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A P-loop Mutation in Gα Subunits Prevents Transition to the Active State: Implications for G-protein Signaling in Fungal Pathogenesis

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Abstract

Heterotrimeric G-proteins are molecular switches integral to a panoply of different physiological responses that many organisms make to environmental cues. The switch from inactive to active Gαβγ heterotrimer relies on nucleotide cycling by the Gα subunit: exchange of GTP for GDP activates Gα, whereas its intrinsic enzymatic activity catalyzes GTP hydrolysis to GDP and inorganic phosphate, thereby reverting Gα to its inactive state. In several genetic studies of filamentous fungi, such as the rice blast fungus *Magnaporthe oryzae*, a G42R mutation in the phosphate-binding loop of Gα subunits is assumed to be GTPase-deficient and thus constitutively active. Here, we demonstrate that Gα(G42R) mutants are not GTPase deficient, but rather incapable of achieving the activated conformation. Two crystal structure models suggest that Arg-42 prevents a typical switch region conformational change upon Gαi(G42R) binding to GDP-AlF4- or GTP, but rotameric flexibility at this locus allows for unperturbed GTP hydrolysis. Gα(G42R) mutants do not engage the active state-selective peptide KB-1753 nor RGS domains with high affinity, but instead favor interaction with Gβγ and GoLoco motifs in any nucleotide state. The corresponding Gα(G48R) mutant is not constitutively active in cells and responds poorly to aluminum tetrafluoride activation. Comparative analyses of *M. oryzae* strains harboring either G42R or GTPase-deficient Q/L mutations in the Gα subunits MagA or MagB illustrate functional differences in environmental cue processing and intracellular signaling outcomes between these two Gα mutants, thus demonstrating the in vivo functional divergence of G42R and activating G-protein mutants.


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Introduction

G protein-coupled receptors (GPCRs) convert extracellular signals to intracellular responses, primarily by stimulating guanine nucleotide exchange on heterotrimeric G-protein Gα subunits [1]. Upon receptor-stimulated exchange of GTP for GDP, Gα subunits undergo a conformational change, dominated by three mobile switch regions, resulting in separation of Gα from the obligate Gβγ heterodimer [2]. Switches one and two directly contact the bound guanine nucleotide and include residues critical for catalyzing GTP hydrolysis, while switch three contacts switch two in the activated conformation [3]. The nucleotide-dependent conformational shift of Gα subunits can be monitored biochemically by differential resistance to proteolysis by trypsin or altered tryptophan fluorescence spectra [4,5]. The switch mechanism of activation is highly conserved among the mammalian Gα subunit family members, as well as in those found in fungi [6,7]. The activated Gα and free Gβγ subunits propagate signals through numerous effectors, including adenyl cyclases, phospholipases, ion channels, and phosphodiesterases [8]. Mammals express multiple Gα subunits which can be classified into subfamilies according to function. For example, members of the Gαq, i/o subfamily have inhibitory effects on adenyl cyclase and stimulatory effects on cGMP-phosphodiesterase, while Gα1 family members stimulate phospholipase C isoforms, promoting hydrolysis of phosphatidylinositol bisphosphate to produce diacylglycerol and inositol trisphosphate [9,10]. Gα signaling is terminated by intrinsic hydrolysis of bound GTP to GDP, a reaction accelerated by regulators of G-protein signaling (RGS proteins), and reversion of the Gα switch conformation to the inactive, GDP-bound state [9,11]. Gα-GDP can then re-assemble a heterotrimer with Gβγ or, in the case of the Gα1 subfamily, engage GoLoco motif
Author Summary

Heterotrimeric G-proteins function as molecular switches to convey cellular signals. When a G-protein coupled receptor encounters its ligand at the cellular membrane, it catalyzes guanine nucleotide exchange on the Gα subunit, resulting in a shift from an inactive to an active conformation. G-protein signaling pathways are conserved from mammals to plants and fungi, including the rice blast fungus *Magnaporthe oryzae*. A mutation in the Gα subunit (G42R), previously thought to eliminate its GT-Pase activity, leading to constitutive activation, has been utilized to investigate roles of heterotrimeric G-protein signaling pathways in multiple species of filamentous fungi. Here, we demonstrate through structural, biochemical, and cellular approaches that G42R mutants are neither GT-Pase deficient nor constitutively active, but rather are unable to transition to the activated conformation. A direct comparison of *M. oryzae* fungal strains harboring either G42R or truly constitutively activating mutations in two Gα subunits, MagA and MagB, revealed markedly different phenotypes. Our results suggest that activation of MagB is critical for pathogenic development of *M. oryzae* in response to hydrophobic surfaces, such as plant leaves. Furthermore, the lack of constitutive activity by Gα(G42R) mutants prompts a re-evaluation of its use in previous genetic experiments in multiple fungal species.

proteins that are also selective for the inactive Gα state [12]. In addition to naturally occurring conformationally selective binding partners, phage display peptides have also been engineered to discriminate between Gα-GDP and Gα-GTP. For example, the peptides KB-752 and KB-1753 selectively interact with the inactive GDP-bound and active GTP-bound states of Gαi1, respectively [13].

Heterotrimeric G-protein signaling components are well-characterized regulators of mammalian biology and are also utilized as sensors for extracellular cues in non-mammalian organisms, such as fungi, plants, and yeast [7,14,15]. The rice blast fungus, *Magnaporthe oryzae*, forms infection structures known as appressoria in response to specific environmental surface signals [16]. For example, hydrophobic, but not hydrophilic surfaces, promote appressorium formation [17–19]. Genetic studies have implicated a number of G-protein signaling pathway components in the regulation of *M. oryzae* growth, sexual reproduction, and appressorium formation [20–22]. Additionally, an RGS protein (Rgs1) catalyzes guanine nucleotide exchange on the Gα subunit, *M. oryzae* Rgs1(Q204L) and the RGS-insensitive Gα(G42R) mutants suggest a role for heterotrimeric G-protein signaling in *M. oryzae* appressorium formation [24,25].

Among the most stringently conserved motifs of Gα subunits is the phosphate-binding loop (P-loop) (Figure S1). Very little variation in the P-loop sequence is seen across Gα subunits in distantly related species, including plants, fungi, and metazoans [27]. In fact, the P-loop is also conserved as a key phosphoryl group-interacting motif in ATP-binding kinases and members of the Ras GT-Pase superfamily [28].

A P-loop mutation to human Ras isoforms, Gly-12 to valine, is frequently found in human cancers. Ras G12V mutants are GT-Pase deficient, and thus constitutively active, leading to aberrant signaling and oncogenesis [29]. In fact, mutation of H-Ras Gly-12 to any residue other than proline results in constitutive activity [30]. Mutation of the corresponding P-loop residue in Gαi1, Gly-42 to valine, also drastically reduces its GT-Pase activity [31]. Structural studies of Gαi1(G42V) suggest that the introduced valine side chain sterically prevents appropriate positioning of Gln-204, a residue that coordinates a nucleophilic water molecule during GT-Pase hydrolysis [31]. This glutamine is highly conserved and critical for GT-Pase activity; its mutation to leucine (“Q/L”) in Ras GT-Pases or Gα subunits also leads to constitutive activity [11,29].

To understand how the G42R P-loop substitution affects Gα subunit structure and function, we obtained a 3.0 Å resolution crystal structure model of Gαi1(G42R) bound to GDP using the...
inactive state-selective phage display peptide KB-752 as a crystallography tool [49]. The asymmetric unit contained three \( \alpha_i(\text{G42R}) \) subunits bound to GDP and Mg\(^{2+} \); two of three monomers were bound to the KB-752 peptide, while the third (chain C) lacked electron density for the peptide and instead displayed switch region disorder characteristic of free, GDP-bound G\(\alpha\) subunits [31]. For data collection and refinement statistics, see Table S1. A comparison of our model with that of wild type G\(\alpha_i\)-GDP/KB-752 (PDB id 1Y3A) revealed minor perturbations to the inactive state upon introduction of Arg-42 (Figure 1A). The side chain of Arg-42 projects away from the nucleotide-binding pocket, making no direct contacts with other G\(\alpha_i\)(G42R) residues. Switch 1 and the adjacent \( \beta_2 \) strand adopt slightly different conformations in the mutant G\(\alpha_i\) (C\(\alpha\) atoms r.m.s.d. 1.3 \( \text{Å} \)), likely

![Figure 1](https://example.com/f1.png)

**Figure 1. A crystal structure of G\(\alpha_i\)(G42R)-GDP in complex with the phage display peptide KB-752.** (A) The overall structure of G\(\alpha_i\) (cyan) with switch regions in dark blue, bound to KB-752 (red) (current study; PDB 3QE0), is overlaid on the wild type G\(\alpha_i\)-GDP/KB-752 complex (wheat/red transparency) (PDB 1Y3A). GDP is represented by green sticks and magnesium by an orange sphere. (B) The Arg-42 side chain extends from the P-loop, making no polar contacts with other G\(\alpha_i\)(G42R) residues, but preventing the wild type (transparent) switch conformation. G\(\alpha_i\)(G42R) residues Arg-178 and Lys-180 are displaced relative to wild type due to steric and electrostatic repulsion by Arg-42. The G42R \( \beta_2 \) strand and switch 2 also adopt slightly different conformations. For crystallographic data collection and refinement statistics, see Table S2.

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because the basic residues Arg-178 and Lys-180 are electrostatically and sterically repelled from their wild type orientations by the positively charged Arg-42 side chain (Figure 1B). Arg-178 is known to stabilize the leaving phosphate group during GTP hydrolysis [11]; its perturbation in the Gαi1(G42R) structure model is consistent with the previously assumed GTPase deficiency of G42R mutants.

Gα(G42R) is not GTPase deficient

Substitution of the corresponding Gly-12 in H-Ras for any amino acid other than proline yields GTPase deficiency and constitutive activity [30]. Thus it was previously reasoned that Gα(G42R) mutants were also incapable of GTP hydrolysis [26]. Binding of GTP by purified Gα subunits can be assessed with the non-hydrolyzable GTP analog, the radionucleotide GTP\(^{35}\)S. Similarly, GTPase activity can be quantified by tracking release of radioactive inorganic phosphate from \([^{32}\text{P}]\text{GTP}\). GTP\(^{35}\)S radionucleotide binding and \([^{32}\text{P}]\text{GTP}\) single turnover hydrolysis assays indicated that the kinetics of GTP binding and hydrolysis by the equivalent G42R mutant GαoA(G42R), in the most frequent splice variant of the mammalian adenylyl cyclase inhibitory Gαo1, are not significantly different from wild type GαoA (Figure 2A,B). Since the nucleotide binding and hydrolysis rate of this G42R mutant was unexpectedly not perturbed, we further examined the effect of the G42R mutation on Gα interactions with known protein binding partners.

The G42R mutation disrupts Gα interactions with RGS domains

RGS proteins accelerate the intrinsic GTPase activity of Gα subunits by stabilizing the transition state for GTP hydrolysis, a conformation mimicked by Gα binding to GDP, AlF\(^{4}\)-, and Mg\(^{2+}\) [11]. Surface plasmon resonance (SPR) was utilized to detect optical changes upon injection of wild type or G42R mutant GαoA over a surface coated with immobilized GST-RGS12 in the presence of either GDP, GTP, the non-hydrolyzable GTP analog GTP\(^{35}\)S, or the hydrolysis transition state-mimetic GDP-\(\text{AlF}^{4}\)- [50]. The RGS domain of RGS12 bound selectively to wild type GαoA in its GDP-\(\text{AlF}^{4}\)-bound state (K\(_D\) = 1.27 ± 0.06 \(\mu\)M), as measured by surface plasmon resonance (SPR) [50]. However, GαoA(G42R) did not engage the RGS domain in any nucleotide state at concentrations up to 25 \(\mu\)M (Figure 2C,D), suggesting that G42R mutants do not adopt a typical GTP hydrolysis transition.

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![Figure 2](https://www.plospathogens.org/content/pat/8/2/e1002553.t002.large)

**Figure 2. GαoA(G42R) is not GTPase deficient, but retains a normal nucleotide cycle and does not interact with RGS domain.** (A) A comparison of radiolabeled GTP\(^{35}\)S binding by wild type GαoA (k\(_{\text{on}}\) = 0.087 ± 0.020 min\(^{-1}\) (s.e.m.)) and GαoA(G42R) (k\(_{\text{on}}\) = 0.062 ± 0.010 min\(^{-1}\) (s.e.m.)) identified no significant difference in the rate of GDP release and subsequent GTP analog binding. (B) GαoA(G42R) retained the ability to hydrolyze GTP (k\(_{\text{cat}}\) = 0.18 ± 0.05 min\(^{-1}\) (s.e.m.)) at a rate indistinguishable from wild type GαoA (k\(_{\text{cat}}\) = 0.19 ± 0.02 min\(^{-1}\) (s.e.m.)), as determined by single turnover hydrolysis assays. (C) Surface plasmon resonance (SPR) experiments demonstrated selective binding of the transition state-mimetic, GDP-\(\text{AlF}^{4}\)-bound form of GαoA to the RGS domain of RGS12. GαoA(G42R) did not interact with the RGS12 RGS domain in any nucleotide state at concentrations up to 25 \(\mu\)M (D). An equilibrium binding isotherm allowed quantification of wild type GαoA affinity for RGS12 (K\(_D\) = 1.27 ± 0.06 \(\mu\)M (s.e.m.)).

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state in the presence of AlF$_4^-$ and Mg$^{2+}$ (AMF), or alternatively that Arg-42 directly interferes with RGS domain binding. A superimposition of G$\alpha_i$(G42R)/KB-752 and the G$\alpha_i$/RGS complex (PDB 1AGR; not shown) indicated that the mutant arginine side chain likely directly perturbs the RGS-binding surface. To further characterize nucleotide state-dependent interactions of G$\alpha$(G42R), we measured binding affinity toward three additional state-selective G$\alpha$-binding partners: G$\beta\gamma$ subunits, a GoLoco motif, and a phage display peptide, KB-1755 [13].

G$\alpha$(G42R) preferentially engages inactive conformation-selective binding partners in any nucleotide state

G$\alpha$ subunits in their GDP-bound, inactive conformations form heterotrimers with G$\beta\gamma$ subunits [6], and the interaction is disrupted by AlF$_4^-$ or GTP binding to the G$\alpha$ subunit. As expected, wild type G$\alpha_i$:GDP bound G$\beta\gamma_1$, as measured by SPR, but activation of the G$\alpha$ subunit with GDP-AlF$_4^-$ prevented association with G$\beta\gamma$ (Figure 3A). However, G$\alpha_i$(G42R) engaged G$\beta\gamma_1$ in both nucleotide states. Interaction of G$\alpha$ subunits with fluorophore-labeled peptides was assessed by detecting differences in fluorescence polarization between low molecular weight free peptide and the higher molecular weight G$\alpha$/peptide complex [51]. Similar to G$\beta\gamma$, the GoLoco motif of RGS14 was highly selective for binding the GDP-bound, inactive state of wild type G$\alpha_i$(GDP) over the activated GDP-AlF$_4^-$-bound form, as determined by fluorescence polarization (Figure 3B). G$\alpha_i$(G42R) displayed a much reduced selectivity for RGS14 GoLoco motif binding between the GDP and AlF$_4^-$ nucleotide states, being only 3-fold selective for the GDP form, whereas wild type G$\alpha_i$ is >1000-fold selective. Finally, we tested two G42R mutant nucleotide states for interaction with the active conformation-selective phage display peptide KB-1753 using fluorescence polarization [13]. As expected, KB-1753 selectively interacted with wild type G$\alpha_i$:GDP-AlF$_4^-$ (K$_D$ = 470 ± 40 nM) relative to GDP-bound G$\alpha_i$ (Figure 3C). In contrast, G$\alpha_i$(G42R) displayed only weak affinity for KB-1753 in either nucleotide state, as measured by fluorescence polarization. Together these data indicate that G$\alpha_i$(G42R) mutants preferentially engage inactive conformation-selective binding partners regardless of the bound nucleotide. To assess the conformational shift of G$\alpha$(G42R) mutants upon activation with AlF$_4^-$ or a non-hydrolyzable GTP analog, we utilized intrinsic tryptophan fluorescence and limited trypsin proteolysis.

G$\alpha$(G42R) cannot assume the transition state-mimetic or activated conformations

Upon binding GDP-AlF$_4^-$ or GTP analogs, G$\alpha$ subunits undergo conformational changes dominated by the three switch regions [52]. A tryptophan residue (Trp-211 in G$\alpha_i$) within switch 2 is shifted from a solvent-exposed to a buried orientation, resulting in a reduced efficiency of tryptophan fluorescence quenching that can be detected upon excitation of the G$\alpha$ protein with light at 284 nm wavelength [5]. Wild-type G$\alpha_i$ displayed a large increase in tryptophan fluorescence upon exposure to AlF$_4^-$, indicative of a shift to the activated conformation. In contrast, the shift in tryptophan fluorescence of G$\alpha_i$(G42R) at the same concentration was blunted relative to wild type and occurred with faster kinetics ($k_{obs}$ = 0.19 ± 0.01 s$^{-1}$ [95% C.I.], compared to $k_{obs}$ = 0.05 ± 0.01 s$^{-1}$ for wild type G$\alpha_i$; Figure 4A).

The active and inactive states of G$\alpha$ subunits are also differentially sensitive to proteolysis by trypsin, the more flexible loop conformations of G$\alpha$-GDP promote cleavage [4]. While the flexible N-terminus of wild type G$\alpha_i$ was cleaved in all three nucleotide states, the resulting ~38 kDa fragment was resistant to limited trypsin proteolysis in the GDP-AlF$_4^-$ or GTP-bound conformations relative to the inactive, GDP-bound form (Figure 4B). G$\alpha_i$(G42R), however, was readily proteolysed in any nucleotide state. Addition of AlF$_4^-$ had no detectable effect on G$\alpha_i$(G42R) resistance to trypsin proteolysis, while GTP/S provided only mild protection of the ~38 kDa species compared to that of wild type G$\alpha_i$. These data further support the hypothesis that the switch regions of G$\alpha$(G42R) mutants do not assume appropriate transition state-mimetic or activated state conformations in the presence of AlF$_4^-$ and GTP/S, respectively.

The Arg-42 side chain prevents transition of the switch regions to an active conformation

We next sought a structural explanation for the disrupted conformational switch of G$\alpha$(G42R) mutants. As previously mentioned, the Arg-42 side chain conformation, as modeled in the free GDP-bound G$\alpha_i$(G42R), would not allow glutamine-204 to assume its critical position for orienting the nucleophilic water required for GTP hydrolysis (Figure 1). However, unlike the G42V mutant of G$\alpha$ subunits, the G42R mutant retains normal GTP hydrolysis kinetics (Figure 2). Positioning of Glu-204 for hydrolysis may be possible if the Arg-42 side chain adopts an alternate rotamer. We also crystallized G$\alpha_i$(G42R)/GDP in complex with the GoLoco motif from RGS14 and derived an independent structural model at 2.8 Å resolution (Table S2). In one of the two monomers of the asymmetric unit, Arg-42 adopts an alternative rotamer that would allow Glu-204 to orient the nucleophilic water for hydrolysis (Figures 4C and S2).

Since we are presently unable to crystallize G$\alpha_i$(G42R) in either its GDP-AlF$_4^-$ or GTP analog-bound states, we superimposed our structural model of G$\alpha_i$(G42R)-GDP (excluding the RGS14 GoLoco peptide) with the previously described, wild type G$\alpha_i$(GTPyS) (PDB id 1GIA) (Figure 4C,D). In the activated, GTP/S-bound state of wild type G$\alpha_i$, switches 1 and 2 converge on the nucleotide γ-phosphoryl group, while Glu-236 of switch 3 forms a new polar contact with the backbone of switch 2 [3]. The result is a convergence of the three switch regions near the P-loop to form a stable interface recognized by effector molecules. Superposition of G$\alpha_i$(G42R)-GDP suggests that the bulky Arg-42 side chain would not be easily accommodated by the active switch conformations observed in wild type G$\alpha_i$, switches 1 and 2 converge on the nucleotide γ-phosphoryl group, while Glu-236 of switch 3 forms a new polar contact with the backbone of switch 2 [3]. The result is a convergence of the three switch regions near the P-loop to form a stable interface recognized by effector molecules. Superposition of G$\alpha_i$(G42R)-GDP suggests that the bulky Arg-42 side chain would not be easily accommodated by the active switch conformations observed in wild type G$\alpha_i$, switches 1 and 2 converge on the nucleotide γ-phosphoryl group, while Glu-236 of switch 3 forms a new polar contact with the backbone of switch 2 [3]. The result is a convergence of the three switch regions near the P-loop to form a stable interface recognized by effector molecules.

The G42R mutant is not constitutively active and displays a blunted response to stimulation by AlF$_4^-$

To investigate the effects of G42R mutants in a signaling pathway context, we introduced the corresponding P-loop mutation into the phospholipase C stimulating mammalian G$\alpha$ subunit, G$\alpha_q$(G48R). Wild-type G$\alpha_q$:GTP activates phospholipase Cβ (PLCβ), which in turn hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to yield diacyl glycerol (DAG) and inositol

G$\alpha$(G42R) Fails to Attain an Active Conformation

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triphosphate (IP3) [10]. Phospholipase C activity can be quantified by measuring accumulation of radioactive IP3 in cells pre-treated with tritiated inositol. Overexpression of wild type Gαq in COS-7 cells had little effect on inositol phosphate accumulation, while the GTPase-deficient and constitutively active Gαq(Q209L) markedly stimulated PLCb activity in a dose-dependent fashion (Figure 5A,B). Gαq(G48R), however, had no significant effect on PLCβ activity when overexpressed, confirming its lack of constitutive activity. Activation of PLCβ by endogenous and overexpressed Gαq can be stimulated by exposure to AlF4−, since Gαq-GDP-AlF4− has high affinity for PLCβ [33]. As expected, endogenous Gαq was activated by AlF4−, and the effect was enhanced by overexpression of wild type Gαq. However, overexpressed Gαq(G48R) did not respond to AlF4− stimulation to the same extent as wild type Gαq, reflecting its inability to assume a fully-activated conformation (Figure 5C,D).

Figure 3. Gαi1(G42R) engages inactive conformation-selective binding partners in two nucleotide states. (A) Wild type Gαi1 binds Gβ1γ1 only in the GDP-bound state, as determined by SPR, while Gαi1(G42R) displayed no nucleotide state-selectivity of Gβ1γ1 binding when liganded with either GDP or GDP-AlF4−. (B) Similarly, fluorescence polarization experiments showed highly nucleotide state-selective binding of the RGS14 GoLoco motif to wild-type Gαi1-GDP (Kd = 9.0±1.1 nM (s.e.m.)) compared to the AlF4−-bound form (Kd = 8.7±1.0 μM (s.e.m.)), but both nucleotide states of Gαi1(G42R) interacted with the GoLoco motif peptide, with affinity constants of 45±7 nM (s.e.m.) and 168±27 nM (s.e.m.) for GDP and AlF4−, respectively. (C) The activated state-selective peptide KB-1753 preferentially bound the AlF4−-bound form of wild-type Gαi1 (Kd = 470±40 nM (s.e.m.)) compared to the GDP-bound form (Kd = 6.7±0.4 μM (s.e.m.)), but had low affinity for Gαi1(G42R) in both nucleotide states.

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The Gα(G42R) mutant utilized in genetic studies of fungal species, such as Aspergillus nidulans and the rice blast fungus Magnaporthe oryzae, was assumed to be GTPase deficient and thus constitutively active [26,32], and has been used extensively to understand the biology of fungal G-protein signaling [19,26,32–41]. Since the biochemical and structural characterization of such G42R mutants (Figures 1–4 above) indicate intact GTPase activity and, instead of constitutive activity, an inability to assume the activated conformation, we sought to clarify the behavior of G42R mutations in the Gα subunits of M. oryzae. G42R and Q204L mutants of M. oryzae Gα subunits exhibit different effects on appressorium formation. We directly compared strains of M. oryzae harboring mutations in the Gα subunits MagA or MagB. Since both Gα subunits are known to regulate appressorium formation in response to inductive, hydrophobic surfaces [24], we assessed appressorium formation by GTPase-deficient Q/L and non-activatable G42R mutant strains on both hydrophobic and hydrophilic surfaces. The magA(G45R) mutant formed slightly fewer appressoria on hydrophobic, inductive surfaces than wild-type M. oryzae, but maintained the differential response to surface hydrophobicity (Figure 6A,B). In contrast, approximately 35% of magA(Q208L) conidia formed highly pigmented appressoria, albeit aberrant, after 16 hours, regardless of surface hydrophobicity. The magB(G42R) mutant strain resembled magA(Q208L), with 30% appressorium formation independent of surface hydrophobicity (Figure 6C,D). The magB(Q204L) strain, however, formed very few appressoria on either surface. To further characterize differences between magA and magB G42R and Q/L mutant strains of M. oryzae, we compared colony

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and conidia morphology, as well as conidiation, to the wild type fungus. Both the magA and magB G42R mutants displayed different overall morphology from the corresponding Q/L mutants (Figure S3). In the case of magA(G45R), morphology was indistinguishable from the wild type. Upon exposure to light, the magA(G45R) also produced slightly fewer conidia when compared to the wild-type *M. oryzae*, but magA(Q208L) formed very few heavily pigmented, aberrant conidia (Figure 6A, inset and S4A). Both magB(G42R) and magB(Q204L) displayed enhanced conidiation relative to wild type, but those of magB(Q204L) were of a distinct morphology, with longer and thinner dimensions than either magB(G42R) or wild type (Figure S4B, C).

These data indicate that fungal Gα G42R mutants exhibit markedly different phenotypes from truly GTPase-deficient Q/L mutants, consistent with aforementioned structural, biochemical, and cellular experiments that indicate an intact GTPase activity, but a marked inability to achieve an activated conformation.

*M. oryzae* expressing either G42R or Q204L mutant Gα subunits have differential effects on pathogenesis

We next determined what effect the introduction of the non-activatable G42R mutant Gα subunits has on fungal infection of barley leaves compared to constitutively active Q/L mutants. As expected, barley leaves inoculated with wild type *M. oryzae* showed the characteristic dose-dependent formation of disease lesions (Figure 7). The magA(G45R) strain showed similar pathogenicity as the wild type, consistent with intact surface-inducible appressorium formation (Figure 6B). magB(G42R) displayed a reduced ability to cause disease, although small lesions were observed at the highest inoculations tested. Both magA(Q208L) and magB(Q204L) showed drastically reduced lesion formation relative to wild type and the corresponding G42R mutants. These data indicate that constitutive activity of either MagA or MagB can suppress the ability of *M. oryzae* to penetrate and infect the plant tissue. Additionally, we conclude that the ability of MagB to achieve its activated conformation is critical for *Magnaporthe* pathogenesis.

**Discussion**

Mutant Gα subunit strains have provided excellent tools for probing the functions of heterotrimeric G-proteins in many fungal species, including *Aspergillus nidulans* and *Magnaporthe oryzae* (Table S1) [19,26,32–41]. Here, we have demonstrated that the P-loop mutant, G42R, is neither GTPase deficient nor constitutively active as assumed in previous studies. Rather, Gα(G42R) is unable to undergo a typical conformational change upon binding GTP, reflected by its inability to engage RGS domains or effector-like molecules. Consistent behavior of Gα(G42R) muta-
Figure 6. M. oryzae strains expressing G42R or GTPase-deficient Q204L mutant Gα subunits show disparity in appressoria formation. (A) Conidia harvested from the magA<sup>G45R</sup>, magA<sup>G208L</sup> and WT strains were inoculated on inductive (plastic cover slips) or non-inductive surfaces (GelBond membrane) and assessed for the ability to form appressoria after 16 hpi (hours post inoculation). The 2-celled conidia (white arrow) of the magA<sup>G208L</sup> produced aberrant appressorium (white asterisk) on both inductive and non-inductive surfaces. Insets represent the highly pigmented structures (black arrowhead) made by the magA<sup>G208L</sup> strain. Scale bars = 10 μm. (B) Bar graph illustrating the efficiency of appressorium formation in the magA<sup>G45R</sup>, magA<sup>G208L</sup> and WT strains on inductive (black bar) or non-inductive surfaces (gray bar) respectively. Values represent mean ± S.E from three independent replicates involving 300 conidia per sample. (C) Identical experiments were conducted on the corresponding magB wild type and mutant strains. Unlike the wild type, the majority of conidia from the magB<sup>G42R</sup> strain failed to produce melanized appressoria efficiently on inductive surfaces. A small proportion of the magB<sup>G42R</sup> conidia produced mature appressoria on the non-inductive surface (indicated by the white arrow). Conidia from the magB<sup>G204L</sup> failed to produce appressoria on both inductive and non-inductive surfaces. (D) Bar graph showing quantification of appressorium formation, as in (B).

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Despite the retained ability of Gα(G42R) mutants to exchange and hydrolyze nucleotide, they favor an inactive state-like conformation, likely forming a less-dissociable heterotrimer with Gβγ in a cellular context, thereby reducing Gβγ/effector interactions. Since Gα(G42R) does not engage effectors with high affinity, it may be expected to behave as a dominant negative mutation; the Gα(G42R)/Gβγ heterotrimer may serve as a substrate for receptor-stimulated exchange but fail to activate downstream signaling pathways. In Magnaporthe oryzae, it was previously unclear why strains with magB deleted or expressing the assumedly constitutively active magB<sup>G142R</sup> exhibited similar phenotypes regarding conidiation, sexual reproduction, and virulence on plant leaves [26]. The present study resolves this issue by demonstrating that the G42R mutant is not constitutively active, but likely exerts a dominant negative effect. The distinct behaviors of Gα(G42R) mutants are highlighted by a direct comparison to the truly GTPase-deficient and constitutively active Q/L mutants.

Although the magA<sup>G145R</sup> and magB<sup>G42R</sup> mutant strains do not reflect constitutive Gα subunit activity, as previously assumed [26,32], they do provide insight into fungal pathogenic development. A phenotypic deficiency upon expression of a Gα(G42R) mutant suggests that specific activation of the Gα of interest and subsequent engagement of its downstream effectors is necessary for a particular function of a cell or organism. For instance, both magB deletion [24] and magB<sup>G42R</sup> mutant strains display...
drastically reduced induction of appressoria by hydrophobic surfaces, while magA deletion [24] and magA G45R mutations each have minimal effects. Thus, it is likely that MagB transduces an external surface hydrophobicity signal, presumably through a GPCR. Use of the magB G42R mutant suggests that the conformational change accompanying MagB activation is necessary for the selective development of appressoria on hydrophobic surfaces (Figure S6). It remains to be determined whether the Gα or Gβγ subunits or both propagate signals required for appressorium formation and disease lesion formation in M. oryzae.

In conclusion, Gα(G42R) mutants are incapable of assuming a typical activated conformation, but their retained ability to hydrolyze GTP indicates an uncoupling of conformational change and enzymatic activity. Since G42R mutants are unable to separate from Gβγ or to activate effectors, they provide tools for dissecting the functions of Gα subunits in cellular contexts. Utilizing both G42R and constitutively active Q/L mutants of two Gα subunits, we postulate a critical role for MagB activation in response to growth on hydrophobic surfaces, leading to appressorium formation in the rice blast fungus, M. oryzae.

**Materials and Methods**

**Chemicals and other assay materials**

Unless otherwise noted, all chemicals were the highest grade available from Sigma or Fisher Scientific. Peptides were synthesized by Fmoc (N-(9-fluorenyl)methoxycarbonyl) group protection, purified by HPLC, and confirmed using mass spectrometry by the Tufts University Core Facility (Medford, MA). Peptides used for crystallography and biophysical studies have been previously reported: FITC-RGS14 GoLoco [55], RGS14 GoLoco [56], FITC-KB-1753 [13], and KB-752 [49].
Protein purification

Although we were unable to obtain properly folded, purified M. oryzae Gα subunits, the P-loop and surrounding switch regions are highly conserved from mammals to fungi (Figures S1). Thus, we utilized the readily available purified Gαi and GαN and corresponding GαR mutants. For biochemical experiments, full-length, hexahistidine-tagged Gαi and GαN and GαR mutants thereof, were purified from E. coli by NTA affinity and gel filtration chromatography as previously described [57] (see Figure S5). A GST fusion of the RGS12 RGS domain (aa 664–885) was purified as described [58]. Biotinylated Gβγ was purified as described [59]. For crystallization, an N-terminally affinity chromatography; the hexahistidine tag was cleaved by TEV protease, and the Gα subunit further purified by ion exchange (SourceQ, GE Healthcare) and gel filtration chromatography. Purified Gαi(G42R) was loaded with excess GppNHp or GDP for 3 hours at room temperature and concentrated to 15 mg/mL in GppNHp crystallization buffer (50 mM HEPES pH 8.0, 10 mM MgCl2, 10 mM GppNHp, 1 mM EDTA, 5 mM DTT) or GDP crystallization buffer (10 mM Tris pH 7.5, 1 mM MgCl2, 5% v/v glycerol, 5 mM DTT).

Crystallization and structure determination

The complex of Gαi(G42R) and synthetic KB-752 peptide was obtained by mixing a 1:1.5 molar ratio of protein to peptide in GppNHp crystallization buffer. Despite loading of Gαi(G42R) and crystallization in the presence of GppNHp, the crystal lattice contained Gαi(G42R) liganded with GDP and bound to KB-752. The selectivity of KB-752 for the GDP bound state [49] may account for the apparent absence of GppNHp. Crystals of Gαi(G42R)-GDP/KB-752 were obtained by vapor diffusion from hanging drops containing a 1:1 (v/v) ratio of protein/peptide solution to well solution (17% v/v PEG MME 5000, 200 mM MgCl2, 100 mM HEPES pH 7.0). Hexagonal rod crystals (~300×100×100 μm) formed in 5 days at 18°C exhibited the symmetry of space group P6122 \( (a = b = 106.6, c = 455.1, \text{and } a = b = 90°, c = 120°) \) and contained two Gαi(G42R)-GDP/KB-752 dimers and one Gαi(G42R)-GDP monomer in the asymmetric unit. For data collection at 100K, crystals were serially transferred into well solution supplemented with 30% saturated sucrose in 10% increments for ~30 s, followed by plunging into liquid nitrogen. A native data set was collected at the SER-CAT 22-ID beamline at the Advanced Photon Source (Argonne National Laboratory). Data were processed using the HKL-2000 program [60]. The crystal structure of the wild type Gαi/KB-752 heterodimer (PDB 1Y3A [49]), excluding the peptide, nucleotide, and waters was used as a molecular replacement search model. All structural images were made with PyMOL (Schrodinger LLC, Portland, OR).

Nucleotide binding and hydrolysis assays

The \([^35]S\)GTPγS filter-binding assay used to measure rates of spontaneous GDP release from wild type and mutant GαN was conducted as described previously [64]. Intrinsic GTP hydrolysis rates of GαN and mutants were assessed by monitoring \[^32P\]-labeled inorganic phosphate production during a single round of GTP hydrolysis as described previously [65].

Surface plasmon resonance assays

Optical detection of protein/protein interactions by surface plasmon resonance was performed using a Biacore 3000 (GE Healthcare). Carboxymethylated dextran (CM5) sensor chips (GE Healthcare) with covalently bound anti-GST antibody surfaces were created as described previously [50]. The GST-RGS12 RGS domain protein and GST alone (serving as a negative control) were separately immobilized on SPR chip surfaces. Biotinylated Gβγ1 and mNOTCH peptide (serving as a negative control) were separately immobilized on a streptavidin (SA) sensor chip (GE Healthcare) as described previously [50].

Fluorescence polarization measurements

All polarization experiments were conducted using a PHER-Astar microplate reader (BMG Labtech, Offenburg, Germany), essentially as described previously [51].

Intrinsic tryptophan fluorescence measurements of Gα activation

Changes in tryptophan fluorescence of Gαi subunits were measured to assess activation by GDP-AlF4⁻, as described previously [51]. Activation of Gαi subunits resulted in translocation of a conserved switch 2 tryptophan into a hydrophobic pocket, increasing the quantum yield of tryptophan fluorescence [5]. Fluorescence intensity traces shown are representative of triplicate experiments.

Limited trypsin proteolysis

Gα subunits are relatively protected from trypsin-mediated proteolysis in the GDP-AlF4⁻ and GTP analog-bound, activated states [4]. Ten μg of wild type or mutant Gαi in 50 mM HEPES (pH 8.0), 1 mM EDTA, 5 mM DTT, 0.05% CM12E10, and 10 mM MgCl2 were incubated for three hours at room temperature with either 100 μM GDP, 100 μM GTPγS, or 100 μM GDP, 20 mM NaF, and 60 μM AlCl3. Five hundred ng of N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin was added to each reaction, followed by a 10-minute incubation at room temperature. Proteolysis was stopped by addition of SDS-PAGE sample buffer and boiling. Samples were subjected to SDS-PAGE and stained with Coomassie Blue.

Quantitation of phospholipase C (PLC) activity

COS-7 cells in 12-well culture dishes were transfected with KT3-tagged wild type or mutant Gαq, metabolically labeled with 1 μCi of \([^3]H\)inositol/well and assayed for inositol phosphate production during a single round of receptor-mediated phospholipase C activation.
accumulation using Dowex chromatography as described previously [66]. For AlF₄⁻ stimulation experiments, final concentrations of 10 mM NaF and 30 μM AlCl₃ were added to cell media. To determine wild type and mutant Gα₁₄ expression levels, cells were lysed in SDS-PAGE sample buffer. Proteins separated by electrophoresis were immunoblotted with anti-KT3 antibody (Covance) or anti-actin antibody (Sigma).

Fungal strains, growth, and culture conditions

The *M. oryzae* wild-type strain B157 was obtained from the Directorate of Rice Research (Hyderabad, India). *Magnaporthe* strains carrying individual point mutations in the Gα₁₄ subunits, namely: *magA*<sup>G42R</sup>, *magA*<sup>G204R</sup>, *magB*<sup>i1(G42R)</sup>, *magB*<sup>G42R</sup>, have been described previously together with the *rgs1Δ* mutant [19]. Wild type and mutant strains cultures were maintained at 28°C in the dark on Prune Agar medium plates (PA; per L: 40 mL prune juice, 5 g lactose, 5 g Sucrose, 1 g yeast extract and 20 g agar, pH 6.5). Assessment of the radial growth, aerial hyphae and colony characteristics was carried out as previously described [22]. Conidiation was induced in the *Magnaporthe* colonies through exposure to continuous incandescent light at room temperature for 6 days.

Evaluation of conidiation status

Conidia were harvested by scraping the surface growth in water with an inoculation loop. The suspension was filtered through two layers of Miracloth (Calbiochem, San Diego, USA), collected in Falcon tubes (BD Biosciences, USA), vortexed for a minute to ensure complete detachment of conidia from the mycelia, and then pelleted by centrifugation at 3,000 rpm for 15 minutes. The conidia were washed twice and re-suspended in a fixed volume of sterile water. Prior to harvesting the spores, the radius of each colony was measured to calculate the surface area of the colony. Conidia produced by a given colony were quantified using a hemocytometer and reported as the total number of conidia present per unit area of the colony.

Appressoria formation assays

Droplets (20 μL containing 500 conidia) of conidal suspension were placed on plastic cover slips (hydrophobic surface) or hydrophilic side of GelBond membrane (Lonza Walkersville Inc., USA) and incubated in a humid chamber at room temperature. The total number of appressoria formed by each strain on either surface was quantified at 16 hpi (hours post inoculation).

Evaluation of pathogenicity in *Magnaporthe* strains

For pathogenicity assays, leaves from two week old barley seedlings were cut into smaller pieces (2–3 cm long) and washed in sterile water, following which the leaf bits were rinsed for 45 seconds in 40% ethanol. The leaf pieces were then washed twice with sterile antibiotic-containing distilled water. The washed leaves were placed on kinetin agar plates (2 mg/mL kinetin, 1% agar). Conidia were quantified and a dilution series of the conidial suspension was inoculated on detached barley leaves at the required concentrations. The samples were incubated in a humidified growth chamber with a 16 h light/8 h dark cycle at 22°C. Disease symptoms were assessed 5–7 days post inoculation.

Microscopic analysis

Samples were observed on a BX51 (Olympus, Japan) microscope equipped with UPlan FL N 60X/1.25 Oil objective with appropriate filter sets. Bright field images were captured using a Cool SNAP HQ camera (Photometrics, USA) and processed using Image J (National Institutes of Health, USA), MetaVue (Universal Imaging, USA) and Adobe Photoshop 7.0 (Adobe Inc, USA).

Supporting Information

**Figure S1** The Gα₁₄ subunit P-loop is highly conserved in fungi and mammals. The β1 strands, z1 helices, and intervening P-loops (grey), as well as the three switch regions of selected Gα subunits from humans and fungi are aligned. Nucleotide contacting residues are highlighted by black circles, and the mutated glycine by an arrowhead. (EPS)

**Figure S2** Arg-42 adopts an alternate rotamer in the crystal structure model of Gα₁₄(G42R)-GDP/RGS14 GoLoco motif. Gα₁₄(G42R) is shown in cyan with switch regions in dark blue and selected side chains in sticks. GDP is represented as green sticks, and a portion of the RGS14 GoLoco motif is orange. GoLoco motif residues 311 and 312 were disordered in the crystal structure; the cartoon shown is truncated at residue 510 (PDB 3QJ2). The side chain of Arg-42 adopts a different rotamer than that seen in Gα₁₄(G42R)-GDP/KB-752 (magenta sticks). Instead, the Arg side chain forms direct polar contacts with Glu-245 of Gα₁₄(G42R) and the backbone carbonyl group of Val-507 from the RGS14 GoLoco motif. Arg-42 also coordinates a well-ordered water molecule (yellow sphere) with Arg-242 and Gln-147 of Gα₁₄(G42R). This Arg-42 rotamer would sterically prevent switch 3 from approaching the nucleotide upon binding to GTP. However, there is room for Arg-178 and Gln-204 to potentially assume their critical positions for GTP hydrolysis, providing a possible rationale for the normal GTPase activity of Gα₁₄(G42R). (EPS)

**Figure S3** *M. oryzae* colony and growth characteristics. Morphology of the *magA*<sup>G42R</sup>, *magA*<sup>G204R</sup>, *magB*<sup>i1(G42R)</sup>, *magB*<sup>B</sup>, WT (wild-type) and *rgs1Δ* colonies. The indicated strains were grown in the dark on prune agar medium for a week and photographed (upper panels). The *magB*<sup>B</sup> mutation lead to reduced rate radial growth. The radius of the *magB*<sup>B</sup> colony was 2.24±0.03 cm compared to 2.52±0.03 cm in the *magB*<sup>B</sup> or the WT strain, when grown under identical conditions for a period of seven days at 28°C in the dark. Values represent the mean ± SE (n=7 colonies per strain; p<0.001). The lower panels represent cross sections at near-medial planes. The *magB*<sup>B</sup> showed dramatic reduction in aerial hyphal growth, compared to the *magB*<sup>B</sup> and WT. The *magB*<sup>B</sup> and *magB*<sup>B</sup> mutants showed reduced aerial hyphal growth compared to the WT strain. (EPS)

**Figure S4** *M. oryzae* conidiation defects and conidial morphology. Comparative quantitative analysis of conidiation in the *magA*<sup>G42R</sup>, *magA*<sup>G204R</sup>, *magB*<sup>i1(G42R)</sup> and wild type strains. The indicated strains were initially grown in the dark for a day and then exposed to constant illumination for 6 days. Data represents mean ± SE based on three independent replicates. (A) Conidia per surface area unit were quantified for all five strains. Both *magA*<sup>G42R</sup> and *magA*<sup>G204R</sup> produced fewer conidia than wild type fungi, although *magA*<sup>G204R</sup> produced statistically significantly fewer conidia than *magA*<sup>G42R</sup>. The asterisk indicates the heavily pigmented aberrant structures and conidia with a single septum produced predominantly by the *magB*<sup>B</sup> mutant. *magB*<sup>B</sup> and *magB*<sup>B</sup> both displayed an increased number of conidia compared to wild type. (B) Conidia from *magB*<sup>B</sup> displayed a thin, elongated morphology, while those of *magB*<sup>B</sup> were similar to wild type. (C) The dimensions (length and width) of conidia
from the indicated strains were quantified. Values represent the mean ± SE (n = 200 conidia per strain).

**References**


