Hydrophobicity of Residue^{351} of the G Protein $G_{i1}\alpha$ Determines the Extent of Activation by the α_{2A}-Adrenoceptor†

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ABSTRACT: Cysteine^{351} is the site for pertussis toxin-catalyzed ADP-ribosylation in the G protein $G_{i1}\alpha$. Alteration of this residue, or the equivalent cysteine in other G_i-family G proteins, has been used to examine specific interactions between receptors and these G proteins. However, no systematic analysis has been performed to determine the quantitative effect of such alterations. To address this we mutated cysteine^{351} of $G_{i1}\alpha$ to all other possible amino acids. Each of the G protein mutants was transiently coexpressed along with the porcine α_{2A}-adrenoceptor in HEK 293/*T* cells. Following pertussis toxin treatment of the cells, membranes were prepared and the capacity of the agonist UK14304 to stimulate the binding of [^{35}S]GTPγS to the modified G proteins was measured. A spectrum of function was observed. The presence of either a charged amino acid or a proline at this position essentially attenuated agonist regulation. The wild-type G protein did not result in maximal stimulation by agonist. The presence of certain branched chain aliphatic amino acids or bulky aromatic R groups at amino acid^{351} resulted in substantially greater maximal stimulation by the α_{2A}-adrenoceptor than that achieved with the wild-type sequence. The degree of activation of the forms of $G_{i1}\alpha$ correlated strongly with the octanol/water partition coefficient of the amino acid at residue^{351}. Variation in EC_{50} values for agonist-induced stimulation of binding of [^{35}S]GTPγS to the mutant G proteins also correlated with the octanol/water partition coefficient. **T**hese results define a central role for hydrophobicity of this residue in defining productive receptor-G protein interactions.

A large number of seven-transmembrane element G protein-coupled receptors (GPCRs) are known which mediate their effects via activation of members of the family of heterotrimeric G proteins. The α subunits of all of the G proteins release GDP and exchange it for GTP upon effective interaction with an agonist-occupied GPCR (*1*−*3*). As such, assays which record agonist-induced binding of a poorly hydrolyzed analogue of GTP have been widely used in studies to examine agonist function or in searches for novel agonists (*4*−*7*). The G_i subfamily of G proteins is particularly suitable for such studies as they possess a markedly greater guanine nucleotide exchange rate than other G proteins (*1*−*3*). In addition, these G proteins act as substrates for pertussis toxin-catalyzed ADP-ribosylation due to the presence of a conserved cysteine residue four amino acids from the C-terminus (*8*). This covalent modification attenuates functional interactions between GPCRs and the G_i-like G proteins (*8*). However, as a number of G_i-family G proteins are routinely coexpressed, analysis of the interactions of a GPCR with specific G_i isoforms is often difficult to achieve in simple assays based on inhibition of function by pertussis toxin. One strategy to overcome this involves modification of the conserved cysteine such that the mutated G protein is no longer a substrate for pertussis toxin (*9*−*12*). Thus, following pertussis toxin treatment of cells, the interaction of an agonist-liganded GPCR with the mutated G protein can be examined in isolation without potential contributions to the signal from the population of endogenously expressed G_i-like G proteins. This experimental strategy has been successfully used to examine coupling of G_i-family G proteins to GPCRs including the α_{2A}-adrenoceptor (*11*) and the dopamine D2 receptor (*9*) following mutation of this cysteine to glycine and for the muscarinic M2 receptor (*10*) and the dopamine D4 receptor (*12*) following mutation of the cysteine to serine. However, quantitative analysis of the variation in effectiveness of GPCR regulation of the modified G proteins has not been reported. It might be anticipated that GPCR regulation of these modified G proteins would result in poor agonist responses. This is based on analysis of the interactions of rhodopsin with a combinatorial library of peptides representing variants of the C-terminus of the α subunit of the G protein transducin which suggested that the presence of the pertussis toxin-sensitive cysteine is essential to promote high-affinity interactions between the GPCR and this G protein (*13*). By contrast, studies with mutationally modified forms of transducin have indicated this not to be an absolute (*14*−*15*).

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Table 1: Primers Used for Construction of Cys^{351}Xaa Mutants of $G_{i1}\alpha$[a]

common 5′ primer
AGCT GAA TTC GCC ACC ATG GGC TGC ACA CTG AGC GC
3′ primers
primer 1: Cys^{351} to Ile, Met, Thr, Asn, Lys, Ser, Arg
ACGT GAA TTC TTA GAA GAG ACC (G/C)NT GTC TTT TAG G
primer 2: Cys^{351} to Pro, Arg, Leu
ACGT GAA TTC TTA GAA GAG ACC (C/G)(A/G/C)G GTC TTT TAG G
primer 3: Cys^{351} to Val, Ala, Asp, Glu
ACGT GAA TTC TTA GAA GAG ACC (C/G)(T/G/A)C GTC TTT TAG G
primer 4: Cys^{351} to Phe, Tyr, Trp, Leu
ACGT GAA TTC TTA GAA GAG ACC (C/G)(C/T/A)A GTC TTT TAG G
primer 5: Cys^{351} to His
ACGT GAA TTC TTA GAA GAG ACC GTG GTC TTT TAG G
primer 6: Cys^{351} to Gln
ACGT GAA TTC TTA GAA GAG ACC CTG GTC TTT TAG G
primer 7: Cys^{351} to Lys
ACGT GAA TTC TTA GAA GAG ACC CTT GTC TTT TAG G
primer 8: Cys^{351} to Thr
ACGT GAA TTC TTA GAA GAG ACC TGT GTC TTT TAG G
primer 9: Cys^{351} to Ala
ACGT GAA TTC TTA GAA GAG ACC CGC GTC TTT TAG G

[a] The above primers (all in 5′ to 3′ notation) were generated and used as described in Experimental Procedures. The degenerate 3′ primers (1−4) were designed to introduce all possible mutants at Cys^{351} of $G_{i1}\alpha$ except His^{351} and Gln^{351}. As such, specific primers (5 and 6) were generated for these two mutants. Ala^{351}, Thr^{351}, and $Lys^{351}G_{i1}\alpha$ were not easily generated by the degenerate 3′ primers and thus specific 3′ primers (7−9) were also produced.

As such, in the current study we have examined the quantitative details of agonist-mediated binding of [^{35}S]GTPγS to mutationally modified forms of $G_{i1}\alpha$ in which cysteine^{351} has been converted to all of the other amino acids to eliminate any potential inherent bias in selection of the mutation. We demonstrate a spectrum of responsiveness in which the presence of branched chain aliphatic amino acids and certain hydrophobic aromatic amino acids at position^{351} improve the agonist-induced signal compared to the wild-type sequence and that charged residues, whether positive or negative, attenuate the signal. Agonist EC_{50} was altered only to a limited degree by the identity of the mutation, but hydrophobicity of amino acid^{351} was associated with a lower requirement for the agonist.

EXPERIMENTAL PROCEDURES

Materials. All materials for tissue culture were supplied by Life Technologies, Inc. (Paisley, Strathclyde, Scotland, U.K.). [^{3}H]RS-79948-197(90 Ci/mmol) was purchased from Amersham International. [^{35}S]GTPγS (1020 Ci/mmol) was obtained from DuPont/NEN. Pertussis toxin (240 μg/mL) was purchased from Speywood. All other chemicals were from Sigma or Fisons plc and were of the highest purity available. Oligonucleotides were purchased from Genosys (Cambridge, U.K.).

Methods. Construction of Cys^{351}Xaa Mutants of $G_{i1}\alpha$. Cys^{351}Xaa pertussis toxin-resistant forms of rat $G_{i1}\alpha$ were generated by PCR amplification of Nru1 linearized wild-type sequence in pBluescript KS- (Stratagene). The PCR reaction consisted of a 50 ng template, 100 pmol of a common 5′ sense primer, 100 pmol of one of four degenerate 3′ antisense primers (Table 1), dNTP's (0.2 mM each dATP, dCTP, dGTP, dTTP (Pharmacia)), 1 unit of *Pfu* DNA polymerase (Stratagene), and *Pfu* buffer in a total volume of 50 μL. The reaction conditions were as follows: 1 cycle at 95 °C for 2 min and 30 cycles consisting of a 95 °C 45 s denaturation step, a 55 °C 45 s annealing step, and a 72 °C 3 min extension step. The reaction was completed by a single 5 min extension at 72 °C. All reactions were performed using a Perkin-Elmer 9600 thermal cycler.

Resulting amplification products were run on a 1% (w/v) agarose gel. DNA bands corresponding to the anticipated 1065 bp length were excised and purified from the agarose gel using a Wizard DNA clean up kit (Promega). Three microliters of each purified amplification product was ligated into the pCR−Script SK(+) vector (Stratagene).

Plasmids containing rat $G_{i1}\alpha$ Cys^{351}Xaa were identified by restriction analysis to confirm the presence of $G_{i1}\alpha$ followed by DNA sequencing to identify the amino acid residue incorporated at position^{351}. Using this approach 13 of the required amino acid substitutions were identified. Rat $G_{i1}\alpha$ Cys^{351}Thr, Cys^{351}His, Cys^{351}Gln, Cys^{351}Lys, and Cys^{351}Ala were generated from the wild-type sequence by PCR using specific primers (Table 1). $Cys^{351}GlyG_{i1}\alpha$ had previously been generated (*11*). All the $G_{i1}\alpha$ Cys^{351}Xaa mutants were subsequently transferred from pCR−Script SK(+) into the mammalian expression vector pCDNA3.

Cell Culture and Transfection. HEK 293/T cells (HEK293 cells stably expressing the SV40 large T-antigen) were maintained in DMEM containing 10% (v/v) newborn calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded in 60 mm culture dishes and grown to 60−80% confluency (18−24 h) prior to transfection with pCDNA3 containing the relevant cDNA species using lipofectamine reagent (Life Technologies, Inc.). For transfection, 3 μg of DNA was mixed with 10 μL of lipofectamine in 0.2 mL of Opti-MEM (Life Technologies, Inc.) and incubated at room temperature for 30 min prior to the addition of 1.6 mL of Opti-MEM. Cells were exposed to the DNA/lipofectamine mixture for 5 h. Two milliliters of 20% (v/v) newborn calf serum in DMEM was then added to the cells. Cells were harvested 48 h after transfection. In the bulk of experiments cells were treated for the final 16 h prior to cell harvest with pertussis toxin (50ng/mL) to cause ADP-ribosylation of the endogenous G_i-family G proteins

(*8*) and thus prevent potential interactions between these and the receptor.

Preparation of Membranes. Plasma membrane-containing P2 particulate fractions were prepared from cell pastes that had been stored at −80 °C following harvest as described previously (*11*).

[^{3}H]RS-79948-197 Binding Studies. Binding assays were initiated by the addition of 5 μg of protein to assay buffer (10 mM Tris-HCl, 50 mM sucrose, 20 mM $MgCl_2$, pH 7.5) containing [^{3}H]RS-79948-197 (*16*) (0−1 nM). Nonspecific binding was determined in the presence of 100 μM idazoxan. Reactions were incubated at 30 °C for 45 min, and bound ligand was separated from free by vacuum filtration through GF/C filters. The filters were washed with 3 × 5 mL of assay buffer, and bound ligand was estimated by liquid scintillation spectrometry.

Immunological Studies. Antiserum I1C (*17*) was produced in a New Zealand White rabbit, using a conjugate of a synthetic peptide corresponding to amino acids 160−169 of the $G_{i1}\alpha$ subunit and keyhole limpet hemocyanin (Calbiochem) as antigen. The specificity of this antiserum for $G_{i1}\alpha$ has been demonstrated previously (*17*). Membrane samples were resolved by SDS−PAGE using 10% (w/v) acrylamide gels. Proteins were subsequently transferred to nitrocellulose (Schleicher and Schuell), probed with relevant antiserum, and visualized as described (*18*).

Binding of [^{35}S]GTPγS. This binding was performed using a modified method from those described in refs *4* and *19*. Assays were performed in 96-well format. Membranes (5 μg/assay point) were diluted to 0.083 μg/μL in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4), supplemented with saponin (10 mg/L), and preincubated with 40 μM GDP. Agonist and [^{35}S]GTPγS (1020 Ci/mmol, Amersham) at 0.3 nM was added (total volume of 100 μL), and binding was allowed to proceed at room temperature for 30 min. Nonspecific binding was determined by the inclusion of 0.6 mM GTP. Wheatgerm agglutinin SPA beads (Amersham) (0.5 mg) in 25 μL of assay buffer were added, and the whole was incubated at room temperature for 30 min with agitation. Plates were centrifuged at 1500*g* for 5 min and [^{35}S]GTPγS bound was determined by scintillation counting on a Wallac 1450 microbeta Trilux scintillation counter.

Correction of Agonist-Stimulated [^{35}S]GTPγS Binding for Levels of Expression of $G_{i1}\alpha$ Mutants. Both within sets of transfections and between different transfections, levels of expression of the individual mutants of $G_{i1}\alpha$ varied (see Results for details). As such, a strategy was required to normalize the levels of agonist-stimulated [^{35}S]GTPγS binding observed within and between experiments. We have previously demonstrated that expression of differing levels of $Gly^{351}G_{i1}\alpha$ results in a linear increase of agonist-induced G protein activation with detected G protein immunoreactivity (*11*). Differing levels of the mutant G proteins were expressed and quantitated by immunoblot (an example for $Ala^{351}G_{i1}\alpha$ is displayed as Figure 3) along with wild-type $G_{i1}\alpha$, and agonist-stimulation of the binding of [^{35}S]GTPγS was assessed. The relative stimulation of the two forms of $G_{i1}\alpha$ was then assessed at equal expression levels. This ratio was used to correct for differences in levels of expression in individual experiments.

RESULTS

We have previously constructed a pertussis toxin-insensitive form of the G protein $G_{i1}\alpha$ by alteration of Cys^{351} to Gly (*11*). The selection of this alteration was based on earlier observations by Senogles on the capacity of the D_2-dopamine receptor to interact with such a modified G protein (*9*). GTPase activity of this form of $G_{i1}\alpha$ was stimulated following coexpression with the porcine α_{2A}-adrenoceptor in COS-7 cells and addition of agonist to membranes prepared from these cells (*9*). However, the EC_{50} for agonist stimulation of $Cys^{351}Gly G_{i1}\alpha$ was some 10-fold higher than when using wild-type $G_{i1}\alpha$ (*11*). As others have used related strategies to modify G_i-like G proteins by replacement of the equivalent of Cys^{351} with amino acids such as serine (*10*, *12*), we decided to examine the effect of substitution of Cys^{351} of $G_{i1}\alpha$ on receptor regulation of the G protein without making inherent *a prioiri* decisions on the likely effects of the alteration. As such, Cys^{351} of $G_{i1}\alpha$ was converted to all of the other potential amino acids (Table 1).

Confirmation of the pertussis toxin insensitivity of each of the mutant forms of $G_{i1}\alpha$ was obtained by resolving membranes of either untreated or pertussis toxin-pretreated (50 ng/mL, 24 h) HEK 293/T cells expressing either wild-type $G_{i1}\alpha$ or the various mutants in SDS−PAGE containing 6 M urea followed by immunoblotting with the $G_{i1}\alpha$ specific antiserum I1C (*17*). ADP-ribosylation of $G_{i1}\alpha$ by pertussis toxin results in a retardation of mobility of $G_{i1}\alpha$ through such gels as we have previously shown for the wild-type protein (*11*, *20*). None of the $G_{i1}\alpha$ mutants showed a similar alteration in mobility (data not shown but see ref *11*).

Each of these forms of $G_{i1}\alpha$ was transiently coexpressed in HEK 293/T cells along with the porcine α_{2A}-adrenoceptor. The receptor was expressed to high (10−15 pmol/mg of membrane protein) and equivalent levels in all sets of transfections as measured by the specific binding of the high affinity and α_2-adrenoceptor selective ligand [^{3}H]RS-79948-197, and this was not altered by coexpression of any of the mutated forms of $G_{i1}\alpha$ (data not shown).

The capacity of varying concentrations of the α_2-adrenoceptor agonist UK14304 to stimulate binding of [^{35}S]GTPγS to the individual forms of $G_{i1}\alpha$ was then assessed in membranes derived from these cells (Figure 1). Following expression of wild-type (Cys^{351}) $G_{i1}\alpha$ pertussis toxin treatment (50 ng/mL, 24 h) abolished the capacity of UK14304 to stimulate binding of [^{35}S]GTPγS (data not shown). Therefore, with the exception of those expressing wild-type $G_{i1}\alpha$, cells were treated with pertussis toxin prior to cell harvest and membrane preparation to prevent any potential for interactions of the receptor with endogenously expressed forms of $G_i\alpha$ and to ensure that signals emanated from activation of the mutant forms of $G_{i1}\alpha$. Maximal UK14304-induced binding of [^{35}S]GTPγS varied widely with the individual $G_{i1}\alpha$ mutants (Figure 1). For Pro^{351}, Met^{351}, Arg^{351}, and Lys^{351} $G_{i1}\alpha$, no significant effect of agonist could be recorded. By contrast, although a number of mutants including Thr^{351}, His^{351}, Gln^{351}, Ser^{351}, Asn^{351}, and Ala^{351} produced degrees of agonist-stimulated binding of [^{35}S]-GTPγS lower than or similar to those of the wild-type G protein α subunit, a number of the mutant forms of $G_{i1}\alpha$, especially Leu^{351}, Ile^{351}, and Trp^{351} produced substantially greater [^{35}S]GTPγS binding than the wild type (Figure 1).

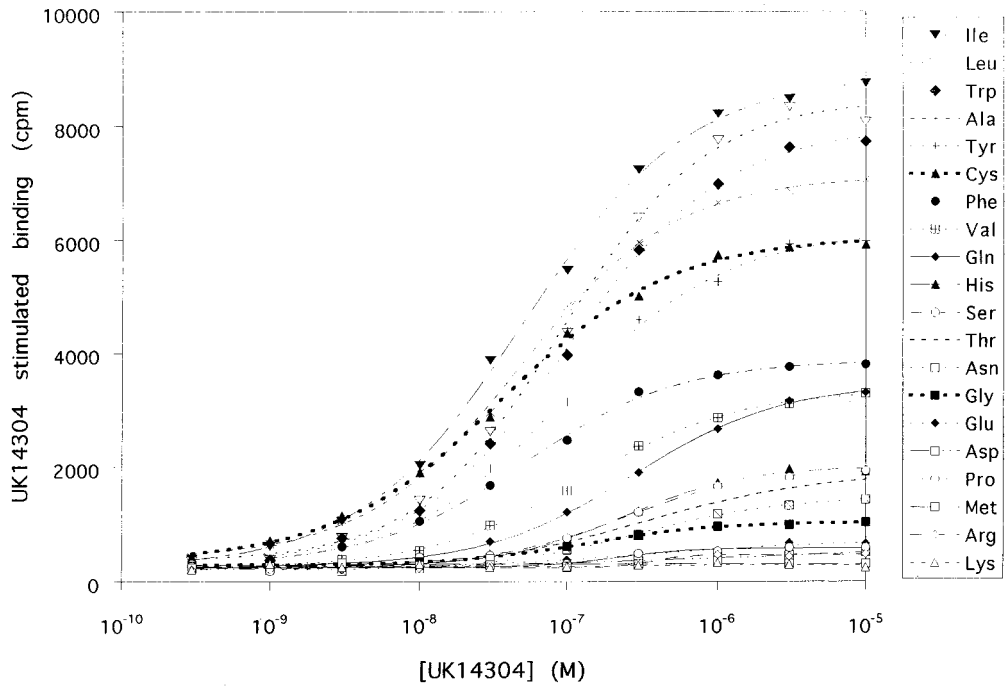

FIGURE 1: The capacity of Cys^{351}Xaa mutants of $G_{i1}\alpha$ to be activated by the α_{2A}-adrenoceptor. The porcine α_{2A}-adrenoceptor was expressed in combination with mutants of $G_{i1}\alpha$ containing every possible amino acid (denoted by their conventional 3 letter abbreviations) at $residue^{351}$ in HEK 293/T cells. Except where the receptor was expressed in combination with wild-type (Cys^{351}) $G_{i1}\alpha$, the cells were treated with pertussis toxin (50 ng/mL) for 24 h prior to cell harvest. Following membrane preparation the capacity of varying concentrations of UK14304 to stimulate binding of [^{35}S]GTPγS was assessed as in the Methods section. Data are taken from a typical experiment.

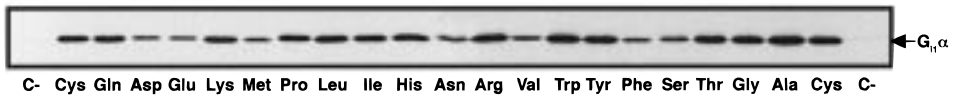

FIGURE 2: Expression of Cys^{351}Xaa mutants of $G_{i1}\alpha$ in HEK 293/T cells: Immunodetection. HEK 293/T cells were transiently transfected with the α_{2A}-adrenoceptor alone (C−) or in combination with cDNA encoding forms of $G_{i1}\alpha$ in which $residue^{351}$ was any of the possible amino acids as in Figure 1. Following membrane preparation and SDS−PAGE, the samples were probed for $G_{i1}\alpha$ immunoreactivity using antiserum I1C. The amino acid at $residue^{351}$ is identified by the standard three letter abbreviation.

Coexpression of $Val^{351}G_{i1}\alpha$, although now possessing a branched chain amino acid at this position, did not allow the same degree of stimulated [^{35}S]GTPγS binding as the Leu^{351} and $Ile^{351}G_{i1}\alpha$ mutants, for example, and indeed appeared to be poorer than wild-type $G_{i1}\alpha$ (Figure 1). Assays were performed over a 30 min period as preliminary time course experiments demonstrated that agonist-induced binding of [^{35}S]GTPγS reached maximal levels between 20 and 40 min (data not shown). At longer time periods the agonist-induced binding was reduced, due to increasing levels of agonist-independent binding of [^{35}S]GTPγS. This presumably reflects the well appreciated, relatively high rates of spontaneous guanine nucleotide exchange of G_i-family G proteins. No differences were noted in the time course of maximal agonist-induced binding of [^{35}S]GTPγS when using each of Cys^{351}, Ile^{351}, or $Gly^{351}G_{i1}\alpha$ (data not shown). To assess whether the variation in maximal response to α_{2A}-adrenoceptor stimulation might be related to the levels of expression of the $G_{i1}\alpha$ mutants, this was measured by immunoblotting with antiserum I1C (Figure 2). Twelve of the mutant forms of $G_{i1}\alpha$ which covered the full spectrum of functional response to UK14304 (Ile^{351}, Leu^{351}, Trp^{351}, Tyr^{351}, Ala^{351}, Thr^{351}, Pro^{351}, Gln^{351}, His^{351}, Gly^{351}, Arg^{351}, and Lys^{351}) expressed to essentially the same high levels as wild-type (Cys^{351}) $G_{i1}\alpha$ in membranes of these cells (Figure 2), while the others, Phe^{351}, Val^{351}, Ser^{351}, Asn^{351}, Met^{351}, Glu^{351}, and Asp^{351}, although clearly expressed were present at significantly lower levels (Figure 2). As these mutant forms of $G_{i1}\alpha$ did not express as highly as wild-type $G_{i1}\alpha$ a variant strategy was required to assess their absolute effectiveness. $Ala^{351}G_{i1}\alpha$ bound [^{35}S]GTPγS in response to addition of UK14304 to a similar extent as wild-type $G_{i1}\alpha$ (Figure 1) and was as effectively expressed (Figure 2). We coexpressed varying amounts of $Ala^{351}G_{i1}\alpha$ cDNA along with the α_{2A}-adrenoceptor in HEK 293/T cells and examined the stimulation of binding of [^{35}S]GTPγS in response to a maximally effective concentration of UK14304 in membranes of pertussis toxin-treated cells. Over the range of cDNA amounts used, this was linear with the degree of expression of $Ala^{351}G_{i1}\alpha$ quantitated by immunoblotting the membranes with antiserum I1C (Figure 3) as we have previously noted for $Gly^{351}G_{i1}\alpha$ (*11*). Parallel expression and measurement of agonist activation of a level of wild-type $G_{i1}\alpha$ within the linear range for agonist activation of the mutant allowed direct comparison of the effectiveness of the two proteins. Construction of equivalent titration curves also allowed correction for expression of Phe^{351}, Val^{351}, Ser^{351}, Asn^{351}, Met^{351}, Glu^{351}, and $Asp^{351}G_{i1}\alpha$ to produce a final analysis of the effects of mutation of $residue^{351}$ (Figure 4).

We have previously demonstrated that α_{2A}-adrenoceptor stimulation of the high-affinity GTPase activity of $Gly^{351}G_{i1}\alpha$ is some 10-fold less potent than for wild-type $G_{i1}\alpha$ (*11*). Although the EC_{50} for UK14304 did not vary by more than

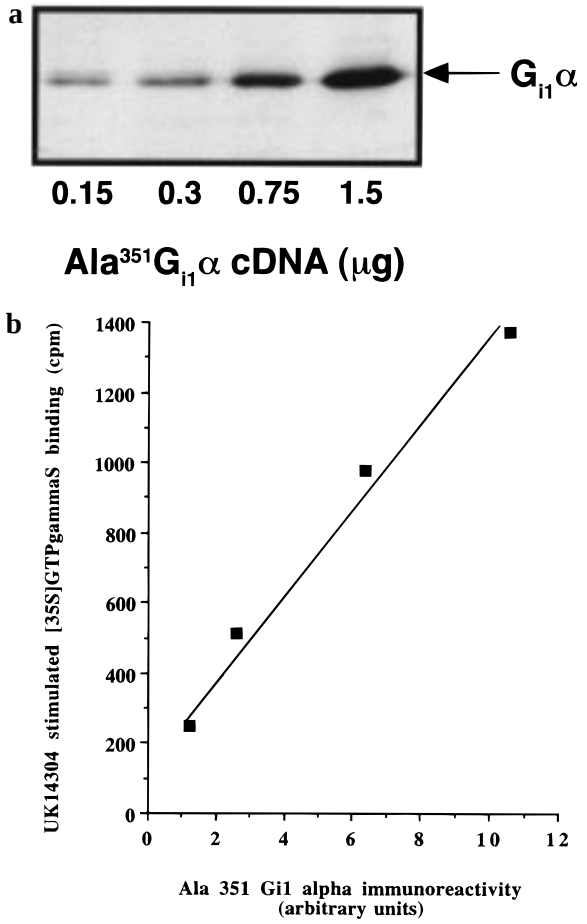

FIGURE 3: A linear relationship between expression levels of $Ala^{351}G_{i1}\alpha$ and UK14304-mediated stimulation of [^{35}S]GTPγS binding. Varying amounts of $Ala^{351}G_{i1}\alpha$ cDNA were coexpressed with the α_{2A}-adrenoceptor in HEK293/T cells. Following pertussis toxin treatment, membranes were prepared which were either immunoblotted with antiserum I1C (a) or used to measure the capacity of UK14304 (10 μM) to stimulate binding of [^{35}S]GTPγS (b).

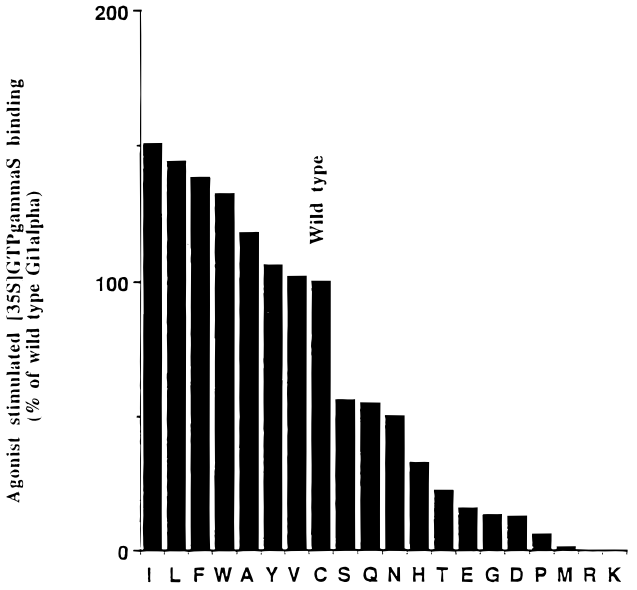

FIGURE 4: The relative effectiveness of the Xaa^{351} forms of $G_{i1}\alpha$ to bind [^{35}S]GTPγS in response to UK14304. The capacity of UK14304 (10 μM) to stimulate binding of [^{35}S]GTPγS to Xaa^{351} forms of $G_{i1}\alpha$ following their coexpression with the α_{2A}-adrenoceptor and correction for variation in expression of the forms of $G_{i1}\alpha$. Values are presented relative to that of wild-type $G_{i1}\alpha$. The identity of amino acid at residue^{351} in the forms of $G_{i1}\alpha$ is noted using the single letter amino acid symbol.

Table 2: Agonist-Stimulated [^{35}S]GTPγS Binding, EC_{50} for UK14304, and Partition Coefficient Octanol/Water

amino acid ^{351}	GTPγS[a]	EC_{50} (M)	log *P* (octanol/water)[b]
I	0.51	$1.6 \times 10^{-8} \pm 8.2 \times 10^{-10}$	−1.72
L	0.44	$2.9 \times 10^{-8} \pm 2.5 \times 10^{-9}$	−1.61
F	0.38	$1.6 \times 10^{-8} \pm 1.9 \times 10^{-9}$	−1.63
W	0.32	$3.1 \times 10^{-8} \pm 1.6 \times 10^{-9}$	−1.75
A	0.18	$4.8 \times 10^{-8} \pm 2.9 \times 10^{-9}$	−2.89
Y	0.06	$3.2 \times 10^{-8} \pm 2.3 \times 10^{-9}$	−2.42
V	0.01	$4.2 \times 10^{-8} \pm 1.6 \times 10^{-9}$	−2.08
C	0.00	$3.7 \times 10^{-8} \pm 3.2 \times 10^{-9}$	−2.49
S	−0.44	$7.3 \times 10^{-8} \pm 7.5 \times 10^{-9}$	−3.30
Q	−0.45	$8.2 \times 10^{-8} \pm 5.7 \times 10^{-9}$	−3.15
N	−0.50	$9.1 \times 10^{-8} \pm 9.1 \times 10^{-9}$	−3.41
H	−0.68	$8.4 \times 10^{-8} \pm 1.1 \times 10^{-8}$	−3.56
T	−0.78	$5.4 \times 10^{-8} \pm 2.9 \times 10^{-8}$	−2.91
E	−0.84	$7.8 \times 10^{-8} \pm 3.5 \times 10^{-8}$	−4.19
G	−0.87	$1.4 \times 10^{-7} \pm 9.9 \times 10^{-9}$	−3.25
D	−0.87		−4.25
P	−0.94		−2.50
M	−0.99		−1.84
R	−1.00		−4.20
K	−1.00		−4.44

[a] The GTPγS index was obtained as (cpm mutant − cpm wild type (Cys^{351}))/cpm wild type, where cpm mutant and cpm wild type are the agonist-stimulated [^{35}S]GTPγS binding data values for each mutant and for the wild-type $G_{i1}\alpha$, respectively. Values for the mutants were corrected for levels of expression of the individual forms of $G_{i1}\alpha$ (see Methods for details). [b] Taken from ref *36*.

10-fold between the forms of $G_i\alpha$ which produced sufficient agonist-induced binding of [^{35}S]GTPγS to allow analysis (Table 2), there was a distinct trend in which the mutants including Phe^{351}, Ile^{351}, and Leu^{351} which allowed greater maximal binding were more sensitive to agonist and displayed lower EC_{50} values than mutants such as Gln^{351}, His^{351}, and Asn^{351} which allowed lower maximal binding of [^{35}S]GTPγS in response to the agonist. As noted previously in experiments measuring agonist regulation of high-affinity GTPase activity (*11*) the EC_{50} for UK14304 activation of binding of [^{35}S]GTPγS to $Gly^{351}G_{i1}\alpha$ was clearly greater than for activation of wild-type $G_{i1}\alpha$ (Table 2).

It was possible that the mutant forms of $G_{i1}\alpha$ which did not display significantly enhanced binding of [^{35}S]GTPγS in response to the agonist were simply incapable of exchanging guanine nucleotide and hence binding GTPγS rather than being inherently unresponsive to the agonist-occupied receptor. To examine this possibility we used the known capacity of GTPγS-bound forms of $G_{i1}\alpha$ to display reduced tryptic sensitivity compared to GDP-bound forms of the same protein (*21*). Following expression of wild-type $G_{i1}\alpha$ in HEK 293/T cells and membrane preparation, addition of GTPγS resulted in the production of a stable trypsin cleaved I1C reactive polypeptide. In the absence of GTPγS this immunoreactive fragment was further proteolyzed to forms which could not be detected by the antiserum (Figure 5). Equivalent expression and analysis of $Arg^{351}G_{i1}\alpha$, which displayed no significant capacity to be activated by the α_{2A}-adrenoceptor, and $Ile^{351}G_{i1}\alpha$, which produced greater stimulated [^{35}S]GTPγS binding than wild-type $G_{i1}\alpha$, both resulted in production of similar levels of a cleaved fragment following addition of GTPγS which was resistant to further proteolysis

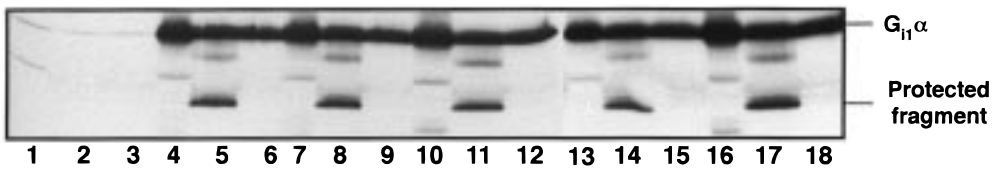

FIGURE 5: Poorly activated Cys^{351}Xaa mutants of $G_{i1}\alpha$ can still bind GTPγS. HEK 293/T cells were either mock transfected (1−3) or transfected with wild-type (Cys^{351}) $G_{i1}\alpha$ (4−6), Gly$^{351}G_{i1}\alpha$ (7−9), Arg$^{351}G_{i1}\alpha$ (10−12), Phe$^{351}G_{i1}\alpha$ (13−15), or Ile$^{351}G_{i1}\alpha$ (16−18). Membranes (100 μg) from these cells were untreated (1, 4, 7, 10, 13, 16) or treated with trypsin in the presence (2, 5, 8, 11, 14, 17) or absence (3, 6, 9, 12, 15, 18) of GTPγS as described in the Methods section. Following the incubations, the samples were resolved by SDS−PAGE and immunoblotted with antiserum I1C.

and was indistinguishable from that produced from wild-type $G_{i1}\alpha$ (Figure 5).

DISCUSSION

Mutations of pertussis toxin-sensitive G proteins which render them insensitive to the actions of this toxin by alteration of the acceptor cysteine residue have been used in a range of studies to allow examination of interactions between GCPRs and specific G proteins (*9*−*12*). Inherently this strategy might be considered to have the possibility of disrupting substantially functional interactions between GPCRs and these G proteins as ADP-ribosylation of this cysteine attenuates such interactions (*8*). However, the addition of ADP-ribose can be anticipated to have an extreme effect as a large and charged side group is added to the protein and because information transfer can be achieved using mutants containing relatively conservative substitutions at this site (*9*−*12, 14*−*15*). As such, the cysteine side chain at position^{351} is not inherently required for GPCR-G protein interactions, and the effect of addition of ADP-ribose is presumably a combination of steric hindrance and the introduction of charge (see below). Despite this, little attention has been paid to the quantitative alterations in signal transduction which might derive from the use of G proteins with mutations in this position. Furthermore, there has been no systematic analysis of the effect of the identity of the alteration on the effectiveness of signal transduction between a GPCR and G protein. These are both important practical issues, as a range of strategies both to examine GPCR pharmacology and to screen for novel agonist ligands has utilized agonist-mediated stimulation of either the binding of [^{35}S]GTPγS or the hydrolysis of γ[^{32}P]GTP by G_i-like G protein α subunits as robust assays. As such, it is clearly of importance to know how severely a particular mutation might affect the sensitivity of the assay or the potency for agonist ligands and also whether this might alter the steady-state levels of expression of the protein.

Although likely conservative mutations have been used previously it is unwise to predict *a prioiri* the likely outcome of such mutations. Therefore in the current studies we simply replaced cysteine^{351} with all other potential amino acids and then examined the capacity of each mutant to be activated by the α_{2A}-adrenoceptor (Figure 1). The majority of the mutant forms of $G_{i1}\alpha$ were expressed at levels similar to that of the wild-type protein (Figure 2), but perhaps surprisingly, the wild-type protein was not the most effectively activated by the receptor (Figures 1 and 4). Replacement of cysteine^{351} either with branched chain aliphatic amino acids or with those containing an aromatic side chain allowed greater levels of agonist-stimulated binding of [^{35}S]GTPγS with the most effective alterations resulting in some 50% higher specific binding (Figure 4). These observations have substantial implications for agonist screening studies as well as basic understanding of the activation of G proteins by GPCRs. In screening systems it is vital to configure assays to maximize sensitivity of response so that the lowest levels of cell protein can be used to minimize cost. These findings indicate that judicious mutation (at least in $G_{i1}\alpha$) may substantially increase sensitivity to agonist, even though this could probably not have been predicted from any previous mutational studies on this or other G proteins. Previous alanine-scan mutational studies on transducin α have indicated important roles in receptor interactions for the leucine residues in the C-terminal decapeptide of this G protein (*15*). Leucine residues are conserved in equivalent positions across the G protein family, and it has been argued that they may contribute substantially to interactions with receptors as hydrophobic amino acids often play crucial roles in protein−protein contacts (*22*).

Less surprisingly, other mutations decreased the capacity of the agonist-occupied GPCR to activate the G protein. Indeed, certain alterations which resulted in the presence of either a fixed positive or negative charge essentially eliminated receptor-mediated activation of the G protein (Figures 1 and 4). It was a formal possibility that these mutations lacked the capacity to exchange guanine nucleotide. However, addition of GTPγS stabilized an immunodetectable, proteolytically clipped, fragment of Arg$^{351}G_{i1}\alpha$, a mutant which displayed no capacity to bind [^{35}S]GTPγS in response to agonist, from extensive tryptic cleavage to the same extent as observed for both wild-type $G_{i1}\alpha$ and mutants of $G_{i1}\alpha$ which produced greater maximal [^{35}S]GTPγS binding in response to agonist than the wild-type G protein (Figure 5).

As the experiments discussed above used a single high concentration of GTPγS, it was also possible that the individual forms of $G_{i1}\alpha$ displayed difference affinities for guanine nucleotides. We have recently approached this issue in a novel manner. By constructing fusion proteins between the α_{2A}-adrenoceptor and $G_{i1}\alpha$ we have shown that these can be be treated as agonist-activated enzymes with Michaelis−Menten characteristics (*23*−*25*). This has allowed us to measure directly the K_m for GTP. No matter whether the fusion proteins contain Cys^{351}, Ile^{351}, or Gly$^{351}G_{i1}\alpha$ the K_m for GTP is close to 0.4 μM (ref *25* and data not shown). Such studies define that GTP affinity is not modified by the modification of residue^{351}.

In an attempt to rationalize the variation of agonist stimulation of [^{35}S]GTPγS binding shown by the individual residue^{351} mutants of $G_{i1}\alpha$, we searched for quantitative relationships between empirical descriptors of the physicochemical properties of natural amino acids and the functional

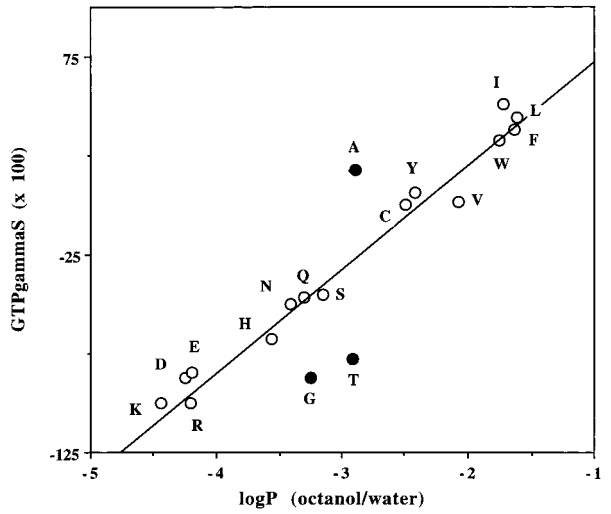

FIGURE 6: Correlation of amino acid hydrophobicity and maximal agonist stimulation of [^{35}S]GTPγS binding to Cys^{351}Xaa mutants of $G_{i1}\alpha$. GTPγS (= (cpm mutant − cpm wild type)/cpm wild type, where cpm mutant and cpm wild type are the agonist-stimulated [^{35}S]GTPγS binding data values for each mutant and for the wild type, respectively (see Table 2 for details) was plotted against log *P*(octanol/water) (*36*). The linear regression equation is GTPγS = 1.237 (±0.167) + 0.522 (±0.054)log *P*(octanol/water), $n = 18$, $r = 0.924$, and $s = 0.213$, where n is the number of forms of $G_{i1}\alpha$, r is the correlation coefficient, s is the standard deviation, and the numbers in parentheses give the 95% confidence intervals. Proline and methionine were excluded from the correlation. Amino acids are denoted by the standard one letter symbol. Elimination of alanine, threonine, and glycine (filled circles) from the analyses results in the above equation becoming GTPγS = 1.242 (±0.065) + 0.513 (±0.02)log *P*(octanol/water), $n = 15$, $r = 0.989$, and $s = 0.083$.

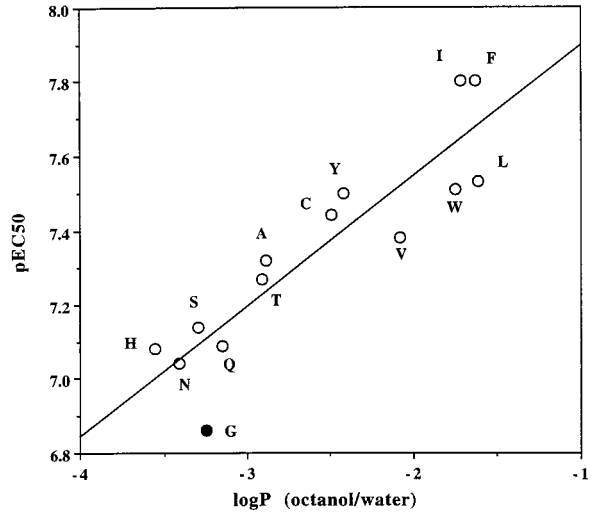

FIGURE 7: Correlation between the pEC_{50} for UK14304 measured for activation of mutants at residue^{351} of $G_{i1}\alpha$ and the hydrophobicity parameter log *P*(octanol/water) of the modified amino acids. Amino acids are denoted by the standard one letter symbol. The linear regression equation is $pEC_{50} = 8.246$ (±0.133) + 0.351 (±0.055)log *P*(octanol/water), $n = 14$, $r = 0.90$, and $s = 0.129$, where n is the number of forms of $G_{i1}\alpha$, r is the correlation coefficient, s is the standard deviation, and the numbers in parentheses give the 95% confidence intervals. Leaving out glycine (filled circle), the above equation becomes $pEC_{50} = 8.194$ (±0.112) + 0.323 (±0.04)log *P*(octanol/water), $n = 13$, $r = 0.916$, and $s = 0.106$.

properties of the mutants. The effectiveness of applying a similar quantitative structure−activity relationship analysis to investigate the intrinsic process of the activation of the α_{1B}-adrenoceptor has recently been shown (*26*).

In the present study, we analyzed a large number of descriptors of the physicochemical properties of natural amino acids, including a variety of hydrophobicity and hydrophilicity parameters (*27*−*29*), size descriptors (*30*), volume and surface area values (*27*, *31*−*33*), solution properties (*26*), *R* values (*26*, *34*), and polarity and polarizability indices (*26*, *30*). Moreover, the three principal components (z1, z2, and z3) identified by principal component analysis of 29 different experimental properties of the 20 coded amino acids were also considered (*35*).

A dimensionless descriptor accounting for the relative agonist stimulation of the [^{35}S]GTPγS binding for each mutant with respect to the wild type was used in the correlation analysis. This index was obtained from the formula: GTPγS = (cpm mutant − cpm wild type)/cpm wild type, where cpm mutant and cpm wild type are the agonist-stimulated [^{35}S]GTPγS binding data values for each mutant (following correction of the levels of agonist-stimulated [^{35}S]GTPγS binding for the expression levels of the mutant) and for the wild-type $G_{i1}\alpha$, respectively (Table 2). Moreover, the pEC_{50} for UK14304 measured for the mutants and wild-type $G_{i1}\alpha$ (Table 2) was also used in this analysis. Simple regression analysis revealed that the most significant linear trends with the GTPγS and the pEC_{50} values are related to the hydrophobicity parameters. The partition coefficient octanol/water (log *P*(octanol/water)) (*36*) gave the best correlations in this analysis. In fact, when eliminating methionine and proline, this index gives a strong correlation with the GTPγS binding ($r = 0.92$, Figure 6). The correlation coefficient becomes close to 1 ($r = 0.99$) if alanine, threonine, and glycine (labeled by filled circles in Figure 6) are additionally left out from the correlation. A potential explanation for the observation that methionine and proline behave as marked outliers in this relation is the following. For methionine, it is possible that this residue was present as methionine sulfoxide in the G protein which would severely affect its hydrophobic character. For proline, this amino acid may affect the conformation of the C-tail of the G protein. A requirement for flexibility at Gly^{348} in transducin α for its binding to light-exposed rhodopsin was previously hypothesized on the basis of the observation that proline is more effective at disrupting rhodopsin binding than other amino acids at this position (*15*). It is worth noting that Gly^{348} of transducin α corresponds to Gly^{352} of $G_{i1}\alpha$ which is adjacent to the residue targeted in these studies. A linear relation was also found between the log *P*(octanol/water) and the pEC_{50} values (Figure 7).

The very good linear relationships between the hydrophobicity parameters and the functional response expressed as agonist stimulation of [^{35}S]GTPγS binding have important mechanistic implications, strengthening the hypothesis that the C-terminal tail of G proteins plays a fundamental role in GPCR/G protein interaction (*37*). In fact, on the basis of the results of our analysis, it can be inferred that the C-terminal portion of the $G_{i1}\alpha$ carrying the mutated residue becomes buried with respect to the cytosolic water to interact with the receptor. The higher the hydrophobic character of the amino acid at residue^{351} the higher the propensity to escape from water and the higher is the intermolecular stabilization due to the short-range acting intermolecular

interactions established between G protein and activated receptor.

On the basis of the effects observed herein and the low maximal stimulation of function of $Gly^{351}G_{i1}\alpha$ by the agonist-occupied α_{2A}-adrenoceptor, it is clear that this particular mutation was far from ideal for analysis of this receptor in our earlier experiments (*11*). Whether this will vary between different receptors cannot be considered without direct experimentation, and this will be an interesting issue to examine in the future. Whatever the outcome of such future studies, the random mutagenesis approach for $G_{i1}\alpha$ used herein has provided a range of novel insights into the interaction interface between this G protein and the α_{2A}-adrenoceptor as well as demonstrating that certain mutations at position 351 can result in the generation of forms of $G_{i1}\alpha$ which can produce greater agonist stimulation of [^{35}S]GTPγS binding via activation of the α_{2A}-adrenoceptor.

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