Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2009;23:01-08

Accepted: January 05, 2009

The Cationic Region of Rhes Mediates its Interactions with Specific Gβ Subunits

Claire Hill[3,*], Alan Goddard[1,*], Graham Ladds[2,#] and John Davey[3,#]

[1]Department of Biochemistry, University of Oxford, [2]Division of Clinical Sciences, Warwick Medical School and [3]Department of Biological Sciences, University of Warwick, Coventry, *These authors contributed equally to the work, #These authors contributed equally to the work

Key Words
AGS1 • Cationic region • Gβ subunit • Rhes

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 Gβ1 subunit of heterotrimeric G proteins and we have used yeast two-hybrid studies to show that Rhes binds selectively to Gβ1, Gβ2 and Gβ3 subunits. Binding to the Gβ subunits involves the cationic regions of AGS1 and Rhes, and we used Rhes-AGS1 chimeras to show that their different cationic regions determine the Gβ-specificity of the interactions. Possible implications of this interaction for the activity of Rhes are discussed.

Introduction

The Ras superfamily of small GTPases comprises several protein subfamilies (Figure 1, [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. 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 Gα, Gβ and Gγ subunits in which the Gα subunit is bound to GDP. Activation of the receptor stimulates the exchange of GDP for GTP, releasing the Gα-GTP and Gβγ subunits to activate a variety of downstream effectors that are responsible for

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/09/0233-0001$26.00/0

Accessible online at:
www.karger.com/cpb

Dr Graham Ladds
Division of Clinical Sciences, Warwick Medical School
University of Warwick, Coventry, CV4 7AL (Great Britian)
Tel. +44 02476 528361, E-Mail Graham.Ladds@Warwick.ac.uk

bringing about the changes in cell behaviour. Gα 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 Gα subunits and promoting nucleotide exchange [10, 11], and is consistent with *in vivo* evidence that AGS1 activates both Gα- [12, 13] and Gβγ-mediated signalling pathways [11, 14] in the absence of ligand. However, the ability of AGS1 to inhibit receptor-activated Gβγ-mediated signalling [11, 14-16] suggested additional activities and we previously used yeast two-hybrid analysis to reveal a novel interaction between AGS1 and Gβ1 [17]. The interaction between the C-terminal cationic region of AGS1 and blades 3-to-7 of the Gβ1 propeller structure might explain how AGS1 promotes Gαβγ dissociation in the absence of receptor stimulation but selectively inhibits Gβγ-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 β2-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 Gα subunit or a directly activated effector protein (protein kinase A activated by forskolin) [18] suggests that Rhes might be acting on the heterotrimeric Gαβγ 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 Gβ subunits. Using the yeast two-hybrid system, we demonstrate that Rhes interacts selectively with Gβ1, Gβ2 and Gβ3 and that the interaction, like that between AGS1 and Gβ1 [17], involves the cationic region of Rhes and blades 3-to-7 of the Gβ propellers. Analysis of AGS1-Rhes chimeras suggests that the different cationic regions specify their interactions with the Gβ subunits. The interaction between Rhes and Gβ subunits revealed in this study could explain how Rhes can influence signalling through heterotrimeric G proteins.

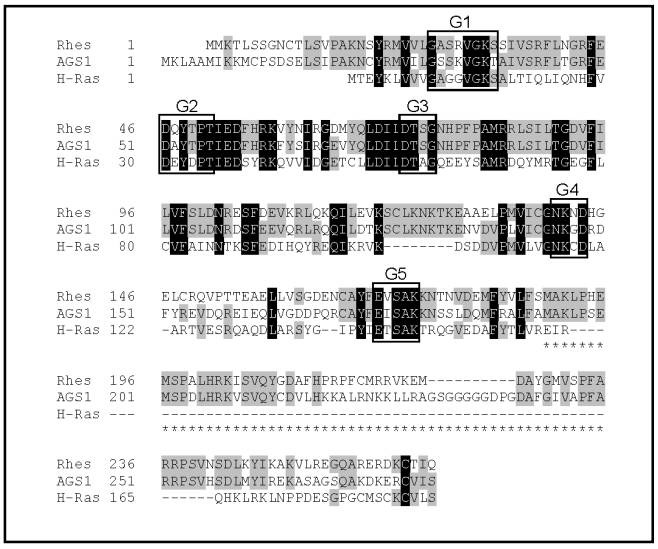

Fig 1. Sequence comparison of Rhes, AGS1 and mammalian H-Ras. Sequences were aligned using MultAlin version 5.4.1. [36] using symbol comparisontable blosum62, gap weight 12 and gap length weight 2. Gaps introducedto maximise the alignment are indicated by '-'. Residues shaded in black are conserved in 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 Met189-Ala235 of Rhes) is indicated by *.

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 µl 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 Gβ subunits (Gβ1, Gβ2, Gβ3, Gβ4 and Gβ5) and the deleted Gβ1 constructs ($G\beta1^{WD1-7}$, $G\beta1^{WD3-7}$, $G\beta1^{WD5-7}$) 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' ggg catATG ATG AAG ACT TTG TCC AGC, sense primer containing an *Nde*I site and initiator codon in bold, lowercase letters indicate sequence not found within the gene being amplified) and JO1940 (5' ggg gat ccTCA CTG GAT GGT GCA CTT GTC CCT CT; antisense primer containing a *Bam*HI site and TCA stop anticodon) and cloned into the *Nde*I-*Bam*HI sites of the yeast two-hybrid bait vector pGBKT7 to generate pGBKT7-Rhes.

$Rhes\Delta^{CR}$ (Rhes construct lacking the region Met^{189}-Ala^{235}) was generated by inverse PCR on pGBKT7-Rhes using JO2179 (5' CGC CGC CCC AGC GTC AAC AGT GAC CTC AAG; sense primer containing the CGC codon corresponding to Arg^{236} of Rhes) and JO2178 (5' GCT GAA GAG CAC GTA GAA CAT CTC G; antisense primer containing the GCT anticodon corresponding to Ser^{188} of Rhes) and ligating the product to itself.

$Rhes^{189-235}$ (construct corresponding to the region Met^{189}-Ala^{235} of Rhes) was amplified using JO2184 (5' ggg catATGGCCAAGCTGCCCCACGAGAT; sense primer containing an *Nde*I site and the ATG codon corresponding to Met^{189} of Rhes) and JO2185 (5' ggg gat cct caGGC GAA GGG CGA GAC CAT GCC AT; antisense primer containing a *Bam*HI site and a stop anticodon immediately downstream of the GGC anticodon corresponding to Ala^{235} of Rhes) with pGBKT7-Rhes as template.) and cloned into the *Nde*I-*Bam*HI sites of the yeast two-hybrid bait vector pGBKT7 to generate pGBKT7-$Rhes^{189-235}$.

$Rhes^{CR-AGS1}$ (construct in which the cationic region of Rhes [Met^{189}-Ala^{235}] was replaced by the equivalent region of AGS1 [Met^{194}-Ala^{250}]) was created by ligating the inverse PCR product generated for the construction of $Rhes\Delta^{CR}$ (see above) to the PCR product obtained on pGBKT7-AGS1 [17] using JO2175 (5' ATG GCC AAG CTG CCC AGC GAG; sense primer containing the ATG codon corresponding to Met^{194} of AGS1) and JO2174 (5' CGC GAA GGG TGC CAC GAT GCC AAA GGC; antisense primer containing the CGC anticodon corresponding to Ala^{250} of AGS1).

$AGS1^{CR-Rhes}$ (construct in which the cationic region of AGS1 [Met^{194}-Ala^{250}] was replaced by the equivalent region of Rhes [Met^{189}-Ala^{235}]) was created by ligating the product generated by PCR on pGBKT7-Rhes with JO2181 (5' ATG GCC AAG CTG CCA CAC GAG; sense primer containing the ATG codon corresponding to Met^{189} in Rhes) and JO2180 (5' GGC GAA GGG CGA GAC CAT GCC; antisense primer containing the GGC anticodon corresponding to Ala^{235} of Rhes) to the product obtained by inverse PCR on pGBKT7-AGS1 using JO2173 (5' CGC CGG CCC AGC GTA CAC AGC; sense primer containing the CGC codon corresponding to Arg^{251} in AGS1) and JO2172 (5' GGC GAA GAG CGC GCG GAA CAT CTG; antisense primer containing the GGC anticodon corresponding to Ala^{193} 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 Gβ subunits

Yeast two-hybrid analysis was used to investigate interactions between Rhes and each of the five human Gβ subunits (Figure 2). The yeast screening strain AH109 was transformed first with pGBKT7-Rhes and then with the various pGADT7-Gβ 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+Xαgal (MM lacking histidine and adenine but incorporating 5-bromo-4-chloro-3-indolyl-α-D-galactoside [Xαgal]) indicated that interaction between Rhes and the Gβ1, Gβ2 and Gβ3 subunits (but not Gβ4 nor Gβ5) 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 Gβ 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-

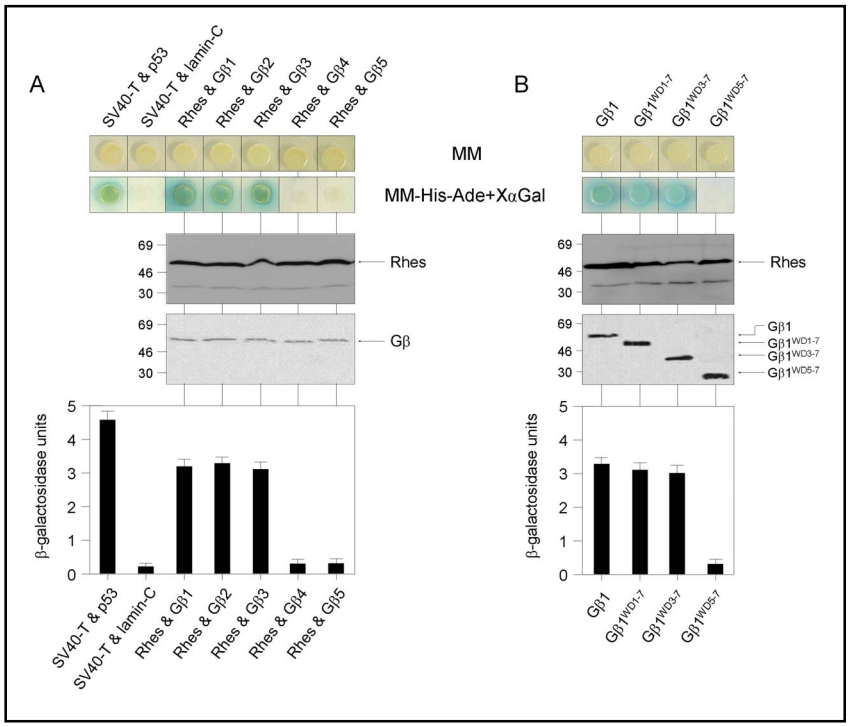

Fig 2. Rhes interacts specifically with Gβ1, Gβ2 and Gβ3. A) Rhes (expressed from pGBKT7-Rhes) was co-expressed with each of the human Gβ subunits (expressed from pGADT7). All transformants grew on MM but only those containing Gβ1, Gβ2 or Gβ3 grew on MM-His-Ade+XαGal, suggesting that only these Gβ subunits 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 $G\beta1^{WD5-7}$ 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 Gβ constructs were confirmed by immunoblotting with an antibody to the GAL4-AD (the Gβ 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 β-galactosidase results are presented as the mean ± SD of three different isolates of the relevant strain, each with duplicate determinations.

BD detects a faint product at ~35 kDa in all samples, including those not expressing the fusion protein), fusion of the Gβ 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 β-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 β-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 Gβ1, Gβ2 or Gβ3 produced ~3 Units of activity, suggesting a reasonably strong interaction. In contrast, strains expressing Rhes and either Gβ4 or Gβ5 produced less than 0.5 Units, indicating these proteins do not interact.

Neither Rhes nor any of the Gβ subunits supported growth on the MM-His-Ade+Xαgal 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 Gβ 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 Gβ1, Gβ2 and Gβ3 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 Gβ subunit was able to interact in the two-hybrid system with Gγ4 [22], a human Gγ subunit that binds to Gβ subunits *in vivo* [23].

Mapping the Rhes interaction site on Gβ1

Gβ subunits contain two structurally distinct domains, an N-terminal α-helix of ~20 amino acids which forms a coiled-coil interaction with Gγ subunits, and a seven-bladed propeller structure formed from the repeating WD repeat motifs that is believed to interact with binding partners [17, 24-26]. Each propeller blade consists of a single WD repeat organised into four twisted β-strands, and variable regions between each repeat make it straightforward to generate constructs containing different numbers of

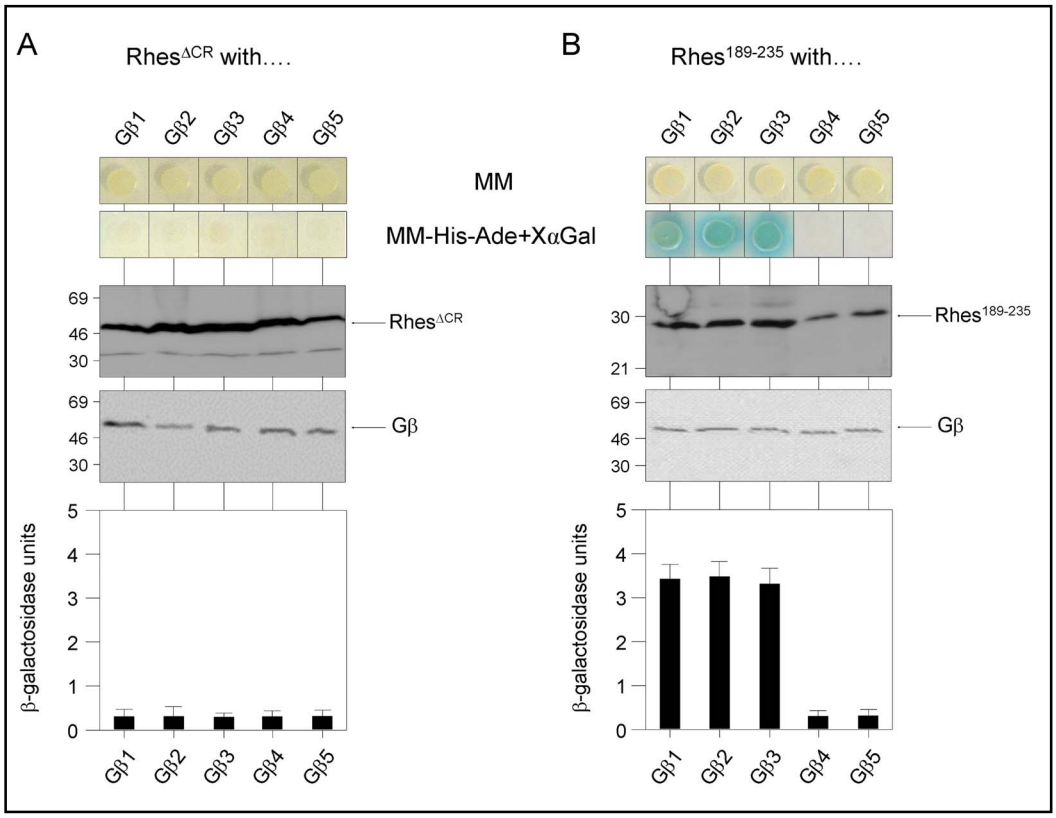

Fig 3. Binding of Gβ subunits to the cationic region of Rhes. A) Rhes lacking the cationic region (expressed from pGBKT7-RhesΔ^{CR}, lacks residues Met^{189}-to-Ala^{235}) was co-expressed with each of the Gβ subunits (expressed from pGADT7). All transformants grew on MM but none grew on MM-His-Ade+XαGal, suggesting that the cationic region of Rhes is essential for interaction with the Gβ subunits. The liquid-based β-galactosidase assay confirms that none of the combinations induced expression of the *LacZ* reporter. B) The cationic region of Rhes (expressed from pGBKT7-Rhes$^{189-235}$, contains only those residues from Met^{189}-to-Ala^{235}) was co-expressed with 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 the cationic region of Rhes is sufficient for the interaction with these Gβ subunits. The liquid-based β-galactosidase assay confirms that co-expression of Rhes$^{189-235}$ and Gβ1, Gβ2 or Gβ3 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 Gβ constructs were confirmed by immunoblotting with an antibody to the GAL4-AD (the Gβ 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 β-galactosidase results are presented as the mean ± SD of three different isolates of the relevant strain, each with duplicate determinations.

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 Gβ1 constructs were tested for their ability to interact with Rhes (Figure 2B). Immunoblotting demonstrated expression of Rhes and each of the Gβ1 constructs (Gβ1 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 Gβ1 constructs), while control experiments confirmed that neither Rhes nor the Gβ1 constructs were able to induce reporter genes when expressed on their own (not shown). Removal of either the N-terminal α-helix or the first two propeller blades of Gβ1 did not affect its interaction with Rhes. However, Gβ1^{WD5-7} (lacks blades 1-to-4) failed to interact with Rhes, suggesting that this region is required for the interaction. The same region of Gβ1 was previously identified as being required for its interaction with AGS1 [17].

Mapping the Gβ interaction site on Rhes

Rhes is similar to Ras and other small monomeric G proteins except for a C-terminal cationic region (residues Met^{189}-Ala^{235}; Figure 1). A similar cationic region in the highly homologous AGS1 mediates its interaction with both the neuronal nitric oxide adaptor protein CAPON [27] and Gβ1 [17], and we used two-hybrid analysis to investigate the role of this region in the interaction of Rhes with the Gβ 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

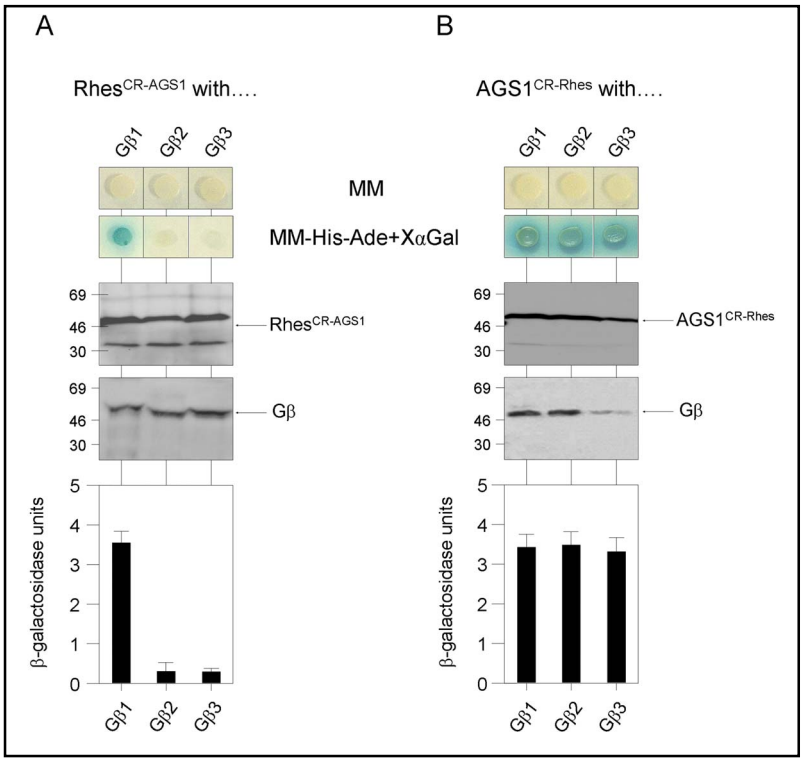

Fig 4. The cationic regions of Rhes and AGS1 determine the specificity of their binding to Gβ subunits. A) Rhes containing the cationic region from AGS1 (pGBKT7-Rhes$^{CR-AGS1}$, residues Met^{189}-to-Ala^{235} of Rhes replaced by residues Met^{194}-to-Ala^{250} of AGS1) was co-expressed with the human Gβ1, Gβ2 and Gβ3 subunits (expressed from pGADT7). All transformants grew on MM but only the transformant containing Gβ1 grew on MM-His-Ade+XαGal and induced expression of the LacZ reporter, suggesting that Rhes$^{CR-AGS1}$ interacts with only Gβ1. B) AGS1 containing the catioinic region of Rhes (pGBKT7-AGS1$^{CR-Rhes}$, residues Met^{194}-to-Ala^{250} of AGS1 replaced by residues Met^{189}-to-Ala^{235} of Rhes) was co-expressed with the human Gβ1, Gβ2 and Gβ3 subunits (expressed from pGADT7). All transformants grew on MM and MM-His-Ade+XαGal, and all induced expression of the LacZ reporter, suggesting that AGS1$^{CR-Rhes}$ interacts with all three Gβ subunits. Expression of Rhes$^{CR-AGS1}$ and AGS1$^{CR-Rhes}$ were confirmed by immunoblotting with an antibody to the GAL4-BD (the constructs are fused to the GAL4 binding domain), and expression of the Gβ 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 β-galactosidase results are presented as the mean ± SD of three different isolates of the relevant strain, each with duplicate determinations.

lacking the cationic region (Rhes$^{\Delta CR}$, lacks residues Met^{189}-Ala^{235}) failed to interact with any of the Gβ subunits (Figure 3A), whereas a construct containing just this region of Rhes (fused to the GAL4 binding domain) interacted with Gβ1, Gβ2 and Gβ3 (Figure 3B). Thus, the cationic region of Rhes is not only required for its interactions with Gβ 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 1) 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$^{CR-AGS1}$, which has residues Met^{189}-Ala^{235} of Rhes replaced by residues Met^{194}-Ala^{250} 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$^{CR-Rhes}$, (Met^{194}-Ala^{250} of AGS1 replaced by residues Met^{189}-Ala^{235} 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 Gβ1, Gβ2 and Gβ3 subunits of heterotrimeric G proteins, but not Gβ4 or Gβ5. The interaction involves the C-terminal cationic region of Rhes and blades 3-to-7 of the Gβ subunits, and appears similar to the interaction between the cationic region of AGS1 and blades 3-to-7 of Gβ1 [17]. Indeed, exchanging the cationic regions between Rhes and AGS1 alters the Gβ-binding specificities of the chimeras.

The interaction of Rhes with the Gβ 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 Gβ1 [17]. Despite both binding to Gβ1, 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 Gβ1 while the cationic region of Rhes also interacts with Gβ2 and Gβ3. 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-Gβ1 interaction requires blades 3-to-7 of the Gβ β-propeller. Given the conservation of sequence and structure between Gβ subunits, it seems likely that the same regions of Gβ2 and Gβ3 are involved in their interaction with Rhes. Our previous study identified the same region of Gβ1 to be involved in its interaction with AGS1 [17]. This region of Gβ1 is not generally thought to be involved in interactions with its other binding partners. Gγ subunits, for example, form a coiled-coil interaction with the N-terminal α-helix of the Gβ subunit [28], while Gα subunits are believed to interact with blades 1-to-3 of the β-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 Gβ 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 Gα subunits. It is also possible that binding of a protein to one part of the Gβ subunit could affect the binding of a second protein to a different part of the same Gβ molecule.

Rhes has complex effects on GPCR signalling [18, 19]. It attenuates signalling by GPCRs that function through Gαs but does not appear to affect signalling through Gαi subunits. The effect on Gαs signalling appears to be at the level of activation of the heterotrimer Gαβγ complex as constitutively active receptors are affected, but constitutively active Gαs 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 Gαs subunits. The interaction of Rhes with Gβ 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 affects. 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]. Gγ subunits are also C-terminally prenylated, Gαs subunits are N-terminally palmitoylated, and Gαi subunits are both palmitoylated and myristoylated at the N-terminus (reviewed by [31]). Gβ subunits associate indirectly with membranes though interaction with the Gγ subunits, and this could be sensitive to inhibition by the membrane localised Rhes.

Why Rhes affects only Gαs signalling, and not Gαi signalling, is an interesting challenge [18]. One possibility could relate to the Gβ-specificity of Rhes (Figure 2A) and it might be, for example, that Gαs subunits interact preferentially with Gβ1-3 (and thus be affected by Rhes) whereas Gαi subunits are more often complexed with Gβ4-5 (and not affected by Rhes). Alternatively, Gαi may be less susceptible than Gαs to disruption by Rhes because Gαi has a stronger interaction with the plasma membrane and may be better able to interact with Gβγ subunits and/or receptors. Gαi is both myristoylated and palmitoylated whereas Gαs is only palmitoylated and requires interaction with Gβγ subunits for full membrane association, the prenylated Gγ subunit providing a second membrane anchor [32] (reviewed by [33]). Indeed, Gαs associates poorly with membranes in the absence of Gβγ subunits, and requires this interaction for efficient palmitoylation [34]. The greater membrane association of Gαi may overcome the inhibitory effects of Rhes more readily than Gαs.

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.

Acknowledgements

We thank Juan Bernal (CSIC-UAM, Madrid) for reagents. This work was supported by the Biotechnology and Biological Sciences Research Council (UK) (CH, AG) and by the University Hospitals of Coventry and Warwickshire NHS Trust (GL).

References

1 Reuther GW, Der CJ: The Ras branch of small GTPases: Ras family members don't fall far from the tree. Curr Opin Cell Biol 2000;12:157-165.

2 Vargiu P, Morte B, Manzano J, Perez J, de Abajo R, Gregor Sutcliffe J, Bernal J: Thyroid hormone regulation of rhes, a novel Ras homolog gene expressed in the striatum. Brain Res Mol Brain Res 2001;94:1-8.

3 Kemppainen RJ, Behrend EN: Dexamethasone rapidly induces a novel Ras superfamily member-related gene in AtT-20 cells. J Biol Chem 1998;273:3129-3131.

4 Krupnick JG, Benovic JL: The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 1998;38:289-319.

5 De Vries L, Zheng B, Fischer T, Elenko E, Farquhar MG: The regulator of G protein signalling family. Ann Rev Pharmacol Toxicol 2000;40:235-271.

6 Ross EM, Wilkie TM: GTPase-activating proteins for heterotrimeric G proteins: Regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem 2000;69:795-827.

7 Lanier SM: AGS proteins, GPR motifs and the signals processed by heterotrimeric G proteins. Biol Cell 2004;96:369-372.

8 Blumer JB, Cismowski MJ, Sato M, Lanier SM: AGS proteins: receptor-independent activators of G-protein signalling. Trends Pharmacol Sci 2005;26:470-476.

9 Hamploez B, Knoblich JA: Heterotrimeric G proteins: new tricks for an old dog. Cell 2004;119:453-456.

10 Cismowski MJ, Takesono A, Ma C, Lizano JS, Xie X, Fuernkranz H, Lanier SM, Duzic E: Genetic screens in yeast to identify mammalian nonreceptor modulators of G-protein signalling. Nat Biotechnol 1999;17:878-883.

11 Cismowski MJ, Ma C, Ribas C, Xie X, Spruyt M, Lizano JS, Lanier SM, Duzic E: Activation of heterotrimeric G protein signaling by a Ras-related protein. J Biol Chem 2000;275:23421-23424.

12 Graham TE, Key TA, Kilpatrick K, Dorin RI: Dexras1/AGS-1, a steroid hormone-induced guanosine triphosphate-binding protein, inhibits 30,50-cyclic adenosine monophosphate-stimulated secretion in AtT20 corticotroph cells. Endocrinology 2001;142:2631–2640.

13 Graham TE, Oiao Z, Dorin RI: Dexras1 inhibits adenylyl cyclise. Biochem Biophys Res Commun 2004;316:307-312.

14 Graham TE, Prossnitz ER, Dorin RI: Dexras1/AGS-1 inhibits signal; transduction from the Gi-coupled formyl peptide receptor to Erk1/2 MAP kinases. J Biol Chem 2002;277:10876-10882.

15 Nguyen CH, Watts VJ: Dexras1 blocks receptor-mediated heterologous sensitization of adenylyl cyclase 1. Biochem Biophys Res Commun 2005;332:913-920.

16 Takesono A, Nowak MW, Cismowski M, Duzic E, Lanier SM: Activator of G-protein signaling-1 blocks GIRK channel activation by a G-protein-coupled receptor. J Biol Chem 2002;277:13827-13830.

17 Hiskens R, Vatish M, Hill C, Davey J, Ladds G: Specific *in vivo* binding of activator of G protein signalling 1 to the Gb1 subunit. Biochem Biophys Res Commun 2005;337: 1038-1046.

18 Vargiu P, De Abajo R, Garcia-Ranea JA, Valencia A, Santisteban P, Crespo P, Bernal J: The small GTP-binding protein, Rhes, regulates signal transduction from G protein-coupled receptors. Oncogene 2004;23:559-568.

19 Agretti P, De Marco G, Pinchera A, Vitti P, Bernal J, Tonacchera M: Ras homolog enriched in striatum inhibits the functional activity of wild type thyrotropin, follicle-stimulating hormone, luteinizing hormone receptors and activating thyrotropin receptor mutations by altering their expression in COS-7 cells. J Endocrinol Invest 2007;30:279-284.

20 Errico F, Santini E, Migliarini S, Borgkvist A, Centonze D, Nasti V, Carta M, De Chiara V, Prosperetti C, Spano D, Herve D, Pasqualetti M, Di Lauro R, Fisone G, Usiello A: The GTP-binding protein Rhes modulates dopamine signalling in striatal medium spiny neurons. Mol Cell Neurosci 2008;37:335-245.

21 Li B, Fields S: Identification of mutations in p53 that affect its binding to SV40 T antigen by using the yeast two-hybrid system. FASEB J 1993;7:957-963.

22 Goddard A, Ladds G, Forfar R, Davey J: Identification of Gnr1p, a negative regulator of Gα signalling in *Schizosaccharomyces pombe*, and its complementation by human Gβ subunits. Fungal Genet Biol 2006;43:840-851.

23 Yan K, Kalyanaraman V, Gautam N: Differential ability to form the G protein bg complex among members of the band g subunit families. J Biol Chem 1996;271:7141-7146.

24 Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR: The structure of the G protein heterotrimer Giα1β1γ2. Cell 1995;83:1047-1058.

25 Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE, Sigler PB: The 2.0 A crystal structure of a heterotrimeric G protein. Nature 1996;379:311-319.

26 Sondek J, Bohm A, Lambright DG, Hamm HE, Sigler PB: Crystal structure of a G-protein bg dimer at 2.1 Å resolution. Nature 1996;379:369-374.

27 Fang M, Jaffrey SR, Sawa A, Ye K, Luo X, Snyder SH: Dexras1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. Neuron 2000;28:183-193.

28 Clapham DE, Neer EJ: G protein bg subunits, Annu Rev Pharmacol Toxicol 1997;37:167-203.

29 Albsoul-Younes AM, Sternweis PM, Zhao P, Nakata H, Nakajima S, Nakajima Y, Kozasa T: Interaction sites of the G protein b subunit with brain G protein-coupled inward rectifier K^+ channel, J Biol Chem 2001;276:12712-12717.

30 Zhao Q, Kawano T, Nakata H, Nakajima Y, Nakajima S, Kozasa T: Interaction of G protein b subunit with inward rectifier K^+ channel Kir3. Mol Pharmacol 2001;64:1085-1091.

31 Wedegaertner PB, Wilson PT, Bourne HR: Lipid modifications of trimeric G proteins, J Biol Chem 1995;270:503-506.

32 Resh MD: Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochem Biophys Acta 1999;1451:1-16.

33 Marrari Y, Crouthamel M, Irannejad R, Wedegaertner PB: Assembly and trafficking of heterotrimeric G proteins. Biochemistry 2007;46:7665-7677.

34 Evanko DS, Thiyagarajan MM, Wedegaertner PB: Interaction with Gbg is required for membrane targeting and palmitoylation of Gαs and Gαq. J Biol Chem 2000;275:1327-1336.

35 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG: The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 1997;24:4876-4882.

36 Corpet F: Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 1998;16:10881-10890.