

## The cationic region of Rhes mediates its interactions with specific G $\beta$ subunits

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## **Abstract**

Ras homologue enriched in striatum (Rhes) is a small monomeric G protein which functions in a variety of cellular processes, including attenuation of G protein-coupled receptor (GPCR) signalling. There have been many studies into the effects of Rhes, but there is no molecular information about how Rhes might bring about these effects. Rhes shares striking sequence homology to AGS1 (activator of G protein signalling 1) and we considered whether the two proteins function in similar ways. AGS1 binds to the G $\beta$ 1 subunit of heterotrimeric G proteins and we have used yeast two-hybrid studies to show that Rhes binds selectively to G $\beta$ 1, G $\beta$ 2 and G $\beta$ 3 subunits. Binding to the G $\beta$  subunits involves the cationic regions of AGS1 and Rhes, and we used Rhes-AGS1 chimeras to show that their different cationic regions determine the G $\beta$ -specificity of the interactions. Possible implications of this interaction for the activity of Rhes are discussed.

**Key words:** AGS1 / cationic region / G $\beta$  subunit / Rhes

## Introduction

The Ras superfamily of small GTPases comprises several protein subfamilies (Figure 1A, [1]). Sequence analysis identifies two family members, Rhes and AGS1, as a distinct subclass with an additional domain in the carboxyl terminal region of the protein. This domain has been termed the “cationic region” as it is enriched in positively charged residues (Figure 1B). As well as their sequence similarity, both proteins are expressed in response to hormones; Rhes expression is regulated by thyroid hormone during the postnatal period [2], whereas AGS1 is induced by dexamethasone (as indicated by its original name Dexras1) [3]. Both proteins have also been implicated in signalling pathways downstream of G protein-coupled receptors (GPCRs).

GPCRs represent the largest family of cell surface receptors and are characterised by seven transmembrane helices. In the unstimulated state, the receptor is associated with a heterotrimeric complex of  $G\alpha$ ,  $G\beta$  and  $G\gamma$  subunits in which the  $G\alpha$  subunit is bound to GDP. Activation of the receptor stimulates the exchange of GDP for GTP, releasing the  $G\alpha$ -GTP and  $G\beta\gamma$  subunits to activate a variety of downstream effectors that are responsible for bringing about the changes in cell behaviour.  $G\alpha$  proteins possess an intrinsic GTPase activity and GTP hydrolysis returns the heterotrimer to its inactive state. The specificity, magnitude and duration of GPCR signalling is further regulated by accessory proteins that interact with the receptors, the G proteins or the effectors. These include receptor kinases (reviewed by [4]), regulator of G protein signalling (RGS) proteins (reviewed by [5,6]), and activator of G protein signalling (AGS) proteins (reviewed by [7,8]). The realisation that heterotrimeric G proteins can function in cellular processes independently of the receptors [9] illustrates how significant the regulation by these accessory proteins can be in some circumstances.

AGS1/DexRas1 was first identified in mice [3] and its human homologue was isolated in a yeast screen for receptor-independent activators of heterotrimeric G proteins [10,11]. *In vitro* analyses suggested that AGS1 mimics activated receptors by binding to  $G\alpha$  subunits and promoting nucleotide exchange [10,11], and is consistent with *in vivo* evidence that AGS1 activates both  $G\alpha$ - [12,13] and  $G\beta\gamma$ -mediated signalling pathways [11,14] in the absence of ligand. However, the ability of AGS1 to inhibit receptor-activated  $G\beta\gamma$ -mediated signalling [11,14,15,16] suggested additional activities and we previously used yeast two-hybrid analysis to reveal a novel interaction between AGS1 and  $G\beta 1$  [17]. The interaction between the C-terminal cationic region of AGS1 and blades 3-to-7 of the  $G\beta 1$  propeller structure might explain how AGS1 promotes  $G\alpha\beta\gamma$  dissociation in the absence of receptor stimulation but selectively inhibits  $G\beta\gamma$ -mediated signalling in receptor-stimulated pathways [8].

There is increasing evidence that Rhes regulates signalling pathways involving GPCRs and heterotrimeric G proteins; it attenuates signalling via the cAMP/PKA pathway mediated by both wild-type thyroid stimulating hormone (TSH) receptors and constitutively active  $\beta 2$ -adrenergic receptors [18], inhibits signalling from a variety of other receptors [19], and modulates dopamine signalling [20]. How Rhes achieves this is unclear, although its lack of effect on either a constitutively active  $G\alpha$  subunit or a directly activated effector protein (protein kinase A activated by forskolin) [18] suggests that Rhes might be acting on the heterotrimeric  $G\alpha\beta\gamma$  complex. Given the functional similarity between Rhes and AGS1, and the striking sequence homology between the two proteins (particularly with regards to their novel cationic regions), we investigated whether Rhes, like AGS1, interacts with  $G\beta$  subunits. Using the yeast two-hybrid system, we demonstrate that Rhes interacts selectively with  $G\beta 1$ ,  $G\beta 2$  and  $G\beta 3$  and that the interaction, like that between AGS1 and  $G\beta 1$  [17], involves the

cationic region of Rhes and blades 3-to-7 of the G $\beta$  propellers. Analysis of AGS1-Rhes chimeras suggests that the different cationic regions specify their interactions with the G $\beta$  subunits. The interaction between Rhes and G $\beta$  subunits revealed in this study could explain how Rhes can influence signalling through heterotrimeric G proteins.

## Materials and methods

### *Yeast two-hybrid assays*

The AH109 *Saccharomyces cerevisiae* strain, pGADT7-SV40, pGBKT7-p53, and pGBKT7-lamin-C constructs used in this study were supplied with the Matchmaker yeast two-hybrid kit and all yeast-based methods were carried out as detailed by the manufacturer (BD Biosciences Clontech, Oxford, UK; kit version PT13529-1). Two-hybrid assays were carried out following sequential transformations of AH109 with pGBKT7-based plasmids and then pGADT7-based plasmids. Co-transformants were maintained on MM (a defined minimal medium lacking leucine and tryptophan) prior to screening for interactions. Cell concentrations were determined using a Coulter Channelyser (Beckman-Coulter, Luton, UK). All experiments to investigate interactions and protein expression were repeated at least three times with different isolates.

### *DNA constructs*

DNA manipulations were performed by standard methods. Amplification by the polymerase chain reaction (PCR) was performed in 70  $\mu$ l reactions using Pwo DNA polymerase from *Pyrococcus woesei* according to the supplier's instructions (Roche Molecular Biochemicals, Lewes, UK). Oligonucleotides were synthesised by TAGN Newcastle (Gateshead, Northumbria, UK). All DNA constructs were sequenced to confirm that no errors had been introduced during amplification.

The full-length G $\beta$  subunits (G $\beta$ 1, G $\beta$ 2, G $\beta$ 3, G $\beta$ 4 and G $\beta$ 5) and the deleted G $\beta$ 1 constructs (G $\beta$ <sup>1WD1-7</sup>, G $\beta$ <sup>1WD3-7</sup>, G $\beta$ <sup>1WD5-7</sup>) were expressed as fusions with the GAL4 Activation Domain from the pGADT7 vector as described previously [17].

The Rhes open reading frame (ORF) was amplified by PCR from placental cDNA using JO1939 (5' gggcat**ATGATGAAGACTTTGTCCAGC**, sense primer containing an *NdeI* site and initiator codon in bold, lowercase letters indicate sequence not found within the gene being amplified) and JO1940 (5' ggggatcc**TCACTGGATGGTGCACCTGTCCCTCT**; antisense primer containing a *BamHI* site and **TCA** stop anticodon) and cloned into the *NdeI*-*BamHI* sites of the yeast two-hybrid bait vector pGBKT7 to generate pGBKT7-Rhes.

Rhes<sup>ACR</sup> (Rhes construct lacking the region Met<sup>189</sup>-Ala<sup>235</sup>) was generated by inverse PCR on pGBKT7-Rhes using JO2179 (5' **CGCCGCCCCAGCGTCAACAGTGACCTCAAG**; sense primer containing the **CGC** codon corresponding to Arg<sup>236</sup> of Rhes) and JO2178 (5' **GCTGAAGAGCACGTAGAACATCTCG**; antisense primer containing the **GCT** anticodon corresponding to Ser<sup>188</sup> of Rhes) and ligating the product to itself.

Rhes<sup>189-235</sup> (construct corresponding to the region Met<sup>189</sup>-Ala<sup>235</sup> of Rhes) was amplified using JO2184 (5' gggcat**ATGGCCAAGCTGCCCCACGAGAT**; sense primer containing an *NdeI* site and the **ATG** codon corresponding to Met<sup>189</sup> of Rhes) and JO2185 (5' ggggatcctca**GGCGAAGGGCGAGACCATGCCAT**; antisense primer containing a *BamHI* site and a stop anticodon immediately downstream of the **GGC** anticodon corresponding to Ala<sup>235</sup> of Rhes) with pGBKT7-Rhes as template.) and cloned into the *NdeI*-*BamHI* sites of the yeast two-hybrid bait vector pGBKT7 to generate pGBKT7-Rhes<sup>189-235</sup>.

Rhes<sup>CR-AGS1</sup> (construct in which the cationic region of Rhes [Met<sup>189</sup>-Ala<sup>235</sup>] was replaced by the equivalent region of AGS1 [Met<sup>194</sup>-Ala<sup>250</sup>]) was created by ligating the inverse PCR product generated for the construction of Rhes<sup>ACR</sup> (see above) to the PCR product obtained on pGBKT7-AGS1 [17] using JO2175 (5' **ATGGCCAAGCTGCCCCAGCGAG**; sense primer containing the **ATG** codon corresponding to Met<sup>194</sup> of AGS1) and JO2174 (5' **CGCGAAGGGTGCCACGATGCCAAAGGC**; antisense primer containing the **CGC** anticodon corresponding to Ala<sup>250</sup> of AGS1).

AGS1<sup>CR-Rhes</sup> (construct in which the cationic region of AGS1 [Met<sup>194</sup>-Ala<sup>250</sup>] was replaced by the equivalent region of Rhes [Met<sup>189</sup>-Ala<sup>235</sup>]) was created by ligating the product generated by PCR on pGBKT7-Rhes with JO2181 (5' **ATGGCCAAGCTGCCACACGAG**; sense primer containing the **ATG** codon corresponding to Met<sup>189</sup> in Rhes) and JO2180 (5' **GGCGAAGGGCGAGACCATGCC**; antisense primer containing the **GGC** anticodon corresponding to Ala<sup>235</sup> of Rhes) to the product obtained by inverse PCR on pGBKT7-AGS1 using JO2173 (5' **CGCCGGCCCAGCGTACACAGC**; sense primer containing the **CGC** codon corresponding to Arg<sup>251</sup> in AGS1) and JO2172 (5' **GGCGAAGAGCGCGGGAACATCTG**; antisense primer containing the **GGC** anticodon corresponding to Ala<sup>193</sup> of AGS1).

#### *Western blotting*

Western blotting was performed as described previously [17]. Mouse monoclonal anti-GAL4 binding domain (BD) and mouse monoclonal anti-GAL4 activation domain (AD) were used according to manufacturers' instructions (BD Biosciences, Oxford, UK). HRP-conjugated secondary antibodies were from either Amersham plc (Little Chalfont, UK) or Roche Diagnostics (Lewes, UK) and used according to the manufacturer's instructions.

## Results

### *Rhes binds selectively to human G $\beta$ subunits*

Yeast two-hybrid analysis was used to investigate interactions between Rhes and each of the five human G $\beta$  subunits (Figure 2). The yeast screening strain AH109 was transformed first with pGBKT7-Rhes and then with the various pGADT7-G $\beta$  constructs and co-transformants isolated on selective medium. Growth on MM (a defined minimal medium lacking leucine and tryptophan) confirmed the presence of the complementing plasmids in each isolate. The formation of blue colonies on MM-His-Ade+X $\alpha$ gal (MM lacking histidine and adenine but incorporating 5-bromo-4-chloro-3-indolyl- $\alpha$ -D-galactoside [X $\alpha$ gal]) indicated that interaction between Rhes and the G $\beta$ 1, G $\beta$ 2 and G $\beta$ 3 subunits (but not G $\beta$ 4 nor G $\beta$ 5) had resulted in expression of the *HIS3*, *ADE2* and *MEL1* reporter genes. A positive control for interacting proteins was provided by transformants expressing SV40-T antigen and p53, proteins that interact strongly [21]. In contrast, SV40-T antigen does not interact with lamin-C and co-transformants were unable to grow on plates lacking histidine and adenine. Immunoblotting confirmed expression of Rhes and each of the G $\beta$  subunits in the various transformants; fusion of Rhes to the GAL4 DNA binding domain (BD) in pGBKT7 increases its MW from ~30 kDa to ~53 kDa (note that the antibody to GAL4-BD detects a faint product at ~35 kDa in all samples, including those not expressing the fusion protein), fusion of the G $\beta$  subunits to the GAL4 activation domain (AD) in pGADT7 increases their MWs from ~37 kDa to ~55 kDa. The interactions were quantitated using a liquid-based  $\beta$ -galactosidase assay for expression of the *LacZ* reporter gene. Under the conditions used, strongly interacting proteins (such as SV40-T antigen and p53) generate ~4.5 Units of  $\beta$ -galactosidase activity, while non-interacting proteins (such as SV40-T antigen and lamin-C) generate less than 0.5 Units. Co-transformants expressing Rhes and G $\beta$ 1, G $\beta$ 2 or G $\beta$ 3 produced ~3 Units of activity,

suggesting a reasonably strong interaction. In contrast, strains expressing Rhes and either G $\beta$ 4 or G $\beta$ 5 produced less than 0.5 Units, indicating these proteins do not interact.

Neither Rhes nor any of the G $\beta$  subunits supported growth on the MM-His-Ade+Xgal plates when expressed on their own (not shown), demonstrating they have no inherent ability to activate transcription of the reporter constructs. Furthermore, transferring Rhes and the G $\beta$  subunits from their original two-hybrid plasmids to the complementing plasmids to provide the reciprocal pairing for analysis generated interactions that were indistinguishable from those in Figure 2, confirming that induction of reporter activity was due to an interaction between Rhes and G $\beta$ 1, G $\beta$ 2 and G $\beta$ 3 and not to non-specific effects arising from expression of these proteins as fusions with either the GAL4 binding domain or GAL4 activation domains. We have shown previously that each G $\beta$  subunit was able to interact in the two-hybrid system with G $\gamma$ 4 [22], a human G $\gamma$  subunit that binds to G $\beta$  subunits in vivo [23].

#### *Mapping the Rhes interaction site on G $\beta$ 1*

G $\beta$  subunits contain two structurally distinct domains, an N-terminal  $\alpha$ -helix of ~20 amino acids which forms a coiled-coil interaction with G $\gamma$  subunits, and a seven-bladed propeller structure formed from the repeating WD repeat motifs that is believed to interact with binding partners [17,24,25,26]. Each propeller blade consists of a single WD repeat organised into four twisted  $\beta$ -strands, and variable regions between each repeat make it straightforward to generate constructs containing different numbers of blades. As constructs require an odd number of blades to be stable, and blade 7 provides a 'velcro snap' to close the propeller, only a limited series of G $\beta$ 1 constructs were tested for their ability to interact with Rhes (Figure 2B). Immunoblotting demonstrated expression of Rhes and each of the G $\beta$ 1 constructs (G $\beta$ 1

constructs are expressed as fusions to the activation domain from pGADT7 and were detected using an antibody to the activation domain, and the activation domain adds ~18 kDa to the MW of the G $\beta$ 1 constructs), while control experiments confirmed that neither Rhes nor the G $\beta$ 1 constructs were able to induce reporter genes when expressed on their own (not shown). Removal of either the N-terminal  $\alpha$ -helix or the first two propeller blades of G $\beta$ 1 did not affect its interaction with Rhes. However, G $\beta$ 1<sup>WD5-7</sup> (lacks blades 1-to-4) failed to interact with Rhes, suggesting that this region is required for the interaction. The same region of G $\beta$ 1 was previously identified as being required for its interaction with AGS1 [17].

#### *Mapping the G $\beta$ interaction site on Rhes*

Rhes is similar to Ras and other small monomeric G proteins except for a C-terminal cationic region (residues Met<sup>189</sup>-Ala<sup>235</sup>; Figure 1B). A similar cationic region in the highly homologous AGS1 mediates its interaction with both the neuronal nitric oxide adaptor protein CAPON [27] and G $\beta$ 1 [17], and we used two-hybrid analysis to investigate the role of this region in the interaction of Rhes with the G $\beta$  subunits (Figure 3). Immunoblotting confirmed that all of the constructs were expressed, and none of the constructs induced expression of the reporter genes when present on their own (not shown). A Rhes construct lacking the cationic region (Rhes <sup>$\Delta$ CR</sup>, lacks residues Met<sup>189</sup>-Ala<sup>235</sup>) failed to interact with any of the G $\beta$  subunits (Figure 3A), whereas a construct containing just this region of Rhes (fused to the GAL4 binding domain) interacted with G $\beta$ 1, G $\beta$ 2 and G $\beta$ 3 (Figure 3B). Thus, the cationic region of Rhes is not only required for its interactions with G $\beta$  subunits but appears to be sufficient for these interactions.

*The cationic region determines the specificity of the Gβ interactions*

The cationic regions of both AGS1 [17] and Rhes (Figure 3) are required for their interactions with Gβ subunits. However, the two regions are very different in the two proteins (Figure 1B) and we wondered whether these differences were responsible for their different Gβ specificities; both AGS1 and Rhes bind to Gβ1, but only Rhes also binds to Gβ2 and Gβ3. We therefore generated constructs that exchanged the cationic regions between the two proteins, and used two-hybrid analysis to examine their ability to interact with the various Gβ subunits (Figure 4). Immunoblotting confirmed that all of the constructs were expressed, and none of the constructs induced expression of the reporter genes when present on their own (not shown). Rhes<sup>CR-AGS1</sup>, which has residues Met<sup>189</sup>-Ala<sup>235</sup> of Rhes replaced by residues Met<sup>194</sup>-Ala<sup>250</sup> of AGS1, interacted with only Gβ1 and, unlike wild type Rhes, was unable to interact with either Gβ2 or Gβ3 (Figure 4A). In contrast, AGS1<sup>CR-Rhes</sup>, (Met<sup>194</sup>-Ala<sup>250</sup> of AGS1 replaced by residues Met<sup>189</sup>-Ala<sup>235</sup> of Rhes) interacted with Gβ1, Gβ2 and Gβ3 (Figure 4B). These results suggest that the different cationic regions of Rhes and AGS1 interact with different Gβ subunits.

## Discussion

Our data suggest that Rhes interacts specifically with the G $\beta$ 1, G $\beta$ 2 and G $\beta$ 3 subunits of heterotrimeric G proteins, but not G $\beta$ 4 or G $\beta$ 5. The interaction involves the C-terminal cationic region of Rhes and blades 3-to-7 of the G $\beta$  subunits, and appears similar to the interaction between the cationic region of AGS1 and blades 3-to-7 of G $\beta$ 1 [17]. Indeed, exchanging the cationic regions between Rhes and AGS1 alters the G $\beta$ -binding specificities of the chimeras.

The interaction of Rhes with the G $\beta$  subunits requires its C-terminal cationic region. This region identifies Rhes and AGS1 within the Ras-like family of small G proteins and was previously shown to mediate the interaction of AGS1 with G $\beta$ 1 [17]. Despite both binding to G $\beta$ 1, there is no obvious sequence homology between the cationic regions of Rhes and AGS1, and any shared activities are presumably not dependent upon precise residues. But there is a level of specificity in the interaction in that the cationic region of AGS1 interacts with G $\beta$ 1 while the cationic region of Rhes also interacts with G $\beta$ 2 and G $\beta$ 3. The cationic region of AGS1 also interacts with the neuronal nitric oxide adaptor protein CAPON [27]. It is therefore possible that this domain allows both AGS1 and Rhes to interact with a wide variety of other molecules. These may be responsible for the effects of Rhes and AGS1 that do not appear to be mediated through G proteins.

The Rhes-G $\beta$ 1 interaction requires blades 3-to-7 of the G $\beta$   $\beta$ -propeller. Given the conservation of sequence and structure between G $\beta$  subunits, it seems likely that the same regions of G $\beta$ 2 and G $\beta$ 3 are involved in their interaction with Rhes. Our previous study identified the same region of G $\beta$ 1 to be involved in its interaction with AGS1 [17]. This

region of G $\beta$ 1 is not generally thought to be involved in interactions with its other binding partners. G $\gamma$  subunits, for example, form a coiled-coil interaction with the N-terminal  $\alpha$ -helix of the G $\beta$  subunit [28], while G $\alpha$  subunits are believed to interact with blades 1-to-3 of the  $\beta$ -propeller [28], and target effector proteins primarily contact the outer segments of blades 1 and 2 [29,30]. However, overlapping binding sites and more complex interactions, especially between G $\beta$  subunits and target effectors, might further complicate the interactions. We cannot, for example, rule out a requirement for blade 3 in binding Rhes, or AGS1, as well as its role in binding G $\alpha$  subunits. It is also possible that binding of a protein to one part of the G $\beta$  subunit could affect the binding of a second protein to a different part of the same G $\beta$  molecule.

Rhes has complex effects on GPCR signalling [18,19]. It attenuates signalling by GPCRs that function through G $\alpha$ s but does not appear to affect signalling through G $\alpha$ i subunits. The effect on G $\alpha$ s signalling appears to be at the level of activation of the heterotrimer G $\alpha\beta\gamma$  complex as constitutively active receptors are affected, but constitutively active G $\alpha$ s subunits are not, and Rhes has no effect on direct activation of the cAMP/PKA pathway by forskolin. These results suggest that Rhes can affect the coupling of GPCRs to G $\alpha$ s subunits. The interaction of Rhes with G $\beta$  subunits revealed in our study could provide a mechanism for this inhibition, either by disrupting the formation of heterotrimeric G proteins or inhibiting the interaction of the heterotrimer with the receptor. Rhes appears perfectly located within the cell to have such effects. Like the G protein subunits, Rhes is not an integral membrane protein but associates with the plasma membrane through post-translational modifications. For Rhes, this is via prenylation of a conserved CAAX motif at its C-terminus [18]. G $\gamma$  subunits are also C-terminally prenylated, G $\alpha$ s subunits are N-terminally palmitoylated, and G $\alpha$ i subunits are both palmitoylated and myristoylated at the N-terminus (reviewed by [31]). G $\beta$  subunits associate

indirectly with membranes through interaction with the  $G\gamma$  subunits, and this could be sensitive to inhibition by the membrane localised Rhes.

Why Rhes affects only  $G_{\alpha s}$  signalling, and not  $G_{\alpha i}$  signalling, is an interesting challenge [18]. One possibility could relate to the  $G\beta$ -specificity of Rhes (Figure 2A) and it might be, for example, that  $G_{\alpha s}$  subunits interact preferentially with  $G\beta_{1-3}$  (and thus be affected by Rhes) whereas  $G_{\alpha i}$  subunits are more often complexed with  $G\beta_{4-5}$  (and not affected by Rhes). Alternatively,  $G_{\alpha i}$  may be less susceptible than  $G_{\alpha s}$  to disruption by Rhes because  $G_{\alpha i}$  has a stronger interaction with the plasma membrane and may be better able to interact with  $G\beta\gamma$  subunits and/or receptors.  $G_{\alpha i}$  is both myristoylated and palmitoylated whereas  $G_{\alpha s}$  is only palmitoylated and requires interaction with  $G\beta\gamma$  subunits for full membrane association, the prenylated  $G\gamma$  subunit providing a second membrane anchor [32] (reviewed by [33]). Indeed,  $G_{\alpha s}$  associates poorly with membranes in the absence of  $G\beta\gamma$  subunits, and requires this interaction for efficient palmitoylation [34]. The greater membrane association of  $G_{\alpha i}$  may overcome the inhibitory effects of Rhes more readily than  $G_{\alpha s}$ .

As a final consideration, Rhes functions independently of G proteins, binding to effector proteins such as Raf and phosphoinositide 3-kinase [18]. It will be interesting to determine if these more traditional targets for Ras-like proteins also interact with the cationic region of Rhes or whether they interact with other parts of the Rhes molecule.

## Figure legends

### Figure 1

Rhes and AGS1 comprise a distinct sub-family of the Ras G protein family. A) Schematic phylogenetic tree of selected members of the Ras superfamily, scale bar represents 0.1 nucleotide substitutions per base. The tree was constructed using ClustalX [35] and visualised using TreeView version 1.6.6. B) Sequence comparison of Rhes, AGS1 and mammalian H-Ras. Sequences were aligned using MultAlin version 5.4.1. [36] using symbol comparison table blosum62, gap weight 12 and gap length weight 2. Gaps introduced to maximise the alignment are indicated by '-'. Residues shaded in black are conserved in all three proteins, those in grey are in two of the proteins. Boxed regions correspond to interactions for the phosphate moieties of GTP/GDP (G1, G3), the GAP effector (G2), and the guanine nucleotide moiety (G4, G5). The cationic region (residues Met<sup>189</sup>-Ala<sup>235</sup> of Rhes) is indicated by \*.

### Figure 2

Rhes interacts specifically with Gβ1, Gβ2 and Gβ3. A) Rhes (expressed from pGBKT7-Rhes) was co-expressed with each of the human Gβ subunits (expressed from pGADT7). All transformants grew on MM but only those containing Gβ1, Gβ2 or Gβ3 grew on MM-His-Ade+XαGal, suggesting that only these Gβ subunits interact with Rhes to induce expression of the reporter genes. Control strains expressing the strongly interacting SV40-T antigen and p53 or the non-interacting SV40-T and lamin-C are included for comparison. A liquid-based β-galactosidase assay confirms that co-expression of Rhes and Gβ1, Gβ2 or Gβ3 induces expression of the *LacZ* reporter. B) Rhes was expressed separately with various Gβ1 constructs lacking different parts of the protein. All transformants grew on MM but those containing Gβ1<sup>WD5-7</sup> were unable grow on MM-His-Ade+XαGal, suggesting that this

construct was unable to interact with Rhes. Expression of Rhes in each strain was confirmed by immunoblotting with an antibody to the GAL4-BD (Rhes was fused to the GAL4 binding domain; note that the antibody to GAL4-BD detects a faint product at ~35 kDa in all samples, including those not expressing the Rhes fusion protein), and expression of the various G $\beta$  constructs were confirmed by immunoblotting with an antibody to the GAL4-AD (the G $\beta$  constructs are fused to the GAL4 activation domain). At least three separate isolates of each strain were subjected to immunoblotting to confirm that the results shown are representative. The  $\beta$ -galactosidase results are presented as the mean  $\pm$  SD of three different isolates of the relevant strain, each with duplicate determinations.

### Figure 3

Binding of G $\beta$  subunits to the cationic region of Rhes. A) Rhes lacking the cationic region (expressed from pGBKT7-Rhes <sup>$\Delta$ CR</sup>, lacks residues Met<sup>189</sup>-to-Ala<sup>235</sup>) was co-expressed with each of the G $\beta$  subunits (expressed from pGADT7). All transformants grew on MM but none grew on MM-His-Ade+X $\alpha$ Gal, suggesting that the cationic region of Rhes is essential for interaction with the G $\beta$  subunits. The liquid-based  $\beta$ -galactosidase assay confirms that none of the combinations induced expression of the *LacZ* reporter. B) The cationic region of Rhes (expressed from pGBKT7-Rhes<sup>189-235</sup>, contains only those residues from Met<sup>189</sup>-to-Ala<sup>235</sup>) was co-expressed with human G $\beta$  subunits (expressed from pGADT7). All transformants grew on MM but only those containing G $\beta$ 1, G $\beta$ 2 or G $\beta$ 3 grew on MM-His-Ade+X $\alpha$ Gal, suggesting that the cationic region of Rhes is sufficient for the interaction with these G $\beta$  subunits. The liquid-based  $\beta$ -galactosidase assay confirms that co-expression of Rhes<sup>189-235</sup> and G $\beta$ 1, G $\beta$ 2 or G $\beta$ 3 induces expression of the *LacZ* reporter. Expression of the various Rhes constructs were confirmed by immunoblotting with an antibody to the GAL4-BD (the

Rhes constructs were fused to the GAL4 binding domain), and expression of the various G $\beta$  constructs were confirmed by immunoblotting with an antibody to the GAL4-AD (the G $\beta$  constructs were fused to the GAL4 activation domain). At least three separate isolates of each strain were subjected to immunoblotting to confirm that the results shown are representative. The  $\beta$ -galactosidase results are presented as the mean  $\pm$  SD of three different isolates of the relevant strain, each with duplicate determinations.

#### Figure 4

The cationic regions of Rhes and AGS1 determine the specificity of their binding to G $\beta$  subunits. A) Rhes containing the cationic region from AGS1 (pGBKT7-Rhes<sup>CR-AGS1</sup>, residues Met<sup>189</sup>-to-Ala<sup>235</sup> of Rhes replaced by residues Met<sup>194</sup>-to-Ala<sup>250</sup> of AGS1) was co-expressed with the human G $\beta$ 1, G $\beta$ 2 and G $\beta$ 3 subunits (expressed from pGADT7). All transformants grew on MM but only the transformant containing G $\beta$ 1 grew on MM-His-Ade+X $\alpha$ Gal and induced expression of the LacZ reporter, suggesting that Rhes<sup>CR-AGS1</sup> interacts with only G $\beta$ 1. B) AGS1 containing the cationic region of Rhes (pGBKT7-AGS1<sup>CR-Rhes</sup>, residues Met<sup>194</sup>-to-Ala<sup>250</sup> of AGS1 replaced by residues Met<sup>189</sup>-to-Ala<sup>235</sup> of Rhes) was co-expressed with the human G $\beta$ 1, G $\beta$ 2 and G $\beta$ 3 subunits (expressed from pGADT7). All transformants grew on MM and MM-His-Ade+X $\alpha$ Gal, and all induced expression of the LacZ reporter, suggesting that AGS1<sup>CR-Rhes</sup> interacts with all three G $\beta$  subunits. Expression of Rhes<sup>CR-AGS1</sup> and AGS1<sup>CR-Rhes</sup> were confirmed by immunoblotting with an antibody to the GAL4-BD (the constructs are fused to the GAL4 binding domain), and expression of the G $\beta$  constructs were confirmed by immunoblotting with an antibody to the GAL4-AD (the constructs are fused to the GAL4 activation domain). At least three separate isolates of each strain were subjected to immunoblotting to confirm that the results shown are representative. The  $\beta$ -galactosidase

results are presented as the mean  $\pm$  SD of three different isolates of the relevant strain, each with duplicate determinations.

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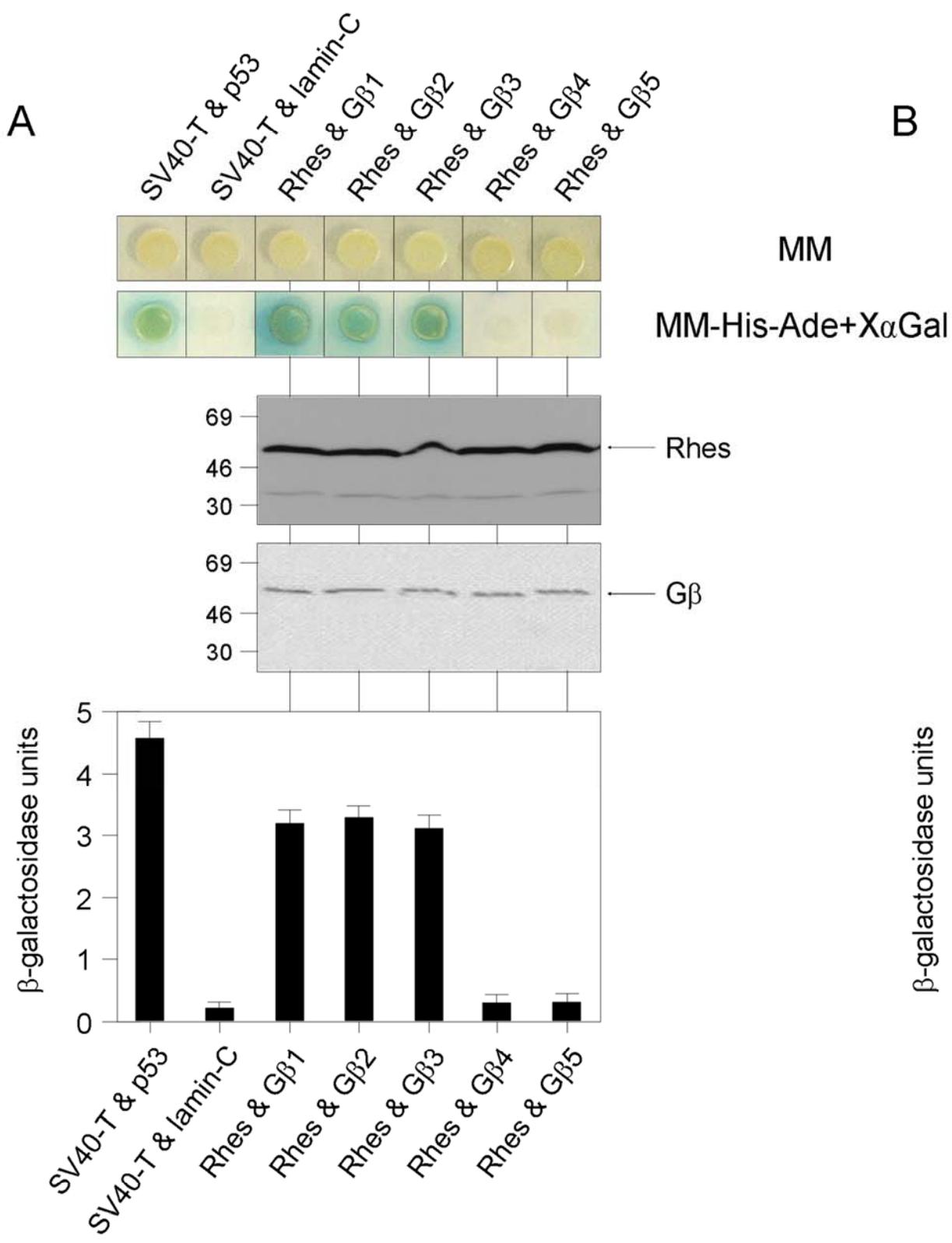
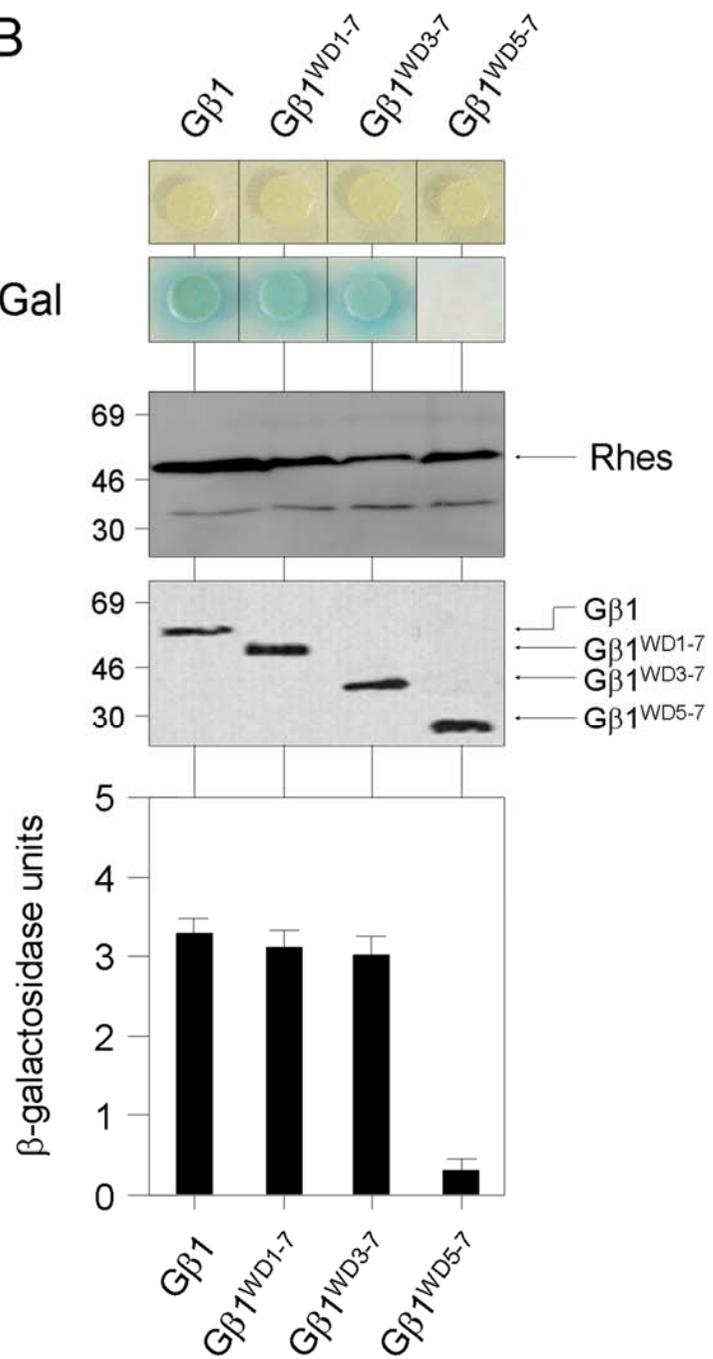
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**A****B**

**A**Rhes<sup>ΔCR</sup> with....

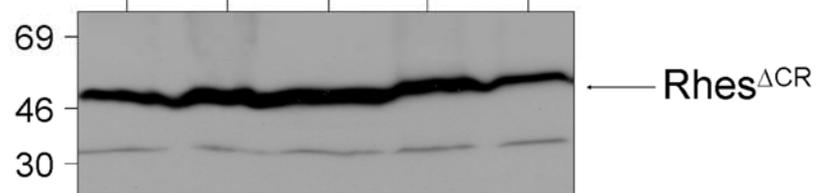
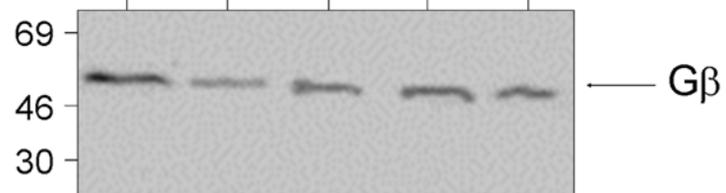
Gβ1 Gβ2 Gβ3 Gβ4 Gβ5



MM

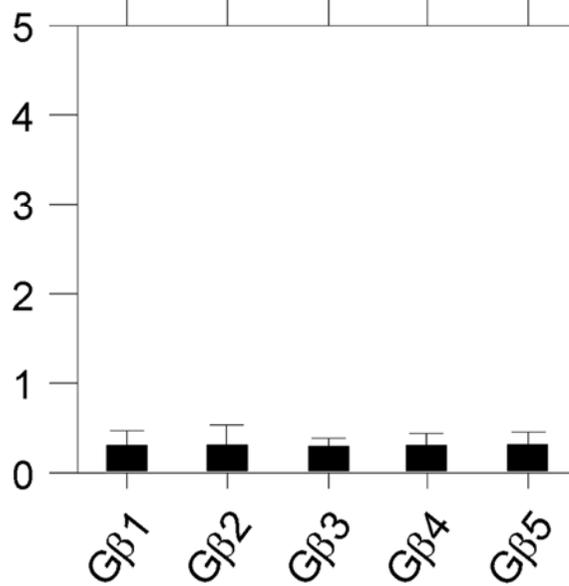


MM-His-Ade+XαGal

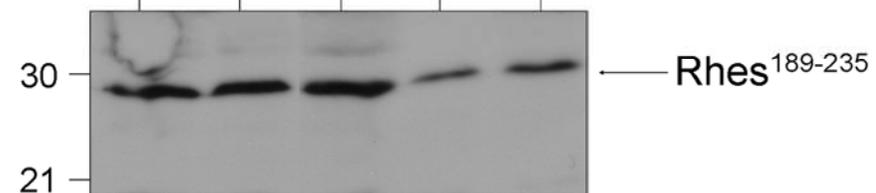
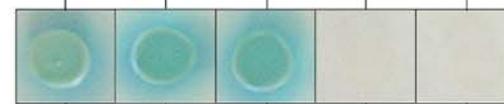
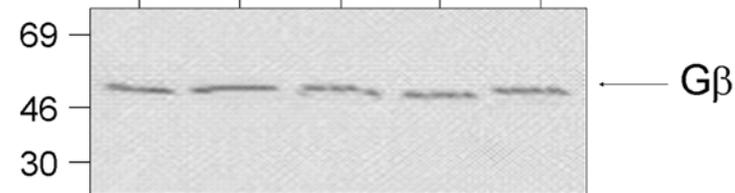
Rhes<sup>ΔCR</sup>

Gβ

β-galactosidase units

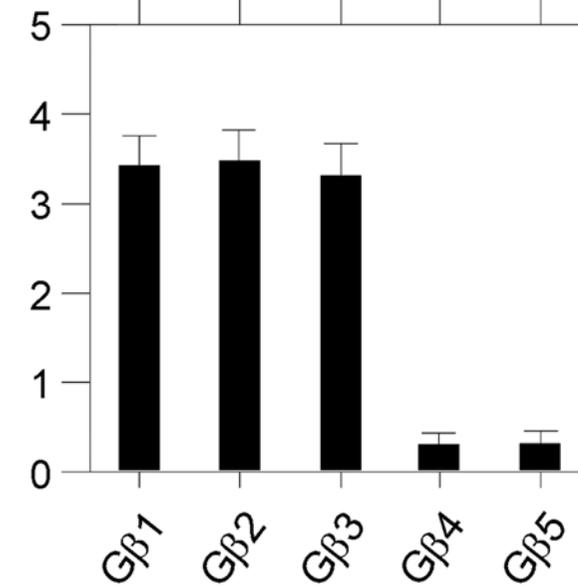
**B**Rhes<sup>189-235</sup> with....

Gβ1 Gβ2 Gβ3 Gβ4 Gβ5

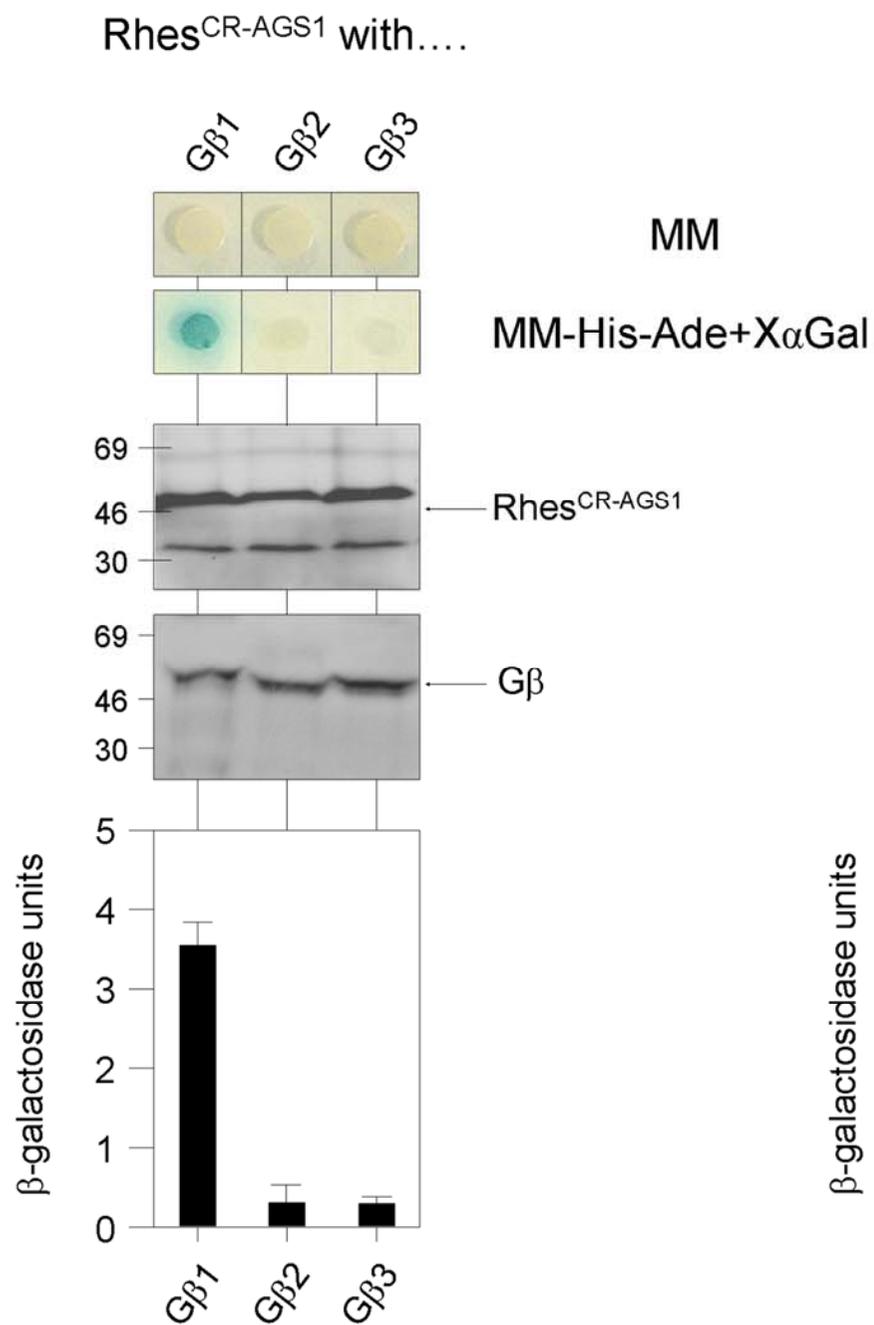
Rhes<sup>189-235</sup>

Gβ

β-galactosidase units



A



B

