Intragenic suppression of a constitutively active allele of $G_s\alpha$ associated with McCune–Albright syndrome

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Abstract

McCune–Albright syndrome (MAS) is a human genetic disorder caused by a mutation that constitutively activates the $G_s\alpha$ subunit by abolishing GTP hydrolysis. MAS patients suffer from a range of endocrinopathies as well as polyostotic fibrous dysplasia of bone. We previously identified an intragenic suppressor of the MAS mutation in a yeast system, which substituted two residues in the GTP-binding site of Gpa1: L318P and D319V to suppress the constitutive activity of an R297H mutation, corresponding to the human F222P, D223V, and R201H mutations respectively. To extend these studies, the human *GNAS* gene was subjected to site-directed mutagenesis. Constructs expressing the MAS mutation (R201H), the MAS mutation plus the mutations homologous to the yeast suppressors (R201H, F222P/D223V), or the yeast suppressor mutation alone (F222P/D223V) were transfected into HEK293 cells, and basal and receptor-stimulated cAMP levels were measured. Expression of R201H increased the basal cAMP levels and decreased the EC_{50} for hormone-stimulated cAMP production. These effects were dependent on the amount of R201H protein expressed. R201H, F222P/D223V abolished the constitutive activity of the MAS mutation and caused responses to hormone that were not different from those measured in cells expressing WT $G_s\alpha$. Interestingly, F222P/D223V behaved similar to R201H in causing increases in basal cAMP production, thus demonstrating constitutive activity. Substitution of another acidic (E) or polar (N, T, and G) amino acid at position 223 caused no suppression of R201H activity, while substitution of a second nonpolar amino acid (A) at this position partially suppressed, and the larger polar I residue completely suppressed the effects of R201H.

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Introduction

McCune–Albright syndrome (MAS) is a disease of somatic genetic mutations. This syndrome was first described by Drs McCune and Albright in the 1930s (McCune 1936, Albright *et al.* 1937), and a classic triad of symptoms is associated with the disease: polyostotic fibrous dysplasia,

precocious puberty, and café-au-lait hyperpigmentation of the skin. While the presenting symptom for children affected with MAS is almost always precocious puberty, the fibrous dysplasia of bone is responsible for severe pathologies in many cases. McCune also reported

hyperthyroidism in his first case, and a wide variety of hyperfunctioning endocrine disorders may accompany the classic triad of symptoms, including GH excess, renal phosphate wasting, and Cushing syndrome (Collins 2006).

The etiology of MAS explains the wide variety of symptoms and range of disease severity in the patient population. MAS is caused by a mutation in *GNAS*, the gene which encodes $G_{s\alpha}$. Like all G-proteins, $G_{s\alpha}$ exchanges GDP for GTP when activated by a receptor and then goes on to activate the plasma membrane enzyme adenylyl cyclase. The α subunit also hydrolyzes GTP to GDP+P_i, resulting in its inactivation. If the G-protein then encounters another active receptor, it can be reactivated by nucleotide exchange of GDP to GTP. This built-in 'off switch' is essential for proper $G_{s\alpha}$ function (Neves *et al.* 2002). Substitution of arginine 201 with another amino acid abolishes the GTPase activity of the $G_{s\alpha}$ protein, rendering it constitutively active. The literature reports mostly histidine and cysteine substitutions (Weinstein *et al.* 1991, Schwindinger *et al.* 1992, Lumbroso *et al.* 2004), although serine (Candeliere *et al.* 1997) and glycine (Riminucci *et al.* 1999) have also been found at codon 201 in MAS isolates. ADP-ribosylation of this residue by cholera toxin also abolishes the GTPase activity of the subunit. All MAS patients exhibit mosaicism for their mutation; the affected tissues carry the mutation while the unaffected tissues do not. Because of the importance of $G_{s\alpha}$ in so many different signaling pathways, it is not surprising that activating (and thus dominant, gain-of-function) mutations in this gene are only seen as somatic mutations. Germline mutation of this essential signaling protein would certainly result in failure of one or more organ systems to develop properly.

Current therapies for MAS focus on preventing or delaying the responses to elevated cAMP in the affected tissues. Fibrous dysplasia requires surgery in almost all cases, either for repair of broken long bones or for correction of facial deformities caused by thickening of the bones of the face (Collins 2006). The endocrine disorders are generally treated with drugs that alleviate each specific endocrinopathy. The consequences of precocious puberty in MAS patients are delayed until the patient approaches a normal age for puberty using aromatase inhibitors and/or tamoxifen (Eugster *et al.* 2003, Feuillan *et al.* 2007). The rational design of more effective drugs to treat MAS and other disorders caused by constitutively active proteins requires detailed structural information about the protein which will serve as the drug target.

We previously used yeast genetics in a search for regions of $G_{s\alpha}$, which, when altered, can suppress the constitutive activity of MAS mutations. In this project, we constructed a small library of random mutations in constitutively active GPA1 (the yeast α subunit) carrying a mutation homologous to the R201H seen in MAS patients. The library was screened for plasmids that could support colony formation under conditions where the library plasmid was the only copy of GPA1 available. These experiments identified an intragenic suppressor of the constitutively active GPA1 (Ooms *et al.* 2006). We now extend the previous work by analyzing the suppressor mutation in the human *GNAS* gene, expressed in cultured human cells.

Materials and methods

Mutagenesis

pCDNA3.1+GNASL (Guthrie cDNA Resource Center) was subjected to site-directed mutagenesis using the Stratagene QuickChange II XL site-directed mutagenesis kit. Mutagenic primers introduced or eliminated unique restriction sites (silent mutations) into the cDNA encoding GNASL for primary screening of the mutagenized clones. Table 1 gives the amino acid substitutions encoded by each of the new clones, the sequence of the forward primer, and the identity of a change in restriction enzyme sites that were caused by the mutagenesis. All novel restriction sites were produced through the use of silent mutations in the DNA sequence. The reverse primers were direct complements to the forward primers. The altered restriction sites were used for first-round screening of mutagenized clones. The complete coding sequences of all mutagenized clones were confirmed by dideoxy sequencing, performed by SeqWright (Houston, TX, USA), or ACGT, Inc. (Morton Grove, IL, USA).

Cell culture and transfection

HEK293 cells were grown in bicarbonate-buffered DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere. Cells from 80–90% confluent plates were split 1:5 into 100 mm plates 1 day before transfection. Cells were transiently transfected using either Trans-fast reagent (Promega) at a 1:1 ratio of lipid:DNA or with jetPRIME transfection reagent (Polyplus, New York, NY, USA). Cells were analyzed 48 h post-transfection.

Table 1 Primer sequences for site-directed mutagenesis of human *G_sα*_L

Amino acid substitutions	Forward primer sequence	Restriction site change
R201H	CCTGCTTCGCTGCCACGTGCTGACTTCTGG	PmlI added
F222P/D223V	AGTCAACTTCAACATGCCTGTCGTGGGTGGCCAGCGC	PciI removed
F222P	AAGTCAACTTCCACATGCCTGACGTGGGTGGCCAGC	PciI removed
D223V	GTGGACAAAGTCAACTTCCATATGTTTGTCTGGGTGGCC	PciI removed
D223E	CAACTTCCACATGTTTGAAGTGGGTGGCCAGCGCG	BstBI added
D223A	GTCAACTTCCATCTGTTTGGCGTGGGTGGCCAGCGCG	PciI removed
D223N	GTCAACTTCCACATGTTTAACTTGGTGGCCAGCGCG	AclI added
D223I	GTCAACTTCCATATGTTTATCGTGGGTGGCCAGCGCGATG	PciI removed
D223L	GGACAAAGTCAACTTCCACATGTTACTAGTGGGTGGCCAGCGCG	SpeI added
D223T	GGACAAAGTCAACTTCCATATGTTTACCGTGGGTGGCCAGCGCG	NdeI added
D223G	GGACAAAGTCAACTTCCATATGTTTGGCGTGGGTGGCCAGCGCG	NdeI added

cAMP assays

Transiently transfected HEK293 cells were moved to wells of a 24-well plate 24 h after transfection. Cells from half of one 60 mm diameter culture plate were divided evenly among six wells of a 24-well plate. The remaining cells were replated on the 60 mm plate for immunoblot analysis. Levels of cellular cAMP were measured by treating the cells for 15 min at 37 °C in serum-free medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) and indicated concentrations of human chorionic gonadotropin (hCG), aspirating the media, then lysing the cells in 0.1 M HCl, and analyzing the lysates using an ELISA (EIA) Kit for cAMP (ENZO/Assay Designs, Farmingdale, NY, USA).

Cell lysate preparation

Whole-cell lysates of transfected cells were prepared by removing the transfected cells from the culture plate in PBS, pelleting the cells with a low-speed centrifuge, and lysing the pellet directly with 100 µl Laemmli sample buffer in boiling water for 2 min. Proteins were sonicated at 50% power in a Misonix XL-2000 sonicator for 2- to 15-s exposures, spun in at 15 000 *g* in a microcentrifuge, and then analyzed by western blot immediately or stored in a –20 °C freezer until analysis.

Western blot

Lysate (25 µl) was applied to each well of an 8–14% polyacrylamide Tris–tricine gel (Bio-Rad or Lonza, Rockland, ME, USA) and proteins were separated by electrophoresis in a Bio-Rad tetra cell chamber. The proteins were transferred to nitrocellulose for 1 h at 100 V. The membrane was blocked for 30 min with 5% Coffeemate reconstituted in Tris-buffered saline (TBS). Mouse anti-human *G_sα* MAB

((12), Santa Cruz Biotechnology) was diluted 1:2000 in 5% milk and incubated with the membrane for 2 h at room temperature with rocking. The membrane was washed five times for 5 min each with TBS and then incubated overnight at 4 °C with a 1:5000 dilution of HRP-coupled goat anti-mouse IgG (Bio-Rad) in 5% milk for ECL detection or with a 1:10 000 dilution of IRDye 800CW Goat anti-Mouse IgG (Li-COR, Lincoln, NE, USA). The membranes were washed five times for 5 min each in TBS before detection. For ECL, the bands were visualized using Immun-Star HRP ECL substrate (Bio-Rad) and detected using Hyperfilm ECL (Amersham). **Figures 2 and 4** show westerns imaged with a Li-COR Odyssey Fc for infrared detection of the reactive bands.

Statistical analysis

Differences between means for a group of cAMP experiments were determined using ANOVA analysis. When ANOVA results that indicated significant differences among groups, selected groups were compared using a Student's *t*-test. Significant differences between groups were defined as *P* values <0.05. Linear and nonlinear regressions were performed using GraphPad Prism Software.

Results

Previous work from this laboratory constructed a yeast model for MAS, where constitutively active alleles of the yeast *Gpa1* protein were identified on the basis of their inability to support colony formation. This system successfully identified an intragenic suppressor mutation capable of suppressing the growth arrest phenotype of the R297H mutation in the yeast *Gpa1* protein, L319P/D320V (Ooms *et al.* 2006). Because R297 is homologous to the arginine residue mutated in MAS (**Fig. 1A**), and the amino

Figure 1

Structure of $G_{s\alpha}$. (A) The amino acid sequences of a portion of the GTP-binding site were aligned for the human *GNAS* gene that encodes $G_{s\alpha}$ and the yeast G_{α} subunit Gpa1. The location of R201 is marked with an open arrow. F222 and D223 are marked with gray arrows. Switch I is outlined in a solid rectangle, Switch II is outlined with a dotted line. Residues shown to make direct contact with the β subunit are marked by asterisks. (B) The structure of the GTP-binding site of $G_{s\alpha}$ is modeled from the coordinates in PDB (1AZT). Cn3D was used to illustrate the locations of the guanine nucleotide (GTPγS in this model), the R201 residue mutated in MAS, and the suppressor mutation sites F222 and D223. The backbone of the protein is shown as a wire.

acids of the suppressor mutation are also identical or conserved in the human G-protein, we postulated that the intragenic suppressor mutation would be capable of suppressing the constitutive activity of the human MAS allele of $G_{s\alpha}$ as well. All three of these residues are found in the GTP-binding pocket of $G_{s\alpha}$ and other G-proteins (Fig. 1B). R201 is found on the conformationally flexible Switch I domain, and D223 is on the Switch II domain (Sprang 1997, Johnston & Siderovski 2007). To test our hypothesis, site-directed mutagenesis was used to introduce combinations of the homologous mutations into the human *GNASL* gene and the resulting protein isoforms were analyzed in signaling assays in transiently transfected HEK293 cells.

To examine the effects of heterologous expression of the R201H allele of *GNAS*, increasing amounts of plasmid DNA were transiently transfected into HEK293 cells along with 2 μ g plasmid DNA encoding the human LH receptor (LHR)/hCG, which signals through G_s pathways. Basal levels of cAMP were measured in the presence of 1 mM IBMX $\pm$ 10 μ M forskolin (Fig. 2A) and levels of G_s protein were detected by immunoblot (Fig. 2A, inset). Because forskolin-stimulated cAMP levels varied from experiment to experiment, the basal cAMP data are expressed as a

percentage of the forskolin response to control for changes in cell numbers. The basal cAMP levels of $0.4 \pm 0.2\%$ forskolin response (mean $\pm$ s.e.m.) for control cells were slightly elevated to $7.4 \pm 2.0\%$ of the forskolin response for cells transfected with 0.001 μ g R201H plasmid. This difference is not statistically significant. Transfection with 0.01 μ g plasmid increased basal cAMP levels (Fig. 2A) to $34.9 \pm 3.4\%$ of forskolin levels, significantly higher than the control ($P < 0.001$). Even higher basal levels were observed after transfection with 0.1 μ g ($71.7 \pm 10.5\%$ of forskolin) or 1.0 μ g of plasmid ($98.5 \pm 10.8\%$ of forskolin). Signaling through the G_s -coupled LHR was examined by exposing cells to increasing doses of hCG + 1 mM IBMX

Figure 2

Expression of G_s -R201H elevates basal cAMP levels and increases LHR sensitivity to hormone. (A) Basal levels of cAMP in HEK293 cells transiently transfected with the indicated amounts of plasmid encoding G_s -R201H. Inset: immunoblot of lysates from cells in one representative experiment. The bars represent the mean $\pm$ s.e.m. for three to four independent experiments, each performed in duplicate. (B) Dose-response relationship in cells transiently transfected with LHR and varying amounts of G_s -R201H. Open circles, no R201H; filled boxes, 0.001 μ g R201H plasmid; open triangles, 0.01 μ g R201H; filled triangles, 0.1 μ g R201H; open box, 1.0 μ g R201H. Points represent the mean $\pm$ s.e.m. for three to four independent experiments, each performed in duplicate. Data were fit to a four-parameter dose vs response curve using GraphPad Prism Software.

(Fig. 2B). The EC_{50} for hCG-stimulated cAMP production was 0.10 U/ml (95% CI=0.02–0.55 U/ml) for control cells expressing only endogenous G_s . Transfection with 0.001 mg R201H plasmid DNA shifted the EC_{50} to the left by eightfold to a value of 0.013 U/ml (95% CI=0.003–0.049 U/ml). At transfection levels of plasmid higher than 0.001 μ g/plate, the data did not conform to a sigmoidal dose–response relationship, although for 0.01 mg/plate there was a significant increase in cAMP levels from basal to maximally stimulated (10 U/ml) cells ($P<0.05$).

Basal levels of cAMP in cells transfected with 5 μ g of various $G_{s\alpha}$ alleles were measured to investigate whether or not the suppressor mutations isolated from the yeast system (Ooms *et al.* 2006) were able to suppress the constitutive activity of the R201H mutation in the human context (Fig. 3A). This amount of plasmid was chosen because it produced strong and consistent overexpression

of all G_s alleles. Basal cAMP levels were very low in mock-transfected cells at $0.18 \pm 0.04\%$ of the forskolin response. Expression of WT $G_{s\alpha}$ slightly elevated the basal cAMP level to $12.3 \pm 4.7\%$ of the forskolin response, indicating a low level of activation of the overexpressed $G_{s\alpha}$ protein. Expression of the MAS allele of $G_{s\alpha}$ greatly raised basal cAMP levels, resulting in a fivefold increase in cAMP levels to $59.8 \pm 13.4\%$ of forskolin. The triple mutant did not raise basal cAMP levels over those seen with WT $G_{s\alpha}$ expression, producing $6.9 \pm 5.2\%$ of forskolin levels. Single mutations of either F222P or D223V were both able to completely suppress the activity of the R201H mutation back to levels seen with the WT allele, rendering basal levels of cAMP at 0.6 ± 0.7 and $3.2 \pm 2.1\%$ of forskolin responses respectively. All suppressing mutations resulted in basal cAMP levels that were not significantly different from levels in cells expressing the WT allele of $G_{s\alpha}$ by ANOVA analysis. For all these $G_{s\alpha}$ constructs, transfection in the HEK293 cells resulted in measurable immunoreactivity at least equivalent to R201H levels on immunoblots (Fig. 2B).

$G_{s\alpha}$ transmits signals from G-protein-coupled receptors to the plasma membrane enzyme adenylyl cyclase. To investigate the ability of these $G_{s\alpha}$ alleles to function in signaling pathways, HEK cells were transiently cotransfected with 5 μ g plasmid encoding various $G_{s\alpha}$ alleles plus 2 μ g cDNA encoding the LHR/hCG. This cotransfection technique has been previously used to study the interactions between heterologously expressed GPCRs and signaling proteins in transient transfection systems (Pals-Rylaarsdam *et al.* 1995). The LHR was chosen because it couples to $G_{s\alpha}$, is not endogenously expressed in HEK cells, and has an economically priced agonist (hCG) available. Cells were treated with 10 U/ml hCG in 1 mM IBMX for 15 min at 37 °C, lysed, and cAMP levels were measured with the EIA assay. Basal cAMP levels in cells coexpressing the LHR and G_s alleles were found to exhibit the same pattern as seen in Fig. 3: low levels for cells transfected with vector, WT, or the triple mutant (Fig. 4A, first data point). In cells that overexpressed the R201H allele of $G_{s\alpha}$, there was no statistical difference between basal ($90.3 \pm 7.0\%$) vs hCG-stimulated cAMP levels ($88.4 \pm 3.6\%$). Interestingly, cells transfected with a G_s allele carrying only the suppressor mutations (F222P/D223V) exhibited constitutive activity of the G_s protein. Basal cAMP levels ($87.9 \pm 6.7\%$ of forskolin) and hCG-stimulated levels ($75.9 \pm 11.0\%$ of forskolin) were statistically indistinguishable from R201H values. Treatment with the hormone hCG caused a dose-dependent rise in intracellular cAMP levels in cells transfected with

Figure 3

Expression of $G_{s\alpha}$ alleles and their effects on basal cAMP levels in HEK293 cells. (A) Basal cAMP levels in HEK293 cells transiently transfected with the indicated $G_{s\alpha}$ constructs were measured using an EIA kit. The bars represent the mean $\pm$ s.e.m. for three to five independent experiments, each performed in triplicate. (* $P<0.01$ vs WT, Student's *t*-test). (B) A representative ECL western blot of lysates from cells transiently transfected with the indicated $G_{s\alpha}$ constructs.

Figure 4

Effects of $G_{s\alpha}$ isoforms on receptor-activated cAMP production. (A) Dose-response relationship in cells transiently transfected with LHR and different alleles of G_s . Open circles, vector; filled boxes, WT; closed circles, R201H; open triangles, R201H, F222P/D223V; open diamonds, F222P/D223V. Points represent the mean $\pm$ s.e.m. for three to four independent experiments, each performed in duplicate. Data were fit to a four-parameter dose vs response curve using GraphPad Prism Software. (B) A representative western blot of lysates from cells transiently transfected with the indicated $G_{s\alpha}$ constructs.

just the hCG receptor. Responses to a maximal dose of 10 U/ml hCG in cells transfected with vector were measured at $64.6 \pm 3.5\%$ of the forskolin response, in cells overexpressing the WT allele of $G_{s\alpha}$ to $63.0 \pm 4.8\%$ of forskolin, and in cells overexpressing the triple mutant allele of $G_{s\alpha}$ to $72.7 \pm 1.0\%$ of forskolin (Fig. 4A). The data show no statistical differences in either the EC_{50} or the maximal response to 10 U/ml hCG for cells transfected with vector (95% CI=0.03–0.11 U/ml), WT (0.01–0.43 U/ml), or the triple mutant (0.01–0.08 U/ml) alleles of G_s . The levels of hCG-stimulated cAMP were slightly but significantly higher in cells expressing R201H than in control cells or cells overexpressing WT G_s ($0.01 < P < 0.001$). The differences in the responses to hCG were not due to changes in protein expression of the $G_{s\alpha}$ proteins, as equivalent levels of $G_{s\alpha}$ were seen in western blots of transfected cell lysates (Fig. 4B).

Aspartic acid 223 is an absolutely conserved residue in all heterotrimeric $G\alpha$ subunits, as well as in small

G-proteins and other GTP-binding proteins (Sprang 1997). Because of the evolutionary significance of this residue, we chose to examine more closely which types of amino acid substitutions could suppress the R201H constitutive activity when introduced at position 223. We constructed alleles of $G_{s\alpha}$ with a conservative substitution (D223E), substitutions of the acidic group with a nonpolar groups (D223A, D223I, and D223L), and substitution of the acidic group with its polar uncharged side chains (D223N, D223T, and D223G). These alleles of $G_{s\alpha}$ were transfected into HEK cells, and basal cAMP levels were measured 48 h post-transfection (Fig. 5A). Neither the D223E nor the D223N mutation had any effect on blocking the activity of R201H, showing 46.4 ± 5.8 and $49.4 \pm 5.4\%$ of the forskolin response compared with $40.9 \pm 2.6\%$ of the forskolin response in R201H-transfected cells. Similar observations were made for D223T and D223G, which exhibited 44.1 ± 1.9 and $37.2 \pm 3.6\%$ of the forskolin-stimulated levels of cAMP in the absence of other stimulation. By contrast, D223A exhibited a partial

Figure 5

Nonpolar residues, but not polar or acidic residues, at position 223 suppress the R201H constitutive activity. (A) Basal cAMP levels in HEK293 cells transiently transfected with the indicated $G_{s\alpha}$ constructs were measured using an EIA kit. All substitutions of D223 also carry the R201H mutation. The bars represent the mean $\pm$ s.e.m. for three to six independent experiments, each performed in triplicate ($*P < 0.005$ vs R201H, $**P < 0.05$ vs R201H, and $P < 0.05$ vs WT, Student's *t*-test). (B) A representative ECL western blot of lysates from cells transiently transfected with the indicated $G_{s\alpha}$ constructs. R201H/D223I was analyzed in a separate western blot from the rest of the mutants, as indicated by the separate box for that gel.

suppression of R201H, with basal cAMP levels significantly lower than the R201H allele itself at $25.2 \pm 3.4\%$ of the forskolin response ($P < 0.001$) but also statistically higher than the WT allele that showed only $11.6 \pm 2.9\%$ of the forskolin response ($P < 0.001$). D223I reduced basal cAMP levels back to $10.1 \pm 1.7\%$ of the forskolin response, statistically indistinguishable from WT levels and significantly less than R201H levels response ($P < 0.001$). D223L was transfected into HEK cells five independent times, all without any detectable protein expression. Equivalent expression of the alleles of $G_{s\alpha}$ alleles other than D223L was again confirmed by immunoblot (Fig. 5B).

Discussion

This study investigated the ability of two previously undescribed mutations in $G_{s\alpha}$ to suppress the constitutive activity of a mutation (R201H) associated with MAS. In our expression system, low levels of R201H produced an elevation in basal cAMP and increased the sensitivity of a heterologously expressed G-protein-coupled receptor to activation by agonist. Higher levels of R201H expression resulted in greater basal cAMP production, to the point where receptor activation could not produce further increases in the second messenger. The lack of further increase is likely due to the experimental overexpression of the proteins and not to the inability of these alleles to couple to receptors. The data in this paper confirm that mutation of two residues near the GTP-binding site of $G_{s\alpha}$ suppresses the constitutive activity caused by an R201H mutation, also found in the GTP-binding site. This triple mutation (R201H, F222P/D223V) was indistinguishable in its signaling behavior from overexpressed WT $G_{s\alpha}$. Basal cAMP levels, EC₅₀ values for the LHR response to hCG, and maximum cAMP production in response to hCG were statistically not different in cells expressing these two forms of $G_{s\alpha}$. A variety of mutations of D223 were constructed to examine the structural requirements for suppression of R201H. While acidic and polar substitutions had no effect on the constitutive activity caused by R201H, substitution with bulky nonpolar residues (V and I) for aspartic acid at position 223 was effective in counteracting the effects of R201H in causing constitutive activity of $G_{s\alpha}$. The small nonpolar amino acid alanine was partially effective in blocking constitutive activity.

Interestingly, the suppressor mutations by themselves produced a novel allele of $G_{s\alpha}$ that itself acts in a constitutively active manner. The F222P/D223V mutant G-protein caused a dramatic elevation in basal cAMP levels and mirrored the activity of the original R201H mutation

in agonist activity assays. The mutations described in this paper have not been identified in other constitutively active G-proteins, either isolated from patient samples or found through *in vitro* mutagenesis of other α subunit family members. Nearby residues in switch II, namely G226 (Gao *et al.* 1995) and Q227 (Masters *et al.* 1989), are well-established residues that, when mutated, can abolish GTPase activity of the α subunit, rendering it constitutively active. Homologous mutations to Q227 in many other α subtypes have been shown to be constitutively active and have been used in numerous studies of the role of α subunits in diverse cell signaling pathways (for example, Lo *et al.* (2003), Slessareva *et al.* (2006), Ogata *et al.* (2007), Yevenes *et al.* (2011), and Jeon *et al.* (2012)).

X-ray crystal structures are solved for $G_{s\alpha}$ in its active conformation (Sunahara *et al.* 1997), as well as structures of $G_{s\alpha}$ associated with adenylyl cyclase (Tesmer *et al.* 1997) and bound to the agonist-occupied β_2 -adrenergic receptor (Rasmussen *et al.* 2011). Crystal structures of other α subunits in both the active and inactive conformations are also available for comparison (for example, Coleman *et al.* (1994) and Coleman & Sprang (1998)). Heterotrimeric G-proteins have a highly conserved GTPase fold, the region where R201, F222, and D223 lie in $G_{s\alpha}$. The homologous arginine residue to R201 is found not only in all heterotrimeric α subunits but also in the translation elongation factors EF-Tu and EF-G (Sprang 1997). In a crystal structure of GDP·AlF₄⁻·Mg²⁺ bound to G_iα1, a structure thought to correspond to the transition state of GTP hydrolysis, this arginine stabilizes the negative charge on the γ -phosphate of GTP during the S_N2 hydrolysis reaction (Sprang 1997). The $G_{s\alpha}$ crystal structure is also consistent with this role for R201 (Sunahara *et al.* 1997), and the loss of GTP hydrolysis activity in MAS alleles of $G_{s\alpha}$ can thus be explained by a reduction in the stability of the transition state. The D223 residue is also indirectly involved in the coordination of the Mg²⁺ cofactor, via a water molecule. Indeed, the loop where R201 is found (switch I, G-2 effector loop) and the loop where D223 is found (switch II, G-3 effector loop) are two regions of the G-protein that exhibit large conformational changes upon GTP binding and hydrolysis to GDP (Sprang 1997). Thus, it is not unexpected that altering these residues alters the functioning of the G-protein. The role of the F222P mutation in contributing to the suppression effects is more difficult to explain. Proline residues are associated with terminating α -helix structures; however, F222 is found on a β -strand, not an α -helix. It is possible that the proline residue contributes only minimally to the phenotype of the constitutively active mutant. Neither of

these residues is directly involved in binding to adenylyl cyclase or the β_2 -adrenergic receptor (Tesmer *et al.* 1997, Rasmussen *et al.* 2011).

Using a yeast model system to identify potential suppressors of constitutive activation has been used by other laboratories with success for other G_{α} subunits activated at the glutamine residue homologous to Q227 in G_{α} (Apanovich *et al.* 1998). Interestingly, this group observed that the suppressor mutation alone had dominant-negative properties in G_{α} , in this case blocking interaction with $\beta\gamma$ subunits but having no effect on the inhibition of adenylyl cyclase through the α subunit. It will be interesting to note whether as more intragenic suppressor mutations of R201H and/or Q227L are identified, if a pattern emerges of suppressors of one site being constitutively active while suppressors of the other are dominant-negative alleles. Taken together, these papers and our current report validate the utility of yeast models for identifying potential suppressor mutations of constitutively active alleles of G_{α} subunits, work that may contribute to rational drug design for diseases caused by constitutive activation of G_{α} subunits as more suppressor alleles are identified and mapped onto the G-protein structure.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R P-R designed the experiments, supervised the laboratory work, conducted many of the experiments, analyzed the data, and wrote the manuscript. R T-R did the site-directed mutagenesis for some of the constructs in Fig. 5 and conducted cAMP measurements for that figure. She was assisted in the cAMP assays by D J. D S conducted cAMP measurements for Fig. 3. K T and J C conducted experiments for Figs 2 and 4. L O did the site-directed mutagenesis for the constructs in Figs 2 and 3.

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