The Nucleotide Exchange Factor Ric-8A Is a Chaperone for the Conformationally Dynamic Nucleotide-Free State of Ga1

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Abstract

Heterotrimeric G protein α subunits are activated upon exchange of GDP for GTP at the nucleotide binding site of Gα, catalyzed by guanine nucleotide exchange factors (GEFs). In addition to transmembrane G protein-coupled receptors (GPCRs), which act on G protein heterotrimers, members of the family cytosolic proteins typified by mammalian Ric-8A are GEFs for Gi/q/12/13-class Gα subunits. Ric-8A binds to Gα-GDP, resulting in the release of GDP. The Ric-8A complex with nucleotide-free Gα1 is stable, but dissociates upon binding of GTP to Gα1. To gain insight into the mechanism of Ric-8A-catalyzed GDP release from Gα1, experiments were conducted to characterize the physical state of nucleotide-free Gα1 (hereafter referred to as Gα1*) in solution, both as a monomeric species, and in the complex with Ric-8A. We found that Ric-8A-bound, nucleotide-free Gα1* is more accessible to trypsinolysis than Gα1+GDP, but less so than Gα1[ ]. The TROSY-HSQC spectrum of [15N]Gα1* bound to Ric-8A shows considerable loss of peak intensity relative to that of [15N]Gα1+GDP. Hydrogen-deuterium exchange in Gα1* bound to Ric-8A is 1.5-fold more extensive than in Gα1+GDP. Differential scanning calorimetry shows that both Ric-8A and Gα1+GDP undergo cooperative, irreversible unfolding transitions at 47°C and 52°C, respectively, while nucleotide-free Gα1* shows a broad, weak transition near 35°C. The unfolding transition for Ric-8A:Gα1* is complex, with a broad transition that peaks at 50°C, suggesting that both Ric-8A and Gα1* are stabilized within the complex, relative to their respective free states. The C-terminus of Gα1 is shown to be a critical binding element for Ric-8A, as is also the case for GPCRs, suggesting that the two types of GEF might promote nucleotide exchange by similar mechanisms, by acting as chaperones for the unstable and dynamic nucleotide-free state of Gα.

Introduction

As members of the Ras superfamily of regulatory GTP binding proteins, heterotrimeric G protein α subunits (Gα) undergo cycles of activation and deactivation driven by binding and hydrolysis of GTP [1]. Conversion to the basal, inactive state results from the intrinsic GTP hydrolyase activity of the G protein. Reactivation is achieved by replacement of GDP by GTP at the nucleotide binding site, catalyzed by guanine nucleotide exchange factors (GEFs). Although the structural events that accompany GEF-catalyzed nucleotide exchange on small, Ras-like G proteins are relatively well understood [2], the mechanism of heterotrimeric G protein activation remains enigmatic. Agonist-activated, transmembrane G protein-coupled receptors (GPCRs) [3] are the best characterized heterotrimeric G protein GEFs. GPCRs act on plasma membrane-localized G protein heterotrimers that consist of GDP-bound Gα tightly associated with heterodimers of Gβ and Gγ subunits. Recently, members of a family of predominantly cytosolic proteins, typified by mammalian Ric-8A, were identified as non-receptor GEFs that catalyze nucleotide exchange directly on Gα subunits of the Gi/o/q/12/13 families [4]. Across phylogeny, Ric-8A paralogs act in GPCR-independent pathways to orient mitotic spindles in asymmetric cell division, as demonstrated in C. elegans [5,6], Drosophila [7], and mammalian cells [8].

Ric-8A is a soluble 59.7 kDa protein predicted to adopt a superhelical structure composed of α-helical armadillo repeats [9]. In contrast to GPCRs, Ric-8A catalyzes the release of GDP directly on Gα subunits, but has markedly weak affinity for Gα bound to GTP or non-hydrolyzable GTP analogs [4]. Upon binding to Gα1+GDP, Ric-8A catalyzes GDP release and forms a stable nucleotide-free Ric-8A-Gα1 [ ] complex (empty brackets: Gα1[ ]).
"[ ]", denote absence of bound nucleotide). In the presence of GTP, the complex dissociates to yield free Ric-8A and Gz11-GTP [4].

Using limited proteolysis, circular dichroism (CD) spectroscopy, heteronuclear NMR spectroscopy, hydrogen-deuterium exchange mass spectrometry (HD-MS), and differential scanning calorimetry (DSC), we have found that Ric-8A stabilizes Gz11 in a conformationally dynamic and heterogeneous state which we propose, facilitates GDP release and subsequent GTP binding. We show that the C-terminus of Gz11 is a critical binding element for Ric-8A recognition and activity, as is also the case for GPCRs [10,11], suggesting that the two GEFs may act by convergent mechanisms.

**Results**

**The smallest fragment of Ric-8A with full GEF activity encompasses most of the protein**

We conducted limited trypsin proteolysis, together with mass spectrometric and secondary structural analysis, to define a minimal fragment of Ric-8A that retained the activity of the full-length protein (Fig. 1A–C). The fragment encompassing residues 1–492 (Ric-8AΔC492) exceeded full-length Ric-8A in GEF activity, while C-terminal truncations of successive predicted helical regions (ΔC426, ΔC453) or truncation of the N-terminus (ΔN12, ΔN38) in the background of ΔC492 retained GDP release activity that was uncoupled from GTP/S binding stimulatory activity (Fig. 1D,E). Truncated proteins (ΔC402, ΔC374) bound Gz11-GDP weakly (data not shown) but had no nucleotide release or GEF activity. Because it is both more abundantly expressed in *Escherichia coli* and appears to biochemically more stable as well as more active than the full-length protein, we chose to conduct subsequent experiments with Ric-8AΔC492. To enhance the sensitivity of tryptophan fluorescence assays of GEF activity, we used a non-musylated Gz11 mutant in which Trp 258 was substituted with alanine. The W258A mutation did not impair GTP binding, GTPase activity, or susceptibility to the GEF activity of Ric-8A (Fig. 2) [12]. For brevity, we refer to ΔC492Ric-8A and W258AGz11 as Ric-8A and Gz11, respectively.

**Relative to Gz11-GDP, nucleotide-free Gz11 is more accessible to protease digestion, and deficient in secondary structure**

Trypsinolysis experiments demonstrated that Gz11-[ ] was substantially more protease-sensitive than Gz11-GDP (Figures 3A and 3B) and was more rapidly degraded into <20 kDa fragments. The distribution of cleavage products is different in the free and GDP-bound states. Normalized as mean residue elipticity, the CD spectrum of Gz11-[ ] showed an overall reduction of regular secondary structure relative to Gz11-GDP (Figure 4). These results accord with earlier findings that Gz11-[ ] is converted into a misfolded species with low affinity for guanine nucleotides [13]. Gz11-[ ] bound to Ric-8A was more resistant to trypsinolysis than free Gz11-[ ], as indicated by the persistence of fragments labeled 2 though 4 at the 10 minute time point in Figure 3E. Note, for example, that band 1, visible at the 5 minute time point of Gz11-[ ], is degraded after 10 minutes of protease exposure (Figure 3B). The same fragment persisted after 10 minutes in the complex with Ric-8A (Figure 3E, band 3). Fragments 2–4 encompass the N-terminal residues of the Ras domain beyond the P-loop, together with most or all of the helical domain of Gz11 [14]. Nevertheless, Ric-8A-bound Gz11-[ ] was still more susceptible to proteolysis than Gz11-GDP (Figure 3A). In contrast, if bound to Gz11, Ric-8A was more sensitive to protease digestion than free Ric-8A (Figures 3D and 3E). After 10 minutes of protease digestion of Ric-8A-Gz11-[ ], all Ric-8A fragments with molecular weights greater than ~24 kDa were degraded, yet several fragments of greater length remained intact after free Ric-8A was exposed to trypsin for the same duration. For both free and Gz11-bound Ric-8A, residues 141–340 appears to constitute a relatively protease-resistant protein core (bands 5 and 1 in Figure 3D and 3E, respectively; for reference to the predicted secondary structure of Ric-8A see Figure 1C). Note that no protease inhibitors were present in the trypsin preparation used to generate the data shown in Figure 3, so the extent of Ric-8A degradation is greater than that shown in Figure 1A.

The mass-normalized CD spectra of Ric-8A and Ric-8A-Gz11-[ ] show similar degrees of secondary structure formation. Therefore, we infer that Ric-8A-bound Gz11-[ ] possesses higher secondary structure content than free Gz11-[ ]. Both spectra are indicative of predominantly α-helical structure, whereas Ric-8A-GDP shows evidence of both α-helical and β-sheet structure, which is characteristic of the Ras-like domain of this and other G proteins [1] (Figure 4). The near absence of β-sheet structure estimated from the CD spectrum of Ric-8A-Gz11-[ ] suggests that changes in secondary structure may occur in the α/β Ras-like domain of Gz11 upon binding to Ric-8A and subsequent release of GDP.

**Peaks in the 15N-H HSQC spectrum of Gz11 are severely attenuated upon binding to Ric-8A**

To elucidate the biochemical properties of Gz11 bound either to nucleotides or to Ric-8A, we acquired 1H-15N Transverse Relaxation Optimized (TROSY) Heteronuclear Single Quantum Coherence (HSQC) spectra [15] of [15N]Gz11. The 1H-15N TROSY-HSQC spectrum of Gz11-GDP (Figure 5A) and Gz11-GTP/S (data not shown) showed ~300 moderately well resolved and dispersed peaks, comparable in quality to spectra reported by Abdulaev, *et al.* [16] for a GDP-bound chimera (Gz11i of transducin α (Gz11i) and Gz11i). In contrast, the spectrum of [15N]Gz11:Ric-8A showed considerable diminution in the amplitude of many peaks, indicative of extensive line broadening (Figure 5B). Significant changes in chemical shift were not observed upon binding to Ric-8A. Gel filtration of the sample after data collection confirmed that Ric-8A-[15N]Gz11-[ ] remained a homogeneous heterodimer over the time-scale of the NMR experiment, and did not aggregate (data not shown). Peak broadening observed in the spectrum of Ric-8A-[15N]Gz11-[ ] could result from exchange among conformational states of Ric-8A-bound Gz11-[ ] in the intermediate t/μs time scale, and/or from slow tumbling of the 96 kDa complex. Subsequent re-acquisition of the 1H-15N TROSY-HSQC spectrum after addition of GTP/S to dissociate the complex and removal of free Ric-8A, afforded a [15N]Gz11-GTP/S spectrum identical to that of [15N]Gz11-GDP prepared in the absence of Ric-8A (Figure 5C), thus demonstrating that Gz11 bound to Ric-8A in the NMR sample retained biochemical activity.

**The population of rapidly exchanging Gz11 protons doubles upon Ric-8A binding and release of GDP**

To test the hypothesis that Ric-8A-bound Gz11-[ ] adopts a state of high conformational flexibility, we conducted HD-MS exchange experiments [17] to directly assess changes in structural dynamics of Gz11 upon formation of the Ric-8A-Gz11-[ ] complex. Hydrogen-deuterium exchange in either Gz11-GDP or Ric-8A-Gz11-[ ] was initiated by rapid dilution of the proteins into D2O buffer. To determine the rate and extent of HD exchange,
Figure 1. GEF activity of purified Ric-8A fragments defined by limited trypsinolysis and secondary structure analysis. (A) Coomassie-stained SDS PAGE analysis of Ric-8A after trypsinization for the times indicated below each lane; unique fragments are identified by colored asterisks. (B) Electrospray mass spectrometric analysis of Ric-8A tryptic digest fragments extracted from the SDS PAGE gel shown in panel A; peaks identified by asterisks refer to corresponding bands shown in panel A. Fragment masses (Da) are indicated at each peak position. (C) Amino acid sequence of rat Ric-8A; cylinders indicate helical segments predicted using JPRED [51]. Residue codes colored red indicate sites of proteolytic cleavage (see panel A). Residue codes in green indicate N or C-termini of recombinant Ric-8A fragments engineered to coincide approximately with proteolytic sites or predicted secondary structure boundaries: ΔC492 denotes the Ric-8A fragment comprising residues 1–492. Both N-terminal truncations ΔN12 and ΔC530 were made to facilitate expression of recombinant Ric-8A.
Ric-8A and Ric-8A truncation mutants (200 nM). Error bars represent ± determined in three replicates. doi:10.1371/journal.pone.0023197.g001

The exchange reaction was quenched with formic acid/acetonitrile at successive time intervals, and the products analyzed by electrospray mass spectrometry (ES-MS). For each time-point, the mass distribution of Gα1 was determined by deconvolution of the raw m/z spectrum (Figure 6A,B). The mass distribution of Gα1⋅GDP remained unimodal throughout the 60 minute exchange period (Figure 6A), whereas that for Gα1 alone to Ric-8A evolved into a multimodal distribution, suggestive of conformational heterogeneity (Figure 6B). Analysis of these data revealed a nearly four-fold greater initial rate of deuterium exchange in Ric-8A-bound Gα1 than in Gα1⋅GDP (Figure 6C). After 60 minutes of exposure to D2O, the mass of Gα1 alone in the complex with Ric-8A increased by ~340 Da, accounting for more than half of all the exchangeable Gα1 protons, versus a ~210 Da mass increase in Gα1⋅GDP alone. The enhanced rate and extent of deuterium substitution is indicative of greater solvent accessibility at exchangeable sites in Ric-8A-bound Gα1 than in Gα1⋅GDP, most likely due to amplified breathing motions in the Ric-8A⋅Gα1 complex [18].

Intrinsic and Ric-8A-catalyzed GTP/βS binding rates of the Gα1 proteins used in this study. Intrinsic and Ric-8A-catalyzed kinetics of binding of GTP/βS to wild-type Gα1, W258A-Gα1, N252G-Gα1, Gα1Cα9 and Gα1-G552C were measured using a fluorescence binding assay [12,47]. 400 μl of protein (1 μM) in the GDP bound form was preincubated for 10–15 min at 25°C in a cuvette. A 10-fold excess of GTP/βS was added and fluorescence at 340 nm upon excitation at 290 nm was monitored in the absence (open bars) or presence (filled bars) of Ric-8A (1 μM). Error bars represent ± one standard deviation apparent first-order rate constants determined in three replicates. doi:10.1371/journal.pone.0023197.g002

Figure 2. Intrinsic and Ric-8A-catalyzed GTP/βS binding rates of the Gα1 proteins used in this study. Intrinsic and Ric-8A-catalyzed kinetics of binding of GTP/βS to wild-type Gα1, W258A-Gα1, N252G-Gα1, Gα1Cα9 and Gα1-G552C were measured using a fluorescence binding assay [12,47]. 400 μl of protein (1 μM) in the GDP bound form was preincubated for 10–15 min at 25°C in a cuvette. A 10-fold excess of GTP/βS was added and fluorescence at 340 nm upon excitation at 290 nm was monitored in the absence (open bars) or presence (filled bars) of Ric-8A (1 μM). Error bars represent ± one standard deviation apparent first-order rate constants determined in three replicates. doi:10.1371/journal.pone.0023197.g002

Thermodynamic stability of both nucleotide-free Gα1 and Ric-8A increase upon complex formation

We used differential scanning calorimetry (DSC), by which change in heat capacity (Cp) is measured as a function of

Figure 3. Ric-8A provides limited protection of nucleotide-free Gα1 from trypsin digestion. Samples were incubated with TPCK treated trypsin at a 1:1000 molar ratio (trypsin:sample) at 4°C, withdrawn at the indicated time points, separated by SDS-PAGE and visualized by Coomassie blue staining. (A) Gα1-GDP: lanes from left to right: molecular weight markers, M; untreated Gα1-GDP, U; samples digested for 5 and 10 minutes. Mass spectroscopic analysis identifies band 1 as Gα1 residues 21–179: observed/calculated mass 17,761/17,774 Da. (B) Ric-8A: markers, M; untreated, U; Ric-8A: after 5, 10 and 15 minutes of trypsin digestion. Mass analysis identifies band 1 as Ric-8A residues 1–408: 46,218/46,207 Da; band 2, residues 72–378: 34,815/34,799; band 3, residues 141–348: 23,834/23,804 Da; band 4, residues 62–178: 13,563/13,523 Da. (E) Ric-8A: Gα1[ ] complex: markers, M; untreated Ric-8A-Gα1[ ] complex; R and G indicate bands for intact Ric-8A and Gα1, respectively; Ric-8A-Gα1[ ] complex digested for 10 and 25 minutes. Mass analysis identifies band 1 as Ric-8A residues 141–348: 23,834/23,804 Da (present also as band 3 in Panel D); band 2, Gα1 residues 17–191: 19,646/19,652 Da; band 3, Gα1 residues 21–179: 17,753/17,761 Da (present as band 1 in panel B); band 4: Gα1 residues 10–141: 14,532/14,520 Da. doi:10.1371/journal.pone.0023197.g003

Figure 3. Ric-8A provides limited protection of nucleotide-free Gα1 from trypsin digestion. Samples were incubated with TPCK treated trypsin at a 1:1000 molar ratio (trypsin:sample) at 4°C, withdrawn at the indicated time points, separated by SDS-PAGE and visualized by Coomassie blue staining. (A) Gα1-GDP: lanes from left to right: molecular weight markers, M; untreated Gα1-GDP, U; samples digested for 5 and 10 minutes. Mass spectroscopic analysis identifies band 1 as Gα1 residues 21–179: observed/calculated mass 17,761/17,774 Da. (B) Ric-8A: markers, M; untreated, U; Ric-8A: after 5, 10 and 15 minutes of trypsin digestion. Mass analysis identifies band 1 as Ric-8A residues 1–408: 46,218/46,207 Da; band 2, residues 72–378: 34,815/34,799; band 3, residues 141–348: 23,834/23,804 Da; band 4, residues 62–178: 13,563/13,523 Da. (E) Ric-8A: Gα1[ ] complex: markers, M; untreated Ric-8A-Gα1[ ] complex; R and G indicate bands for intact Ric-8A and Gα1, respectively; Ric-8A-Gα1[ ] complex digested for 10 and 25 minutes. Mass analysis identifies band 1 as Ric-8A residues 141–348: 23,834/23,804 Da (present also as band 3 in Panel D); band 2, Gα1 residues 17–191: 19,646/19,652 Da; band 3, Gα1 residues 21–179: 17,753/17,761 Da (present as band 1 in panel B); band 4: Gα1 residues 10–141: 14,532/14,520 Da. doi:10.1371/journal.pone.0023197.g003
temperature, to determine the modality and mid-point temperatures ($T_m$) for the unfolding transitions of $G_{ai1}$N-GDP, $G_{ai1}[]$, Ric-8A and Ric-8A:$G_{ai1}[]$ [19]. At temperatures below and above the thermal unfolding transition of a protein, the $C_p$ exhibits a linear, typically positive, dependence on the temperature of the native and unfolded states, respectively. In the region of the thermal transition, $C_p$ exceeds that of both the denatured and native states as hydrophobic groups are increasingly exposed to the aqueous solvent, and reaches a maximum value at $T_m$ [20,21,22].

$G_{ai1}$-GDP underwent an irreversible cooperative unfolding transition with $T_m = 52^\circ C$ (Figure 7, blue trace). The irreversible nature of the transition of this and the other proteins and protein complexes reported here precludes accurate determination of the enthalpy of unfolding, but allows comparison of the significant thermal features of the four species when measured at equivalent scan rates. Only a weak transition near $33^\circ C$ was observed for $G_{ai1}[]$ (Figure 7, dashed blue trace), which exhibited changes in $C_p$ that were close to the detection limits of the instrument. The nucleotide-free protein thus appears to be conformationally heterogeneous or disordered [23], consistent with its high protease sensitivity (Figure 3B) and CD spectrum (Figure 4). Ric-8A itself underwent an irreversible cooperative folding transition with $T_m = 47^\circ C$ (Figure 7, black trace). Thermal denaturation of Ric-8A:$G_{ai1}[]$ was characterized by a nearly linear increase in heat capacity, suggestive of non-cooperative unfolding, followed by a discrete cooperative transition at $50^\circ C$ (Figure 7, green trace), higher than the melting temperature of free Ric-8A. This latter $T_m$ is invariant with protein concentration (data not shown), indicating that the complex remains intact throughout the transition. The denaturation profile of Ric-8A:$G_{ai1}[]$ complex cannot be modeled as weighted average of the profiles of Ric-8A and $G_{ai1}[]$ (Figure 7, red dashed line), hence the complex has unique thermodynamic properties relative to $G_{ai1}[]$ and Ric-8A.

The C-terminus of $G_{ai1}$ is a specific and critical recognition element for Ric-8A binding and GEF activity

The mechanism by which Ric-8A catalyzes nucleotide exchange is similar in some respects to the analogous reaction catalyzed by GPCR at G$\beta$y-bound $G_{\alpha}$ subunits. Experimental evidence
indicates that specific recognition and binding of the C-terminus of Ga is crucial to the action of Ric-8A, just as it is for GPCRs [10,11]. First, a yeast two-hybrid screen of a rat brain library using a bait construct comprising Ric-8A residues 1–297 yielded a prey clone expressing the C-terminal 81 residues of Ga, that also interacted with a full-length Ric-8A bait construct (Figure 8A). Second, the peptide GaC18, which is composed of a sequence of amino acid residues identical to that of the 18 C-terminal residues of Ga, inhibited Ric-8A-catalyzed exchange of GTPc for GDP with an IC50 of 23 μM (Figure 8B). Isothermal calorimetric measurements indicated that GaC18 binds directly to Ric-8A with a Kd of 12 μM (Figure 8C). Third, GaCA9, a Ga truncation mutant lacking the nine C-terminal residues of the native protein, fails to serve as a substrate for Ric-8A although it retains GTP binding and hydrolytic activity [24] (Figure 2). Finally, substitution of the C-terminal twelve residues of Ga with the corresponding residues of Gs, a Ga protein that does not bind to Ric-8A [4], abrogated susceptibility to the GEF activity of Ric-8A (Figure 2) but did not impair GTP binding activity. These results are in accord with recent findings that pertussis toxin-

![Figure 6](https://example.com/figure6.png)

**Figure 6. Nucleotide-free Ga bound to Ric-8A exhibits rapid hydrogen/deuterium exchange kinetics relative to the Ga-GDP complex.** (A) Mass distribution for Ga-GDP measured at fixed time points (see panel C) after dilution into D2O; the mass distribution at the zero time point, before exchange was initiated, corresponds to the red peak centered at 40.1 kDa. The average of the Ga mass distribution increases as H/D exchange reaction proceeds. (B) Mass distribution for Ga in complex with Ric-8A measured at fixed time points after dilution into D2O; note that the Ga mass distribution becomes multimodal as the H/D exchange reaction proceeds. (C) The increase in mass (Da), determined at the centroid of the mass distribution of Ga-GDP (black squares) and Ga derived from the complex with Ric-8A (red circles) is plotted as a function of time after rapid dilution from aqueous buffer into D2O. doi:10.1371/journal.pone.0023197.g006

![Figure 7](https://example.com/figure7.png)

**Figure 7. Thermal denaturation properties of Ga and Ric-8A are affected by their mutual interaction.** Temperature-dependence of heat capacity was measured by differential scanning calorimetry. Buffer baseline-corrected thermograms were recorded for Ric-8A (black trace), Ga (dashed blue trace), Ga-GDP (blue trace) and Ric-8A-Ga (green trace). The weighted average of the thermograms for Ric-8A and Ga (red dashed line) overlaps that of Ga in the temperature range below −37°C and that of Ric-8A above that temperature, and is distinct from the thermogram of Ric-8A-Ga. The inset shows a magnified view of the four thermograms and the weighted average function in the 20°C–45°C range. doi:10.1371/journal.pone.0023197.g007
catalyzed ADP ribosylation at the C-terminus of Gαi1 [8] and that truncation of the twelve Gαi1 C-terminal residues [25] blocks Ric-8A binding and GEF activity. On the other hand, truncation of 25 residues from the N-terminus of Gαi1 did not affect its susceptibility to the GEF activity of Ric-8A (Figure 2).

Discussion

The experiments described in this report provide insight into the mechanism of Ric-8A-catalyzed exchange of GDP for GTP on Gαi1. In this reaction, Ric-8A:Gαi1[ ] is a stable intermediate that does not readily dissociate in the absence of GTP or non-hydrolysable GTP analogs. We have shown that, within this complex, Gαi1[ ] adopts a considerably more dynamic conformation than nucleotide-bound Gαi1, but is more structured and less susceptible to proteolysis than free Gαi1[ ]. This is in sharp contrast to most nucleotide-free complexes of small G proteins with cognate GEFs, in which both the GEF and G protein components are typically well ordered structures [2].

Ric-8A-catalyzed nucleotide exchange proceeds through a stable (in the absence of GTP) but loosely structured intermediate. Examples of enzymes that stabilize proteins in unfolded or disordered states include chaperones such as GroEL [26], and AAA+ ATPase unfoldases of ClpXP proteases and related proteases that degrade mis-folded proteins [27]. However, Ric-8A functions differently from these, in that it is not coupled to an exergonic reaction (e.g. ATPase activity), but does exhibit high substrate specificity and catalyzes a discrete chemical transformation. We propose that the catalytic power of Ric-8A derives, in part, from its ability to act (in rough analogy with GroEL and other unfoldases) as a chaperone for a partially unfolded or disordered conformation Gαi1[ ], thereby reducing the activation energy barrier to GDP release and GTP binding, while disfavoring unproductive side reactions that would lead to Gαi1 deactivation and aggregation. In the partially unstructured state induced and stabilized by Ric-8A, the nucleotide binding site of Gαi1 may be more solvent-accessible than in the nucleotide-bound state.

The mechanism by which Ric-8A catalyzes nucleotide exchange may be similar in some respects to the analogous reaction catalyzed by GPCR at Gβγ-bound Gα subunits. Recognition and
binding of the Gz C-terminus is crucial to the action of both exchange factors. In a manner analogous to that proposed for GPCRs, Ric-8A could promote nucleotide release by gripping, and perhaps tensioning, the C-terminus of Gz and thereby weaken local tertiary structure (85 helix, β5 and β6 strands) that is allosterically coupled to the purine binding site and switch regions [11,20,29]. Indeed, the substantial reduction in fluorescence emission of Trp 211 in switch II of Gz1 upon binding to Ric-8A provides evidence for such perturbations [12]. Whether Ric-8A directly engages the switch regions of Gz is uncertain. Such interactions might be precluded since Ric-8A is able to form a transient ternary complex with Gz1i1GDP:AGS3 [12], AGS3, a guanine nucleotide dissociation inhibitor, comprises GPR/GoLoco motifs [30,31] that partially block the switch I/switch II interface [32]. Similarly, direct interactions between GPCRs and the switch regions of Gz are problematic on stereochemical grounds [33]. It is also noteworthy in this context that importin-β, a protein involved in the transport of protein cargo into the nucleus, and also a presumptive structural analog of Ric-8A, has been shown to act as a GEF for Ran1-GDP [34]. Crystallographic analysis demonstrates that importin-β induces conformational changes in the switch regions of Ran1-GDP [35].

Both GPCRs and Ric-8A appear to induce or maintain a dynamic state of Gz. NMR studies of complexes between [15N]Gz1i1GDP and rhodopsin mimetics show that resonances in the Gz subunit are highly broadened [36], as we have observed for Ric-8A: Gz1i1 [29]. In contrast, the HSQC spectrum of [15N]Gz1i1 bound to Gz1i1GDP, a complex with a molecular mass comparable to Ric-8A: [15N]Gz1i1, is well defined and similar to that of Gz1i1GDP [37]. Recent evidence obtained from double electron electron resonance (DEER) spectroscopy shows that, in the activated rhodopsin-heterotrimer complex, nucleotide-free Gz1i1 is conformationally heterogeneous, and that the Ras-like and helical domains of Gz1i1, between which nucleotide is bound, swing away from each other [38]. The NMR and HD-MS data presented here suggest that nucleotide-free Gz1i1 bound to Ric-8A undergoes conformational exchange, with interconversion times that are possibly in the μs-range. The DSC melting profile of Ric-8A:Gz1i1 is suggestive of a non-cooperative unfolding transition at lower temperature followed by a discrete transition near 50°C. It seems reasonable to attribute the former to a conformationally heterogeneous and dynamic Gz1i1 and the latter to Ric-8A, which in the complex with Gz1i1 is more thermostable yet also more protease accessible than unbound Ric-8A, suggesting that Ric-8A itself may undergo some structural change upon binding to Gz1i1. However, structural assignment of transitions in the DSC spectra is speculative. It remains to be determined which segments of Gz1i1 become mobile within the nucleotide-free complex with Ric-8A, and importantly, to confirm that induction or stabilization of a partially disordered or conformationally flexible state in Gz1i1 in fact reduces the kinetic energy barrier to GDP release and GTP binding.

Materials and Methods

Molecular Cloning and Protein Expression

The open reading frame of rat Ric-8A and truncation variants (comprising residues 1–492, 1–492, 1–453, 1–426, 1–402, 1–374, 12–492 and 38–492) were amplified by PCR and subcloned into the pET-28a vector for expression as N-terminally hexa-histidine tagged proteins. Proteins were expressed in Escherichia coli BL21 (DE3)-RIPL cells in LB media containing ampicillin (120 mg/L) and induced with 300 μM isopropyl β-D-thiogalactopyranoside (IPTG) at 20°C. After overnight growth at 20°C, cells were lysed by sonication at 20°C in lysis buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 2 mM DT, and 2 mM PMSF. The cell lysate was clarified by centrifugation and loaded onto a column containing 5 ml of nickel NTA-agarose (GE Healthcare). After extensive washing with lysis buffer, proteins were eluted from the resin with buffer (30 mM Tris, pH 8.0, 150 mM NaCl and 2 mM PMSF) containing 250 mM imidazole and dialyzed in a low ionic strength buffer (50 mM Tris, pH 8.0, 2 mM DT, and 2 mM PMSF). The dialyse was loaded onto an UNO-Q matrix (Bio-Rad) and eluted with a 0–500 mM NaCl gradient on an AKTA FPLC system (GE Healthcare). Pure Ric-8AΔC492 eluted from the matrix at 165–175 mM NaCl.

Rat Gz1i1 was expressed as a tobacco etch virus protease (TEV)-cleavable, N-terminal glutathione-S-transferase (GST) fusion protein as described [12]. W258A-Gz1i1, in which the tryptophan residue at position 258 is substituted by alanine, was generated by use of the QuickChange (Stratagene) kit according to the manufacturer’s protocol, using the pDEST-15 vector harboring wild type GST-Gz1i1 as a template. To generate NA25-Gz1i1, from which the N-terminal 25 residues of the native protein are deleted, attB-modified primers corresponding to amino acids 25 to 35 and 343 to 353 of Gz1i1 were used for PCR amplification and cloning of the fragment into the pDEST15 vector. W258A-Gz1i1 and NA25-Gz1i1 were expressed and purified as described [12].

The plasmid pBN905, which expresses rat Gz1i1AC9, lacking the C-terminal nine residues of the native protein, fused in-frame to intein-CBD cDNA in the pTxB3 expression vector (New England Biolabs), was a kind gift from Dr. T.J. Baranski, Washington University, St. Louis, MO. Gz1i1AC9 was expressed and purified as described [39]. With the exception of experiments summarized in Figure 1, 2 and 8A, all other experiments were performed with Ric-8AΔC492 and W258A-Gz1i1, which we henceforth refer to as Ric-8A and Gz1i1, respectively.

Nucleotide-free Gz1i1 proteins were prepared by the method of Ferguson and Higashijima [40], using exchange and dialysis buffers composed of 50 mM Tris.HCl, pH 8.1, 2 mM Tris [2-carboxyethyl]phosphene (TCEP) with 20% glycerol (v/v) and 150 mM NaCl. GTPγS-bound Gz1i1 was prepared as described [41].

Preparation of [15N]-labeled proteins

[15N]Gz1i1 was prepared as described with minor modifications [42]. Briefly, transformed E.coli cells were grown in minimal media supplemented with [15N]NH4Cl (Cambridge Isotopes, 99.8% purity) and [15N]Bioexpress Cell Growth media (10 ml of 10x concentrate/liter of media) (Cambridge Isotope Labs), induced with 500 μM IPTG at 20°C and allowed to express Gz1i1 overnight at the same temperature. The purification protocol was identical to that used for native proteins and the yields were approximately one third lower.

Preparation of Ric-8A:Gz1i1 complexes

Nucleotide-free Ric-8A-Gz1i1 and Ric-8A(r1)[15N]Gz1i1 complexes were generated by incubating equimolar concentrations of Ric-8A (500 μl of 150 μM protein) with Gz1i1-GDP or [15N]Gz1i1 (500 μl of 150 μM protein) overnight in sample buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM DT, and 5 mM EDTA) containing 50 μl of immobilized alkaline phosphatase (Sigma) to hydrolyze released nucleotide, and gently rocked at 4°C. The immobilized alkaline phosphatase was removed by centrifugation, and complex was gel-filtered over tandem Superdex 200/75 gel filtration columns pre-equilibrated in 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM DT and eluted at a flow rate of 0.4 ml/min using an AKTA FPLC (GE Healthcare).
Trp-8A Binds Dynamic Nucleotide-Free Gz1

Yeast two hybrid experiments

A rat brain yeast two-hybrid prey library [43] was screened with a pVJL1 [44] bait construct encoding the amino-terminal 297 amino acids of Ric-8A (1–297) in the L40 yeast strain [45] with a prey clone consisting of the carboxyl-terminal 81 amino acids of Gz1 (Gz1 C-term). Full-length Gz1 and Gαq, and the Gz1 C-term preys, in pGADGH (Clonetech) were then tested pair-wise continuously flushed with a nitrogen flow throughout the course of the experiment. Secondary structure analysis was performed using K2D2 [48].

NMR spectroscopy

Protein samples for NMR spectroscopy ([15N]Gz1-GDP, [15N]Gz1-Ric-8A or [15N]Gz1-GTPγS) were dialyzed against 20 mM sodium phosphate, pH 6.8, 75 mM NaCl, and 2 mM DTT in 10% 2H2O/90% H2O, and concentrated to 250 mM. The [15N]Gz1-GTPγS sample was prepared from the [15N]Gz1-Ric-8A complex by addition of five molar excess of GTPγS and incubation for 10 minutes at 25°C. Free Ric-8A, and non-dissociated [15N]Gz1:Ric-8A complex were then removed with Na2 IMAC (BioRad). 1H-15N TROSY-HSQC spectra [49] were acquired at 25°C on a 600 MHz Varian NMR System equipped with a salt-tolerant cold probe and processed with Felix 2004 (Felix NMR, Inc.).

Hydrogen-Deuterium Exchange Mass Spectrometry

Hydrogen-deuterium exchange of the Gz1-GDP or Ric-8A:Gz1 [41] was analyzed by automated reverse-phase HPLC coupled to electrospray ionization TOF mass spectrometry. The HPLC consisted of an Agilent 1100 HPLC with a G1377a autosampler, and the ESI-TOF was a Bruker microTOF. Following initiation of the reaction by ten-fold dilution of protein stock (1 mg/ml Gz1-GDP or Ric-8A:Gz1 [41], in 20 mM sodium phosphate, pH 6.8, 100 mM NaCl and 2 mM DTT) into D2O, the reaction mixture was pipetted into a sealed autosampler vial and the autosampler was used to draw aliquots at regular time intervals. Quenching of the exchange reaction was achieved by rapid binding of the protein onto a C4 reverse phase cartridge from Michrom Bioreources (5x1 mm) and subsequent washing and elution. The column and autosampler were pre-equilibrated with 20% (v/v) acetonitrile, 80% H2O and 0.1% formic acid (w/v), pH 2.2, prior to sample loading. Immediately following sample (0.5 μl) injection, the solvent composition was changed to 100% acetonitrile, 0.1% formic acid. By using a rapid step gradient and
very high flow rates of 600 μl/min, the sample was minimally delayed in the flow path to the mass spectrometer, eluting at approximately 0.4 minutes. The column system was equilibrated at 4°C to minimize back-exchange. Data processing was performed with the Bruker Data Analysis software package, version 4.0. The Maximum Entropy deconvolution routine was used to perform charge-deconvolution for the spectral range of 700 m/z to 1400 m/z, which encompassed the majority of the observed distribution of protein signal. The deconvoluted spectra were exported to ORIGIN software and the centroid masses for Gzii were calculated and plotted as a function of time.

Differential Scanning Calorimetry (DSC)

For DSC analysis, Gzii-GDP, Ric-8A and Ric-8A-Gzii[1] were dialyzed against degassed DSC sample buffer: 25 mM PIPES pH 7.2, 150 mM NaCl and 1 mM TCEP, and additionally for the Gzii-GDP sample, 20 μM GDP. DSC buffer for Gzii[1] contained 20% glycerol (v/v). Immediately before DSC analysis, protein samples were clarified by centrifugation at 14,000 RPM for 10 min in a bench-top Eppendorf microfuge. Protein concentrations after dilution, if required, were determined by least squares fitting of predicted protein extinction coefficients to spectra in the 220–420 nm range measured on a HP diode array instrument. The measured values were 3.6 μM for Gzii-GDP, 5.9 μM for Ric-8A, 5.1 μM for Ric-8A-Gzii[1] and 7.9 μM for Gzii[1]. DSC measurements were conducted using a Microlcal capDSC with autosampler (MicroCal, GE Healthcare). After establishing a thermal history by running water vs. water scans, three buffer against water scans were conducted for each sample using the corresponding dialyse solution to obtain the buffer Cp over the experimental temperature range. Following this, two buffer vs protein scans were performed. Protein samples were rescanned once to check for thermal reversibility.

A typical thermal cycle involved cooling the instrument to 20°C after which a 10 min. thermal equilibration was initiated.

Data analysis was performed using Origin 7.0 by first subtracting the last buffer scan from the protein thermal scan. After normalizing the data to the protein concentration, a progressive baseline estimation was performed by calculating the fractional contribution of the native and denatured state to the sample Cp at each point beneath the excess heat capacity function, thus producing a smoothly varying function of temperature [50]. Data presented in Figure 7 were corrected by subtraction of the temperature-dependent change in Cp of the buffer. A weighted average thermal profile for Gzii[1] and Ric-8A was computed using the expression

\[ \frac{C_p}{C_p}^\text{excess}(T) = \frac{w_{\text{Ric-8A}}^\text{Cp} \text{Ric-8A}(T)}{w_{\text{Gzii}}^\text{Cp} \text{Gzii}(T)} \]

where \( w_{\text{Ric-8A}} \) and \( w_{\text{Gzii}} \) are weighting factors for Ric-8A and Gzii[1] contributions to the heat capacity at temperature T, and \( C_p^\text{Ric-8A} \) and \( C_p^\text{Gzii} \) are the heat capacities measured for free Ric-8A and Gzii[1] at that temperature.

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Author Contributions

Conceived and designed the experiments: SRS CJT JPS BB KB JH GGT. Performed the experiments: CJT JPS KB NM JH GGT. Analyzed the data: SRS KB CJT JPS JH GGT. Contributed reagents/materials/analysis tools: KB JPS BB. Wrote the paper: SRS CJT JPS JH.

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