# Differential effects of RGS proteins on $G\alpha_q$ and $G\alpha_{11}$ activity

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**ABSTRACT**

Heterotrimeric G proteins play a pivotal role in GPCR signalling; they link receptors to intracellular effectors and their inactivation by RGS proteins is a key factor in resetting the pathway following stimulation. The precise GPCR:G protein:RGS combination determines the nature and duration of the response. Investigating the activity of particular combinations is difficult in cells which contain multiples of each component. We have therefore utilised a previously characterised yeast system to express mammalian proteins in isolation. Human $G\alpha_q$ and $G\alpha_{11}$ spontaneously activated the yeast pheromone-response pathway by a mechanism which required the formation of Gα-GTP. This provided an assay for the specific activity of human RGS proteins. RGS1, RGS2, RGS3 and RGS4 inhibited the spontaneous activity of both $G\alpha_q$ and $G\alpha_{11}$ but, in contrast, RGS5 and RGS16 were much less effective against $G\alpha_{11}$ than $G\alpha_q$. Interestingly, RGS2 and RGS3 were able to inhibit signalling from the constitutively active $G\alpha_q^{QL}$/$G\alpha_{11}^{QL}$ mutants, confirming the GAP-independent activity of these RGS proteins. To determine if the RGS-Gα specificity was maintained under conditions of GPCR stimulation, minor modifications to the C-terminus of $G\alpha_q$/$G\alpha_{11}$ enabled coupling to an endogenous receptor. RGS2 and RGS3 were effective inhibitors of both Gα subunits even at high levels of receptor stimulation, emphasising their GAP-independent activity. At low levels of stimulation RGS5 and RGS16 retained their differential Gα activity, further highlighting that RGS proteins can discriminate between two very closely related Gα subunits.

## 1. INTRODUCTION

G protein-coupled receptors (GPCRs) are a diverse family of integral membrane proteins that enable cells to respond to a wide variety of extracellular signals. The receptors act through heterotrimeric G proteins composed of Gα, Gβ and Gγ subunits. In unstimulated cells the Gα subunit is bound to GDP and associated with the Gβγ dimer. An activated receptor stimulates the exchange of GDP for GTP and the Gα-GTP and Gβγ subunits regulate the activity of effector proteins to bring about changes in cell behaviour. One mechanism that contributes to the recovery of the cell following stimulation involves regulator of G protein signalling (RGS) proteins that act as GTPase-activating proteins (GAPs) for Gα-GTP, leading to the formation of Gα-GDP and reassociation of the inactive heterotrimer.

The relative simplicity of the GPCR-Gαβγ-RGS signalling unit belies a diversity of function. The human genome encodes approximately 360 non-sensory GPCRs (and a similar number of olfactory and gustatory receptors), 16 Gα subunits, 5 Gβ subunits, 12 Gγ subunits and at least 20 RGS proteins [1]. They are not all expressed in every cell type, but many cells express multiple examples of each component, and these can interact in various combinations to generate a multitude of different signalling pathways. It is the precise combination of GPCR, G protein, and RGS protein that determines which effectors are activated and for how long. This, in turn, determines the nature of the response to a particular stimulus. There is consequently a great deal of interest in identifying how GPCRs and RGS proteins target individual G proteins.

While much information has been obtained regarding GPCR-Gα specificity, demonstrating RGS-Gα selectivity has proven more of a challenge. However, some RGS proteins have been shown to act in a restricted manner; for example, RGS-PX1 against $G\alpha_s$ [2], and RGS20 against $G\alpha_z$ [3]. Results for members of the $G\alpha_q$ family have been more difficult to achieve due to problems associated with the experimental measurement of $G\alpha_q$ GTPase activity. Despite this, sufficient evidence exists to suggest that RGS2, RGS3, RGS4, RGS18 and GAIP-RGS19 directly exhibit GAP activity toward $G\alpha_q$ [4]. Other RGS proteins (RGS1, RGS5, and RGS16) have been

demonstrated to bind $G\alpha_q$, but their GAP activity has been difficult to establish [4]. We therefore sought to investigate the selectivity of the R4 family (RGS4-like) of RGS proteins against $G\alpha_q$-mediated signalling. Due to the presence of multiple components with overlapping functions within mammalian cells, we have employed a model organism.

Yeast are an attractive system in which to analyse individual GPCR signalling pathways. Mechanisms of GPCR signalling in yeast are similar to those in higher eukaryotes but there are many fewer components and they can be easily manipulated. Most studies use the pheromone-response pathway within the budding yeast *Saccharomyces cerevisiae* and have made a number of significant contributions to our understanding of GPCR signalling [5-7]. Although many components can be functionally replaced by their mammalian counterparts [8], few studies have reported the expression of non-yeast Gα proteins in *Sc. cerevisiae* [9-11]. This is primarily due to the Gα subunit in *Sc. cerevisiae* acting as a negative regulator of pheromone signalling (it is the Gβγ dimer that propagates the signal) [12] and, exchanging it with a human Gα which has low affinity for the yeast Gβγ will cause constitutive activation of the signalling machinery, leading to an arrest of the cell cycle.

To allow investigation of mammalian Gα-signalling, we utilised a yeast in which the Gα is a positive effector of signalling [13]. The pheromone-response pathway of the fission yeast *Schizosaccharomyces pombe* provides an alternative system for analysing GPCR signalling, since strains have been modified to provide assays for G protein activation [14,15]. Studies in *Sz. pombe* have included isolation and characterisation of signal regulators [16], RGS proteins [14], and analysis of constitutively active receptors [17]. Non-yeast receptors have also been introduced into these strains and coupled to the pheromone-response pathway [15].

Here we demonstrate that human $G\alpha_q$ and $G\alpha_{11}$ are expressed in *Sz. pombe*. Both subunits activate the morphology-response pathway normally stimulated by the yeast Gα protein (Gpa1), providing an assay for their activity. We demonstrate that Gα signalling was dependent on GTP-binding and that members of the R4 family of RGS proteins can selectivity inhibit $G\alpha_q$- but not

$G\alpha_{11}$-mediated signalling. Furthermore, expression of constitutively active versions of the Gα subunits illustrates that some RGS proteins are capable of modulating Gα signalling in a GAP-independent manner. Finally, by modification of the last 5 residues of $G\alpha_q$ and $G\alpha_{11}$, we have functionally coupled them to the yeast pheromone receptor, reconstituting the GPCR-Gα-RGS signalling unit and allowing RGS specificity against receptor-activated Gα proteins to be investigated.

## 2. MATERIALS AND METHODS

*2.1 Strains, reagents and general methods* - The yeast used in this study were derived from strain JY546 (*mat1-M, Δmat2/3::LEU2⁻, leu1⁻, ura4-D18, cyr1-D51, sxa2>lacZ*) [16]. The *gpa1* gene was disrupted in a two-step process in which the open reading frame (ORF) was first replaced with a 1.8kb $ura4^+$ cassette. JY1285 (*mat1-M, Δmat2/3::LEU2⁻, leu1⁻, ura4-D18, cyr1-D51, gpa1-D12, sxa2>lacZ*) was subsequently generated by the removal of the $ura4^+$ cassette from the *gpa1* locus. A similar process was used to disrupt the *rgs1* gene in JY1285 to generate JY1287 (*mat1-M, Δmat2/3::LEU2⁻, leu1⁻, ura4-D18, cyr1-D51, gpa1-D12, rgs1-D14, sxa2>lacZ*) and the *mam2* gene in JY1287 to generate JY1286 (*mat1-M, Δmat2/3::LEU2⁻, leu1⁻, ura4-D18, cyr1-D51, gpa1-D12, rgs1-D14, mam2-D10, sxa2>lacZ*). All gene replacements were confirmed by polymerase chain reaction (PCR) and Southern blot analysis. General yeast procedures were performed as described previously [18]; yeast extract medium was used for routine cell growth and defined minimal medium for selective growth and all assays. Cell concentrations and median cell volumes were determined using a Coulter Channelyser (Beckman Coulter, Luton, UK) [19]. DNA manipulations were performed by standard methods. Oligonucleotides were synthesised by TAG Newcastle Ltd. (Gateshead, UK). Amplification by PCR used *Pwo* DNA polymerase (from *Pyrococcus woesei*) (Boehringer Mannheim Biochemicals, Lewes, East Sussex, UK). All constructs generated by PCR were confirmed by sequencing.

*2.2 Yeast expression constructs* - The pREP series of *Sz. pombe* vectors allow expression of genes under the control of the thiamine-repressible *nmt1* promoter [20]; pREP3X contains the *LEU2* gene for nutritional selection and was used for expression of all Gα subunits, pREP4X contains the *ura4* gene and was used for expression of all RGS proteins. All wild-type ORFs were amplified by PCR and cloned into the pREP expression vectors. *Sz. pombe* genomic DNA was used as template for Gpa1 and Rgs1. All human ORFs were amplified using cDNA clones from the Guthrie cDNA Resource Center (www.cdna.org, University of Missouri at Rolla, USA). Most Gα mutants were produced by bipartite PCR on the wild-type constructs [21]. In this technique, two PCR reactions are performed on a plasmid containing the Gα ORF. Each PCR reaction amplifies approximately half of the target plasmid, from the site of mutagenesis in the Gα ORF to a site within the $Amp^R$ gene that confers ampicillin resistance on the host plasmid. Ligating the two PCR products in the correct orientation recreates the original vector, but containing the mutagenised Gα ORF. Human $G\alpha_q^{QL}$ and $G\alpha_{11}^{QL}$ were cloned into the pREP3X expression vector as described for the wild-type ORFs. Gα-transplants were created by bipartite PCR; a Gα construct used as template for the major part of the protein and a Gpa1 construct as template for the C-terminal fragment.

*2.3 Immunoblotting* - Cell extracts containing human Gα subunits and RGS proteins were prepared from duplicated cultures of *Sz. pombe* cells as described previously [17]. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, Herts, UK). Samples containing equal amounts of protein were separated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) under standard conditions. Separated proteins were transferred to polyvinyl difluoride (Bio-Rad). Western blotting was performed using primary antibodies against the proteins of interest, and horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) were used to visualise bound primary antibodies by enhanced chemiluminescence reagents (ECL) (Amersham). All primary antibodies were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) with the exception of RGS20 which was

supplied by Abcam Plc. (Cambridge, UK) and an anti-Gq antibody used for detection of Gq[5C] which was a gift from Professor Graeme Milligan (University of Glasgow). All secondary antibodies were supplied by Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

## 3. RESULTS

*3.1 Yeast reporter strains for assaying signalling activity of human Gα subunits* - We have previously constructed *Sz. pombe sxa2>lacZ* reporter strains in which the chromosomal copy of the *sxa2* ORF is replaced by the ORF of the bacterial *lacZ* gene (encodes β-galactosidase). Sxa2 is a carboxypeptidase that is only expressed following stimulation with the pheromone P-factor [22] and expressing *lacZ* under the transcriptional control of the *sxa2* promoter provides a convenient readout for signalling through the pheromone-response pathway [16]. We sought to further modify this strain to provide a host for assaying human Gα subunits. The activity of a Gα subunit is regulated by interacting proteins. A stimulated GPCR induces the release of GDP from the Gα, forming Gα-GTP, while RGS proteins promote GTP hydrolysis to return the activated Gα to Gα-GDP. Therefore, we removed not only Gpa1 (the endogenous Gα subunit) but also Mam2 (the P-factor receptor) and Rgs1 (the RGS protein for Gpa1) (Fig. 1). The resulting strain (JY1286; *Δmam2, Δrgs1, Δgpa1*) had almost no signalling activity, as demonstrated by very low expression of β-galactosidase from the *sxa2>lacZ* reporter construct (Fig. 2*A*).

Expressing the *Sz. pombe* Gα subunit (Gpa1) in JY1286, from the thiamine-repressible *nmt1* promoter on the pREP3X plasmid [20], induced transcription of the *sxa2>lacZ* reporter (Fig. 2*A*). This is presumably caused by spontaneous nucleotide exchange on Gpa1, leading to signalling through the pheromone-response pathway. Such activation of Gα subunits in the absence of an appropriate receptor has been reported in both *Sz. pombe* [15,24] and *Sc. cerevisiae* [25,26], and is consistent with a role for unoccupied receptors in maintaining G proteins in their inactive state. It is likely that this spontaneous activity is accentuated by the absence of an RGS protein from our strain.

We next investigated the ability of non-yeast Gα subunits to signal in JY1286. Mammalian Gα proteins are grouped into classes based upon structural and functional similarity. We initially chose to analyse prototypical members of the $G\alpha_q$ sub-family ($G\alpha_q$ and $G\alpha_{11}$), as they are ubiquitously expressed, and couple to a wide range of GPCRs [4]. Expression of the Gα subunits in JY1286 was confirmed by immunoblotting and strains were assayed for induction of the *sxa2>lacZ* reporter (Fig. 2*A*). $G\alpha_q$, but not $G\alpha_{11}$, induced a low but reproducible level of β-galactosidase, demonstrating the first functional activity of a human Gα subunit in *Sz. pombe*.

*3.2 Shmoo formation* - In addition to the transcription of pheromone-dependent genes, such as *sxa2* (the transcriptional response), *Sz. pombe* undergoes a change in cell morphology following pheromone stimulation. Responding cells continue to grow from the cell tip and elongate towards the source of the pheromone forming a shmoo [27,28]. Both pathways are mediated by Ras1 (Fig. 1) and diverge immediately downstream of this molecular switch. Microscopic examination of JY1286 strains expressing $G\alpha_q$ or $G\alpha_{11}$ revealed elongated cells reminiscent of shmoos (Fig. 2*B*). Cells cultured in the presence of thiamine, to repress expression of $G\alpha_q$/$G\alpha_{11}$, were not elongated, providing evidence that these Gα subunits can spontaneously activate the pheromone-response pathway leading to shmoo formation.

The increase in cell volume during shmoo formation provides a quantitative assay for signalling activity [19,28]. Briefly, because a non-synchronous culture contains cells of various sizes, it is not appropriate to monitor changes in individual cells. Instead, shmoo formation can be measured as an increase in the median cell volume of a culture. A strain lacking a Gα subunit had a median cell volume of ~63 fl (Fig. 2*C*). Expression of Gpa1 increased this to ~95 fl, and expression of either $G\alpha_q$ or $G\alpha_{11}$ increased the median cell volume to greater than 80 fl (Fig. 2*C*). This demonstrates that both human Gα subunits can initiate the shmoo response in *Sz. pombe*.

To confirm that the responses in Fig. 2 were dependent upon the activated GTP-bound form of the Gα subunits, we constructed a series of Gα mutants and assayed their ability to signal in

JY1286 (Fig. 3). Mutation of a conserved glycine in the switch II region of Gα subunits inhibits the release of GDP and locks the Gα subunit in its inactive form [29]. The corresponding $Gpa1^{IN}$ mutant (containing a $Gly^{243}$ to Ala mutation) was unable to induce either expression of the *sxa2>lacZ* reporter (Fig. 3*A*) or an increase in cell volume (Fig. 3*B*). Likewise, neither of the inactivated human Gα subunits $G\alpha_q^{IN}$ ($Gly^{208}$ to Ala) (Fig. 3*C*) nor $G\alpha_{11}^{IN}$ ($Gly^{208}$ to Ala) (Fig. 3*D*) induced shmoo formation.

The activity of Gα subunits is regulated by RGS proteins that serve as GAPs for Gα-GTP. As demonstrated previously [14], *Sz. pombe* Rgs1 reduced the ability of Gpa1 to induce expression of the *sxa2>lacZ* reporter and prevented shmoo formation (Fig. 3*A* and 3*B*). This suggests that there is sufficient Rgs1 to ensure that almost all of the Gpa1 is in the inactive GDP-bound form. To confirm that the reduction in Gpa1 activity was due to the effect of Rgs1, we created a mutant Gpa1 ($Gpa1^{rgs}$) that was expected to have a much reduced interaction with RGS proteins. Mutation of a conserved glycine in the switch I region of Gα subunits ($Gly^{223}$ in Gpa1) blocks the interaction with RGS proteins, but leaves intact the ability of Gα to couple to receptors and downstream effectors [30]. $Gpa1^{rgs}$ (containing a $Gly^{223}$ to Ser mutation) activated both the transcription- and morphology-response pathways and was insensitive to co-expression of Rgs1 (Fig. 3*A* and 3*B*).

Expression of *Sz. pombe* Rgs1 markedly reduced the cell volume increase due to $G\alpha_q$ (Fig. 3*C*) and $G\alpha_{11}$ (Fig. 3*D*), suggesting that the yeast RGS protein can function as a GAP for human Gα subunits. This is not surprising as many RGS proteins function against more than one Gα subunit and, in a reciprocal arrangement, several mammalian RGS proteins have been shown to act as GAPs for GPA1 in the *Sc. cerevisiae* pheromone-response pathway [6]. However, this is the first demonstration that the *Sz. pombe* Rgs1 protein can function on human Gα subunits. We confirmed that the yeast Rgs1 protein directly reduced $G\alpha_q$ and $G\alpha_{11}$ activity by constructing RGS-insensitive versions of the human subunits $G\alpha_q^{rgs}$ ($Gly^{188}$ to Ser) and $G\alpha_{11}^{rgs}$ ($Gly^{188}$ to Ser). These mutant Gα subunits displayed comparable activity in the presence or absence of Rgs1 (Fig. 3*C* and 3*D*).

*3.3 Investigating the activity of human RGS proteins* - Some 20 human RGS proteins have been identified and can be grouped into subfamilies based on sequence similarity [31]. The R4 family (those with similarity to RGS4) are the simplest in terms of structure, containing minimal protein motifs in addition to the signature RGS domain. Many R4 members can reduce signalling through $G\alpha_{q/11}$-dependent pathways [4,31,32] but in vivo studies can be complicated by overlapping activities of the Gα subunits and RGS proteins, coupled to the technical difficulties in measuring GTPase activity associated with these G proteins. We therefore investigated whether the yeast-based system could better define the interaction between the various Gα subunits and RGS proteins.

The strain JY1286 lacks Mam2, Rgs1 and Gpa1 and had a median cell volume of ~63 fl. This increased to ~85 fl following expression of $G\alpha_q$ and to ~83 fl following expression of $G\alpha_{11}$ (Fig. 2*C*). Using a second plasmid, which has previously been demonstrated to express proteins at levels equivalent to pREP3x [17], we co-expressed various human RGS proteins with $G\alpha_q$ (Fig. 4*A*) or $G\alpha_{11}$ (Fig. 4*B*). Immunoblotting confirmed that each RGS protein was expressed to the same level in the presence of either $G\alpha_q$ or $G\alpha_{11}$. RGS3 migrates as a larger species than the other RGS proteins due to expression of the long form of the protein (accession number: **AAM12641**). Determination of the median cell volume for each culture revealed that the RGS proteins reduced shmoo formation to different extents (Fig. 4*C* and 4*D*). To compare the Gα-RGS combinations, we normalised the results such that the cell volume in the absence of a Gα subunit (representing the minimum Gα activity that could be obtained) was set as 0% signalling, and the cell volume in the presence of Gα but the absence of an RGS protein (minimum RGS activity allowing maximum Gα signalling) was set as 100% signalling. The percentage signalling activity for each Gα in the presence of different RGS proteins was then calculated (Fig. 4*E*). All of the R4 RGS proteins tested reduced signalling by $G\alpha_q$. In contrast, despite similar expression levels to the Gq strain, RGS16 was unable to reduce signalling for $G\alpha_{11}$, while RGS5 had an intermediate effects, reducing signalling to about 40% of that seen for $G\alpha_{11}$ in the absence of an RGS. The differential effects of

RGS16, and to a lesser extent RGS5, suggest that some RGS proteins can discriminate between these two highly related Gα subunits.

To further investigate the RGS specificity within the yeast system we assayed the ability of RGS20 to reduce signalling via $G\alpha_q$ and $G\alpha_{11}$. RGS20 has previously been shown to be highly selective for $G\alpha_z$ and displays very low activity towards members of the $G\alpha_{q/11}$ family [3]. Its inability to affect signalling of either $G\alpha_q$ or $G\alpha_{11}$ in yeast is consistent with earlier work in other systems; experiments demonstrating that RGS20 completely inhibits signalling through the *Sz. pombe* Gpa1 confirm that the protein is functional in yeast (not shown).

*3.4 Inhibitory effect of RGS proteins on constitutively active Gα subunits* - RGS proteins can reduce signalling through Gα-GTP by direct GAP activity and/or by acting as effector antagonists that inhibit the interaction of the Gα subunit with its downstream effector [33]. To examine whether the relative contribution of GAP and effector antagonistic effects differs among RGS proteins, we examined RGS activity under conditions where they cannot promote Gα-GTP hydrolysis. Mutation of $Gln^{209}$ to Leu within the switch II region of both $G\alpha_q$ and $G\alpha_{11}$ destroys their GTPase activity and renders them constitutively active, even in the presence of a GAP [34]. $G\alpha_q^{QL}$ or $G\alpha_{11}^{QL}$ were therefore expressed in JY1286 (*Δmam2, Δrgs1, Δgpa1*) in the presence of the different RGS proteins. Immunoblotting confirmed expression of all RGS proteins and $G\alpha_q^{QL}$ (Fig. 5*A*) or $G\alpha_{11}^{QL}$ (Fig. 5*B*) before the level of signalling was determined as previously, from 0% in the absence of $G\alpha^{QL}$ to 100% in the presence of $G\alpha^{QL}$ but absence of an RGS (Fig. 5*C*). In contrast to the results with the wild-type Gα subunits, only RGS2 and RGS3 caused a marked reduction in signalling by the constitutively active mutants. RGS1 and RGS4 were unable to exert an inhibitory effect in a setting where they could not act as GAPs. Furthermore, neither RGS5 nor RGS16 were able to reduce signalling through $G\alpha_q^{QL}$.

*3.5 GPCR-mediated activation of human Gα subunits* - To determine if RGS-$G\alpha_q$/$G\alpha_{11}$ specificity was maintained under conditions of GPCR stimulation, we coupled the human subunits to the yeast pheromone receptor. The yeast strain JY1287 lacks Gpa1 and Rgs1 but contains the Mam2 receptor. Expressing Gpa1 in JY1287 recreates a functional signalling pathway but, in contrast to the earlier situation in which Gpa1 expression in JY1286 (*Δmam2, Δrgs1, Δgpa1*) increased the median cell volume to ~95 fl (Fig. 2*C*), the cell volume remained at ~63 fl (Fig. 6). Presumably, the presence of the receptor in JY1287 ensures that the expressed Gpa1 is maintained in the inactive conformation. Stimulation with P-factor activated the morphology-response pathway, increasing the cell volume to ~95 fl.

In contrast to Gpa1, neither $G\alpha_q$ nor $G\alpha_{11}$ coupled to the Mam2 receptor and their spontaneous activation of the pheromone-response pathway produced cells with a volume of ~85 fl and ~83 fl, respectively (Fig. 6). There was no further increase in cell volume following exposure to mating pheromone, again consistent with the failure of the human Gα subunits to couple to the receptor. GPCR-Gα interactions can be specific and a given Gα subunit does not necessarily interact with all receptors. One of the major factors determining whether a GPCR will couple to a G protein involves the C-terminus of the Gα. Switching as few as 5 amino acids from one subunit to another can have dramatic effects on which receptors couple to a particular G protein [35]. These Gα-transplants have previously been used to alter the coupling specificity of the *Sz. pombe* Gα subunit [15]. We therefore created the reciprocal human-yeast transplants, in which the last 5 residues of Gpa1 (QSLMF) were used to replace the equivalent residues of $G\alpha_q$ (EYNLV) and $G\alpha_{11}$ (also EYNLV). JY1287 cells expressing the modified constructs, $G\alpha_q^{[5C]}$ and $G\alpha_{11}^{[5C]}$, were smaller than those expressing the unmodified human Gα subunits, and were approximately the same size as cells expressing the endogenous Gpa1. This would suggest that the $G\alpha^{[5C]}$-transplants couple to the Mam2 receptor and there is a concomitant reduction in their spontaneous activation of the response pathway. Consistent with this, pheromone stimulation of these cells induced shmoo formation (Fig. 6), suggesting that the $G\alpha^{[5C]}$-transplants were able to interact with the Mam2

receptor and activate downstream signalling components. Immunoblotting confirmed that the $G\alpha^{[5C]}$-transplants were expressed to equivalent levels as for wild type $G\alpha_q$ and $G\alpha_{11}$.

To examine the ability of the different RGS proteins to regulate receptor-activated $G\alpha_q^{[5C]}$ and $G\alpha_{11}^{[5C]}$, the various Gα-RGS combinations were expressed in JY1287 (*Δgpa1, Δrgs1*). Immunoblotting confirmed expression of each RGS protein with either $G\alpha_q^{[5C]}$ (Fig. 7*A*) or $G\alpha_{11}^{[5C]}$ (Fig. 7*B*) before median cell volumes in the presence of low-level stimulation ($10^{-8}$ M P-factor) or high-level stimulation ($10^{-6}$ M) were determined (Fig. 7*C* and 7*D*). The pattern of activity of the RGS proteins at low-level stimulation was similar to that observed for the wild-type Gα subunits in strains lacking the receptor (compare Fig. 7*C* with Fig. 4*E*); all of the R4 proteins reduced signalling by $G\alpha_q^{[5C]}$, but only RGS1, RGS2, RGS3 and RGS4 reduced signalling by $G\alpha_{11}^{[5C]}$ as RGS5 had only partial effects and RGS16 had almost no effect. Neither $G\alpha_q^{[5C]}$ nor $G\alpha_{11}^{[5C]}$ were inhibited by RGS20. The pattern of activity of the RGS proteins at high-level stimulation was similar to that observed for the constitutively active $G\alpha^{QL}$ subunits (compare Fig. 7*D* with Fig. 5*C*); signalling was most reduced by RGS2 and RGS3, although the difference between these and other RGS proteins was less distinct than for $G\alpha^{QL}$.

## 4. DISCUSSION

*4.1 Human Gα subunits and RGS proteins function in Sz. pombe* - Our results demonstrate that human $G\alpha_q$ and $G\alpha_{11}$, along with members of the R4 family of RGS proteins, are active in *Sz. pombe*. In strains lacking the pheromone receptor and Rgs1, an RGS protein that normally reduces Gα signalling, the human subunits activate the morphology-response pathway (shmoo formation) usually regulated by Gpa1 (Fig. 2). In *Sz. pombe* both the receptor [15,24] and RGS protein [14] reduce Gpa1 signalling, and we presume that removing these negative regulators increases the rate at which Gα subunits become spontaneously activated to Gα-GTP. Consistent with this suggestion, the activity of both $G\alpha_q$ and $G\alpha_{11}$ was dependent upon binding of GTP and reduced by expression

of Rgs1 (Fig. 3). Replacement of Rgs1 with human members of the R4 family of RGS proteins enabled determination of RGS-G$\alpha_q$ and RGS-G$\alpha_{11}$ specificity under a number of activating conditions.

*4.2 Differences in activity between the human Gα subunits and Gpa1* - Although the human Gα subunits were able to activate the morphology-response pathway, they were unable to induce the pheromone-dependent transcription pathway. In contrast, endogenous Gpa1 activates both pathways (Fig. 1). The pathways separate immediately downstream of Ras1, a homologue of the mammalian RAS proto-oncoprotein. Like Gα subunits, Ras1 functions as a molecular switch, with a GTP-bound 'on' state and a GDP-bound 'off' state, in a cycle that is regulated by guanine-nucleotide exchange factors (GEFs) and GAPs. The ability of Ras1 to activate the different effectors, Byr2 and Scd1, is regulated by different GEFs; Ste6 directs it to the transcription-response pathway while Efc25 directs it towards the morphology response. The functions of Ste6 and Efc25 are not interchangeable. Indeed, the two appear to act in competition [36] and this might explain how Ras1 is regulated.

Our results suggest that, whereas Gpa1 can activate both Byr2 and Scd1, G$\alpha_q$ and G$\alpha_{11}$ are unable to interact with Ras1 to activate the transcription-response pathway. The most likely explanation is that the human Gα subunits lack the structural requirements to create a productive Gα-Ras1-Byr2 interaction. Identifying these regions will not be straightforward, as effector domains are usually a feature of the folded Gα subunit, and the contributing residues are often distributed throughout the protein [37,38].

*4.3 The involvement of Gβγ subunits* - An interesting aspect of our study concerns the involvement, or otherwise, of a Gβγ dimer. *Sz. pombe* expresses a single Gβ subunit, Gpb1/Git5 [39,40], and a single Gγ subunit, Git11 [37]. Despite claims to the contrary [39], the Git5-Git11 dimer does not interact with Gpa1 but is the Gβγ partner for a second Gα subunit, Gpa2, involved in the glucose-

sensing pathway [41]. The *Sz. pombe* genome does not appear to encode any other Gβγ subunits that could interact with Gpa1, but this does not preclude the possibility that Gpa1 interacts with other partners. For example, in *Sc. cerevisiae*, the GPA2 subunit interacts with one of two kelch-repeat proteins and a single Gγ-like subunit [42]. The resulting complexes have some, but not all, of the properties of Gαβγ heterotrimers.

The human Gα subunits in this study obviously do not have access to their normal Gβγ partners (these are absent from our strains) and it will be interesting to discover whether they hijack the Git5-Git11 subunits from the glucose-sensing pathway or whether they interact with some other Gβγ-like partners in the yeast. Alternatively, the human Gα subunits may be operating as monomers, a result that would have wide ranging repercussions for G protein signalling in higher eukaryotes.

*4.4 Selectivity of human RGS proteins* - Consistent with results *in vitro* and in mammalian cells [32], we find that RGS1, RGS2, RGS3 and RGS4 exert an inhibitory effect on both $G\alpha_q$- and $G\alpha_{11}$-mediated signalling (Fig. 4). In contrast, RGS20, which is highly selective for $G\alpha_z$ [3], has no effect on either Gα subunit. These results suggest that the specificity of Gα-RGS interactions in yeast is broadly similar to that in mammalian cells.

Interestingly, our results suggest that RGS16 and, to a lesser extent, RGS5 are more effective against $G\alpha_q$ than $G\alpha_{11}$ at the expression levels achieved in our strains. The ability of these two RGS proteins to inhibit $G\alpha_q$-mediated signalling is well documented [43-45] but their reduced effect on $G\alpha_{11}$-mediated signalling has not been described previously. $G\alpha_q$ and $G\alpha_{11}$ are structurally similar, sharing 89% of their amino acid residues, and are often considered to have similar activities. However, although they share the ability to activate phospholipase-Cβ, they can differ in tissue distribution and ability to interact with downstream effectors and adaptors [4]. Few studies have attempted to investigate these differences at the molecular level. An exception is the activation of $G\alpha_q$, but not $G\alpha_{11}$, by the *Pasteurella multocida* toxin (PMT), which is due to the interaction of the

toxin with residues 105-113 of $G\alpha_q$ [46,47]. Identifying which regions are responsible for the interactions between $G\alpha_q$/$G\alpha_{11}$ and RGS5/RGS16 will be a challenge as multiple residues throughout the RGS-Gα interface are likely to be involved. Our *Sz. pombe* strains will provide the ideal system in which to perform these studies.

To further investigate the mechanism of action of the various RGS proteins, we assayed their effects against constitutively active Gα subunits lacking GTPase activity (Fig. 5). RGS1, RGS4, RGS5 and RGS16 were much less effective at reducing signalling through the constitutively active $G\alpha^{QL}$ subunits than the wild-type, suggesting that their inhibitory properties rely on GAP activity. In contrast, and in accordance with results seen in COS-7 cells [48], RGS2 and RGS3 reduced signalling from both $G\alpha_q^{QL}$ and $G\alpha_{11}^{QL}$. This suggests that they do not rely entirely on GAP activity and other mechanisms, such as effector antagonism, are sufficient to mediate their inhibitory effects. This difference in the mode of action of RGS2 and RGS3 to other members of the R4 subfamily has also been observed in mammalian cells [48,49].

Small changes to the C-terminus of the human Gα subunits enabled them to couple to an endogenous receptor. We utilised these modified subunits to compare the effects of the different RGS proteins on Gα signalling under conditions of low and high stimulation with pheromone (Fig. 7). The pattern of activity for the RGS proteins at low-level stimulation was remarkably similar to that observed for the spontaneously active Gα subunits, and the pattern at high-level stimulation was similar to that observed for the constitutively active $G\alpha^{QL}$ subunits (compare Fig. 7*C* and 4*E*, and Fig. 7*D* and 5*C*). A simple explanation could be that the activity of Gα subunits at low-level stimulation is reduced by RGS proteins acting as GAPs, but that the reduction of signalling at high levels of stimulation requires the RGS proteins to have additional inhibitory functions, such as effector antagonism. It will be interesting to investigate this aspect further.

While this study has demonstrated RGS-Gα specificity, it remains poorly understood how RGS proteins target particular activated signalling cascades in vivo. Evidence has suggested a potential role for the activated GPCR in this process [50,51]. Since non-yeast GPCRs have been

demonstrated to function in *Sz. pombe* [15], our next challenge will be to generate yeast cells that express a complete mammalian signalling unit (GPCR-Gα-RGS). These strains should greatly contribute to our understanding of the complex interactions that occur between these three signalling components.

## 5. CONCLUSION

It has previously been demonstrated that certain members of the B/R4 family (RGS4-like) of RGS proteins are able to modulate $G\alpha_q$ signalling in vitro [4]. Our studies have extended this research by investigating the ability of these RGS protein to attenuate $G\alpha_q$ and $G\alpha_{11}$ signalling in vivo. By using a model yeast system, where it is possible to express individual RGS-Gα combinations, we have demonstrated that two specific RGS proteins (RGS5 and RGS16) are able to discriminate between these highly related Gα subunits. This represents the first demonstration of RGS-Gα selectivity between these two members of the $G\alpha_q$ subfamily of G proteins. Since many GPCRs couple to $G\alpha_q$/$G\alpha_{11}$ our results could provide a mechanism for selectively modulating the stimulated GPCRs. Expression of different Gα and RGS combinations, within our yeast cells, should provide further insights into the selectivity that RGS proteins display for specific G proteins.

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**FIGURE LEGENDS**

**Fig. 1.** Signalling pathways that regulate mating responses in *Sz. pombe*. Mating responses are initiated by the binding of the mating pheromones to receptors on the surface of target cells (23); P-factor binds to a 7-span receptor, on the cell surface, that is coupled to the Gα subunit Gpa1. Pheromone stimulation leads to the formation of Gpa1-GTP. One target for Gpa1-GTP is the mitogen-activated protein (MAP) kinase cascade comprised of Byr2 (a MAP kinase kinase kinase [MAP3K]), Byr1 (a MAP2K), and Spk1 (a MAPK). Substrates of Spk1 include the Ste11 transcription factor that regulates the expression of proteins required for mating. Gpa1-GTP also activates a second signalling pathway, by activation of Scd1, a protein which is involved in controlling cell morphology. Both the transcription and morphology pathways are mediated by Ras1. Signalling is terminated within these cells by the action of a number of proteins including Rgs1, a GTPase-activating protein for Gpa1.

**Fig. 2.** Assays for Gα activity. *A*, JY1286 cells (*Δmam2, Δgpa1, Δrgs1, sxa2>lacZ*) were transformed with pREP3X constructs containing the *Sz. pombe* Gpa1 or human $G\alpha_q$ and $G\alpha_{11}$ subunits under the control of the thiamine-repressible *nmt1* promoter. Cells grown in the presence (repressed, *nmt1* promoter is off) or absence (induced, *nmt1* promoter is on) of thiamine were assayed for β-galactosidase activity (16). Data shown are averages of duplicate determinations of three independent isolates (± SD). Expression of $G\alpha_q$ and $G\alpha_{11}$ was monitored by immunoblotting using a monoclonal rabbit anti-$G\alpha_{q/11}$. *B*, JY1286 cells (*Δmam2, Δgpa1, Δrgs1*) expressing $G\alpha_q$ or $G\alpha_{11}$ from the pREP3X vector were grown in the presence (repressed) or absence (induced) of thiamine. The elongated cell morphology is reminiscent of the shmoo formation observed when wild-type *Sz. pombe* cells are exposed to mating pheromone (19). *C*, JY1286 cells (*Δmam2, Δgpa1, Δrgs1*) transformed with pREP3X constructs containing the *Sz. pombe* Gpa1, human $G\alpha_q$ or human $G\alpha_{11}$ were grown in the presence (repressed) or absence (induced) of thiamine. Median cell

volumes were determined using a Coulter Channelyser (19) and the results shown are averages of duplicate determinations of three independent isolates (± SD). Cells containing the empty pREP3X vector (No Gα) provided a control for the effects of thiamine on cell volume.

**Fig. 3.** Signalling requires GTP exchange and is inhibited by RGS activity. Mutant Gα subunits expressed in JY1286 (*Δmam2, Δgpa1, Δrgs1*) from the pREP3X vector were assayed for their ability to activate the transcription response (*A* - Gpa1) and/or the morphology response (*B* - Gpa1; *C* - $G\alpha_q$; *D* - $G\alpha_{11}$). Mutation of a conserved glycine in the switch II region inhibits the release of GDP and locks the Gα in an inactive form ($G\alpha^{IN}$). The effects of co-expression of the *Sz. pombe* Rgs1 protein (from the pREP4X vector) on the activity of wild-type Gα subunits and mutant Gα subunits, in which mutation of a glycine in the switch I region blocks the interaction with RGS proteins ($G\alpha^{rgs}$), were also monitored. Median cell volumes were determined using a Coulter Channelyser and the results shown are averages of duplicate determinations of three independent isolates (± SD).

**Fig. 4.** Effects of different RGS (R4) proteins on $G\alpha_q$ and $G\alpha_{11}$ signalling. JY1286 cells (*Δmam2, Δgpa1, Δrgs1*) expressing (*A*) $G\alpha_q$ or (*B*) $G\alpha_{11}$ from pREP3X, were transformed with pREP4X constructs directing the expression of various RGS proteins. Expression of $G\alpha_q$ or $G\alpha_{11}$ was monitored by immunoblotting using a monoclonal rabbit anti-$G\alpha_{q/11}$, while expression of individual RGS proteins was determined by using antibodies specific to the relevant RGS protein. Median volumes of cultures grown in the absence of thiamine (to induce expression of both the Gα and RGS proteins) were determined using a Coulter Channelyser, (*C*) $G\alpha_q$ or (*D*) $G\alpha_{11}$, and the results shown are averages of duplicate determinations of three independent isolates (± SD). (*E*) - comparison of the different Gα-RGS combinations. Results were normalised such that the median cell volume in the absence of a Gα subunit was set at 0% Gα signalling, and the median cell

volume in the presence of the Gα but the absence of an RGS was set at 100% Gα signalling (dashed lines highlighting 10%, 50% and 100% signalling are included to aid comparison). Data in (*E*) are from the average values in (*C*) and (*D*).

**Fig. 5.** Effects of RGS (R4) proteins on signalling by constitutively active Gα subunits. JY1286 cells (*Δmam2, Δgpa1, Δrgs1*) expressing constitutively active versions of $G\alpha_q$ or $G\alpha_{11}$ (contain the $Gln^{209}Leu$ mutation within the switch II region) from pREP3X were transformed with pREP4X constructs directing the expression of various RGS proteins. Expression of (*A*) $G\alpha_q^{QL}$ and (*B*) $G\alpha_{11}^{QL}$ was monitored by immunoblotting using a monoclonal rabbit anti-$G\alpha_{q/11}$, while expression of individual RGS proteins was determined as for Fig. 4. (*C)* - median volumes of cultures grown in the absence of thiamine (to induce expression of both the $G\alpha^{QL}$ and RGS proteins) were determined using a Coulter Channelyser. The signalling activity of each $G\alpha^{QL}$-RGS combination was determined as in Fig. 4.

**Fig. 6.** Receptor-dependent stimulation of $G\alpha_q$ and $G\alpha_{11}$. JY1287 cells (*Δgpa1, Δrgs1*, but express the Mam2 pheromone receptor) were transformed with pREP3X constructs expressing Gpa1, $G\alpha_q$, $G\alpha_{11}$ or modified human subunits containing the last 5 residues of the *Sz. pombe* Gpa1 ($G\alpha^{[5C]}$). Expression of $G\alpha_q/G\alpha_q^{[5C]}$ and $G\alpha_{11}/G\alpha_{11}^{[5C]}$ was monitored by immunoblotting using individual monoclonal rabbit anti-$G\alpha_q$ or anti- $G\alpha_{11}$ respectively each generated to a central portion of the proteins. Cells were grown in thiamine-free medium and exposed to P-factor mating pheromone for 16 h. Median cell volumes were determined using a Coulter Channelyser and the results shown are averages of duplicate determinations of three independent isolates (± SD).

**Fig. 7.** Effects of RGS (R4) proteins on receptor-dependent $G\alpha_q$ and $G\alpha_{11}$ activity. JY1287 cells (*Δgpa1, Δrgs1*, but express the Mam2 pheromone receptor) expressing (*A*) $G\alpha_q^{[5C]}$ and (*B*) $G\alpha_{11}^{[5C]}$

from pREP3X were transformed with pREP4X constructs directing the expression of various RGS proteins. Cells were grown in thiamine-free medium and exposed to P-factor mating pheromone for 16 h; (*C*) low ($10^{-8}$ M P-factor), (*D*) high ($10^{-6}$ M). Median cell volumes were determined using a Coulter Channelyser and the signalling activity of each Gα-RGS combination was determined as in Fig. 4. Expression of $G\alpha_q^{[5C]}$ and $G\alpha_{11}^{[5C]}$ was monitored as for Fig. 6, while expression of individual RGS proteins was determined as for Fig. 4.

Figure 1

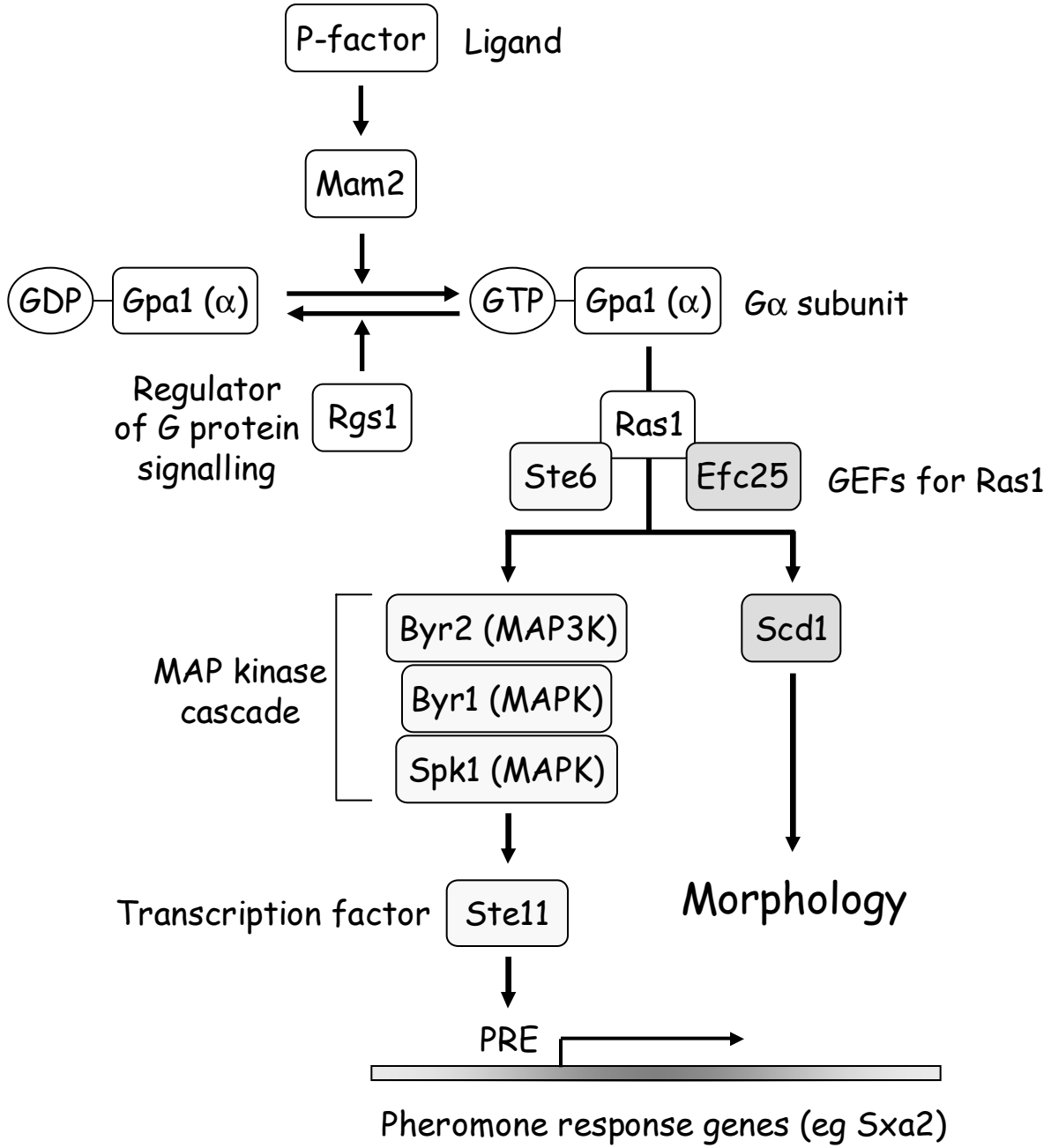

P-factor
Ligand
Mam2
GDP
Gpa1 (α)
GTP
Gpa1 (α)
Gα subunit
Regulator of G protein signalling
Rgs1
Ras1
Ste6
Efc25
GEFs for Ras1
Byr2 (MAP3K)
Byr1 (MAPK)
Spk1 (MAPK)
MAP kinase cascade
Scd1
Transcription factor
Ste11
Morphology
PRE
Pheromone response genes (eg Sxa2)

Figure 2

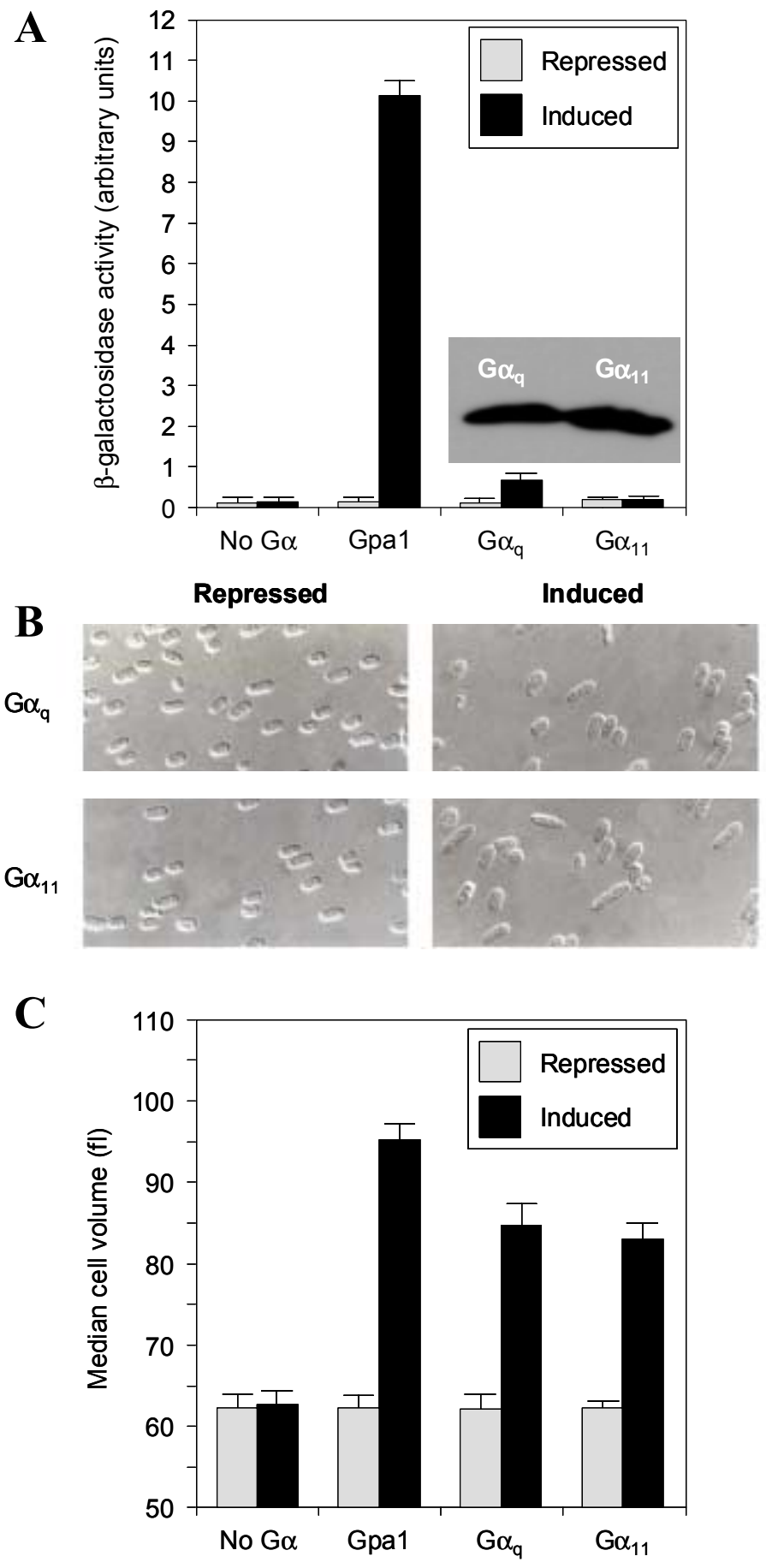

A
β-galactosidase activity (arbitrary units)
12
11
10
9
8
7
6
5
4
3
2
1
0
Repressed
Induced
Gαq
Gα11
No Gα
Gpa1
Gαq
Gα11
Repressed
Induced
B
Gαq
Gα11
C
Median cell volume (fl)
110
100
90
80
70
60
50
Repressed
Induced
No Gα
Gpa1
Gαq
Gα11

**Figure 3**

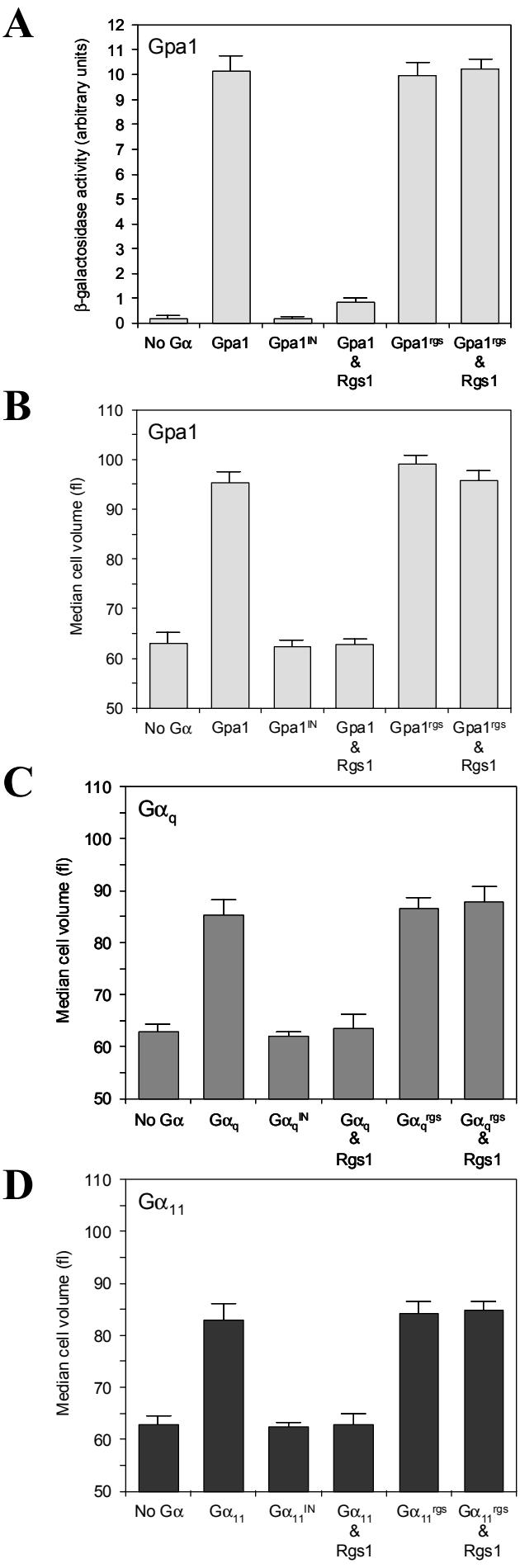

A
Gpa1
β-galactosidase activity (arbitrary units)
12
11
10
9
8
7
6
5
4
3
2
1
0
No Gα
Gpa1
Gpa1IN
Gpa1 & Rgs1
Gpa1rgs
Gpa1rgs & Rgs1
B
Gpa1
Median cell volume (fl)
110
100
90
80
70
60
50
No Gα
Gpa1
Gpa1IN
Gpa1 & Rgs1
Gpa1rgs
Gpa1rgs & Rgs1
C
Gαq
Median cell volume (fl)
110
100
90
80
70
60
50
No Gα
Gαq
Gαq IN
Gαq & Rgs1
Gαq rgs
Gαq rgs & Rgs1
D
Gα11
Median cell volume (fl)
110
100
90
80
70
60
50
No Gα
Gα11
Gα11 IN
Gα11 & Rgs1
Gα11 rgs
Gα11 rgs & Rgs1

Figure 4

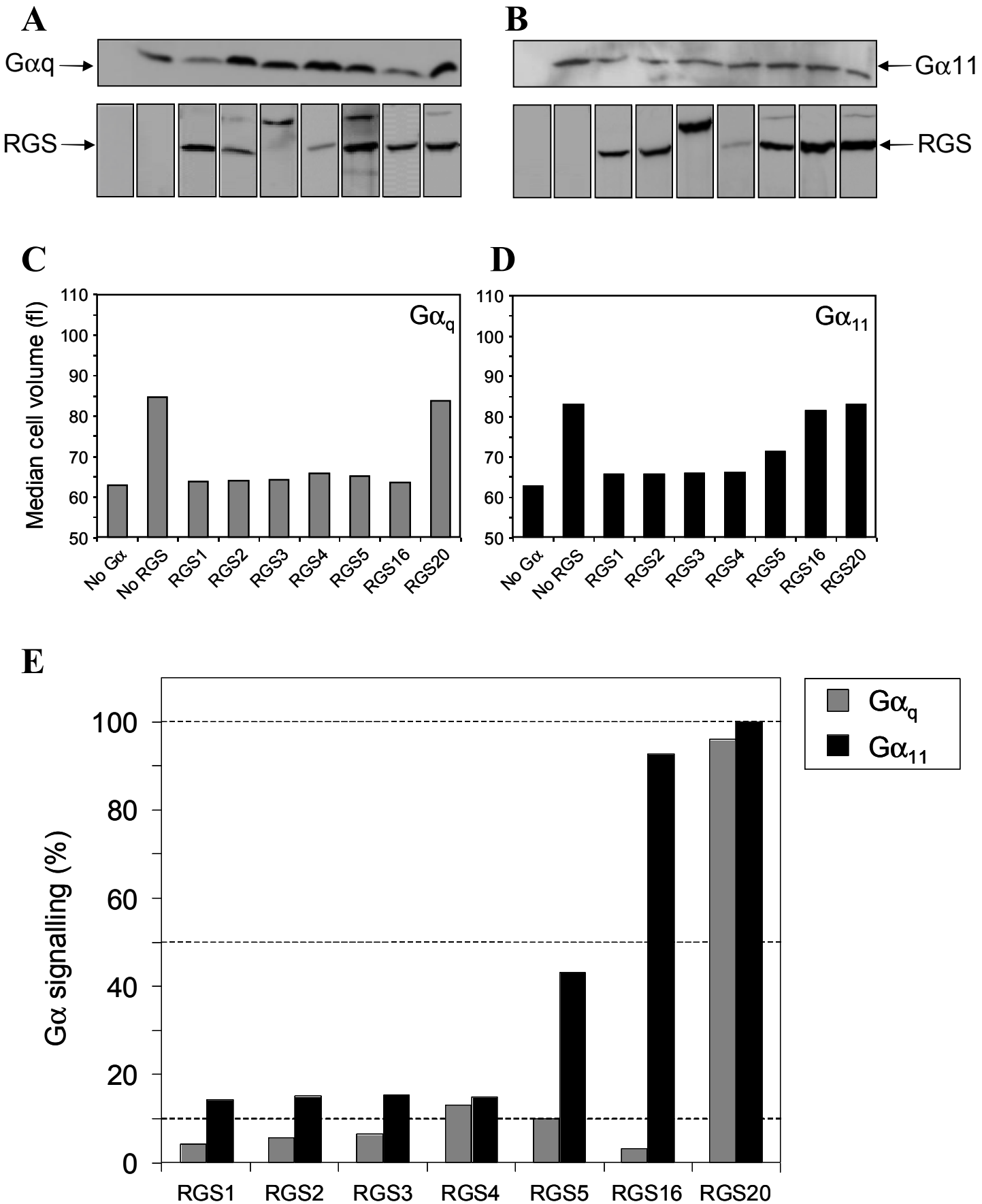

A
Gαq
RGS
B
Gα11
RGS
C
Median cell volume (fl)
Gαq
110
100
90
80
70
60
50
No Gα
No RGS
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
D
Gα11
110
100
90
80
70
60
50
No Gα
No RGS
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
E
Gα signalling (%)
100
80
60
40
20
0
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
Gαq
Gα11

Figure 5

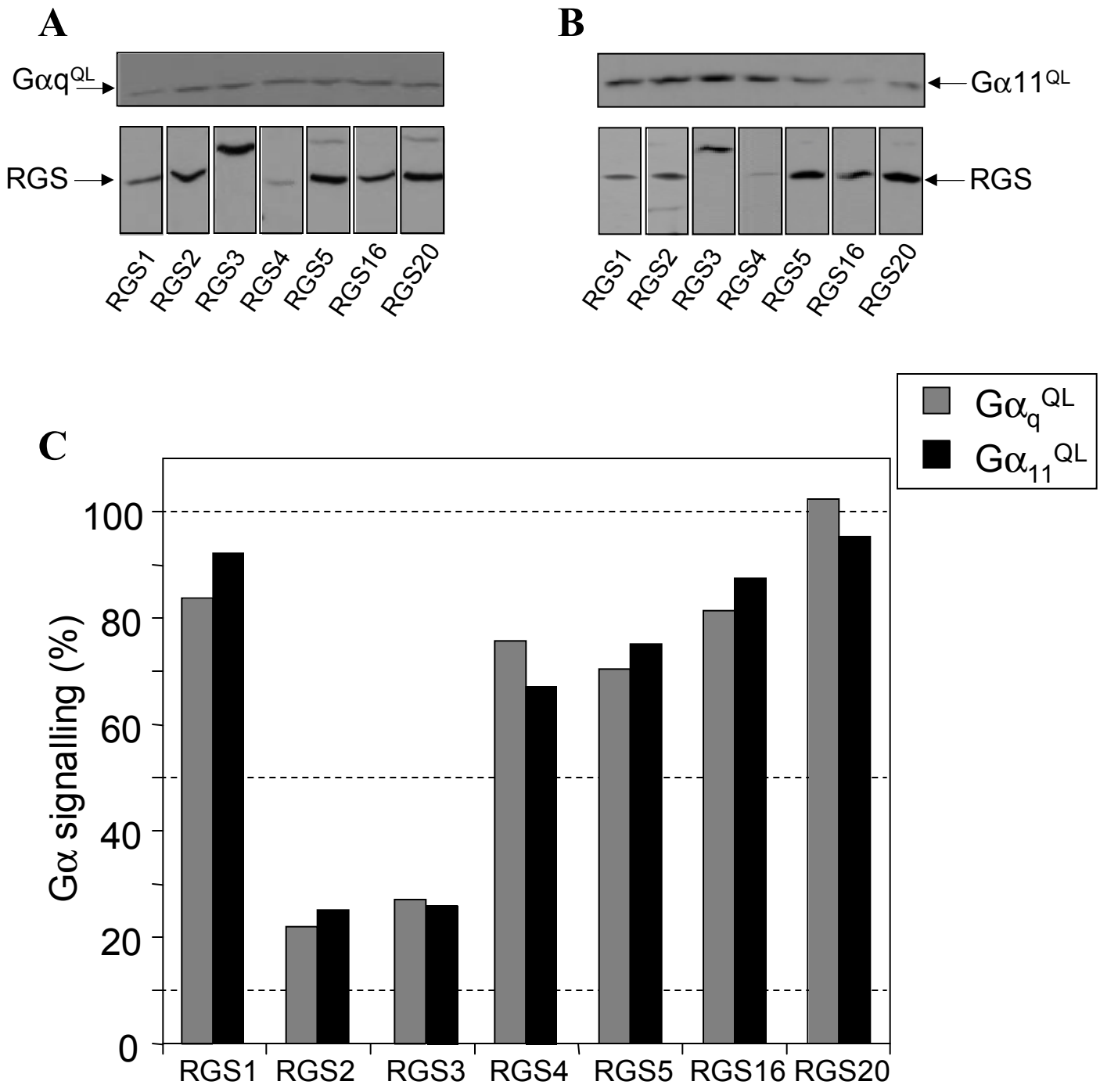

A
Gαq QL
RGS
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
B
Gα11 QL
RGS
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
C
Gαq QL
Gα11 QL
Gα signalling (%)
100
80
60
40
20
0
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20

Figure 6

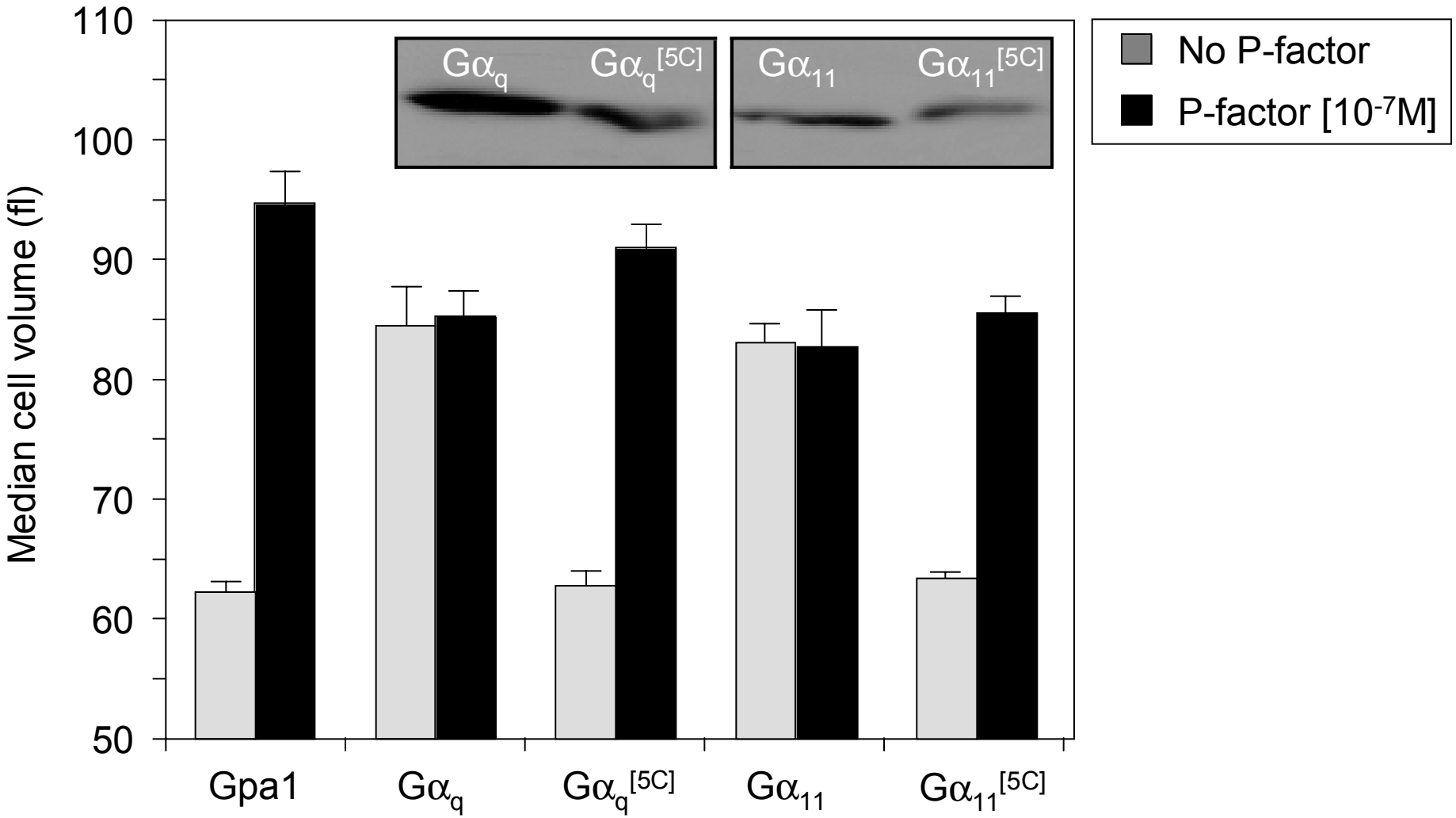
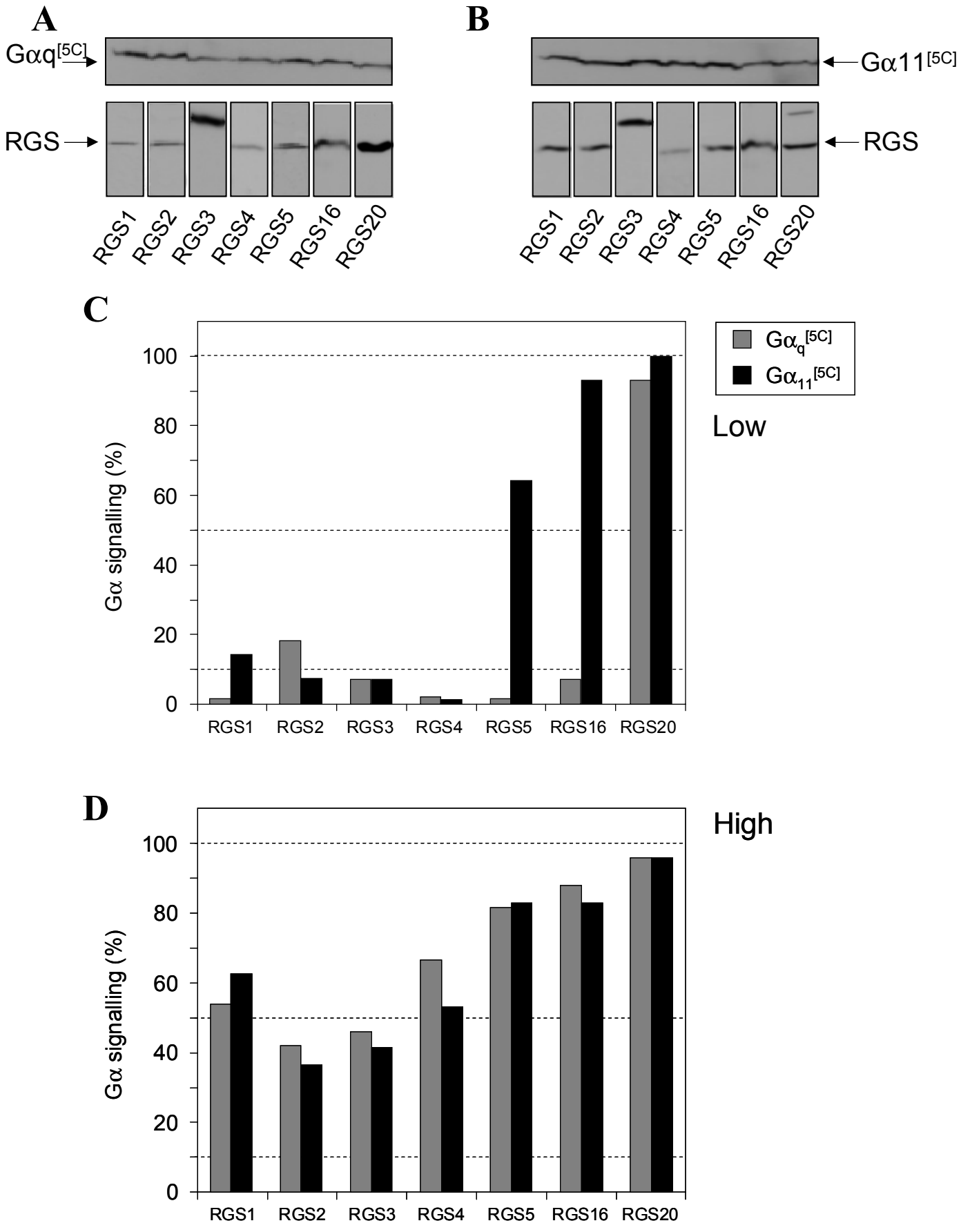

110
100
90
80
70
60
50
Median cell volume (fl)
Gα$_q$
Gα$_q$[5C]
Gα$_{11}$
Gα$_{11}$[5C]
No P-factor
P-factor [10$^{-7}$M]
Gpa1
Gα$_q$
Gα$_q$[5C]
Gα$_{11}$
Gα$_{11}$[5C]

Figure 7


A
Gαq[5C]
RGS
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
B
Gα11[5C]
RGS
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
C
Gαq[5C]
Gα11[5C]
Low
Gα signalling (%)
0
20
40
60
80
100
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20
D
High
Gα signalling (%)
0
20
40
60
80
100
RGS1
RGS2
RGS3
RGS4
RGS5
RGS16
RGS20