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# A bifunctional $G\alpha_i/G\alpha_s$ modulatory peptide that attenuates adenylyl cyclase activity

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*Abbreviations:*

**AC** adenylyl cyclase [E.C. 4.6.1.1]

**AMP** adenosine monophosphate

**cAMP** cyclic AMP

**FITC** fluorescein isothiocyanate

**FSK** forskolin

**GDI** guanine nucleotide dissociation inhibitor

**GEF** guanine nucleotide exchange factor

**GPCR** G-protein coupled receptor

**GTPγS** gamma-thiol guanosine triphosphate

**SPR** surface plasmon resonance

## Abstract

Signaling via G-protein coupled receptors is initiated by receptor-catalyzed nucleotide exchange on Gα subunits normally bound to GDP and Gβγ. Activated Gα·GTP then regulates effectors such as adenylyl cyclase. Except for Gβγ, no known regulators bind the adenylyl cyclase-stimulatory subunit $G\alpha_s$ in its GDP-bound state. We recently described a peptide, KB-752, that binds and enhances the nucleotide exchange rate of the adenylyl cyclase-inhibitory subunit $G\alpha_i$. Herein, we report that KB-752 binds $G\alpha_s$·GDP yet slows its rate of nucleotide exchange. KB-752 inhibits GTPγS-stimulated adenylyl cyclase activity in cell membranes, reflecting its opposing effects on nucleotide exchange by $G\alpha_i$ and $G\alpha_s$.



### Keywords

Adenylyl cyclase; Biosensors; G-proteins; Phage display; Signal transduction; Surface plasmon resonance


## 1. Introduction

G-proteins serve as crucial intermediaries of extracellularly-evoked signaling cascades critical to cellular physiology [1]. In the conventional model of heterotrimeric G-protein signaling, extracellular cues such as hormones and neurotransmitters activate seven transmembrane domain receptors (GPCRs) coupled to heterotrimers consisting of Gα·GDP bound to Gβγ in the inactive state [2]. Gβγ stabilizes the GDP-bound state and prevents spontaneous nucleotide exchange, thus serving as a guanine nucleotide dissociation inhibitor (GDI). In contrast, GPCR activation leads to exchange of GDP for GTP on Gα, thus serving as a guanine nucleotide exchange factor (GEF). Binding of GTP alters the conformation of three flexible “switch” regions within Gα, causing Gβγ dissociation [3]. Both Gα·GTP and freed Gβγ can subsequently regulate several downstream signaling components including adenylyl cylcases (ACs), phospholipases, and ion channels responsible for the elicited cellular responses [2]. Signal termination is achieved by the intrinsic GTP hydrolysis activity of Gα, regenerating Gα·GDP capable of reassociation with Gβγ and thereby preventing effector interactions. Given this bimodal nucleotide cycle, the lifetime of activated signaling is reliant on the duration of Gα in the GTP-bound state. In this fashion, G-proteins serve as critical temporal regulators of these pathways and thus understanding the molecular determinants underlying their nucleotide cycle is of particular interest.

Phage display is a powerful technique for identifying small peptides capable of binding protein targets in an unbiased manner. These peptides can then serve as tools for studying target protein binding surfaces, protein-protein interaction sites, and protein function and regulation [reviewed in [4]]. Phage display, along with similar approaches, has been used to investigate G-protein binding interfaces on GPCRs and effector binding regions on Gβγ subunits, as well as to identify peptides with regulatory properties including GEF and GDI activities [5-9]. We recently used phage display to identify a peptide that interacts with the AC inhibitory Gα subunit $G\alpha_i$, specifically in its GDP-bound state. This peptide, KB-752, exerts GEF activity on $G\alpha_i$, the molecular determinants for which were ascertained by the $G\alpha_{i1}$·GDP/KB-752 crystal structure [10].

Here, we demonstrate that KB-752 is capable of binding the AC stimulatory Gα subunit, $G\alpha_s$, in a GDP-selective manner similar to its interaction with $G\alpha_i$. Analysis of mutations perturbing the $G\alpha_{i1}$/KB-752 binding interface revealed a mutation retaining activity for $G\alpha_s$, thus improving its Gα selectivity profile. Via N-terminal conjugation with the fluorescent dye FITC, KB-752 was found to act as a sensor for the GDP-bound conformation of $G\alpha_s$. However, in contrast to its GEF activity toward $G\alpha_i$, binding of KB-752 to $G\alpha_s$·GDP was found to slow

the rate of spontaneous nucleotide exchange *in vitro*, indicating that KB-752 is a GDI for $G\alpha_s$. Furthermore, KB-752 was capable of inhibiting both GTPγS- and forskolin-stimulated generation of cyclic AMP by AC in cell membranes, consistent with the GDI activity of this peptide towards $G\alpha_s$, a central participant in AC regulation. Our findings suggest that the KB-752 peptide, and variants thereof, will serve as valuable molecular tools to modulate cellular AC activity.

## 2. Materials and Methods

### 2.1. Materials.

Unless otherwise noted, all reagents were purchased from Sigma. Peptides were synthesized by Anaspec (San Jose, CA), except for the N-terminally FITC-labelled KB-752, the N-terminally palmitoylated KB-752 and its scrambled and W5A mutant counterparts which were synthesized by Dr. Michael Berne and the Tufts University Core Facility (www.tucf.org).

### 2.2. Protein purification.

Recombinant $G\alpha_{i1}$ and $G\alpha_o$ proteins were expressed and purified as described [10]. $His_6$-tagged bovine $G\alpha_s$ (short splice variant) was purified from BL21(DE3) *E. coli* essentially as previously described [11]. Briefly, protein expression from the prokaryotic plasmid pPRO-EXHTb-$G\alpha_s$ was induced at an $OD_{600}$ = 0.6 with 0.1 mM IPTG for 16-18 hours at 20°C. $G\alpha_s$ was then purified by sequential $Ni^{2+}$ nitrilotriacetate, anion exchange, and size exclusion chromatographies [11].

### 2.3. Surface plasmon resonance (SPR) biosensor measurements.

All SPR binding assays were performed at 25°C on a BIAcore 3000. To analyze nucleotide-dependent binding of KB-752 to $G\alpha_s$, an N-terminally biotinylated KB-752 (diluted to 0.1 μg/ml in BIA running buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and 0.005 % NP40]) was coupled to separate flow cells of streptavidin biosensor chips to a surface density of approximately 250, 500, or 1000 response units (this variation of surface density was used as an internal control to ensure accuracy in binding affinity calculations). Prior to injection, $G\alpha_s$ was diluted in BIA running buffer containing 100 μM GDP, 100 μM GDP plus 30 μM $AlCl_3$ and 10 mM NaF, or 100 μM GTPγS and allowed to incubate at room temperature for 2-3 hours. Thirty microliters of Gα subunits were then simultaneously injected over flow cells at 5 μL/min followed by a 300 sec dissociation phase. Binding to a non-Gα-interacting peptide (C-tail of mNOTCH1, PSQITHIPEAFK; [12]) was subtracted from all binding curves to correct for nonspecific binding and buffer shifts created during injection. Surfaces were regenerated between each injection with two injections of 10 μL regeneration buffer (500 mM NaCl and 25 mM NaOH) at 20 μL/min. Binding curves and kinetic analyses were conducted using BIAevaluation software version 3.0. Binding affinities were calculated with the simultaneous association and dissociation rate analysis parameter using generated curves.

### 2.4. Fluorimetric binding assays.

Assays were conducted in buffer containing 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 10 mM $MgCl_2$, 150 mM NaCl, and 50 μM GDP. Experiments conducted in the presence of $AlF_4^-$ were performed by supplementing the above buffer with 10 mM NaF and 30 μM $AlCl_3$. $G\alpha_s$·GDP and $G\alpha_s$·GDP·$AlF_4^-$ were prepared as described for SPR. Fluorescence measurements were made using a LS-55B spectrofluorimeter (Perkin Elmer). Timecourse measurements were made at 5 second intervals using excitation and emission wavelengths of 494 nm and 515 nm, respectively, and slit widths of 5 nm. Emission scans were performed at a rate of 20 $nm \cdot min^{-1}$ using an excitation wavelength of 440 nm, with slit widths of 5 nm.

### 2.5. GTPγS exchange and steady-state GTPase assays.

GTPγS binding assays were conducted at 20 °C using a nitrocellulose filter binding method detailed previously [13]. Steady-state GTPase assays were carried out at 20 °C using a charcoal precipitation as described previously [14].

### 2.6. Adenylyl cyclase assays.

HEK293 cell monolayers were lysed with ice-cold hypotonic buffer (1 mM Na+-HEPES, pH 7.4, 2 mM EDTA). Scraped cell lysates were centrifuged at 30,000 × *g* for 20 min. The resulting crude membrane fraction was resuspended (1 mg/ml) with a glass-teflon handheld homogenizer (8-10 strokes) in storage buffer (15 mM $Na^+$-HEPES, pH 7.4, 1 mM EDTA) and frozen at -70° C until assayed. Total protein levels were determined with a BCA protein assay kit from Pierce (Rockfield, IL). AC assays were carried out as described previously [15] with modifications. Briefly, frozen membranes were thawed and added (15-40 μg of protein/tube) to duplicate assay tubes containing the reaction mixture (15 mM Na+-HEPES, pH 7.4, 4.5 mM phosphocreatine, 5 mM $MgCl_2$, 0.25 mM ATP, 0.5 mM isobutylmethylxanthine, 3 units of creatine phosphokinase) in a final volume of 100 μL. Incubations were carried out at 30° C, then terminated by the addition of 200 μL 3% trichloroacetic acid. Tubes were vortexed and centrifuged for 10 min at 14,000 x *g*. cAMP in the supernatant was quantified using a competitive binding assay previously described [15] with minor modifications. Duplicate samples of supernatants (15 μl) were added to reaction tubes. [$^3$H]cAMP (~1 nM final concentration) and cAMP binding protein (ca. 150 mg) were diluted in cAMP assay buffer (100 mM Tris/HCl pH 7.4, 100 mM NaCl, 5 mM EDTA) and then added to each well for a total volume of 550 μL. The tubes were incubated on ice for 2 hr and harvested by filtration (Packard Unifilter GF/C) using a 96-well Packard Filtermate Cell harvester (Meriden, CT). The filters were allowed to dry, and Microscint O scintillation fluid was added. Filter radioactivity was determined using a Packard TopCount scintillation/luminescence detector. cAMP concentrations in each sample were estimated in duplicate from a standard curve ranging from 0.1 to 300 pmol of cAMP per assay.

## 3. Results and Discussion

### 3.1 Nucleotide selective interaction between KB-752 and $G\alpha_s$:

We previously demonstrated that the phage display-derived peptide KB-752 (SRVTWYDFLMEDTKSR) interacts preferentially with the GDP-bound conformation of $G\alpha_{i1}$ ($K_D$ of ~4 μM; [10]). We have since found that KB-752 also interacts with $G\alpha_s$ in a GDP-selective manner (Figure 1). In SPR binding assays, immobilized KB-752 peptide showed a robust interaction with recombinant $G\alpha_s$ in the presence of GDP; no detectable interaction was seen with $G\alpha_s$ in either the GDP·$AlF_4^-$- or GTPγS-bound states (Figure 1A). Using simultaneous $k_{on}$ and $k_{off}$ rate calculations, the apparent dissociation constant ($K_D$) for the KB-752/$G\alpha_s$·GDP interaction was found to be 5.1 ± 0.9 μM (Figure 1B). These results indicate that KB-752 interacts selectively with the inactive, GDP-bound state of $G\alpha_s$ with an affinity similar to that for its original target interactor, $G\alpha_{i1}$·GDP.

The nucleotide state-selective nature of the Gα/KB-752 interaction suggests a possible application of peptides such as KB-752 as specific sensors for Gα conformational state. As proof-of-principle for such an application, we tested a KB-752 variant containing a covalently-linked N-terminal fluorescein isothiocyanate (FITC) group. Adding $G\alpha_s$·GDP to a solution containing FITC-KB-752 resulted in an increase in fluorescence intensity above baseline (Figure 1C). Importantly, addition of $G\alpha_s$·GDP·$AlF_4^-$ showed no change in fluorescence response. These results suggest that fluorescent dye-labelled KB-752 can serve as a biosensor for the GDP-bound conformation of $G\alpha_s$. Analysis of the emission spectrum of FITC-KB-752 prior to and following the addition of $G\alpha_s$·GDP indicates an ~20% increase in overall

fluorescence intensity yield (Figure 1C inset). Thus, while this modified peptide represents a potentially novel tool for studying $G\alpha_s$ activation/deactivation dynamics *in vitro* [16], further development strategies to increase the change in fluorescence quantum yield will be required for its utility in *in vivo* applications.

### 3.2 GDI activity of KB-752 toward $G\alpha_s$:

KB-752 serves as a guanine nucleotide exchange factor for $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ subunits [10]. In contrast, KB-752 slowed the rate of spontaneous nucleotide exchange by $G\alpha_s$, as measured by the binding of [$^{35}$S]GTPγS (Figure 2A). The effective concentration for 50% of maximal GDI activity ($EC_{50}$) toward $G\alpha_s$ was 4.5 ± 1.9 μM (Figure 2B), agreeing closely with the observed binding affinity. To confirm this GDI activity, we also measured the effect of KB-752 on the steady-state GTPase rate of $G\alpha_s$. Given that GDP release is the rate-limiting step in the guanine nucleotide cycle for isolated Gα subunits [17], any modulation of nucleotide exchange is reflected in the overall steady-state rate [14]. KB-752 association was found to reduce the steady-state GTPase rate of $G\alpha_s$ (Figure 2C), further supporting the discovery of GDI activity for this peptide/Gα pairing.

Gβγ serves as the prototypical GDI in the conventional model of GPCR-mediated signal transduction. Gβγ binds Gα·GDP and couples the resulting heterotrimer to receptor, as well as preventing spontaneous GDP release [18,19]. More recently, we and others have described the GoLoco motif within several proteins that binds $G\alpha_i$ family members and slows spontaneous release of GDP [11,20-22]. However, the GoLoco motif does not interact with nor serve as a GDI for $G\alpha_s$ [20], and no similar protein domain has been identified for $G\alpha_s$ to date. Thus, KB-752 serves as a unique peptide capable of serving as a GDI for $G\alpha_s$.

### 3.3 Inhibition of AC activity by KB-752:

$G\alpha_s$·GTP stimulates the ability of AC to generate cAMP from ATP, whereas $G\alpha_i$ family members inhibit AC activity when in their GTP-bound state. Thus, we hypothesized that KB-752, by virtue of its GDI activity on $G\alpha_s$ and GEF activity on $G\alpha_i$ [10], could inhibit AC activity by modulating the balance of stimulatory and inhibitory inputs from Gα subunits. To test this idea, we incubated HEK293 cell membranes with an N-terminally palmitoylated version of KB-752 and examined cAMP accumulation. Palmitoylated KB-752 peptide was found to inhibit GTPγS-stimulated cAMP accumulation, whereas a sequence-scrambled, control palmitoylated peptide did not significantly affect cAMP accumulation (Figure 3). KB-752 had no effect on basal cAMP accumulation. These results suggest that KB-752 can modulate G-protein activation in a cellular context and in a manner consistent with its effects measured using purified Gα subunits.

### 3.4 Generation of a KB-752 variant with improved Gα selectivity:

Our study of the structural determinants of KB-752 binding to $G\alpha_{i1}$·GDP indicated that residues W5, F8, and E11 within KB-752 make critical contacts with $G\alpha_{i1}$; for example, mutating either the W5 or F8 positions to alanine results in a loss of $G\alpha_{i1}$ binding and GEF activity on $G\alpha_{i1}$ [10]. We investigated the effects of these two mutations on the KB-752/$G\alpha_s$ interaction. Whereas the W5A mutant peptide retained $G\alpha_s$ association and GDI activity (albeit with an accelerated off-rate; Figure 4A), the F8A mutation abrogated both binding and GDI activity toward $G\alpha_s$ (Figure 4A,B). These results suggest that F8, but not W5, is critical to the interaction of KB-752 with $G\alpha_s$. Our findings identify KB-752(W5A) as a $G\alpha_s$-selective variant of KB-752 that retains the ability to inhibit GTPγS-stimulated AC activation (Figure 4C), although more weakly than that of wildtype, presumably reflecting its loss of Gαi-directed GEF activity and/or reduction in $G\alpha_s$ affinity. Both wildtype and W5A mutant palmitoylated KB-752 peptides were also capable of inhibiting forskolin-stimulated AC activity (Figure 4C); this inhibition is

likely related to $G\alpha_s$-directed GDI activity, as $G\alpha_s$ and forskolin are known to activate various AC isoforms in a synergistic fashion [23,24].

Although attempts to crystallize a $G\alpha_s$·GDP/KB-752 complex have not yet succeeded, several predictions can be made as to the structural determinants of this interaction. First, in light of its nucleotide-dependent nature, the $G\alpha_s$·GDP/KB-752 interaction presumably involves one or more of the three switch regions of $G\alpha_s$ that undergo conformational changes throughout the nucleotide cycle [25]. Second, while the structural determinants for KB-752 binding to $G\alpha_s$ may partially overlap with those for $G\alpha_{i1}$ binding (*e.g.*, F8 in KB-752 is critical for binding both targets), the exact interactions and relative positions of the KB-752 binding interface likely differs. This is underscored by the W5A mutant which retains activity towards $G\alpha_s$. This tryptophan residue participates in critical $G\alpha_{i1}$ contacts and underlies the GDP-dependent binding [10]. The hydrophobic binding pocket in $G\alpha_{i1}$ responsible for KB-752 binding (*i.e.*, the cleft between the α2 and α3 helices) represents a highly conserved region within Gα subunits, including $G\alpha_s$. Thus, while the F8 residue of KB-752 is still critical for interaction with $G\alpha_s$ (Figure 4), the dispensability of W5 suggests a different interaction site with $G\alpha_s$ versus $G\alpha_{i1}$, perhaps a shift in peptide orientation within the α2/α3 hydrophobic groove. Third, KB-752 may make direct contact with the bound GDP within $G\alpha_s$ and/or bind in a manner that occludes its release. This mode of binding is seen in GoLoco-induced inhibition of GDP release from $G\alpha_{i1}$ [21]. Alternatively, KB-752-mediated GDI activity could arise by the repositioning of the switch regions such as to inhibit GDP release.

In conclusion, we have found that KB-752 binds to the AC-stimulatory $G\alpha_s$ protein in a GDP-selective manner. We had previously shown that KB-752 interacts with $G\alpha_{i1}$ in a similar nucleotide-dependent manner [10]. Whereas KB-752 has GEF activity for $G\alpha_{i1}$, we demonstrate here that KB-752 possesses the diametrically-opposite biochemical activity for $G\alpha_s$, namely GDI activity. These opposing activities on $G\alpha_i$ and $G\alpha_s$ result in an inhibition of cAMP production via G protein-modulated AC activity in cell membrane preparations. We serendipitously found a mutation (W5A) that prevents binding of KB-752 to $G\alpha_{i1}$, thereby creating a more selective peptide that could be used to perturb $G\alpha_s$ signaling without affecting $G\alpha_i$-mediated processes. Our observation that the $G\alpha_s$-selective KB-752(W5A) variant retains the ability to decrease GTPγS- and forskolin-stimulated AC activity suggests that the predominant mechanism of action of KB-752 as an AC inhibitor resides in its GDI activity towards $G\alpha_s$. Although the sequence of KB-752 does not correspond to any naturally-occurring sequence [10], its nucleotide-dependent binding and GDI activity make it a potentially powerful molecular tool for studying $G\alpha_s$ signaling.

#### 4. Acknowledgements

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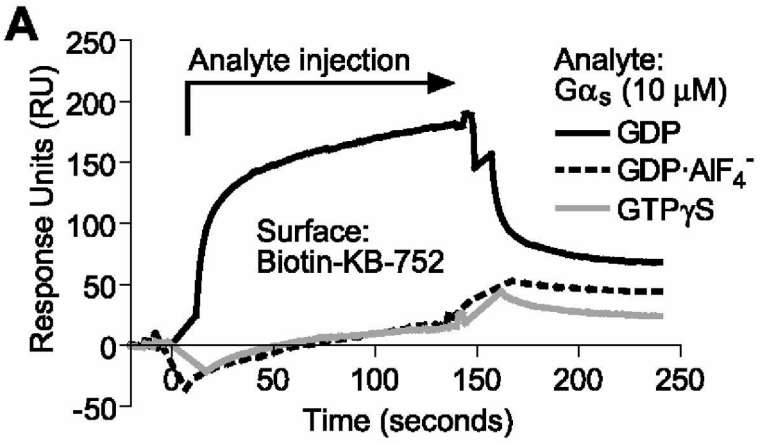


B

| $K_D$ (μM): | GDP | GDP·$AlF_4^-$ | GTPγS |
|---|---|---|---|
| $G\alpha_s$ | 5.1 ± 0.9 | >1000 | >1000 |
| $G\alpha_o$ | 18.2 ± 3.0 | >1000 | >1000 |
| $G\alpha_{i1}$ | 3.9 ± 0.6 | >1000 | 28.0 ± 3.2 |

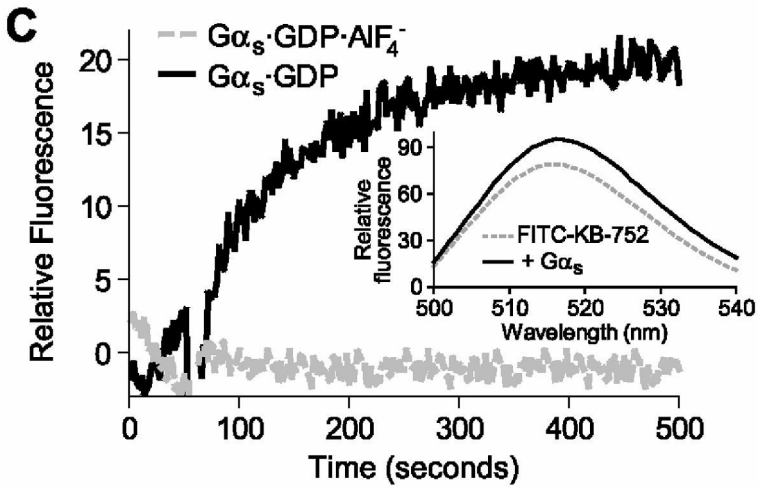


**Figure 1.**

Nucleotide-dependent binding of KB-752 to $G\alpha_s$. (**A**) 10 μM of $G\alpha_s$ ("Analyte"), in each of the indicated nucleotide bound states, was injected over immobilized KB-752 and binding measured by SPR. Non-specific binding to a control biotinylated peptide surface was subtracted from each curve. (**B**) $G\alpha_s$, $G\alpha_o$, and $G\alpha_{i1}$ were separately injected at increasing concentrations (0.01 to 50 μM) over immobilized KB-752 to determine the dissociation constants ($K_D$) for each interaction pair as obtained from simultaneous $k_{on}/k_{off}$ analyses (n = 4-6 for each state). (**C**) Time course of FITC-KB-752 binding to $G\alpha_s$. 100 nM FITC-KB-752 was equilibrated in buffer containing either GDP (black trace) or GDP·$AlF_4^-$ (grey trace) and background fluorescence subtracted. At 50 seconds, 1 μM $G\alpha_s$·GDP (black trace) or 1 μM Gα·GDP·$AlF_4^-$ (grey trace) was added. (**Inset**) Emission scans of FITC-KB-752 in the absence and presence of $G\alpha_s$·GDP obtained using an excitation wavelength of 440 nm.

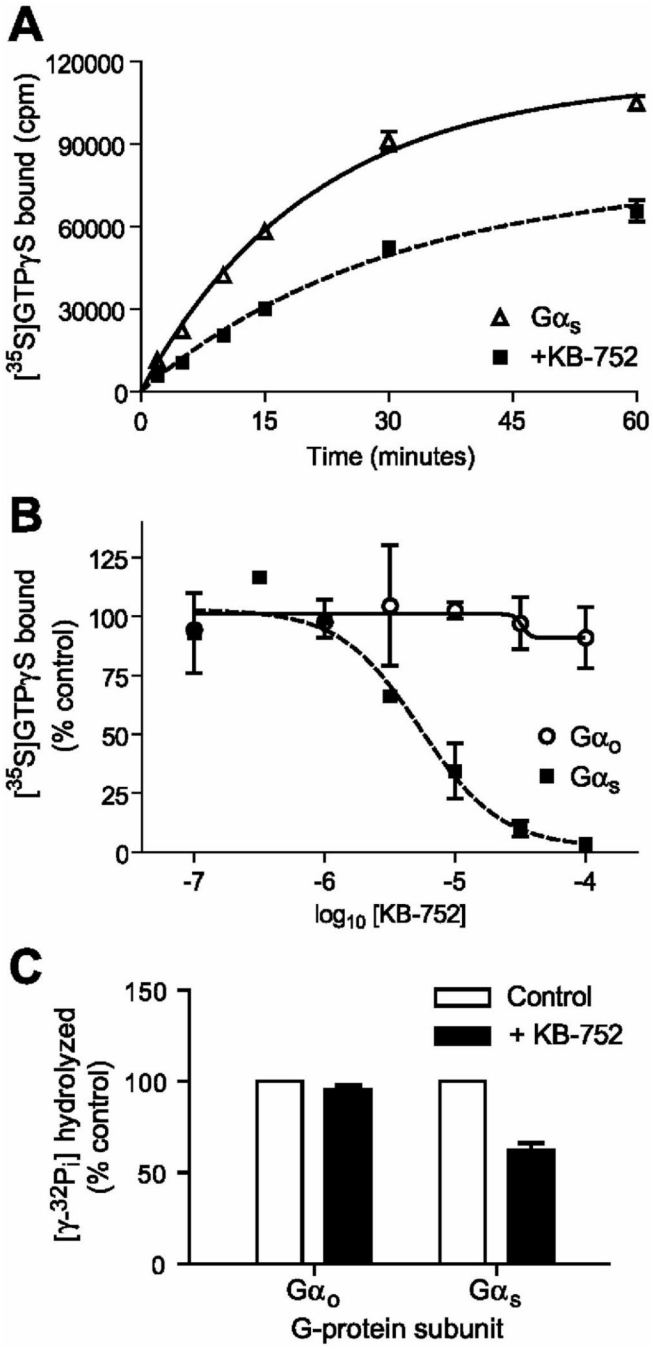


**Figure 2**

. GDI activity of KB-752 on $G\alpha_s$ assessed by GTPγS binding and steady-state GTPase measurements. (**A**) 50 nM $G\alpha_s$ was incubated in the absence or presence of 5 μM KB-752 for 15 minutes at room temperature prior to addition of [$^{35}$S]GTPγS. Duplicate reaction aliquots were removed at indicated times and protein-bound radioactivity counted. (**B**) 50 nM $G\alpha_s$ or $G\alpha_o$ was incubated with the indicated concentrations of KB-752 as in (**A**) and GTPγS binding reactions were incubated at 20 °C for 5 minutes. (**C**) 100 nM $G\alpha_o$ or $G\alpha_s$ was incubated with 10 μM KB-752 for 15 minutes at room temperature prior to the addition of [γ-$^{32}$P]GTP and measurement of released [$^{32}$P] inorganic phosphate.

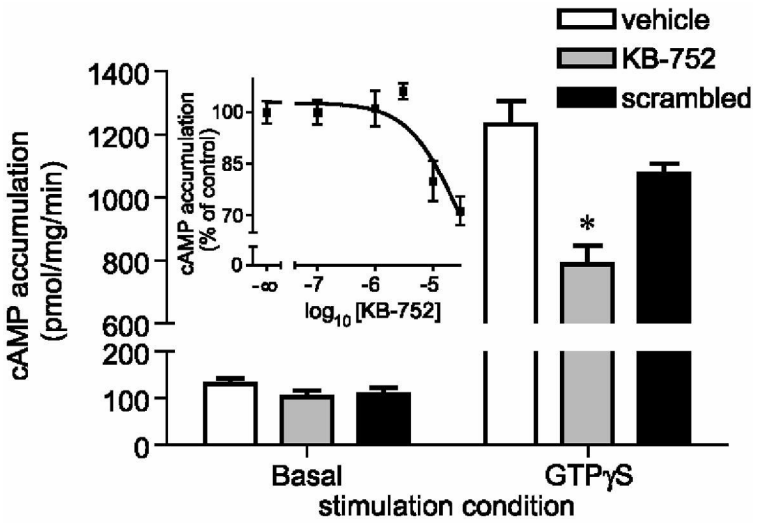


**Figure 3**

. Effect of KB-752 on adenylyl cyclase activity. Basal or 10 μM GTPγS-stimulated cAMP accumulation over 10 minutes was measured from HEK293 cell membrane preparations in the absence (vehicle) or presence of 30 μM wildtype, palmitoylated KB-752 or a sequence-scrambled version. *, $p < 0.05$ compared to vehicle; one-way ANOVA followed by Dunnett's ($n = 4$). *Inset:* HEK293 membrane preparations were stimulated with 10 μM GTPγS in the presence of indicated molar concentrations of wildtype palmitoylated KB-752 and cAMP accumulation was measured following 10 minutes. Data are shown as the mean ± SEM of 4 independent experiments and expressed as percent of cAMP generated in the absence of peptide.

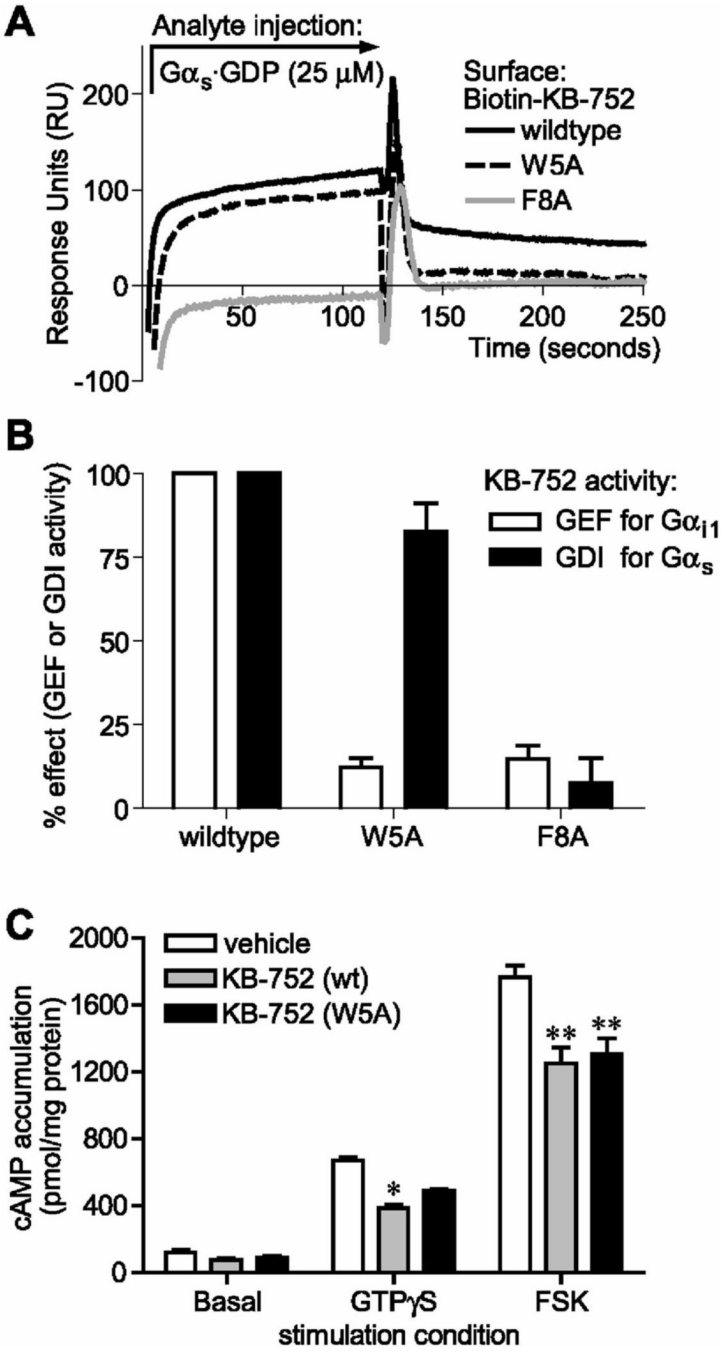


**Figure 4**

. Effects of alanine substitutions to KB-752 binding, GDI activity, and attenuation of AC activation. (**A**) N-terminally biotinylated KB-752 mutant (W5A, F8A) or wildtype peptides were each immobilized to a density of ~1000 response units (RUs) on separate streptavidin-coated flow cells and 25 μM of GDP-bound $G\alpha_s$ ("Analyte") was injected simultaneously over all surfaces. (**B**) Purified $G\alpha_s$ or $G\alpha_{i1}$ was incubated with the indicated KB-752 peptide and [$^{35}$S]GTPγS binding was measured following incubation for 5 minutes at 20 °C. (**C**) cAMP accumulation over 10 minutes at 30 °C was measured from HEK293 cell membrane preparations stimulated with 10 μM GTPγS or 30 μM forskolin (FSK) in the absence (vehicle)

or presence of 30 μM wildtype, palmitoylated KB-752 (wt) or the tryptophan-5 substituted version (W5A). *, $p < 0.05$; **, $p < 0.01$ compared to vehicle; one-way ANOVA followed by Dunnett's ($n = 3$).