Guanine nucleotide dissociation inhibitor activity of the triple GoLoco motif protein G18: alanine-to-aspartate mutation restores function to an inactive second GoLoco motif

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GoLoco ('G$\alpha_{i/o}$–Loco' interaction) motif proteins have recently been identified as novel GDIs (guanine nucleotide dissociation inhibitors) for heterotrimeric G-protein α subunits. *G18* is a member of the mammalian GoLoco-motif gene family and was uncovered by analyses of human and mouse genomes for anonymous open-reading frames. The encoded G18 polypeptide is predicted to contain three 19-amino-acid GoLoco motifs, which have been shown in other proteins to bind Gα subunits and inhibit spontaneous nucleotide release. However, the G18 protein has thus far not been characterized biochemically. Here, we have cloned and expressed the G18 protein and assessed its ability to act as a GDI. G18 is capable of simultaneously binding more than one Gα_{i1} subunit. In binding assays with the non-hydrolysable GTP analogue guanosine 5′-[γ-thio]triphosphate, G18 exhibits GDI activity, slowing the exchange of GDP for GTP by Gα_{i1}. Only the first and third GoLoco motifs within G18 are capable of interacting with Gα subunits, and these bind with low micromolar affinity only to Gα_{i1} in the GDP-bound form, and not to Gα_o, Gα_q, Gα_s or Gα_{12}. Mutation of Ala-121 to aspartate in the inactive second GoLoco motif of G18, to restore the signature acidic-glutamine-arginine tripeptide that forms critical contacts with Gα and its bound nucleotide [Kimple, Kimple, Betts, Sondek and Siderovski (2002) Nature (London) **416**, 878–881], results in gain-of-function with respect to Gα binding and GDI activity.

Key words: fluorescence spectroscopy, GoLoco motif, G-protein regulatory motif (GPR motif), guanine nucleotide dissociation inhibitor, heterotrimeric G-protein, surface plasmon resonance.

INTRODUCTION

In the conventional model of heterotrimeric G-protein signalling, seven-transmembrane-domain GPCRs (G-protein-coupled receptors) are functionally linked to a membrane-associated heterotrimer composed of Gα, Gβ and Gγ subunits [1]. Gα subunits bind guanine nucleotides and alternate between a GDP-bound ground state and a GTP-bound active conformation. Gβ and Gγ form a heterodimer that binds with high affinity to GDP-bound Gα, slowing its release of GDP and facilitating its coupling to the receptor [2,3]. Upon agonist binding, the GPCR acts as a guanine nucleotide exchange factor, promoting the release of GDP from the Gα subunit, which then binds GTP. The binding of GTP changes the conformation of three flexible 'switch' regions within Gα, allowing dissociation of G$\beta\gamma$. Both GTP-bound Gα and free G$\beta\gamma$ are capable of initiating signals by interacting with downstream effector proteins. Finally, the intrinsic GTPase activity of the Gα subunit causes the hydrolysis of GTP to GDP, returning the Gα subunit to its inactive state. Reassociation of G$\beta\gamma$ with inactive Gα terminates all effector interactions by obscuring critical effector interaction sites on both Gα and G$\beta\gamma$ [4,5]. Hence the duration of heterotrimeric G-protein signalling is thought to be controlled by the lifetime of the Gα subunit in the GTP-bound state. This lifetime is dramatically shortened by RGS (regulator of G-protein signalling) proteins, which accelerate the intrinsic GTPase activity of Gα subunits [6].

We and others have recently identified a diverse family of Gα-interacting proteins, all of which contain one or more conserved GoLoco ('G$\alpha_{i/o}$–Loco' interaction) motifs [otherwise known as GPR (G-protein regulatory) motifs] [7–9]. GoLoco motif-containing proteins bind specifically to GDP-bound Gα subunits of the G$_i$ ('adenylate cyclase inhibitory') class and act as GDIs (guanine nucleotide dissociation inhibitors), slowing the spontaneous exchange of GDP for GTP [10–14]. Recent determination of the crystallographic structure of GDP-bound Gα_{i1} in complex with the GoLoco motif of RGS14 has revealed critical determinants of Gα subunit specificity and GDI activity [14]. In particular, the highly conserved Asp-Gln-Arg tripeptide that defines the final residues of the conserved GoLoco signature are important for orienting the arginine to contact the α- and β-phosphates of GDP [14].

The family of known GoLoco motif-containing proteins represents a diverse set of signalling regulators [9]. One subset of GoLoco proteins includes the R12 subfamily of RGS proteins: RGS12, RGS14 and the *Drosophila* protein Loco [6]. In addition to an RGS box and tandemly repeated Ras-binding domains, each of these proteins contains a single, C-terminal GoLoco motif that is a GDI for Gα_i subunits [12]. A second subset, consisting of AGS3, LGN and the *Drosophila* protein PINS, all contain a tandem array of three or four GoLoco motifs at their C-terminus, along with an N-terminal region of multiple tetratricopeptide repeats. The mammalian protein LGN is essential for the assembly and organization of the mitotic spindle [15]. Studies in *Drosophila* indicate that PINS is required for asymmetrical division of neuroblasts and for regulating the planar polarity of developing sensory organ precursor cells (reviewed in [9]). We and others

Abbreviations used: BODIPY®, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene; EST, expressed sequence tag; Gα_{i1}-ΔN, N-terminal 30-amino-acid truncation mutant of Gα_{i1}; G18-GL1 (etc.), protein containing only the first GoLoco motif of G18 (etc.); G18-GL123, protein containing all three GoLoco motifs of G18; GDI, guanine nucleotide dissociation inhibitor; GoLoco, G$\alpha_{i/o}$–Loco interaction; GPCR, G-protein-coupled receptor; GPR, G-protein regulatory; GST, glutathione S-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; ORF, open-reading frame; RFU, relative fluorescence units; RGS, regulator of G-protein signalling; SPR, surface plasmon resonance; TEV, tobacco etch virus.

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have also recently reported that the *Caenorhabditis elegans* proteins GPR-1 and GPR-2, each of which contain a single GoLoco motif C-terminal to tandem tetratricopeptide repeats, are required for proper generation of pulling forces required for asymmetrical cell division in the *C. elegans* early embryo [16–18]. It is thus becoming clear that GoLoco proteins play important roles in regulating cell division processes. Yet, our understanding of the biochemical properties of many GoLoco proteins, especially those with multiple GoLoco motifs, remains incomplete. In the present paper, we describe a series of biochemical and biophysical studies designed to address whether the three GoLoco motifs within the hitherto uncharacterized G18 protein are each capable of interacting with Gα subunits and of affecting their guanine nucleotide cycle.

EXPERIMENTAL

Materials

BODIPY® (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) FL–GTP[S] (guanosine 5′-[γ-thio]triphosphate) (BODIPY® FL thioether) was purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). His_6-tagged mouse $G\alpha_{oA}$ protein, expressed and purified from a pET15b-based *Escherichia coli* expression vector, was kindly provided as a gift by Dr Laurie Betts and Dr John E. Sondek (UNC-Chapel Hill).

Preparation of recombinant Gα proteins

His_6-tagged human $G\alpha_{i1}$ (full-length), $G\alpha_{i1}$-ΔN (an N-terminal 30-amino-acid truncation mutant) and His_6–$G\alpha_{i1}$–KT3 [full-length human $G\alpha_{i1}$ containing an N-terminal TEV (tobacco etch virus protease)-cleavable His_6 tag and a C-terminal KT3 (simian virus-40 large T antigen C-terminal epitope) tag of PPEPET] were expressed and purified as described previously [12,14].

A cDNA encoding the short isoform of bovine $G\alpha_s$ in the pNpT7-5 vector was a gift from Dr Maurine Linder (Washington University, St. Louis, MO, U.S.A.). The $G\alpha_s$ cDNA was excised from pNpT7-5 using *Nco*I and *Hin*dIII double digestion and ligated into *Nco*I/*Hin*dIII-digested pProEX-HTb vector (Invitrogen) using the Rapid Ligation Kit (Roche) as per manufacturer's instructions. The sequence fidelity of the resultant vector was confirmed at the UNC Automated DNA Sequencing Facility. Expression and purification of $G\alpha_s$ protein was performed as described previously [19,20], except for the following differences: (1) *E. coli* strain BL21(DE3) RIL (Stratagene) was transformed with the pProEX-HTb-$G\alpha_s$ expression vector, and (2) His_6–$G\alpha_s$ protein was first purified by Ni^{2+}-nitrilotriacetate chromatography via batch loading, followed by further purification by ion exchange and size exclusion chromatography. All proteins used in this study were concentrated in Centriprep® YM-30 or YM-10 centrifugal filter devices (Millipore). The concentrations of all proteins were determined by A_{280} measurements upon denaturation in guanidine hydrochloride and calculation based on predicted molar absorption coefficients. The functionality of recombinant Gα subunits was confirmed by assessing their ability to spontaneously exchange guanine nucleotide using a fluorescence-based BODIPY®–GTP[S] loading assay [21].

Preparation of recombinant GST (glutathione S-transferase)–G18 fusion proteins

pGEX4T2 (Amersham Bioscience) was engineered to contain a TEV protease cleavage site by insertion of an annealed TEV-site linker (yielding the plasmid pGEX4TEV2). Overlapping primers (sense, 5′-GATCCGAAAATCTGTATTTCCAGGGG-3′; antisense, 5′-AATTCCCCTGGAAATACAGATTTTCG-3′) were heated to 94 °C for 5 min, allowed to cool to room temperature, and ligated into pGEX4T2 digested with *Bam*HI and *Eco*RI restriction enzymes. The full-length human G18 ORF (open-reading frame) of 160 amino acids was subcloned out of IMAGE EST (expressed sequence tag) clone #5213618 (GenBank accession number BI906830) (sense primer, 5′-TGAATTCGATGG-AGGCTGAGAGACCCCAG-3′; antisense primer, 5′-TGCG-GCCGCTTTGAGACCAGTGGCAAGGAAG-3′), trapped in the pCR2.1-Topo vector (Invitrogen), and subcloned in-frame using the *Eco*RI and *Not*I sites of pGEX4TEV2. The full-length 159-amino-acid ORF of mouse G18, described previously as the anonymous ORF NG1 [7], was retrieved similarly from IMAGE EST clone #1528349 using PCR and subcloned into pGEX4TEV2. To create human G18 GoLoco fragments for insertion into pGEX4TEV2, we used a 'heterostagger' PCR method as described previously [22]. All expression plasmids were sequence-verified prior to transformation into *E. coli* strain BL21(DE3). Bacteria were grown to a D_{600} of 0.6–0.8 at 37 °C before induction with 1 mM isopropyl β-D-thiogalactoside. After an additional 4 h at 37 °C, cell pellets were lysed and GST fusion proteins were purified by gluthatione–agarose and size exclusion chromatography as described previously [12].

SPR (surface plasmon resonance) biosensor measurements

SPR binding assays were performed at 25 °C on a BIAcore 3000 (BIAcore Inc., Piscataway, NJ, U.S.A.) in the UNC Department of Pharmacology Protein Core Facility. For screening and kinetic analyses of G18 binding to Gα subunits, carboxymethylated-dextran sensor chips with covalently bound anti-GST antibody surfaces were created as described previously [10,12]. Recombinant GST or GST fusion proteins were bound to separate flow cells of anti-GST antibody surfaces to a density of ~800 resonance units.

As recommended by Lenzen and colleagues [23], binding analyses were performed using buffer W [150 mM NaCl, 5 mM $MgCl_2$, 0.005 % (v/v) Nonidet P-40, 20 mM Hepes, pH 7.4) as the running buffer to stabilize the anti-GST antibody surface. Recombinant Gα subunits were initially diluted to 2 μM in buffer W containing 32 μM GDP alone, 32 μM GDP plus 36 μM $AlCl_3$ and 10 mM NaF, or 32 μM GTP[S], and incubated for 90 min at 30 °C. To screen for Gα binding, 25 μl aliquots of Gα protein were injected at a flow rate of 5 μl/min over four flow-cell surfaces simultaneously using the KINJECT command. For saturation binding analyses, 60 μl of $G\alpha_{i1} \cdot GDP$ at concentrations ranging from 1 nM to 20 μM was injected at a flow rate of 5 μl/min over four flow-cell surfaces simultaneously using the KINJECT command. Surface regeneration was performed by serial injections of 10 μl of 10 mM glycine, pH 2.2, and 10 μl of 0.05 % (w/v) SDS at a 20 μl/min flow rate. Background binding to a GST-coated surface (acquired simultaneously) was subtracted from all binding curves using BIAevaluation software version 3.0 (BIAcore Inc.) and plotted using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). For saturation binding analyses, the average response from 660 to 680 s was used to calculate specific binding for each GST–G18 fusion protein. The response at each concentration was plotted as percentage of B_{max} (maximal binding), and the resulting curve was fitted to a hyperbolic binding model by GraphPad Prism.

Gα/G18 co-immunoprecipitation

pcDNA3.1-based eukaryotic expression vectors encoding human $G\alpha_{i1}$, $G\alpha_q$, $G\alpha_s$ and $G\alpha_{12}$ were purchased from the Guthrie cDNA

collection (Sayre, PA, U.S.A.; http://www.cdna.org). Gα subunit variants encoding the KT3 tag were created by QuickChange (Stratagene) insertional site-directed mutagenesis to introduce nucleotides coding for the amino acid sequence PPEPET at the C-terminus of each Gα subunit. A mammalian expression vector encoding full-length G18 with an N-terminal Myc tag was created by subcloning the ORF of human G18 into the *Eco*RI and *Not*I sites of the plasmid pcDNA3.1/myc-His (Invitrogen).

Transient co-transfections of HEK 293T cell monolayers with expression constructs for Myc-tagged human G18 and KT3-tagged Gα were performed using FuGENE-6 (Roche Applied Science) according to the manufacturer's instructions. Cells were harvested 48 h post-transfection by scraping into 1 ml of lysis buffer [20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 100 μM GDP, 5 mM $MgCl_2$, 1 mM EGTA, 1 % (v/v) Triton X-100, and Complete™ Mini EDTA-free protease inhibitor mixture (Roche)]. Lysates were sonicated for 5 min and clarified by centrifugation at 16 000 ***g*** for 20 min at 4 °C. Supernatants were pre-cleared with 50 μl of Protein A/G–agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). Beads were removed by centrifugation at 16 000 ***g*** for 1 min, and aliquots of the supernatant were removed for immunoblot analysis. The remaining lysate was then immunoprecipitated overnight at 4 °C using anti-KT3 monoclonal antibody (Covance Research Products, Inc., Berkeley, CA, U.S.A.) or anti-Myc monoclonal antibody 9E10 (Roche). Immune complexes were precipitated using 50 μl of Protein A/G–agarose beads, washed twice with wash buffer [50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 100 μM GDP, 1 % (v/v) Triton X-100], and washed twice with detergent-free wash buffer. Immune complexes were eluted from the beads by adding 50 μl of Laemmli sample buffer and boiling for 5 min. Proteins were separated by SDS/PAGE, electroblotted on to nitrocellulose, and detected with appropriate antisera, secondary antibodies conjugated to horseradish peroxidase (Amersham), and enhanced chemiluminescence (Amersham).

Fluorescence-based GTP[S] binding assay

Measurements of BODIPY® fluorescence were performed in the UNC Department of Pharmacology Protein Core Facility using a PerkinElmer LS55 Luminescence Spectrometer (PerkinElmer Analytical Instruments, Shelton, CT, U.S.A.) with excitation at 485 nm and emission at 530 nm (slitwidths each at 3.0 nm). BODIPY® FL–GTP[S] was diluted to 1 μM in 10 mM Tris, pH 8.0, 1 mM EDTA and 10 mM $MgCl_2$, and equilibrated to 30 °C in 2 ml cuvettes. The fluorescence of BODIPY® FL–GTP[S] alone was measured prior to addition of Gα protein. $G\alpha_{i1}$ (200 nM), incubated with 1 μM of GST-fusion protein, was allowed to equilibrate at 25 °C for 10 min before addition to the cuvette. The change in fluorescence was measured over time and normalized to baseline BODIPY® FL–GTP[S] fluorescence. The rate of nucleotide exchange is presented as the initial reaction rate in RFU (relative fluorescence units)/s, and was calculated over the first 750 s following addition of Gα protein mixture to the cuvette. Statistical significance was determined by the one-sample *t* test (GraphPad Prism) with a *P* value of < 0.003.

Multiple Gα subunit interaction assay

GST–G18-GL123 (a fusion protein comprising GST and all three GoLoco motifs of G18), GST–G18-GL1 (containing only the first GoLoco motif of G18) or GST was incubated with glutathione–agarose resin for 1 h at 4 °C in TEV buffer [20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM dithiothreitol and either 40 μM GDP alone or 40 μM GDP + 20 mM NaF + 30 μM $AlCl_3$ (to form aluminium tetrafluoride)]. The resin was collected by brief centrifugation at 5000 ***g*** and washed twice with 1 ml of TEV buffer. Aliquots of 25 μg of the truncation mutant $G\alpha_{i1}$-ΔN and 25 μg of KT3-tagged $G\alpha_{i1}$ were incubated together with appropriate nucleotide-containing TEV buffer for 1 h at 25 °C prior to their addition to resin-bound GST fusion proteins. After incubation at 4 °C for 1 h, the resins were washed twice with 1 ml of TEV buffer, resuspended in 200 μl of TEV buffer, and incubated overnight at 4 °C with 50 μl of recombinant TEV protease (Invitrogen). The resins were collected by centrifugation at 5000 ***g***, and the soluble fraction was removed and 4 μl of mouse monoclonal anti-KT3 antibody added (Covance). Following incubation for 1 h at 4 °C, 25 μl of Protein A/G–agarose beads was added and incubated for 1 h at 4 °C. The resin was collected by centrifugation and washed twice with 1 ml of TEV buffer. Finally, 25 μl of 3 × SDS/PAGE loading buffer was added to the resin and the mixture was boiled for 5 min before dilution with 50 μl of water. Proteins were resolved by SDS/12 %-PAGE before being electroblotted on to nitrocellulose and probed with anti-KT3, anti-$G\alpha_i$ (internal epitope; BioDesign International, Saco, ME, U.S.A.) or anti-$G\alpha_i$ (C-terminal epitope; NEN Life Science Products, Inc. Boston, MA, U.S.A.) antibodies. To visualize the immunoprecipitated proteins, the blots were processed with appropriate secondary antibody conjugated to horseradish peroxidase and treated with enhanced chemiluminescent reagent (Amersham Bioscience).

RESULTS AND DISCUSSION

G18 contains three GoLoco motifs and binds $G\alpha_{i1}$

In our original discovery of the GoLoco motif [7], we described an anonymous ORF from the human genome (Swiss-Prot accession number P78548) as containing three 19-amino-acid GoLoco motifs in a tandem array. The full-length 160-amino-acid ORF (GenBank accession number NP_071390; Figure 1) was originally called G18 by Kendall et al. [24] and uncovered as one of seven novel genes (*G12* to *G18*) within a 160 kb gene cluster found between the human major histocompatibility complex HLA-D and complement C4 loci on human chromosome 6. The mouse G18 orthologue (GenBank accession number NP_598877), described previously in the literature as 'NG1' [7], contains 159 amino acids and shares 88 % identity (93 % similarity) with the human G18 polypeptide sequence (Figure 1A).

Secondary structure prediction [25] of the G18 ORF suggests that the majority of the protein consists of random coil (Figure 1A). However, the regions corresponding to the first 15 residues of the three GoLoco motifs are predicted to form α helices. These predicted α helices correspond to the α helix seen by X-ray crystallography in the N-terminal portion of the RGS14 GoLoco motif bound to $G\alpha_{i1} \cdot GDP$ [14]. The crystal structure of the RGS14–$G\alpha_{i1}$ complex suggests that the minimal Gα-binding region is 36 amino acids, including not only the originally identified 19-amino-acid GoLoco consensus signature but also a C-terminal extension that is less conserved among GoLoco family members [7,14]. Given this extended definition of a minimal functional GoLoco motif, the second and third GoLoco motifs of G18 clearly overlap (Figures 1A and 1B). This overlap suggests that both of these GoLoco motifs would be incapable of binding Gα subunits simultaneously. In addition, the second GoLoco motif (amino acids 104–138) deviates from the consensus sequence [7], in that residue 121 is an alanine rather than the conserved aspartate or glutamate (Figure 1A).

Nonetheless, the full-length G18 protein, containing all three GoLoco motifs, was found to interact with GDP-bound $G\alpha_{i1}$ both *in vitro* and upon cellular co-transfection (Figure 2). Human

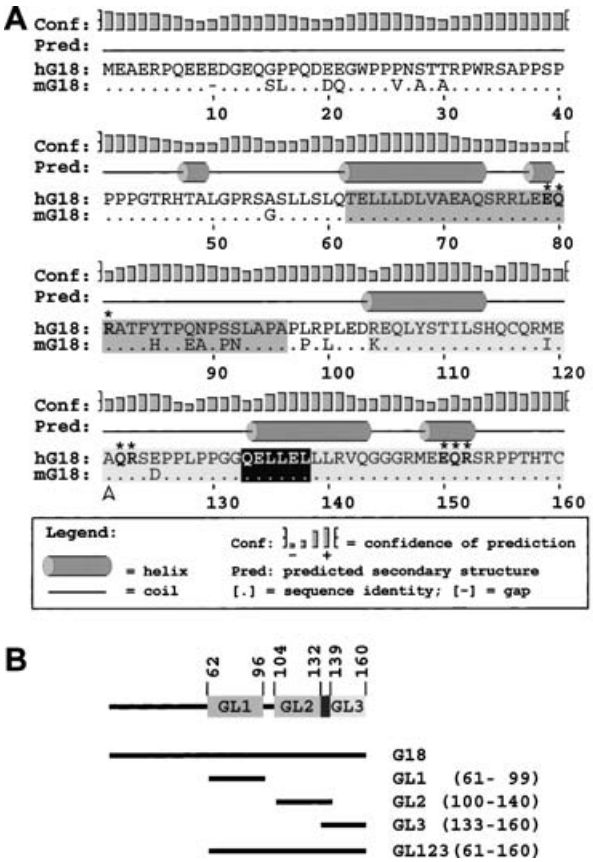

Figure 1 Secondary structure prediction of the triple GoLoco motif human G18 protein and schematic of the protein constructs used in the present study

(**A**) Predicted amino acid sequence and secondary structure of the ORF of human *G18* (hG18; GenBank accession number NP_071390) and mouse *G18* (mG18; GenBank NP_598877). Secondary structure prediction was performed using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred) [25]. GoLoco motifs are boxed in grey, and the highly conserved acidic-glutamine-arginine tripeptide within each GoLoco motif [14] is denoted by asterisks. Ala-121 is highlighted with an arrowhead. Overlap between the second and third GoLoco motifs is denoted in black. (**B**) Polypeptide spans of human G18, expressed and purified as GST-fusion proteins for *in vitro* characterization, are denoted below the architectural schematic of the G18 ORF (amino acid boundaries of cloned polypeptide spans are indicated in parentheses).

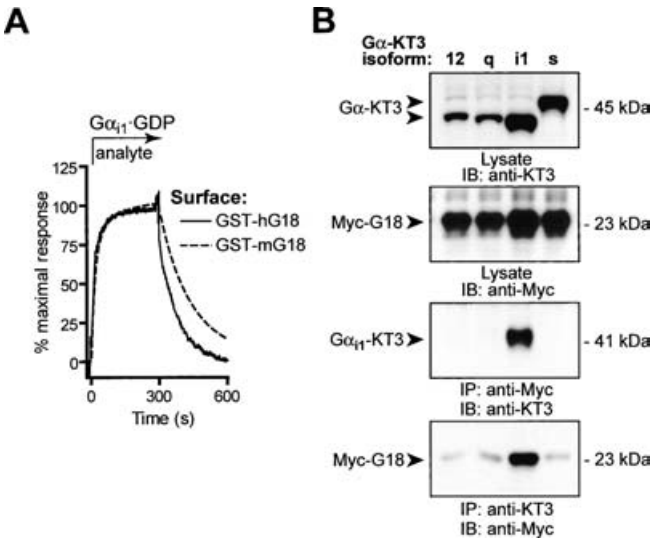

Figure 2 Full-length G18 binds $G\alpha_{i1}$ *in vitro* and in cells

(**A**) Binding of recombinant, GDP-loaded $G\alpha_{i1}$ protein to SPR surfaces preloaded to saturation with the indicated GST–G18 fusion proteins (h, human; m, mouse). Relative responses of each surface are plotted over time upon injection of 25 μl of 2 μM $G\alpha_{i1} \cdot GDP$ (0–300 s; flow rate 5 μl/min) followed by a 300 s dissociation period. Both Gα interaction curves were subtracted from response curves generated simultaneously on a separate control surface bound to GST alone. (**B**) Reciprocal co-immunoprecipitation of Myc-epitope-tagged full-length human G18 solely with KT3-epitope tagged $G\alpha_{i1}$, and not three other Gα–KT3 isoforms tested ($G\alpha_{12}$, $G\alpha_q$ and $G\alpha_s$). Lysates of transiently co-transfected HEK 293T cell monolayers (Lysate) were immunoblotted (IB) separately with anti-KT3 and anti-Myc monoclonal antibodies to verify protein expression of Gα subunits and Myc–G18 protein respectively. Cell lysates were immunoprecipitated (IP) with anti-KT3 or anti-Myc antibodies as indicated, and Myc-G18–Gα-KT3 complex formation was detected by immunoblotting with the reciprocal antibody. Faint, non-specific bands detected in the $G\alpha_{12}$, $G\alpha_q$ and $G\alpha_s$ lanes of the bottom anti-Myc immunoblot represent immunoglobin light chains of the immunoprecipitating anti-KT3 antibody, which resolves at the same mobility on SDS/PAGE as Myc–G18 protein.

and mouse G18 were each expressed in and purified from *E. coli* as recombinant GST-fusion proteins and bound to anti-GST-antibody-coated SPR biosensor surfaces. Recombinant $G\alpha_{i1}$ protein was injected for 300 s over these GST–G18 surfaces, as well as a surface coated with GST alone, and found to bind specifically to the two G18 fusion protein surfaces (Figure 2A). To test Gα binding specificity, human G18 was also expressed as an N-terminally Myc-epitope-tagged protein in HEK 293T cells, along with one canonical member of each of the four Gα subclasses [26]. In these co-transfection experiments, Myc-tagged G18 selectively co-immunoprecipitated KT3-tagged $G\alpha_{i1}$, but not $G\alpha_{12}$, $G\alpha_q$ or $G\alpha_s$ subunits (Figure 2B). In the reciprocal experiment, KT3-tagged $G\alpha_{i1}$ subunit, but not $G\alpha_{12}$, $G\alpha_q$ or $G\alpha_s$ subunits, co-immunoprecipitated Myc-tagged G18 (Figure 2B), thus indicating that G18 is a $G\alpha_i$-selective GoLoco motif protein in a cellular context. The Gα-binding specificity of G18 is thus similar to that of AGS3 and the isolated GoLoco motifs of RGS12 and RGS14 [8,10–12,14]; indeed, no GoLoco motif has yet been shown to bind Gα subunits outside of the adenyate cyclase inhibitory subclass [9].

Selectivity of G18 GoLoco motifs for GDP-bound $G\alpha_{i1}$

To assess the Gα selectivity and nucleotide dependence of the individual GoLoco motifs of G18, real-time binding assays of multiple Gα subunits in three nucleotide states were performed using SPR. Polypeptides corresponding to each of the three GoLoco regions of human G18 (as illustrated in Figure 1B) were separately purified as GST-fusion proteins and bound to anti-GST-antibody-coated SPR biosensor surfaces. Recombinant $G\alpha_{i1}$, $G\alpha_{oA}$ and $G\alpha_s$ were separately injected for 300 s over these biosensor surfaces, having first been incubated with GTP[S] to mimic the activated GTP-bound form, with $GDP \cdot AlF_4^-$ to mimic the transition state of GTP hydrolysis, or with GDP alone to preserve the ground state of the Gα subunit.

Individual GoLoco motifs of G18 interacted exclusively with $G\alpha_{i1}$ in its GDP-bound, inactive state (Figure 3), a selectivity identical to that seen both for full-length G18 (Figure 2) and for an N-terminally truncated, triple-GoLoco motif fusion protein (G18-GL123; Figure 3D). $G\alpha_{oA}$ and $G\alpha_s$ proteins did not bind to any of the G18 fusion proteins tested (Figure 3). Both the N-terminal (G18-GL1) and C-terminal (G18-GL3) GoLoco motifs bound to $G\alpha_{i1} \cdot GDP$ (Figures 3A and 3C). However, the second GoLoco motif (G18-GL2) exhibited no detectable binding upon injection of either 1 μM or 20 μM $G\alpha_{i1} \cdot GDP$ (Figure 3B and results not shown). The existence of non-functional GoLoco motifs within either single- or multi-GoLoco proteins has not been observed or reported previously.

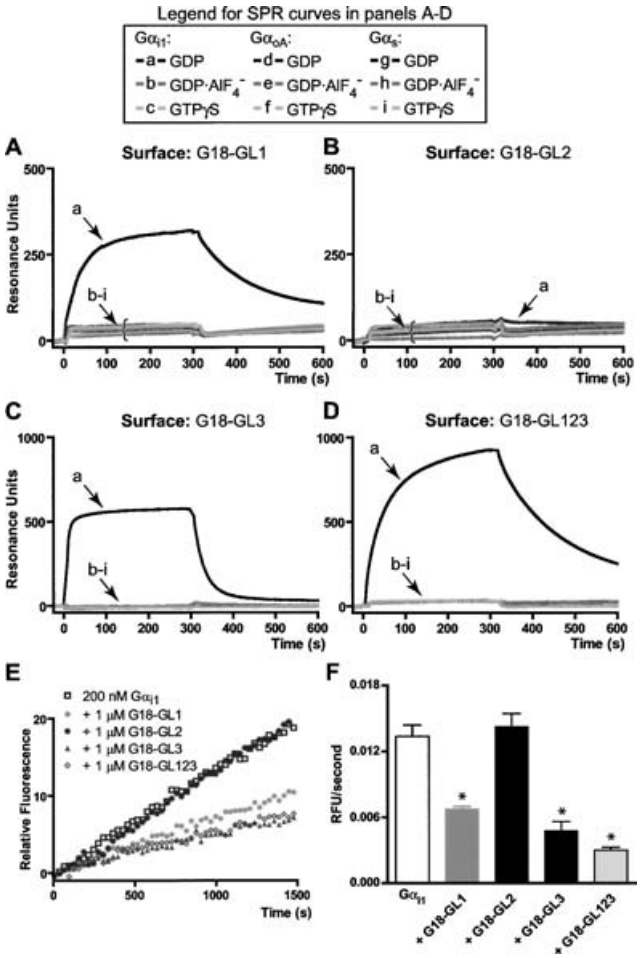

Figure 3 Specific binding and GDI activity of the first and third, but not the second, GoLoco motifs of human G18

(**A**)–(**D**) Responses of anti-GST-antibody-coated SPR biosensor surfaces, preloaded to saturation with individual GST–G18 GoLoco fusion proteins as indicated, upon injection (0–300 s; flow rate 5 μl/min) of 25 μl of 2 μM recombinant $G\alpha_{i1}$, $G\alpha_{oA}$ or $G\alpha_s$ subunits prebound with GDP, GDP · AlF_4^- or GTP[S] (GTPγS). All Gα interaction curves were subtracted from response curves generated simultaneously on a separate control surface bound to GST alone. (**E**) Time course of BODIPY®–GTP[S] binding to 200 nM $G\alpha_{i1}$ · GDP in the presence or absence of the indicated GST–G18 GoLoco fusion proteins. (**F**) As assessed by initial rates of BODIPY®–GTP[S] binding-induced fluorescence increase (RFU/s), a 5-fold molar excess of GST–G18 fusion proteins encompassing the first (GL1), third (GL3) or all three (GL123) GoLoco motifs exhibits significant GDI activity. In contrast, the initial rate of BODIPY®–GTP[S] binding by $G\alpha_{i1}$ · GDP is unaltered by preincubation with GST–G18 fusion protein encompassing the second GoLoco motif (GL2). Results shown are means and S.D. for at least three separate experiments ($^*P < 0.003$).

GDI activity of the GoLoco motifs of G18

We previously identified the GoLoco motifs of RGS12, RGS14 and AGS3 as GDIs for their Gα targets [10,12]. To test for any GDI activity of G18, we used a real-time, fluorescent GTP[S] binding assay [21] to monitor the spontaneous nucleotide exchange rate of $G\alpha_{i1}$ · GDP. As predicted by the Gα binding studies, GST fusion proteins possessing the first and/or third, but not the second, GoLoco motif of G18 exhibited GDI activity towards GDP-bound $G\alpha_{i1}$ (Figure 3E). Preincubation of $G\alpha_{i1}$ · GDP with a 5-fold molar excess of G18-GL1, G18-GL3 or G18-GL123 fusion protein inhibited the initial rate of GTP[S] binding by 50–80 % (Figure 3F). This level of GDI activity is similar to that observed previously for the RGS12 and RGS14 GoLoco motifs [12], i.e. a 50–70 % inhibition of the initial GTP[S] binding rate at a 2-fold excess of RGS12/14 GoLoco peptide relative to uncomplexed $G\alpha_{i1}$. Consistent with its lack of demonstrable binding affinity towards $G\alpha_{i1}$ (Figure 3B), the second GoLoco motif failed to slow the GTP[S] binding rate of $G\alpha_{i1}$ · GDP at a 5-fold molar excess (Figures 3E and 3F).

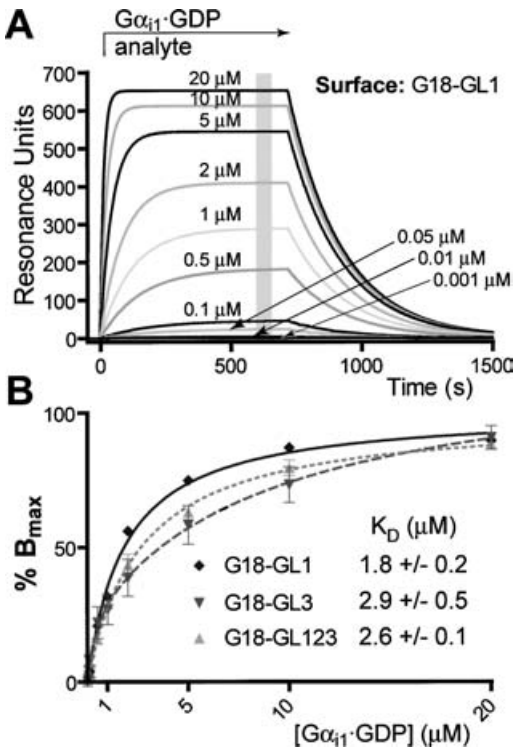

Figure 4 Human G18 GoLoco motifs bind with low micromolar affinity to $G\alpha_{i1}$ · GDP

(**A**) Sample response curves from a saturation binding SPR experiment. GST–G18-GL1 fusion protein was pre-bound to the SPR biosensor surface and the indicated concentrations of $G\alpha_{i1}$ · GDP protein (from 0.001 to 20 μM) were injected as described in the Experimental section ('analyte'). The shaded region within the association phase denotes the time period used for calculation of binding affinity. (**B**) Various concentrations of recombinant $G\alpha_{i1}$ · GDP were injected over the indicated GST–G18 fusion protein surfaces. The average response from 660–680 s during $G\alpha_{i1}$ injection was normalized as a percentage of maximum binding (%B_{max}) and fitted to a hyperbolic binding model in GraphPad Prism version 4.0 to calculate the apparent dissociation constant (K_D).

Binding affinities of the first and third GoLoco motifs

To quantify the Gα-binding affinity of the G18 GoLoco motifs capable of interacting with $G\alpha_{i1}$ · GDP, various concentrations of GDP-bound $G\alpha_{i1}$ protein (from 1 nM to 20 μM) were separately injected over each SPR biosensor surface until a steady-state binding level was reached (e.g. association curves for G18-GL1 fusion protein are shown in Figure 4A). At each concentration, the average response from 660 to 680 s was used to plot saturation binding curves for each surface (Figure 4B). The apparent dissociation constant (K_D) for the triple-GoLoco-motif G18 fusion protein was calculated to be 2.6 ± 0.1 μM, with the first and third GoLoco motifs alone exhibiting apparent K_D values of 1.8 ± 0.2 and 2.9 ± 0.5 μM respectively. These affinities are weaker by one to two orders of magnitude than those ascertained previously for the single GoLoco motifs within RGS12 (19 nM) and RGS14 (65 nM) [12], perhaps reflecting the fact that G18 bears more than one functional GoLoco motif in tandem and thus may require less avid single Gα-binding sites.

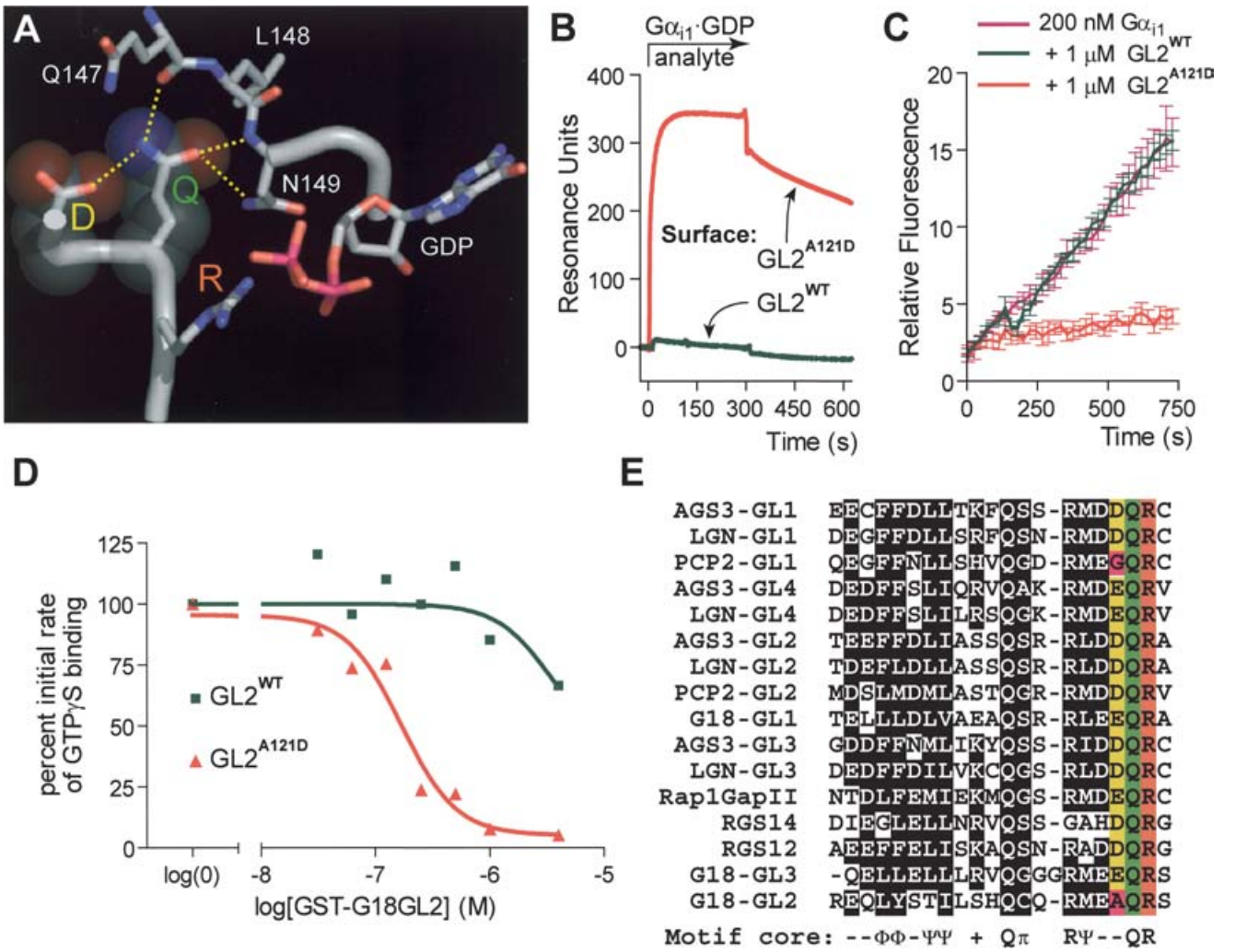

Figure 5 Mutation of Ala-121 to aspartate restores Gα binding and GDI activity to the second GoLoco motif of human G18

(**A**) Positioning of the GoLoco motif 'arginine finger' by the preceding aspartate and glutamine residues is important for GDI activity [14]. The Arg-516 residue (R) of the Asp-Gln-Arg tripeptide within the RGS14 GoLoco motif is shown in its position contacting the α- and β-phosphates of GDP bound within $G\alpha_{i1}$, as derived from the crystal structure of the RGS14–$G\alpha_{i1}$ · GDP complex (PDB accession number 1KJY). The side chains of Asp-514 (D) and Glu-515 (Q) are illustrated as transparent space-filling models. Asp-514 is involved in orienting Arg-516 by hydrogen-bonding to the neighbouring Glu-515 side chain that also makes hydrogen-bond contacts to the peptide backbone of $G\alpha_{i1}$ between residues Glu-147, Leu-148 and Asn-149. Within the second GoLoco motif of G18, the analogous residue to Asp-514 of RGS14 is Ala-121, which has a methyl side chain that lacks the ability to hydrogen bond with its neighbouring glutamine side chain. (**B**) Relative binding of recombinant, GDP-loaded $G\alpha_{i1}$ protein to SPR surfaces preloaded to saturation with the indicated GST–G18 fusion proteins ($GL2^{WT}$, wild-type second GoLoco motif; $GL2^{A121D}$, second GoLoco motif with aspartate replacing Ala-121). Relative responses of each surface are plotted over time upon injection of 25 μl of 2 μM $G\alpha_{i1}$ · GDP (0–300 s; flow rate 5 μl/min) followed by a 300 s dissociation period. Both $G\alpha$ interaction curves were subtracted from response curves generated simultaneously on a separate control surface bound to GST alone. (**C**) Time course of BODIPY®–GTP[S] binding to 200 nM $G\alpha_{i1}$ · GDP in the presence or absence of GST–G18-GoLoco fusion proteins. Pre-incubation with 1 μM wild-type GST–G18-GL2 protein fails to alter the initial rate of BODIPY®–GTP[S] binding, whereas replacement of Ala-121 with aspartate yields a GoLoco motif exhibiting significant GDI activity. (**D**) By measuring the initial rates of BODIPY–GTP[S] binding after preincubating 200 nM $G\alpha_{i1}$ · GDP with various concentrations (5 μM to 30 nM) of GST–G18-GL2 fusion proteins, a submicromolar IC_{50} value of 169 nM was observed for GST–G18-$GL2^{A121D}$ (95 % confidence interval of 82–350 nM). (**E**) By analogy with the second GoLoco motif of G18, the first GoLoco motif of human PCP2 (Purkinje cell-specific protein-2) is predicted to also lack robust $G\alpha$ binding and GDI activity, as the first residue of its highly conserved Asp-Gln-Arg tripeptide (shaded in colour) is glycine rather than an acidic residue. GoLoco motif residues illustrated in the structural model in (**A**) are highlighted in their respective colours. For each representative human GoLoco motif sequence in the alignment, Swiss-Prot ID or GenBank accession numbers and sequence ranges are as follows: AGS3 (AA017260; GL1 = residues 472–492, GL2 = 525–545, GL3 = 573–593 and GL4 = 607–627); LGN (AAH27732; GL1 = 482–502, GL2 = 537–557, GL3 = 587–607, GL4 = 621–641); Pcp2 (AAN52488; GL1 = 7–27, GL2 = 63–83); G18 (AAH18724; GL1 = 62–82, GL2 = 104–124, GL3 = 133–153); Rap1GapII (BAA83674; GL1 = 26–46); RGS14 (RGSE_HUMAN; GL1 = 497–517); RGS12 (RGSC_HUMAN; GL1 = 1187–1207).

Mutation of Ala-121 restores function to the second GoLoco motif

Our results from SPR and GTP[S] binding studies (Figures 3C and 3E) suggest that the second GoLoco motif of G18 exhibits little binding affinity or GDI activity towards $G\alpha_{i1}$ · GDP. Comparison of the polypeptide sequence of this GoLoco motif with the consensus GoLoco signature reveals that the first residue (amino acid 121) of the highly conserved acidic-glutamine-arginine tripeptide is alanine rather than an aspartic acid or glutamic acid residue (Figure 1A). Recent structural determination of the RGS14 GoLoco motif bound to $G\alpha_{i1}$ · GDP has helped to underscore the critical nature of this Asp-Gln-Arg tripeptide for GDI function [14]; namely, hydrogen bonding between the aspartate and glutamine side chains and backbone interactions with the $G\alpha$ subunit serve to position the tripeptide arginine into the guanine nucleotide binding cleft (Figure 5A).

Therefore, to test whether this single position (Ala-121) is solely responsible for the lack of function of the second GoLoco

motif within G18, we created an alanine-to-aspartate point mutant of the GST–G18-GL2 fusion protein by site-directed mutagenesis. The resultant purified protein ($GL2^{A121D}$) was tested in parallel with wild-type GST–G18-GL2 fusion protein in the SPR and GTP[S] binding assays. Significant binding to $G\alpha_{i1} \cdot GDP$ (Figure 5B) and a significant decrease in spontaneous GDP release by $G\alpha_{i1} \cdot GDP$ (IC_{50} 169 nM; Figures 5C and 5D) were both observed with the $GL2^{A121D}$ mutant protein, in contrast with the apparent lack of activity of the wild-type protein (Figures 5B–5D).

At a 50-fold molar excess (5 μM), wild-type GST–G18-GL2 fusion protein exhibited weak inhibitory activity ($\sim$33 % inhibition) on spontaneous GDP dissociation (Figure 5D). We are unable to test higher concentrations of this fusion protein for GDI activity, given limitations in protein purification and concentration. Nevertheless, it is apparent that the gain-of-function Ala-121-to-aspartate mutation in the second GoLoco motif increases its potency of GDI activity by at least 20-fold.

Based on these findings with the second GoLoco motif of G18, we predict that other naturally occuring GoLoco sequences lacking an acidic residue in the first position of the Asp-Gln-Arg tripeptide lack robust Gα binding and GDI activities. For example, the longer alternatively spliced isoform of human PCP2 (Purkinje cell-specific protein-2), recently shown to contain not one but two GoLoco motifs [27], has a glycine rather than an aspartate or glutamate at this critical position within its first GoLoco motif (PCP2-GL1; Figure 5E), suggesting that it too may possess weak to nil Gα binding and GDI activities.

Ternary complex formation between G18 and two Gα subunits

The existence of two functional GoLoco motifs (GL1 and GL3) within the wild-type G18 polypeptide suggests that it may act as a Gα 'scaffold' [7] by binding more than one Gα subunit simultaneously. To test the ability of G18 to form a ternary complex with more than one Gα subunit, we incubated two identifiably different $G\alpha_{i1}$ subunits, $G\alpha_{i1}$–KT3 and $G\alpha_{i1}$-ΔN (Figure 6B), in either GDP-bound or GDP-AlF_4^--bound states, with GST alone, a GST fusion of a single GoLoco motif (GST–G18-GL1), or GST–G18-GL123 fusion protein. After incubation, the GST–G18 fusion proteins were precipitated, washed extensively to remove unbound Gα subunits, and cleaved by TEV protease to release the C-terminal G18 GoLoco polypeptides [and any bound Gα partner(s)] from their N-terminal GST tags (Figure 6A). Protein complexes released into the supernatant upon cleavage by TEV protease were then immunoprecipitated with an anti-KT3 epitope monoclonal antibody (Figure 6A) and co-immunoprecipitated proteins were blotted and probed for the presence of both $G\alpha_{i1}$–KT3 and $G\alpha_{i1}$-ΔN (Figure 6C). The smaller $G\alpha_{i1}$-ΔN protein lacks the C-terminal KT3-epitope tag, and therefore can only be precipitated indirectly with $G\alpha_{i1}$–KT3 if both are bound to the same scaffold, namely G18 (Figure 6A).

$G\alpha_{i1}$-ΔN protein was observed to be co-immunoprecipitated with $G\alpha_{i1}$–KT3 only when using GST–G18-GL123 as the protein scaffold (Figure 6C, lower panel); this interaction is dependent on nucleotide state and was not seen using GDP-AlF_4^--bound Gα subunits. This result indicates that the triple GoLoco motif C-terminus of human G18 is capable of forming a ternary complex with two GDP-bound $G\alpha_i$ subunits, presumably via the first and third GoLoco motifs, as the second motif is non-functional. This ability of G18 to bind more than one $G\alpha_i \cdot GDP$ subunit simultaneously is similar to the findings of Bernard et al. with the tetra-GoLoco-motif protein AGS3 [28]. The next challenge will be to establish a clearly delineated role for multiple (as opposed to single) $G\alpha_i \cdot GDP$-binding sites in GoLoco-protein-mediated modulation of GPCR signalling and/or regulation of mitotic spindle force generation and cell division processes.

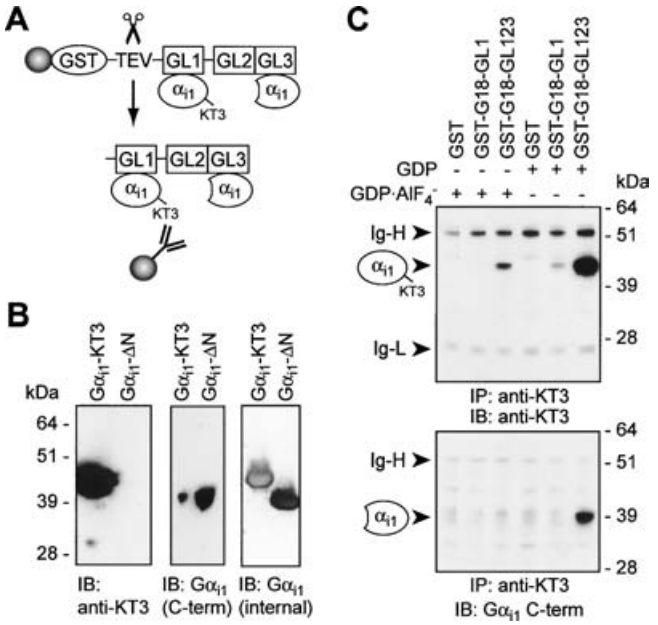

Figure 6 Human G18 protein can bind more than one Gα subunit at a time

(**A**) Schematic representation of the multiple $G\alpha_{i1}$ subunit interaction assay. GST–G18 fusion proteins, or GST alone, were bound to glutathione–agarose resin and incubated with two different forms of recombinant $G\alpha_{i1} \cdot GDP$: one form ($G\alpha_{i1}$–KT3) bears a C-terminal PPEPET extension that comprises the minimal KT3-epitope tag, and the other form ($G\alpha_{i1}$-ΔN) lacks the first 30-amino-acid α-helical extension, but remains folded and functional [14]. Upon release from resin binding by TEV protease-mediated cleavage, the resultant protein complexes are immunoprecipitated with anti-KT3 monoclonal antibody. (**B**) Validation of the specific immunodetection of each $G\alpha_{i1}$ isoform. Recombinant, purified $G\alpha_{i1}$–KT3 and $G\alpha_{i1}$-ΔN proteins were resolved by SDS/PAGE, electroblotted on to nitrocellulose and immunoblotted (IB) with the indicated antibodies. Anti-KT3 antibody does not recognize $G\alpha_{i1}$-ΔN protein (left panel), whereas an antibody directed to the wild-type $G\alpha_{i1}$ C-terminus (C-term) does not recognize the higher-molecular-mass $G\alpha_{i1}$–KT3 protein (middle panel), since the KT3 tag is located at the C-terminus. An antibody directed to an internal peptide epitope of $G\alpha_{i1}$ recognizes both $G\alpha_{i1}$–KT3 and $G\alpha_{i1}$-ΔN isoforms. (**C**) Following glutathione–agarose resin precipitation of the indicated GST–G18 fusion proteins (or GST alone), incubated previously with both $G\alpha_{i1}$–KT3 and $G\alpha_{i1}$-ΔN in the indicated guanine nucleotide state, protein complexes were liberated from resin by TEV protease cleavage and immunoprecipitated (IP) with anti-KT3 antibody. Immunoblotting with anti-$G\alpha_{i1}$ C-terminal antibody reveals that $G\alpha_{i1}$-ΔN protein is only detectable in protein complexes formed using GDP-bound $G\alpha_{i1}$ subunits and GST–G18-G123 protein that contains all three GoLoco motifs (lower panel, rightmost lane). Ig-H and Ig-L indicate the locations of immunoglobulin heavy and light chains respectively of the immunoprecipitating anti-KT3 antibody.

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