

G-protein-directed ligand discovery with peptide combinatorial libraries

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Modulators of G-protein signaling have a central role in controlling cell physiology and represent over half of all marketed prescription drugs. G-protein pathways have traditionally been targeted by developing ligands to the extracellular surface of a small subset of the estimated ~1000 G-protein-coupled receptors in humans. The intracellular machinery, consisting of the cytosolic receptor surfaces and heterotrimeric G proteins, provides an equivalent diversity of targets that has remained relatively unexplored until now. This review summarizes recent efforts using combinatorial peptide libraries to develop new G-protein signaling modulators targeting intracellular components.

Introduction

G-protein-coupled receptors (GPCRs) relay diverse extracellular signals to intracellular signal transduction pathways through heterotrimeric G proteins [1,2]. Whereas drug discovery efforts have primarily focused on GPCRs, ligands for intracellular G proteins that modulate signaling directly have been increasingly regarded as potential drugs [3–5]. Short peptides, both naturally occurring and synthetically derived from segments of GPCRs, G proteins and effectors, have been used extensively to map crucial interaction sites and antagonize or activate G proteins [4–9]. Although successful, most of these peptides are weak modulators of signaling, exhibiting their activities at μM to mM concentrations. Combinatorial methods have the potential to substantially increase the potency of known ligands and to identify novel peptides with new functions from diverse, random libraries [10,11]. Here, we review several examples of *in vitro* selection applied to the isolation of peptide modulators of G-protein signaling.

G-protein signaling cycle

In the classical G-protein signaling model, an inactive GPCR is coupled to a GDP-bound, $G_{\alpha\beta\gamma}$ heterotrimer (Figure 1). $G_{\beta\gamma}$ binds tightly to $G_{\alpha}\text{-GDP}$, which enhances coupling of the inactive heterotrimer to specific GPCRs and acts as a guanine nucleotide dissociation inhibitor (GDI) by preventing GDP release [12]. Activation by an extracellular agonist causes the GPCR to act as a guanine nucleotide exchange factor (GEF), exchanging GDP for GTP in the G_{α} subunit. Binding of GTP to G_{α} induces $G_{\beta\gamma}$ release and subsequently both $G_{\alpha}\text{-GTP}$ and $G_{\beta\gamma}$ can interact with downstream effectors. The intrinsic GTPase

activity of G_{α} results in the eventual hydrolysis of GTP, leading to reformation of the inactive $G_{\alpha\beta\gamma}$ heterotrimer. GTPase-activating proteins (GAPs) accelerate the hydrolysis of $G_{\alpha}\text{-GTP}$, leading to shorter activation times and/or lower basal activities. This simple model of G-protein signaling has grown increasingly complex because of: (i) the numerous regulatory proteins that modulate or attenuate signaling by acting as GEFs, GDIs or GAPs and/or by directly competing with receptor, G protein or effector interactions [13,14]; (ii) the immense diversity and crosstalk of signal transduction pathways controlled by heterotrimeric G-protein activation [2,15]; and (iii) the growing number of intracellular receptor partners discovered that activate signals through means other than classical G-protein pathways [16].

In humans, there are 20 distinct, but highly homologous, G_{α} subunits that are divided into four classes based on their sequence and function: (i) $G_{i/o}$, (ii) G_s , (iii) $G_{q/11}$ and (iv) $G_{12/13}$ [2]. Despite their similarity, the G_{α} families can elicit different functions and have distinct and

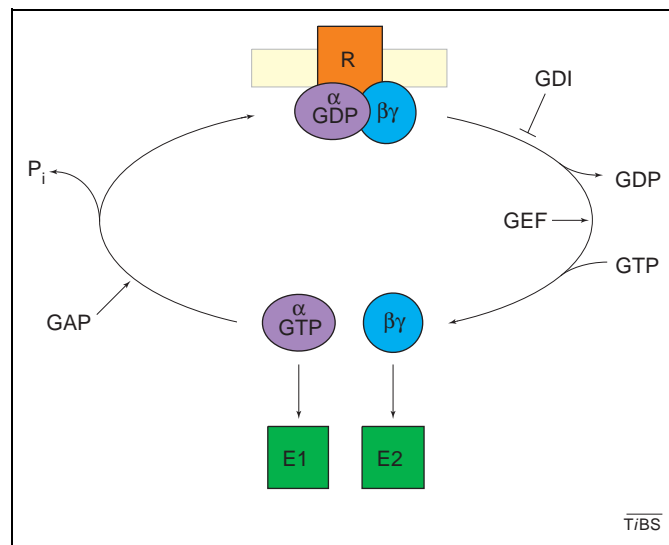


Figure 1. Classical G-protein signaling. An intracellular, GDP-bound $G_{\alpha\beta\gamma}$ heterotrimer is coupled to a membrane-spanning GPCR (R). $G_{\beta\gamma}$ acts as a GDI for $G_{\alpha}\text{-GDP}$, inhibiting nucleotide exchange and maintaining the inactive state. Extracellular agonists cause the GPCR to act as a GEF, catalyzing the exchange of GDP for cytosolic GTP in the G_{α} subunit. $G_{\alpha}\text{-GTP}$ and $G_{\beta\gamma}$ subsequently dissociate and are free to signal downstream effectors (E1 and E2). Hydrolysis of $G_{\alpha}\text{-GTP}$ to the GDP-bound state, a reaction that is catalyzed by GAPs, results in re-association with $G_{\beta\gamma}$ and termination of signaling. Potential modulators of G-protein signaling might interfere with protein–protein interactions at any part of the signaling cycle (e.g. receptor coupling of G proteins, $G_{\alpha\beta\gamma}$ heterotrimer formation or effector-G protein) and/or act as GDIs, GEFs or GAPs, thereby modifying the G_{α} nucleotide-bound state.

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sometimes overlapping specificities for their binding partners [2]. There are currently at least seven known human G_{β} and 12 known G_{γ} isoforms, making a large number of $G_{\beta\gamma}$ heterodimers possible. G_{α} and $G_{\beta\gamma}$ pairs can each interact with a wide variety of effectors. Whereas classical drugs typically target GPCRs, mimicking or antagonizing natural agonist responses, direct G-protein ligands can potentially modulate individual effector pathways, alter signals specifically from particular G-protein classes or subclasses and/or modify the kinetics of G-protein signaling. Hence, there is a large degree of selectivity that can be conferred by drugs that directly interact with G proteins or interfere with G-protein signaling [5].

Peptide selection with combinatorial libraries

Naturally occurring peptides, as well as peptides derived from portions of GPCRs, G proteins and effectors, have been used effectively to study the interactions between these proteins both *in vitro* and *in vivo* [6]. These peptides are able to modulate G-protein signaling in different ways (e.g. by antagonizing G-protein interactions or directly stimulating GDP exchange in G_{α} subunits). Methods for the directed evolution of peptides can optimize these ligands for higher affinity and activity or isolate novel sequences with desired properties from random libraries [10,11]. A typical selection experiment involves: (i) construction of a DNA library, (ii) expression of the library using a method that physically links each peptide with its encoding nucleic acid sequence (e.g. peptides-on-plasmids [17], phage display [18] and mRNA display [19]), (iii) affinity selection against an immobilized target to retain functional peptides, and (iv) amplification of the recovered nucleic acid sequences to produce an enriched library, which can be used for a subsequent round of selection. Each selection cycle generates a new library that is further enriched with functional members, eventually resulting in a pool that is dominated by active peptides. Individual peptides can then be identified by cloning and DNA sequencing. Typical selection libraries examine 10^8 – 10^9 unique molecules, whereas purely *in vitro* methods that do not require an *in vivo* transformation step can access even greater pool complexities ($>10^{13}$) [10,20].

The receptor–G-protein interface

Although a complete structural characterization of GPCR–G-protein coupling and activation has not yet been described, biochemical analyses have established that the receptor– G_{α} interface involves several regions on G_{α} , including the N and C termini, and the intracellular loops and C terminus of the GPCR [13,21]. It has been proposed that individual receptors can recognize different combinations of regions of the same G_{α} subunit [13,22]. Hence, although hundreds of GPCRs signal through a limited set of G proteins, molecules targeting the receptor–G-protein interface could be highly specific for a particular receptor pathway.

Synthetic peptides corresponding to the last 11 amino acids in the C terminus of several G_{α} subunits have been shown to block G-protein–receptor coupling with low potency (μM IC_{50} values); in addition, they have been

shown to stabilize active forms of the GPCR, presumably by mimicking the conformational effects of heterotrimeric G proteins [4–6,13]. These C-terminal-derived peptides generally demonstrate receptor selectivity similar to the full-length G_{α} subunit. To enhance the potency of a rhodopsin-binding peptide derived from the C terminus of G_{tz} (transducin), a ‘doped’ library – where a percentage of changes were permitted at each position in a single, functional sequence – was constructed using the ‘peptides-on-plasmids’ approach [17]. In this selection method, peptides are expressed as LacI fusions, which bind stably to *lacO* DNA sequences on the plasmid encoding the peptide. Peptide–LacI–plasmid complexes were affinity purified on activated rhodopsin and recovered plasmids encoding functional peptides were subsequently amplified [23]. Selected peptides were significantly more potent than the wild-type sequence (by several orders of magnitude) and the amino acid conservation highlighted several crucial residues (Table 1). Subsequent work demonstrated that the G_{tz} peptide analogs are able to modulate high and/or low affinity states of the A_1 adenosine receptor and reduce GPCR signaling responses in a receptor-selective fashion [24]. These results suggest that selections targeting other GPCRs might produce specific ligands, even though many receptor– G_{α} contacts are shared.

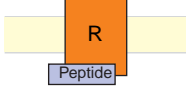



Interestingly, although a consensus was not observed in the random region of the library (Table 1), full-length, 15-residue peptides were significantly more potent than synthetic, C-terminal 11-mers derived from the selected sequences [23], suggesting that the structural context of the synthetic peptides is important for the high affinity interaction with rhodopsin. Indeed, recombinant maltose-binding protein (MBP)–peptide fusions were several orders of magnitude more potent than their synthetic peptide counterparts. These fusion proteins might present the selected peptides in a manner similar to the LacI fusion used in the peptides-on-plasmids approach, thereby more closely approximating the original selection vehicle.

The receptor–G-protein interface can also be disrupted using peptides derived from the intracellular regions of the GPCR, which presumably bind to G proteins and prevent coupling [6,21]. Previously, peptides derived from different intracellular regions of rhodopsin were shown to inhibit G-protein coupling [25]. These peptides demonstrated synergistic inhibition; the addition of multiple peptides dramatically decreased G-protein coupling by binding to multiple contact sites on the G_{α} subunit. Hence, selection libraries based on protein scaffolds that present several receptor-derived loops, thereby mimicking the intracellular face of a GPCR, might be more effective for isolating potent ligands.

G-protein activators

Random peptide libraries have been an effective tool in the isolation of novel sequences with desired properties. Recently, $G_{i\alpha 1}$ was directly targeted in a phage display selection using a commercially available, 7-mer (x_7) peptide library [26]. In phage display, peptide sequences are expressed on the surface of filamentous phages and selected against an immobilized target [18]. Three classes of peptides with short consensus motifs were identified from the

Table 1. Peptide selections against G-protein-related targets

Target	Library	Diversity	Selected peptide(s) ^a	Potency	Refs
GPCR (light-activated rhodopsin)	 x ₄ -IKENLKDCGLF ^b	2 × 10 ⁹	x ₄ -hxxxLKDCGLF	IC ₅₀ ^c 140 nM	[23]
 G _{12z1} ^d	x ₇	20 ⁷ = 10 ⁹	(i) aPxxaHP (ii) QxPxSxP (iii) LPaxxxH	EC ₅₀ ^e (i) 16 μM (ii) > 1000 μM (iii) 17 μM	[26]
 G _{β1γ2}	x _m ^f xCx _n Cx x ₅ Cx ₃ Cx ₄ x ₄ Cx _p Cx ₄ x ₈ Cx ₈ xCx ₁₅ x ₁₅ Cx xCx ₃ Cx ₅ C ₄ GIEGRG	10 ⁸ –10 ⁹ (each library)	(i) KAxxLLG (ii) KaxxaaG (iii) CEKRxGxxxC (iv) Cx ₅ C	IC ₅₀ ^g (i) ~ 5 μM	[29]
 G _{12z1} -GDP	MSQSKRLDDQR-x ₆	20 ⁶ = 6 × 10 ⁷ ^h	MSQTKRLDD <u>QLYWWEYL</u> ⁱ	K _D ^j 60 nM	[40]

^aAmino acid types: a = aromatic or aliphatic; h = hydrophobic; x = any amino acid. Multiple sequences represent consensus classes.

^bEach of the wild-type residues was mutagenized (doped) at a 50% rate.

^cIC₅₀ of competition with G_{12z1} for binding to light-activated rhodopsin (Meta II). Activity is for the most potent, full-length, synthetic peptide. MBP fusion proteins were several orders of magnitude more potent [23].

^dSelection buffer was apparently not supplemented with nucleotide. Hence, the G_α nucleotide state is unclear, although it probably consisted of a mix between GDP-bound and nucleotide-free subunits.

^eEC₅₀ of rate enhancement of GTPγS binding to G_{12z1}.

^fSubscripts m = 6, 15 or 30; n = 4, 6, 8, 10 or 12; and p = 4, 5 or 6.

^gIC₅₀ of G_{βγ}-mediated phospholipase C activation. Peptides also disrupt G_{αβγ} heterotrimer formation at similar concentrations [32].

^hSelected peptides encoded crucial mutations in the constant region. The presence of these mutations implies that the initial diversity of the library was actually higher than indicated. The total number of molecules in the initial mRNA display pool was ~ 10¹². Hence, at least 10⁴ copies of each unique (random region) peptide were present. This over-representation, coupled with a finite error-rate during PCR amplification, is most likely what permitted access to extremely rare sequences derived from mutations in the constant region.

ⁱUnderlined region represents the minimal active peptide (K_D = 200 nM to G_{12z1}-GDP).

^jK_D for binding to G_{12z1}-GDP. Peptides also exhibited GDI activity and competed with G_{βγ} for binding to G_{12z1}.

selection (Table 1). Because the consensus sequences were short, database searches identified many (250–1000) proteins containing the motifs, only a few of which were implicated or known to be involved in signal transduction [26].

The authors did not report any direct binding assays to assess peptide affinity or specificity for G_α subunits. However, two of the peptide classes exhibited GEF activity, increasing the rate of binding of GTP to G_{12z1}, G_{αo} and G_{so} subunits [26]. These peptides bear little similarity to other known G-protein activators that have cationic, amphipathic structures, such as mastoparan [27]. Functionally, the active peptides were shown to increase the sensitivity of A₁ adenosine receptor agonist-binding to GTP in a reconstituted GPCR membrane assay [26]. The mechanism by which these agonistic peptides function will be of great interest, in comparison with the presumed receptor-analogous model of activators such as mastoparan. In addition, selections against various G_α subunits with new libraries based on the selected peptides could identify more potent molecules with increased specificity.

G_α-G_{βγ} interface

Activation of effectors by either G_α-GTP or G_{βγ} is effectively blocked by formation of the GDP-bound heterotrimer, G_{αβγ}. Hence, individual effectors probably share overlapping binding sites at the G_α-G_{βγ} interface. Extensive mapping of key residues for effector binding on

G_{βγ}, for example, has shown that various signaling partners for G_{βγ} rely on different, but partially overlapping, subsets of residues for interaction [28]. Hence, by targeting different sites on or adjacent to the G_α-G_{βγ} interface, individual pathways might be affected.

Phage display peptides against G_{βγ}

Recently, phage display was used to identify peptides that bind to G_{βγ} [29]. A variety of libraries were used, both linear and constrained with disulfide bridges (Table 1). Approximately 250 copies of peptide were displayed per phage, permitting the recovery of peptides with even very low affinity due to avidity effects (although higher affinity peptides might be more difficult to isolate due to the narrower dynamic range of binding). The authors cleverly modified G_{βγ} with an amine-specific biotinylation reagent in the presence of G_α, thereby 'protecting' the G_α-G_{βγ} interface from modification. After G_α was removed by affinity chromatography, biotinylated G_{βγ} was immobilized on streptavidin and used as the selection target.

The selected peptides were grouped into four consensus classes, one of which had significant homology to peptides derived from phospholipase C-β (PLC-β) and to a short motif in phosphocucurbitin that binds to G_β subunits [29]. Peptides from all four classes appeared to bind to a single site on G_{βγ} based on competition experiments, suggesting a

'hot spot' for binding interaction [30,31]. A crystal structure of one of the selected peptides in complex with $G_{\beta 1\gamma 2}$ confirmed that the binding site includes residues sampled by other $G_{\beta\gamma}$ -interacting partners (T. Davis *et al.*, unpublished).

Functionally, the selected peptides inhibited activation of PLC- β by $G_{\beta\gamma}$, but not $G_{\beta\gamma}$ -mediated inhibition of voltage-gated calcium channels or adenylyl cyclase [29]. Subsequent studies also demonstrated an *in vivo* response to the application of cell-permeable versions of the peptides, which presumably resulted from the disruption of heterotrimers and activation of downstream MAP kinase pathways in the absence of receptor activation [32]. One synthesized peptide (SIGK) was shown to actively promote G_{α} dissociation from $G_{\beta\gamma}$, whereas other peptides were competitive with G_{α} for binding to $G_{\beta\gamma}$ subunits but did not actively increase G_{α} dissociation rates [32,33]. Recent studies with site-specific mutants of G_{β} show that although the peptides share a common binding site and can interfere with $G_{\beta\gamma}$ effectors, the ability to actively promote G_{α} dissociation from $G_{\beta\gamma}$ is dependent on whether particular contacts are made in the binding 'hot spot' (T.M. Bonacci *et al.*, unpublished). Hence, it might be possible to develop peptides that selectively activate or inhibit $G_{\beta\gamma}$ signaling.

The more recent description of an N-terminal, single-site biotinylation tag on G_{β} [32] suggests that homogeneously oriented, immobilized $G_{\beta\gamma}$ could be used in the future as a selection target. This could provide access to additional protein interaction sites that were blocked by biotinylation, due to protection of only the G_{α} -binding surface. Various sets of effectors might also be useful as competitors during selection experiments to identify rare peptides with highly specific functions.

mRNA display with the GPR motif

mRNA display is a completely *in vitro* method for selection where individual peptides are covalently coupled to the 3'-end of their encoding mRNA, resulting in stable RNA-peptide fusions [19]. Pools of fusions are selected for binding against an immobilized target and recovered sequences are amplified by RT-PCR. The G-protein regulatory (GPR) – or $G_{\alpha i/o}$ -Loco interaction (GoLoco) – motif is a ~20-residue peptide sequence that binds selectively to $G_{i\alpha}$ and $G_{o\alpha}$ subunits, acting as a GDI and actively promoting $G_{\beta\gamma}$ dissociation from heterotrimers [8,33–39]. The ability to differentiate between $G_{i\alpha}$ and $G_{o\alpha}$ family members is remarkable because $G_{i\alpha}$ and $G_{o\alpha}$ subunits share significant (>70%) protein sequence identity, and within each subclass (e.g. $G_{i\alpha 1}$, $G_{i\alpha 2}$ and $G_{i\alpha 3}$ or $G_{o\alpha A}$ and $G_{o\alpha B}$) the identity is even stronger. mRNA display libraries based on the C-terminal half of a GPR consensus sequence [8], a segment that lacks detectable binding and GDI activities, were selected against $G_{i\alpha 1}$ specifically biotinylated at the N or C terminus [40]. A strongly conserved motif was identified and the dominant peptide after selection (named R6A) demonstrated high affinity ($K_D=60$ nM) and GDI activity for $G_{i\alpha 1}$ (Table 1).

The R6A peptide was subsequently minimized to a 9-residue sequence (R6A-1) that retained high affinity and GDI activity and also competed with $G_{\beta\gamma}$ for binding to

$G_{i\alpha 1}$ [40]. The Arg finger has been a common structural motif for interaction in the nucleotide-binding pocket of G proteins [41] and the conserved Arg residue at the C terminus of the GPR motif has been shown to be crucial for binding and/or GDI activity by both mutagenesis [42] and crystallography [43]. The R6A-1 peptide retains only two residues from the original GPR consensus motif and contains no Arg or Lys residues. Hence, R6A-1 probably exerts its effects through a different mechanism than the GPR consensus peptide. Recent results have also demonstrated that the minimal peptide is able to bind to different G_{α} subunits representing all four G protein families (W.W. Ja and R.W. Roberts, unpublished). Hence, this peptide serves as a core motif for G-protein binding and probably interacts with a conserved region in all G_{α} subunits. By starting with doped libraries based on this consensus sequence, peptides could be selected against various G_{α} subunits to identify novel molecules with class- and/or subclass-specificity.

G_{α} specificity using adaptor peptides

The high sequence and structural similarity between the various G_{α} subunits makes it difficult to isolate small ligands that can distinguish between G-protein classes. When comparing G_{α} subunits, the helical domain stands out as the most obvious target for developing class-specific molecules because of the high variability between all four G-protein classes in this region (Figure 2a). Several natural proteins attain G_{α} class-specificity in this manner. The ~200-residue core domain of regulator of G-protein signaling 9 (RGS9) is a GAP for $G_{t\alpha}$, a G_{α} subunit that belongs to the $G_{i/o}$ family. Assays using chimeric $G_{t\alpha}$ - $G_{i\alpha 1}$ proteins demonstrate that RGS9 differentiates between the two G_{α} subunits through the helical domain, presumably by recognizing subclass-specific residues [44].

RGS14 is another GAP that, in addition to its core domain, contains a GPR motif. Recently, the crystal structure of $G_{i\alpha 1}$ in complex with a 36-residue peptide (R14GL) derived from the GPR motif of RGS14 revealed how a short sequence can selectively bind to a G_{α} subunit [43]. R14GL acts as a GDI for $G_{i\alpha 1}$ with no apparent activity for $G_{o\alpha}$ [35]. The poorly conserved region C-terminal to the GPR motif makes numerous contacts with residues in the helical domain of $G_{i\alpha 1}$ that differ in $G_{o\alpha}$, thereby imparting increased affinity and subclass-specificity (Figure 2b). RGS14 specificity has been extended recently to $G_{i\alpha 1}$ over $G_{i\alpha 2}$, which is remarkable due to the high protein sequence identity (88%) between these two isoforms [45].

The C-terminal region of the R14GL peptide essentially acts as an efficient payload delivery system for directly affecting G-protein signaling. Whereas the GPR motif (the 'payload') interacts with regions that interfere with nucleotide exchange and $G_{\beta\gamma}$ -binding, the C-terminal region acts as an adaptor peptide that delivers the required functional groups to a specific G_{α} target. Indeed, replacing the R14GL peptide C terminus with the corresponding sequence from Pep2 (a GPR protein that recognizes both $G_{i\alpha}$ and $G_{o\alpha}$) enables the resulting chimeric peptide to act as a GDI for $G_{o\alpha}$ [43]. Hence, it might be possible to design specific G_{α} ligands using

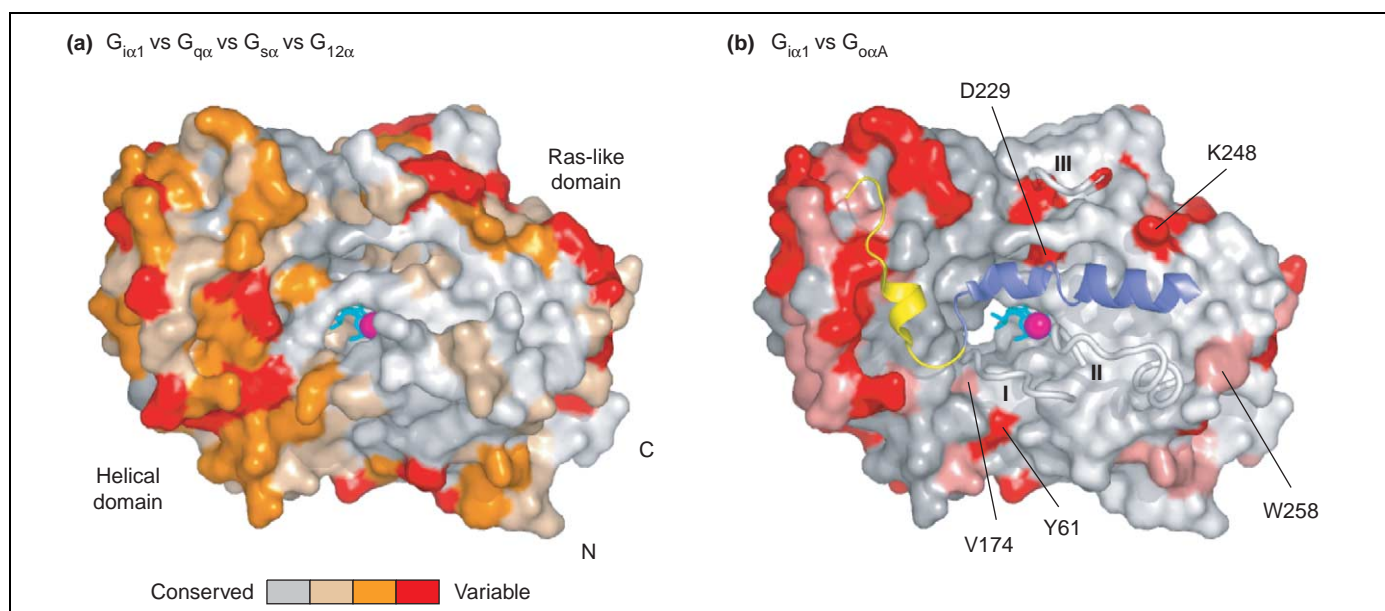


Figure 2. Conservation between G_α subunits. **(a)** Amino acid conservation between a representative G_α subunit from each family (human $G_{i\alpha 1}$, $G_{q\alpha}$, $G_{s\alpha}$ and $G_{12\alpha}$), overlaid on a surface representation of $G_{i\alpha 1}$. Amino acid differences between the G_α subunits are colored on a scale from gray, for highly conserved or identical residues, to red, for highly variable residues. The helical and Ras-like domains are in different shades of gray and are approximately the left and right halves of the structure. Gaps in the protein sequence alignment generally appear in surface loops (not shown). GDP and Mg^{2+} (in the center of the structure) are colored cyan and magenta, respectively. The N and C termini are labeled. **(b)** Structure of $G_{i\alpha 1}$ -GDP in complex with the RGS14-GPR peptide (R14GL) [43]. The conserved GPR motif (blue) and extended C-terminal region (yellow) make extensive contacts through the Ras-like and helical domains of $G_{i\alpha 1}$, respectively. Non-identical amino acids between $G_{i\alpha 1}$ and $G_{o\alpha A}$ are colored in pink or red for conserved or non-conserved differences, respectively. Specific contact residues in the helical domain that differ between $G_{i\alpha 1}$ and $G_{o\alpha}$ have been described previously [43]. Residues in the Ras-like domain that might be important for the specific binding of the GPR consensus peptide [8,42], a sequence that lacks an extended C terminus, are marked. Protein alignments were performed using ClustalW [70] from human cDNA sequences obtained from the UMR cDNA Resource Center (<http://www.cdna.org>). Both structure images were made from Protein Data Bank file 1KJY [43] using PyMOL (<http://www.pymol.org>). Approximately 30 residues from the N terminus and five residues from the C terminus are not present in the crystal structure.

various adaptor peptides to deliver small molecules or functional peptide motifs that modulate signaling activity.

Although the G_α helical domain is an attractive target for designing specific ligands, several selective peptides have been characterized that probably interact primarily with the Ras-like domain of G_α subunits. A GPR consensus peptide that binds to $G_{i\alpha}$ without the presence of an extended C-terminal region retains a strong preference for $G_{i\alpha}$ over $G_{o\alpha}$ subunits [8,42]. Assuming that the consensus peptide binds similarly to the strongly related GPR motif of R14GL in the $G_{i\alpha 1}$ complex crystal structure [43], specificity for $G_{i\alpha 1}$ over $G_{o\alpha}$ probably results from different conformations of the G_α binding surface rather than the identity of specific residue contacts (Figure 2b). Although $G_{i\alpha 1}$ has been characterized extensively by crystallography, structures of other $G_{i/o\alpha}$ isoforms are not yet available. These structures might reveal subtle conformational differences of interaction sites that establish subclass specificity between these highly related proteins.

Several peptide activators of G_α subunits have also been studied. Mastoparan and its analogs demonstrate varying specificities for the $G_{i/o\alpha}$ and $G_{s\alpha}$ families [27,46,47]. Competition binding studies suggest that mastoparan interacts with the C terminus of $G_{i\alpha}$, which is located in the Ras-like domain [48]. A 14-residue peptide derived from the IGF-II receptor demonstrates remarkable specificity and preferentially activates $G_{i\alpha 2}$ over $G_{i\alpha 1}$ and $G_{i\alpha 3}$ [49]. Although its binding site is unknown, competition studies suggest that the peptide also interacts with the G_α C terminus [50]. These examples show that class-specific peptide modulators of G-protein signaling

targeting the Ras-like domain can be developed, although the molecular design and mechanism of achieving this specificity is much less clear.

Future directions

The increasingly complex model for G-protein signaling drives the need for new tools for probing G-protein structure and function. Selection techniques have already enabled the discovery of novel peptide ligands with unique properties. The targeting of different G-protein states (nucleotide-free [51], GDP, GDP- AlF_4^- [52,53] or GTP) could facilitate the isolation of various G-protein modulators that act as GEFs, GDIs or GAPs. Altering the G-protein nucleotide-bound state might also be achieved by targeting natural G-protein regulators (e.g. RGS proteins [54,55]). The Ras superfamily of small, monomeric G proteins represents another class of related, intracellular drug targets. Ras itself, for example, is a bona fide cancer target [56,57] that has been used in previous phage display selections [58]. Additional structural work on protein-ligand complexes (e.g. through crystallography or NMR) will be necessary for understanding the effects that these molecules have on G-protein signaling.

Of the techniques already used for peptide selections against G-protein-related targets, mRNA display will be an important tool for the rapid isolation of potent ligands. mRNA display has significant advantages over other peptide selection techniques, including access to higher complexity libraries and monovalent display of peptides, resulting in the identification of high affinity sequences [59]. Access to extremely large libraries, comprising $>10^{12}$

molecules, probably led to the successful isolation of high affinity $G_{i\alpha 1}$ -binding peptides that contain a crucial mutation in the peptide constant region [40]. The recent incorporation of unnatural amino acids into mRNA display libraries using sense [60,61] and nonsense [62] suppression schemes provides further diversity to exploit in the construction of molecular libraries. In addition, mRNA display libraries of peptide–drug conjugates [63] might be useful in the selection of fusion molecules consisting of nucleotide analogs or other G-protein-interacting ligands covalently coupled to peptides optimized for selectivity. Other established selection techniques, such as ribosome display [64,65], as well as screening methods with synthetic small molecule libraries [66,67], are promising alternatives for developing G-protein ligands. These techniques are also amenable to the use of unnatural or novel functional groups.

Assaying the effect *in vivo* of potential signal modulators will be crucial towards their use as drugs or drug leads. The use of direct peptide modulators of G-protein signaling has been demonstrated in model systems using peptide expression constructs [68,69] or the application of cell-permeable peptides [7,32]. These examples confirm that if peptide ligands can overcome the plasma membrane barrier and avoid proteolysis, they could indeed be useful as drugs *in vivo*.

New discoveries of GPCR and G-protein activation through non-traditional means continue to add complexity to the classical G-protein signaling model [16]. Several diverse proteins (e.g. arrestins, GPCR kinases and small GTP-binding proteins) have been found that associate with activated GPCRs and could represent additional targets for selection. Inhibition of G proteins could attenuate these alternate modes of signaling and demonstrate whether targeting G proteins for pharmaceutical purposes will be viable. Although further advances are required for the facile conversion of peptide leads into drugs, ligand discovery aimed at G-protein-related targets will continue to provide new tools for studying biological signaling pathways.

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