators of G-protein signaling). This family of proteins has been divided into three groups, based on the postulated mechanism by which they activate G-protein signaling in the functional screen. The G-protein activation mechanism of AGS1 (group I), is similar to that of GPCRs in terms of its ability to function as a guanine nucleotide exchange factor for Gαi/Gαo, increasing GTPγS binding to free Gαi2 and to purified brain G-proteins [Cismowski et al., 2001]. Group II comprises AGS 3–6, which are characterized by the presence of one or more 20–25 amino-acid repeats termed GPR or GoLoco motifs. This group of AGS proteins binds to Gαi and Gαt with higher affinity than to Gαo, but do not bind to Gαs. The GPR motif stabilizes the GDP-bound conformation of Gα, promotes the subunit dissociation from preformed G-protein heterotrimers and competes with Gβγ for binding to Gα. Therefore, a GPR protein can form a stable complex with free GαiGDP behaving as a GDI with binding affinities at the nanomolar range. AGS 2, 7, and 8 belong to group III, and in contrast to group I and II proteins, they bind to Gβγ and not to Gα [Blumer et al., 2006a]. The role of this group of proteins in mammalian G-protein signaling has not been established, although it has been suggested that they may serve as binding partners for a number of Gβγ molecules within cells that function independently of Gα. In light of these new findings, one interesting hypothesis to consider is that AGS proteins may be involved in receptor-independent G-protein regulation at different intracellular compartments, were Gα and Gβγ function independently of GPCR activation and heterotrimer formation.

Ric-8, A NON-CANONICAL GEF

Among the newly identified proteins that may also function as positive modulators of heterotrimeric G-protein complexes is Ric-8 (also known as synembryn). Ric-8 gene was initially identified during two independent studies: (a) a genetic screening in *C. elegans* designed to identify mutants that were resistant to the cholinesterase inhibitor Aldicarb [Miller et al., 1996] and (b) a yeast two-hybrid screen to search for partners interacting with mammalian heterotrimeric Gα subunits [Klattenhoff et al., 2003; Tall et al., 2003]. In contrast to *C. elegans* and *Drosophila* where a single Ric-8 gene has been identified, two ortologs have been described in mammals (Ric-8A and Ric-8B). The Ric-8A and Ric-8B proteins were shown to preferentially interact with the GDP-bound form of Gα subunits and to stimulate their nucleotide exchange activity, therefore acting as receptor-independent GEFs. Ric-8 proteins differ from traditional GPCR-ligand complexes as they are unable to stimulate the guanine nucleotide exchange activity of G-proteins while they remain part of the heterotrimeric complex. Hence, Ric-8 proteins can only function on dissociated monomeric Gα subunits [Tall et al., 2003]. Importantly, both isoforms of Ric-8 display different specificities; Ric-8A functions as a GEF on Gαi/o/q but not on Gαs whereas Ric-8B functions on Gαs/q but not on Gαi/o [Romo et al., 2008; Chan et al., 2011a]. As a cytoplasmic molecule, Ric-8 has been shown to contribute to the synaptic transmission process in *C. elegans* [Miller and Rand, 2000]. In this nematode, neurotransmitter release at the neuromuscular junction is controlled by the

Gαq-Gαo signaling pathway, where EGL-30 (Gαq) activates EGL-8 (PLCβ), leading to the production of diacylglycerol (DAG). This DAG then positively regulates neurotransmitter secretion, in part via an interaction with the DAG binding protein UNC-13 [Miller and Rand, 2000]. On the other hand, the EGL-30 pathway is negatively regulated by GOA-1 (Gαo), which stimulates a DAG kinase to reduce functional DAG levels [Matsuki et al., 2006]. As Ric-8 and EGL-30 mutants show similar phenotypes, it has been suggested that both proteins function in the same pathway to positively regulate synaptic transmission. Genetic epistasis analyses indicate that Ric-8 functions upstream of or in conjunction with EGL-30. The guanine nucleotide exchange activity reported for Ric-8 over Gαq is in agreement with these data and supports a model where Ric-8 protein exhibits a positive role in this signaling pathway (Fig. 1).

FUNCTIONS OF Ric-8

RIC-8 IN THE CONTROL OF MITOTIC CELL DIVISION

Ric-8 has been recently reported as a regulatory component of an evolutionarily conserved heterotrimeric G-protein-mediated mechanism that controls asymmetric cell division in *C. elegans* embryos [Miller and Rand, 2000] and *Drosophila* neuroblasts [Wang et al., 2005]. Asymmetric cell division is a critical process during the generation of cell diversity, where cell-fate determinants often cluster at specific locations, frequently at opposite poles of the parent cell. When the cell divides, its two daughter cells inherit different sets of fate determinants and go on to adopt their respective identities. However, it is not sufficient that the determinants are just distributed asymmetrically; cell cleavage must also occur such that the determinants can be efficiently segregated to the appropriate daughter cells. Therefore, proper orientation and positioning of the mitotic spindle is essential for the correct segregation of these fate determinants. Studies in model systems have shown that spindle positioning is regulated by a non-canonical, heterotrimeric G-protein-mediated signaling pathway. This pathway is GPCR-independent and activated by two types of Gα-binding proteins; GDIs containing one or more GoLoco motifs (GPR1/2 in *C. elegans* and Pins/Loco in *Drosophila*) and a non-receptor GEF (Ric-8) promoting GDP-GTP exchange in the absence of any membrane receptor binding.

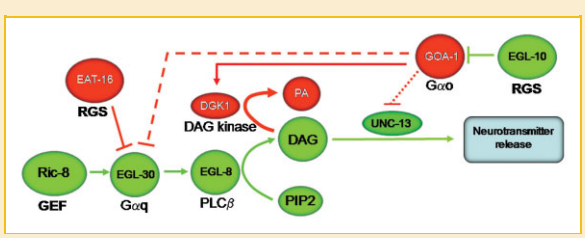

Fig. 1. Signaling network model that shows regulation of neurotransmitter release in *C. elegans*. Solid lines indicate known or highly likely direct interactions, while dashed lines indicate poorly understood interactions or missing components. Proteins or molecules that promote neurotransmitter release are shown in green, while proteins that inhibit the process are shown in red. Adapted from Reynolds et al. [2005].

In the early embryo of *C.elegans*, diverse cortical factors that constitute the Par complex, Par3-Par6-aPKC, localize to the anterior cortex before the first cleavage, setting up a series of events that results in the generation of an anterior daughter cell that is larger than its posterior sister. The difference in sibling size is the result of a shift in the cleavage plane toward the posterior pole, caused by the migration of the posterior centrosome to that direction, due to an imbalance in cortical force generators that act on astral microtubules that pull on spindle poles [Grill et al., 2001]. The asymmetric activation of receptor-independent G-protein signaling, downstream of the Par complex, is critical in tipping the balance. Two highly similar Gα binding proteins, called GPR1/2, function as GDIs, by stably binding the GDP forms of GOA-1 (Gαo) and GPA-16 (Gαi) and causing the heterotrimeric complex to split into its Gα and Gβγ components. Whereas the two Gα subunits are evenly distributed around the entire cortex, cortical GPR1/2 is concentrated at the posterior pole. On the other hand, the division of *Drosophila* embryonic neuroblasts is an apico-basal, rather than anterior-posterior, event. In this process, the Par complex and several G-protein signaling factors—Gαi and two GDIs (Pins and Loco)—are also involved [Yu et al., 2006]. A neuroblast specific protein, inscuteable, binds the two GDIs and forms a complex with the apically situated Par3 homologue Bazooka [Kuchinke et al., 1998]. The Gαi-GDI complex is important in orienting the mitotic spindle along the apico-basal axis, allowing basal daughter cells to inherit a set of determinants. The enhanced development of the apical spindle shifts the cleavage plane basally, resulting in smaller size basal daughter cell. Interestingly, the development of the astral microtubules is more robust from the centrosome proximal to the GDI-enriched cortical domain [Yu et al., 2006]. This is in contrast with the *C. elegans* zygote where cleavage occurs more proximal to the GDI side [Grill et al., 2001]. Taken together these findings indicate that a conserved mechanism operates in these two organisms. This mechanism involves a receptor-independent G-protein signaling pathway that controls the interactions between the cell membrane and microtubules, affecting spindle orientation and the generation of pulling force. An important question that then arises is: Does the GDP-GTP exchange cycle also take place in this receptor-independent G-protein signaling pathway?; the discovery of Ric-8, functioning as a GEF for several Gα species provided a new component to efficiently control this GDP-GTP exchange [Tall et al., 2003; Romo et al., 2008]. Ric-8 protein has been shown to function as a GEF for the Gα subunit of *C. elegans* GOA-1 as well as to be indispensable for the formation of the GOA-1-GPR1/2 complex and the generation of pulling forces [Miller and Rand, 2000]. These findings, together with the discovery that the GAP molecule RGS7 has a role in this process, strongly support the notion that GDP-GTP cycles are also required for receptor-independent G-protein signaling [Hess et al., 2004]. In *Drosophila* neuroblasts and sensory organ precursor cells, Ric-8 was also found to be essential for proper spindle orientation, differences in daughter cell size and in asymmetric localization of cell-fate determinants [Matsuzaki, 2005]. In addition, Ric-8 was found to be important during gastrulation, a process that requires highly coordinated rearrangements and movement of cells that are known to rely on receptor-dependent G-protein signaling. Unexpectedly, in the

absence of Ric-8, all G-proteins subunits, including Gαi, Gαo, Gβ and by extension, presumably Gγ, fail to reach the cell cortex. This discovery points to an additional new function of Ric-8, in which it may not necessarily acts only as a GEF, but also as a facilitator of Gα function by promoting heterotrimer assembling.

In mammals, two proteins that are related to *Drosophila* Pins have been identified, called LGN and AGS3 [Mochizuki et al., 1996]. Members of this class of cytoplasmic non-receptor-regulators of G-protein signaling generally contain two types of repeats; seven tetratricopeptide repeats (TPR) at the amino-terminus and three GoLoco repeats at the carboxy-terminus. TPR motifs usually mediate protein–protein interactions, whereas GoLoco motifs are responsible for association with Gα subunits of heterotrimeric G proteins. In addition to Gα, another binding partner for LGN identified is NuMA, a nuclear protein that is required for spindle organization during mitosis [Du and Macara, 2004]. NuMA is a large coiled-coil protein that contains at its C-terminus a microtubule binding domain, which partially overlaps with the LGN binding region. Hence, NuMA is unable to bind LGN and microtubules simultaneously [Du and Macara, 2004]. Using a FRET biosensor, these authors demonstrated that LGN behaves as a conformational switch: In its closed state, the N- and C-termini interact, but NuMA or Gαi can disrupt this association, allowing LGN to interact with both proteins and resulting in their cortical localization. Therefore, at the onset of mitosis, when the nuclear envelope breaks down, NuMA can associate with the N-terminal region of LGN, triggering the release of the C-terminus, such that LGN switches to a partially open state. Much of the LGN/NuMA complex associates with the spindle poles, but some bind with high affinity to GαiGDP at the cell cortex to form a ternary complex. Overexpression of either LGN or Gαi, induces a pronounced rocking motion in the mitotic apparatus of epithelial MDCK cells, which is reminiscent of motions observed in *C. elegans* zygotes during anaphase prior to asymmetric cell division [Blumer et al., 2006b]. It has been proposed, that the ternary NuMA/LGN/Gαi complex perturbs the microtubules cortical attachment, causing transient changes in the pulling forces by the asters microtubules on the spindle poles. Unbalance pulling forces produce then the rotation of the mitotic apparatus. However, it remains unclear how the LGN complex alters the attachment of microtubules to the cell cortex and how the GTP-binding-hydrolysis cycle of Gαi functions in the context of this pathway. It is possible that the LGN complex might either increase the dynamic instability of microtubules or modulate the binding of attachment proteins to the plus ends, such as dynein/dynactin.

In this context, alternative models that describe the interplay of Ric-8, RGS, GoLoco, Gα and their accessory proteins that regulate aster microtubule forces during cell division, have been postulated [Srinivasan et al., 2003; Afshar et al., 2004; Hampoelz and Knoblich, 2004; Hess et al., 2004]. One model predicts that NuMA-LGN-GαiGDP is the active complex that regulates the microtubule pulling forces. A second model suggests that NuMA-LGN-GαiGDP is a substrate for the guanine nucleotide exchange activity of Ric-8, which leads to the production of free GαGTP and signals force generation [Manning, 2003]. This hypothesis is very attractive, given the opposed phenotypes of *C. elegans* rgs7 and Ric-8 mutants and due to the finding that mammalian Ric-8A does not activate Gαβγ heterotrimers, but only free Gα subunits in vitro.

One major unresolved question regarding to these two models, was to determine whether GoLoco-bound Gαi is a substrate for Ric-8A-mediated guanine nucleotide exchange. Tall and Gilman [2005] took a biochemical approach using purified components to demonstrate that mammalian Ric-8A recognizes LGN- and AGS3-Gαi-GDP complexes as substrates in vitro. It was found that Ric-8A was able to dissociate GoLoco protein/Gαi-GDP complexes while activating Gαi catalytically in the presence of GTPγS. Activation of the Gαi/LGN/NuMA protein complex by Ric-8A also stimulated the release of NuMA from LGN, an event that has been proposed to be a mechanism of aster microtubule regulation during cell division [Du and Macara, 2004]. Cycling of Gαi between its GDP- and GTP-bound forms is evidenced by the fact that both, Ric-8 and RGS7, influence the pathway in opposed fashion [Hess et al., 2004]. The resulting Gαi-GDP could rebind to GoLoco (and not to Gβγ) to complete one round of the cycle. Rapid cycling of this process may be necessary to regulate the pulling forces on microtubules appropriately during a round of chromosome segregation (Fig. 2).

The role of Ric-8A and Gαi in mammalian mitotic spindle orientation during mitosis was recently addressed in HeLa and MDCK cells [Woodard et al., 2010]. The localization of Ric-8A at the cell cortex, spindle poles, centromeres, central spindle, and midbody was prevented by *Pertussis* toxin and by reduction of Gαi and Ric-8A intracellular levels. All these treatments reduced the localization of LGN, NuMA, and dynein at the metaphase cell cortex and also disturbed the mitotic spindle orientation, leading to mitotic arrest and expanded mitosis. These findings indicate that Ric-8A participates in building of the cortical complex which orients the mitotic spindle. Recently, Cho and Kehrl [2007] observed that the three isoforms of Gαi (Gαi1, Gαi2 and Gαi3) and RGS14, a RGS family member that displays GAP activity over Gαi, colocalize in the centrosomes and at the midbody of non-polarized HeLa cells. They also observed that simultaneous depletion of Gαi1, Gαi2, and Gαi3

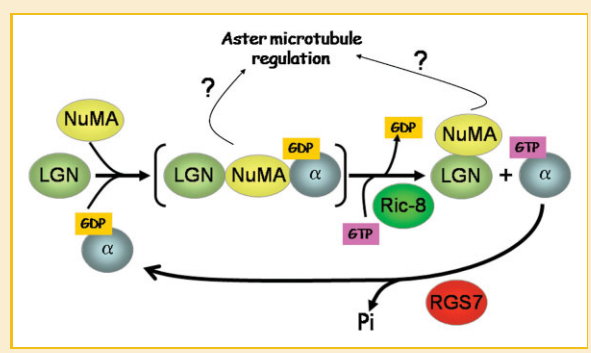

Fig. 2. Proposed mechanism for Gαi guanine-nucleotide-cycling regulation of aster microtubule pulling forces during cell division. At the onset of mitosis, Gαi-GDP/LGN/NuMA complexes are formed at the sites of aster microtubule regulation. Afterwards, the guanine nucleotide activity of Ric-8 stimulates the release of GDP and GTP binding on Gαi, which produces the dissociation of the complex into Gαi-GTP, LGN, and NuMA. RGS activity stimulates then the hydrolysis of GTP on Gαi and the resultant Gαi-GDP could reform the active Gαi-GDP/LGN/NuMA complex. Adapted from Tall and Gilman [2005].

or RGS14 by siRNA, induces cytokinesis failure, revealing a requirement for these proteins during normal symmetric cell division. Since RGS14 contains a GoLoco domain in addition to its RGS domain, it posses GDI and GAP activities, allowing interaction with both the GDP-bound form (through GoLoco) and with the GTP-bound form (through RGS) of Gαi. The presence of all these components at the centrosomes and midbody suggests that the GDP/GTP cycle is necessary for non canonical G-protein signaling at these sites. A missing GEF function component in this system may likely then be attributed to Ric-8. The presence of a [RGS14-Gαi-GDP-Ric-8A] complex in mouse brain has been recently reported by Vellano et al. [2011], therefore providing further support to the essential role of Ric-8 as regulator of RGS14-Gαi signaling functions in cell division control (Fig. 3).

RIC-8 IN CELL SIGNALING

In addition to their role in non-receptor G-protein signaling, Ric-8 proteins have also been involved in receptor-dependent signaling processes. While studying odorant signal transduction mechanisms von Dannecker et al. [2005] searched for potential regulators of the olfactory G protein Gαolf (which is highly homologous to Gαs) in the olfactory epithelium. They not only identified Ric-8B through a two-hybrid screen analysis, but also found that Ric-8B and Gαolf are expressed in the same territories in the brain, in the olfactory epithelium, and in regions of the brain such as the striatum, nucleus accumbens, and olfactory tubercle. Interestingly, Gαs, which is widely expressed in the brain, shows little or no expression in the brain areas were Gαolf is detected, suggesting that Gαolf can be the physiological target of Ric-8B. Consistent with its role as a GEF over monomeric Gα subunits and not over heterotrimers [Tall et al., 2003], Ric-8B was found only capable of stimulating cAMP production when a Gαs/olf-GPCR (β2 AR) was previously activated [von Dannecker et al., 2005]. This indicates that activation of Gα subunits by Ric-8 depends on the heterotrimer dissociation that can be induced by a GPCR-ligand complex that generates Gα—GTP and Gβγ. Following Gα-induced hydrolysis of GTP, Ric-8 could bind to Gα-GDP, thus competing with Gβγ, catalyzing guanine nucleotide exchange, and potentiating GPCR signaling. Therefore, in the olfactory system signaling, Ric-8B can be functioning as an amplifier. Whether this function of Ric-8B represents a critical component within the olfactory system of animals that need detecting with extremely high sensitivity, traces of scents generated by a predator or a prey, remains to be determined. Importantly, this function of Ric-8B is in agreement with findings from our group that show that translocation of Ric-8B to the plasma membrane of mammalian cells is only observed after GPCR stimulation [Klattenhoff et al., 2003]. In this same context Ric-8A, that was identified in a two-hybrid screen search for novel effectors or regulators of Gα subunits, has been found to be located in the cytosol and to partly translocate to the plasma membrane in response to Gαq-coupled receptor stimulation. Moreover, the role of Ric-8A on Gαq-mediated signaling has been further confirmed as the depletion of Ric-8A expression results in a diminished Gq-coupled receptor-mediated ERK activation and reduced intracellular calcium mobilization. Expression of myristoylated Ric-8A, in contrast, showed enhanced Gq-coupled receptor-mediated ERK activation, consistent with a role of Ric-8A at the plasma membrane. Importantly, Ric-8A-mediated enhancement of ERK activation and guanine nucleotide exchange activity through Gαq, were inhibited by the Gαq selective inhibitor YM-254890. Taken together, these findings indicate that both Ric-8A and Ric-8B positively regulate GPCR-mediated signaling pathways at the plasma membrane, functioning as signal amplifiers to potentiate the signal initiated by a ligand-GPCR complex (see Fig. 4).

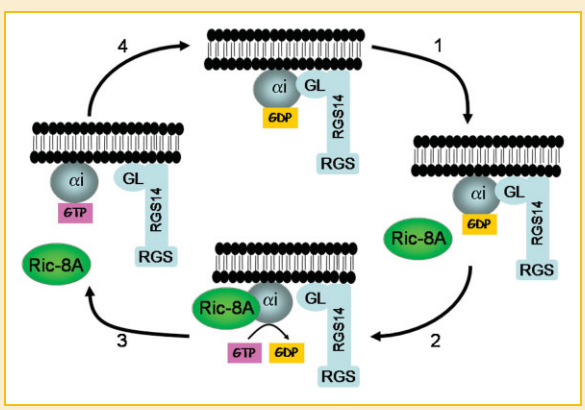

Fig. 3. Proposed mechanism of RGS14-Gαi1 cycle regulation by Ric-8A. At the resting state, RGS14 binds to Gαi-GDP through its GoLoco (GL) domain. 1: Stimulation of signaling recruits Ric-8A to the Gαi-GDP complex. 2: Ric-8A induces, through its GEF activity, the GDP/GTP exchange in Gαl, generating the Gαi-GTP active specie. 3: Ric-8A dissociates from the active Gαi-GTP form initiating the intracellular signaling. 4: The GAP activity of RGS14 turns-off the signaling through its RGS domain, generating the inactive Gαi-GDP form and the resting state [Gαi-RGS14] complex. Adapted from Vellano et al. [2011].

There are a number of additional reports that also support a relevant role of Ric-8 in signal transduction. In *C. elegans*, the G-protein regulators AGS-3 and Ric-8 function in chemosensory neurons in a mutually dependent manner to activate Gαo, which is the signaling molecule activated in response to food deprivation [Hofler and Koelle, 2011]. During plant infection by the fungus *Magnaporthe oryzae*, Ric-8 has been recently identified as a relevant component within the cAMP-mediated signaling pathway generated in response to the infection. Ric-8 interacts with the Gα subunit MagB and hence regulates the pathway that allows *M.oryzae* to form a peg-shaped hypha that penetrates the hydrophobic surfaces from the point where the infection occurs [Li et al., 2010]. Loss of Ric-8 in the fungi *Neurospora Crassa* leads to phenotypes that are similar to those displayed by mutants lacking the Gs-protein genes *gna-1* and *gna-3* that exhibit impaired growth and loss of female fertility [Wright et al., 2011]. In mammals, novel regulation of adenylyl cyclases (ACs) by direct protein–protein interactions between the isoform AC5 with Ric-8A has been described [Wang et al., 2007]. This interaction leads to suppression of AC5 activity in a Gαi-dependent and GPCR-independent manner. As Ric-8A also enhances Gβγ-evoked signaling [Malik et al., 2005] and because Gβγ can modulate AC5 function, it will be important to clarify whether this regulation of AC5 activity occurs through an

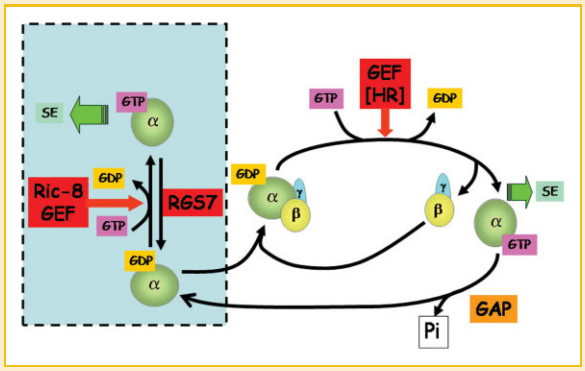

Fig. 4. Canonical and non-canonical G-protein signal transduction regulation. The right part of the diagram shows a canonical signaling pathway initiated by the GEF activity of the GPCR, that generates the active free species Gα-GTP and Gβγ, which in turn regulate different effector systems (ES). After GTP has been hydrolyzed to GDP by the intrinsic GTPase activity of the Gα subunit (GAP), the inactive Gα-GDP form reassociates with Gβγ to regenerate the heterotrimer Gα-GDPGβγ. In the non-canonical signaling pathway (left part of the diagram), Gα-GDP interacts with the GEF Ric-8 which stimulates the exchange of GDP for GTP. This exchange activates the pathway in a receptor-independent manner, potentiating the signal initiated by the ligand-GPCR complex. The GTPase activity of RGS7 deactivates Gα-GTP.

interaction with a putative Ric-8A-Gβγ complex. Recent reports also demonstrate that in mouse embryonic fibroblasts (MEFs), down regulation of Ric-8A by RNA interference inhibits PDGF-induced cell migration and prevented Ga13 translocation to the cell cortex [Wang et al., 2011]. These results indicate that Ric-8A may be critical for the PDGF-induced signaling that leads to cytoskeletal reorganization [Wang et al., 2011]. Together, these results illustrate the relevant functions of Ric-8 in the regulation of G-protein signaling within eukaryotic cells.

RIC-8 IN DEVELOPMENT

Although the critical role of Ric-8 in *C. elegans* and *Drosophila* asymmetric cell division during early embryogenesis has been valued [Couwenbergs et al., 2004], its main function during embryogenesis has not been characterized in depth. Initial studies in mice showed that Ric-8A is expressed during early development in the nervous system, mainly in areas such as cranial ganglia, neural tube, and dorsal root ganglia [Tõnissoo et al., 2003]. The same studies also provided evidence for Ric-8A expression in adult brain. The importance of Ric-8A in embryogenesis is supported by results indicating that the homozygous Ric-8 (−/−) mutant mice is not viable as the embryos die early during development [Tõnissoo et al., 2006]. In the heterozygous mutant mice (+/−), the spatial memory and anxiety are affected, indicating the importance of Ric-8A in neural functions. The activity of Ric-8A protein is relevant for a correct gastrulating of a mouse embryo, as Ric-8$^{-/-}$ embryos are capable of completing gastrulation but are unable to develop further [Tõnissoo et al., 2010]. Additionally, Ric-8B knockout mice were also found not viable, dying before embryonic day 8.5. Therefore, in mouse the study of G-protein function in the total absence of Ric-8A or Ric-8B has been forced to be performed in embryonic stem cells derived from live blastocysts obtained from homozygous mutant mice embryos [Gabay et al., 2011]. An insertion mutagenesis analysis in zebrafish genes also showed that Ric-8 is required during the embryo life and that the disruption of the gene causes edema, embryonic lethality, and changes in the pigment phenotype [Nagayoshi et al., 2007]. Interestingly, the latter effect can be rescued by addition of forskolin, a well-known activator of adenylyl cyclase. This suggests that the small pigment spots observed in the Ric-8 mutants are caused by a decrease in adenylyl cyclase activity due to Gαs uncoupling. Nevertheless, both the edema and embryonic lethality were not rescued by forskolin, perhaps indicating the involvement of other G proteins such as Gαq in the processes. In *Xenopus tropicalis*, Ric-8 is expressed as maternal mRNA in the oocyte and as development proceeds it becomes expressed in the animal hemisphere and subsequently restricted to the neural tube, brain, neural crest-derived structures, and craniofacial region [Maldonado-Agurto et al., 2011].

RIC-8 IN PROTEIN SYNTHESIS

An additional role for Ric-8, in which it may not essentially function as a GEF, has been described during the protein synthesis process. Previous observations in *Drosophila* indicated that Ric-8 may serve as a chaperone that promotes heterotrimer G-protein mRNA translation [Wang et al., 2005]. Similar results were obtained in *C. elegans*, in which the depletion of Ric-8 by RNA interference led to a significant decrease in GPA-16 levels. Interestingly, the synthesis of another *C. elegans* G-protein, GOA-1, was not affected, indicating a degree of specificity for Ric-8 in this regulatory function [Afshar et al., 2004]. The presence of Ric-8B also promotes efficient expression of non-tagged olfactory receptors in heterologous cell systems [von Dannecker et al., 2006]. Although the mechanism underlaying this stabilization of G-proteins and GPCRs by Ric-8 remains unknown, other recent studies have demonstrated that Ric-8B stabilizes the Gαs subunit by preventing its ubiquitination and subsequent proteosome-mediated degradation [Nagai et al., 2010]. These data have led to the use of this approach, co-express Ric-8 proteins, as an improved method for Gα purification from cell systems [Chan et al., 2011b]. Importantly, two recent reports strongly support this putative role of Ric-8A and Ric-8B as molecular chaperones, providing functional evidence for a role of these proteins in protecting Gα subunit from degradation, facilitating its appropriate folding and contributing to its proper membrane targeting [Gabay et al., 2011; Thomas et al., 2011].

CONTROL OF Ric-8 GENE EXPRESSION

Proper regulation of gene expression is essential for normal development, cellular growth, and differentiation. In situ hybridization analyses of mRNA coding for either Ric-8A or Ric-8B vertebrate orthologues have shown different expression profiles during embryo and adult stages, indicating an important control of Ric-8 gene expression in neural and olfactory tissues [Tõnissoo et al., 2003; von Dannecker et al., 2005; Tõnissoo et al., 2006; Tõnissoo et al., 2010]. The "Brain Atlas" (www.brain-map.org, Allen

Institute for Brain Science) indicates that Ric-8A mRNA shows a ubiquitous expression profile and that Ric-8B mRNA exhibits a localized expression pattern within the cerebral nuclei area, specifically at the caudoputamen, nucleus accumbens, and olfactory tubercle striatum sub areas. We have recently determined that *Xenopus tropicalis* Ric-8 (xtRic-8) is also expressed in the otic capsule and craniofacial arches, which derive from neural crest cells [Maldonado-Agurto et al., 2011]. Interestingly, neural crest-derived cells give rise to several cell lineages, including those forming the cartilage and connective tissue of the head [Trainor, 2005]. We have recently described that mammalian Ric-8B gene expression is up-regulated during proliferation of osteprogenitor cells and down-regulated during osteoblast differentiation [Grandy et al., 2011]. This repression involves an epigenetic control mechanism that is mediated by the transcription factor C/EBPβ-LAP* in association with the SWI/SNF chromatin remodeling complex, which bind to the Ric-8B gene promoter, mobilize nucleosomes, and reduces accessibility at relevant transcription regulatory sequences [Grandy et al., 2011]. Down-regulation of Ric-8 expression might represent an important step to allow cell differentiation as it may reduce cell signaling associated with high cell proliferation or precursor cells.

An association between differential expression of Ric-8 and specific human pathologies has also been proposed. In a model of pre-eclampsia induced by mutation in the $p57^{Kip2}$, a cell-cycle inhibitor, full genome expression profile analysis at placenta revealed Ric-8 as one of the two most consistently down-regulated genes [Knox and Baker, 2007]. Also, analysis of expression profile data obtained through microarray studies of primary human breast tumors showed reduced Ric-8A mRNA expression [Miller et al., 2005]. Moreover, an integrated genomic approach, combining a genome-wide copy number data with gene expression profiles and non-sense-mediated mRNA decay rates, led to the identification of Ric-8A as a candidate target gene in breast cancer cell lines. Interestingly, a subset of clinical breast cancer samples associated with aggressive features showed reduced levels of Ric-8A expression, indicating that repression of this gene may play a role for cancer-relevant signaling pathways [Muggerud et al., 2008]. In human colorectal cancer cells, Ric-8A gene expression has been found to be down-regulated by the anti-cancer ginseng-derived compound Rg3 [Luo et al., 2008]. Although the mechanism of action of Rg3 is not known, it is tempting to speculate that the anti-proliferative effect may be associated with attenuation of the expression of genes involved in the regulation of cell division, including Ric-8. On the other hand, it has been reported that in nasal biopsies from patients with mental illnesses the expression of Ric-8B is significantly reduced [Tajinda et al., 2010]. Interestingly, individuals suffering from schizophrenia also show impaired odor identification, an effect that may be hypothesized to be a reflection of deficiencies in odorant signal transduction pathways due to the absence of an efficient Ric-8B activation of the olfactory-specific G-protein Gαolf [von Dannecker et al., 2005]. Nevertheless, although very exciting, all of these associations between Ric-8 expression/function and human pathologies will require extensive further research before any more definitive correlations can be established.

POST-TRANSLATIONAL MODIFICATION OF Ric-8 PROTEIN

Ric-8A has been identified as a phosphoprotein in human cells going through mitosis [Yang et al., 2007]. Interestingly, Ric-8B was also identified as a phosphoprotein, although in contrast with Ric-8A its peptide sequence does not contain a serine residue within a context sequence for MAPK phosphorylation, suggesting that a different kinase may be catalyzing Ric-8B post-translational modification. Other serine, threonine, and tyrosine residues that are potentially modifiable by phosphorylation have been identified in both Ric-8A and Ric-8B proteins trough large-scale quantitative phosphoproteomic analysis coupled to mass spectrometry [Dai et al., 2007; Mayya et al., 2009]. In this study, the Ric-8 aminoacid peptide sequence GLMAGGRPEGQYSEDEDTDTEEYR came out as the target domain containing the three residues that underwent phosphorylation in response to signaling activation. The exact roles of these phosphorylations at Ric-8A and Ric-8B remain to be investigated through hypothesis-driven experiments. One possibility is that changes in Ric-8 phosphorylation could represent a feedback regulation mechanism, important to subsequent transfer of the signal through new protein interactions and as a result additional regulatory roles for Ric-8.

Ric-8 PROTEIN STRUCTURE

One way of understanding the different functions associated with a regulatory protein is by knowing its three-dimensional structure. Nevertheless, until now no group has experimentally assessed and reported with significant detail the structural features of the Ric-8 proteins. Recently, our research team carried out a structural characterization of Ric-8B in silico by building a model of its putative three-dimensional structure. Because Ric-8B has no homology to any other known protein, we utilized different bioinformatic methods that are based on folding recognition motifs (threading) to construct a structural model for *Xenopus laevis* Ric-8 (xRic-8) in the absence of a template. The structural model obtained for Ric-8B shows an alpha–alpha superhelix folding that corresponds to the armadillo structure according to SCOP classification [Andreeva et al., 2008]. Based on this folding, we subsequently built a refined model using as templates proteins that are known to contain the armadillo structure [Coates, 2003]. We propose that the xRic-8 structure is formed by 10 armadillo folding motifs, organized in a right-twisted alpha–alpha super helix (Fig. 5).

To further validate this structural model, xRic-8 was expressed in bacteria, purified by affinity and anion exchange chromatography, and then subjected to circular dichroism analysis (CD) and thermo stability studies. It was found that xRic-8 structure contains an approximately 80% of alpha helix domains, with a Tm of 43°C, which is almost identical to the Tm value reported for α-importin, a protein composed also of 10 armadillo repeats. Importantly, armadillo proteins are known to function as scaffold proteins, capable of interacting with multiple partners and hence participating in the regulation of diverse cellular functions. A human protein–protein interaction network was recently annotated and used to

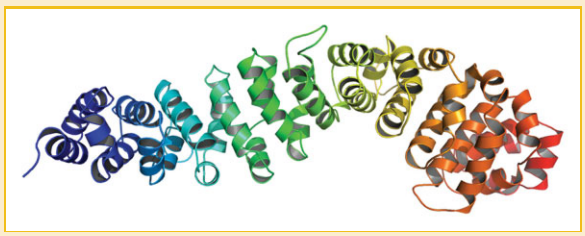

Fig. 5. Proposed 3D model for the Ric-8 protein. Blue ribbons represent the amino- and red ribbons the carboxilic-terminal. Adapted from Figueroa et al. [2009].

search for interactions that link uncharacterized gene products. In this in silico study, four putative novel Ric-8 interacting proteins were identified: MAPK8IP3, TERF1, TUBB, and ZNF585B [Stelzl et al., 2005]. Interestingly, two of these new potential partners are proteins involved in cell focal adhesion and actin cytoskeleton organization. We believe that our proposed structural model of Ric-8 opens a possibility for exploring molecular mechanisms in which the interaction with these new as well as other relevant protein partners can be addressed. Assessing these protein–protein interactions between Ric-8 and partner proteins may also allow to model and experimentally address the role of specific post-translation modifications (e.g., phosphorylation) in cell function.

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