Mutation of cysteine 214 in G_{i1} α subunit abolishes its endogenous GTPase activity

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The functional consequences of the mutation of a conserved Cys-214 in G_{i1} have been investigated. We reported herein that substitutions of Cys-214 of G_{i1} to either alanine or tryptophan abolished the intrinsic GTPase activity. Free phosphate release from [³²P]GTP-bound G_{i1} C214A or [³²P]GTP-bound G_{i1} C214W was at least 30-fold lower than that of the wild-type G_{i1} in single-turnover GTPase assays. Consistently, tryptic proteolysis of C214A and C214W proteins showed that they were partially protected by GTP, further confirming that the GTPase activity in both mutant proteins was impaired. Expression of C214A or C214W mutants in Chinese hamster ovary K1 cells caused significant inhibition of forskolin-stimulated adenylate cyclase activity. However, the mutations did not significantly affect the GTP[S]

(guanosine 5'-[γ -[³⁵S]thio]triphosphate)-binding activity. Both C214A and C214W mutants serve as good substrates for pertussis toxin-catalysed ADP ribosylation, indicating that they interact well with $\beta\gamma$ subunits. Moreover, RGS4 protein, a GTPase-activating protein for G_{i1} , cannot interact with Cys-214 mutants even in the presence of AlF_4^- , which induces the transition state of G_{i1} . In summary, our findings suggest that C214A or C214W are GTPase-deficient mutants and can functionally serve as constitutively active forms of G_{i1} in cells.

Key words: cysteine, G-protein, GTPase, GTP hydrolysis, site-directed mutagenesis.

INTRODUCTION

The heterotrimeric G-protein transmits signals from cell membrane receptors to a variety of intracellular effectors, such as adenylate cyclase, phospholipase and ion channels [1–3]. The α subunit of the G-protein has guanine nucleotide-binding activity and undergoes dramatic conformational changes upon the binding of GTP or GDP. The G_{i1} subunit is in an active state when it binds GTP, following receptor activation. Deactivation occurs thereafter by G_{i1} endogenous GTPase activity that hydrolyses the GTP to GDP and the G-protein converts back to an inactive state.

Much of the understanding of the mechanism of GTP hydrolysis by G_{i1} subunits comes from the three areas: the early studies of the ADP ribosylation of G_{i1} subunits by bacterial toxins [4–7], site-specific mutagenesis, which was largely based upon analogies to the related small G-proteins, such as p21^{ras} proteins and translation elongation factor EF-Tu [8–12], and the recent information obtained from high-resolution crystal structures of the G_{i1} proteins under different conditions [13–16]. Most data, thus far, indicate that the mechanism underlying the hydrolysis of GTP by G_{i1} subunits occurs by an S_N2 in-line attack of a water molecule on the γ phosphate of the nucleotide. This is convincingly demonstrated by a crystallographic study that the G_{i1} -GDP- AlF_4^- complex mimics the transition state of the S_N2 reaction and a well-ordered water molecule that is positioned to carry out a nucleophilic attack has been identified near the γ phosphate group [13]. Attempts have been made to identify the residues that play an essential role in the catalysis of GTP hydrolysis. Although many residues have been indicated to be critical for binding to guanine nucleotides [17–19], only a few showed a change in GTPase activity [20–22]. Attention has previously focused on two amino acids. The arginine located at position 178 in G_{i1} was originally identified

as the ADP-ribosylation site in G_{i1} (Arg-201) and G_{i2} (Arg-174) by cholera toxin and leads to irreversible inhibition of GTPase activity [4,7]. Another amino acid of interest is Gln-204 (in G_{i1}). This Gln has been speculated previously to be the critical catalytic residue required for the de-protonation or polarization of the water molecule for in-line attack on the γ phosphate group of GTP. However, recent studies suggest that this residue may exert its influence in the stabilization of the transition state of the S_N2 reaction [23]. Mutations at either of these two residues Gln-204 and Arg-178 largely abolish GTP hydrolytic activity and thus stabilize the GTP-bound state [23]. Gln-204 is located in the Switch II region which spans the α_2 helix and the preceding loop (residues 199–219 of G_{i1}) [14]. The Switch II region also undergoes substantial rearrangement upon GTP hydrolysis which implies an involvement in the formation of the transition state [13–15]. Several residues around the Gln-204 in the Switch II region, such as Gly-203, Glu-207 and Arg-208 of G_{i1} , have been exploited and mutagenesis of these sites did not show any effects on the GTPase activity [10,17,23,24]. The Cys-215 in G_{i1} (analogous to Cys-214 in G_{i1}), is also conserved in the Switch II region and has been shown previously to be involved in the chemical crosslinking of G_{i1} with $\beta\gamma$ subunits by 1,6-bismaleimido hexane [25,26]. However, the role of this Cys in the GTP hydrolysis of G_{i1} is unknown. In the present work, we found that the mutation of Cys-214 to Ala or Trp in the G_{i1} subunit caused a large decrease in the endogenous GTPase activity. The C214A and C214W mutants can functionally serve as constitutively active G_{i1} mutants for inhibiting adenylate cyclase activity in cells. Calculations of the thermodynamic parameters of binding for C214A and C214W in both GTP[S] (guanosine 5'-[γ -[³⁵S]thio]triphosphate)-bound ground state and G_{i1} -GDP- AlF_4^- -bound transition state indicate that the transition-state

Abbreviations used: GST, glutathione S-transferase; CHO, Chinese hamster ovary; GTP[S], guanosine 5'-[γ -[³⁵S]thio]triphosphate; PTX, pertussis toxin; DTT, dithiothreitol.

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energy relative to the ground-state energy is much greater for C214A and C214W mutants than it is for the wild type, and thus both C214A and C214W mutants raise the energy barrier for the GTP hydrolysis by ≈ 3 -fold. We therefore propose that the mutation of Cys-214 to either alanine or tryptophan impair the GTPase activity of G_{α_i} by destabilizing the transition state.

EXPERIMENTAL

Materials and cDNA constructs

The Cys-214 of $G_{\alpha_{i1}}$ was individually mutated to either alanine or tryptophan by overlap extensions employing the PCR with primers encoding the mutations. This method involves amplifying two overlapping fragments that contain the mutation site in their overlapping region. The overlapping regions of the oligonucleotides were annealed and the full-length fragment was obtained by amplification using the outside primers. The final PCR products were subcloned into the *Nco*I and *Bam*HI restriction sites of the pQE60 vector (Qiagen) for protein expression and purification. For mammalian cell study, the wild-type and mutant $G_{\alpha_{i1}}$ cDNAs were subcloned into *Hind*III and *Xho*I restriction sites of the pcDNA3.1 (+) vector (Invitrogen). All constructs were verified directly by DNA sequencing.

The $G_{\alpha_{i1}}$ -His₆, $G_{\alpha_{i1}}$ C214A-His₆ and $G_{\alpha_{i1}}$ C214W-His₆ proteins were purified from *Escherichia coli* via a Ni²⁺-nitrilotriacetate resin-based chromatography (QIAexpress System from Qiagen). GST (glutathione S-transferase)-RGS4 fusion protein was purified as described previously [27].

Single-turnover GTPase assay

The GTPase activity of $G_{\alpha_{i1}}$ was measured by monitoring free γ -P_i release from the $G_{\alpha_{i1}}$ -bound GTP as described previously [27]. Briefly, 1 μ M $G_{\alpha_{i1}}$ protein was incubated with 1 μ M [γ -³²P]GTP in assay buffer containing 10 mM Hepes (pH 7.5), 5 mM EDTA, 2 μ M GTP, 1 mM DTT (dithiothreitol) and 0.05 % Lubrol, at 30 °C for 30 min. The [γ -³²P]GTP-loaded $G_{\alpha_{i1}}$ was separated from unbound [γ -³²P]GTP by passing through a gel-filtration column (Princeton Separations, Princeton, NJ, U.S.A.), and diluted to 200 nM with ice-cold assay buffer. The $G_{\alpha_{i1}}$ GTPase was initiated by adding 10 mM MgCl₂ and 100 μ M GTP. The reaction was stopped by adding 5 % Norit-activated charcoal in 50 mM sodium phosphate (pH 3.0). Following centrifugation, the supernatant containing free ³²Pi was counted. All assays were performed at 0 °C.

GTP[S]-binding assay

GTP-binding activity of $G_{\alpha_{i1}}$ was measured by incubation of $G_{\alpha_{i1}}$ protein (20 μ g/ml) with [³⁵S]GTP[S] (2500 c.p.m./pmol) in the buffer for this assay (10 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 % Lubrol, 1 μ M GTP[S] and 20 mM MgCl₂). Reactions were stopped by passing aliquots (40 μ l) of the reaction mixture through the gel-filtration column in a centrifuge (900 g) for 2 min. The radioactivity in eluates was determined by liquid scintillation counting.

Tryptic proteolysis

Tryptic digestion was performed according to Kleuss et al. [28]. Briefly, 2 μ g of wild-type or mutant $G_{\alpha_{i1}}$ protein was incubated for 30 min at 30 °C in 50 μ l of buffer containing 20 mM Hepes, pH 8.0, 10 mM MgCl₂, 1 mM EDTA, 0.64 % Lubrol PX and 1 mM DTT, in the presence of either 100 μ M GDP, GTP or GTP[S] or 30 μ M AlCl₃/10 mM NaF. Trypsin was then added

to a final concentration of 50 μ g/ml and incubated for 10 min at 30 °C. Digestions were stopped by adding 0.5 μ l of PMSF (1 mg/ml) and boiled for 5 min. The digestion products were visualized by Western-blot analysis using a monoclonal antibody against $G_{\alpha_{i1}}$ (Upstate Biotechnology, Lake Placid, NY, U.S.A.).

cAMP measurement

CHO-K1 (Chinese hamster ovary K1) cells maintained in Dulbecco's modified Eagle's medium with 10 % fetal bovine serum were transfected with empty pcDNA3.1 (+) vector, wild-type $G_{\alpha_{i1}}$, or C214A, C214W, Q204L or R178C mutants in pcDNA3.1 using Lipofectamine PLUSTM (Invitrogen). After 24 h post-transfection, cells were incubated in Krebs buffer (120 μ M KH₂PO₄, 2.5 mM NaHCO₃, pH 7.0, 11.8 mM NaCl, 0.5 mM KCl, 1.1 mM glucose, 0.12 mM MgSO₄ and 0.12 mM CaCl₂) for 15 min. Cells were treated with 500 μ M 3-isobutyl-1-methyl-xanthine for 5 min, and then treated with 10 μ M forskolin for a further 10 min. The reactions were stopped by 0.5 M perchloric acid. The cAMP levels were measured using a cAMP SPA screening kit (Amersham Biosciences) following the manufacturer's instructions.

PTX (pertussis toxin)-catalysed ADP ribosylation

PTX-catalysed ADP ribosylation was conducted by the method of Carty [29]. CHO-K1 cells were transfected with either empty pcDNA3.1 (+) vector, wild-type $G_{\alpha_{i1}}$, C214A or C214W in pcDNA3.1 using Lipofectamine PLUSTM. Cell-membrane fractions were prepared 24 h after the transfection as described previously [30]. Cell membranes were re-suspended in buffer containing 10 mM Hepes, pH 8.0, 1 mM DTT, 1 mM EDTA, 10 mM thymidine and 5 μ M [³²P]NAD. Reactions were started by adding 0.1 μ g of activated PTX. The reactions were stopped after 1 h at 30 °C by adding 4 $\times$ SDS sample buffer and boiled for 5 min. The [³²P]ADP-ribosylated $G_{\alpha_{i1}}$ proteins were visualized by autoradiography following SDS/PAGE.

Calculations of ground- and transition-state energies using MOE (Molecular Operating Environment) software

Dynamics calculations were performed on $G_{\alpha_{i1}}$ complexed with either the non-hydrolysable analogue GTP[S] (ground state) [13] or GDP-AlF₄⁻ (transition state) [13]. Three dynamics calculations were run individually on C214A, C214W and wild-type $G_{\alpha_{i1}}$ protein for both the ground-state and transition-state complexes. The dynamics calculations were run under the following conditions: temperature, 300 K; time step, 0.001 ps; 120 000 equilibrium steps and 120 000 averaging steps. During the dynamics run the switch region residues were allowed to move (Val-34–Ala-59, Pro-169–Glu-216), and the outer protein and the ligands (GTP[S] or GDP-AlF₄⁻) were held fixed. At various points during the dynamics run (every 10 000 steps after equilibration, 12 geometries in all) the interaction energy of residue 214 with the rest of the protein ($E_{\text{int},i}$) and the residue 214 strain energy ($E_{\text{strain},i}$) were determined. The binding energy of residue 214 to the protein ($E_{\text{bind},i}$) was then evaluated as the sum of the interaction and strain terms.

At the end of the dynamics run, the average residue 214 binding energy was determined as:

$$\langle E_{\text{bind}} \rangle = \sum_{i=1}^{12} E_{\text{bind},i} \quad (1)$$

where $i=1-12$ specifies each of 12 geometries pulled from the dynamics run after equilibration (every 10 000 steps of a

120 000-step run). The energy barriers were determined individually for wild-type protein, C214A and C214W mutants. The barrier heights were calculated as:

$$\Delta E_{\text{barrier}} = \langle E_{\text{bind}}(\text{TST}) \rangle - \langle E_{\text{bind}}(\text{GS}) \rangle$$

where TST indicates the transition state and GS the ground state. Also, the root-mean-square deviation of each of the mutant proteins from the wild-type Cys-214 structure was determined at the last step of the dynamics run.

Miscellaneous methods

GST fusion protein pull-down was carried out as described previously [27]. Western-blot analysis using ECL (Amersham Biosciences) was accomplished following the manufacturer's instructions.

RESULTS

Mutation of Cys-214 of G_{i1} impaired its endogenous GTPase activity

To examine the role of Cys-214 in G_{i1} endogenous GTPase activity, we substituted the Cys-214 with alanine (C214A). We also mutated Cys-214 into tryptophan (C214W), since it has similar hydrophobic properties to alanine, but has the largest average mass and accessible surface of all the amino acids. The GTPase activities of G_{i1} C214A and G_{i1} C214W, as well as of the wild-type G_{i1} proteins, were compared in single-turnover GTPase assays. As shown in Figure 1(A), the wild-type G_{i1} protein indicated an intrinsic basal GTPase activity with an observed rate constant K_{obs} of 0.591 s^{-1} at 0°C , whereas the GTPase activities of C214A and C214W were too low to be detected under the same conditions. When the assay temperature was increased to 30°C (Figure 1B), the GTPase activities of both mutant proteins become detectable. The K_{obs} values for GTP hydrolysis by C214A and C214W (≈ 0.02 and 0.0194 min^{-1} respectively) were ≈ 30 -fold lower than that of G_{i1} wild-type ($\approx 0.63 \text{ min}^{-1}$) at this temperature.

To confirm that the C214A and C214W are GTPase-deficient mutants, tryptic proteolysis of the protein was pursued. This method utilizes the phenomenon that the patterns of the GDP- and GTP-bound forms of G_{i1} subjected to trypsin digestion are distinctly different [31]. The GTP-bound α subunits are cleaved specifically near the N-terminus and render a $\approx 39 \text{ kDa}$ fragment ($\approx 2 \text{ kDa}$ shorter than the wild-type G_{i1}), while GDP-bound α subunits are fully hydrolysed by trypsin to smaller fragments and no 39 kDa fragment accumulates. Therefore the GDP- or GTP-bound state of the α subunit can be easily determined by the accumulation of the 39 kDa fragment after digestion by trypsin [31]. As expected, tryptic proteolysis of GDP-treated wild-type G_{i1} did not result in a 39 kDa fragment (Figure 2, lane 2). GTP-treated wild-type G_{i1} also did not produce a 39 kDa band following digestion (Figure 2, lane 3), since the normal endogenous GTPase in G_{i1} sufficiently hydrolysed the GTP to GDP. However, the GTP[S]- or AlF_4^- -treated G_{i1} resulted in the detection of a 39 kDa fragment (Figure 2, lanes 4 and 5), since both agents can stabilize the protein in an active GTP-bound conformation. In contrast to the wild-type G_{i1} , significant amounts of a 39 kDa fragment accumulate in the C214A and C214W mutants following incubation with GTP (Figure 2, lane 3), indicating that the endogenous GTPase of both mutant G_{i1} are diminished and can not hydrolyse GTP as efficiently as the wild-type. Furthermore, both C214A and C214W mutants are also fully protected by GTP[S], but not by AlF_4^- , suggesting that AlF_4^- either does not

Figure 1 GTPase activity of wild-type, C214A and C214W mutants of G_{i1} in single-turnover GTPase assays

(A) GTPase assay at 0°C . The individual 500 nM [^{32}P]GTP-bound protein was added with 10 mM MgCl_2 and reactions stopped at the indicated times (0, 20, 40, 60, 90, 120 and 180 s). The released ^{32}P -labelled free phosphates were determined at the different time points. (B) GTPase assay at 30°C . Data are presented as the percentage of the released ^{32}P -labelled free phosphates from the maximum of [^{32}P]GTP. The data shown here represent at least three individual experiments.

bind to or cannot activate these G_{i1} mutants. Interestingly, the tryptic digestion patterns of C214A and C214W mutants are similar to that of Q204L mutant of G_{i1} , a well-known GTPase-deficient mutant [28].

G_{i1} C214A and C214W mutations bind GTP[S] at a similar rate to the wild-type G_{i1}

To determine whether guanine nucleotide-binding activity was changed in C214A and C214W mutants, the wild-type and mutant G_{i1} proteins were incubated with GTP[S] in the presence of Mg^{2+} and their GTP[S]-binding activities were measured. The results showed that the three proteins bound GTP[S] to a similar level at equilibrium (Figure 3). The K_{obs} values of GTP[S] binding to the wild-type and the mutant G_{i1} proteins were similar and varied from 0.014 to 0.02 min^{-1} . These K_{obs} values are quite consistent

Figure 2 Tryptic proteolysis of wild-type and mutant forms of $G\alpha_{i1}$

Wild-type (wt) or mutant $G\alpha_{i1}$ was incubated with either GDP (100 μ M), GTP (100 μ M), GTP[S] (100 μ M) or AlF_4^- (10 mM NaF + 100 μ M $AlCl_3$) and trypsin where indicated. Digestion products were separated by SDS/PAGE and visualized in a Western blot using a monoclonal antibody against the $G\alpha_{i1}$.

with the previous reports of the recombinant $G\alpha_{i1}$ proteins [28]. The result indicated that the mutations at Cys-214 did not perturb the active site or the binding mode of the GTP[S] and thus further imply that C214A and C214W mutations may not cause improper folding or general dysfunction of the protein.

C214A and C214W inhibit forskolin-stimulated adenylate cyclase activity in CHO-K1 cells

Negative regulation of the effector enzyme, adenylate cyclase, by $G\alpha_{i1}$ has been well demonstrated [32]. Transient transfection with

Figure 3 Determination of [35 S]GTP[S] binding kinetics of the wild-type and mutant $G\alpha_{i1}$ proteins

Proteins were incubated with [35 S]GTP[S] (GTPgammaS) for indicated times (0, 10, 20, 30, 60, 90, 120, 150 and 180 min). Data were normalized to the binding activity at 180 min, which represented the maximum binding. The observed rate constants (K_{obs}) for each protein were calculated using GraphPad Prism2 based on a one-phase exponential association equation $Y = Y_{max}[1 - \exp(-KX)]$.

Figure 4 Effects of wild-type and mutant $G\alpha_{i1}$ proteins on cAMP accumulation in CHO-K1 cells

CHO-K1 cells were transfected individually with either empty vector, wild type (wt), C214A, C214W, Q204L or R178C $G\alpha_{i1}$ plasmids. 24 h post-transfection, cells were treated with or without forskolin (10 μ M) for 10 min. Levels of cAMP were measured using the cAMP SPA Screening Assay Kit (Amersham Biosciences). Data shown represent means $\pm$ S.E. from triplicate measurements from three independent experiments. * $P < 0.05$, significantly different from the untransfected control.

the constitutively active Q204L mutant of $G\alpha_i$ has been previously reported to substantially decrease cAMP production in cells [9]. Detecting inhibition of adenylate cyclase by $G\alpha_i$ requires prior activation of the cyclase either by stimulation of $G\alpha_s$ or treatment by forskolin [33]. Since C214A and C214W hydrolyse GTP extremely poorly, we would expect that these mutants demonstrate constitutive activity to inhibit adenylate cyclase activity in cells. As shown in Figure 4, treatment of cells with forskolin (10 μ M) caused ≈ 4 -fold increases in cAMP accumulation in cells compared with that of untreated cells. Expression of exogenous wild-type $G\alpha_{i1}$ did not significantly affect both forskolin-treated and -untreated cAMP levels. However, overexpression of $G\alpha_{i1}$ C214A or C214W mutants resulted in ≈ 22 –31% inhibition of the forskolin-stimulated cAMP accumulation in cells. As positive controls, $G\alpha_{i1}$ Q204L and R178C mutants inhibited almost 40% of the forskolin response in cAMP production (Figure 4). The overnight treatment with PTX (100 ng/ml) did not significantly affect the inhibition of adenylate cyclase by these $G\alpha_i$ mutants (results not shown). Western-blot analysis confirmed that expression of the wild-type, C214A, C214W and Q204L $G\alpha_{i1}$ in transfected CHO-K1 cells were quantitatively similar (results not shown).

C214A and C214W still bind to $\beta\gamma$ subunits

To determine whether $G\alpha_{i1}$ C214A and C214W still retain the capacity to interact with $\beta\gamma$ subunits, we examined the modification of $G\alpha_{i1}$ mutants by ADP ribosylation with PTX. $G\alpha_{i1}$ is a substrate for PTX only when bound to $\beta\gamma$ subunits as a heterotrimer [34]. The results indicated that both $G\alpha_{i1}$ C214A and C214W mutants were still good substrates for PTX, although a little less than that observed for wild-type $G\alpha_{i1}$ (Figure 5). Overall, these results suggest that a reasonable fraction of the C214A and C214W mutants expressed in CHO cells can still bind GDP and associate with $\beta\gamma$ subunits. This conclusion is consistent with previous observations that both the wild-type and the C215A mutant of $G\alpha_o$ had similar binding activities to $\beta\gamma$ subunits in a sucrose-gradient-sedimentation analysis [26].

Figure 5 PTX-catalysed ADP ribosylation of wild-type and mutant G_{i1}

Indicated amounts (5, 10 and 20 μ g) of membrane proteins from CHO-K1 cells expressing either wild-type (wt) or mutant G_{i1} were incubated with PTX and [32 P]NAD as described in the Experimental section. [32 P]ADP-ribose incorporation into the G_{i1} proteins was evaluated by SDS/PAGE and autoradiography with 24 h exposure.

Figure 6 *In vitro* GST pull-down assay for wild-type and mutant G_{i1} with RGS4

GST–RGS4 protein (2 μ g) was individually incubated with 2 μ g of wild-type (wt), C214A or C214W G_{i1} protein in the absence or presence of GDP (100 μ M), GTP (100 μ M), GTP[S] (100 μ M) or AlF_4^- (10 mM NaF + 100 μ M $AlCl_3$) for 4 h at 4 °C. The wild-type or mutants of G_{i1} that bound to GST–RGS4 were pulled down by glutathione–agarose beads, separated by SDS/PAGE and visualized by Western blotting using a monoclonal antibody against G_{i1} protein.

C214A and C214W mutants cannot bind to RGS4 protein

We were also interested in determining the capacity of G_{i1} C214A and C214W to interact with the RGS4, a GTPase-accelerating protein for G_{i1} . Since RGS4 binds only to the transition state of G_{i1} [35], the binding activity of G_{i1} to RGS4 may therefore reflect the capacity of mutant G_{i1} to convert to a transition state. The results of an *in vitro* GST fusion protein pull-down assay are shown in Figure 6. A GST–RGS4 fusion protein was attached to glutathione–agarose beads and incubated individually with wild-type G_{i1} , C214A or C214W protein under different conditions. As shown in Figure 6, RGS4 bound to wild-type G_{i1} only in the presence of AlF_4^- (Figure 6, lane 5, top

Table 1 Residue 214 binding free energies (kcal/mol) for ground and excited state complexes

Ground-state structure, 1GIA.pdb [13]; transition-state structure, 1GFI.pdb [13]. RMS refers to root-mean-square deviation of the mutant complexes relative to the wild-type complex at the end of the dynamics run.

Step	Binding free energy* (kcal/mol)					
	Ground state (residue 214 $E_{bind,i}$)			Transition state (residue 214 $E_{bind,i}$)		
	Wild type	C214A	C214W	Wild type	C214A	C214W
1	–15.99	–17.34	–16.82	–19.68	–7.65	–11.24
1×10^5	–17.77	–14.28	–22.23	–28.17	–12.47	–10.83
2×10^5	–19.84	–18.11	–25.65	–19.77	–14.17	–8.76
3×10^5	–22.04	–13.07	–27.07	–12.95	–9.74	–8.50
4×10^5	–18.01	–15.91	–14.64	–15.74	–6.33	–8.96
5×10^5	–25.02	–18.02	–8.47	–21.18	–16.08	–6.51
6×10^5	–13.98	–19.40	–11.95	–17.02	–11.02	–11.41
7×10^5	–14.50	–21.68	–14.14	–20.02	–8.13	–12.09
8×10^5	–22.82	–14.40	–11.96	–17.13	–15.20	–10.59
9×10^5	–24.97	–13.72	–11.54	–22.08	–15.44	–23.51
10×10^5	–20.72	–13.51	–10.27	–16.55	–10.68	–11.73
11×10^5	–23.41	–14.84	–14.54	–15.89	–16.57	–13.23
12×10^5	–21.92	–13.20	–19.74	–15.21	–9.92	–1.22
Average†	–20.08	–15.96	–16.08	–18.57	–11.80	–10.66
$\Delta E_{barrier}^\ddagger$	–	–	–	+1.51	+4.16	+5.42
RMS	–	0.322	0.343	–	0.450	0.448

* $E_{bind,i} = E_{int,i} + E_{strain,i}$.

† See eqn (1).

‡ $\Delta E_{barrier} = \langle E_{bind}(TST) \rangle - \langle E_{bind}(GS) \rangle$.

panel) which induces a transition state of the protein. However, RGS4 did not bind to C214A (Figure 6, middle panel) and C214W (Figure 6, lower panel) mutants under any conditions tested, not even in the presence of AlF_4^- (Figure 6, lane 5). This is in agreement with our previous observation that neither C214A nor C214W of G_{i1} were activated by AlF_4^- (Figure 2). Consistently, we observed that the diminished GTPase activities of C214A and C214W could not be rescued by RGS4 protein in single-turnover GTPase assays (results not shown).

Mutation of Cys-214 into Ala or Trp dramatically increases the energy barriers for the GTP hydrolysis

To explore the mechanism of C214A and C214W mutations in the abolishment of GTPase activity, dynamics calculations were first performed on the transition-state structure of G_{i1} complexed with GDP and AlF_4^- [13]. It was found that the geometries of the transition-state structures of G_{i1} mutant were virtually identical to that of wild-type G_{i1} (root mean square < 0.5 Å; see last row of Table 1). Thus the loss in GTPase activity of the mutant proteins could not be explained in terms of structural changes in the catalytic site.

To explore the possibility that the loss in GTPase activity of mutants may be due to changes locally to the Cys-214 site, attention was focused on the binding energy of residue 214 (Cys, Ala or Trp) to the protein in the transition state. These binding energies are given in Table 1. It was found that the binding energy of Cys in wild-type G_{i1} (–18.57 kcal/mol) was significantly lower than that of Ala in C214A (–11.80 kcal/mol), or Trp in C214W (–10.66), implying that mutation of Cys-214 to either Ala or Trp may energetically destabilize the G_{i1} –GDP transition-state complex.

To further obtain the activation energy barriers (height) required for the GTP hydrolysis, dynamics calculations were performed

also on the ground state of $G\alpha_{i1}$ complexed with the non-hydrolysable analogue GTP[S] (Table 1). The ground-state binding energies were then subtracted from the transition-state binding energies to obtain activation energies or barrier heights (Table 1, $\Delta E_{\text{barrier}}$). The calculations show that the activation energy heights for GTP hydrolysis are significantly larger for both mutant proteins (4.16 kcal/mol for C214A and 5.42 kcal/mol for C214W) than that for the wild-type protein (1.51 kcal/mol). These results indicate that the loss in GTPase activity of C214A and C214W mutants relative to the wild type is most probably due to an energetic destabilization of the $G\alpha_{i1}$ –GDP transition state relative to the $G\alpha_{i1}$ –GTP ground state.

DISCUSSION

The Cys-214 in $G\alpha_{i1}$ is one of the most conserved residues located in the α_2 helix of the Switch II region, which is known to undergo a dramatic conformational change upon shifting from the GTP- to the GDP-bound form during hydrolysis. Thomas et al. [26] reported previously that the conserved Cys-215 in $G\alpha_o$ (analogous to Cys-214 in $G\alpha_{i1}$) can be specifically crosslinked to $\beta\gamma$ subunits by 1,6-bis(maleimido)hexane. However, it is still not clear what function this conserved cysteine has in GTP hydrolysis. Here we report that mutations of Cys-214 in $G\alpha_{i1}$ to either Ala or Trp caused a significant reduction of the intrinsic GTPase activity. These GTPase-deficient mutants constitutively inhibit adenylate cyclase activity in CHO cells. However, mutations of Cys-214 in the $G\alpha_{i1}$ do not affect their capability to bind to GTP[S] and $\beta\gamma$ subunits, but do abolish the interaction with RGS4 protein, a GAP (GTPase-activating protein) for $G\alpha_{i1}$.

It is still elusive how the GTPase activity of the $G\alpha_{i1}$ is impaired by the Cys-214 mutations. The mutations of Cys-214 into Ala or Trp apparently do not cause an overall disruption of the functional structure of $G\alpha_{i1}$. C214A and C214W still bind well to GTP[S] and $\beta\gamma$ subunits (Figures 3 and 5), and functionally interact with their effector, adenylate cyclase (Figure 4). We also found that AlF_4^- can induce a significant change of Trp fluorescence on C214W mutant, implying that the mutation of Cys-214 may not abolish its binding activity to AlF_4^- (results not shown). The involvement of cysteine residue in the catalysis of GTP hydrolysis has been proposed in the tubulin GTPase [36]. A possible mechanism involves a thiol group from the cysteine in the GTP hydrolysis. This thiol group may attack the γ phosphate of GTP by transferring a proton to the phosphoryl oxygen and hydrolysing the thiophosphoryl intermediate [36]. However, so far there is no evidence suggesting that the cysteine residue contributes directly to the catalysis of the GTP hydrolysis in $G\alpha$ subunits. In the crystal structure of the $G\alpha_{i1}$ bound to GDP– AlF_4^- , Cys-214 does not contact with either GTP or the water molecule [37]. Actually, in this model, Cys-214 is located in a distance of ≈ 16 Å to the guanine nucleotide-binding site and is obviously not directly involved in the GTP binding and water nucleophile attack. There is also no evidence suggesting that the Cys-214 forms a disulphide bond with other internal cysteines of $G\alpha_{i1}$ to maintain a structural integrity that may be required for the GTP hydrolysis. The GTPase activity of $G\alpha_{i1}$ is not significantly affected by the reducing agent DDT. The thiol group of Cys-215 in $G\alpha_o$ is chemically available to link with 1,6-bis(maleimido)hexane in the physiological buffer with DTT [26].

Several observations suggest that the C214A and C214W mutations may exert their effects on GTP hydrolysis by blocking the transition state of $G\alpha_i$. This is supported herein by an observation that C214A and C214W mutants cannot convert to the transition state activated by the AlF_4^- (Figure 2). AlF_4^- can

mimic the γ phosphate in its pentavalent transition state during hydrolysis and thus stabilize the $G\alpha$ subunit in the transition state [13]. Furthermore, both C214A and C214W mutants fail to bind RGS4 protein, even in the presence of AlF_4^- . This is in line with previous evidence that RGS4 binds only to the transition state (AlF_4^- -bound) of $G\alpha_{i1}$, and does not bind to GDP-, GTP- or GTP[S]-bound $G\alpha_{i1}$ [35]. Consistently, the defective GTPase activities of C214A and C214W cannot be stimulated by RGS4 (results not shown), and is in striking contrast to that of another GTPase-deficient mutant, R178C. The Arg-178 is not critically required in the stabilization of transition state of $G\alpha_{i1}$, and thus its defective GTPase can be mostly rescued by RGS4 [38]. Interestingly, characteristics of the C214A and C214W are strikingly similar to that of G204L mutant in $G\alpha_{i1}$, in that the Q204L mutant's diminished GTPase activity cannot be rescued by RGS4 [28]. This implies that both Gln-204 and Cys-214 mutants may share the same mechanism in their defective GTPase activity. The essential role of Gln-204 in the GTP hydrolysis by $G\alpha$ subunits has been well documented. Gln-204 performs a dramatic re-orientation induced by GDP– AlF_4^- at the catalytic centre, and appears to be the major residue capable of interacting with GTP and nucleophilic water in the transition state of $G\alpha$ subunits [13–15]. Previously, Gln-204 had been speculated upon to act as a general base to deprotonate the water nucleophile. However, more recent evidence suggests that Gln-204 is involved in stabilizing the transition state for GTP hydrolysis. In the crystal structure of $G\alpha_{i1}$ complex with GDP– AlF_4^- , Gln-204 has significant changes from its ground-state position in the GTP structure to a position in which its amino group stabilizes the negative character on the equatorial oxygen of the transition state and its carbamoyl oxygen stabilizes the attacking nucleophilic water [39].

To explore the mechanism of C214A and C214W mutations in the abolition of GTPase activity, dynamics-based computer models derived from the published X-ray crystal structures of $G\alpha_{i1}$ with either GTP[S] (ground state) or GDP– AlF_4^- (transition state) were calculated. No significant differences in the conformations between the mutants and the wild-type protein were found in the ground state or transition state. However, the activation energy barriers for the GTP hydrolysis in the case of C214A and C214W mutants were calculated to be ≈ 2.75 – 3.5 -fold higher than that of wild-type $G\alpha_{i1}$. This indicates destabilization of the mutant transition-state structures relative to the wild-type and therefore blockage of GTP hydrolysis.

In summary, we reported herein the characterization of new constitutively active mutations of $G\alpha_{i1}$, C214A or C214W, which shows defective endogenous GTPase activity. Neither mutation causes general disruption of the protein conformation and both interact well with $\beta\gamma$ subunits and adenylate cyclase. We propose that these mutants abolished their endogenous GTPase activity by destabilizing formation of the transition state during GTP hydrolysis.

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REFERENCES

- Gilman, A. G. (1987) G proteins: transducers of receptors-generated signals. *Annu. Rev. Biochem.* **56**, 615–649.
- Neer, E. J. (1994) G proteins: critical control points for transmembrane signals. *Protein Sci.* **3**, 3–14.
- Sprang, S. R. (1997) G protein mechanisms: Insights from structural analysis. *Annu. Rev. Biochem.* **66**, 639–678.
- Freissmuth, M. and Gilman, A. G. (1989) Mutations of $G_{s\alpha}$ designed to alter the reactivity of the protein with bacterial toxins. Substitutions at ARG187 result in loss of GTPase activity. *J. Biol. Chem.* **264**, 21907–21914.

- 5 Hoshino, S.-I., Kikkawa, S., Takahashi, K., Itoh, H., Kaziro, Y., Kawasaki, H., Suzuki, K., Katada, T. and Ui, M. (1990) Identification of sites for alkylation by N-ethylmaleimide and pertussis toxin-catalyzed ADP-ribosylation on GTP-binding proteins. *FEBS Lett.* **276**, 227–231
- 6 Katada, T. and Ui, M. (1982) Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3129–3133
- 7 Van Dop, C., Tsubokawa, M., Bourne, H. and Ramachandran, J. (1984) Amino acid sequence of retinal transducin at the site ADP-ribosylated by cholera toxin. *J. Biol. Chem.* **259**, 696–698
- 8 Graziano, M. P., Freissmuth, M. and Gilman, A. G. (1991) Purification of recombinant $G_{s\alpha}$. *Methods Enzymol.* **195**, 192–202
- 9 Hermouet, S., Merendino, Jr, J., Gutkind, J. and Spiegel, A. (1991) Activating and inactivating mutations of the α subunit of G_{12} protein have opposite effects on proliferation of NIH 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10455–10459
- 10 Lee, E., Taussig, R. and Gilman, A. G. (1992) The G226A mutant of $G_{s\alpha}$ highlights the requirement for dissociation of G protein subunits. *J. Biol. Chem.* **267**, 1212–1218
- 11 Masters, S., Miller, R., Chi, M., Chang, F., Beiderman, B., Lopez, N. and Bourne, H. (1989) Mutations in the GTP-binding site of $G_{s\alpha}$ alter stimulation of adenylyl cyclase. *J. Biol. Chem.* **264**, 15467–15474
- 12 Osawa, S. and Johnson, G. (1991) A dominant negative $G\alpha_s$ mutant is rescued by secondary mutation of the α chain amino terminus. *J. Biol. Chem.* **266**, 4673–4676
- 13 Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G. and Sprang, S. R. (1994) Structures of active conformations of $G_{i1\alpha}$ and the mechanism of GTP hydrolysis. *Science* **265**, 1405–1412
- 14 Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E. and Sigler, P. B. (1996) The 2.0 Å crystal structure of a heterotrimeric G protein. *Nature (London)* **379**, 311–319
- 15 Mixon, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G. and Sprang, S. R. (1995) Tertiary and quaternary structural changes in $G_{i1\alpha}$ induced by GTP hydrolysis. *Science* **270**, 954–960
- 16 Noel, J. P., Hamm, H. E. and Sigler, P. B. (1993) The 2.2 Å crystal structure of transducin- α complexed with GTP- γ S. *Nature (London)* **366**, 654–663
- 17 Apanovitch, D. M., Iiri, T., Karasawa, T., Bourne, H. R. and Dohlman, H. G. (1998) Second site suppressor mutations of a GTPase-deficient G-protein α -subunit. Selective inhibition of $G\beta\gamma$ -mediated signaling. *J. Biol. Chem.* **273**, 28597–28602
- 18 Posner, B. A., Mixon, M. B., Wall, M. A., Sprang, S. R. and Gilman, A. G. (1998) The A326S mutant of $G_{i1\alpha}$ as an approximation of the receptor-bound state. *J. Biol. Chem.* **273**, 21752–21758
- 19 Slepak, V., Quick, M., Aragay, A., Davidson, N., Lester, H. and Simon, M. (1993) Random mutagenesis of G protein α subunit $G_{o\alpha}$. Mutations altering nucleotide binding. *J. Biol. Chem.* **268**, 21889–21894
- 20 Denker, B., Neer, E. and Schmidt, C. (1992) Mutagenesis of the amino terminus of the α subunit of the G protein G_o . *In vitro* characterization of α_o and $\beta\gamma$ interactions. *J. Biol. Chem.* **267**, 6272–6277
- 21 Lan, K.-L., Sarvazyan, N. A., Taussig, R., Mackenzie, R. G., DiBello, P. R., Dohlman, H. G. and Neubig, R. R. (1998) A point mutation in $G\alpha_o$ and $G\alpha_{i1}$ blocks interaction with regulator of G protein signaling proteins. *J. Biol. Chem.* **273**, 12794–12797
- 22 Raw, A. S., Coleman, D. E., Gilman, A. G. and Sprang, S. R. (1997) Structural and biochemical characterization of the GTP- γ S-, GDP-Pi-, and GDP-bound forms of a GTPase-deficient Gly42 $\rightarrow$ Val mutant of $G_{i1\alpha}$. *Biochemistry* **36**, 15660–15669
- 23 Kleuss, C., Raw, A. S., Lee, E., Sprang, S. R. and Gilman, A. G. (1994) Mechanism of GTP hydrolysis by G-protein α subunits. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9828–9831
- 24 Ueda, N., Iniguez-Lluhi, J. A., Lee, E., Smrcka, A. V., Robishaw, J. D. and Gilman, A. G. (1994) G protein $\beta\gamma$ subunits. Simplified purification and properties of novel isoforms. *J. Biol. Chem.* **269**, 4388–4395
- 25 Garcia-Higuera, I., Thomas, T. C., Yi, F. and Neer, E. J. (1996) Intersubunit surfaces in G protein $\alpha\beta\gamma$ heterotrimers. *J. Biol. Chem.* **271**, 528–535
- 26 Thomas, T., Schmidt, C. and Neer, E. (1993) G-protein α_o subunit: mutation of conserved cysteines: identifies a subunit contact surface and alters GDP affinity. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10295–10299
- 27 Wang, Y., Ho, G., Zhang, J. J., Nieuwenhuijsen, B., Edris, W., Chanda, P. K. and Young, K. H. (2002) Regulator of G protein signaling Z1 (RGSZ1) interacts with $G\alpha_i$ subunits and regulates $G\alpha_i$ -mediated cell signaling. *J. Biol. Chem.* **277**, 48325–48332
- 28 Linder, M. E., Ewald, D. A., Miller, R. J. and Gilman, A. G. (1990) Purification and characterization of $G_{o\alpha}$ and three types of $G_{i\alpha}$ after expression in *Escherichia coli*. *J. Biol. Chem.* **265**, 8243–8251
- 29 Carty, D. J. (1994) Pertussis toxin-catalyzed ADP-ribosylation of G proteins. *Methods Enzymol.* **237**, 63–71
- 30 Wang, Y., Windh, R. T., Chen, C. A. and Manning, D. R. (1999) N-Myristoylation and $\beta\gamma$ play roles beyond anchorage in the palmitoylation of the G protein α_o subunit. *J. Biol. Chem.* **274**, 37435–37442
- 31 Fung, B. and Nash, C. (1983) Characterization of transducin from bovine retinal rod outer segments. II. Evidence for distinct binding sites and conformational changes revealed by limited proteolysis with trypsin. *J. Biol. Chem.* **258**, 10503–10510
- 32 Wong, Y. H., Federman, A., Pace, A. M., Zachary, L., Evans, T., Pouyssegur, J. and Bourne, H. R. (1991) Mutant α subunits of G_{12} inhibit cyclic AMP accumulation. *Nature (London)* **351**, 63–65
- 33 Taussig, R., Iniguez-Lluhi, J. A. and Gilman, A. G. (1993) Inhibition of adenylyl cyclase by $G_{i\alpha}$. *Science* **261**, 218–221
- 34 Neer, E., Lok, J. and Wolf, L. (1984) Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. *J. Biol. Chem.* **259**, 14222–14229
- 35 Berman, D. M., Kozasa, T. and Gilman, A. G. (1996) The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *J. Biol. Chem.* **271**, 27209–27212
- 36 Mejillano, M. R., Shivanna, B. D. and Himes, R. H. (1996) Studies on the nocodazole-induced GTPase activity of tubulin. *Arch. Biochem. Biophys.* **336**, 130–138
- 37 Coleman, D. E., Lee, E., Mixon, M. B., Linder, M. E., Berghuis, A. M., Gilman, A. G. and Sprang, S. R. (1994) Crystallization and preliminary crystallographic studies of $G_{i1\alpha}$ and mutants of $G_{i1\alpha}$ in the GTP and GDP-bound states. *J. Mol. Biol.* **238**, 630–634
- 38 Berman, D. M., Wilkie, T. M. and Gilman, A. G. (1996) GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein α subunits. *Cell* **86**, 445–452
- 39 Coleman, D. E. and Sprang, S. R. (1999) Structure of $G_{i1\alpha}$ -GppNHp, autoinhibition in a $G\alpha$ protein-substrate complex. *J. Biol. Chem.* **274**, 16669–16672

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