Self-activating G protein α subunits engage seven-transmembrane regulator of G protein signaling (RGS) proteins and a Rho guanine nucleotide exchange factor effector in the amoeba *Naegleria fowleri*

The free-living amoeba *Naegleria fowleri* is a causative agent of primary amoebic meningoencephalitis and is highly resistant to current therapies, resulting in mortality rates >97%. As many therapeutics target G protein–centered signal transduction pathways, further understanding the functional significance of G protein signaling within *N. fowleri* should aid future drug discovery against this pathogen.

We report that the *N. fowleri* genome encodes numerous transcribed G protein signaling components, including G protein–coupled receptors, heterotrimeric G protein subunits, regulator of G protein signaling (RGS) proteins, and candidate Gα effector proteins. We found *N. fowleri* Gα subunits have diverse nucleotide cycling kinetics; Nf Gα5 and Gα7 exhibit more rapid nucleotide exchange than GTP hydrolysis (i.e., “self-activating” behavior). A crystal structure of Nf Gα7 highlights the stability of its nucleotide-free state, consistent with its rapid nucleotide exchange. Variations in the phosphate binding loop also contribute to nucleotide cycling differences among Gα subunits. Similar to plant G protein signaling pathways, *N. fowleri* Gα subunits selectively engage members of a large seven-transmembrane RGS protein family, resulting in acceleration of GTP hydrolysis.

The free-living amoeba *Naegleria fowleri* is the causative agent of primary amoebic meningoencephalitis, a rare infection with mortality rates >97% in the United States (cdc.gov, (1, 2)). Human infection is established by intranasal exposure, typically during swimming in warm freshwater bodies, although ritual nasal cleansing (ablation) and the use of medical sinonasal rinsing devices have also been implicated (5, 6). *N. fowleri* trophozoites access the cranial cavity by tracking along olfactory neurons and crossing the cribriform plate (1, 7). The amoebae incite a robust and destructive neutrophilic inflammatory response in the meninges and brain, in contrast to the type IV hypersensitivity response elicited in the brain by other free living amoebae such as *Balamuthia* or *Acanthamoeba* (8). The resulting devastating brain injury is thought to result primarily from an amplified immune response, rather than direct toxicity or phagocytosis by the parasite, as implied by the misnomer “brain-eating amoeba” (8).

Primary amoebic meningoencephalitis progresses rapidly, leading almost invariably to death within ~5 days (9). Symptoms of *N. fowleri* infection may mimic the more common etiologies of meningitis (bacterial and viral), complicating diagnosis and potentially delaying therapy (9, 10). A review of confirmed *N. fowleri* cases revealed a myriad of treatment approaches including combinations of antifungal and antiparasitic drugs that unfortunately lack significant impact on survival (<3% in the US) (2). Investigation of potential therapeutic targets is therefore critically needed for this rare but nearly universally fatal disease.

G protein signaling pathway modulators comprise approximately one-fourth of all currently FDA-approved drugs, with the most frequent targets being the seven-transmembrane G protein–coupled receptors (GPCRs) at the top of the pathway (11). GPCRs are specifically activated by a wide variety of extracellular cues such as hormones, neurotransmitters, chemokines, and photons and activate cytoplasmic heterotrimeric G proteins composed of Gα, Gβ, and Gγ subunits (12). Receptor-catalyzed exchange of GDP for GTP on the Gα subunit induces a conformational change dominated by three mobile switch regions (13). The activated Gα-GTP separates from the Gβγ heterodimer, both of which engage effector proteins and promote second messenger signaling (12). Signaling is terminated by GTP hydrolysis on the Gα subunit, a reaction accelerated by regulators of G protein signaling (RGS proteins)
and leading to the re-formation of the $\alpha\beta\gamma$ heterotrimer (14, 15). Canonical RGS proteins serve as GTPase-accelerating proteins (GAPs) by stabilizing the switch regions of GTP-bound $\alpha$ subunits in the transition state (16). Within the animal kingdom, $\alpha$ subunits can be classified into four subfamilies: $\alpha_1$, and $\alpha_{12/13}$ subfamilies stimulate and inhibit adenyl cyclase, respectively; $\alpha_0$ family members engage phospholipase C$\beta$ isoforms; and the $\alpha_{12/13}$ subfamily activates a family of Rho GTPase guanine nucleotide exchange factors (GEFs) containing RGS-like domains (RGS-RhoGEFs) (17–19). In contrast to the animal kingdom, plant, fungal, and protozoan $\alpha$ subunits exhibit greater sequence divergence and, correspondingly, diverse interactions with signaling partners (20). For instance, Saccharomyces cerevisiae GPA1 and Arabidopsis thaliana GPA1 do not engage homologs to mammalian $\alpha$ subunit effectors; instead, the G$\beta$Y dimer is thought to play a dominant role promoting downstream signaling in fungi and plants (21, 22).

An additional important difference of many plant $\alpha$ subunits such as A. thaliana GPA1 (23, 24) and some protozoan $\alpha$ subunits like those of Trichomonas vaginalis (25) is relatively rapid nucleotide exchange activity in the absence of receptor inhibition. In these $\alpha$ subunits, GTP hydrolysis rather than nucleotide exchange is the rate-limiting step of the nucleotide cycle, allowing accumulation of the activated $\alpha$-GTP species in the cytoplasm, independent of a GPCR or other GEF, referred to elsewhere as “self-activation” (24, 26). Plant $\alpha$ subunits are known to engage seven-transmembrane GRS proteins (7TM GRS) that accelerate the rate-limiting GTP hydrolysis step, likely with modulation by extracellular cues, as exemplified by the glucose-responsive A. thaliana 7TM GRS protein AtRGS1 (23, 24). Other protozoan $\alpha$ subunits exhibit the typical nucleotide cycle pattern of rate-limiting nucleotide exchange and thus presumably require activation by a GEF such as a GPCR (27). One such $\alpha$ subunit from the enteric pathogen Entamoeba histolytica, EhG$\alpha$1, engages the G$\alpha$ effector EhRGS-RhoGEF, leading to Rho family GTPase activation and modulation of pathogenic behaviors such as migration, extracellular matrix invasion, and host cell killing (28, 29). A more extensive array of G protein signaling components, including cyclic AMP receptors typified by cAR1, are utilized in the slime mold Dictostelium discoideum for processes such as chemotaxis, development, and quorum-sensing (30, 31).

The availability of N. fowleri genome sequences (32, 33) along with publicly available RNAseq transcriptome data has provided opportunities for the identification and validation of potential therapeutic targets. Of note, there is substantial divergence at the genome sequence level from the nonpathogenic-related species Naegleria gruberi (32, 34). In the present study, we identify and characterize G protein signaling components encoded by the N. fowleri genome that may be amenable to future pharmacological manipulation.

**Results**

**Putative G protein signaling components encoded by the N. fowleri genome**

Heterotrimeric G protein subunits, their nucleotide cycle regulators, and candidate $\alpha$ effector proteins were identified by bioinformatic interrogation of the N. fowleri genome (32) using hidden Markov models. Thirteen putative $\alpha$, two G$\beta$, and one G$\gamma$ subunits were identified (Fig. 1), and the majority are apparently expressed in trophozoites (Table S1) as evidenced by publicly available RNAseq data (32, 35). A single expressed gene with low homology (29% identity) to D. discoideum cyclic AMP receptor-like proteins (36) was identified as a candidate GPCR (AmoebaDB accession NF0059410). Other predicted seven-transmembrane proteins with some features of GPCRs, despite no significant sequence similarity to known receptors, were identified within the N. fowleri proteome using 7TMRmine (data not shown) (37). The presence of transcripted arrestin-like genes supports the hypothesis of at least one functional GPCR protein in N. fowleri, given that the encoded arrestin-like proteins are predicted to have roles in GPCR desensitization, internalization, and recycling (38, 39). N. fowleri also expresses a relatively large family of 28 putative seven-transmembrane proteins with GRS domains at the C terminus (Fig. 1)—a fused protein construction as also seen in plants and some other protists such as Trichomonas (20, 25, 40). Probably best characterized is the 7TM GRS protein from A. thaliana, AtRGS1, that modulates cellular responses to glucose, in part by accelerating GTPase activity on the “self-activating” $\alpha$ subunit AtGPA1 (23, 39, 42). A number of the 7TM GRS proteins in N. fowleri harbor GPCR proteolytic site motifs (Fig. 1), reminiscent of the adhesion GPCRs that are activated via cell–cell or cell–matrix contact (43). Consistent with this suggested function, several Nf 7TM GRS proteins have complex extracellular N termini with predicted epidermal growth factor–like repeats and leucine domains (e.g., Nf 7TM GRS2, Nf 7TM GRS3, and Nf 7TM GRS4; Fig. 1). In addition to 7TM GRS proteins, the N. fowleri genome encodes a large GRS protein family, with 79 additional GRS domain-containing proteins (beyond the 7TM GRS protein class) and a single RGS-RhoGEF protein with a multidomain structure (Fig. 1) similar to the G$\alpha$ effector in E. histolytica, EhRGS-RhoGEF (27, 29), despite low protein sequence similarity (20%). Three phospholipase C (PLC) genes are present within the N. fowleri genome, although none encodes sufficient protein sequence similarity with mammalian PLCs to allow subclassification, such as among the PLCB isozymes that are G$\alpha_q$ effectors in mammals (19). Relatively simplified PLC domain structures with catalytic X-box and Y-box domains, and EF hands suggest calcium regulation (Fig. 1). A remarkably large family of 80 putative adenyl/guanine cyclase proteins containing a catalytic CYcc domain are present in the N. fowleri genome (Fig. 1), 62 of which are apparently simultaneously expressed in trophozoites by RNAseq (FPKM > 20th percentile) (32, 35). These putative cyclic nucleotide-forming enzymes exhibit diverse topologies and domain combinations, including predicted cytoplasmic proteins (e.g., Nf AC5) and proteins with variable predicted transmembrane helices (Fig. 1).

**Phylogenetic analyses and nucleotide exchange characteristics of N. fowleri $\alpha$ subunits**

$\alpha$ subunits are signaling hubs with distinct downstream effectors that can be predicted in mammals and higher
eukaryotes based on sequence similarity (17). Phylogenetic analyses were performed based on multiple sequence alignments (MSAs) of the Ga subunits encoded within the N. fowleri genome, as compared to Ga MSAs from humans and select model organisms (Fig. S1). Clear phylogenetic relationships were apparent among subfamilies of Ga subunits from humans, Dictyostelium rerio, Dictyostelium melanogaster, and C. elegans; in contrast, those from protists such as N. fowleri, D. discoideum, T. vaginalis, and E. histolytica, from fungi such as S. cerevisiae, and from the plants A. thaliana and O. sativa are more distantly related. Of note, "self-activating" Ga subunits (those with known rapid nucleotide exchange rates, such as A. thaliana GPA1 and T. vaginalis Ga1 (23, 24, 27)) do not show clear phylogenetic relationships (Fig. S1).

To examine the functionality and nucleotide cycling characteristics of Ga subunits in N. fowleri, six family members were produced as recombinant proteins from Entamoeba coli (Fig. S2). Conformational change upon activation of many Ga subunits can be detected as changes in intrinsic tryptophan fluorescence, primarily effected by a tryptophan on switch 2 (44, 45), a fluorescent residue which is universally conserved among the N. fowleri Ga subunits (Fig. S3). When purified from E. coli, five N. fowleri Ga subunits exhibited the expected increase in tryptophan fluorescence upon nucleotide activation (Fig. 2). Three of these Ga subunits (Nf Ga1–3) exhibited typical activation upon binding to the nonhydrolyzable GTP analog GppNHp (Fig. 2, A–C), while GTP was insufficient for detectable activation, consistent with nucleotide exchange being the rate limiting step in the nucleotide cycle. Observed kinetics of activation for Nf Ga1–3 ranged over an approximate order of magnitude (0.03–0.3 min⁻¹; Fig. 2, D–F). In contrast, Nf Ga5 and Nf Ga7 each achieved the active conformation in the presence of either GppNHp or GTP (Fig. 2, G and I), consistent with "self-activation" and GTP hydrolysis being the rate limiting step of nucleotide cycling. As observed for other Ga subunits with rapid nucleotide exchange (e.g., ref. (24)), the intrinsic tryptophan fluorescence of Nf Ga5 returned slowly to near-baseline levels after multiple

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**Figure 1.** Candidate heterotrimeric G protein signaling components encoded by the Naegleria fowleri genome. Homologs of known G protein signaling components were identified by hidden Markov model searches of the N. fowleri genome (32). Green text indicates successful (and red text indicates attempted) PCR-based cloning of open reading frames from genomic DNA and expression as recombinant proteins in E. coli. Domain abbreviations are as follows: Arr-N and Arr-C, N- and C-terminal arrestin-like domains; GPS, GPCR proteolytic site motif; EGF, epidermal growth factor-like domain; RGS, regulator of G protein signaling domain or "RGS-box"; EF hand, calcium binding motif; X, phospholipase C X catalytic domain; Y, phospholipase C Y catalytic domain; CYCc, adenylyl/guanosyl cyclase catalytic domain; PAS, signal sensor motif; LisH, lissencephaly type 1-like homology motif; RasGGEF, Ras GT Pase guanine nucleotide exchange factor; LRRs, leucine rich repeats; DH, Dbl homology domain; PH, pleckstrin homology domain.
Figure 2. Nucleotide-dependent activation of *N. fowleri* Gα subunits. Indicated recombinant purified Gα subunits were mixed with nucleotide at time zero, and the intrinsic tryptophan fluorescence monitored to detect conformational change. Nf Gα1, Nf Gα2, and Nf Gα3 underwent conformational change detected in the presence of the nonhydrolyzable GTP analog GppNHp (A–C). Activation rates were obtained from data shown in panels D–F. Nf Gα5 and Nf Gα7 exhibited “self-activation” (G and I) and assumed the active conformation in the presence of GTP, indicating that GTP hydrolysis, rather than nucleotide exchange, is rate limiting under these conditions. Activation rates were obtained from data shown in panels J and L. Nf Gα6 was not activated by guanine nucleotides, but conformational change was detected upon addition of NaF and AlCl3 in the presence of magnesium (AMF; H). Deletion of a predicted low complexity loop in Nf Gα6 (a.a. 148–222) abolished activation by AMF (K). All traces are a single representative from three independent experiments, except panel J which reflects mean and standard deviation of three independent experiments. AMF, aluminum magnesium and fluoride; RFU, relative fluorescence units.
rounds of hydrolysis and exhaustion of available GTP (Fig. 2G). Nf Ga5 and Nf Ga7 also exhibit significantly more rapid activation kinetics (0.7 and 1.3 min⁻¹) as compared to other Nf Ga subunits tested (Fig. 2, J and L). Nf Ga6 did not display a significant change in tryptophan fluorescence upon addition of either GTP or GppNHp (Fig. 2H). However, activation was achieved by addition of GDP, NaF, and AlCl₃ in the presence of Mg²⁺ (i.e., forming aluminum magnesium and fluoride [AMF]) to mimic the hydrolysis transition state. This pattern of activation is reminiscent of human Ga₉, which exhibits negligible nucleotide exchange activity in vitro in the absence of a ligand-activated GPCR but is rapidly activated by addition of AMF (46). Nf Ga6 contains a relatively low-complexity polypeptide insertion N terminal to the predicted switch 1 region, as highlighted by MSA (Fig. S3). To examine the potential role of this insertion in modulating nucleotide exchange, a deletion mutant (Δ148–222) was constructed and produced as a recombinant protein from E. coli (Fig. S2). However, Nf Ga6Δ148–222 was not activated by any nucleotide or AMF (Fig. 2K). Two of several possible explanations are that residues 148 to 222 are required for Nf Ga6 to assume the active conformation or that deletion of these residues results in loss of specific activity (e.g., misfolding).

Structure of a self-activating Ga in the nucleotide-free state

To better understand the nucleotide cycling characteristics of the “self-activating” N. fowleri Ga proteins, crystallographic studies were attempted on both Nf Ga5 and Nf Ga7. A structural model based on diffraction data (1.7 Å resolution) was obtained for the self-activating Nf Ga7 crystallized in the presence of GDP (Fig. 3; PDB id 6NE6; ref. (47)). The structural data resulting from collaborative efforts with the Seattle

Figure 3. Structural model of Nf Ga7 in the nucleotide-free state as obtained by X-ray crystallography. A, the overall structure of Nf Ga7 exhibited domain architecture and secondary structure highly similar to mammalian, plant, and protozoan Ga subunits despite low protein sequence identity. Although GDP was present in the crystallization conditions, electron density for nucleotide was absent. Switch 2 is extended away from the nucleotide binding site, which is typical of other Ga subunit structures in inactive states. B, the overall structure of Nf Ga7 is highly similar to human Ga₉ (PDB id 1GIT), and key nucleotide-interacting residues are well conserved. Important conformational differences in the nucleotide-free Nf Ga7 include rotation (~90°) away from the nucleotide binding site of Asp275, the key residue in the highly conserved guanine binding NKxG motif, and distinct backbone positioning and side chain rotamer of Arg181 partially obstructing the nucleotide binding site. C, contacts of the switch regions with the neighboring asymmetric unit may influence their conformation in the structural model.
Structural Genomics Center for Infectious Disease were made publicly available in 2019 and briefly mentioned in a prior Seattle Structural Genomics Center for Infectious Disease (SSGCID) publication (47). However, depictions, comparisons, and analyses in the current work have not been published elsewhere. The overall structure was highly similar to mammalian Ga subunits such as Ga11 (PDB id 1GIT, DALI server z score 34, Ca r.m.s.d. 2.2 Å, protein sequence identity 37%), the protozoan EhGa1 from *E. histolytica* (PDB id 4FID, z score 30, Ca r.m.s.d. 2.3 Å, protein sequence identity 31%), and the plant AtGPA1 from *A. thaliana* (PDB id 2XTZ, z score 25, Ca r.m.s.d. 2.3 Å, protein sequence identity 34%) (48). Among the three switch regions that dominate nucleotide-dependent conformational changes in other Ga structures and their interactions with effectors, Nf Ga7 switch 2 (a.a. 204–222) is modeled in a position that is extended away from the nucleotide binding site, similar to previous structural models of other Ga subunits in the inactive (GDP-bound) state (Fig. 3).

Within the electron density data, no nucleotide was observable in the catalytic site of Nf Ga7, which was seen instead to be occupied by solvent and an ordered ethylene glycol, a chemical present in the cryoprotectant solution (Fig. 3). To our knowledge, this is the first crystallographic snapshot of an isolated nucleotide-free Ga subunit, although GPCR/G protein heterotrimer complex structures have also lacked nucleotide (49, 50). The marked shift in the spatial relationship between the all helical and Ras-like domains and the shift of the α5 helix observed in GPCR/G protein complex structures is absent in nucleotide-free Nf Ga7. However, the possibility of similar conformational changes in solution cannot be excluded based on this crystallographic snapshot.

A comparison of the Nf Ga7 nucleotide binding site with the structurally similar GDP-bound human Ga1 (PDB id 1GIT) revealed highly conserved nucleotide-interacting residues (Fig. 3B). Asp275 of the NKxD motif stringently conserved across GTPases (51) was rotated away from the nucleotide binding site (D275 in light blue within Fig. 3B); furthermore, Arg181 of Nf Ga7, a conserved switch 1 residue required for efficient GTP hydrolysis (52), adopted a side-chain rotamer that partially obstructs the nucleotide binding site (R181 in *dark blue* in Fig. 3B). However, the conformations of switch region residues within Nf Ga7, including Arg181, may be influenced by crystallographic contacts observed with the neighboring asymmetric unit (Fig. 3C). Previous structural and molecular dynamics studies of the “self-activating” GPA1 from *A. thaliana* have suggested that heightened mobility of the all-helical domain, reflected as high B factors in the crystal structure, serves as a principal mechanism of rapid nucleotide exchange (53, 54). In contrast, the structural model of Nf Ga7 has no significant average B factor differences between all helical and Ras-like domains.

**N. fowleri RGS proteins accelerate Ga GTP hydrolysis**

To identify potential transmembrane interaction partners and downstream effectors for Nf Ga7 and other Nf Ga subunits, the isolated RGS domains from four 7TM RGS proteins (named Nf 7TM RGS1–4; Fig. 1) and Nf RGS-RhoGEF were produced as recombinant proteins from *E. coli* (Fig. 52). Each Ga/RGS domain combination was screened for a functional interaction using a previously described fluorescent nucleotide (BODIPYFL-GTP) hydrolysis assay for GAP activity (55). The RGS domain of Nf 7TM RGS1 interfered with baseline fluorescence of BODIPYFL-GTP, rendering uninterpretable fluorescence time courses; it was therefore excluded from further study. Similarly, Nf Ga6 was excluded because no appreciable nucleotide binding or hydrolysis was detected by incubation with BODIPYFL-GTP, consistent with the inability to activate Nf Ga6 with GTP or GppNHp in intrinsic tryptophan fluorescence assays (Fig. 2H). Each of the remaining four RGS domains were mixed with Nf Ga1–3, Nf Ga5, or Nf Ga7, and GAP activity by these RGS domains subsequently detected as a significant decrease in area under the fluorescence time course curve (Fig. 4) (55). No functional interaction of Nf Ga1 was observed with RGS domains, while both Nf 7TM RGS2 and Nf RGS-RhoGEF served as GAPs for Nf Ga2 (Fig. 4, A, B and I). Each of the RGS domains exhibited significant GAP activity on Nf Ga3, suggesting promiscuous coupling of this particular Nf Ga subunit to RGS proteins (Fig. 4, C and D). Nf 7TM RGS2 and Nf 7TM RGS3 served as GAPs for Nf Ga5, while Nf Ga7 engaged Nf 7TM RGS2 and Nf 7TM RGS4 (Fig. 4, E–H).

A subtle mutation of a conserved Ga switch 1 glycine to serine, known as the “RGS insensitivity” mutation (56, 57), disrupts interactions of canonical RGS domains with their Ga subunit partners, while mammalian RGS-RhoGEF “RGS-like” domain/Ga interactions are not affected by this G-to-S mutation. The switch 1 glycine is conserved across *N. fowleri* Ga subunits (Fig. 5A), and mutation of this position to serine in Nf Ga2(G181S) and NfGa3(G184S) disrupted GAP activity by all tested RGS domains (Fig. 5, B–E). Of note, interaction of both NfGa2 and NfGa3 with the RGS domain of Nf RGS-RhoGEF was disrupted by the RGS insensitivity mutation, indicating canonical RGS domain/Ga interactions rather than an interface akin to mammalian RGS-RhoGEF/Ga pairs. This mode of Ga and effector interaction (i.e., using a canonical RGS domain rather than an “RGS-like” or “RGS-homology” domain) was previously observed in *E. histolytica*, suggesting a shared evolutionary origin (29).

Direct binding interactions between selected *N. fowleri* Ga subunits and purified recombinant RGS domains were also examined by surface plasmon resonance (SPR), and binding affinities quantified (Fig. 6; additional data in Figs. S4–S6). All observed Ga/RGS interactions were selective for the transition state mimetic (GDP and AlF4⁻ bound) form of Ga, consistent with prior studies of RGS domain binding selectivity (e.g., ref. (29, 58)) (Figs. S4–S6). No significant binding to RGS domains within physiologically relevant concentration ranges was detected for either NfGa6 or NfGa1 (Fig. 6, A and C), consistent with a lack of measurable GAP activity on these two Ga subunits (e.g., Fig. 4I). Nf Ga2 exhibited preferential binding to the RGS domain of Nf 7TM RGS2 (KD = 630 ± 190 nM) and lower affinity interaction with Nf RGS-RhoGEF (KD = 2.4 ± 0.5 μM). Increased resonance of the Nf Ga2 surface with high concentrations of Nf 7TM RGS4 likely represents nonspecific binding, as indicated by the atypical, approximately linear binding curve (Fig. S5). In support of this.
hypothesis, no significant GAP activity was observed for this Gα/RGS domain pair at 5 μM RGS protein concentration (Fig. 4B). Nf Ga2 showed accelerated GTP hydrolysis in the presence of Nf 7TM RGS2 and Nf RGS-RhoGEF. A significant reduction in area under the curve (AUC) indicates GAP activity (B, D, F and H). E, Nf 7TM RGS2 and Nf 7TM RGS3 had GAP activity on Nf Ga3. C, Nf Ga3 showed accelerated GTPase activity in the presence of all RGS domains tested, while hydrolysis on Nf Ga1 (I) was unaffected by each. G, Nf 7TM RGS2 and Nf 7TM RGS4 had GAP activity on Nf Ga7. Time course and AUC error bars reflect standard deviation for independent experiments (n = 3–10). 7TM RGS, seven-transmembrane RGS proteins; RGS, RGS, regulator of G protein signaling domain.

Figure 4. An RGS-RhoGEF effector and 7TM RGS proteins are selective GTPase accelerating proteins for N. fowleri Ga subunits. Four recombinant N. fowleri RGS domains (at 5 μM concentration) were tested consecutively against five Ga subunits for GTPase accelerating protein (GAP) activity using a fluorescent nucleotide substrate (55). A, Nf Ga2 showed accelerated GTP hydrolysis in the present of Nf 7TM RGS2 and Nf RGS-RhoGEF. A significant reduction in area under the curve (AUC) indicates GAP activity. A, B, D, F and H, E, Nf 7TM RGS2 and Nf 7TM RGS3 had GAP activity on Nf Ga3. C, Nf Ga3 showed accelerated GTPase activity in the presence of all RGS domains tested, while hydrolysis on Nf Ga1 (I) was unaffected by each. G, Nf 7TM RGS2 and Nf 7TM RGS4 had GAP activity on Nf Ga7. Time course and AUC error bars reflect standard deviation for independent experiments (n = 3–10). 7TM RGS, seven-transmembrane RGS proteins; RGS, RGS, regulator of G protein signaling domain.

Phosphate binding loop variation among N. fowleri Ga subunits contributes to nucleotide cycling characteristics and RGS domain interactions

The phosphate binding loop (P-loop) is a highly conserved motif among G proteins, as well as within ATP-binding kinases (59). The P-loop is intimately associated with the bound nucleotide in Gα subunits; as such, mutations in this region are known to reduce nucleotide hydrolysis or impair activation (45, 60). A MSA of selected N. fowleri Ga subunits with human and other protist Gα subunits revealed a high degree of overall conservation (Fig. 7B). However, the Nf Ga1 sequence deviates significantly in positions 23 to 25, suggesting a role for this region in modulating nucleotide cycling. The Nf Ga1 alanine 24 corresponds to a position with high conservation of glycine among heterotrimeric G proteins and Ras superfamily GTPases, interacting directly with the γ-phosphoryl group of GTP. Missense mutations at this locus to essentially any other residue disrupt GTPase activity in Ras GTPases, rendering them constitutively active, as commonly seen in the oncogenic Ras G12V mutation (61). Mutation of this residue in heterotrimeric G proteins has variable effects on nucleotide cycling. For example, human Gα11 G42V is GTPase deficient and constitutively active, while Gα11 G42R is unable to transition to the active state conformation (45, 60).
A switch 1 RGS insensitivity mutant eliminates GAP activity and demonstrates canonical RGS/Go interations. A, a highly conserved glycine residue in switch 1, when mutated to serine (the "RGS insensitivity" mutation; ref. 57) eliminates GAP activity of canonical Go/RGS pairs. B and C, GTP hydrolysis on Nf Go3 G184S is unaffected by presence of 7TM RGS domains and the Nf RGS-RhoGEF putative effector. D and E, similarly, no RGS-domain-mediated GAP activity was observed on Nf Go2 G181S. Time course and AUC error bars reflect standard deviation for independent experiments (n = 4). 7TM RGS, seven-transmembrane RGS proteins; GAP, GTPase-accelerating proteins; RGS, RGS, regulator of G protein signaling domain.
position corresponding to Nf Gα1 glutamine 25 is well conserved as a negatively charged residue (typically glutamate) that interacts with bound nucleotide and exhibits interactions with the conserved “arginine finger” (e.g., R181 in Nf Gα7, Fig. 3B), implicated in control of nucleotide exchange (62).

To test this hypothesis, P-loop residues 23 to 25 of Nf Gα1 were substituted for the corresponding residues 36 to 38 of Nf Gα2 (“Nf Gα2 P1” chimera), and the converse substitution was also generated to create the “Nf Gα1 P2” chimera (Fig. 7). Wildtype Nf Gα1 and the Nf Gα1 P2 chimera were each activated by GppNHp with indistinguishable kinetics (Fig. 7D and E), suggesting similar rates of nucleotide exchange. However, Nf Gα1 P2 exhibited more rapid GTP hydrolysis than wildtype protein (e.g., Fig. 7, G and H; also compare buffer-only conditions of Fig. 4I with Fig. 7A). Like wildtype Nf Gα1 (Fig. 4I), the Nf Gα1 P2 chimera was not a substrate for any RGS domains examined (Fig. 7A). In contrast, the Nf Gα2 P1 chimera displayed impaired GTP hydrolysis compared to wildtype (Fig. 7, H and I). The Nf Gα2 P1 chimera also exhibited significantly more rapid activation by GppNHp (Fig. 7E; 0.10 ± 0.01 min⁻¹ compared to 0.058 ± 0.002 min⁻¹) and assumed an activated conformation in the presence of GTP (Fig. 7F). Unlike wildtype Nf Gα2 (Fig. 4, A and B), Nf Gα2 P1 did not functionally engage Nf 7TM RGS2 or Nf RGS-RhoGEF in GAP activity assay (Fig. 7C). Together these findings indicate that the unusual P-loop of Nf Gα1 (23-TAQ-25) confers a relatively slow GTP hydrolysis rate to the Gα subunit possessing it and likely also contributes to selective engagement of RGS domains.

Discussion

The N. fowleri genome encodes a relatively complex set of heterotrimeric G protein signaling components, many of which are apparently simultaneously expressed in single-celled trophozoites. Given the amenability of G protein signaling to pharmacologic manipulation (11, 15), these pathways provide ample opportunity for the development of chemical probes and (potentially) therapeutics. Of particular interest are the candidate GPCRs in the N. fowleri genome; homologs in other organisms respond to extracellular cues including, but not limited to, small molecules (11, 43, 50). Although one candidate N. fowleri GPCR (AmoebaDB accession NF0059410) exhibits some similarity to the D. discoideum cAR family cyclic AMP receptors with known importance for functions such as chemotaxis and quorum sensing (63); the low overall sequence identity of the N. fowleri homolog (29%) limits speculation about potential ligands. The 7TM RGS proteins may provide a
similar mode of transmembrane signal transduction, as previously discovered for the plant 7TM RGS protein AtRGS1 that regulates heterotrimeric G protein signaling in response to glucose (64, 65). The remarkable diversity of 28 different 7TM RGS proteins encoded by the *N. fowleri* genome suggests adaptive radiation of this particular signaling modality for detecting extracellular cues. The substantial overlap of RGS domain/Gα subunit interaction specificity in the present study of four Nf 7TM RGS proteins (summarized in Fig. 8) suggests a level of redundancy of downstream signaling for these proteins, should they be responsive to extracellular/environmental agonist cues. A sizable number of 7TM RGS proteins in *N. fowleri*, including Nf 7TM RGS1–4 as illustrated in Figure 1, exhibit extracellular domain structures with epidermal growth factor–like repeats, putative carbohydrate-binding domains, and GPCR proteolytic site motifs reminiscent of the adhesion GPCRs that respond to cell–cell or cell–matrix interactions (43). Whether these (and/or other) 7TM RGS proteins in *N. fowleri* function as cell surface receptors and how extracellular cues may alter GAP activity remains to be determined.

The presence of both “self-activating” Gα subunits rate-limited by GTP hydrolysis (e.g., Nf Gα7) and conventional Gα subunits rate-limited by nucleotide exchange (e.g., presumably catalyzed by ligand-activated GPCR GEF activity) is unique to *Naegleria* among biochemically characterized species to date (20). A previously conducted evolutionary analysis of heterotrimeric G protein signaling led to a hypothesis that GPCR-mediated activation of slow exchanging Gα subunits arose relatively late in evolutionary history among unikonts, which include animals, fungi, and amoebazoans such as Dictyostelium (20). In contrast, the more primitive system of “self-activating” Gα subunits coupling to 7TM and other RGS proteins is dominant among the bikonts, which include Excavata such as Trichomonas and Naegleria. Indeed, biochemical testing of this division has held true, including
demonstrations of the “self-activating” properties of four Trichomonas Gα subunits that interact with 7TM RGS proteins (20, 25). However, the mixture of Gα subunits with either slow or fast nucleotide exchange in Naegleria, and both types functionally interacting with 7TM RGS proteins (Fig. 8), suggest an earlier evolutionary origin of exchange factor–dependent G protein signaling. The Excavata supergroup containing Naegleria (one of six total supergroups) is a highly diverse group of organisms, considered to be of closest relationship to the ancestor of all eukaryotes (66). Although an early horizontal gene transfer event cannot be entirely excluded, our data here indicate the presence of both “self-activating” Gα/7TM RGS signaling and exchange-factor dependent G protein signaling in early eukaryotic evolution.

At this time, the specific biological functions of heterotrimeric G protein signaling pathways in Naegleria species remain unknown. However, the nucleotide state–dependent interaction of Nf Gα2 and Nf Gα3 with an RGS-RhoGEF candidate effector suggests crosstalk between at least some aspects of Naegleria heterotrimeric G protein signaling and Rho family GTPase signaling, the latter which is conserved across species for regulation of the actin cytoskeleton, transcription, and cell division (67–70). The domain structure of Nf RGS-RhoGEF containing a canonical RGS domain (not an “RGS-like” domain) and a DH-PH tandem that mirrors the domain organization of the RGS-RhoGEF from E. histolytica, although the Naegleria protein also contains putative leucine-rich repeats at its N terminus (29, 71). Eh RGS-RhoGEF activates Rho family GTPases downstream of Eh Gα1, which modulates trophozoites behaviors such as migration, invasion, and host cell killing that are dependent on a highly dynamic actin cytoskeleton (29, 72, 73). In contrast with the mammalian RGS-like domain-containing RGS-RhoGEFs that serve as Gα12/13 effectors, Naegleria and Entamoeba RGS-RhoGEF proteins interact with Gα subunits though a canonical RGS/ Gα interface, a difference that can be distinguished by the RGS insensitivity mutation on switch 1 (Fig. 5) (29). This finding supports a shared evolutionary origin for Gα interactions with RGS-RhoGEFs in Naegleria and Entamoeba, likely separate from Gα12/13 signaling in the animal kingdom.

**Experimental procedures**

**Identification and comparison of putative G protein signaling components**

The *N. fowleri* genome sequence (32, 33) was obtained through publicly available databases (NCBI). Open-reading frames were predicted using Augustus (74). Candidate G proteins, RGS proteins, arrestins, and G protein effectors were identified using hidden Markov models (HMMer 3.0, (75)) generated from MSAs (Clustal Omega, (76)) of mammalian homologs. Publicly available RNAseq data (NCBI, (32)) was aligned to the *N. fowleri* reference genome sequence and candidate open reading frames using TopHat 2.1.0 and
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Cufflinks 2.2.0 (77). Read counts and percent sequence coverage were calculated for select loci to determine relative expression levels. Additional candidate G protein signaling components were identified and expression levels assessed by BLAST searching (78) of N. fowleri RNAseq data available through AmoebaDB (amoebadb.org, (35)). The N. fowleri Ga subunits and RGS proteins were aligned using T-coffee (79), and BLOSUM62-based neighbor-joining dendrograms derived using Jalview 2.10 (80). Phylogenetic analysis of selected Ga subunits was carried out with Phylogeny.fr (81).

Cloning of G protein signaling components

Heterotrimeric G protein subunits and isolated RGS domains from candidate RGS proteins were cloned by PCR amplification from genomic DNA of the N. fowleri Carter strain (ATCC) and inserted into modified pET-15b E. coli expression vectors (pET-His-LIC, e.g., (28)) using ligation-independent cloning to form N-terminal tobacco etch virus protease-cleavable, hexahistidine-tagged fusions, as previously described (69). The predicted flexible N-terminal helices (~25–35 amino acids) of all Ga subunits were deleted prior to cloning. The putative G protein γ subunit, NfGy1, was not found within the AmoebaDB RNAseq data; it was cloned de novo from N. fowleri genomic DNA with sequence 5’-ATGAATAAAAATGGCAAAACGGTATGAGCATT TGTTGGCAACTATTGGGCAAGAATTCAACAGTTTGA GAAAGTTTGGAAAGTTCGAAAAAGCCATCCCCAATT TCTGAAAGCATGTCGAACCTCATAATTGATTCGAAT GTACCAAAATGGCAAGGATATGCTGATGATGGGAC CGCAACCAATTCTGAAATTCACCACAGATGGGC GGTTGTTGTACCATCATTGAA-3’. Primer sequences, AmoebaDB gene identifiers, and fragments used for biochemical experiments are detailed in Table S1. Introns were removed, and mutations generated using an overlap extension PCR method (82).

Protein purification

N. fowleri Ga subunits were expressed and purified from E. coli, essentially as we have previously described for E. histolytica EhGa1 (28). For hexahistidine-tagged Ga subunits and RGS domains, transformed B834 E. coli were grown to an A600nm of 0.7 to 0.8 at 37 °C before induction with 500 μM isopropyl-β-D-thiogalactopyranoside for 14 to 16 h at 20 °C. Cell pellets were resuspended in N1 buffer (for Ga subunits: 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM MgCl2, 10 mM NaF, 30 μM AlCl3, 50 mM GDP, 30 mM imidazole, 5% [w/v] glycerol; for RGS domains: 50 mM Hepes pH 8.0, 150 mM NaCl, 30 mM imidazole, 5% [w/v] glycerol) and lysed at 10,000 kPa using an Avestin Emlulsiflex. Cleared lysates were applied to nickel–nitritotriacetic acid resin (GE Healthcare), washed, and eluted with N1 buffer containing 300 mM imidazole. Eluted protein was resolved using a calibrated size exclusion column (GE Healthcare) with S200 buffer for Ga subunits (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM DTT, 5% [w/v] glycerol, and 50 μM GDP) or RGS domains (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM DTT, 5% [w/v] glycerol). Recombinant proteins were analyzed by SDS-PAGE electrophoresis, concentrated to 0.5 to 1.5 mM, and snap frozen in a dry ice and ethanol bath prior to long-term cryostorage.

Crystallization and structure determination

Crystallization and structure determination were accomplished in collaboration with the SSGCID (47). DEB initiated collaboration toward Naegleria G protein signaling component structures with SSGCID and provided plasmids, protein purification methods, and preliminary biochemical data. Hexahistidine-tagged NF Ga7 in crystallization buffer (25 mM Heps pH 7.5, 500 mM NaCl, 5% glycerol, 2 mM DTT, 0.025% sodium azide, 10 mM MgCl2, 10 mM NaF, 30 μM AlCl3, 5 μM GDP) was mixed 1:1 with crystallization solution (16% [w/v] PEG-800, 40 mM potassium phosphate monobasic, 20% [v/v] glycerol). The resulting crystals were cryoprotected with 20% ethylene glycol. Diffraction data collection from a single crystal was performed at the Advanced Photon Source (beamline 21-ID-F), data reduced with XDS, and model refined with Phenix (83). The structural model was refined to a 1.7 Å resolution, with Rwork 0.161 and Rfree 0.195 (PDB id 6N6E). Crystallographic data collection and refinement statistics have been described in the previous publication (47).

Intrinsic tryptophan fluorescence measurements

The key tryptophan residue allowing detection of activation (44) was located within the switch 2 regions of N. fowleri Ga subunits 1 through 8 (Fig. S3). Tryptophan fluorescence (excitation and emission wavelengths 284 and 340 nm, respectively) was measured at 20 °C in exchange buffer (100 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM MgCl2, and 5% glycerol) using a FluoroLog modular spectrofluorometer (Horiba) (28). Recombinant purified N. fowleri Ga subunit was added to 500 nM concentration, and a baseline fluorescence established. Guanine nucleotide (1 μM) was then added, and fluorescence monitored at 20 to 30 s intervals. For Ga subunits without measurable activation by a non-hydrolyzable GTP analog (GppNHp), 20 mM NaF and 50 μM AlCl3 were added to reaction mixtures containing GDP to assemble the transition-state mimetic form (i.e., GDP–aluminum tetrafluoride). Activation rate constants (kobs) were estimated by modeling observed fluorescence using one-phase association in GraphPad Prism 7. All experiments were performed in at least biological triplicate (three or more independent experiments).

Fluorescent GTP hydrolysis and acceleration by RGS proteins

Fluorescent detection of GTP binding and hydrolysis was conducted essentially as described previously (55). Fluorescence measurements (excitation 485 nm and emission 530 nm) were made at a constant temperature of 20 °C and 30 s intervals using a FluoroLog modular spectrofluorometer (Horiba). All experiments were conducted with constant stirring by magnetic stir bars. Experiments were conducted in TEM buffer (20 mM Tris pH 8.0, 1 mM EDTA, 10 mM
MgCl₂). Recombinant purified RGS protein (5 μM) or an equivalent volume of buffer was diluted in TEM buffer. 100 nM nucleotide, BODIPYFL-GTP (ThermoFisher) was added and allowed to equilibrate for at least 10 min, with stabilization of the fluorescent signal. Baseline fluorescence was indistinguishable across experiments, indicating no effect of RGS proteins on nucleotide fluorescence, with the exception of 7TM RGS1 (excluded from further study). After equilibration, recombinant N. fowleri Ga subunits were added to a final concentration of 200 nM. Fluorescence time courses were monitored over 40 to 60 min. Relative fluorescence units were derived by subtraction of baseline fluorescence in the absence of Ga subunit. Area under the curve (AUC) was calculated using GraphPad Prism 7. Reduction in AUC was interpreted as reduced time of the Ga subunit in the GTP-bound state, consistent with GTPase activity acceleration (GAP), as previously described (55). All experiments were conducted with biological replicates (3–10 independent experiments). Statistical significance was defined as p < 0.05 using a two-tailed t test in GraphPad Prism 7.

**SPR binding measurements**

SPR-based measurements of protein–protein interaction were performed on a Proteon XPR36 (Bio-Rad) at the UNC Macromolecular Interactions Facility, essentially as described previously (29). Approximately 5000 resonance units of purified hexahistidine-tagged N. fowleri Ga subunits were separately immobilized on a nickel-NTA biosensor chip (Bio-Rad) using covalent capture coupling as previously described (84). Two surfaces with irrelevant proteins, one denatured by injection of sodium hydroxide, served as negative controls. Experiments were performed in running buffer containing 50 mM Heps (pH 7.4), 150 mM NaCl, 0.05% NP-40 alternative (Calbiochem), 50 μM EDTA, and 1 mM MgCl₂. Three nucleotide states of the Ga subunits were generated by addition of GDP (100 μM), GppNHP (100 μM), or AMF (100 μM GDP, 20 mM NaF, and 30 μM AlCl₃) to the running buffer, respectively, and then equilibration with this addition over 2 h. Increasing concentrations of RGS proteins were separately injected at 20 μl/min. Equilibrium affinity constants (Kₒ) and kinetic parameters of binding (kₐ [association constant] and k₅ [dissociation constant]) were derived using Proteon Manager software (Bio-Rad) and GraphPad Prism 7. All experiments were conducted with three analyte injections (technical replicates) and performed at least twice on separate surfaces.

**Data availability**

All data are contained within the manuscript, except genomic and transcriptomic data which are publicly available at amoebadb.org. Structure coordinates and structure factors were deposited in 2019 and available in the PDB (accession 6NE6).

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**Abbreviations**—The abbreviations used are: AMF, aluminum magnesium and fluoride; GAP, GTPase-accelerating protein; GEF, guanine nucleotide exchange factor; GPCR, G protein–coupled receptor; MSA, multiple sequence alignment; P-loop, phosphate binding loop; PLC, phospholipase C; RGS, regulator of G protein signaling protein; r.m.s.d, root mean square deviation; SPR, surface plasmon resonance.

**References**

1. Roy, S. L., Metzger, R., Chen, J. G., Laham, F. R., Martín, M., Kipper, S. W., et al. (2014) Risk for transmission of Naegleria fowleri from solid organ transplantation. *Am. J. Transplant.* 14, 163–171
2. Pugh, J. J., and Levy, R. A. (2016) Naegleria fowleri: diagnosis, pathophysiology of brain inflammation, and antimicrobial treatments. *ACS Chem. Neurosci.* 7, 1178–1179
3. Goudot, S., Herbelin, P., Mathieu, L., Soreau, S., Banas, S., and Jorand, F. P. (2014) Biocidal efficacy of monochloramine against planktonic and biofilm-associated Naegleria fowleri cells. *J. Appl. Microbiol.* 116, 1055–1065
4. Siddiqui, R., Ali, I. K., Cope, J. R., and Khan, N. A. (2016) Biology and pathogenesis of Naegleria fowleri. *Acta Trop.* 164, 375–394
5. Siddiqui, R., and Khan, N. A. (2014) Primary amoebic meningoencephalitis caused by Naegleria fowleri: an old enemy presenting new challenges. *PloS Negl. Trop. Dis.* 8, e2617
6. Centers for Disease Control and Prevention (CDC) (2013) Notes from the field: primary amebic meningoencephalitis associated with ritual nasal rinsing—St. Thomas, U.S. Virgin islands, 2012. *MMWR Morb. Mortal. Wkly. Rep.* 62, 903
7. Komarov, R. N., and Belov, I. V. (2008) [Approaches to the thoracoabdominal aorta]. *Angiol. Sovus. Khir.* 14, 125–128
8. Baig, A. M. (2015) Pathogenesis of amoebic encephalitis: are the amoebae being credited to an ‘inside job’ done by the host immune response? *Acta Trop.* 148, 72–76
9. Zaheer, R. (2013) Naegleria fowleri—the brain-eating amoeba. *J. Pak. Med. Assoc.* 63, 1456
10. Parsonson, F., and Nicholls, C. (2016) Primary amoebic meningoencephalitis in North Queensland: the diagnostic challenges of Naegleria fowleri. *Pathology* 48 Suppl. 1, S105–S106
11. Williams, C., and Hill, S. J. (2009) GPCR signaling: understanding the pathway to successful drug discovery. *Methods Mol. Biol.* 552, 39–50

*J. Biol. Chem.* (2022) 298(8) 102167
12. Mahoney, J. P., and Sunahara, R. K. (2016) Mechanistic insights into GPCR-G protein interactions. Curr. Opin. Struct. Biol. 41, 247–254
13. Dessauer, C. W., Posner, B. A., and Gilman, A. G. (1996) Visualizing signal transduction: receptors, G-proteins, and adenylate cyclases. Clin. Sci. (Lond.) 91, 527–537
14. Siderovski, D. P., and Willard, F. S. (2005) The GAPs, GEFS, and GDIs of heterotrimeric G-protein alpha subunits. Int. J. Biol. Sci. 1, 51–66
15. Kimple, A. J., Bosch, D. E., Giguère, P. M., and Siderovski, D. P. (2011) Regulators of G-protein signaling and their Gsa substrates: promises and challenges in their use as drug discovery targets. Pharmacol. Rev. 63, 728–749
16. Tesner, I. J., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) G protein signaling pathways in mammals. J. Cell Sci. 120, 1359–1367
17. Gresset, A., Sondek, J., and Harden, T. K. (2012) The phospholipase C isozymes and their regulation. Subcell Biochem. 58, 61–94
18. Bradford, W., Buckholz, A., Morton, J., Price, C., Jones, A. M., and Urano, D. (2013) Eukaryotic G protein signaling evolved to require G protein-coupled receptors for activation. Sci. Signal. 6, ra37
19. Wilkie, T. M., and Yokoyama, S. (1994) Evolution of the G protein alpha subunit multigene family. Science 265, 576–583
20. Clement, S. T., Dixit, G., and Dohlman, H. G. (2013) Regulation of yeast G protein signaling by the kinases that activate the AMPK homolog Snf1. Sci. Signal. 6, ra78
21. Johnston, C. A., Taylor, J. P., Gao, Y., Kimp, A. J., Grigston, J. C., Chen, J. G., et al. (2007) GTPase acceleration as the rate-limiting step in Arabidopsis G protein-coupled sugar signaling. Proc. Natl. Acad. Sci. U. S. A. 104, 17317–17322
22. Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Holm, L., and Laakso, L. M. (2016) Dali server update.
23. Bosch, D. E., Willard, F. S., Ramananjum, R., Kimple, A. J., Willard, M. D., Naqvi, N. I., et al. (2012) A P-loop mutation in Gsa subunits prevents transition to the active state: implications for G-protein signaling in fungal pathogenesis. PLoS Pathog. 8, e1002553
24. Koch, M., Fisk, J. E., Ramananjum, R., Willard, M. D., Naqvi, N. I., et al. (2011) Crystal structure of the B2 adrenergic receptor-Gs protein complex. Nature 477, 549–555
25. Der, T. E., Glynias, M. J., and Merrick, W. C. (1987) GTP-binding domain: three consensus sequence elements with distinct spacing. Proc. Natl. Acad. Sci. U. S. A. 84, 1014–1018
26. Mann, D., Tuch, C., Tammis, E. A., Schröter, G., Gerwert, K., and Küting, C. (2016) Mechanism of the intrinsic arginine finger in heterotrimeric G proteins. Proc. Natl. Acad. Sci. U. S. A. 113, E8041–E8050
27. Jones, I. C., Duffy, J. W., Machius, M., Temple, B. R., Dohlman, H. G., and Jones, A. M. (2011) The crystal structure of a self-activating G protein alpha subunit reveals its distinct mechanism of signal initiation. Sci. Signal. 4, ra8
28. Jones, I. C., Temple, B. R., Jones, A. M., and Dohlman, H. G. (2011) Functional reconstitution of an atypical G protein heterotrimer and regulator of G protein signaling protein (RGS1) from Arabidopsis thaliana. J. Biol. Chem. 286, 13143–13150
29. Willard, F. S., Kimple, A. J., Johnston, C. A., and Siderovski, D. P. (2005) A direct fluorescence-based assay for RGS domain GTPase accelerating activity. Anal. Biochem. 340, 341–351
30. Meigs, T. E., Junjea, J., DeMarco, C. T., Stemme, L. N., Kaplan, D. D., and Casey, P. J. (2005) Selective uncoupling of G alpha 12 from Rho-mediated signaling. J. Biol. Chem. 280, 18049–18055
Heterotrimeric G protein signaling in Naegleria fowleri

57. Lan, K. L., Sarvasyan, N. A., Taussig, R., Mackenzie, R. G., DiBello, P. R., Dohlman, H. G., et al. (1998) A point mutation in Galphao and Galphlb blocks interaction with regulator of G protein signaling proteins. *J. Biol. Chem.* 273, 12794–12797

58. Soundararajan, M., Willard, F. S., Kimple, A. J., Turnbull, A. P., Ball, L. J., Schoch, G. A., et al. (2008) Structural diversity in the RGS domain and its interaction with heterotrimeric G protein alpha-subunits. *Proc. Natl. Acad. Sci. U. S. A.* 105, 6457–6462

59. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* 15, 430–434

60. Raw, A. S., Coleman, D. E., Gilman, A. G., and Sprang, S. R. (1997) Structural and biochemical characterization of the GTPgammaS-, GDP- and GDP-bound forms of a GTPase-deficient Gly42-> Val mutant of Gialpha1. *Biochemistry* 36, 15660–15669

61. Garcia-Marcos, M., Ghosh, P., and Farquhar, M. G. (2011) Molecular basis of a novel oncogenic mutation in GNAO1. *Oncogene* 30, 2691–2696

62. Alvarez-Curto, E., Rozen, D. E., Ritchie, A. V., Fouquet, C., Baldauf, S. L., and Schap, P. (2005) Evolutionary origin of cAMP-based chemotraction in the social amoebae. *Proc. Natl. Acad. Sci. U. S. A.* 102, 6385–6390

63. Urano, D., Phan, N., Jones, J. C., Yang, J., Huang, J., Grigston, J., et al. (2012) Endocytosis of the seven-transmembrane RGS1 protein activates G-protein-coupled signalling in Arabidopsis. *Nat. Cell Biol.* 14, 1079–1088

64. Grigston, J. C., Osuna, D., Scheible, W. R., Liu, C., Stitt, M., and Jones, A. M. (2008) D-Glucose sensing by a plasma membrane regulator of G signaling protein, AtRGS1. *FEBS Lett.* 582, 3577–3584

65. Dawson, S. C., and Paredez, A. R. (2013) Alternative cytoskeletal landscapes: cytoskeletal novelty and evolution in basal excavate protists. *Curr. Opin. Cell Biol.* 25, 134–141

66. Brembu, T., Wings, P., Bones, A. M., and Yang, Z. (2006) A RHOse by any other name: a comparative analysis of animal and plant Rho GTPases. *Cell Res.* 16, 435–445

67. Vlahou, G., and Rivero, F. (2006) Rho GTPase signaling in Dictyostelium discoideum: insights from the genome. *Eur. J. Cell Biol.* 85, 947–959

68. Bosch, D. E., Wittchen, E. S., Qiu, C., Burridge, K., and Siderovski, D. P. (2011) Unique structural and nucleotide exchange features of the Rho1 GTPase of Entamoeba histolytica. *J. Biol. Chem.* 286, 39236–39246

69. Bosch, D. E., Yang, B., and Siderovski, D. P. (2012) Entamoeba histolytica Rho1 regulates actin polymerization through a divergent, diaphanos-related form. *Biochemistry* 51, 8791–8801

70. Rossman, K. L., Der, C. J., and Sondek, J. (2005) GEF means go: turning on RHO GTpases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* 6, 167–180

71. Dufour, A. C., Olivo-Marin, J. C., and Guillaum, N. (2015) Amoeboid movement in protozoan pathogens. *Semin. Cell Dev. Biol.* 46, 128–134

72. Maguis, B., Brugués, J., Nassoy, P., Guillaum, N., Sens, P., and Amblard, F. (2010) Dynamic instability of the intracellular pressure drives bleb-based motility. *J. Cell Sci.* 123, 3884–3892

73. Stanke, M., and Morgenstern, B. (2005) AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. *Nucleic Acids Res.* 33, W465–W467

74. Finn, R. D., Clements, J., Arndt, W., Miller, B. L., Wheeler, T. J., Schreiber, F., et al. (2015) HMMP web server: 2015 update. *Nucleic Acids Res.* 43, W30–W38

75. Sivers, F., and Higgins, D. G. (2014) Clustal omega. *Curr. Protoc. Bioinformatics* 48, 3.13.1–3.13.16

76. Ghosh, S., and Chan, C. K. (2016) Analysis of RNA-Seq data using TopHat and Cufflinks. *Methods Mol. Biol.* 1374, 339–361

77. Madden, T. (2013) The BLAST sequence analysis tool. In: Beck, J. E. A., ed. *The NCBI Handbook [Internet]*, National Center for Biotechnology Information, Bethesda, MD: 1–15

78. Magis, C., Taly, I. F., Bussotti, G., Chang, J. M., Di Tommaso, P., Erb, I., et al. (2014) T-Coffee: tree-based consistency objective function for alignment evaluation. *Methods Mol. Biol.* 1079, 117–129

79. Rivier, C., Corrigan, A., and Vale, W. (1991) Effect of recombinant human inhibin on gonadotropin secretion by the male rat. *Endocrinology* 129, 2155–2159

80. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469

81. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77, 51–59

82. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Echols, N., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* 66, 213–221

83. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res.* 36, W465–W469

84. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Echols, N., et al. (2011) The Phenix software for automated determination of macromolecular structures. *Methods Mol. Biol.* 55, 94–106

85. Kimple, A. J., Muller, R. E., Siderovski, D. P., and Willard, F. S. (2010) A capture coupling method for the covalent immobilization of hexahistidine tagged proteins for surface plasmon resonance. *Methods Mol. Biol.* 627, 91–100