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The Gβ3 splice variant associated with the C825T gene polymorphism is an unstable and functionally inactive protein

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Abstract

A spice variant of Gβ3, termed Gβ3s, has been associated with the C825T polymorphism in the Gβ3 gene and linked with many human disorders. However, the biochemical properties and functionality of Gβ3s remain controversial. Here, using multidisciplinary approaches including co-immunoprecipitation analysis and bioluminescence resonance energy transfer (BRET) measurements, we showed that unlike Gβ3, Gβ3s failed to form complexes with either Gγ or Gα subunits. Moreover, using a mutant Gγ2 deficient in lipid modification to purify Gβ3s from Sf9 cells without the use of detergents, we further showed that the failure of Gβ3s to form dimers with Gγ was not due to the instability of the dimers in detergents, but rather, reflected the intrinsic properties of Gβ3s. Additional studies indicated that Gβ3s is unstable, and unable to localize properly to the plasma membrane and to activate diverse Gβγ effectors including PLCβ2/3, PI3Kγ, ERKs and the Rho guanine exchange factor (RhoGEF) PLEKHG2. Thus, these data suggest that the pathological effects of Gβ3 C825T polymorphism may result from the downregulation of Gβ3 function. However, we found that the chemokine SDF1α transmits signals primarily through Gβ1 and Gβ2, but not Gβ3, to regulate chemotaxis of several human lymphocytic cell lines, indicating the effects of Gβ3 C825T polymorphism are likely to be tissue and/or stimuli specific and its association with various disorders in different tissues should be interpreted with great caution.

Keywords

Heterotrimeric G protein; GPCR; GNB3; signalling; polymorphism; chemotaxis

1. Introduction

G protein coupled receptors (GPCRs) are the largest family of cell surface receptors and play a critical role in many physiological processes, including neurotransmission, metabolism, cardiovascular regulation and leukocyte migration [1, 2]. Dysregulation of GPCRs has been associated with many diseases, including hypertension, obesity, diabetes and tumorigenesis [3-8].

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GPCRs transmit extracellular signals through the heterotrimeric G protein, which is composed of the Gα subunit and the obligate Gβγ dimer. Multiple isoforms of each subunit have been identified, including twenty-three Gα, six Gβ, and thirteen Gγ subunits [1, 2]. These different subunits can pair to form heterotrimeric G proteins with unique compositions that may define their specificity and selectivity in coupling to receptors and activation of downstream effectors [9]. Activation of G proteins is initiated by receptor-catalyzed exchange of GDP for GTP on Gα subunits, and the dissociation of Gα from Gβγ subunits. Both the activated Gα and Gβγ subunits can stimulate distinct downstream signaling cascades through diverse effectors, including phospholipases, kinases, ion channels and interacting proteins [1].

Six well characterized Gβ isoforms are encoded by five distinct genes, Gβ1, Gβ2, Gβ3, Gβ4 and Gβ5 [9]. Alternate splicing of the Gβ5 gene generates two Gβ5 isoforms, the long and short Gβ5. Additionally, a number of genetic polymorphisms in Gβ3 have been identified and are associated with the occurrence of various shorter Gβ3 splice variants [10-13]. One of the best characterized Gβ3 polymorphisms is a C825T polymorphism, which is associated with an alternative splicing of exon 9 in the Gβ3 gene, resulting in an in-frame deletion of 123-bp that encodes a WD40-repeat domain in Gβ3 [10]. The resultant Gβ3 splice variant is 41 amino acids shorter than the wild-type Gβ3 and is termed Gβ3s. The Gβ3 C825T polymorphism was originally identified in cultured lymphocytes from patients with essential hypertension and enhanced Na^+/H^+ exchange activity [10]. Subsequent studies found that the C825T allele occurs most frequently in Black Africans (79%), followed by Mongloids (46%) and Caucasians (36%) [14]. In population-based association studies, the Gβ3 C825T polymorphism has been associated with an increased risk for diverse disorders, including hypertension, obesity, diabetes, depression, and tumorigenesis [15-17]. It may also serve as a pharmacogenetic marker to predict treatment responses for various diseases [14]. Nevertheless, conflicting data regarding the association of C825T polymorphism with various disorders have also been reported [18-22].

Despite the lack of one WD40 domain, initial studies from Siffert's group showed that Gβ3s can still form dimers with various Gγ subunits in heterologous expression systems [23]. Moreover, as compared to the wild-type Gβ3, Gβ3s was found to display enhanced ability to mediate GPCR-stimulated GTP/GDP exchange on Gαi subunits and to stimulate a Gβγ effector, ERKs [13]. Based on these findings, it has been speculated that many disorders associated with the Gβ3 C825T polymorphism are mediated by the gain-of-function of Gβ3s signaling. However, in contrast to these findings, Ruiz-Velasco et al. reported that, when overexpressed in rat sympathetic neurons, Gβ3s failed to either form a complex with Gγ2 or Gαi2, or modulate N-type Ca^{2+} and G protein-gated inwardly rectifying K^+ channels [24]. Using a rabbit reticulocyte lysate expression system in vitro, Dingus et al. also reported that unlike Gβ3, Gβ3s did not form dimers with various Gγ subunits [25]. Nevertheless, whether Gβ3s can form complexes with Gγ subunits and stimulate many other Gβγ effector in cells has not been systematically evaluated. Moreover, the function of Gβ3 and Gβ3s has not been established in cells/tissues endogenously expressing these proteins. Thus, despite the fact that the physiological and pathological implications of Gβ3s have been extensively studied, the biochemical properties and functionality of Gβ3s remains uncertain. This may in part account for the current confusion about the significance of Gβ3 C825T allele in multiple disorders.

In this study, we used multiple approaches to systematically assess the ability of Gβ3s to function as a canonical Gβ protein. We show here that Gβ3s was deficient in interaction with either Gα or Gγ in various expression systems, using multi-disciplinary approaches including co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) assays. We further show that Gβ3s failed to couple to GPCRs and to stimulate diverse Gβγ

effectors including PLCβ, PI3Kγ, ERKs and RhoGEFs. Moreover, as compared to Gβ3, Gβ3s was less stable and did not localize properly to the plasma membrane. Accordingly, although the Gβ3s transcript can be identified in several human lymphocytic cell lines containing the C825T allele, these cells did not express detectable Gβ3s protein. Intriguingly, we found that SDF 1α-stimulated signaling and chemotaxis of these cells were not affected by downregulation of Gβ3/Gβ3s but abolished by inhibition of Gβ1 and Gβ2, suggesting that the function of Gβ3 and Gβ3s is stimuli and/or cell type dependent. Together, our findings demonstrate that Gβ3s is an unstable and functionally inactive protein. Therefore, it is likely that the pathological effects of Gβ3 C825T polymorphism result from a functional downregulation of Gβ3 activity. Nevertheless, out data indicate that the function of Gβ3 is stimuli and/or tissue specific. Thus, great caution should be exerted when interpreting the functional consequence of Gβ3 C825T polymorphism in different tissues.

2. Material and methods

2.1. Reagents

Mouse anti-FLAG (M2) antibody was from Sigma-Aldrich. Rat anti-HA antibody was from Roche Applied Science (Indianapolis, Indiana, USA). Rabbit anti-Gαi1, anti-Gα12 and anti-Gβ (T20) antibodies were from Santa Cruz Biotechnology (Santa Cruz). Rabbit anti-Gαq/11 antibody was from EMD Millipore. Mouse anti-Gαs and anti-His antibodies were from UC Davis/NIH NeuroMab Facility. AKT, phospho-AKT, ERK1/2, and phospho-ERK1/2 antibodies were from Cell Signaling Technology, Inc. Human SDF-1α was from PeproTech. Fura-2/AM, Alexa 488- and Alexa 568-conjugated secondary antibodies and Dynabeads protein G were from Life Technologies. Nickel -nitrilotriacetic acid-agarose (Ni-NTA) beads were from Qiagen. siRNAs against Gβ1, Gβ2 and Gβ1/2 were from Thermo Fisher Scientific Dharmacon. A siRNA against Gβ3 was from Life Technologies. All other materials were obtained with highest quality available.

2.2. Cell Culture

HEK293 cells and COS-7 cells were obtained from ATCC and grown at 37°C, 5% CO2 in DMEM (Life Technologies) containing 10% fetal bovine serum (FBS). Jurkat T cells were maintained in RPMI (Life Technologies) supplemented with 10% FBS. B lymphocytic cell lines GM19116C and GM18500B were from Coriell Cell Repositories and maintained in RPMI supplemented with 10% FBS. Sf9 insect cells were grown at 28 °C in Sf900 II serum-free medium (Life Technologies).

2.3. Plasmid and Viral Constructs

FLAG-tagged or untagged human Gβ3, Gβ3s, Gγ2, Gγ5, Gγ8, Gγ10 and Gγ12 in pcDNA3.1 were obtained from Missouri S&T cDNA Resource Center. For the bioluminescence resonance energy transfer (BRET) assay the cDNAs encoding Gβ3 and Gβ3s were cloned into pRluc-C (PerkinElmer), while the cDNAs encoding various Gγ subunits were cloned into pGFP^2-C (PerkinElmer). Baculoviruses encoding FLAG-tagged Gβ3 and Gβ3s, and 6 × His-tagged Gγ2 and Gγ5 were generated using the Gateway cloning and the Bac-To-Bac baculovirus expression systems (Life Technologies) as described. Lentiviruses encoding FLAG-tagged Gβ3 and Gβ3s were generated by cloning FLAG-tagged Gβ3 and Gβ3s into the destination vector pLenti-PGK-Puro-DEST vector (Addgene) by the Gateway cloning system and packaged in HEK293FT cells as described [26].

2.4. Transfection

Transient transfection of HEK293 cells was performed using PolyJet DNA *in vitro* transfection reagent (Signagen). Stable expression of FLAG-tagged Gβ3 or Gβ3s in HEK293

cells was performed by transducing cells with lentiviruses encoding FLAG-tagged Gβ3 or Gβ3s and selecting with 2-20 μg/ml puromycin for about 2 weeks. The puromycin-resistant cells were pooled and maintained in the medium containing 10 μg/ml puromycin.

Transient transfection of COS-7 was performed by using Lipofectamine 2000 or the Neon transfection system (Life Technologies) according to the manufacture's protocol. Transient transfection of Jurkat T cells with siRNAs (2 nmol/100 μl) against Gβ1 (5′-ggataacatttgctccatttt-3′), Gβ2 (5′-actgggtacctgtcgtgtttt-3′), a common sequence of Gβ1 and Gβ2 (5′-tacgacgacttcaactgcatt-3′) or Gβ3 (5′-tcgcaagatgggaagctgatcgtgt-3′) was performed by using the Neon transfection system as described previously [26].

2.4. Immunoprecipitation

The interaction of Gβ3 or Gβ3s with various Gγ subunits was determined using co-immunoprecipitation assays in HEK239 cells co-transfected with FLAG-tagged Gβ3 or Gβ3s and HA-tagged Gγ subunits. Two days post-transfection, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) containing protease inhibitors. HA-Gγ was then immunoprecipitated by protein G Dyna beads pre-coupled with the rat anti-HA antibody. Protein complexes were resolved by SDS-PAGE and analyzed by Western blot analyses. To determine the interaction of Gβ3 or Gβ3s with endogenous Gα, immunoprecipitation was performed in cell lysates prepared from HEK239 cells transfected with FLAG tagged Gβ3 or Gβ3s and Gγ2, using an anti-FLAG antibody.

2.5. Analysis of Gβ/Gγ Interactions in Sf9 Cells

Subconfluent Sf9 insect cells were infected with baculoviruses encoding FLAG-Gβ3 or -Gβ3s and 6 × His-Gγ2, -Gγ5 or Gγ2C68S. Two days post-infection, cell lysates were prepared using HEPES buffer (20 mM HEPES, pH 8.0, 200 mM NaCl, 10 mM Imidazole, pH 8.0, 0.5% Thesit, 5 mM β-mercaptoethanol) containing protease inhibitors, and were incubated with Ni-NTA beads for 2 hours at 4°C. Protein complexes were washed three times with the HEPES buffer containing 500 mM NaCl, and then resolved by SDS-PAGE and analyzed by Western blotting.

2.6. BRET Assays

HEK293 cells were co-transfected with 100 ng pRluc-Gβ3 or –Gβ3s and different amount of pGFP^2-Gγ subunits in 12-well plates. 48 hr post-transfection, cells were trypsinized and resuspended in Hank's balanced salt solution containing magnesium and calcium (Life Technologies) and 0.1% glucose. Cells (1×10^5) were distributed in triplicate into opaque 96-well plates. Subsequently, the luciferase substrate coelenterazine 400a (5 μM final concentration) was added, and luminescence and fluorescence were measured at 410 ± 80 nm and 515 ± 30 nm with the Biotek Synergy 4 microplate reader [27, 28]. BRET signals were determined by calculating the 510/410 nm ratio of light intensity. Net BRET values were determined by subtracting the background signal detected from expression of the pRluc-tagged construct alone.

2.7. Immunofluorescence Staining

The cellular localization of FLAG-Gβ3 and FLAG-Gβ3s was determined in HEK293 cells stably expressing these proteins. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-100 for 5 min. Cells were then stained with mouse anti-FLAG (1:500) and rabbit anti-Gβ (T20; 1:800) at room temperature for 1 hr, followed by incubation with the secondary antibodies Alexa 488- and Alexa 568-conjugated anti-mouse and anti-rabbit IgG, respectively. Images were acquired with a LSM510 Meta inverted

confocal microscope (Carl Zeiss) with an argon/krypton laser and a Plan Apo 40× 1.3 NA oil immersion lens, and processed with Adobe Photoshop [26].

2.8. Analysis of Gβ3 and Gβ3s Stability

HEK293 cells stably expressing FLAG-Gβ3 and FLAG-Gβ3s were treated with cycloheximide (50 μM), and the expression of FLAG-Gβ3 and FLAG-Gβ3s was analyzed by Western blotting after treatment at different time points.

2.9. Measurement of PLCβ2 Activity

Gβγ-mediated PLCβ2 activation was determined in COS-7 cells as described previously [29]. Briefly one day post-transfection, cells were labeled for 48 h with *myo*-[^{3}H]inositol at 2 μCi/ml in inositol-free DMEM containing 1% dialyzed FBS. After serum starvation for 4 h, 10 mM LiCl was added to the cells to initiate inositol phosphate (IP) accumulation for 1 h. Total IPs were separated by AG 1-X8 columns and determined by β-spectrometry. To eliminate difference in IP accumulation caused by different cell numbers, total IPs were expressed as percentage of total [^{3}H]-inositol incorporated into the intact cells.

2.10. Measurement of PI3Kγ Activity

Gβγ-mediated PI3Kγ activation assays were performed in COS-7 cells co-transfected with myc-tagged PI3K p110γ and myc-tagged p101 together with FLAG-Gβ3γ2 or FLAG-Gβ3sγ2 using Lipofectamine 2000. 4 h post-transfection, the media were replaced by serum-free DMEM containing 0.1% BSA. 24 h later, cell lysates were prepared in lysis buffer containing protease inhibitors and phosphatase inhibitors (5 mM NaF, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 5 μM cantharidin). 100 μg of proteins were then subjected to SDS-PAGE and Western blotting analyses of AKT phosphorylation.

2.11. Measurement of ERK Activity

Gβγ-mediated ERK activation was assessed in COS-7 cells co-transfected with HA-ERK1 and Gβ3γ2 or Gβ3sγ2, using the same procedure as the PI3Kγactivation assays. 24 hr post-transfection, HA-ERK1 was immunoprecipitated with protein G Dynabeads preincubated with rat anti-HA antibody and analyzed for phosphorylation by Western blotting.

Receptor mediated ERK1/2 activation was determined in HEK293 cells transiently transfected with Gβ3γ2 or Gβ3sγ2 in the presence of absence of HA-α2A-adrenergic receptor (α2A-AR). 24 hr post-transfection, cells were serum-starved overnight, and then stimulated with epinephrine (10 μM) in the presence of 10 μM *dl*-propranolol) for 5 min at 37°C. Cell lysates were then prepared and used for detection of ERK1/2 phosphorylation by Western blotting.

2.12. Measurement of PLEKHG2 Activity

HEK-293 cells seeded on 24-well plates were co-transfected with Gβ3γ2 or Gβ3sγ2 together with the pSRE-luciferase reporter plasmid and the pMaxGFP control vector. 4 hr post-transfection, cells were serum-starved overnight, washed twice with saline, and then lysed with lysis buffer (100 mM potassium phosphate, pH7.8, 0.2 % Triton X-100) containing protease inhibitors. Luciferase activities were determined using 200 μM luciferin as a substrate as described [30]. The activity of the experimental reporter was normalized against the fluorescent intensity of GFP measured with the Biotek Synergy 4 microplate reader.

2.13. DNA Genotyping

Genomic DNA was extracted from lymphocytic cells using Wizard genomic DNA purification kit (Promega), and used for PCR with oligonucleotide primers 5′-TGACCCACTTGCCACCCGTGC-3′ (sense) and 5′-GCAGCAGCCAGGGCTGGC-3′ (antisense) encompassing the genomic sequence of human Gβ3 from nucleotide 5348 to 5614. The resulting PCR amplicon was digested with *Bse*DI [10]. After *Bse*DI digestion, homozygous C825C genotypes generate bands of 115 bp and 152 bp, while homozygous C825T genotypes generate a band of 267 bp.

2.14. Detection of Gβ3s Transcripts

RNA was prepared from leukocytes for RT-PCR using oligonucleotide primers 5′-CGGGAGCTTTCTGCTCACAC -3′ (sense) and 5′-TGTTCACTGCCTTCCACTTCC-3′ (anti-sense), as described [31]. The resulting RT-PCR products encoding Gβ3 and Gβ3s are 651 bp and 528 bp, respectively. They were size-fractionated and purified from 1.5% agarose gels. The purified products were cloned onto pCR4-TOPO vector using the TOPO TA Cloning Kit (Life Techologies), followed by DNA sequencing.

2.15. Quantitative Real-time PCR

Relative quantification of mRNA levels in siRNA-transfected Jurkat T cells was achieved using quantitative RT-PCR (qPCR) and comparative *Ct* methods. 24 hr post-transfection, total RNA was isolated from cells using Trizol (Life Technologies), and 500 ng of total RNA was reversed transcribed into cDNAs using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using primers specific for the target gene and the iQSYBR green supermix (Bio-Rad) with the C1000 thermal cycler (Bio-Rad). mRNA expression levels of each gene were normalized with GAPDH mRNA.

2.16. Chemotaxis Assay

Chemotaxis of Jurkat T cells stimulated by chemoattractant SDF1α was determined as previously described [26, 29, 32].

2.17. Measurement of AKT, ERK1/2 phosphorylation and Cytosolic Ca^{2+} Concentration in Jurkat T cells

AKT and ERK1/2 phosphorylation were determined by Western blotting, and the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$) in Jurkat T cells was measured using Fura 2/AM as previously described [26, 32].

2.18. Statistical analysis

Data were expressed as mean ± SE. Statistical comparisons between two groups were analyzed by two tail Student's *t* test ($P<0.05$ was considered significant).

3. Results

3.1. Gβ3s does not interact with either Gγ or Gα subunits

We initially attempted to purify Gβ3s for functional studies as a dimer with His-tagged Gγ2 or Gγ5 from Sf9 cells using nickel column but failed to achieve that, although we can easily purify Gβ3γ2 or Gβ3γ5 (data not shown). We verified these findings by Western blot analysis of the proteins isolated by Ni-NTA agarose (Fig. 1 A, B). Except for non-specific binding to Ni-NTA agarose, no specific binding of Gβ3s to His-Gγ2 or His- Gγ5 can be detected as compared to Gβ3. The failure of Gβ3s to form dimers with Gγ2 and Gγ5 was not due to its lower expression as Gβ3s was expressed at the similar level as Gβ3 (Fig. 1 A, B). To evaluate the possibility that the Gβ3sy dimer may be unstable in the presence of

detergent, we co-expressed Gβ3 or Gβ3s with a His-tagged Gγ2 mutant (Gγ2C68S) that is deficient in lipid modification [33], and then purified the complex from the cytosol of Sf9 cells after disrupting the cells with sonication in the absence of detergents. Although Gβ3 can be readily purified with His-Gγ2C68S, no complex formation between Gβ3s and Gγ2C68S can be detected (Fig. 1C). These findings suggest that Gβ3s may be deficient in the ability to interact with Gγ.

To verify the findings in mammalian cells, we co-transfected HEK293 cells with FLAG-tagged Gβ3 or Gβ3s and the HA-tagged Gγ subunits previously reported to form complexes with Gβ3 and Gβ3s [13, 23, 34], and then performed co-immunoprecipitation using an anti-HA antibody. As shown in Fig. 2A, FLAG-Gβ3 readily co-immunoprecipitated with each of Gγ subunit tested, including Gγ2, Gγ5, Gγ8, Gγ10 and Gγ12, while FLAG-Gβ3s did not. We further investigated the interaction of Gβ3s with Gγ in intact cells, using a BRET-based assay. Co-expression of a fixed amount of pRluc-Gβ3 (donor) with increasing concentrations of $pGFP^2$-Gγ5 or $pGFP^2$-Gγ8 (acceptor) led to a dose-dependent increase in net BRET values that are significantly larger than those observed with the co-expression of pRluc-Gβ3 with the control $pGFP^2$ alone (Fig. 2B). In contrast, when BRET was measured between pRluc-Gβ3s and $pGFP^2$-Gγ5 or $pGFP^2$-Gγ8, the net BRET values were not significantly different from that between pRluc-Gβ3s and $pGFP^2$ (Fig. 2C). Similar results were observed when pRluc-Gβ3 or pRluc-Gβ3s was co-expressed with a single saturation concentration of $pGFP^2$-Gγ2, $pGFP^2$-Gγ10 or $pGFP^2$-Gγ12 (Fig. 2D). Taken together, these data demonstrate that, as compared to Gβ3, Gβ3s lacks the ability to form a complex with Gγ subunits.

It has been shown previously that expression of Gβ3s facilitated an increase in GTPγs binding to Gα subunits stimulated by the activating peptide mastoparan 7 or GPCRs, implying that Gβ3s can interact with Gα subunits [10, 23]. To directly examine the interaction of Gβ3s with Gα subunits, we immunoprecipitated FLAG-Gβ3 or FLAG-Gβ3s from HEK293 cell lysates after co-expression with Gγ2, and then examined the immunoprecipitates for the presence of endogenous Gα subunits by Western blot. Although there was no interaction with Gαs, FLAG-Gβ3 co-immunoprecipitated with Gαi, Gαq/11 and Gα12. In contrast, none of these Gα subunits was detected in the FLAG-Gβ3s immunoprecipitate (Fig. 3). These findings indicate that Gβ3s is also deficient in interaction with Gα subunits.

3.2. Gβ3s displayed altered cellular localization and a decreased stability

The association of Gβ with Gγ and Gα is known to facilitate the membrane localization of Gβ [33, 35]. Given that Gβ3s fails to bind either Gγ or Gα subunits, it raises the possibility that it may not localize properly to the cell membrane. To test this, we generated stable HEK293 cell lines expressing comparable levels of FLAG-Gβ3 and FLAG-Gβ3s. As expected, like the endogenous Gβ, FLAG-Gβ3 is located primarily in the plasma membrane (Fig. 4A). In contrast, FLAG-Gβ3s is diffusely distributed in the cytosol (Fig. 4A).

The association of Gβ with Gγ is also known to render the stability of Gβ [36]. Therefore, we further investigated if the deficiency of Gβ3s in forming a complex with Gγ subunits affects its stability. We treated HEK293 cells stably expressing FLAG-Gβ3 and FLAG-Gβ3s with the protein synthesis inhibitor cycloheximide (CHX) for different amount of time (0-8 hr) and then analyzed the level of proteins by Western blotting. While more than 80% of FLAG-Gβ3 remained detectable 8 hr after CHX treatment, FLAG-Gβ3s rapidly degraded in 0.5 hr and became almost undetectable in 8 hr (Fig. 4B). The calculated half-life of FLAG-Gβ3 and FLAG-Gβ3s turnover is 18 hr *versus* 0.3 hr.

3.3. Gβ3s does not activate Gβγ effectors

We next determined whether Gβ3s is capable of stimulating Gβγ-dependent effectors. We first evaluated the well-described canonical Gβγ effectors, PLCβ2, ERK1/2 and PI3Ky. As shown in Fig. 5A, co-expression of Gβ3γ2 with PLCβ2 in COS-7 cells dose dependently increased PLCB2 activity 2-5 fold above basal. In contrast, when Gβ3s was co-expressed with Gγ2 at the similar level as Gβ3, the activity of PLCβ2 was not significantly increased. Similar data were obtained with the activation of PLCβ3 (data not shown). Similarly, while overexpression of Gβ3γ2 led to increased phosphorylation of the co-expressed HA-ERK1, and PI3Kγ-mediated phosphorylation of endogenous AKT, overexpression of Gβ3sγ2 had no effects (Fig. 5B, C). The failure of Gβ3s to activate various effectors was not due to its low expression, as the level of Gβ3s expression was comparable to that of Gβ3.

We then examined whether Gβ3s can activate a recently discovered Gβγ effector, PLEKHG2, a RhoGEF [37]. To monitor PLEKHG2 activity, we measured transcription of a reporter gene, luciferase, controlled by the SRE promotor, which is known to be regulated by RhoGTPases [38]. Co-expression of Gβ3γ2 stimulated PLEKHG2-induced luciferase activity in a dose-dependent manner (Fig. 5D), but co-expression of Gβ3sγ2 had no effects on PLEKHG2 activity (Fig. 5D).

3.4. Gβ3s does not modulate GPCR signaling

Heterologous expression of Gβ3s has been shown to enhance GPCR-stimulated GTP binding on Gα subunits and cell migration, implying that Gβ3s may facilitate increased coupling of heterotrimeric G proteins to GPCRs for signal transduction [13, 23, 39]. To test this, we examined if overexpression of Gβ3 or Gβ3s affects ERK1/2 phosphorylation stimulated by co-expressed α2A-AR in HEK293 cells. The α2A-AR stimulated ERK1/2 phosphorylation via Gβγ released from the activated Gi/o proteins, because it is sensitive to inhibition by pertussis toxin treatment or overexpression of Gαt that sequesters free Gβγ (data not shown) [29]. Overexpression of either Gβ3γ2 or Gβ3sγ2 did not affect α2A-AR-stimulated ERK1/2 phosphorylation (Fig. 6), suggesting that neither Gβ3 nor Gβ3s is able to enhance α2A-AR-mediated signal transduction. Similar results were found when LPA-stimulated ERK1/2 phosphorylation via endogenous LPA receptors was tested (data not shown).

3.5. Gβ3s protein is undetectable in human lymphocytic cell lines expressing Gβ3s transcript

To further understand the function of Gβ3s, we screened several human leukocyte cell lines for the presence of Gβ3 C825T polymorphism and the expression of endogenous Gβ3s transcripts. As reported [10], we genotyped the cell lines for the presence of C825T by PCR followed by restriction enzyme digestion (Fig. 7A). We identified homozygous C825C and T825T alleles in B lymphocytic cell lines GM19116C and GM18500B, respectively, and heterozygous C825T allele in Jurkat T cell line. As reported previously [31], RT-PCR analysis followed by DNA sequencing of the RT-PCR product indicate that Gβ3s-specific mRNA transcript was expressed only in cell lines containing the T825 allele, while the wild-type Gβ3 mRNA transcript can be detected in all cell lines regardless of the T825 status (Fig. 7B). To determine the expression of Gβ3s protein, we used a pan anti-Gβ antibody (T20) that recognizes Gβ1, Gβ2, Gβ3, Gβ3s, and Gβ4 but not Gβ5 by Western blotting (Fig. 7C), since we could not identify a commercially available antibody that recognizes Gβ3 and Gβ3s specifically (data not shown). Using this antibody, we detected expression of endogenous Gβ in all cell lines, but could not detect a protein corresponding to the size of Gβ3s in the cell lines expressing Gβ3s mRNA transcripts (Fig. 7D). These findings are consistent with the notion that Gβ3s is an unstable protein, but does not exclude the

possibility that low levels of Gβ3s expression may also occur at the translational and transcription level.

3.6. Gβ3 does not play a role in transmitting chemotactic signal for leukocyte migration

Given our data indicating that Gβ3s is an unstable and functionally inactive protein, it is likely that the pathological effects of Gβ3 C825T result from the downregulation of Gβ3 function, and not the enhanced functionality of Gβ3s. To test this, we further evaluated the role of Gβ3 in mediating chemotaxis in human lymphocytic cell lines, as leukocytes from individuals carrying Gβ3 C825T have been shown to exhibit enhanced chemotactic responses to chemoattractant stimulation. We initially chose Jurkat T cells as a model system for studies because this cell line expresses a substantial amount of Gβ3, although it contains heterozygous C825T allele and also expresses Gβ3s transcripts (Fig. 7A, B). Moreover, Jurkat T cells exhibit robust chemotactic and signaling responses after stimulation with SDF1α. We transiently transfected Jurkat T cells with a siRNA to specifically inhibit Gβ3 and Gβ3s expression (Fig. 8A). Downregulation of Gβ3 and Gβ3s had no effect on SDF1α-stimulated Ca^{2+} signaling and AKT and ERK phosphorylation, nor did it affect SDF1α-stimulated chemotaxis (Fig. 8B, C). These findings indicate that Gβ3 and Gβ3s do not play a major role in SDF1α-stimulated signal transduction and Jurkat T cell migration. In supporting this notion, transfection of Jurkat T cells with a siRNA targeting a common sequence shared by Gβ1 and Gβ2 alleviated SDF1α- activated Ca^{2+} signaling, AKT and ERK phosphorylation, and completely abolished SDF1α-stimulated cell migration (Fig. 8C, D). In contrast, OKT3-stimulated Ca^{2+} signaling via T cell receptors was not affected, indicating that inhibition of Gβ1 and Gβ2 specifically affects GPCR-mediated signal transduction (Fig. 8B, C). Notably, targeting either Gβ1 or Gβ2 alone had no effect, indicating that the function of SDF1α is mediated primarily via Gβ1 and Gβ2 and that these two Gβ isoforms have a redundant role in Jurkat T cells (data not shown).

To determine if Gβ3 has a cell type-specific function, we further evaluated its role in GM19116C cells, a B lymphocytic cell line that contains homozygous C825C allele and expresses only Gβ3 (Fig. 7A, B). As with Jurkat T cells, inhibition of Gβ3 had no effect on SDF1α-induced cell migration, while targeting Gβ1 and Gβ2 abolished chemotaxis (Fig. 8E). Together, these data indicate that Gβ3 does not play a major role in mediating SDF1α-stimulated signal transduction and lymphocyte migration.

4. Discussion

In this study, we have provided unambiguous evidence that the splice variant of Gβ3 associated with C825T polymorphism is a functionally inactive protein. We show that, unlike a canonical Gβ protein, Gβ3s does not form a dimer with Gγ after heterologous overexpression in a mammalian or an insect system. The deficiency in dimerization of Gβ3s and Gγ cannot be explained by instability caused by detergents, which has been previously reported for certain Gβ isoforms such as Gβ5 [40]. This is evident from data showing that Gβ3s does not form a dimer with a Gγ2 mutant deficient in lipid modification in lysis buffer containing no detergents, nor does it interact with various Gγ in intact cells as determined by the BRET-based assays. Co-immunoprecipitation analyses also showed that, unlike Gβ3, Gβ3s does not interact with various Ga subunits. Given that Gγ subunits are obligate partners of Gβ subunits, it is not surprising that Gβ3s does not activate diverse Gβγ effectors, including the classical effectors, PLCβ2/3, PI3Kγ/AKT and ERKs, and the newly identified effector, the RhoGEF PLEKHG2. These findings are consistent with the report of Ruiz-Velasco et al. that Gβ3s does not form a dimer with Gγ2 as determined by fluorescence resonance energy transfer measurements, and does not modulate N-type Ca^{2+} or G protein-gated inwardly rectifying K^+ channels when it was co-expressed with Gγ2 or Gγ5 in rat

sympathetic neurons [24]. Our failure to purify Gβ3s as a dimer with either Gγ2 or Gγ5 from the Sf9 cell expression system is also supported by the reports of other groups [23, 41, 42].

Gβ3 belongs to the superfamily of WD40 repeat-containing proteins. Its C-terminus consists of seven WD40 repeat domains that form a toroidal seven-bladed β propeller structure with each β propeller comprising four anti-parallel β sheets [43]. Each WD40 repeat forms part of two blades within a propeller, including the outer strand of one blade and the three inner strands of another. Gβ3s lacks the entire fourth WD40 repeat domain [10]. Based on sequence analyses, WD40 proteins are predicted to contain 4-16 WD40 repeats [44]. Therefore, it has been proposed that despite the lack of one WD40 repeat, Gβ3s can still form a stable six-blade β propeller structure [13]. However, if this is the case, Gβ3s should still be able to interact with Gγ because a majority of residues involved in binding Gγ are still retained in the N-terminal coiled helix and the WD40 domains 5-7 of Gβ3s [43]. The fact that Gβ3s is deficient in forming a complex with Gγ and Gα indicates that the remaining six WD40 domains of Gβ3s are unable to form a stable β propeller structure. This notion is consistent with modeling prediction that a seven-fold symmetry is preferable to six- or eight-fold symmetry for β propeller assembly [45], and our findings that Gβ3s is highly unstable as compared to Gβ3.

Our findings that Gβ3s is a functionally inactive protein contradict the reports by Siffert's group. They initially proposed that Gβ3s was a functional protein because it was identified in lymphocytes derived from patients with essential hypertension that showed increased G protein signaling, and it has the same ability as Gβ3 to support MAS-7-stimulated GTPγs binding to Gα when co-expressed with Gαi2 and Gγ5 in Sf9 cells [10]. Using the same assay, they later showed that, as compared to Gβ3, Gβ3s displayed an increased ability to mediate receptor-stimulated GTPγs binding to Gα [23]. Moreover, overexpression of Gβ3s alone was sufficient to increase MAS-7-stimulated GTPγs binding in permeabilized COS-7 cells [23]. COS-7 overexpressing Gβ3sγ5 also showed enhanced chemotactic response to LPA stimulation [39]. However, these studies did not directly determine whether Gβ3s indeed formed a heterotrimeric complex with the co-expressed Gαi2 and Gγ5. Our co-immunoprecipitation studies provided the direct evidence that unlike Gβ3, Gβ3s lacks the ability to form a complex with endogenous Gα subunits in HEK293 cells.

Siffert's group also reported that Gβ3s can form dimers with various Gγ after they were translated *in vitro* or co-expressed in HEK293 cells, followed by immunoprecipitation of Gγ [13, 23]. However, the amount of Gβ3s co-immunoprecipitated with Gγ was much less than that of Gβ3. Moreover, as we found here, they also showed that no Gβ3s could be co-purified with Gγ from Sf9 cells [23]. Given our findings that Gβ3s is unstable, it is likely that the small amount of Gβ3s co-immunoprecipitated with Gγ may represent the unstable Gβ3s protein precipitated from solution.

Based on the findings that overexpression of Gβ3s alone was sufficient to significantly enhance the activity of co-expressed ERK1 as compared to Gβ3, Siffert's group proposed that Gβ3s is a gain-of-function protein [13]. However, in the same study it was found that Gβ3s, expressed either alone or in combination with Gγ, was unable to stimulate PLCβ2. It was recently found that Gβ3 preferentially activates PLCβ3 over PLCβ2 [34]. Nevertheless, we found that Gβ3s is deficient in stimulating either PLCβ3 or PLCβ2. Moreover, Gβ3s is unable to activate several other Gβγ effectors. Together with the findings that overexpression of Gβ3s does not affect GPCR-mediated signal transduction, our data strongly indicate that Gβ3s is a loss-of-function protein.

The idea that Gβ3s is a gain-of-function protein has become the dogma in the field of Gβ3 polymorphisms, and has been and continues to be used as the mechanistic basis for the

association of Gβ3 C825T allele with diverse pathophysiological effects in many studies in the literature [14]. However, many of these studies examined neither the expression nor the direct contribution of Gβ3s to the described effects. Our findings that Gβ3s is an unstable and functionally inactive protein indicate that the potential mechanisms associated with Gβ3 C825T allele are probably due to downregulation of the expression and function of Gβ3. Therefore, to fully understand the effect of the Gβ3 C825T polymorphism, it is critical to evaluate the expression level of Gβ3 in the carriers of different genotypes and to define the functionality of Gβ3. Based on Northern blotting analyses, Gβ3 was shown to be widely expressed in many tissues [46]. The expression of Gβ3 protein in several cell types and multiple regions of adult rat brain was also shown by Western blot and immunohistochemistry analyses [10, 47, 48]. It was also reported that the expression of Gβ3 was decreased in the adipocytes of Gβ3 C825T carriers [47]. However, the specificity of Gβ3 antibodies used in these studies was not well characterized. Gβ3 shares a high degree of homology in amino acid sequences to other Gβ isoforms, and consequently, it is hard to identify a region of Gβ3 with amino acid sequences divergent enough to other Gβ isoforms for preparing Gβ3 specific antibodies. Indeed, we found that many commercially available antibodies that are claimed to specifically recognize Gβp3 can also detect other Gβ isoforms (data not shown). Thus, future studies are required to develop specific antibodies for Gβ3 or other approaches to further characterize the distribution of Gβ3 in tissues.

Accumulating evidence indicates that distinct combinations of different isoforms of Gα, Gβ and Gγ may determine the signaling specificity of GPCRs [1, 9]. For example, it has been shown that the mouse macrophage cell line J774A.1 expresses Gβ1, Gβ2 and Gβ4 isoforms, but Gβ2 was the primary Gβ subunit that mediates the signaling and function of the C5a receptor in this cell line [49]. By using antisense oligonucleotides against different Gβ isoforms, it has been established that Gβ1 and Gβ3 subunits selectively mediate the effect of the somatostatin and muscarinic receptors, respectively, to inhibit the voltage-sensitive Ca^{2+} channels [50]. Based on findings that platelets, neutrophils, T lymphocytes and coronary arteries from carriers with Gβ3 C825T allele displayed enhanced responses to GPCR stimulation, it has been suggested that Gβ3s plays a positive role in regulating functions of these cells, although direct evidence is still lacking [39, 51-53]. Our data show that although Gβ3 and Gβ3s transcripts are detected in human lymphocytic cell lines containing Gβ3 C825T allele, SDF1α-stimulated signaling and chemotactic response are not mediated by Gβ3 and Gβ3s, but rather, by the redundant function of Gβ1 and Gβ2 in these cells. These findings indicate that the role of Gβ3 in mediating cell signaling and function likely depends on the receptors and the cell types. Thus, the reported association of Gβ3 C825T polymorphism with various disorders in different organs should be interpreted with great caution. Given that the conversion of Gβ3 to Gβ3s results in the loss of Gβ3 function, clearly, to comprehend the functionality of Gβ3 C825T polymorphism, future work is required to further characterize the role of Gβ3 in mediating the function of diverse GPCRs in different cells and tissues.

5. Conclusions

In conclusion, we results demonstrate that the splice variant of Gβ3, Gβ3s, is a structurally unstable and functionally inactive protein. Moreover, we provided evidence that the role of Gβ3 in mediating functions of GPCRs is likely to be stimuli and/or tissue specific.

Acknowledgments

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Abbreviations

BRET	Bioluminescence resonance energy transfer
GPCR	G protein-coupled receptor
IP	inositol phosphate
PTx	pertussis toxin
RhoGEF	Rho guanine exchange factor

Highlights

- Gβ3s is deficient in interacting with Gγ and Gα.
- Gβ3s is structurally unstable and fails to localize properly to cell membranes.
- No Gβ3s protein can be detected in human lymphocytes expressing its transcript
- Gβ3s fails to active diverse Gβγ effectors

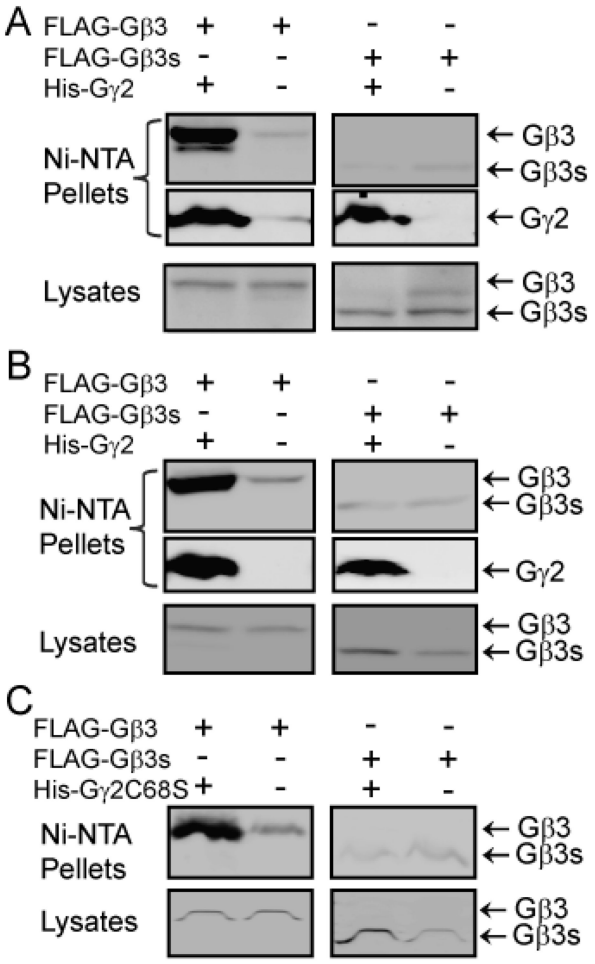

Figure 1. Gβ3s does not form dimers with Gγ in Sf9 cells

Sf9 cells were infected with baculoviruses encoding FLAG-Gβ3 or FLAG-Gβ3s together with baculoviruses encoding His-Gγ2, His-Gγ5 or His-Gγ2C68S mutant. 72 hr post-infection, proteins were isolated with Ni-NTA agarose in the presence (A-B) or absence of the detergent Genapol C-100 (C), and detected by anti-FLAG and anti-His antibodies. Representative images from more than three independent experiments are shown.

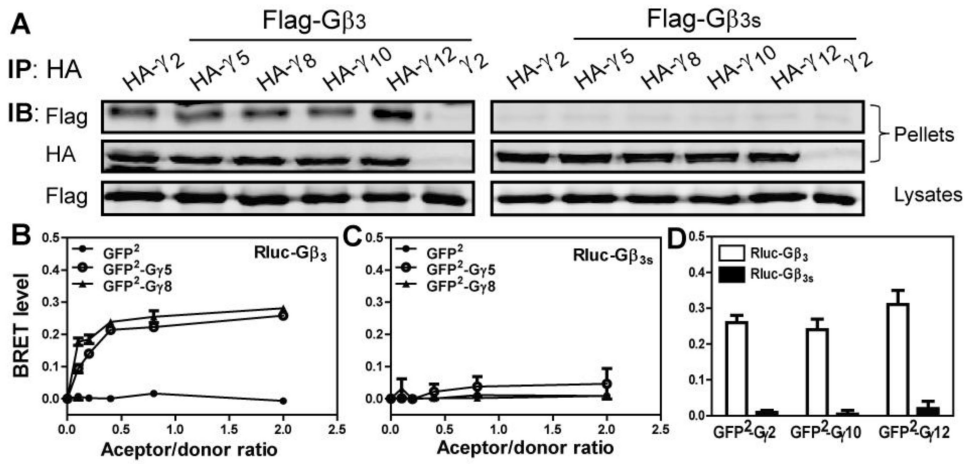

Figure 2. Gβ3s does not form dimers with Gγ in HEK293 cells

A, FLAG-Gβ3 and FLAG-Gβ3s were co-transfected with the indicated HA-tagged Gγ isoforms or untagged Gγ2 in HEK293 cells. Immunoprecipitation assays were performed by anti-HA antibody followed by Western blotting. Representative images from three independent experiments are shown. *B-D*, HEK293 cells were transfected with a fixed amount of Rlu-Gβ3 (100 ng) or Rlu-Gβ3s (150 ng) and increasing concentration (0-300 ng) of GFP^2, GFP^2-Gγ2, GFP^2-Gγ5 (B-C), or a fixed concentration (300 ng) of GFP^2-Gγ2, GFP^2-Gγ10 or GFP^2-Gγl2 (D), and processed for BRET measurement as described under "Experimental Procedures". Results are expressed as the mean ± S.E. (n=3-5).

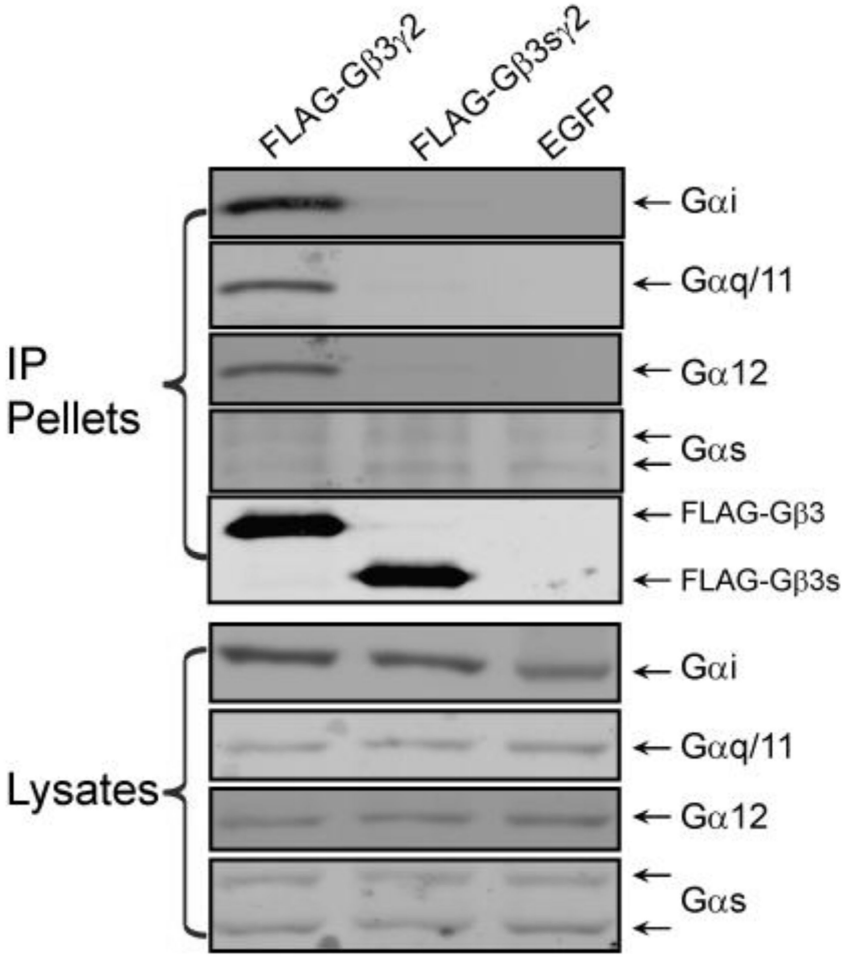

Figure 3. Gβ3s does not interact with endogenous Gα in HEK293 cells

HEK293 cells were co-transfected with EGFP, FLAG-Gβ3 or FLAG-Gβ3s together with HA-Gγ2 and processed for immunoprecipitation with an anti-FLAG antibody. Endogenous Gα subunits co-immunoprecipitated with FLAG-Gβ3 and FLAG-Gβ3s were detected with specific antibodies. Representative images from more than three independent experiments are shown.

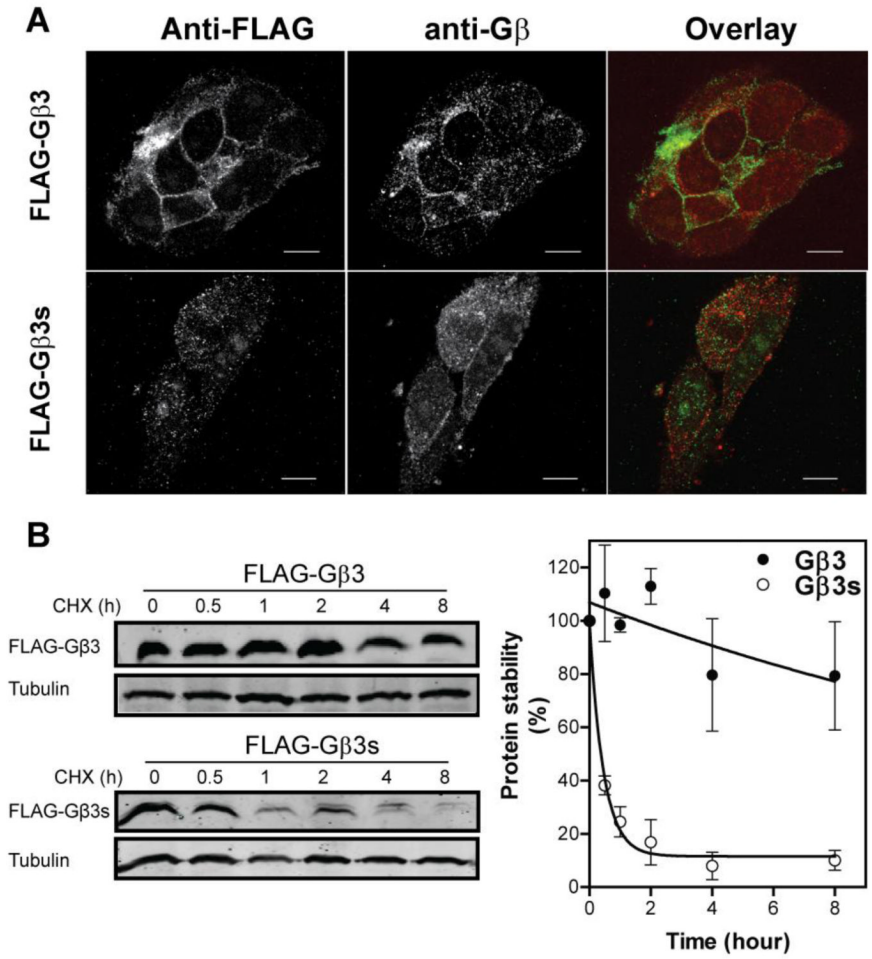

Figure 4. Localization and stability of Gβ3 and Gβ3s in HEK293 cells

A, The cellular localization of FLAG-Gβ3, FLAG-Gβ3s and endogenous Gβ in HEK293 cells stably expressing FLAG-Gβ3 and FLAG-Gβ3s was revealed by staining with a mouse anti-FLAG and a rabbit pan anti-Gβ antibodies followed by an Alexa488-conjugated anti-mouse and an Alex568-conjugated anti-rabbit IgG secondary antibodies. The images are the representatives of more than 20 cells from at least three separate experiments with similar results. *B*, The stability of FLAG-Gβ3 and FLAG-Gβ3s stably expressed in HEK293 cells was determined by treatment with cycloheximide for different amount of time (0-8 hr), followed by Western blotting analyses of protein expression. Displayed are representative images and quantitative data from at least three separate experiments expressed as the mean ± S.E. * $p<0.05$ indicate significance *versus* control.

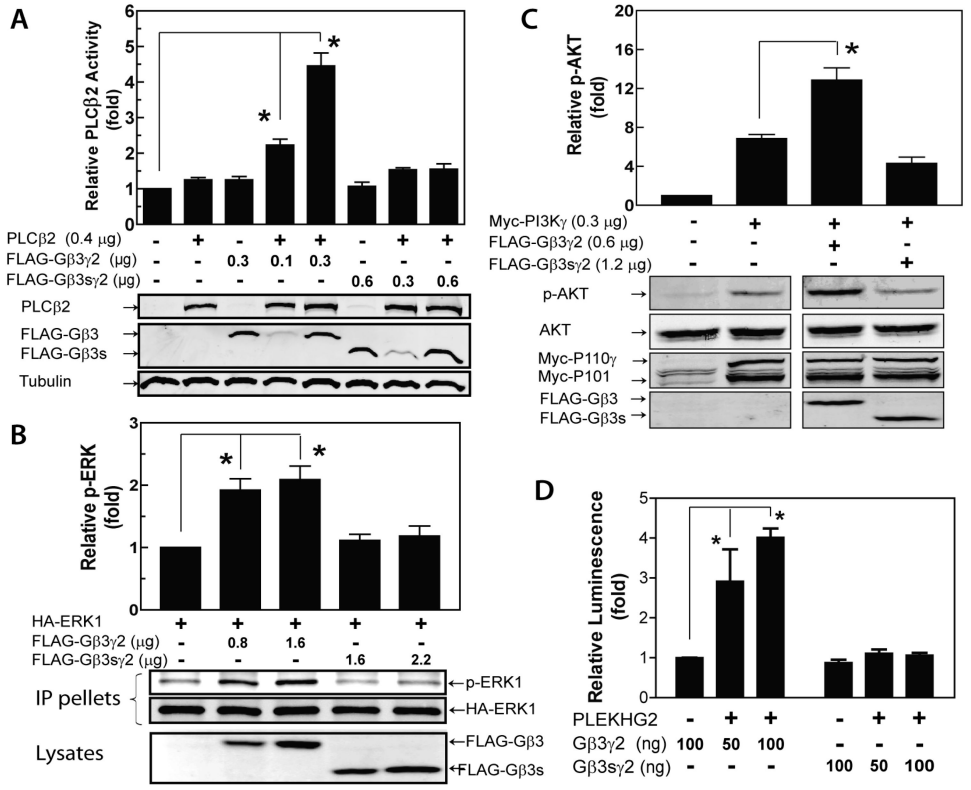

Figure 5. Gβ3s does not activate diverse Gβγ effectors

A, PLCβ2 activation. Total inositol phosphates were measured in COS7 cells transfected with the indicated concentration of vectors encoding PLCβ2 together with FLAG-Gβ3γ2 or FLAG-Gβ3sγ2. *B*, ERK activation. COS7 cells were transfected with HA-ERK1 together with FLAG-Gβ3γ2 or FLAG-Gβ3sγ2 as indicated. 24 hr post-transfection, HA-ERK1 was immunoprecipitated for determining the level of phosphorylation. *C*, PI3Kγ-mediated AKT phosphorylation. COS7 cells were transfected with myc-PI3K p110γ and p101 together with FLAG-Gβ3γ2 or FLAG-Gβ3sγ2 as indicated. 24 hr post-transfection, phosphorylation of endogenous AKT was determined. *D*, PLEKHG2 activation. HEK293 cells were co-transfected with expression vectors for SRE-luciferase, EGFP, PLEKHG2, and different concentration of FLAG-Gβ3γ2 or FLAG-Gβ3sγ2 as indicated. 24 hr post-transfection, luciferase activities were measured. After normalization with the level of GFP expression, relative luciferase activities are shown. Displayed are representative images and quantitative data from three to four experiments expressed as the mean ± S.E. * $p<0.05$ indicate significance.

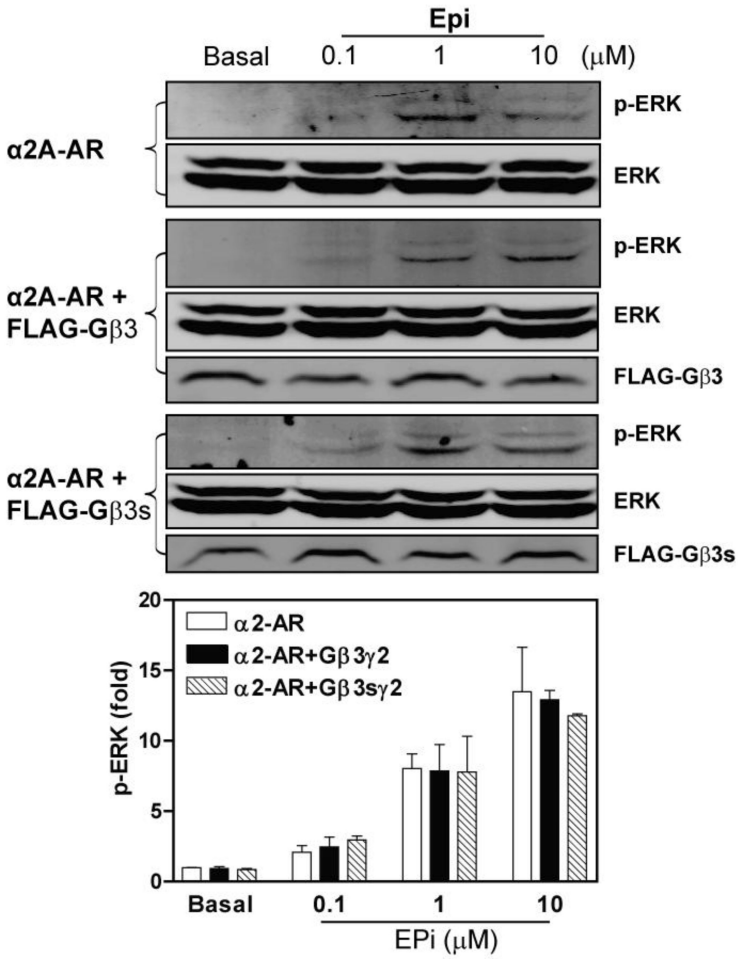

Figure 6. Overexpression of Gβ3 or Gβ3s does not alter GPCR signaling

HEK293 cells were transfected with FLAG-Gβ3 or FLAG-Gβ3s together with Gγ2 and α2A-AR. After serum-starvation overnight, cells were stimulated with epinephrine (Epi) at the indicated concentration for 5 min and then processed for Western blotting. Displayed are representative images and quantitative data from three separate experiments expressed as the mean ± S.E.

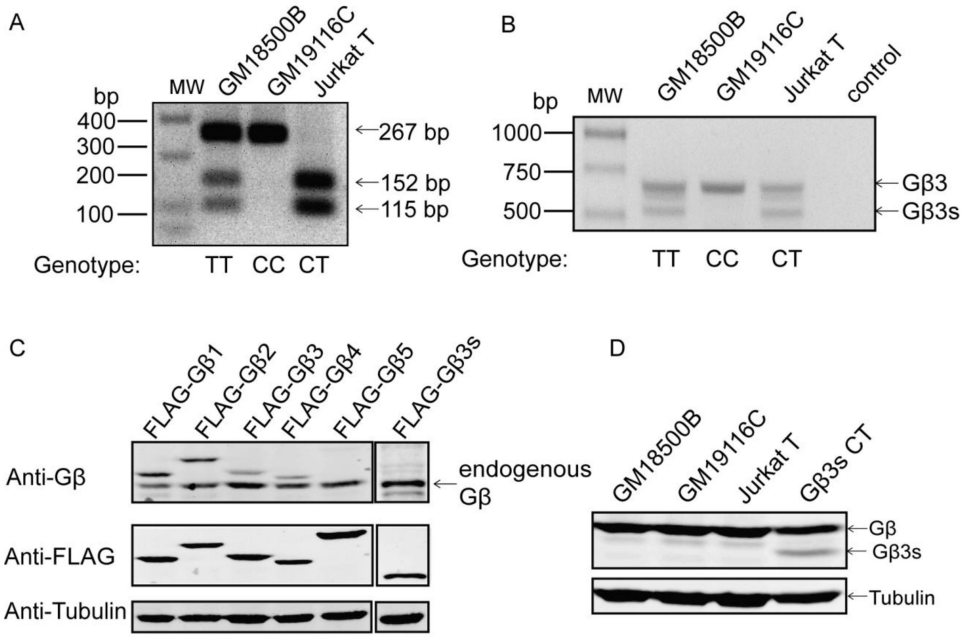

Figure 7. Genotypes of Gβ3 C285T polymorphism and detection of Gβ3- and Gβ3s-specific transcripts and Gβ3s protein in human lymphocytic cell lines

A, genomic DNA was extracted from lymphocytic cell lines. A combination of PCR and digestion of the PCR product with the restriction enzyme BseDI was used for genotyping, and the results are indicated underneath the gel image. CC, C825C homozygous; TT, C825T homozygous; CT, C825T heterozygous. *B*, RT-PCR was performed to identify Gβ3- and Gβ3s-specific transcripts (651bp and 528 bp, respectively) in lymphocytic cell lines. Control was performed in the absence of the template. *C*, the ability of the pan anti-Gβ antibody to detect multiple FLAG-tagged Gβ isoforms expressed in HEK293 cells. *D*, detection of Gβ3s protein in lymphocytic cell lines with a pan anti-Gβ antibody. Lysates from HEK293 cells expressing untagged Gβ3s were used as control (Gβ3s CT). The bands correspond to endogenous Gβ and overexpressed Gβ3s are indicated. Displayed are representative images of at least three separate experiments.

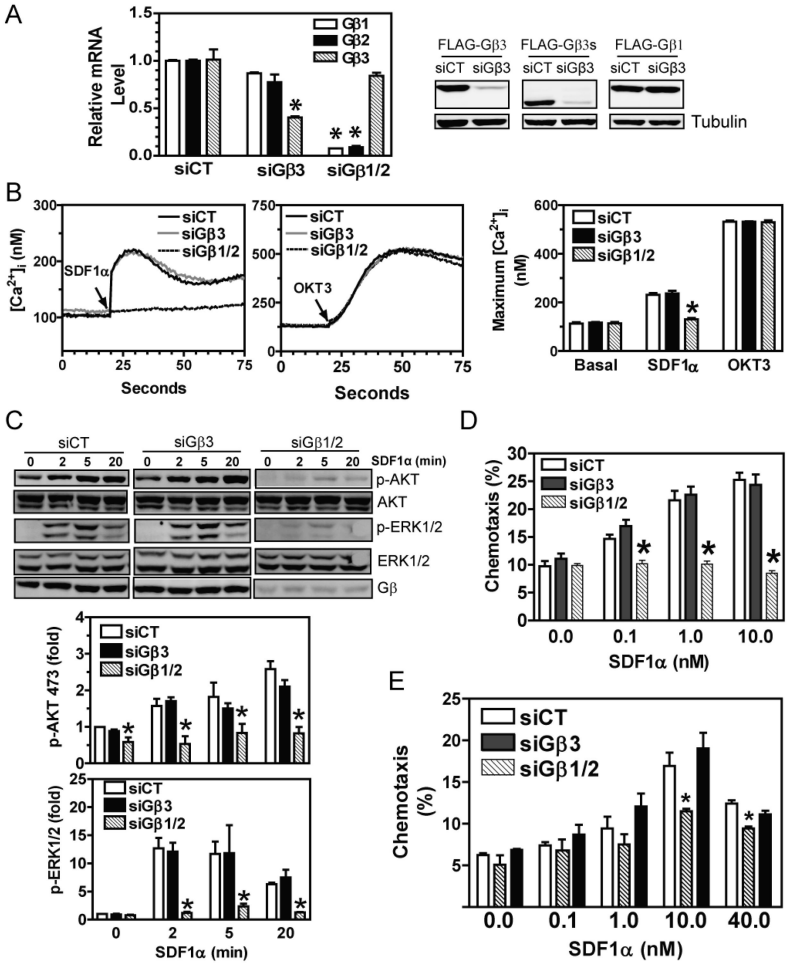

Figure 8. Gβ3 and Gβ3s are not required for SDF1α-stimulated signaling and chemotaxis

A, Jurkat T cells were transiently transfected with a control siRNA (CT) or siRNAs against Gβ3/Gβ3s (siGβ3) or Gβ1/Gβ2 (siGβ1/2) and processed for determining the effects on the level of Gβ3, Gβ1 and Gβ2 mRNAs by qRT-PCR (left panel), and co-expressed Flag-Gβ3 and Gβ1 protein by Western blotting (right panel). *B-D*, effects of inhibiting Gβ3, Gβ1 and Gβ2 in Jurkat T cells on SDF1α- (100 nM) or OKT3 (5 μg/ml)-stimulated Ca^{2+} signaling (B) and the phosphorylation of AKT and ERK1/2 (C), and SDF1α-stimulated chemotaxis (D). Displayed are representative images and quantitative data from at least three independent experiments expressed as the mean ± S.E. *p<0.05 *versus* siCT *E*, effect of inhibiting Gβ3, Gβ1 and Gβ2 on SDF1α-induced chemotaxis of GM19116C cells. * p<0.05 indicates significance *versus* siCT.