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State-selective Binding Peptides for Heterotrimeric G-protein Subunits: Novel Tools for Investigating G-protein Signaling Dynamics

Christopher A. Johnston¹, Francis S. Willard¹, J. Kevin Ramer^{2,§}, Rainer Blaesius^{2,†}, C. Natalia Roques^{2,‡}, and David P. Siderovski^{1,*}

¹ Department of Pharmacology, Lineberger Comprehensive Cancer Center, and UNC Neuroscience Center, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599-7365 USA

² Karo Bio USA, Durham, NC, 27703

Abstract

Heterotrimeric G-proteins, comprising G α , G β , and G γ subunits, are molecular switches that regulate numerous signaling pathways involved in cellular physiology. This characteristic is achieved by the adoption of two principal states: an inactive state in which GDP-bound G α is complexed with the G $\beta\gamma$ dimer, and an active state in which GTP-bound G α is freed of its G $\beta\gamma$ binding partner. Structural studies have illustrated the basis for the distinct conformations of these states which are regulated by alterations in three precise ‘switch regions’ of the G α subunit. Discrete differences in conformation between GDP- and GTP-bound G α underlie its nucleotide-dependent protein-protein interactions (e.g., with G $\beta\gamma$ /receptor and effectors, respectively) that are critical for maintaining their proper nucleotide cycling and signaling properties. Recently, several screening approaches have been used to identify peptide sequences capable of interacting with G α (and free G $\beta\gamma$) in nucleotide-dependent fashions. These peptides have demonstrated applications in direct modulation of the nucleotide cycle, assessing the structural basis for aspects of G α and G $\beta\gamma$ signaling, and serving as biosensor tools in assays for G α activation including high-throughput drug screening. In this review, we highlight some of the methods used for such discoveries and discuss the insights that can be gleaned from application of these identified peptides.

Introduction

Diverse extracellular signals, including hormones, neurotransmitters, growth factors, and sensory stimuli, transmit information intracellularly by activation of plasma membrane-bound receptors. The largest class of such receptors is the superfamily of heptahelical G protein-coupled receptors (GPCRs). GPCRs transmit signals by activating heterotrimeric G-proteins that normally exist in an inactive state of G α -GDP bound to G $\beta\gamma$ subunits. In the traditional model (Figure 1), agonist activation of GPCRs induces incompletely defined conformational changes within the receptor, which subsequently catalyze the exchange of GDP for GTP on the G α subunit by inducing conformational changes within G $\alpha\beta\gamma$ that lower the affinity for GDP allowing for nucleotide release and subsequent GTP binding [1–3]. By this means, GPCRs serve as guanine nucleotide exchange factors (GEFs) for G α -GDP/G $\beta\gamma$ complexes.

* To whom correspondence should be addressed: UNC Department of Pharmacology, CB#7365, 1106 M.E. Jones Bldg., Manning Drive, Chapel Hill, NC 27599-7365 USA; tel 1-919-843-9363; fax 1-919-966-5640; email: dsiderov@med.unc.edu.

§Current addresses: Biogen Idec Inc., 5000 Davis Drive, Research Triangle Park, NC 27709-4627 USA

†Becton Dickinson, 21 Davis Drive, Research Triangle Park, NC 27709 USA

‡Kenan-Flagler Business School, UNC-Chapel Hill, Chapel Hill, NC 27599-2400 USA

Although the exact mechanism by which GPCRs exert their GEF activity remains to be fully elucidated [3], this action is critical to the commencement of G protein signaling, as GDP release is the rate-limiting step of the $G\alpha$ guanine nucleotide cycle [4]. Subsequent to GDP release, GTP, a nucleotide present in a relative excess, binds $G\alpha$ and induces a conformational change in three flexible 'switch regions' of the $G\alpha$ subunit, which deforms the $G\beta\gamma$ binding interface leading to both the dissociation of the $G\beta\gamma$ dimer as well as the adoption of the conformation capable of interacting with effectors [1,5]. Activated $G\alpha$ -GTP and liberated $G\beta\gamma$ both signal to a diverse family of downstream effectors including ion channels, adenylyl cyclases, phosphodiesterases, and phospholipases, producing second messenger molecules that regulate cellular responses underlying physiological processes [2]. Based on their sequence homology and differential regulation of effectors, G-proteins are grouped in four classes: $G\alpha_s$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_{12/13}$ [6]. GPCRs have the ability to couple selectively to members of one or more of these G-protein subfamilies, thus allowing selective modulation of signaling cascades by particular GPCR ligands. G-protein signaling is terminated by the intrinsic GTPase activity of the $G\alpha$ subunit, which occurs at a rate that varies among the G-protein subfamilies. GTP hydrolysis rates can be dramatically enhanced by members of a superfamily of "regulators of G-protein signaling" (RGS) proteins [7–9] that serve as GTPase-accelerating proteins (or "GAPs"). This deactivation reaction results in conversion back to the inactivated, GDP-bound $G\alpha$ that subsequently reassociates with $G\beta\gamma$ to complete the cycle. Because this represents a true *cycle* of activation (by nucleotide exchange and subunit dissociation) and deactivation (by GTP hydrolysis and subunit reassociation), heterotrimeric G-proteins serve as molecular switches and are critical to defining the spatial and temporal aspects of cellular responses to external stimuli.

Biochemical and structural analyses over the past two decades have advanced our understanding of the mechanics underlying G-protein regulation and the guanine nucleotide cycle [3,10]. As previously alluded to, the structural basis for this activation cycle is governed principally by three $G\alpha$ switch regions -- flexible segments that change conformation upon GTP binding and hydrolysis. Indeed, the switch regions contain high sequence conservation among G-proteins, especially in those residue positions that are directly involved in nucleotide binding, GTP hydrolysis, and protein-protein interactions (e.g. $G\beta\gamma$, RGS proteins, effectors) [3,10]. Given that conformational changes to these switch regions are crucial to the G-protein signaling cycle, further defining the structural and biochemical nature of these distinct signaling states of $G\alpha$ should provide further insight into G-protein signaling dynamics and greatly facilitate assay development and target validation for drug discovery.

Screening methods have been developed to identify randomized peptide sequences capable of interacting with target proteins as a means of creating novel binding partners for signaling proteins of interest [11–13]. Recently, such screening methods have been utilized to develop peptides capable of interacting with $G\alpha$ and $G\beta\gamma$ subunits. In the case of $G\alpha$, nucleotide-state specific peptides have been identified by selectively screening against both inactive and active conformations. These peptides have been used to gain additional biochemical and structural insights into the signaling dynamics of heterotrimeric G-proteins. Additionally, several diverse applications have been made of these peptides towards the identification of small molecule drugs that modulate G-protein activity both directly and indirectly, with the ultimate goal of facilitating novel GPCR screening methods. This review will highlight the current successes in the screening for, and early uses of, G-protein binding peptides.

Screening Methods

Several *in vitro* methods have been developed to identify and evolve peptide sequences that interact specifically with target proteins of interest (e.g., refs. [11–13]). Although technically different in several design aspects, these various methods all entail certain fundamental criteria.

Each process begins with the assembly of a diverse, randomized cDNA library suitable for translation into diverse peptide sequences of desired length (although certain *a priori* constraints on sequence complexity can be employed if desired). A method for expressing this library must then be developed to physically link the cDNA sequence to its associated, encoded peptide. Popular methods currently employed for this process include peptides-on-plasmids [14], phage display [13,15,16], and mRNA display [17]. Once such a library of nucleic acid-tethered peptides is generated, the next step involves affinity selection against a desired protein target that is typically immobilized for retaining bound peptides (and their tethered genetic information) and removing non-specific peptides through extensive washing procedures. Sequences found to interact selectively with target proteins are then amplified, thereby producing an enriched library. This library is then typically subjected to further rounds of the selection process to enrich further the target library. Moreover, sequences identified can also be used for further targeted evolution of second-generation libraries. This entire process can be repeated until particular polypeptide sequences of desired quality (e.g., affinity and selectivity) are identified. Cloning and DNA sequencing ultimately defines the amino acid sequence of binding peptides. These *in vitro* techniques can effectively screen libraries as diverse as 10^8 to 10^{13} unique peptide sequences [18,19]. Numerous alternative approaches to identify affinity reagents, such as mass spectrometry/proteomic approaches to peptide library screening, have been discussed in depth elsewhere [20].

The discoveries highlighted herein focus primarily on the techniques of phage display and mRNA display, both recently successful in identifying both $G\alpha$ - and $G\beta\gamma$ -binding peptides. These peptides represent useful new tools for investigating the biochemical and structural dynamics of G-protein signal transduction.

Biochemical Insights

KB-752 – a $G\alpha$ -GDP-selective phage-display peptide

Recent studies using phage display analysis have identified novel $G\alpha$ binding peptides [16, 21–23]. Some of these peptides are capable of interacting selectively with either the inactive (GDP-bound) or active (GTP-bound) conformation of $G\alpha$ [21–23]. A GDP-selective peptide, KB-752 (Table I), was initially demonstrated to interact most avidly with $G\alpha_{i1-3}$ family members [23]. KB-752 interaction with $G\alpha_{i1}$ -GDP results in an enhancement of spontaneous nucleotide exchange; thus, KB-752 serves as a $G\alpha_{i1}$ GEF [23]. This result illustrates the potential usefulness of identified peptides in biochemical studies of G-protein signaling, as the native GEFs for $G\alpha$ (i.e., GPCRs) are notoriously difficult to produce given that they are integral membrane proteins. Subsequent studies revealed that KB-752 was also capable of binding the inactive conformation of $G\alpha_s$ [22]. Interestingly, interaction with $G\alpha_s$ results in guanine nucleotide dissociation inhibitor (GDI) activity, the opposite action to that elicited towards $G\alpha_{i1}$. By virtue of these synergistic activities on both $G\alpha_i$ and $G\alpha_s$, KB-752 inhibits GTP β S-stimulated adenylyl cyclase activation in cell membranes [22]. Together, these results demonstrate several unique biochemical properties of KB-752 potentially useful to the investigation of G-protein mediated signaling pathways.

KB-1753 and two other peptide families with affinity for activated $G\alpha$ subunits

Our phage display screening technique has also yielded peptides that specifically bind the activated conformation of $G\alpha$. We recently published on the identification of KB-1753 (Table I), which interacts with selective $G\alpha$ subunits in both the GTP-bound conformation and (especially) the transition-state mimetic, GDP $\cdot$ AlF $_4^-$ -bound conformation [21]. *In vitro* experiments demonstrated that, by binding to GTP-bound $G\alpha$, KB-1753 blocks the interaction of $G\alpha_t$ with its effector, PDE γ , and prevents stimulation of cyclic GMP degradation. Binding of the transition state $G\alpha$ results in an analogous inhibition of RGS protein interaction and

resultant GAP activity [21]. These results highlight the usefulness of KB-1753 as a potential tool for blocking both $G\alpha$ signaling to effector molecules as well as RGS protein activity for $G\alpha$. Direct inhibition of $G\alpha$ signaling has been suggested as an alternative therapeutic target to traditional modulation of receptor activity [24,25]. Inhibitors of RGS proteins, including both peptidomimetics and small molecules, represent another potentially attractive route of drug discovery targeting G-protein signaling pathways [7,26,27] (cf. [28]). KB-1753 may therefore serve as a useful template for future design of such strategies (see below). In addition to these biochemical properties, KB-1753 has been modified to act as a biosensor for activated $G\alpha$ [21]. By tagging the peptide with YFP, KB-1753-YFP served as the acceptor to the $G\alpha_{i1}$ -CFP donor in a FRET-based biosensor pair *in vitro*. Such probes, along with other dye-based approaches, provide a starting point for the design of *in vivo* applicable probes that may help unravel the spatiotemporal dynamics of G-protein signaling in living cells [29,30].

In our original phage display screen [23], we identified two additional groups of activated-state-dependent, $G\alpha_i$ -binding peptides with distinct sequence homology compared to the KB-1753 group. These two groups have been termed the KB-1746 and KB-1755 groups (Figure 2) after the representative peptide sequence from each that was further characterized (Table I). In contrast to the $G_{V/I}WxG$ motif of the KB-1753 group [21], the KB-1746 and KB-1755 groups display consensus motifs of $CxGWxCY$ and $I/VCPW_{E/D}$, respectively (Figure 2). These results suggest that distinct sequence motifs within multiple peptide groups can each selectively recognize the activated conformation of $G\alpha$ in common.

To quantitate the affinity of the interaction of KB-1746 and KB-1755 with $G\alpha$, we used both fluorescence anisotropy and surface plasmon resonance (SPR) assays. To determine binding affinities in a solution-based assay, we first synthesized N-terminal FITC-labeled KB-1746 and KB-1755 peptides and investigated their interaction with $G\alpha$ using fluorescence anisotropy. KB-1746 and KB-1755 interacted with $G\alpha_{i1}\cdot GDP\cdot AlF_4^-$ and $G\alpha_{t/i1}\cdot GDP\cdot AlF_4^-$ with binding affinities (K_D values) of approximately 1.0 and 1.8 μM , respectively (Figure 3) – both values comparable to the previously determined binding affinity of KB-1753 for its $G\alpha_{i1}$ target (K_D of 1.2 μM ; ref. [21]). Follow-up SPR experiments involved the injection of various $G\alpha$ isoforms (in specific nucleotide-bound forms) over N-terminally biotinylated KB-series peptides immobilized on a streptavidin-coated gold biosensor chip (Figure 4). Observed interactions were in general agreement with the nucleotide-state-selective nature of these binding events first observed in the initial phage screening and phage ELISA results [23], although the KB-1746 peptide displayed more modest nucleotide selectivity in showing some affinity also for the inactive, GDP-bound conformation of $G\alpha_{i1}$ (e.g., Figure 4B). As originally reported [21], KB-1753 interacted solely with $G\alpha$ subunits in their active conformation (Figure 4A,D), including $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha$ -transducin (the latter actually an *E. coli*-purifiable $G\alpha_{t/i1}$ chimera that closely mimics the biology of $G\alpha$ -transducin; refs. [21,31]). KB-1746 displayed a similar $G\alpha$ subfamily selectivity profile to that of KB-1753; however, a robust interaction with $G\alpha_o$ was also detected (Figure 4E). This $G\alpha_o$ /KB-1746 interaction is unique, as neither KB-1753 nor KB-1755 is capable of binding this particular $G\alpha$ subunit despite its high degree of sequence similarity to $G\alpha_i$ subunits in the predicted peptide-binding site [21]. Although KB-1755 showed significant binding to $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, its interaction with $G\alpha_t$ was even more robust (Figure 4F), suggesting that this peptide has a relative $G\alpha_t$ selectivity unseen in the other two peptide groups. Overall, these results highlight the similar preference all three peptide groups possess for the activated $G\alpha$ conformation, as well as differing $G\alpha$ subunit preferences of specific peptides.

Our previous biochemical and structural studies of KB-1753 demonstrated a mode of binding to activated $G\alpha$ analogous to that of effectors, with the specific site of interaction comprising the highly conserved hydrophobic $\alpha 2/\alpha 3$ cleft of $G\alpha$ [21]. To investigate whether KB-1746 and KB-1755 peptides bind to the same site on $G\alpha_{i1}$, we carried out competition binding

analyses again using surface plasmon resonance. SPR surfaces of biotinylated KB-1753 (as a control), KB-1746, and KB-1755 were created and their interaction with activated $G\alpha_{i1}$ was examined in the absence and presence of increasing concentrations of non-biotinylated KB-1753 peptide (Figure 5). As expected, unlabelled wildtype KB-1753 peptide was capable of competing for $G\alpha_{i1}$ binding to the immobilized KB-1753 surface. A loss-of-function mutant KB-1753 peptide (Ile-9 and Trp-10 mutated to alanine; "I9A/W10A"), which does not bind $G\alpha_{i1}$ [21], was ineffective at competing for $G\alpha_{i1}$ binding (Figure 5A). Wildtype KB-1753 peptide was also capable of precluding binding of $G\alpha_{i1}$ to the immobilized KB-1755 surface, suggesting that the binding site for KB-1755 at least partially overlaps with that of KB-1753 (Figure 5C). However, preincubation with KB-1753 did not significantly alter the binding of $G\alpha_{i1}$ to the immobilized KB-1746 surface (Figure 5B), suggesting that KB-1746 interacts through a novel binding site distinct from that of KB-1753.

To more completely investigate the interaction of KB-1746 and KB-1755 with activated $G\alpha$ and map the interaction site, we conducted fluorescence-based binding experiments in the presence of potential competitors for $G\alpha$ binding. Analogous to our prior studies with FITC-labelled KB-752 and R6A-1 peptides [22,32], we observed that FITC-labelled KB-1755 undergoes a significant (~30%) and reproducible enhancement of fluorescence quantum yield upon binding to activated $G\alpha$ (Figure 6). Using this approach, we conducted competition binding studies and found that all three classes of activation-state-selective peptides compete with FITC-KB-1755 for binding to $G\alpha_{i1}\cdot\text{GDP}\cdot\text{AlF}_4^-$ (Figure 6A). The KB-1753 result is in agreement with the SPR analysis described above (Figure 5C). The ability of KB-1746 to compete with FITC-KB-1755, despite its inability to be competed by KB-1753 (Figure 5B), suggests that KB-1755 shares contacts on $G\alpha$ with the two distinct binding sites created by KB-1746 and KB-1753.

To further explore the binding of KB-1755, we also assessed the ability of an RGS protein (RGS16) and an effector peptide (PDE γ) to compete for binding to $G\alpha_{t/i1}$. RGS proteins and effectors both bind the active conformation of $G\alpha$ subunits, but have distinct, non-overlapping binding sites [33,34]. RGS16 and PDE γ each competed with FITC-KB-1755 for $G\alpha_{t/i1}$ binding, reducing nearly equally the observed EC_{50} of the latter, fluorophore-labeled peptide (Figure 6B). Thus, the KB-1755 peptide likely interacts with $G\alpha$ in a mode that extends partially through both the effector- and RGS protein-binding sites. As KB-1753 binds predominantly to the effector binding site of $G\alpha$ [21], this suggests that KB-1746 potentially interacts predominantly with the RGS protein binding site of $G\alpha$.

Derivatives of the GoLoco motif

As an alternative approach to phage display, mRNA display has also been used for identifying novel $G\alpha$ binding sequences. Ja and Roberts recently described a class of $G\alpha$ binding peptides with the prototype sequence termed R6A ([35]; Table I). In contrast to the completely random screening approach employed in our phage display studies, the mRNA display of Ja and Roberts was done using an *a priori* input sequence: namely, that of the consensus motif for the GoLoco motif (also known as the GPR motif) [36]. The naturally-occurring GoLoco motif, found in known GPCR signaling regulators such as RGS12 and RGS14, binds $G\alpha_{i/o}$ subunits preferentially in the GDP-bound form and inhibits spontaneous nucleotide release (i.e., guanine nucleotide dissociation inhibitor or "GDI" activity; [37,38]. Not surprisingly, R6A and other peptides patterned after the GoLoco motif also interact with $G\alpha\cdot\text{GDP}$ and have apparent binding affinities of 60~200 nM [35]. Despite containing a non-conservative replacement of the critical arginine residue in the signature Asp-Gln-Arg motif of the GoLoco motif [36], the R6A family of peptides was initially reported to have GDI activity similar to that of the parent GoLoco motif peptide [35]. However, more careful exploration using multiple modes of measuring nucleotide binding and cycling suggested that, while the R6A-1 peptide does

interact with $G\alpha$ -GDP, it does not elicit any significant GDI activity [32]. The R6A-1 peptide, a minimal 9-mer representing the R6A family (Table I) and possessing sequence similarity to KB-752 [32], was further reported to bind to all $G\alpha$ family members in their GDP-liganded forms and this binding was mutually exclusive with $G\beta\gamma$ binding [39]. One could imagine the R6A family of peptides, as well as their naturally-occurring GoLoco motif progenitors, may prove useful as $G\alpha$ -interacting peptides that occlude $G\beta\gamma$ interaction for studying the regulation of heterotrimer association/dissociation related to the temporal aspects of G-protein signaling.

$G\beta\gamma$ -interacting peptides

In addition to screens for $G\alpha$ -interacting peptides, the isolated $G\beta\gamma$ subunit has also been used as the bait for discovery of novel binding sequences. Smrcka and colleagues used randomized peptide libraries in a phage display screen to identify four distinct groups of $G\beta\gamma$ binding peptides [40]. Peptides in 'group I' had a consensus motif with homology to two native $G\beta\gamma$ binding proteins, PLC β and phosducin, while 'groups II-IV' had little homology to known proteins or to that of group I peptides ([40] and see Table I). Interestingly, a peptide with the strongest correlation to the group I consensus, termed SIRK, was capable of selectively inhibiting $G\beta\gamma$ -mediated activation of PLC β while not affecting $G\beta\gamma$ stimulation of voltage-gated calcium channels or type I adenylyl cyclases. Despite divergent sequences, competition experiments revealed peptides from all four groups bound the same site on $G\beta\gamma$, which was termed the $G\beta\gamma$ 'hot spot' that is likely used for selectivity among effector interactions. [40]. This elegant study has thus produced inhibitory peptides capable of dissecting among several $G\beta\gamma$ -mediated signaling pathways, which will undoubtedly prove to be valuable tools for studying these pathways both *in vitro* and *in vivo*.

Structural Insights

KB-752/ $G\alpha_{i1}$ -GDP complex

We recently elucidated the structural determinants of $G\alpha_{i1}$ binding by the GDP-selective phage peptide KB-752 to high resolution using x-ray diffraction crystallography [23]. In mimicking the biochemical activity of a receptor (i.e., GEF activity), and representing a much more tractable target for structural studies than a membrane-bound GPCR, the KB-752 peptide was instrumental to understanding mechanistic details of $G\alpha$ activation, which remains poorly understood [3]. The crystal structure of the $G\alpha_{i1}$ /KB-752 dimer revealed a peptide binding site centered on the hydrophobic $\alpha 2/\alpha 3$ cleft of the $G\alpha$ Ras-like domain [23] (Figure 7A). Binding of KB-752 levers the conformationally-flexible switch II ($\alpha 2$) helix in a direction away from the GDP binding site. In turn, this alteration causes the preceding $\beta 3/\alpha 2$ loop to assume a novel conformation, stabilized by interactions with KB-752, that removes it from its normal disposition within the $G\alpha_{i1}\beta_1\gamma_2$ heterotrimer. In the heterotrimer structure [41], as well as the $G\alpha_{i1}$ /RGS14-GoLoco structure [38], this $\beta 3/\alpha 2$ loop sits directly in front of the bound GDP molecule. It is thought that this conformation, when bound to either of these GDI proteins, allows the $\beta 3/\alpha 2$ loop to serve as an 'occlusive barrier' to the release of GDP and, furthermore, that receptors use $G\beta\gamma$ as a 'lever' to remove this barrier allowing for nucleotide exchange [3,42,43]. That KB-752 induces such a conformational change in the $\beta 3/\alpha 2$ loop likely explains its GEF activity for $G\alpha_{i1}$. Thus, KB-752, identified using phage display, has provided insight into the mechanism of G-protein activation by giving structural support to a previously proposed model of receptor-mediated activation [42,43].

KB-1753/ $G\alpha_{i1}$ -GDP- AlF_4^- complex

We have also elucidated the high-resolution structural determinants of the interaction of the activation-state-dependent phage peptide KB-1753 bound to $G\alpha_{i1}$ in its transition-state ($GDP\cdot AlF_4^-$) conformation [21]. KB-1753 was found to bind the same $\alpha 2/\alpha 3$ cleft within $G\alpha$ as was seen with KB-752 (Figure 7B). The stringent reversal in nucleotide selectivity

between these two peptides (Table I) is due to the difference in peptide residues that insert in this same hydrophobic groove: the larger tryptophan in KB-752 requires a flexible $\alpha 2$ helix found only in the GDP-bound form, whereas the smaller isoleucine in KB-1753 is perfectly accommodated by the more closed and rigid conformation of the $\alpha 2$ helix found in the active conformation ([21,23] and see Figure 7A,B). The $G\alpha$ /KB-1753 complex revealed a striking similarity to native $G\alpha$ /effector complexes and, thus, represents the first structural glimpse of an 'effector-like molecule' bound to $G\alpha_{i1}$. Although the $G\alpha$ residues contacted by KB-1753 are strictly conserved among all $G\alpha$ families, KB-1753 displays a narrow selectivity profile for specific $G\alpha_i$ subfamily members [21], even discriminating within this subfamily against $G\alpha_o$ (Figure 4D). These results have implications for the nature of specificity determinants within $G\alpha$ /effector interactions, supporting the notion that specificity for particular $G\alpha$ subfamily members can be determined by differences, even subtle, in effector sequence and structure [34]. Structural information on the $G\alpha$ /KB-1753 complex will undoubtedly aid in the rational design of future biosensors as previously described above. Another application made possible by this crystal structure is structure-based design and screening of small molecule inhibitors of $G\alpha$ signaling. Analogous efforts using $G\beta\gamma$ have already proven fruitful ([44] and see discussion below).

SIGK/ $G\beta_1\gamma_2$ complex

A crystal structure of $G\beta_1\gamma_2$ bound to a SIRK peptide derivative (denoted "SIGK"; Table I) has been determined, depicting the molecular basis for this peptide's unique regulatory properties [45]. The SIGK peptide assumes a partial α -helical conformation reminiscent of the switch II helix of $G\alpha$ within the $G\alpha\beta\gamma$ heterotrimer (Figure 7C). Moreover, the SIGK helical peptide binds the same face of $G\beta_1\gamma_2$ as does switch II. This common binding site likely explains the apparent ability of this peptide to induce G-protein heterotrimer dissociation [46,47]. It is interesting to note that $G\beta_1\gamma_2$ uses both polar and nonpolar residues in the overall SIGK binding interface, suggesting that this combination of binding determinants dictates the ability of $G\beta_1\gamma_2$ to recognize a variety of downstream effector binding partners. In support of this notion, mutation of selective $G\beta_1\gamma_2$ residues within the $G\beta_1\gamma_2$ /SIGK interface [45] resulted in loss of binding to different subset(s) of the four distinct groups of $G\beta\gamma$ -binding peptides originally isolated by Smrcka and colleagues [40]. Thus, effectors likely share the common overall SIGK-footprint for interaction with $G\beta_1\gamma_2$, yet distinct sets of interactions within this $G\beta\gamma$ 'hot spot' dictate the specificity of each given complex [45].

'Hot spots' have been proposed to represent unique areas within proteins for guiding targeted drug design efforts [48]. Data from the SIGK family of peptides has defined such an area within $G\beta_1\gamma_2$. The ability of these phage display peptides to selectively perturb $G\beta_1\gamma_2$ signaling nodes [40] further implies that drug design to this particular 'hot spot' may lead to the identification of small molecules capable of inhibiting specific $G\beta\gamma$ -driven signal transduction pathways. Such a discovery has recently been reported using the $G\beta_1\gamma_2$ /SIGK interface as the basis for computational docking screens of virtual compound libraries [44]. Several $G\beta_1\gamma_2$ binding compounds were identified in this *in silico* screen and subsequently shown to selectively modulate $G\beta_1\gamma_2$ signaling both *in vitro* and *in vivo*. M119, a fluorescein-like lead compound from this virtual screening, was capable of blocking $G\beta_1\gamma_2$ /SIGK interaction, inhibiting $G\beta_1\gamma_2$ activation of purified PLC β 2, preventing chemoattractant-induced calcium signaling in intact immune cells, and sensitizing mice to the antinociceptive effects of morphine *in vivo* [44]. These sensational findings demonstrate perhaps the ultimate application of peptide screening studies, given that the original finding of the SIRK phage-display peptide formed the basis for the discovery of the small molecule $G\beta\gamma$ -inhibitor M119. Analogous screens using the $G\alpha_{i1}$ /KB-1753 complex could be envisioned to identify useful small molecule inhibitors of $G\alpha$ signaling. (Note that we are not suggesting that heterotrimeric G-protein alpha subunits will necessarily emerge as drug targets themselves, except possibly in the case of their

mutational activation (as for *GNAS* in endocrine tumors; ref. [49]). However, there is a real need for chemical biology approaches to target heterotrimeric G-protein function and thus further delineate the biology of these signaling molecules as well as facilitate new screening paradigms. A germane example is the G_{aq} -selective inhibitor YM-254890, which is likely to provide novel biological insights [50,51].

Application in High-Throughput Screening for GPCR Modulators

The original intent of our efforts in identifying nucleotide-state-selective $G\alpha$ -binding peptides was to employ them in developing rapid, non-radioactive means for assaying heterotrimeric G-protein activation by GPCRs. In particular, peptides that show specificity towards the active, GTP-bound state of $G\alpha$ subunits (such as KB-1753, KB-1746, and KB-1755) or freed $G\beta\gamma$ subunits (such as SIRC and SIGK) should be valuable tools in detecting receptor-mediated heterotrimer activation when coupled to a real-time, sensitive readout of their specific G-protein subunit interaction (e.g., the FITC-labelled KB-1755 shown in Figure 6A). In pilot studies previously described [52], we have shown that activation-state-selective $G\alpha$ -binding peptides are able to faithfully report on several different GPCR/heterotrimer complexes when their activation status has been modulated by specific agonists and antagonists (Figure 8). In examining the G_i -heterotrimer coupled M2 muscarinic acetylcholine receptor (M2-mAChR), we have observed that binding of the $G\alpha_i$ -GTP-specific peptide KB-1755 to immobilized membrane preparations is significantly higher in the presence of the receptor-specific agonist carbachol than in its absence; furthermore, this binding was inhibited in a dose-dependent manner by the receptor-specific antagonist atropine, a clear indication that the effect is receptor promoted (Figure 8A).

$G\alpha_i$ -GTP-specific peptides, besides being useful in detecting activation or inactivation of a G_i -coupled GPCR, can also be employed in conjunction with chimeric $G\alpha$ subunits (reviewed in [53]) to detect activation or inactivation of a GPCR which normally couples to a different G-protein such as $G\alpha_q$ or $G\alpha_s$. For example, to demonstrate utility in measuring β_2 -adrenergic receptor (β_2 -AR) status upon treatment with agonist isoproterenol and inverse agonist ICI-118,551 (Figure 8B), we employed a $G\alpha_{i6s}$ chimera [54] in which the last 6 residues of $G\alpha_{i1}$ (KDCGLF) were replaced with the last 6 residues of $G\alpha_s$ (RQYELL), thus allowing functional coupling of a $G\alpha_{i6s}/G\beta\gamma$ heterotrimer to the normally G_s -coupled β_2 -AR [55]. We further confirmed the utility of this chimeric $G\alpha$ strategy in detecting dose-dependent activation of the dopamine D1-receptor (another normally G_s -coupled receptor [56]; Figure 8C). We have also used this peptide-based assay to recapitulate the known relative efficacies and rank order of potencies of the β_2 -AR agonists isoproterenol, salbutamol, and dobutamine (refs. [52,57]; Figure 8D); all activation signals from these agonists were inhibited by the inverse agonist ICI-118,551 (data not shown). These latter results indicate that the known pharmacological properties of the β_2 -adrenergic receptor are faithfully preserved in this $G\alpha_i$ -GTP-specific peptide-based activation assay. Additional screening for activation-state-selective phage peptides specifically directed to $G\alpha_s$ and other $G\alpha$ subfamilies (e.g., $G\alpha_q$, $G\alpha_{12/13}$) should obviate the need to reconstitute GPCR/heterotrimer complexes with chimeric $G\alpha_i$ subunits.

We conducted a pilot screening assay in 96-well plates using baculovirus-infected Sf9 cells to investigate the applicability of the M2-mAChR assay for high-throughput screening. The screen was performed on the Library of Pharmacologically Active Compounds (LOPAC; Sigma-Aldrich), with compounds applied at a final concentration of 1 μ M. Twelve documented cholinergic agonists were detected with responses greater than 50% of the signal observed with a saturating dose (10 nM) of oxotremorine. Representative examples including the agonists arecoline, dioxolane, carbachol and the antagonist atropine are annotated in Figure 9. The Z-factor of the assay was calculated as 0.62, indicative of a robust and sensitive screening assay [58]. The compound TMB-8 (3,4,5-trimethoxybenzoic acid 8-[diethylamino]octyl ester) was

also identified as an M2-mAChR agonist in our pilot screen. The peptide-detection assay was then used to directly compare TMB-8 and carbachol for M2-mAChR activation (Figure 10A). In terms of G-protein agonism, the two compounds were similar in potency, while the traditional agonist carbachol had a two-fold greater efficacy. Antagonist competition experiments were consistent with this data (Figure 10B). As a positive control, we examined the guanine nucleotide selectivity of the assay and demonstrated that G-protein activation could only be detected in the presence of micromolar GTP γ S but not GDP (Figure 10C). TMB-8 has previously been characterized as a muscarinic antagonist [59] and an allosteric modulator of the M2 muscarinic acetylcholine receptor [60]. TMB-8 slows the rate of binding and the dissociation of pre-bound acetylcholine from the M2 muscarinic receptor [61]. Our data are interesting in that they demonstrate that TMB-8 has canonical agonist-like properties at the M2-mAChR with regard to G-protein activation (Figure 10A). However, we have not conducted the mechanistic studies necessary to confirm or refute TMB-8 as an allosteric modulator of the M2-mAChR. In this regard, it must be noted that TMB-8 has multiple pharmacologic effects and targets, having been originally characterized as a calcium antagonist [62,63]. Therefore our discovery via this pilot, peptide-based GPCR screen that TMB-8 is an agonist at the M2 muscarinic acetylcholine receptor is a novel pharmacological finding.

Concluding Remarks

G-protein subunit binding peptides, identified by various screening methods, have presented themselves as powerful research tools for studying target proteins within vital signal transduction pathways. As described herein, these peptides have numerous applications including:

1. Serving as direct modulators of target protein activity,
2. Providing structural insights into previously undefined mechanisms of target protein function,
3. Supplying a template for designing biosensors capable of reporting the spatiotemporal dynamics of target protein signaling,
4. Presenting a model for screening small molecule libraries to identify inhibitors of target G-protein subunit activity, and
5. Serving as the starting material for novel, non-radioactive assays for measuring agonist and antagonist activity on GPCR/heterotrimer complexes.

Recently, screening methods including phage-display and mRNA-display techniques have successfully identified such peptides targeting both G α and G $\beta\gamma$ subunits [12,16,21,23,35,39, 40]. These peptides have already given valuable insights into various aspects of G-protein signaling and have been elegantly applied in various scenarios just described. Continued efforts to identify novel peptide sequences to additional G-protein subfamilies should afford further discoveries into heterotrimer nucleotide cycling as well as future assay platforms for receptor-mediated G-protein signaling.

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Abbreviations

GDI

guanine nucleotide dissociation inhibitor

GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
KB	Karo Bio

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Figure 1.

The traditional model of the guanine nucleotide exchange and hydrolysis cycle governing the receptor-mediated activation of heterotrimeric G protein-coupled signal transduction. GPCRs bind, via their intracellular loops, to the heterotrimeric G-protein consisting of $G\alpha$ (with bound GDP) associated with the $G\beta\gamma$ dimer. The isoprenylated $G\beta\gamma$ dimer aids in association of the heterotrimer with the plasma membrane, participates in receptor coupling, and serves as a guanine nucleotide dissociation inhibitor (GDI) preventing spontaneous activation of the $G\alpha$ subunit. Agonist-bound receptors act as guanine nucleotide exchange factors (GEFs) by provoking conformational changes in $G\alpha\beta\gamma$ resulting in the release of GDP and binding of GTP by the $G\alpha$ subunit. Binding of GTP induces changes in three flexible switch regions within the $G\alpha$ subunit, leading to $G\beta\gamma$ dimer dissociation. Both $G\alpha$ -GTP and freed $G\beta\gamma$ dimer subsequently regulate downstream effectors, either alone or in a coordinated fashion. The GPCR/heterotrimer complex returns to the inactive state by the intrinsic GTP hydrolysis activity of $G\alpha$, cleaving the terminal γ -phosphate from GTP and rendering $G\alpha$ again bound to GDP and reassociated with the $G\beta\gamma$ dimer, thus mutually terminating the signaling capacity of both subunits of the heterotrimer. GTP hydrolysis is greatly enhanced by the “regulator of G-protein signaling” (RGS) family of proteins, which serve as GTPase-accelerating proteins (GAPs) for the $G\alpha$ subunit.

Figure 3.

Fluorescence anisotropy was used to measure the equilibrium affinity of interaction between $G\alpha$ subunits and 10 nM of FITC-KB-1746 or FITC-KB-1755 peptides. Equilibrium fluorescence anisotropy was measured in the presence of increasing concentrations of $G\alpha_{i1}\cdot\text{GDP}\cdot\text{AlF}_4^-$ (for KB-1746; *grey*) or $G\alpha_{v11}\cdot\text{GDP}\cdot\text{AlF}_4^-$ (for KB-1755; *black*). 95% confidence intervals for K_D determinations were: KB-1746, 0.86 - 1.2 μM ; KB-1755, 1.4 - 2.1 μM .

Figure 4.

Surface plasmon resonance (SPR) biosensor assays demonstrating nucleotide-selective (A-C) and $G\alpha$ subfamily-selective (D-F) binding to immobilized KB-series peptides. SPR assays were conducted as previously described (refs. [21–23]), with biotinylated KB-series peptides immobilized on separate streptavidin biosensor surfaces to relative surface densities of ~500 RU and indicated $G\alpha$ subunit analytes (each at 5 μ M) injected for 360 seconds (in GDP or GDP·AlF₄⁻ [“AMF”] nucleotide states as indicated in panels A-C; solely in GDP·AlF₄⁻ nucleotide state in panels D-F).

Figure 5.

SPR biosensor competition binding assays, conducted in a similar fashion to panels D-F of Figure 4 and using $1 \mu\text{M } \text{G}\alpha_{i1}\cdot\text{GDP}\cdot\text{AlF}_4^-$ preincubated with the indicated concentrations of wildtype or loss-of-function (I9A/W10A mutant) KB-1753 peptide prior to injection over indicated immobilized peptide surfaces.

Figure 6.

Fluorescence spectroscopic assays were used to measure competitive binding interactions between peptides (and proteins) that bind to activated $\text{G}\alpha$ subunits. (A) FITC-labeled KB-1755 peptide was used as a conformation-sensitive reporter for $\text{G}\alpha$ binding. The fluorescence emission spectrum of 1 μM FITC-KB-1755 was measured in the presence of DMSO vehicle or 10 μM competitor peptides: biotin-KB-1746, biotin-KB-1753, or biotin-KB-1755 (control ["CTRL"] spectra in grey). 3 μM of $\text{G}\alpha_{i1}\cdot\text{GDP}\cdot\text{AlF}_4^-$ was then added and fluorescence emission was re-measured after 5 minutes (black spectra). Data are presented as the average of triplicate determinations. The net fluorescence enhancement was calculated in percent. Percent fluorescence enhancement ($\pm\text{SEM}$): DMSO ($26.2 \pm 1\%$), KB-1746 ($6.9 \pm 1\%$), KB-1753 ($9.4 \pm 1\%$), KB-1755 ($6.0 \pm 1\%$). RFU = relative fluorescence units. (B) Equilibrium fluorescence anisotropy assays were conducted using 10 nM FITC-KB-1755 and increasing concentrations of $\text{G}\alpha_{i1}\cdot\text{GDP}\cdot\text{AlF}_4^-$. Titrations were conducted in the presence of DMSO vehicle or indicated concentration of competitor proteins/peptides: biotin-KB-1746, biotin-KB-1753, biotin-KB-1755, RGS16 (aa 53–190, spanning the minimal RGS domain), or biotin-PDE γ (aa 63–87). Data are presented as EC_{50} values (with 95% confidence intervals) of the fluorescence increase derived from the FITC-KB-1755/ $\text{G}\alpha_{i1}\cdot\text{GDP}\cdot\text{AlF}_4^-$ interaction.

Figure 7.

Structures of G-protein subunit-selective peptides and their G-protein partners (see color version on cover of this issue). (A, B) Overall structural features of the KB-752/ $G\alpha_{i1}$ ·GDP (panel A) and KB-1753/ $G\alpha_{i1}$ ·GDP· AlF_4^- (panel B) complexes as obtained by X-ray diffraction crystallography [21,23]. Both KB-752 and KB-1753 peptides (red) are found situated in a groove between the $\alpha 2$ (“switch II”) and $\alpha 3$ helices of the $G\alpha$ Ras-like domain (blue; switch regions in green); no contacts are made by either peptide to the $G\alpha$ all-helical domain (yellow) nor GDP moiety (magenta). Bound magnesium and aluminum tetrafluoride ion are colored orange and grey, respectively, in panel B. (C) Overall structural features of the SIGK/ $G\beta_1\gamma_2$ complex as obtained by X-ray diffraction crystallography [45]. The SIGK peptide (red) lies in an α -helical conformation over the top of the β -propeller (or “torus”) structure of the $G\beta$ subunit (cyan); no contacts are made to the $G\gamma$ subunit (black).

Figure 8.

Examples of using activation-state-selective, $G\alpha$ -binding peptides to measure GPCR/heterotrimer activation state upon agonist and antagonist treatment. (A) Peptide-based assay of M2-muscarinic acetylcholine receptor (M2-mAChR) stimulation and inhibition.

Membranes of Sf9 insect cells co-infected with baculoviruses expressing M2-mAChR, $G\alpha_{i1}$, and $G\beta_1\gamma_2$ subunits were prepared by nitrogen cavitation; 10 μ g of membrane protein per well (in 100 μ L of immobilization buffer containing 100 mM NaCl, 20 mM HEPES pH 7.4, 5 mM $MgCl_2$, 3 mM EDTA) was covalently immobilized via primary amines using N-oxy succinimide surface chemistry ("DNA-Bind" 96-well plates from Corning Costar; Kennebunk, ME). After five 200 μ L rinses with a wash-buffer containing 150 mM NaCl, 20 mM Tris pH 8, 0.05% Tween-20 and 0.1% BSA, immobilized membranes were incubated with 100 μ L of buffer only (immobilization buffer containing an optimized concentration of GTP γ S) or buffer containing the agonist carbachol (100 μ M) in the absence or presence of indicated concentrations of the antagonist atropine. Guanine nucleotide exchange was allowed to proceed for 20 minutes at room temperature, followed by quenching with the addition of 100 μ L detection buffer (150 mM NaCl, 100 μ M GDP, 0.05% Tween-20, 0.1% BSA, and 1 nM of N-terminally biotinylated KB-1755 peptide pre-adsorbed to Neutravidin-Alkaline Phosphatase (NA-AP; Pierce). After 1 hour incubation, wells were rinsed five times with wash-buffer and bound peptide/NA-AP complex detected with the addition of 100 μ L CDP-Star (Perkin-Elmer) and luminescence detection using a BMG Lumistar plate-reader. (B) Peptide-based assay of β_2 -adrenergic receptor (β_2 -AR) stimulation and inhibition. Membranes of Sf9 insect cells co-infected with baculoviruses expressing β_2 -AR, chimeric $G\alpha_{i6s}$ (see text for details), and $G\beta_1\gamma_2$ subunits were stimulated with the specific adrenergic agonist isoproterenol (0.2 μ M) in the absence or presence of indicated concentrations of the inverse agonist

ICI-118,551. (C) Peptide-based assay of D1-dopamine receptor (D1-R) stimulation. Membranes of Sf9 insect cells co-infected with baculoviruses expressing D1-R, chimeric $G\alpha_{i6s}$, and $G\beta_{1\gamma 2}$ subunits were stimulated with indicated concentrations of the specific agonist CY 208–243. (D) Peptide-based assay of $\beta 2$ -AR stimulation by full and partial agonists at the indicated concentrations, conducted as in panel (B).

Figure 9.

A pilot, peptide-based screening assay for activators of the M2-muscarinic acetylcholine receptor (M2-mAChR). Intact Sf9 cells were used as the source membrane material. Sf9 cells infected with baculoviruses encoding the M2-mAChR receptor, $G\alpha_{i1}$, and $G\beta_1\gamma_2$ subunits were washed with phosphate-buffered saline and applied to DNA-Bind 96-well plates at a density of 500,000 cells per well. Compounds from the LOPAC library (Sigma-Aldrich) were applied at a final concentration of 1 μ M. Compound additions and washing steps were performed with an automated liquid handling device (Tecan, Durham, NC). Results are depicted graphically, with a 'hit' being defined as a response greater than 50% of the signal observed with a saturating dose of oxotremorine (10 nM).

Figure 10.

Peptide-based measurements of M2-mAChR activation confirming agonist activity of TMB-8. (A) TMB-8 is an equipotent partial agonist of the M2-mAChR as compared to carbachol. (B) Dose-dependent atropine antagonism of carbachol- and TMB-8-agonist activity (10 μ M of either agonist). Assays were performed essentially as described in Figure 8A. (C) Nucleotide (and concentration) dependence of the peptide-based measurement of agonist activity on the M2-mAChR by carbachol and TMB-8.

Table I

Specific G-protein subunit binding peptides discussed in this review

Name	Peptide sequence (N-terminal to C-terminal)	Binding specificity and relative affinities	Reference(s)
KB-752	SRVTWYDFLMEDTKSR	$G\alpha_{11/12/13}\cdot\text{GDP} > G\alpha_s\cdot\text{GDP} > G\alpha_q\cdot\text{GDP}$	[22,23,64]
KB-1753	SSRGYYHGIWVGEEGRLSR	$G\alpha_{11/12/13}\cdot\text{GDP}\cdot\text{AlF}_4^-$ (Figure 4A,D)	[21]
KB-1746	SSSYSEHCQRWGCRYARLSR	$G\alpha_{11/12/13/q}\cdot\text{GDP}\cdot\text{AlF}_4^-$ (Figure 4B,E)	This study
KB-1755	SSRLCPEWICPWEWPASSR	$G\alpha_q\cdot\text{GDP}\cdot\text{AlF}_4^- > G\alpha_{11/12/13}\cdot\text{GDP}\cdot\text{AlF}_4^-$ (Figure 4C,F)	This study
R6A	MSQTKRLDDQLYWWEYL	$G\alpha_{11/12/13}\cdot\text{GDP}$	[35]
R6A-1	DQLYWWEYL	$G\alpha\cdot\text{GDP}$	[32,39]
SIRK	SIRKALNILGYPDYD	$G\beta_1\gamma_2$	[40]
SIGK	SIGKAFKILGYPDYD	$G\beta_1\gamma_2$	[45]