Expression, purification and preliminary NMR characterization of isotopically labeled wild-type human heterotrimeric G protein α_{i1}

Authors:

Jan Maly and Karin A. Crowhurst*

Address (for all authors):

Department of Chemistry and Biochemistry, California State University Northridge

18111 Nordhoff St., Northridge CA 91330-8262 USA

* Corresponding author. Contact: karin.crowhurst@csun.edu; phone 818-677-4288; fax 818-677-4068. Postal address listed above.

Abstract

Molecular-level investigation of proteins is increasingly important to researchers trying to understand the mechanisms of signal transmission. Heterotrimeric G proteins control the activation of many critical signal transmission cascades and are also implicated in numerous diseases. As part of a longer-term investigation of intramolecular motions in RGS and Gα proteins in their apo and complexed forms, we have successfully developed a protocol for preparing milligram quantities of highly purified, isotopically labeled wild-type human $G\alpha_{i1}$ ($hG\alpha_{i1}$) subunit for NMR studies. High levels of expression in *E. coli* can be attributed to the use of the SUMO fusion protein system, a bacterial strain that produces rare codons, supplementation of minimal medium with small quantities of isotopically labeled rich medium and a lowered induction temperature. Purification of $hG\alpha_{i1}$ utilized affinity and size exclusion chromatography, and protein activity was confirmed using fluorescence-based GTP-binding studies. Preliminary NMR analysis of $hG\alpha_{i1}$ has shown that high-quality spectra can be obtained at near-physiological temperatures, whereas lower temperature spectra display numerous weak and broadened peaks, providing preliminary evidence for widespread μs-ms timescale exchange. In an effort to further optimize the NMR spectra we prepared a truncated form of $hG\alpha_{i1}$ ($hG\alpha_{i1}$-Δ31) in which the 31-residue unstructured N-terminus was removed. This resulted in further improvements in spectral quality by eliminating high-intensity peaks that obscured resonances from structured segments of the protein. We plan to use $hG\alpha_{i1}$-Δ31 in future investigations of protein dynamics by NMR spectroscopy to gain insight into the role of these motions in RGS/Gα binding selectivity.

Keywords: G protein alpha subunit, signaling protein, protein NMR, SUMO fusion, isotope labeling

Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins with α, β, and γ subunits) comprise a family of intracellular signaling proteins that act as molecular switches to relay chemical and physical signals between G protein coupled receptors (GPCRs)[1] and second messengers downstream of the signal cascade [1]. In its inactive form the GDP-bound α subunit is attached to its obligate βγ dimer near the cell wall. GPCR activation by extracellular stimuli promotes the α subunit to exchange GDP for GTP, causing a change in conformation, destabilizing the heterotrimeric G protein complex and ultimately forcing the dissociation of the Gα subunit from βγ. Each separated component is then able to regulate downstream effectors. The duration of G protein signaling is controlled by the lifetime of the GTP-bound Gα subunit; signal deactivation relies on the efficient hydrolysis of GTP back to GDP by Gα, the rate of which is mediated by RGS (Regulator of G protein Signaling) proteins [2]. These RGS proteins bind Gα adjacent to the GTP binding site via their highly conserved signature domain (the "RGS box") [3].

Although physiological characterization of these systems has been important for advancing our understanding of signal transmission through this complex, it is also important to describe how the proteins participate at the molecular level. Several NMR and crystal structures of RGS and Gα proteins (alone and in complex) have been reported [4-6], but very little investigation of internal protein motions has been done. In recent years it has become clear that protein dynamics are of fundamental importance to protein function; this includes conformational changes, molecular recognition and allosteric processes, all of which are crucial to signal transmission [7, 8]. Investigators have observed evidence for conformational changes that take place between apo and $G\alpha_{i1}$-bound RGS4 [4, 5] as well as changes in flexibility in active versus inactive Gα;

[1] Abbreviations used: BODIPY® FL GTP-γ-S, guanosine 5'-*O*-(3-thiotriphosphate); BSA, bovine serum albumin; ddH_2O, ultrapure water (deionized and then ultrafiltered); GPCR, G-protein coupled receptor; $hG\alpha_{i1}$, wild-type α_{i1} subunit of human heterotrimeric G protein; $hG\alpha_{i1}$-Δ31, α_{i1} subunit of human heterotrimeric G protein with 31 residues removed from the N-terminus; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl β-D-1-thiogalactopyranoside; kan-cam, kanamycin-chloramphenicol antibiotic mixture; NEB, New England Biolabs; NMR, nuclear magnetic resonance; NMRFAM, National Magnetic Resonance Facility at Madison; MM, minimal medium; PIPES, piperazine-N,N′-bis (2-ethanesulfonic acid); RGS, regulator of G protein signaling; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SUMO, small ubiquitin-like modifier; TROSY, transverse relaxation optimized spectroscopy; ULP-1, Ubl-specific protease 1.

however, these motions have not been probed in detail, despite preliminary evidence that protein motions may be key to our understanding of the mechanism of action of RGS and Gα proteins [5]. Our overall research objective is to address this unexplored issue by using NMR spectroscopy to characterize the role played by these motions in the function and selectivity of RGS/Gα interactions.

We are particularly interested in comparing protein motions within wild-type human RGS4, RGS7 and $G\alpha_{i1}$ (denoted hRGS4, hRGS7 and $hG\alpha_{i1}$, respectively) in both their apo and target-bound forms. Thus, the first steps towards achieving these research goals are to successfully express and purify all three proteins as isotopically-labeled samples for NMR analysis. The RGS proteins have been successfully prepared by us and others [4, 5, 9], but until now there has been no published protocol to prepare sufficient quantities of active, isotope-labeled wild-type human heterotrimeric $G\alpha_{i1}$. There are several publications that outline the preparation of $G\alpha_{i1}$ in *E. coli* in rich medium ([5, 10, 11], for example); however, those techniques have not been used successfully to produce large quantities of highly purified and concentrated isotope-labeled protein. The only previously reported preparation of milligram quantities of a Gα-like protein for NMR was a chimera called ChiT, made with an approximate 3:1 sequence ratio of $G\alpha_t$ to $G\alpha_{i1}$ (species undefined) [12]. Although some very interesting research has been performed on ChiT [13, 14], it is a mimic of $G\alpha_t$ and cannot substitute for studies performed on a wild-type human $G\alpha_i$. As far as we are aware, our paper is the first to report on efforts to prepare an isotopically labeled $G\alpha_{i1}$ subunit for NMR studies.

The usual challenges relating to protein expression and purification are augmented when trying to express a correctly folded and soluble 40 kDa human protein in bacterial culture. The difficulty is further enhanced when attempting to produce this protein with isotopic labeling. In order to record heteronuclear NMR experiments, a typical protein sample must be ^{15}N- or ^{13}C-labeled (or both); this requires protein expression to take place in a medium in which all nitrogen and/or carbon atoms are enriched with these isotopes. A minimal medium is most commonly utilized, which is not a favorable environment for bacteria and usually results in a lower protein yield than obtained in rich medium. An additional requirement for the preparation of Gα subunit proteins is that they must be expressed in the soluble fraction; previous researchers have shown that refolding these subunits from inclusion bodies results in GTP-inactive protein [15]. Although it might seem preferable to produce human $G\alpha_{i1}$ in a eukaryotic expression system, this

procedure continues to be very costly for preparing NMR samples, as eukaryotic systems tend to produce low protein yields and the ingredients needed for isotope-labeling are significantly more expensive than what is required for bacterial cultures.

In this paper we describe successful *E. coli* expression and purification of active ^{15}N-labeled wild-type hGα_{i1} in very high yields, as well as optimization of buffer and temperature conditions in order to obtain high-quality NMR spectra. In an effort to further improve NMR spectral quality we also prepared an N-terminally truncated version of the protein, hGα_{i1}-Δ31, in which the first 31 residues (that are known to be unstructured) have been removed. Important contributors to our success included the use of the SUMO fusion protein (for both soluble expression and simplified purification) and a strain of *E. coli* that produces rare codons, as well as the inclusion of a small amount of isotopically labeled rich medium supplement and the use of a decreased temperature for induction of expression. As a result of these efforts we have been able to yield almost 40 mg of soluble isotopically labeled full-length wild-type hGα_{i1} or 15 mg of hGα_{i1}-Δ31 per liter of culture, which has been used to produce NMR samples with concentrations up to 0.5 mM. We are now ready to move on to record chemical shift assignment experiments and protein dynamics experiments on apo- and RGS-bound hGα_{i1}.

Materials and Methods

It should be noted that the expression and purification protocols for both full-length wild type (hGα_{i1}) and truncated (hGα_{i1}-Δ31) forms are identical and that, unless otherwise stated, the methods described herein pertain to both forms of the subunit. Please see Table 1 for some physical properties of the full-length and truncated proteins.

Preparation of the full-length wild type and truncated hGα_{i1} gene

pcDNA3.1+ containing the full-length wild type human G protein alpha i1 subunit (hGα_{i1}) and the 31 residue N-terminally truncated form (hGα_{i1}-Δ31) was obtained from the Missouri S&T cDNA Resource Center and PCR-amplified on a 2720 Thermo Cycler (Applied Biosystems) using oligo primers with ScaI (blunt end) restriction site in the forward (5') direction, and BamHI restriction site in the 3' direction (reverse complement) (Integrated DNA Technologies). Complementary DNA sequences in the forward direction were 5'-

GATATAAGTACTATGGGCTGCACGCTGAGC-3' and 5'-GATATAAGTACTATGCGCGAGGTCAAGCTGC-3' for the full-length and truncated subunits, respectively. Both versions utilized the same reverse complement sequence of 5'-GCAGCCGGATCCTTAAAAGAGACCACAATCTTTTAGAT-3'. Each PCR product was purified using a QIAGEN QIAquick PCR purification kit.

Preparation of pET-SUMO plasmid stock

Approximately 100 ng of bacterial pET-SUMO plasmid (Invitrogen), modified with a multiple cloning site, was obtained from Dr. Rhea Hudson (Hospital for Sick Children, Toronto, ON, Canada) in dried form on filter paper and extracted with 100 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) to make a 1 ng/µL stock. 10 µL of this stock were immediately transformed into electrocompetent XL1-Blue *E. coli* cells (Stratagene) by electroporation. The cells were grown in 450 µL of Luria Broth (LB) media at 37 ^{o}C for 1 hour and transferred onto LB-agar plates containing kanamycin (50 µg/mL). Plates were incubated overnight at 37 °C and one colony was transferred to a snap-cap tube with 5 mL of LB containing 0.1 mM kanamycin for an additional overnight growth. The pET-SUMO plasmid was then isolated using a QIAGEN QIAfilter MidiPrep kit and stored at -20 °C.

Insertion of the hGα_{i1} gene into the pET-SUMO plasmid

Double digests of the purified hGα_{i1} gene and pET-SUMO vector were performed using 10 units each of ScaI and BamHI restriction endonucleases (New England Biolabs, NEB) in 20 µL total volumes of ultrapure sterile H_2O (ddH_2O), NEB buffer 3, and 10x BSA. Because ScaI forms a blunt end, the doubly digested plasmid vector was then dephosphorylated by incubation for one additional hour at 37 ^{o}C with 1 µL (10 units) of calf intestinal phosphatase (CIP) from NEB. Digested DNA samples were run on agarose gels, excised from the gel, and purified using the QIAGEN QIAquick Gel Extraction kit. Purified DNA inserts were ligated into the plasmid using T4 DNA ligase (NEB) in 10 µL volumes of ddH_2O and 10x T4 buffer stock for 24 hours at 16 °C. Ligation ratios were 1:1 and 1:3 (vector : DNA insert). 2 µL of each solution were transformed into XL1-Blue bacterial cells and grown for 1 hour at 37 ^{o}C in 950 µL of LB. The bacterial culture was plated onto LB-agar/kanamycin-chloramphenicol (LB/kan-cam) plates and incubated overnight at 37 ^{o}C. All LB/kan-cam plates contained working

concentrations of 0.1 mM kanamycin and 0.15 mM chloramphenicol. hGα_{i1}-ligated plasmids from viable colonies were amplified using colony PCR, and tested for *hGα_{i1}* gene amplification on an agarose gel. Colonies containing the highest apparent *hGα_{i1}* gene concentrations were each grown 12-16 hours in 5 mL LB/kan-cam media at 37 ^{o}C, and purified with the QIAGEN QIAprep Spin Mini Prep kit. The purified DNA samples were sequenced (Laragen, Inc.) and 20 ng/µL stocks made for transformation into expression hosts and stored at -20 ^{o}C.

Expression of the SUMO-hGα_{i1} fusion protein

The pET-SUMO-hGα_{i1} plasmid was transformed by electroporation into *E. coli* BL21-CodonPlus (DE3)-RIPL cells (Stratagene) and incubated overnight at 37 ^{o}C on LB/agar kan-cam plates.

All 1 L minimal medium (MM) solutions consisted of sterilized ddH_2O and the following: 100 mM Na_2HPO_4, 25 mM KH_2PO_4, 10 mM NaCl, 3 g glucose, 1 g NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, antibiotic (0.1 mM kanamycin and 0.15 mM chloramphenicol) and 10 mL 100X BME (Basal Medium Eagle) vitamins (MP Biomedicals). In the 3-hour and long induction protocols outlined below, the medium was also supplemented with 10% (v/v) BioExpress rich media (Cambridge Isotope Labs). When preparing ^{15}N-labeled Gα, the transformed cells were grown in minimal media using $^{15}NH_4Cl$ and ^{15}N-labeled BioExpress as the sole nitrogen sources.

For 3-hour inductions, a single colony from the transformed plates was picked into 50 mL LB culture containing 0.1 mM kanamycin and 0.15 mM chloramphenicol and shaken at 225 rpm overnight at 37 ^{o}C. This culture was then centrifuged at 5000 rpm for 10 minutes and the pellet was resuspended into 1 L of MM (+ 10% BioExpress). Cultures were grown in an I24 Incubator Shaker (New Brunswick Scientific) to an OD_{600} of 0.7 and induced with 1.0 mM IPTG for 3 hours at 37 °C before harvesting.

For long inductions, a single colony from the transformed plates was picked into 50 mL LB culture containing 0.1 mM kanamycin and 0.15 mM chloramphenicol and shaken at 225 rpm overnight at 37 ^{o}C. In order to delay scale-up to 1 L culture (until later in the day), 0.25 mL of this overnight culture was transferred into a fresh 50 mL LB/kan-cam culture and grown at 37 ^{o}C until OD_{600} ~ 1.0 (3-4 hours). The culture was then centrifuged at 5000 rpm for 10 minutes and the pellet was resuspended into 1 L of MM (+ 10% BioExpress). The MM culture was shaken at

225 rpm at 37 ^{o}C until OD_{600} = 0.5, at which time it was induced with 1.0 mM IPTG, the temperature was lowered to 30 ^{o}C, and the shaker speed lowered to 185 rpm. It was then grown for 15 hours before harvesting.

Purification of hGα_{i1} from the SUMO fusion protein

All chromatographic purification steps were performed on a GE Healthcare ÄKTA$_{FPLC}$ system, equipped with two pumps, a mixer, a UV detector and a fraction collector, and operated in a chromatography refrigerator at 4 ^{o}C.

The cell pellet (from either long or short induction cultures) was resuspended in ice-cold Buffer A1 (20 mM sodium phosphate buffer, pH 7.0, 0.5 M NaCl, 10 mM β-mercaptoethanol) plus 2% (v/v) Triton X-100, 70 mM lysozyme and 6 mM deoxycholic acid, and then lysed using an Aminco French Pressure Cell Press (SLM Instruments, Inc.). The supernatant, obtained by centrifugation at 18,000 rpm for 30 min, was filtered and injected onto a HisTrap HP Ni-NTA column (GE Healthcare) pre-equilibrated with Buffer A1. The N-terminally His-tagged SUMO-hGα_{i1} fusion protein eluted in ~60% Buffer B (20 mM sodium phosphate, pH 7.0, 0.5 M NaCl, 10 mM β-mercaptoethanol, 400 mM imidazole). Fractions containing the fusion protein were pooled and the imidazole and salt concentrations were reduced to ~ 60 and 125 mM, respectively, using two rounds of two-fold dilution and volume reduction. hGα_{i1} was cleaved from the His-tagged SUMO prodomain via incubation with ULP1-protease (obtained as a gift from Dr. Rhea Hudson at the Hospital for Sick Children, Toronto, Canada) at 25 °C for 2 hours. The sample was then dialyzed overnight at 5 °C into Buffer A2 (20 mM sodium phosphate, pH 7.0, 0.5 M NaCl, 10 mM β-mercaptoethanol, 15 mM imidazole). In order to isolate cleaved hGα_{i1} from His-tagged SUMO, the dialyzed sample was again injected onto the Ni-NTA column, this time pre-equilibrated with Buffer A2. The purification run consisted of a step gradient from Buffer A2 to 100% Buffer B. Fractions containing hGα_{i1} (which eluted prior to the imidazole gradient) were pooled, dialyzed at 5 °C into Superdex buffer (50 mM sodium phosphate, pH 7.2, 0.15 M NaCl, 10 mM β-mercaptoethanol), then concentrated to ~2-4 mL and injected in 0.25 mL portions onto a Superdex 75 10/300 GL size exclusion column (GE Healthcare). The concentration and yield of the final pure sample were determined by measuring the absorbance of a small sample denatured in 6 M guanidinium hydrochloride at 280 nm using a BioMate 3 UV-Vis spectrometer (Thermo Scientific) [16].

Fluorescence-based activity assay

BODIPY®FL GTP-γ-S (Life Technologies) was used to monitor the activity of purified $hG\alpha_{i1}$ and $hG\alpha_{i1}$-Δ31, using similar protocols to those described by others [15, 17]. A series of emission spectra were recorded over a wavelength scan range of 500 – 575 nm (λ_{ex} = 485nm) using a Perkin Elmer LS 50B Luminescence Spectrometer. At time = 0 min a wavelength scan of 500 nM BODIPY®FL GTP-γ-S in fluorescence buffer (10 mM tris, pH 8.0, 1 mM EDTA, and 10 mM $MgCl_2$) was recorded. Immediately after that baseline spectrum was obtained, purified protein in fluorescence buffer was added, to a final concentration of 2 µM. Nine more wavelength scans were then recorded, with a ten minute dark period between each scan. Each blank run was performed in the same manner, except that fluorescence buffer-only was mixed with the BODIPY®FL GTP-γ-S solution after the initial scan at t = 0 min. Each experiment was repeated in triplicate. Intensity data were processed using Spekwin32 [18] and Sigmaplot [19] software.

NMR spectroscopy of ^{15}N-labeled $hG\alpha_{i1}$ and $hG\alpha_{i1}$-Δ31

For NMR analysis, the purified ^{15}N isotope-labeled protein was dialyzed against PIPES buffer (20 mM PIPES, pH 7.0, 50 mM NaCl, 3 mM dithiothreitol, 5 mM $MgCl_2$, and 5% glycerol) until the phosphate buffer content was less than 1 µM. GDP was then added to a final concentration of 20 µM, followed by pre-mixed AlF_4^- (made from 10 mM NaF and 300 µM $AlCl_3$). The AlF_4^- serves as an analog of the γ-phosphate of GTP: it binds very tightly in the Gα active site along with Mg^{2+} and GDP, thereby allowing Gα to form a highly stabilized transition state conformational mimic [20]. The final NMR sample also contained 0.2 mM DSS, 2 mM NaN_3, and 10% D_2O. All spectra were recorded on either 800 or 900 MHz Varian spectrometers (equipped with a room temperature probe and a cryoprobe, respectively) at the National Magnetic Resonance Facility at Madison (NMRFAM) in Wisconsin.

For the temperature titrations, 2D [^{15}N,^{1}H] TROSY-HSQC spectra were recorded using a 0.3 mM sample of full-length $hG\alpha_{i1}$ between 20 and 35 °C, in 5 °C increments. Each spectrum utilized 64 scans and 128 x 1024 complex points in t_1 and t_2 respectively. The 900 MHz spectrum of full length $hG\alpha_{i1}$ at 35 °C utilized 32 scans and 128 x 872 complex points. All

spectra of the truncated hGα_{i1}-Δ31 protein were performed on a 0.2 mM sample at 900 MHz and 35 °C, with 24 scans per spectrum and 188 x 1024 complex points.

Spectra were processed and visualized using NMRPipe/NMRDraw and NMRViewJ software [21, 22].

Results and Discussion

Expression of hGα_{i1} and hGα_{i1}-Δ31 in various plasmids and media

As part of a greater effort to perform NMR chemical shift assignment and protein dynamics experiments on apo- and RGS-bound hGα_{i1}, our goal here was to develop a protocol for obtaining high levels of isotopically-labeled recombinant full-length and N-terminally truncated wild-type human Gα_{i1} (named hGα_{i1} and hGα_{i1}-Δ31, respectively, Table 1) in *E. coli* using minimal media, as required for these high resolution studies. Our decision to produce and evaluate a truncated version of the Gα subunit stems from the fact that this region is known to be unstructured in solution, causing intense peaks in the NMR spectra and obstructing other well-resolved peaks (as will be discussed in a following section). Table 2 provides a representative list of the various combinations of expression host, plasmid, and growth conditions that were tested on full-length hGα_{i1}, along with subsequent yields of the purified recombinant protein. Initial expression trials using non-pET-SUMO plasmid expression systems in pure minimal growth media were characterized by low Gα yields and slow growth rates. Media supplemented with various amounts of LB showed accelerated rates, but still low quantities of the soluble protein for NMR characterization (see Figure 1A and B and Table 2 for illustrative examples).

Improved hGα_{i1} yields can be attributed, to a large extent, to the use of the pET-SUMO expression system. pET-SUMO has been used by numerous labs to improve the solubility and/or yield of difficult proteins [23]. In this fusion-based system, we attached His-tagged small ubiquitin-related modifier (SUMO) protein to the N-terminus of hGα_{i1} and hGα_{i1}-Δ31. The SUMO fusion seems to provide only modest improvements in the solubility of hGα_{i1}; however, it significantly increases protein expression (Figure 1C-E), and it aids in simplifying purification. One other major benefit of this expression system is that it utilizes an extremely specific enzyme, ULP-1 protease, to cleave His-tagged SUMO from hGα_{i1} when it is no longer needed, with very

little protein loss (close to 0%). This is compared to other methods used to cleave hexahistidine tags, which can result in losses of 25 – 50% Gα [11].

We did not truly see the benefits of using the pET-SUMO expression system, however, until we combined it with the CodonPlus-RIPL strain of *E. coli* BL21(DE3) cells (Stratagene) and BioExpress rich media (Cambridge Isotope Labs) as a minimal medium supplement (Table 2). BL21-CodonPlus (DE3)-RIPL cells express increased quantities of tRNAs that recognize codons in the heterologous DNA that are rare in bacteria (but common to eukaryotic organisms). These additional tRNAs appear to be important factors in successful expression of h$G\alpha_{i1}$ in minimal medium. However, we have found that the CodonPlus strain works well only when the minimal medium is supplemented with BioExpress, since these particular cells have been observed to express our protein poorly in minimal media alone or with LB (Figure 1C, Table 2). Clearly, our standard minimal medium is missing some ingredients that are critical to the healthy activity of the CodonPlus strain.

Standard versus decreased induction temperatures

Shortly after our transition to using the pET-SUMO expression system we performed a trial expression of h$G\alpha_{i1}$ in which we induced at a lowered temperature (25 ^{o}C) for 15 hours rather than 37 ^{o}C for 3 hours. It had been reported previously that a lower induction temperature resulted in a drastically larger portion of h$G\alpha_{i1}$ in the soluble fraction and a correspondingly increased yield of soluble protein [11]. Our first trial using the lower induction temperature (in regular minimal medium), however, showed only a marginal increase in the yield of purified protein (see Table 2). As a result, we stopped pursuing the lower temperature induction and focused on optimizing the expressions using the shorter timeframes at 37 ^{o}C. Once we observed increased success in using the combination of pET-SUMO vector, BL21-CodonPlus (DE3)-RIPL cells and BioExpress rich media supplement, we decided to revisit the lower temperature induction (30 ^{o}C for 15 hours). This time, the decreased induction temperature resulted in a significant improvement in the yield of purified soluble protein: 39 mg pure ^{15}N h$G\alpha_{i1}$ from a 1 L culture, compared to a maximum of 8 mg from the same type of culture induced at 37 ^{o}C (Table 2). Interestingly, this 5-fold increase in yield seems to be attributed almost exclusively to an increase in the proportion of soluble protein rather than an increase in overall protein expression: compare the sizes of the bands shown from the 3-hour inductions in Figure 1D to

that from the 15-hour induction shown in Figure 1E. Thus, as a result of these expression tests we have successfully developed a method to prepare quantities of isotopically-labeled $hG\alpha_{i1}$ and $hG\alpha_{i1}$-Δ31 that are sufficient to produce several concentrated samples for NMR studies.

Purification of SUMO fusions of $hG\alpha_{i1}$

Despite using combined French press and sonication methods as well as 2% Triton X-100 detergent for cell lysis, SDS-PAGE analysis shows that a considerable amount of $hG\alpha_{i1}$ remains in the insoluble fraction; this is true even for the lower temperature expression protocol, even though the ratio of insoluble to soluble protein appears to be lower (data not shown). Although early attempts were made to extract insoluble SUMO-$hG\alpha_{i1}$ fusion protein from the cell pellet using Triton X and 8 M urea, refolding proved to be difficult using rapid dilution or slower dialysis techniques; in all cases, refolded protein precipitated both before and after SUMO protease cleavage. Because refolded $G\alpha_{i1}$ has been reported to remain in an inactive molten globule rather than its native state [15], further refolding attempts were not pursued.

We have, however, succeeded in purifying the His-tagged SUMO-$hG\alpha_{i1}$ and SUMO-$hG\alpha_{i1}$-Δ31 fusion proteins that are expressed in the soluble fraction. Both full-length and truncated forms can be purified by identical protocols, utilizing Ni-NTA nickel-affinity columns first for separating the His-tagged SUMO fusion construct from other cellular proteins, and second for isolating Gα from SUMO after cleavage by the SUMO-specific protease ULP-1. This is followed by Superdex 75 size exclusion chromatography to further purify the Gα subunits. Because purification procedures are identical for both $hG\alpha_{i1}$ and $hG\alpha_{i1}$-Δ31, the following discussion will focus on the results from purification of $hG\alpha_{i1}$ only.

The fusion protein binds strongly to the first Ni-NTA column, and is successfully eluted over a wide range of fractions between 100 and 250 mM imidazole, with the majority of cellular proteins and DNA eluting prior to SUMO-$hG\alpha_{i1}$ (Figure 2A). In order to prevent precipitation of the fusion protein, it is necessary to keep the sample on ice when diluting (to decrease the imidazole and salt concentrations prior to protease cleavage) and concentrating the pooled fractions containing SUMO-$hG\alpha_{i1}$. Maintaining a low sample temperature during these earlier steps ultimately helps to prevent further precipitation during the subsequent two-hour SUMO protease cleavage at room temperature (25 °C) and dialysis (5 °C) into Buffer A2. In an attempt to further increase the stability of the Gα subunits, we also tried to purify the protein in non-

phosphate buffer containing GDP using 50 mM PIPES, pH 7.2, 10 mM 2-mercaptoethanol, 0.15 M NaCl, 5 mM $MgCl_2$, and 50 µM GDP. When combined with maintaining a low sample temperature, the use of this GDP-PIPES buffer virtually eliminates precipitation of the recombinant protein during the early stages of purification. However, the final purification step does not yield a sample with the same purity as when phosphate buffer is used (without GDP): there are still many impurities remaining, as seen by SDS-PAGE (data not shown). As a result of this low purity, the GDP-PIPES protocol has not been pursued any further.

As expected, cleaved (and now untagged) $hG\alpha_{i1}$ elutes in the column void volume during the second Ni-NTA purification step (Figure 2B). Interestingly, the UV trace shows an elution plateau rather than a Gaussian-shaped peak for $hG\alpha_{i1}$, a result common to all post-cleave Ni-NTA purifications we have performed. His-tagged SUMO, non-specifically bound proteins, and uncleaved fusion protein are eluted from the column using a 400 mM imidazole step gradient. The gel in Figure 2B clearly shows that ULP-1 protease cleavage is essentially 100% complete; this is illustrated by the absence of a band, corresponding to SUMO-$hG\alpha_{i1}$, which would elute at the high imidazole concentration, along with cleaved SUMO (lane 2 of gel). This high efficiency cleavage thereby minimizes protein loss due to the removal of His-tagged SUMO.

In the Superdex (size-exclusion) purification step, $hG\alpha_{i1}$ elutes as a single narrow peak over three or four 0.5 mL fractions (Figure 2C). Please note that while $hG\alpha_{i1}$-Δ31 uses an identical procedure to $hG\alpha_{i1}$, it elutes slightly later from the Superdex column due to its lower molecular weight. The final pooled sample is of high purity as seen by SDS gel (Figure 2C). In early purification trials, the absence of reducing agent in the buffer promoted the dimerization of $hG\alpha_{i1}$, as indicated by multiple poorly resolved peaks on the Superdex UV trace (data not shown). Addition of β-mercaptoethanol (10 mM) was sufficient to prevent oligomerization during all purification steps.

The average yield of the soluble pure WT $hG\alpha_{i1}$ subunit is 6 mg/L of culture using the 3-hour induction protocol, and an impressive 39 mg/L using the long, lower temperature induction protocol. To put these numbers into context, the protocols permit the preparation of one to four 0.5 mM NMR samples. Preparation of the truncated form of the subunit ($hG\alpha_{i1}$-Δ31) consistently yields ~50-60 % less protein than that of full-length $hG\alpha_{i1}$ (using either induction protocol). Specifically, for the low-temperature induction protocol, our yield of pure $^{13}C/^{15}N$ $hG\alpha_{i1}$-Δ31 averages 15 mg from 1 L of culture. This was unexpected, as we anticipated the truncated protein

would be more stable in general, which should have resulted in higher yields than the full-length protein. We also performed test expressions in deuterated medium and have obtained approximately 5-7 mg of pure $^{13}C/^{15}N/^{2}H$ $hG\alpha_{i1}$-Δ31 per liter of culture (corresponding to one ~0.5 mM NMR sample), using the same low-temperature induction and purification protocol as for the non-deuterated samples.

While the methods described work very well for $hG\alpha_{i1}$ and $hG\alpha_{i1}$-Δ31, we have not yet tested our protocol on any other Gα protein classes (such as $G\alpha_o$, $G\alpha_s$, $G\alpha_q$, etc.). However, after observing the significant increase in expression yields resulting from the use of the SUMO fusion, codon-rich *E. coli* cells, specialized rich medium and low temperature induction, it is conceivable that this method would work well for preparing isotopically labeled samples of other types of human Gα proteins.

Activity of $hG\alpha_{i1}$ monitored by fluorescence assay

A series of fluorescence-based experiments were performed to confirm that our purified soluble protein corresponded to correctly-folded active $hG\alpha_{i1}$ (or $hG\alpha_{i1}$-Δ31). A common test for Gα subunit activity is whether the protein is capable of binding GTP (or analogs). One such analog is BODIPY® FL GTP-γ-S, a commercially-available fluorescence probe that has become popular for monitoring GTP binding by Gα subunits [24]. When alone in solution, the fluorescence intensity of the BODIPY® is quenched by the intramolecular guanine base; once the GTP moiety binds to Gα the BODIPY®, quenching is relieved and the fluorescence intensity increases [17].

We performed three sets of binding assays in which our $hG\alpha_{i1}$ protein sample (with no nucleotide in the binding site) was added to buffer containing BODIPY® FL GTP-γ-S and the change in fluorescence was then monitored over 500 – 575 nm for 90 minutes. In the blank experiment, buffer was added to the BODIPY® solution instead of protein. The results show a clear increase in fluorescence with time when $hG\alpha_{i1}$ is present in solution (Figure 3A and B), compared to no change for the blank solution (Figure 3B), thus confirming that the prepared protein is correctly folded and active. Similar results were obtained for fluorescence experiments on $hG\alpha_{i1}$-Δ31, indicating that the removal of the disordered N-terminal residues does not impact the functional activity of the Gα subunit.

NMR analysis and thermal titration of Mg^{2+}/GDP-AlF_4^--bound ^{15}N-labeled h$G\alpha_{i1}$

Our initial attempts at NMR spectroscopic observation of h$G\alpha_{i1}$ were characterized by broad linewidths and spectral overlap (data not shown). A couple of approaches modifying both the acquisition of NMR spectra and the protein sample itself were taken in order to alleviate these issues. Combating the broader linewidths and low intensity peaks of the slowly tumbling 45 kDa protein required the use of high-field spectrometers (800-900 MHz), often equipped with cryoprobes; in addition, the use of 2D [^{15}N,^{1}H] TROSY-HSQC experiments provided a modest but noticeable improvement in the resolution of overlapped peaks over the conventional 2D [^{15}N,^{1}H] HSQC [25].

The issue of protein stability at high concentrations was addressed by the addition of 3 mM dithiothreitol and 5% glycerol, as described previously [12]; the reducing agent prevents dimerization of the alpha subunit, and glycerol provides overall stability in solution through preferential hydration and protein compaction [26]. Interestingly, even with these additives the NMR sample has a tendency to form a very small amount of precipitate. Once that precipitate is formed, however, it does not increase and maintains a stable equilibrium with the soluble state without interference with the NMR experiments. We have maintained the protein concentration in the NMR sample at a maximum of 0.5 mM in order to prevent increased precipitation (although we have not done extensive studies of Gα stability at higher protein concentrations).

In order to determine the optimal temperature for future experiments, a titration was performed on Mg^{2+}/GDP-AlF_4^--bound ^{15}N h$G\alpha_{i1}$, in which 2D [^{15}N,^{1}H] TROSY-HSQC spectra were recorded at 20, 25, 30, and 35 °C. Spectra were not recorded at temperatures higher than 35 ^{o}C due to concerns about long-term protein stability and sample evaporation. The spectrum of h$G\alpha_{i1}$ at 20 °C (Figure 4A) shows relatively well-dispersed peaks, indicating that the protein is folded; however, it is also clear that there are numerous broadened and missing peaks at that temperature. Both the spectral peak widths and intensities improve significantly with increased sample temperature, with the best peak separation observed at 35 °C (Figure 4B, compare also peaks within the ovals in Figure 4A and B). If all spectral resonances were observable, we would expect to see 324 peaks corresponding to NH groups and 54 peaks corresponding to side chain NH_2s. Since it is often difficult to unambiguously determine whether a peak is from an NH_2 or NH within the upper right quadrant of the HSQC spectrum, we have counted all peaks together;

as a result, we can identify 347 peaks out of a possible total of 378, which corresponds to 92% observable resonances in the [^{15}N,^{1}H] HSQC spectrum.

The narrowed linewidths at higher temperatures can be explained in part by an increased tumbling rate of the large protein, but the changes also suggest that several segments of the protein are undergoing μs-ms timescale motions (conformational exchange) at 20 ^{o}C which are accelerated to faster timescales at the higher temperature. The sharpening of peaks at 35 ^{o}C is also accompanied by significant chemical shift changes of some amide groups in hGα_{i1}, further supporting evidence for a change in the distribution of its ensemble of conformations. Because AlF_4^- binds so strongly to the Gα active site in the presence of GDP and Mg^{2+}, apo-holo exchange provides a negligible contribution to the observed chemical shift changes.

These simple experiments illustrate the value of studying the motions within the Gα subunit, in that observed widespread flexibility is likely to play an important role in protein function. Consequently, our results bode well for future NMR relaxation experiments that are planned for this protein, in which we will probe these internal motions in more detail.

Preparation and preliminary NMR characterization of Mg^{2+}/GDP-AlF_4^--bound ^{15}N-labeled hGα_{i1}-Δ31

A persistent issue in all full-length hGα_{i1} spectra is the presence of very intense peaks towards the center of the spectrum (Figure 4, insets). We hypothesized that these peaks were most likely due to the N-terminal region of hGα_{i1}, which is known from crystallography and fluorescence data to be unstructured when Gα is not bound within the heterotrimeric complex [27, 28]. It is for this reason that we prepared hGα_{i1}-Δ31, a truncated form of the protein in which the first 31 residues of the N-terminus are excluded. To test whether the removal of these residues would improve NMR spectra, we recorded a 2D [^{15}N,^{1}H] TROSY-HSQC spectrum of Mg^{2+}/GDP-AlF_4^--bound ^{15}N-labeled hGα_{i1}-Δ31 at 35 °C (Figure 5). The spectrum (in red) clearly shows a significant decrease in the number of high-intensity peaks clustered at the center (compared to hGα_{i1}, in black), as well as an increase in resolution of the remaining peaks. Analysis of a magnified region of the overlaid spectra from hGα_{i1} and hGα_{i1}-Δ31 (Figure 5B) reveals that while most peak positions are unaffected by the truncation, a few chemical shift changes are observed. However, because our fluorescence assays indicate full Gα activity for the truncated protein, it appears that those changes likely correspond to residues local to the

truncated segment and that subunit activity is not negatively impacted. Given that the removal of these residues does not inhibit normal GTP-binding activity and improves the quality of the resulting NMR spectra, we will likely perform our future NMR chemical shift and relaxation studies on hGα_{i1}-Δ31 rather than full-length hGα_{i1}.

Conclusions

In this paper we have presented the first published report of the preparation of functionally active, isotopically-labeled wild-type human Gα_{i1} for NMR studies. Efforts to obtain large quantities of highly purified protein were aided in part by the SUMO fusion protein, as well as a strain of *E. coli* that produces the rare codons required to express human protein, a special isotope-labeled rich medium supplement, and the use of decreased temperature during induction of expression. Preliminary experiments showed that high-quality NMR spectra can be obtained for full-length wild-type hGα_{i1}, with the exception that the center of the spectrum is semi-obscured by very intense peaks corresponding to the unstructured N-terminus. Preparation of an N-terminal truncated variant, hGα_{i1}-Δ31, alleviated this issue and is therefore likely to be chosen for use in future NMR experiments.

Our motivation for producing isotope-labeled human Gα_{i1} subunit is to prepare for NMR studies investigating the differences in protein dynamics in Gα_{i1} and RGS proteins in their apo and complexed forms and the role of these motions in their respective binding selectivities and affinities. Since recent research has shown that allosteric effects and selectivity in protein-protein interactions may be mediated by changes in widespread intramolecular fluctuations [29, 30], we believe that studies of the internal dynamics of RGS and Gα proteins may provide us with crucial information that can improve or change our understanding of the regulation of signal transmission. The evidence we have already observed for significant conformational exchange throughout hGα_{i1} (as seen through our NMR-monitored temperature titration) supports the idea that such studies will provide worthwhile new insight into the functional mechanisms of these proteins.

Acknowledgements

We would like to sincerely thank Drs. Voula Kanelis and Rhea Hudson at the University of Toronto and the Hospital for Sick Children in Toronto, respectively, for introducing the SUMO fusion protein system to us and for providing the starting materials. We are also grateful to Dr. Larry Baresi at California State University, Northridge for the use of his French press. Many thanks to Dr. Marco Tonelli for his assistance in running experiments at NMRFAM, and for his suggestion that we prepare the truncated protein. This work is generously supported by NSF grant MCB-1158177, as well as start-up funds and internal grants from the College of Science and Mathematics and the Office of Sponsored Projects at California State University, Northridge.

References

[1] W.M. Oldham, H.E. Hamm, Structural basis of function in heterotrimeric G proteins, Q. Rev. Biophys. 39 (2006) 117-166.
[2] A.J. Kimple, D.E. Bosch, P.M. Giguere, D.P. Siderovski, Regulators of G-Protein Signaling and Their Gα Substrates: Promises and Challenges in Their Use as Drug Discovery Targets, Pharmacol. Rev. 63 (2011) 728-749.
[3] P. Chidiac, A.A. Roy, Activity, regulation, and intracellular localization of RGS proteins, Receptor. Channel. 9 (2003) 135-147.
[4] F.J. Moy, P.K. Chanda, M.I. Cockett, W. Edris, P.G. Jones, K. Mason, S. Semus, R. Powers, NMR structure of free RGS4 reveals an induced conformational change upon binding Gα, Biochemistry-US 39 (2000) 7063-7073.
[5] M. Soundararajan, F.S. Willard, A.J. Kimple, A.P. Turnbull, L.J. Ball, G.A. Schoch, C. Gileadi, O.Y. Fedorov, E.F. Dowler, V.A. Higman, S.Q. Hutsell, M. Sundstrom, D.A. Doyle, D.P. Siderovski, Structural diversity in the RGS domain and its interaction with heterotrimeric G protein α-subunits, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 6457-6462.
[6] J.J. Tesmer, D.M. Berman, A.G. Gilman, S.R. Sprang, Structure of RGS4 bound to $AlF4^-$-activated $G_{i\alpha1}$: Stabilization of the transition state for GTP hydrolysis, Cell 89 (1997) 251-261.
[7] A. Mittermaier, L.E. Kay, New tools provide new insights in NMR studies of protein dynamics, Science 312 (2006) 224-228.
[8] R.G. Smock, L.M. Gierasch, Sending signals dynamically, Science 324 (2009) 198-203.
[9] H.P. Zhang, T. Nagasima, F. Hayashi, S. Yokoyama, Solution structure of the RGS domain of Regulator of G-protein Signaling 7, unpublished, 2006, Kanagawa, Japan.
[10] W.K. Greentree, M.E. Linder, Purification of recombinant G protein alpha subunits from Escherichia coli, Methods Mol. Biol. 237 (2004) 3-20.
[11] E. Lee, M.E. Linder, A.G. Gilman, Expression of G-protein alpha subunits in Escherichia coli, Method. Enzymol. 237 (1994) 146-164.
[12] N.G. Abdulaev, C. Zhang, A. Dinh, T. Ngo, P.N. Bryan, D.M. Brabazon, J.P. Marino, K.D. Ridge, Bacterial expression and one-step purification of an isotope-labeled heterotrimeric G-protein alpha-subunit, J. Biomol. NMR 32 (2005) 31-40.

[13] N.G. Abdulaev, T. Ngo, E. Ramon, D.M. Brabazon, J.P. Marino, K.D. Ridge, The receptor-bound "empty pocket" state of the heterotrimeric G-protein alpha-subunit is conformationally dynamic, Biochemistry-US 45 (2006) 12986-12997.
[14] K.D. Ridge, N.G. Abdulaev, C. Zhang, T. Ngo, D.M. Brabazon, J.P. Marino, Conformational changes associated with receptor-stimulated guanine nucleotide exchange in a heterotrimeric G-protein alpha-subunit: NMR analysis of GTPgammaS-bound states, J. Biol. Chem. 281 (2006) 7635-7648.
[15] E. McCusker, A.S. Robinson, Refolding of G protein α subunits from inclusion bodies expressed in *Escherichia coli*, Protein Expr. Purif. 58 (2008) 342-355.
[16] C.N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, How to measure and predict the molar absorption coefficient of a protein, Protein Sci. 4 (1995) 2411-2423.
[17] R.J. Kimple, M.B. Jones, A. Shutes, B.R. Yerxa, D.P. Siderovski, F.S. Willard, Established and emerging fluorescence-based assays for G-protein function: heterotrimeric G-protein alpha subunits and regulator of G-protein signaling (RGS) proteins, Comb. Chem. High Throughput Screen. 6 (2003) 399-407.
[18] F. Menges, Spekwin32 - optical spectroscopy software, v.1.71.6, 2012, http://www.effemm2.de/spekwin.
[19] Systat, SigmaPlot, v.10.0.1.2, 2006, Chicago, IL.
[20] J. Bigay, P. Deterre, C. Pfister, M. Chabre, Fluoroaluminates activate transducin-GDP by mimicking the gamma-phosphate of GTP in its binding site, FEBS Lett 191 (1985) 181-185.
[21] F. Delaglio, NMRDraw, v.5.3, 2010, North Potomac, MD.
[22] B.A. Johnson, NMRViewJ, v.8.0.b64, 2007, Newark, NJ.
[23] M. Satakarni, R. Curtis, Production of recombinant peptides as fusions with SUMO, Protein Expr Purif 78 (2011) 113-119.
[24] E.E. Jameson, R.A. Roof, M.R. Whorton, H.I. Mosberg, R.K. Sunahara, R.R. Neubig, R.T. Kennedy, Real-time detection of basal and stimulated G protein GTPase activity using fluorescent GTP analogues, J. Biol. Chem. 280 (2005) 7712-7719.
[25] K. Pervushin, R. Riek, G. Wider, K. Wüthrich, Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 12366-12371.
[26] V. Vagenende, M.G. Yap, B.L. Trout, Mechanisms of protein stabilization and prevention of protein aggregation by glycerol, Biochemistry-US 48 (2009) 11084-11096.
[27] D.E. Coleman, A.M. Berghuis, E. Lee, M.E. Linder, A.G. Gilman, S.R. Sprang, Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis, Science 265 (1994) 1405-1412.
[28] M. Medkova, A.M. Preininger, N.J. Yu, W.L. Hubbell, H.E. Hamm, Conformational changes in the amino-terminal helix of the G protein alpha(i1) following dissociation from Gbetagamma subunit and activation, Biochemistry 41 (2002) 9962-9972.
[29] K.K. Frederick, M.S. Marlow, K.G. Valentine, A.J. Wand, Conformational entropy in molecular recognition by proteins, Nature 448 (2007) 325-329.
[30] S.R. Tzeng, C.G. Kalodimos, Protein dynamics and allostery: an NMR view, Curr. Opin. Struct. Biol. 21 (2011) 62-67.

Figures

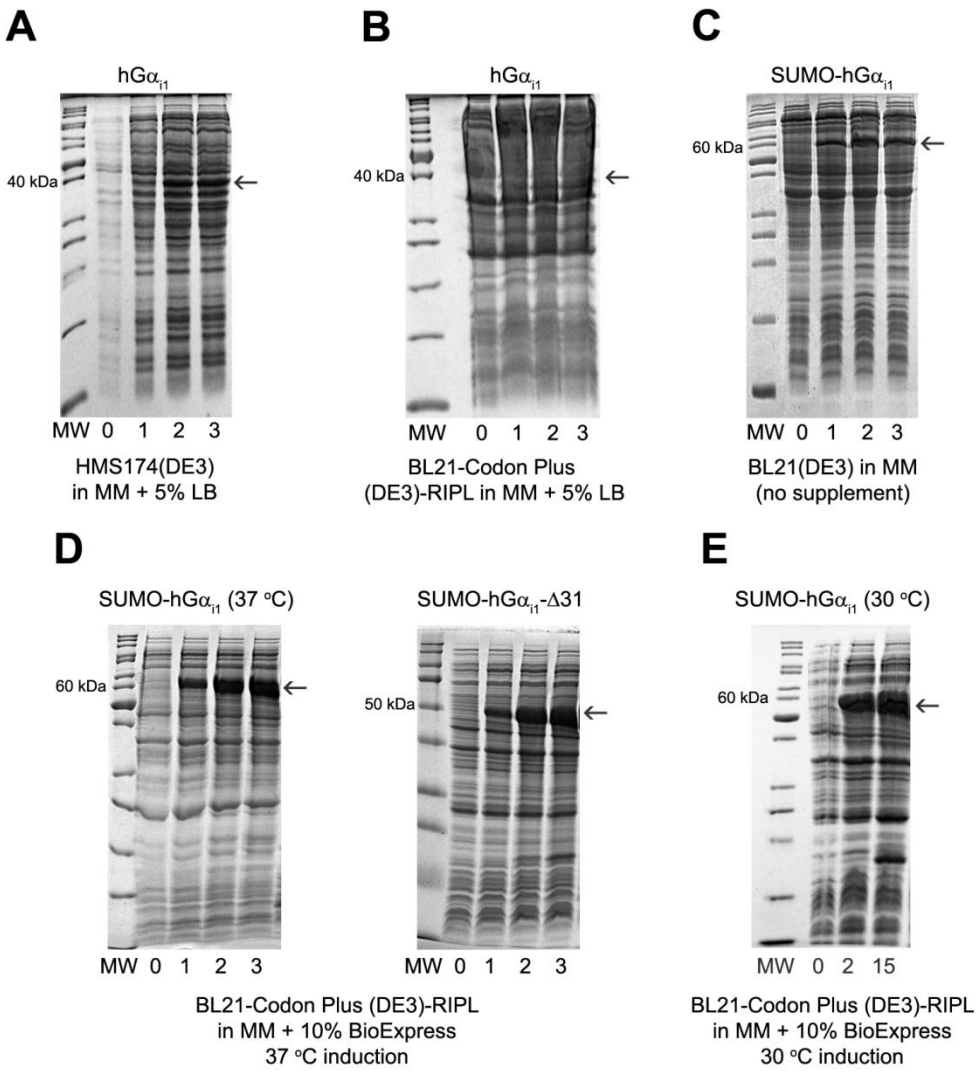

Figure 1: SDS-PAGE gel comparisons of representative expressions of hGα_{i1} and hGα_{i1}-Δ31. All expression strains are from *E. coli*. Numbers shown under each gel lane represent time after induction by IPTG (in hours). Note that gels in A-D show 37 ^{o}C inductions, while E corresponds to a 30 ^{o}C induction. Arrows beside gels are used to indicate the band position of the desired protein. Gels of expressions shown in A and B utilized the pET-15b vector whereas C-E are representative of pET-SUMO expressions. Note that the hGα_{i1} and hGα_{i1}-Δ31 recombinant proteins appear at a higher molecular weight in the gels in C-E (compared to A and B) because they are part of the SUMO fusion (and therefore migrate as ~55 kDa and ~50 kDa proteins, respectively). SDS-PAGE gels show (A) hGα_{i1} expressed using HMS174(DE3) cells in minimal media (MM) and 5% Luria Broth (LB); (B) hGα_{i1} expressed in BL21-CodonPlus (DE3)-RIPL cells in MM and 5% LB; (C) SUMO-hGα_{i1} expressed in BL21(DE3) cells in MM; (D) SUMO-hGα_{i1} and SUMO-hGα_{i1}-Δ31 expressed using BL21-CodonPlus (DE3)-RIPL cells in MM and 10% BioExpress; (E) SUMO-hGα_{i1} expressed using BL21-CodonPlus (DE3)-RIPL cells in MM and 10% BioExpress (with induction at 30^{o}C). Gamma adjustments were made on all gels to increase the image contrast, but they did not alter the appearance of the gel bands.

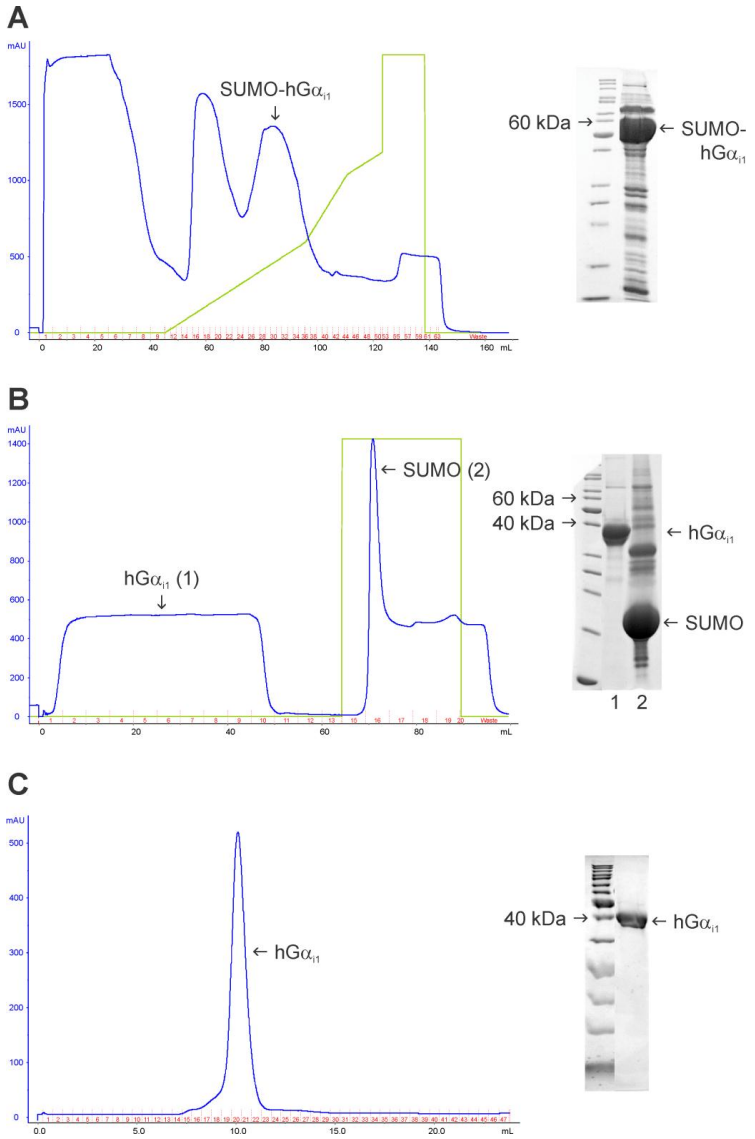

Figure 2: Representative UV traces and SDS-PAGE gel data from FPLC purification of SUMO-hGα_{i1}. Imidazole concentration gradients are indicated by green lines, and blue lines represent UV absorbance, at 280 nm, of fractions eluting from the columns (y-axis). Red tick marks and numbers along the x-axis refer to fraction numbers. The left-most lane in each gel corresponds to a molecular weight marker. (A) First step of Ni-NTA purification of SUMO-hGα_{i1}. A single fraction was sampled for the SDS gel. Fractions encompassing the indicated peak were pooled for cleavage and processed as described. (B) Second Ni-NTA purification step, after cleavage of hGα_{i1} from SUMO. The contents of peak 1 from the UV trace are shown in lane 1 of the gel, confirming that hGα_{i1} elutes prior to most other proteins in 15 mM imidazole. The contents of peak 2 from the UV trace, shown in lane 2 of the gel, indicate that SUMO and numerous impurities elute from the column in 400 mM imidazole. (C) Representative Superdex 75 size exclusion column run used as a final purification step for hGα_{i1}. The UV trace and gel show a highly pure sample at the appropriate molecular weight (~41 kDa). Gamma adjustments were made on all gels to increase the image contrast, but they did not alter the appearance of the gel bands.

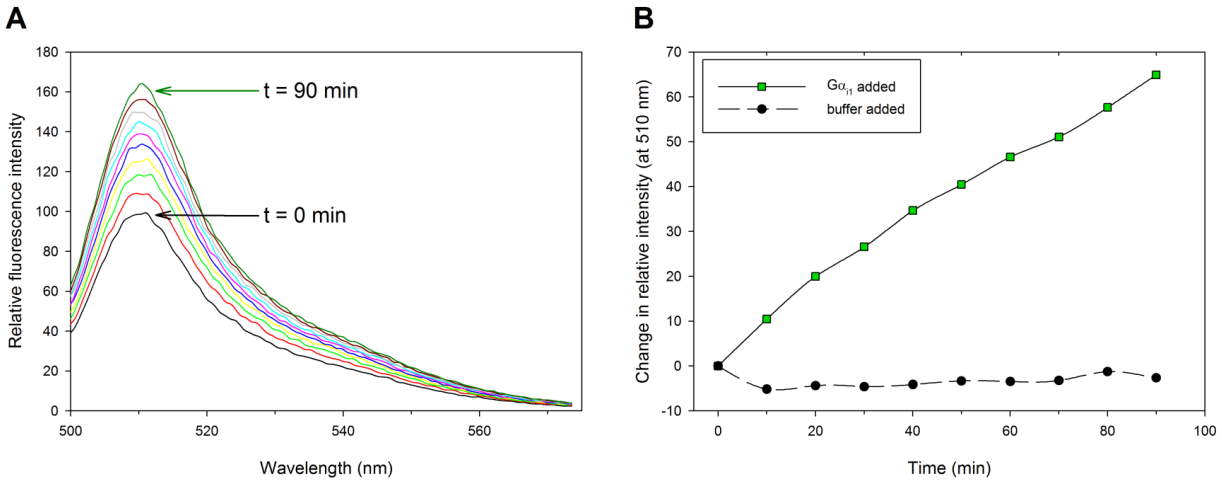

Figure 3: Fluorescence assays of hGα_{i1} activity. (A) Representative set of wavelength scans (from 500 to 575 nm) of BODIPY® FL GTP-γS binding to hGα_{i1}, monitoring increasing fluorescence intensity over time. The scan at time t = 0 min was recorded immediately before hGα_{i1} was added to the cuvette. Wavelength scans were recorded every 10 minutes, to a maximum of 90 minutes. (B) A comparison of the change in relative fluorescence intensity, recorded at 510 nm, as a function of time after adding hGα_{i1} (squares) or buffer (circles) to the cuvette containing BODIPY® FL GTP-γS. Experiments were repeated three times each – all results are consistent with the data set shown here.

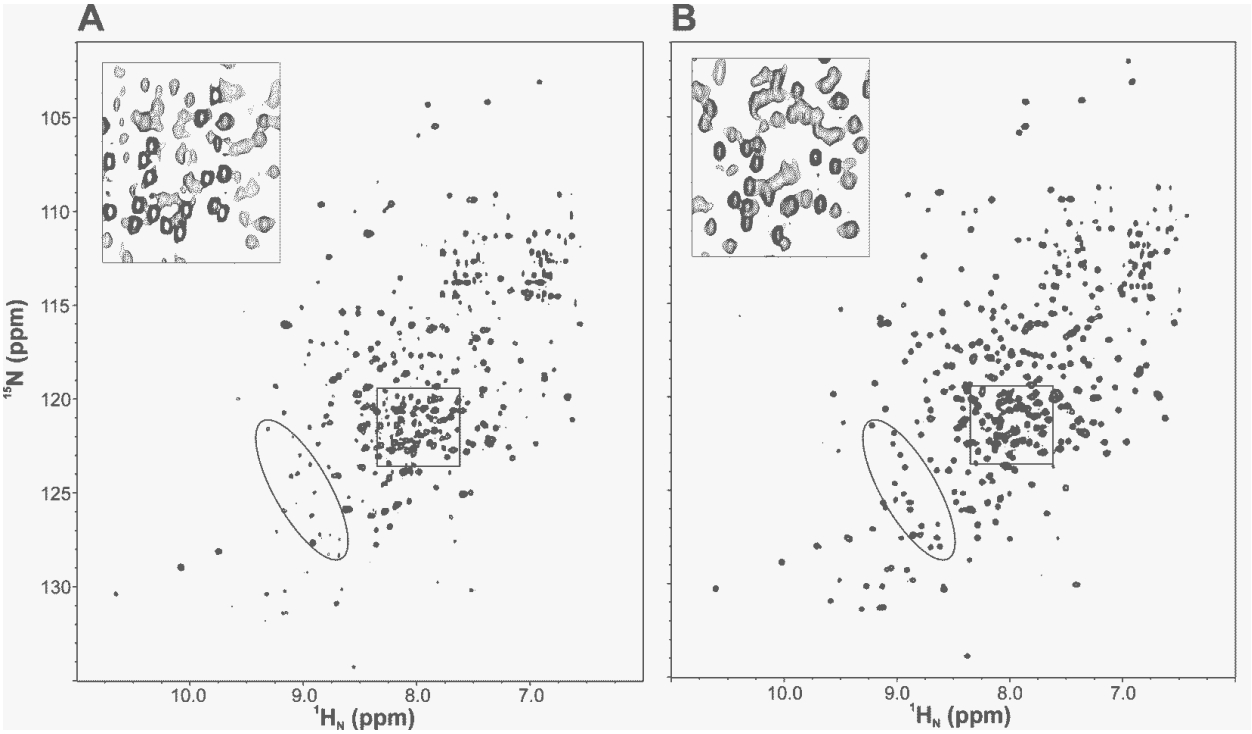

Figure 4: 2D [^{15}N,^{1}H] TROSY-HSQC spectra of Mg^{2+}/GDP-AlF_4^--bound hGα_{i1}, recorded at (A) 20 ^{o}C and (B) 35 ^{o}C on a 800 MHz NMR spectrometer. The boxed area in the center of each spectrum is shown as a magnified inset in the left top corner to illustrate improvements in peak resolution at the higher temperature, and to emphasize the presence of extremely intense peaks (corresponding to the unstructured N-terminus in hGα_{i1}). The regions outlined with ovals are highlighted to show examples of the significant improvement in spectral peak intensities at 35 ^{o}C (B) compared to 20 ^{o}C (A).

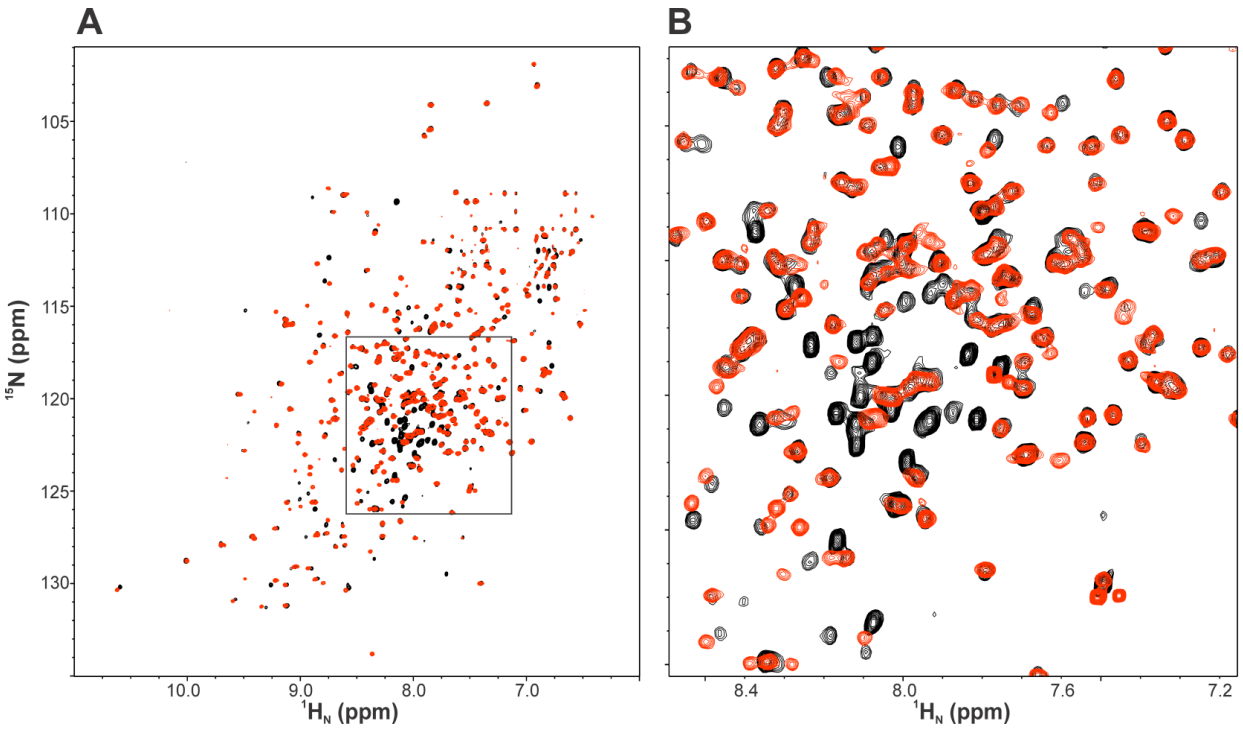

Figure 5: Overlay of 2D [^{15}N,^{1}H] TROSY-HSQC spectra of Mg^{2+}/GDP-AlF_4^--bound hGα_{i1} (black) and hGα_{i1}-Δ31 (red), recorded at 35 ^{o}C on a 900 MHz NMR spectrometer (with cryoprobe). (A) shows the full spectrum and (B) shows a magnified portion (as delineated by the box at the center of the spectrum in (A)). The region magnified in (B) illustrates that the majority of residues removed from hGα_{i1} to make hGα_{i1}-Δ31 correspond to the most intense peaks in the center of the spectrum, confirming that the N-terminal region of hGα_{i1} is unstructured in solution. (B) also shows that most peaks common to both and hGα_{i1} and hGα_{i1}-Δ31 spectra overlay very closely.