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Motion of Carboxyl Terminus of Gα Is Restricted upon G Protein Activation:

A SOLUTION NMR STUDY USING SEMISYNTHETIC Gα SUBUNITS*

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Abstract

The carboxyl terminus of the G protein α subunit plays a key role in interactions with G protein-coupled receptors. Previous studies that have incorporated covalently attached probes have demonstrated that the carboxyl terminus undergoes conformational changes upon G protein activation. To examine the conformational changes that occur at the carboxyl terminus of Gα subunits upon G protein activation in a more native system, we generated a semisynthetic Gα subunit, site-specifically labeled in its carboxyl terminus with ^{13}C amino acids. Using expressed protein ligation, 9-mer peptides were ligated to recombinant $G\alpha_{i1}$ subunits lacking the corresponding carboxyl-terminal residues. In a receptor-G protein reconstitution assay, the truncated $G\alpha_{i1}$ subunit could not be activated by receptor; whereas the semisynthetic protein demonstrated functionality that was comparable with recombinant $G\alpha_{i1}$. To study the conformation of the carboxyl terminus of the semisynthetic G protein, we applied high resolution solution NMR to Gα subunits containing ^{13}C labels at the corresponding sites in $G\alpha_{i1}$: Leu-348 (uniform), Gly-352 (α carbon), and Phe-354 (ring). In the GDP-bound state, the spectra of the ligated carboxyl terminus appeared similar to the spectra obtained for ^{13}C-labeled free peptide. Upon titration with increasing concentrations of AlF_4^-, the ^{13}C resonances demonstrated a marked loss of signal intensity in the semisynthetic Gα subunit but not in free peptide subjected to the same conditions. Because AlF_4^- complexes with GDP to stabilize an activated state of the Gα subunit, these results suggest that the Gα carboxyl terminus is highly mobile in its GDP-bound state but adopts an ordered conformation upon activation by AlF_4^-.

Heterotrimeric G proteins mediate signal transduction between G protein-coupled receptors (GPCRs)[1] and a host of downstream intracellular effectors. The α subunit binds GDP and forms a tight complex with βγ subunits. Activated GPCRs can catalyze the exchange of GDP for

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[1]The abbreviations used are: GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; EPL, expressed protein ligation; HSQC, heteronuclear single quantum correlation; CBD, chitin-binding domain; Fmoc, fluorenylmethoxycarbonyl; CHAPS, 3-[(3-chol-amidopropyl)dimethylammonio]-1-propanesulfonic acid.

GTP, which leads to dissociation of Gα from the complex. The structure of the Gα subunit has been solved bound to GDP (1), GTPγS (2), GDP-AlF_4^- (3), and in complex with Gβγ (4). Gα has two domains, a GTPase domain that is similar to the Ras-like GTPase proteins and an amino-terminal helical domain that is unique to the heterotrimeric G proteins. The GTPase domain consists of five helices surrounding a six-stranded β-sheet, whereas the helical domain has one long helix surrounded by five short helices. In the GTPase domain, GDP to GTP exchange results in conformational changes at three regions near the guanine nucleotide binding pocket, referred to as switch I (11 amino acids), switch II (21 amino acids), and switch III (10 amino acids) (1). The switch regions of Gα subunits either bind to βγ subunits or to downstream effectors, such as adenylyl cyclase and phospholipases, with the specificity of the Gα interactions dictated by GTP exchange and the conformation of the switch regions.

Although the crystal structures reveal the conformational changes in the switch regions and thus provide a structural basis for the specificity of interactions of the GDP-bound *versus* GTP-bound Gα subunits, the structures of Gα subunits and heterotrimeric G proteins provide little information regarding how receptors catalyze GTP exchange. The α subunit of transducin, $G\alpha_t$, has been shown by mutational studies to contain three regions critical for rhodopsin interaction: 1) the amino-terminal 23 residues, 2) an internal sequence consisting of positions 311–329, and 3) the carboxyl-terminal eleven residues, 340–350 (reviewed in Ref. 5). When comparing the heterotrimeric structures *versus* the activated GTPγS-Gα structures, the internal sequence (311–329) does not change conformation significantly in the GDP-*versus* GTP-bound states, and the amino and carboxyl termini are not resolved in most of the structures. The structure of the carboxyl terminus of Gα is of particular interest, because this region confers receptor-specific interactions, and peptide analogs of the tail bind to rhodopsin and stabilize the MII activation state of the receptor. In most structures of G proteins, the carboxyl terminus is not resolved, suggesting that this region is relatively mobile. However, in three independent crystal structures, a structured carboxyl terminus is observed, although the conformations differ. In the structure of RGS 4 bound to AlF_4^--activated $G\alpha_{i1}$ (6), the carboxyl terminus adopts an ordered continuous helix terminated by a carboxyl-terminal capping motif. Of note, this structure is consistent with transferred nuclear Overhauser effect structures of carboxyl-terminal peptides of $G\alpha_t$ bound to photo-activated rhodopsin (7–9). In contrast, the crystal structure of $G\alpha_t$ complexed with GTPγS shows the carboxyl-terminal residues in an extended linear structure, and in one molecule of the asymmetric unit cell, residues 343–349 are in van der Waals contact with residues 212–215 of the α2/β4 loop, which is part of the switch II region of Gα (2). In the crystal structure of $G\alpha_{i1}$ bound to GDP, the carboxyl tail forms an extended α-helical structure that is cradled between the amino terminus and the body of the GTPase domain (10). Although the crystal structures demonstrate disordered termini or different, and seemingly conflicting, structures of the carboxyl tail of Gα subunits, cross-linking and biophysical studies using fluorescent probes provide compelling evidence that the conformation of the carboxyl tail does change upon G protein activation (11,12).

What happens to the Gα structures at the receptor/G protein interface upon receptor activation? Several studies have implicated the carboxyl terminus to be involved in regulating receptor interaction and affecting GDP affinity (13–16) and GDP-GTP exchange (17,18). In addition, carboxyl-terminal peptides from several Gα subunits can both competitively inhibit G protein activation and stabilize the active conformation of G protein-coupled receptors (7,8,19–21). Taken together, these studies suggest that interactions between the Gα carboxyl terminus and GPCRs might play essential roles in communication between the receptor and the GDP binding pocket of the Gα subunit. Further understanding of these conformational changes should thus provide a better understanding of the mechanism of nucleotide exchange on α subunits of G proteins.

To date, most biophysical studies have focused either on peptide segments of the G protein or bulky fluorophores inserted by standard molecular techniques on the receptor or G proteins. Here, we use expressed protein ligation (EPL) to incorporate ^{13}C isotope-labeled amino acids into the carboxyl-terminal tail of Gα. EPL takes advantage of the ability of intein domains to generate thioester intermediates in recombinant proteins, which can then be ligated to peptides containing an amino-terminal cysteine residue. Inteins, the protein equivalents of introns, are found in prokaryotes, archaebacteria, and simple eukaryotes and possess the unique ability to autocatalytically splice themselves from newly translated proteins and, in the process, regenerate a native amide bond at the splice junction (22). By modifying the intein, a thioester intermediate can be trapped and purified for use in native chemical ligations (23). For this study, we ligated a nine-amino-acid carboxyl-terminal peptide to a recombinant Gα (1–345) thioester lacking the carboxyl terminus to create the full-length Gα (1–354). We demonstrated the ability to generate a semisynthetic Gα subunit containing ^{13}C-labeled amino acids in its carboxyl terminus that retains native Gα activity as assayed by its ability to be activated by receptors. Furthermore, we have studied the mobility of the carboxyl terminus of this semisynthetic Gα by high resolution solution NMR. The experiments show that the carboxyl terminus is highly mobile when in the inactive conformation and that binding of AlF_4^- restricts this motion.

MATERIALS AND METHODS

Peptide Synthesis

Peptides were manually synthesized by fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis. ^{13}C-labeled amino acids were obtained from Cambridge Isotope Laboratories (Andover, MA) and were treated with Fmoc-(oxy)succinimide to afford the amino terminus Fmoc protection. After the final cleavage step, crude peptides were purified by preparative high pressure liquid chromatography using a C18 column and characterized by electrospray mass spectrometry.

Cloning, Expression, and Purification of Proteins

A plasmid encoding for $His_6G\alpha_{i1}$ (pQE-6 expression vector) was kindly provided by Dr. M. Linder, Washington University, St. Louis, MO. Plasmid pBN1018, which encodes $His_6G\alpha_{i1}$ containing a N342C mutation, was generated by site-directed mutagenesis with *Pfu* Turbo DNA polymerase (Stratagene). The plasmid pBN905, which expresses $G\alpha_{i1}\Delta CT$, truncated by nine residues at its carboxyl terminus, fused in-frame to intein-CBD cDNA from the pTXB3 expression vector (New England Biolabs) was constructed as follows. Initially, a $His_6G\alpha_{i1}/\alpha_t$ chimera (Chi6, a gift from Dr. H. Bourne, UCLA; originally from Dr. H. Hamm, Vanderbilt University) was digested with PstI/BglII to generate a 3.4-kb fragment. Intein-CBD cDNA from pTXB3 was digested with PstI/SpeI and sub-cloned along with a BglII/SpeI oligonucleotide cassette into the pChi6 expression vector to generate pBN834. To generate pBN905, $pHis_6G\alpha_{i1}$ was digested with EcoRI/AatII and subcloned, along with an AatII/SpeI oligonucleotide cassette into pBN834, which had been digested with EcoRI/SpeI. This strategy resulted in an expression vector containing cDNA encoding $G\alpha_{i1}\Delta CT$ (truncated by nine residues at the carboxyl terminus) fused in-frame with intein-CBD, without the introduction of extra amino acids. Plasmids were transformed into *Escherichia coli* BL21 cells. The cells were grown in 3 liters of T7 medium (20 g of tryptone, 10 g of yeast extract, 5 g of NaCl, 2 ml of glycerol, and 50 ml of 1 M KH_2PO_4, pH 7.2, per liter) in the presence of 50 μg/ml ampicillin at 25 °C up to an A_{600} of 0.5–0.8. The cells were then induced with 50 μM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 4–6 h.

After recovery by centrifugation, the cell pellet was resuspended in 1:20 of cell culture volume with lysis buffer (25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.1 mM EDTA, 5% glycerol, 5 mM

$MgCl_2$, 50 μM GDP, 0.1 mM phenylmethylsulfonyl fluoride). The lysis buffer for $His_6G\alpha_{i1}$ and $His_6G\alpha_{i1}$N342C also contained 5 mM β-mercaptoethanol, and lysis buffer for $His_6G\alpha_{i1}$ΔCT-intein-CBD contained 0.1% Triton X-100. The cell suspension was lysed by sonication, and crude cell lysate was cleared by low speed centrifugation followed by centrifugation at 100,000 × *g* for 60 min.

His_6 purification of $His_6G\alpha_{i1}$ and $His_6G\alpha_{i1}$N342C was performed as described previously (24). Briefly, the supernatant was adjusted to 500 mM NaCl, 20 mM imidazole with the addition of 8× binding buffer (160 mM Tris HCl, pH 8.0, 4 M NaCl, and 160 mM imidazole). The resultant mixture was loaded onto two 5-ml metal chelating columns (Pharmacia Corporation) charged with 100 mM $NiCl_2$ and prepared according to the manufacturer's protocol, at a flow rate of 1 ml/min. The column was washed with 10 volumes of 1× binding buffer, and the bound protein was eluted with 5 column volumes of 1× binding buffer containing 80 mM imidazole (100 mM total). GDP, $MgCl_2$, and β-mercaptoethanol were added to each fraction at a concentration of 25 μM, 2 mM, and 5 mM, respectively. Fractions were analyzed by SDS-PAGE for protein purity, quantitated by comparison to known bovine serum albumin standards, pooled, and dialyzed overnight in dialysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM Nacl, 2 mM $MgCl_2$, 50 μM GDP, and 20% glycerol). Samples were aliquoted and stored at −80 °C. The final yield of Gα subunits was 1–2 mg/liter.

Protein Ligation

Purification of $His_6G\alpha_{i1}$ΔCT-intein-CBD was as follows. After cell lysis, the clarified cell lysate was applied to an affinity column containing chitin matrix that had been equilibrated with 10 column volumes of column buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100). The column was then washed with 20 column volumes of column buffer. Treatment with cleavage buffer (20 mM Tris-Cl, pH 8.0, and 500 mM NaCl) containing 100 mM mercaptoethanesulfonic acid overnight at 4 °C resulted in self-cleavage of the intein, releasing the $G\alpha_{i1}$ΔCT thioester from the chitin-bound intein. Fractions were eluted with 3 column volumes of column buffer (minus Triton X-100). Collected fractions were treated as above, and the $G\alpha_{i1}$ΔCT protein was stored at −80 °C and used as a control in functional assays. Expressed protein ligation of the synthetic peptides to GαΔCT-intein-CBD was carried out as follows. Following loading of the Gα-intein-CBD fusion protein onto the chitin matrix, cleavage was initiated by quickly flushing the column with cleavage buffer containing 1% mercaptoethanesulfonic acid. Synthetic peptides, corresponding to the carboxyl-terminal tail of Gα, were dissolved into cleavage buffer and applied to the chitin column in the presence of 1% mercaptoethanesulfonic acid at an excess of 10–20-fold over protein concentration. The ligation reaction proceeded at 4 °C and was found to be complete within 24 h. The column was eluted with column buffer (minus Triton X-100). The fractions were treated as above, with the exception of an additional reduction step before dialysis. In this step, the pooled fractions were treated with 15 mM dithiothreitol for 30 min at 30 °C to remove peptide linked to the Gα through nonspecific disulfide interaction. The semi-synthetic proteins were analyzed by SDS-PAGE using antibodies corresponding to the carboxyl-terminal synthetic peptide and quantitated on Coomassie-stained gels using bovine serum albumin standards. Protein was concentrated to 5 mg/ml using a Centricon centrifugal device (molecular weight cut off 10,000; Amicon). Ligation was estimated to be 50–70%.

Quantification of Ligation Efficiency

The efficiency of ligation was estimated by quantitative immunoblotting with anti-$G_i\alpha_1$, carboxyl-terminal (residues 345–354), and anti-$G\alpha_i$-(40–54) antibodies (EMD Biosciences) and treatment with goat anti-rabbit IgG conjugated to horseradish peroxidase (Chemicon International), followed by incubation with Lumi-Light^{PLUS} Western blotting substrate (Roche Applied Science). Images were acquired using the ChemiDoc Gel Documentation System

(Bio-Rad) and analyzed with the program Quantity One (version 4). For each set of immunoblots, known amounts of purified $G\alpha_iN346C$ subunit (0, 210, 420, and 630 ng) served as internal standards with which serial dilutions of the ligated sample were compared. To determine the yield of the ligation reaction, the amount of protein determined by blotting with the anti-$G\alpha_i$ antibody (ligated plus unligated protein) was compared with the amount of protein determined using the anti-Gα carboxyl-terminal antibody (ligated protein only). Yields generally ranged from 50 to 70%.

Membrane Preparation

Chinese hamster ovary membranes containing C5aR were prepared as described previously (25,26). Briefly, Chinese hamster ovary cells stably expressing C5aRs were harvested, lysed with buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 20 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride, and homogenized by aspirating through a 27-gauge needle 10 times. The lysed solution was first centrifuged at 960 × *g* for 5 min twice, followed by an additional centrifugation at 217,000 × *g* for 30 min at 4 °C using a sucrose cushion (250 mM sucrose, 50 mM Tris, pH 7.5, 1 mM EDTA). The supernatant was aspirated, and the pellet was resuspended in 1 ml of 6 M urea (10 mM HEPES, pH 7.3, 2.5 mM EGTA), incubated at 4 °C for 30 min, and the membranes were pelleted at 217,000 × *g* for 30 min at 4 °C to strip the membranes of GTP-binding proteins. After a second urea wash and sedimentation, the membranes were resuspended in sucrose buffer, frozen, and stored at −80 °C.

Gβγ Purification

Gβγ was purified from Sf9 insect cells as described previously (27). The baculovirus encoding $G\beta_1His_6\text{-}\gamma_2$ was obtained from M. Linder, Washington University, St. Louis, MO. Peak fractions from the MonoQ were concentrated and exchanged into a buffer containing 20 mM HEPES, pH 8.0, 0.1 mM EDTA, 50 mM NaCl, 2 mM β-mercaptoethanol, and 0.7% CHAPS. The final protein concentration was estimated against the known concentrations of bovine serum albumin.

Receptor-catalyzed GDP/GTPγS Exchange Assay

The receptor-catalyzed exchange of GDP for GTPγS was based on a modification of procedures by Fawzi *et al.* (28). Reactions were performed in a total assay volume of 20 μl. Urea-washed membranes containing C5aR were mixed with G protein subunits on ice in buffer to give a final concentration of 20 mM HEPES, pH 7.6, 100 mM NaCl, 2 mM $MgCl_2$, 0.3 mM β-mercaptoethanol, and 1 μM GDP. The reactions were initiated by the addition of 3 μM [^{35}S] GTPγS (5000 cycles/min/pmol) and 100 nM C5a ligand, reacted at 30 °C for 10 min, and terminated by the addition of ice-cold wash buffer (20 mM Tris-Cl, pH 8.0, 25 mM $MgCl_2$, and 100 mM NaCl) followed by filtration over BA85 nitrocellulose filters. Filters were washed with the ice-cold wash buffer, dried, and radioactivity measured by liquid scintillation counting.

Sample Preparation

The concentration of the protein sample was 50 μM. A total of 1 mg of semisynthetic Gα protein (^{13}C-labeled in the carboxyl terminus) was used. The concentrations of AlF_4^- were 100 and 200 μM (100 μM $AlCl_3$, 10 mM NaF) corresponding to 2 and 4 molar equivalents of AlF_4^-.

NMR Spectroscopy

NMR spectra were collected with a Bruker Avance NMR spectrometer at a ^{1}H frequency of 700.13 MHz using a 5-mm triple resonance HCN probe. The two-dimensional ^{1}H-^{13}C HSQC spectrum was obtained using 256 increments in the t_1 dimension, and 1200 transients were

collected at each increment and averaged. The recycle delay was 1.5 s. The temperature was maintained at 25 °C.

RESULTS

Peptide Design

The scheme for the generation of semisynthetic Gα subunits is shown in Fig. 1. The junction between the recombinant thioester lacking the carboxyl terminus (designated $G\alpha_{i1}\Delta CT$) and the synthetic peptide was selected based on the criteria that a cysteine substitution would likely be tolerated at this position, because the EPL mechanism requires a cysteine at the splice point. An alanine-scanning mutagenesis study of $G\alpha_t$ (13) examined the effects of substitutions throughout the entire protein and demonstrated that an alanine point mutation at Glu-342 within the carboxyl-terminal tail was well tolerated. The Glu-342 position in $G\alpha_t$ corresponds to an Asn-346 residue in $G\alpha_{i1}$; thus the N346C mutation was made, and the peptide synthesized was CNLKDCGLF (residues 346–354 of $G\alpha_{i1}$).

The remaining sequences of the carboxyl termini of $G\alpha_{i1}$ and $G\alpha_t$ are identical after positions Glu-342 and Asn-346, respectively; therefore, we reasoned that previous results of mutational data of $G\alpha_t$ and biophysical studies of transducin peptides could be applied directly to $G\alpha_{i1}$. For NMR studies, we selected which amino acids in the synthetic carboxyl-terminal peptide to ^{13}C label based on the transferred nuclear Overhauser effect structure of the α-peptide (11 carboxyl-terminal residues of $G\alpha_t$) bound to R* (8). In this structure, Gly-348 is critical for forming the C-cap turn observed upon photoactivation, and Leu-344 and Phe-350 are members of the hydrophobic cluster. The peptide synthesized for ligation was labeled at the corresponding sites in $G\alpha_{i1}$: Leu-348 (U-^{13}C), Gly-352 (2-^{13}C), and Phe-354 (ring-^{13}C). The ^{13}C-LGF labels provide a total of 12 unique ^{13}C sites to monitor structural changes in the carboxyl terminus via NMR.

Preparation of Semisynthetic $G\alpha_{i1}$

Initial experiments to optimize peptide ligation and demonstrate functionality of the recombinant Gα subunit were performed using an unlabeled synthetic peptide (CNLKDCGLF) corresponding to the carboxyl terminus of $G\alpha_{i1}$ and recombinant $G\alpha_{i1}\Delta CT$. For the EPL methodology, $G\alpha_{i1}$ offers the advantage of much higher yields in bacterial expression systems when compared with $G\alpha_t$. A recombinant protein consisting of residues 1–345 of $G\alpha_{i1}$ was fused to an intein-CBD. Ligation of the recombinant protein ($G\alpha_{i1}$-(1–345)) to the synthetic peptide was carried out as described under “Materials and Methods.” The following control proteins were also generated: 1) the unligated $G\alpha_{i1}\Delta CT$ protein, obtained by eluting the thioester form of $G\alpha_{i1}$ from the chitin column, and 2) full-length $G\alpha_{i1}$N346C, because ligation of synthetic peptide to the recombinant protein introduces a cysteine at the ligation junction. The ligation products were analyzed using antibodies corresponding to the carboxyl-terminal synthetic peptide (anti-$G\alpha_{i1}$; KNNLKDCGLF). Because there was no cross-reactivity with the recombinant thioester (Fig. 2, *lane 1*), Western blot analysis proved to be a convenient and sensitive method for detecting and monitoring the ligation reaction (Fig. 2). Analysis of the ligation product revealed that the reaction was complete after 24 h at 4 °C (Fig. 2, *lanes 3* and *4*) and that the ligation products remained stable even after 72 h (Fig. 2, *lanes 7* and *8*).

The yield of the ligation reaction was estimated by quantitative Western blot analysis. In this instance, two individual Gα antibodies were used, one to the carboxyl terminus (to monitor ligation efficiency) and the second to an internal site within Gα (to quantify the total amount of Gα subunit present). Direct comparison of the total amount of Gα, including both ligated and unligated components, to the amount of detected ligated product provided an estimated ligation efficiency of 50–70% (data not shown). In other EPL studies, higher yields of protein

ligation (approaching >90%) can be achieved with the use of chaotropic agents (*i.e.* guanidinium-hydrochloride and urea), organic solvents (*i.e.* Me_2SO), or higher temperatures (usually 25–30 °C) (29). However, such agents result in protein denaturation, thereby requiring a strategy for refolding the semisynthetic protein when it is to be used for biological assays. Here, all procedures were carried out at conditions optimal for maintaining stability and function of the Gα subunit, which might have lowered the efficiency of chemical ligation.

Semisynthetic $G\alpha_{i1}$ Is Functional

Using membrane reconstitution assays, we characterized the ability of the semisynthetic $G\alpha_{i1}$ subunits to be activated by GPCRs. Given the large number of studies implicating the carboxyl terminus as essential for the ability of receptors to activate G proteins, we reasoned that this would serve as the strictest test of functionality of ligated G proteins. Intact membranes were isolated from Chinese hamster ovary cells stably expressed with human C5a receptors, a chemoattractant receptor and member of the rhodopsin family of GPCRs. C5a receptors activate $G\alpha_i$ subunits and mediate neutrophil chemotaxis in response to gradients of the C5a ligand, a 74-amino-acid cleavage product of the complement cascade. The receptor-containing membranes were washed with 6 M urea to remove endogenous G proteins. Recombinant Gα proteins, expressed and purified from BL21 (DE3) *E. coli*, and Gβγ, purified from Sf9 insect cells, were incubated with whole membranes, C5a ligand, and ^{35}S-labeled GTPγS. Fig. 3 demonstrates the receptor-catalyzed GDP-GTP exchange. The ligated $G\alpha_i$ bound 4.8 pmol of GTPγS in response to C5a ligand *versus* 3.0 pmol in the absence of ligand (1.6-fold stimulation). The amount of GTPγS binding observed for the native $G\alpha_i$ subunit was 7.2 pmol bound (4.3-fold stimulation). For a 10-min reaction, this amount of GTPγS binding corresponds to a turnover rate of ~50 G proteins/ligand-stimulated receptor. The majority of the basal GTPγS binding that occurs in the absence of ligand most likely represents the intrinsic GTPγS binding activity of the Gα subunit (a function of the GDP off rate), because each of the recombinant G proteins in the absence of membranes bound similar amounts of GTPγS (data not shown). The extent of receptor activation observed for the G proteins in these assays is comparable with those published for activation of purified Gα subunits by other GPCRs in similar membrane reconstitutions systems (30,31).

To ensure that the introduction of a cysteine substitution at Asn-346 did not affect the function of the carboxyl terminus in receptor activation, we also assessed the ability of the recombinant $G\alpha_i$N346C mutant to bind GTPγS in response to ligand stimulation of the C5a receptor in reconstituted membranes. Confirming our prediction that this position should tolerate a cysteine mutation, we found that the C5a receptor activated the $G\alpha_i$N346C mutant to an extent equivalent to the wild-type $G\alpha_{i1}$ subunit (Fig. 3). We also tested $G\alpha_i$ that lacked the terminal nine amino acids. The $G\alpha_i\Delta$CT protein demonstrated increased basal levels of GTPγS binding but did not respond to receptor stimulation. The fact that the truncated Gα subunit $G\alpha_{i1}\Delta$CT displayed increased basal GTPγS binding is consistent with previous work that demonstrates that truncation of the carboxyl-terminal 10 residues of $G\alpha_o$ resulted in decreased affinity of the G protein for GDP but with no change in affinity for GTPγS (14). The complete lack of receptor stimulation of the $G\alpha_i\Delta$CT mutant underscores the importance of the carboxyl terminus for activation of G proteins by receptors.

Truncated $G\alpha_{i1}$ Acts as Competitive Inhibitor for Receptor Activation

Although the ligated $G\alpha_{i1}$ demonstrated receptor-catalyzed nucleotide exchange (1.6-fold stimulation), the activity of the ligated G protein is less than that observed for full-length $G\alpha_{i1}$ or $G\alpha_{i1}$N346C (3–5-fold stimulation). One potential explanation for this decreased activity might be that the unligated component of G proteins present in the semisynthetic $G\alpha_{i1}$ preparations (ranging from 30 to 50% of the total G protein) might compete for receptor sites on our membranes and thereby inhibit receptor activation of full-length ligated $G\alpha_i$

subunits. We tested this possibility by performing competition assays, adding increasing amounts of unligated G protein ($G\alpha_i\Delta CT$) to full-length $G\alpha_{i1}N346C$ and monitoring the receptor-catalyzed nucleotide exchange. We found that the addition of $G\alpha_{i1}\Delta CT$ protein inhibited the receptor-catalyzed exchange of GDP for GTP for $G\alpha_{i1}N346C$ (Fig. 4). Because the efficiency of ligation of our semisynthetic Gα subunits is 50–70%, we can translate our finding to be consistent with what we are observing upon the addition of 125–250 n_M unligated protein in Fig. 4. These data demonstrate that the addition of 125 n_M unligated protein (33% of total) to 250 n_M recombinant $G\alpha_{i1}N346C$ severely masks the full functional activity of the active subunit (8.4-fold stimulation *versus* 1.9-fold stimulation) and implicates the unligated protein as a competitive inhibitor. For the following solution NMR studies, we assumed that the presence of unligated Gα subunit would not affect the conformational changes that might occur during G protein activation. Because the unligated protein does not contain isotope labels, it should not contribute to the obtained spectra. The fact that our semi-synthetic proteins displayed receptor-catalyzed nucleotide exchange even in the presence of a competitive inhibitor was quite compelling. These findings further implicate the carboxyl terminus to be essential for receptor activation but not GTPγS binding.

HSQC NMR Spectrum of ^{13}C-Gα Protein

Fig. 5 presents the ^{1}H-^{13}C HSQC NMR spectrum of the semisynthetic $G\alpha_{i1}$ subunit that has been ^{13}C-labeled at Leu-348, Gly-352, and Phe-54. The Cβ-H, Cδ-H, and Cγ-H resonances from Leu-48 are observed in Fig. 5*A*, the Cα-H resonances of Gly-352 and Leu-348 are observed in Fig. 5*B*, and the Cδ-H resonances of the Phe-354 aromatic ring are observed in Fig. 5*C*. Both the ^{1}H and ^{13}C chemical shifts are close to their positions in the free amino acids, indicating no dispersion due to protein interactions.

The ^{1}H line widths in the HSQC spectrum range from 27 Hz for Leu-348 methyl protons to 50 Hz for the Gly-352 Cα-H protons. For comparison, the methyl line widths in the HSQC spectrum of ubiquitin, a small 76-amino-acid protein, are on the order of 24 Hz. Based on the average line widths in ubiquitin and the Gα subunit, an analysis of the rotational correlation times indicates that the carboxyl terminus in the Gα subunit is much more mobile than expected for a 40-kDa protein.

Mobility of Carboxyl Terminus; Titration with AlF_4^-

Activation of Gα subunits results in characteristic changes in both their structure and function. Such activation can be induced using nonhydrolyzable analogs of GTP (such as GTPγS) or by AlF_4^-, an analog of the γ-phosphate of GTP that coordinates with GDP to mimic the active state of the G protein. To characterize the influence of AlF_4^- on the NMR spectrum of the Gα subunit, the semisynthetic ^{13}C-labeled $G\alpha_{i1}$ protein was titrated with 2 and 4 molar equivalents of AlF_4^-. The addition of AlF_4^- resulted in a loss of intensity in all of the resonances observed in the HSQC spectrum for the ^{13}C-LGF protein. Examination of slices from the two-dimensional HSQC spectra at different concentrations of AlF_4^- showed that there is a loss of total intensity in the lines and not an increase in the broad component at the foot of the peak (Fig. 6). Although all of the resonances decreased, the intensity losses of the Gly-352 and Phe-354 resonances were approximately twice the losses in the Leu-348 resonances. As a control, the addition of AlF_4^- to a solution of the ^{13}C-labeled nine-residue peptide corresponding to the carboxyl terminus of Gα did not influence the line width, consistent with the carboxyl-terminal peptide alone being free and mobile (data not shown). The loss of intensity can only be attributed to loss of mobility of the carboxyl-terminal tail upon the addition of AlF_4^-. The larger influence on Gly-352 and Phe-354 suggests that these carboxyl-terminal residues may become buried upon an AlF_4^--induced conformational change.

Fig. 7 presents the ^{1}H spectrum of the Cα-H region of the ligated Gα protein before and after the addition of AlF_4^-. The absence of any significant change in the proton line widths indicates that the dramatic loss of intensity observed in the ^{13}C resonances of the carboxyl terminus does not represent a global change in the motion of the protein due, for instance, to oligomerization or aggregation but is restricted to a large change in the mobility of the carboxyl-terminal tail.

DISCUSSION

Crystal structures and biochemical studies of G proteins have identified putative receptor interaction sites and regions that undergo conformational changes as a result of nucleotide exchange. The receptor binding regions on Gα are known to be located at some distance (25–30 Å) from the GDP binding pocket. Still, several questions regarding G protein activation remain. For instance, how does receptor binding trigger GDP release? How does GTP binding lead to dissociation of Gα from Gβγ and the receptor?

Here, we probed the conformational changes of the carboxyl terminus of Gα associated with G protein activation using solution NMR. We have developed a strategy, using expressed protein ligation, to site-specifically introduce ^{13}C-labeled amino acids into the carboxyl terminus of a recombinant $G\alpha_{i1}$ subunit. Solution NMR studies on the ligated ^{13}C Gα subunit revealed a loss in intensity of all ^{13}C resonances upon titration with AlF_4^-, which when bound to Gα serves to mimic the activated state of the G protein. This loss in intensity can be directly attributed to loss of mobility of the carboxyl-terminal tail upon the addition of AlF_4^-. The spectra show that there is a loss of total intensity in the relatively narrow NMR resonances (Fig. 6). This would be consistent with the carboxyl terminus being in slow exchange between a free (mobile) conformation and an ordered (bound) conformation. The resonance corresponding to the bound conformation was not observed, even as a broad component in the spectrum. The complete loss of intensity for the bound conformation suggests that the wild-type Gα subunit is oligomeric or aggregated in the absence of the Gβ and Gγ subunits. If the carboxyl terminus is in dynamic equilibrium (*i.e.* in the intermediate exchange regime) between the free and bound conformations, we would expect to see a change in the line width as a function of added AlF_4^-, which we did not observe. Lowering the temperature to 4 °C from 25 °C did not change the observed line widths, suggesting that the carboxyl terminus is not in fast exchange (data not shown). Also, the greater intensity loss observed at Gly-352 and Phe-354 compared with Leu-348 further suggests that the extreme carboxyl-terminal residues become buried upon an AlF_4^--induced conformational change. One can speculate that the carboxyl-terminal aromatic group (Phe-354) finds a binding site in the GTP-stabilized form of Gα. However, we cannot determine this from our study, because the loss of signal intensity that occurs with the addition of AlF_4^- makes an assignment of a specific structure difficult.

Our finding that the carboxyl terminus of Gα subunit becomes ordered upon activation by AlF_4^- is supported by previous work by Hamm and co-workers (11). Using lucifer yellow, an environmentally sensitive fluorescent probe, to label Cys-347 on the carboxyl terminus of $G\alpha_t/G\alpha_i$ chimera, Hamm and co-workers observed an increase in fluorescence upon binding of AlF_4^- to the G protein. The increased fluorescence can be interpreted as the fluorescent label moving from a relatively hydrophilic environment to one that is more hydrophobic. Moreover, the kinetics of the fluorescence change of the lucifer yellow probe correlated well with the rate of formation of the active state, as monitored by the intrinsic fluorescence change of Trp-207 in switch II. Consistent with the idea that Gα activation buries the carboxyl terminus, the ability to label Cys-347 with lucifer yellow decreased after AlF_4^- binding. It is difficult to know what effect, if any, the lucifer yellow probe (M_r 457.2) might introduce on the conformation of the tail. Modification of the Cys-347 by the lucifer yellow would be predicted to disrupt receptor activation, much like ADP-ribosylation of Cys-347 by pertussis toxin. Therefore, the findings of our solution NMR studies demonstrating AlF_4^--dependent ordering of the carboxyl terminus

provide strong validation of the results obtained with fluorescent labeling. Moreover, our studies introduce isotope labels that should not perturb functionality and therefore are more likely to reflect conformational changes that occur in the native Gα subunit.

How do the NMR results of the carboxyl terminus of $G\alpha_i$ compare with crystallography studies? In the GDP-bound state, the carboxyl terminus appeared highly mobile, which is consistent with the observation that the carboxyl terminus is not resolved in all but one of the crystal structures of the heterotrimeric G proteins and Gα subunits bound to GDP. In the one example where the carboxyl terminus is ordered in the GDP-bound state of the Gα subunit ($G\alpha_{i1}$), the tertiary and quaternary structure revealed microdomains composed of regions from the amino terminus of one monomer and the carboxyl terminus of another monomer, suggesting the presence of head-to-tail Gα polymers (10). Based on these data and the observation that the amino and carboxyl termini of Gα subunits appear disordered in many structures of GTP-bound Gα subunits, the authors suggest that polymers of α subunits form as a result of GTP hydrolysis and that the polymers might provide a functional role in signal transduction. The data obtained in our solution NMR studies do not support this model, because the carboxyl terminus of $G\alpha_{i1}$ bound to GDP appeared freely mobile, whereas the tail became ordered upon activation of G protein by the addition of AlF_4^-. In two crystals of activated Gα subunits determined subsequent to the $G\alpha_{i1}$-GDP structure, the carboxyl terminus was ordered; the tail of $G\alpha_t$ bound to GTPγS formed an extended linear structure, and the carboxyl terminus of $G\alpha_{i1}$ bound to regulator of G protein signaling 4 adopted the carboxyl-terminal capping motif (6) observed in the transferred nuclear Overhauser effect structures of carboxyl-terminal peptides bound to photo-activated rhodopsin. We did not observe any spin-spin interactions between Leu-348 and Phe-354, as would be predicted by the carboxyl-terminal capping motif. However, the loss of signal intensity associated with ordering of the carboxyl terminus also decreased the sensitivity of these studies to observe any potential spin-spin interactions or to make any specific assignments to confirm other potential conformations of the carboxyl terminus.

A role for the carboxyl terminus in nucleotide exchange was initially proposed by Denker *et al.* (14), in which a carboxyl-terminal deletion of $G\alpha_o$ resulted in enhanced activation of the subunit in the presence of high GTP concentrations. In their study, truncated $G\alpha_o$ subunits with deletions of 5, 10, or 14 amino acids from the carboxyl terminus of αo are monitored for their ability to bind GTP or GTPγS. Truncation of 5 or 10 amino acids have little effect on the affinity of the G protein for GDP, which is similar to what we observed for the Gα subunit that lacked the carboxyl-terminal nine amino acids (data not shown). However, deletion of 14 amino acids resulted in a diminished affinity for GDP, resulting in enhanced activation of the Gα subunit in the presence of high concentrations of GTP. The region 10–14 amino acids from the carboxyl terminus is located at the end of the α5 helix, which connects the carboxyl-terminal tail and a loop contacting the nucleotide-binding pocket. More recent studies by Marin *et al.* (18) further define a role for the α5 helix in nucleotide exchange. Point mutations at Thr-325, Val-328, or Phe-332 in the α subunit of transducin resulted in dramatic increases in both basal nucleotide exchange and receptor-catalyzed nucleotide exchange (18). These three residues (located 19–26 amino acids from the carboxyl terminus) face inward toward the body of the G protein and likely comprise a functional microdomain in G proteins that affects basal nucleotide release.

To promote nucleotide exchange, conformational changes likely must be transmitted from the carboxyl-terminal tail, an eight-amino-acid region known to be involved in receptor recognition through the α5 helix (18 amino acids) to the nucleotide-binding pocket located 25–30 Å from the membrane. To demonstrate this proposed mechanism, the Artemyev group (32) made recombinant Gα subunits modified between the carboxyl terminus and the α5 helix with either a flexible glycine linker or an extended α helix. They found that mutants containing the flexible linker could not be activated by the receptor, whereas those extending the helix by three turns were capable of activation by photo-excited rhodopsin. These results provide direct

evidence for structural requirements of the carboxyl-terminal tail in G protein activation by GPCRs.

In our membrane reconstitution experiments, we found that the carboxyl terminus is essential for receptor activation, consistent with abundant evidence for essential roles of amino acids in the carboxyl terminus of Gα subunits. Notably, in our membrane reconstitution experiments, the truncated Gα subunit ($G\alpha_{i1}\Delta CT$) acted as a competitive inhibitor for receptor activation, thus demonstrating that the carboxyl terminus is not required for receptor binding, but is likely more important for the receptor-catalyzed exchange of GTP for GDP on the G protein (Fig. 4). The ability of the truncated Gα subunit to compete with the full-length Gα subunit seems paradoxical, because numerous biochemical studies demonstrate the carboxyl-terminal peptides can bind to activated receptors, although with micromolar affinities. Therefore, one would expect that the carboxyl terminus should provide additional binding energy for the full-length G protein and that the truncated Gα subunit would be a poor competitor. How can this be reconciled? One possibility is that the truncated Gα subunit does have reduced binding affinity for the receptor; however, the lack of the carboxyl terminus prevents G protein activation and subsequent release. In this way, the truncated Gα subunit, once bound to a receptor, might act as a dominant negative protein for other G protein activation. Alternately, the peptide binding studies might represent an intermediate in the activation process, such as stabilization of the MII activation state of rhodopsin. Thus, during the activation process, the carboxyl terminus might adopt a conformation that could increase the affinity of the receptor for G protein. Consistent with this notion, G proteins in the empty state have high affinity for receptors (33,34), and it is tempting to speculate that the structures of the carboxyl termini might be functionally linked to the empty state of the G protein. Binding of the GTP by the Gα subunit would then be predicted to remove this interaction, presumably by altering the conformation of the tail.

The results obtained here suggest that activation of Gα subunits alters the conformation of the carboxyl tail. From a mechanistic standpoint, it could be considered that activation of the Gα subunit induces the carboxyl-terminal tail to adopt an ordered conformation in the GTP-bound state. In this conformation, the carboxyl terminus may sterically interfere with binding to the receptor interface, thus decreasing binding affinity for the activated Gα subunit to the receptor and thereby encouraging the release of the Gα subunit from the receptor. Additionally, this model also allows for competition by the truncated G protein for the receptor, because without the carboxyl terminus, the truncated version of the G protein cannot adopt the conformation of the full-length G protein and thus would not reduce receptor-binding affinity.

In addition, we demonstrate the utility of expressed protein ligation for the study of G proteins and signal transduction pathways. The ability to ligate structural probes site-specifically onto proteins provides many applications for this technology. This is particularly important and interesting for G proteins, because they undergo many conformational changes to function in signal transduction. The work presented here provides one example of how one region of a large protein can be monitored, in terms of structural conformation, without the requirement for modifying a large region of the protein. Future directions will focus on performing solid state NMR experiments on isotope-labeled rhodopsin in complex with segment-labeled G protein to allow determination of a structural picture of the docking of the G protein α subunit to the ligand-activated receptor.

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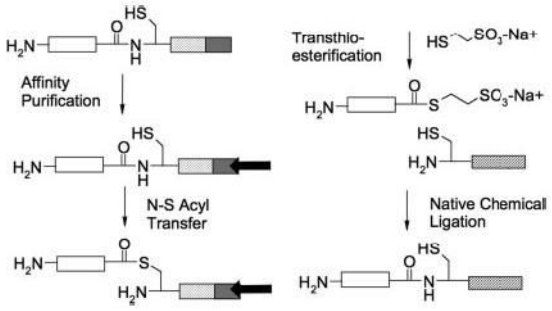

Fig. 1. Expressed protein ligation

$G\alpha_{i1}\Delta CT$ (*white box*) is expressed in *E. coli* as a fusion protein, $G\alpha_{i1}\Delta CT$-intein-CBD. The chitin-binding domain (CBD; *gray box*) allows affinity purification on the chitin matrix (*black arrow*). Intein domain (*diagonal lines*) catalyzes N→S acyl transfer. Incubation with mercaptoethanesulfonic acid results in cleavage of the target protein from the intein-CBD domains, generating recombinant $G\alpha_{i1}\Delta CT$ thioester. The thioester protein thereafter undergoes native chemical ligation with CNLKDCGLF peptide (*cross-hatched*).

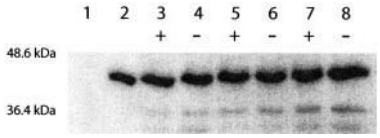

Fig. 2. SDS-PAGE/Western analysis of semisynthetic Gα

Ligated product was observed after 24 h. *Lane 1*, purified $G\alpha_{i1}\Delta CT$ thioester (negative control); *lane 2*, purified $G\alpha_{i1}$ (N346C). Time points were taken at 24 (*lanes 3* and *4*), 48 (*lanes 5* and *6*), and 72 h (*lanes 7* and *8*) and treated with (+) or without (−) 15 m_M dithiothreitol before separation on 10% SDS-polyacrylamide gels. Products were transferred to membranes and blotted with anti-carboxyl-terminal $G\alpha_{i1}$ antibody.

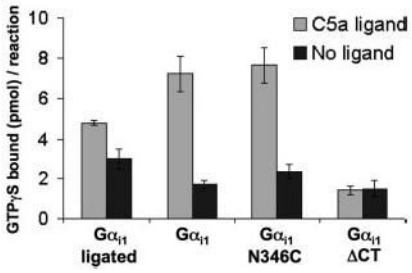

Fig. 3. C5a receptor activation of ligated G proteins

C5a receptors (1–5 nM) in urea-stripped membranes were incubated with C5a ligand (100 nM) and Gα proteins (1 μM) and Gβγ (0.5 μM) and GTPγS (3 μM) in 20-μl reactions for 10 min at 30 °C and then filtered and counted. Data shown are the average ± S.E. of six experiments.

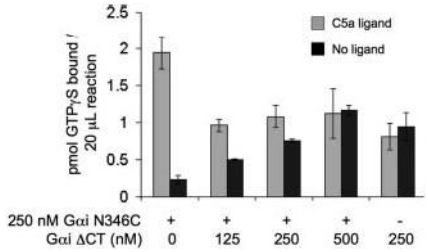

Fig. 4. Competitive inhibition of C5a receptor activation by $G\alpha_i\Delta CT$

C5a receptors (1–5 nM) in urea-stripped membranes were incubated with C5a ligand (100 nM) and $G\alpha_i$N346C proteins (250 nM) and Gβγ (0.5 μM) and GTPγS (3 μM) in 20-μl reactions. Indicated amounts of $G\alpha_i\Delta CT$ were added, and reactions proceeded for 10 min at 30 °C and then filtered and counted. Data shown are the average ± S.E. of three experiments.

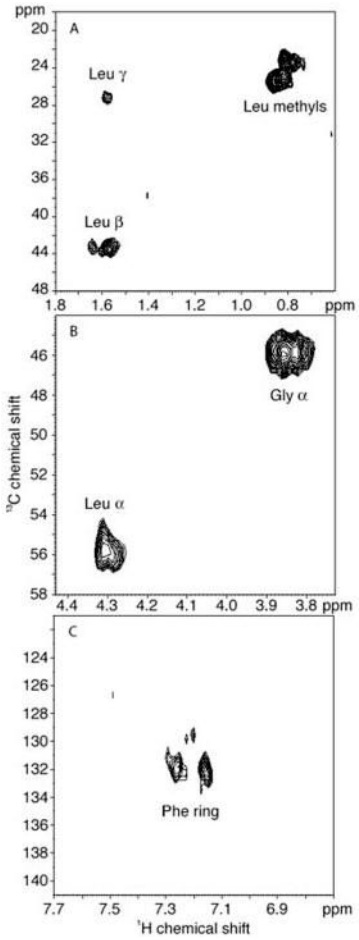

Fig. 5. ^{1}H-^{13}C HSQC spectrum of Gα protein labeled at Leu-348, Gly-352, and Phe-354

The *panels* display the regions containing the Leu-348 methyl Cβ-H and Cγ-H resonances. The Gly-352 (*A*) and Leu-348 (*B*) Cα-H resonances and the Phe354-^{13}C-ring resonances (*C*).

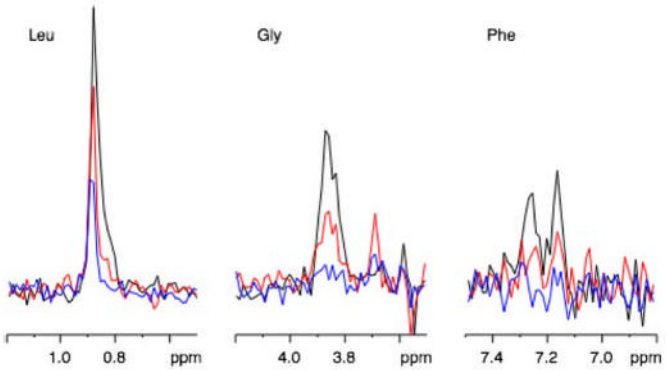

Fig. 6. Intensity of the HSQC NMR resonances as a function of added AlF_4^-

Slices from the two-dimensional HSQC spectra at different concentrations of AlF_4^-. *Black line* is spectra obtained with no additional AlF_4^-. *Red* indicates two molar equivalents of AlF_4^-. *Blue* represents four molar equivalents of AlF_4^-.

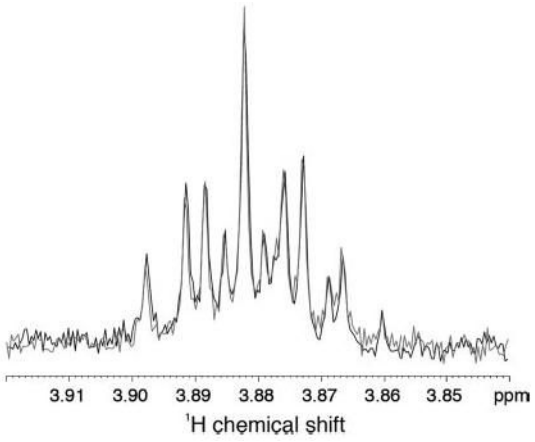

Fig. 7. One-dimensional ^{1}H spectra of the Gα protein before and after the addition of AlF_4^-

The spectra are virtually identical, indicating that there is no overall change in the mobility of the protein due to the binding of AlF_4^-. The changes in the carboxyl-terminal region of the protein are dramatic and localized.