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Dual positive and negative regulation of GPCR signaling by GTP hydrolysis

Benjamin Smith [a,1], Claire Hill [b,1,2], Emma L. Godfrey [c,1], David Rand [d], Hugo van den Berg [d], Steven Thornton [c], Matthew Hodgkin [b], John Davey [b], Graham Ladds [c,*]

[a] Molecular Organization and Assembly of Cells Centre, University of Warwick Coventry CV4 7AL, UK
[b] Department of Biological Sciences, University of Warwick Coventry CV4 7AL, UK
[c] Division of Clinical Sciences, Warwick Medical School, University of Warwick Coventry CV4 7AL, UK
[d] Systems Biology Centre, University of Warwick Coventry CV4 7AL, UK

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ABSTRACT

G protein-coupled receptors (GPCRs) regulate a variety of intracellular pathways through their ability to promote the binding of GTP to heterotrimeric G proteins. Regulator of G protein signaling (RGS) proteins increases the intrinsic GTPase activity of Gα-subunits and are widely regarded as negative regulators of G protein signaling. Using yeast we demonstrate that GTP hydrolysis is not only required for desensitization, but is essential for achieving a high maximal (saturated level) response. Thus RGS-mediated GTP hydrolysis acts as both a negative (low stimulation) and positive (high stimulation) regulator of signaling. To account for this we generated a new kinetic model of the G protein cycle where $G\alpha_{GTP}$ enters an inactive GTP-bound state following effector activation. Furthermore, *in vivo* and *in silico* experimentation demonstrates that maximum signaling output first increases and then decreases with RGS concentration. This unimodal, non-monotone dependence on RGS concentration is novel. Analysis of the kinetic model has revealed a dynamic network motif that shows precisely how inclusion of the inactive GTP-bound state for the Gα produces this unimodal relationship.

1. Introduction

Vast numbers of cellular processes are regulated by the GTPase switch, including trans-plasma membrane signal transduction, control of cellular growth, vesicle and protein transport and cytoskeleton assembly. Increasing the number of GTP-bound G proteins enhances signaling, whereas increasing their intrinsic rate of GTP hydrolysis is believed to regulate the pathway negatively. Within signal transduction pathways, heterotrimeric G proteins (consisting of a nucleotide-binding Gα-subunit plus Gβγ-subunits) couple cell surface-expressed receptors to intracellular effectors, in many different cell types. Active G protein-coupled receptors (GPCRs) promote nucleotide exchange on the Gα-subunit, causing dissociation of the heterotrimer and subsequent effector activation [1,2]. Regulator of G protein signaling (RGS) proteins accelerate GTPase activity of the Gα-subunit (by acting as GTPase activating proteins; GAPs) thereby reducing production of second messengers from the effector [3,4].

The pheromone-response pathway in yeast provides a model G protein-coupled signal transduction pathway that controls cell conjugation and division [5]. The binding of pheromones to receptors activates an effector system via the heterotrimeric G protein, to initiate intracellular changes that regulate the transcription of mating genes [6,7]. Yeast is therefore free from the complication of multiple receptors, effectors and other regulatory proteins [7]. RGS proteins were originally described in *Saccharomyces cerevisiae* as negative regulators of the pheromone-response pathway, where they minimize spontaneous cell activation and enable cells to recover from stimulation in the absence of conjugation [8]. In *S. cerevisiae*, Gβγ-subunits stimulate effector activation, potentially making this pathway less affected by proteins that influence nucleotide hydrolysis on the Gα-subunit [9]. In contrast the fission yeast *Schizosaccharomyces pombe*, utilized the Gα-subunit of a classical heterotrimeric G protein, Gpa1, as its signal propagator of the pheromone-response pathway [10–12]. Briefly *S. pombe* cells, during their mating cycle, grow up a pheromone gradient produced by a cell of the opposite mating type. The pheromones, (P-factor or M-factor) upon detection by mating type specific GPCRs, activates Gpa1 which propagates the signal by promoting the activation of Ras1. This leads to stimulation of a mitogen-activated protein kinase (MAPK) cascade resulting in the activation of the transcription factor Ste11 [6,9]. In addition to the transcription of pheromone-dependent genes, *S. pombe* also undergoes a morphological change in response to pheromone. Responding cells continue to grow from the tip of the cell and elongate towards the source of the pheromone forming a shmoo [13]. It currently remains unknown how Gpa1 activates both pathways.

Deletion of *rgs1* in *S. pombe* increases ligand-independent signaling and reduces mating efficiency [14–16], which suggests that Rgs1 acts as a negative regulator of the pheromone-response as reported in *S. cerevisiae* [7]. Here we demonstrate that at high ligand concentrations, Rgs1 also plays a positive role in regulating the pheromone-response. Through

* Corresponding author. Tel.: +44 2476 528361; fax: +44 2476 523701.
E-mail address: Graham.Ladds@warwick.ac.uk (G. Ladds).
[1] These authors contributed equally to this work.
[2] Present address: Warwick HRI, The University of Warwick, Wellesbourne, Warwickshire CV35 9EF, UK.

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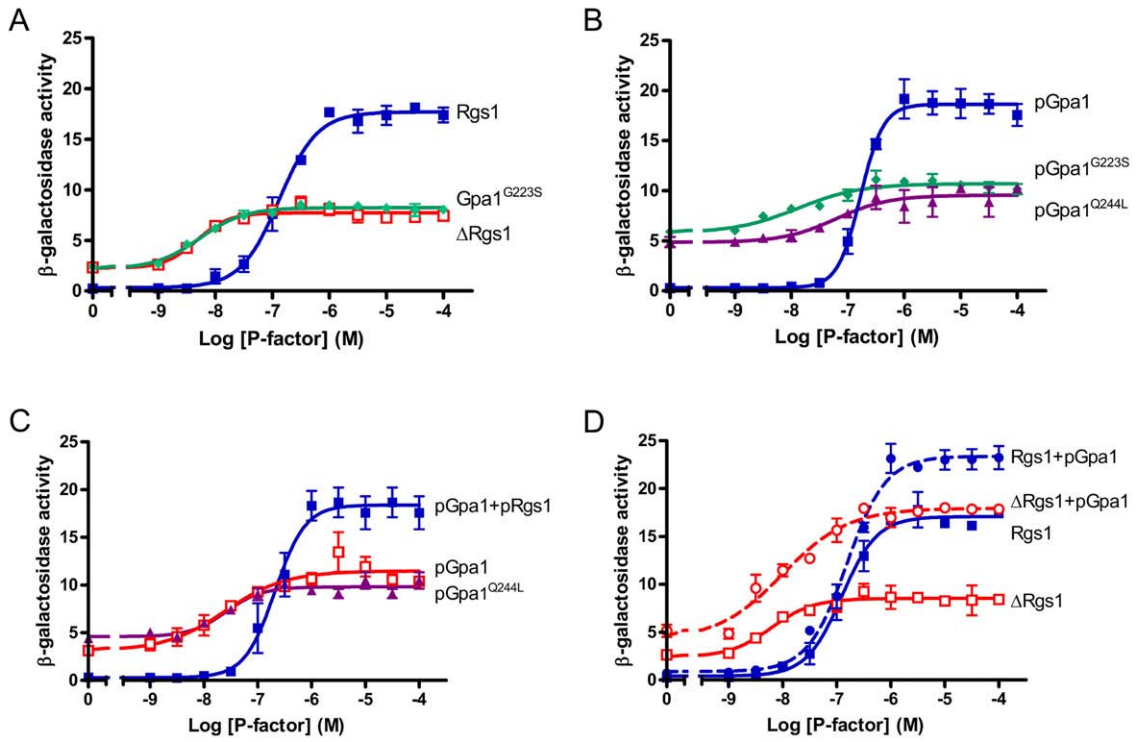

Fig. 1. Mutating residues essential for Gα-mediated GTP hydrolysis reduces maximal pheromone signaling independent RGS activity. Pheromone-dependent transcription of β-galactosidase expressed from the *sxa2>lacZ* reporter construct was measured in yeast strains; (A) expressing wild type Rgs1 (■), a chromosomally expressed RGS-insensitive form of Gpa1 ($Gpa1^{G223S}$; ◆) or deleted for Rgs1 (ΔRgs1; □), (B) deleted for the chromosomal copy of *gpa1* (JY1285) and expressing wild type (■), an RGS-insensitive ($Gpa1^{G223S}$; ◆) or GTPase deficient ($Gpa1^{Q244L}$, ▲) form of Gpa1 from a plasmid, (C) deleted for the chromosomal copies of both *gpa1* and *rgs1* (JY1287) and expressing plasmid-derived copies of Gpa1 (□), GTPase deficient $Gpa1^{Q244L}$(▲) and both wild type Rgs1 with wild type Gpa1 (■), (D) containing wild type Rgs1 with a single chromosomal copy (■) or a chromosomal copy supplemented with a plasmid copy of Gpa1 (●) of Gpa1, and the Rgs1 deleted strain containing a chromosomal copy (□) or chromosomal copy supplemented with a plasmid copy of Gpa1 (○). Results are means ± S.E.M. of triplicate determinations from three independent isolates.

targeted mutational analysis, we alter the GTPase activity on Gpa1 to demonstrate that GTP hydrolysis is absolutely required to achieve maximum signaling.

A mathematical model has been developed that reproduces *in vivo* data, and provides a novel mechanistic description of the G protein signaling pathway. Crucial to the faithful reproduction of the observed phenomena is the assumption that one $G\alpha_{GTP}$ activates only one effector molecule per round of guanosine nucleotide exchange and hydrolysis. Entry into an inactive but GTP-bound state following the activation of an effector removes the $G\alpha_{GTP}$ from the pool of molecules available for signaling, rather than allowing further effector activation by the same molecule. GTP hydrolysis, accelerated by an RGS protein, releases the Gα from this inactive GTP-bound state and allows subsequent reactivation by ligand-bound receptors and then activation of another effector molecule. Parallel *in vivo* and *in silico* experiments reveal a non-monotone relationship with a single maximum (i.e. unimodal) between pathway output and quantity of RGS protein. This non-monotone relationship reflects the dual positive/negative character of RGS regulation. An abstract dynamic network motif that captures the underlying structure of the more complex mathematical model is used to derive the non-monotone relationship explicitly. Finally, comparison of this motif with an alternative motif, whose structure underlies many current models of G protein signaling, shows why these current models are unable to account for non-monotone regulation by the RGS.

2. Materials and methods

2.1. Strains, reagents and general methods

The effector output from the pheromone-response pathway in *S. pombe* can be quantified using pheromone-dependent transcription of β-galactosidase expressed from the *sxa2>lacZ* reporter construct as described previously [16,17]. All yeast strains (Supplementary Table S9) have been described previously [12,16] with the exception of JY1340 which was derived from JY546 but had *gpa1* replaced with $gpa1^{G223S}$. Gene replacements were confirmed by polymerase chain reaction (PCR) and Southern blot analysis. General yeast procedures were performed as described [18,19]. Oligonucleotides were synthesized by Invitrogen Ltd. (Paisley, Scotland, UK). Amplification by PCR used *Pwo* DNA polymerase (Boehringer Mannheim Biochemicals, Lewes, UK) or *KOD* HiFi DNA polymerase (Merck Chemicals Ltd., Nottingham, UK). All constructs generated by PCR were sequenced.

2.2. Assay of β-galactosidase activity

β-galactosidase assays in *S. pombe* cells were performed as described previously [16,20]. *S. pombe* cells were cultured to a density of ~5×10^5 cells ml^{-1} in DMM and 500 μl aliquots transferred to 2-ml Safe-Lock tubes (Eppendorf, Hamburg, Germany) containing 5 μl of P-factor (in HPLC-grade methanol). Tubes were incubated at 29 °C for 16 h on a rotating wheel, and 50 μl transferred to 750 μl Z-buffer containing 2.25 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Reactions were stopped after 90 min by adding 200 μl of 2 M Na_2CO_3 and β-galactosidase activity calculated as optical density at 420 nm (OD_{420}) per 10^6 cells (determined using the Z2 Coulter Channelyzer) (Beckman Coulter, Luton, UK).

2.3. Plasmids

pREP3× contains the *LEU2* gene and pREP4× contains the *ura4* gene, both of which were controlled by thiamine-repressible *nmt1* promoter [21]. The production of all Gpa1 constructs with the exception of $Gpa1^{Q244L}$, have been described [12]. $Gpa1^{Q244L}$ was generated by bipartite PCR [22] on the wild type constructs. Generation of pREP3×-Rgs1 and