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Evolution of class-specific peptides targeting a hot spot of the Gas subunit

Ryan J. Austin¹, William W. Ja², and Richard W. Roberts^{1,*}

¹ Departments Chemistry, Biology, and Mork Family Department of Chemical, Engineering/ Materials Science, University of Southern California, Los Angeles, CA, 90089, USA

² Division of Biology, California Institute of Technology, Pasadena, CA 91125 USA

SUMMARY

The four classes of heterotrimeric G protein α subunits act as molecular routers inside cells, gating signals based on a bound guanosine nucleotide (GTP vs. GDP). Ligands that specifically target individual subunits provide new tools to monitor and modulate these networks, but are challenging to design due to the high sequence homology and structural plasticity of the $G\alpha$ binding surface. Here we have created a mRNA display library of peptides based on the short $G\alpha$ -modulating peptide R6A-1 and selected for variants that target a convergent protein-binding surface of $G\alpha$ •GDP. After selection/evolution, the most $G\alpha$ -specific peptide, GSP ($G\alpha$ specific peptide), was used to design a second-generation library, resulting in several new affinity- and selectivity-matured peptides denoted as mGSPs. The two-step evolutionary walk from R6A-1 to mGSP-1 resulted in an 8000-fold inversion in binding specificity, altered 7 out of 9 residues in the starting peptide core, and incorporated both positive and negative design steps. The resulting mGSP-1 peptide shows remarkable selectivity and affinity, exhibiting little or no binding to 9 homologous $G\alpha$ subunits or human H-Ras and even discriminates the $G\alpha$ splice variant $G\alpha$ (I). Selected peptides make specific contacts with the effector-binding region of $G\alpha$, which may explain an interesting bifunctional activity observed in GSP. Overall, our work demonstrates design of simple, linear, highly specific peptides that target a protein-binding surface of $G\alpha$ and argues that mRNA display-based selection/evolution is a powerful route to target protein families with high class- and state-specificity.

Keywords

mRNA display; directed evolution; G protein; convergent binding site; hot spot; protein interactions; sequence space

INTRODUCTION

The heterotrimeric G protein signaling pathway plays a central role in both biology and human therapies. In this pathway, heterotrimeric G protein subunits (denoted $G\alpha$, $G\beta$, and $G\gamma$) route

*To whom correspondence should be addressed. Telephone: (213) 821-4132. E-mail: richrob@usc.edu.

SUPPORTING INFORMATION AVAILABLE

Tabulation of peptide- $G\alpha$ binding kinetics; $G\beta\gamma$ -heterodimer pull-down assays; mGSP peptide nucleotide exchange measurements; and GSP-library doping distributions.

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information from cell surface G protein coupled receptors (GPCRs) to specific intracellular effectors 1. Presently GPCRs comprise the largest family of receptor drug targets in humans, accounting for 25% of marketed pharmaceuticals 2. Routing of a signal from a GPCR to corresponding effectors is dictated by the identity of the G protein α and $\beta\gamma$ heterodimer subunits associated with the GPCR. In humans, there is substantial combinatorial diversity in the routing partners with 16 distinct $G\alpha$ subunit genes categorized into four classes (i/o, q/11, s, and 12/13) corresponding to their effector coupling, 5 β subunits and 12 receptor-specific γ subunits 3. Combinations of these components enable differentiated cells to respond uniquely to extracellular signals 4.

While drugs targeting effector proteins do exist, so far only one therapy, suramin, appears to target the G protein routers themselves, inhibiting dissociation of nucleotide from $G\alpha\bullet\text{GDP}$ 5; 6. One reason for this paucity of drugs arises from the difficulty of targeting large protein-protein binding interfaces with traditional drug-like molecules 7. Towards this end, our laboratory is interested in exploring whether peptides can be designed that recognize such surfaces with protein-like affinity and selectivity. We have investigated this concept in the context of the G protein α subunits, with the goal of determining whether $G\alpha$ class- and state-specific peptide ligands can be generated that target the protein-binding surface of this router?

$G\alpha$ subunits are comprised of 2 domains, a unique helical domain and a GTPase domain containing the guanosine nucleotide-binding pocket of the protein (Fig. 1a). Identity of the nucleotide bound within this pocket (GTP vs. GDP) is coupled to the conformation of the subunit's binding surface via 3 malleable switch elements in the GTPase domain (SI, SII, SIII). This conformational "switching" provides the basis for $G\alpha$ router activity, allowing the subunit to bind different partners in its "on" ($G\alpha\bullet\text{GTP}$) and "off" ($G\alpha\bullet\text{GDP}$) states.

In the "off" state, $G\alpha\bullet\text{GDP}$ binds $G\beta\gamma$, forming a heterotrimer associated with the cytosolic face of the GPCR. Signal activation of the GPCR triggers exchange of GDP for GTP in $G\alpha$, and causes dissociation of $G\beta\gamma$. Both activated $G\alpha\bullet\text{GTP}$ and $G\beta\gamma$ are then capable of regulating effector response for a period of time dependent upon the GTPase rate of $G\alpha$. GTP hydrolysis returns $G\alpha$ to its "off" state, resulting in reformation of the $G\alpha\bullet\text{GDP}$ - $G\beta\gamma$ heterotrimer and termination of signal 8. Beyond $G\beta\gamma$, which binds the "off" state of $G\alpha$ and various effector proteins that bind the "on" state, additional regulatory proteins also interact with the binding surface of $G\alpha$ in a conformation-specific manner. These include G protein regulatory motifs (GPR/GoLoco-motifs), thought to sequester $G\alpha\bullet\text{GDP}$ and regulator of G protein signaling (RGS) proteins that accelerate the rate of $G\alpha\text{GTPase}$ activity 9.

The $G\alpha$ -binding interfaces of these proteins have been shown to overlap at a convergent binding surface defined by the switch II and α -helix 3 elements of the subunit (SII/ α 3)^{10; 11; 12}. Attributes of convergent binding surfaces, or binding "hot spots" were originally characterized by Wells and coworkers 13, and have since been identified in a growing number of proteins 14. Such sites generally exhibit a hydrophobic character with a high degree of sequence conservation across different members of a protein family, as well as structural plasticity. Sequence conservation of the SII/ α 3 convergent binding site across various classes of the $G\alpha$ family is illustrated in figure 1b. This primary sequence identity results in highly conserved $G\alpha$ protein structures with backbone RMS-deviations of ~ 1 Å in the GTPase domains of $G\alpha$ i1, $G\alpha$ q, $G\alpha$ s(s), and $G\alpha$ 12 subunits^{10; 15; 16}.

Design of specific ligands that target convergent binding surfaces presents an interesting problem due to the sequence conservation and dynamic topography of these sites. Combinatorial approaches such as *in vitro* selection provide a powerful solution for targeting these molecularly compliant surfaces 17. Using such methods, our lab and others have previously developed a number of peptides that bind the SII/ α 3 site of $G\alpha$ in a conformation-

and state-specific manner^{12; 18; 19; 20}. In these selection experiments a library of randomized peptide sequences is selected for the ability to bind the G protein target, typically using affinity chromatography. Functional sequences that bind the target are retained, amplified, and eventually come to dominate the pool after iterative rounds of selection. The selection technique most often employed by our lab is mRNA display, wherein each peptide in a library is covalently coupled to its encoding mRNA as an mRNA-peptide fusion (Fig. 2a)²¹. This approach allows libraries to be created entirely *in vitro*, that are strictly monovalent, and have sequence diversities of $>10^{13}$ independent polypeptide sequences, the most of any available method²². Generally, mRNA display selections result in peptides that bind protein or nucleic acid targets with nanomolar to picomolar affinities^{23; 24; 25; 26; 27, 28}.

In the present work we have set out to determine whether it is possible to generate peptide modulators towards the SII/ $\alpha 3$ site that are capable of discriminating the highly conserved subunit classes (i/o, q/11, s, 12/13). Taking as our starting point a short peptide known to bind the SII/ $\alpha 3$ site of Gai1, we have selected peptides targeting the short isoform of Gas (Gas(s)). Notably, our directed evolution strategy required both positive and negative selection steps, the first step improving affinity for Gas while the second step removing binding to other G α subunits. The resulting peptides are remarkably specific, showing no binding to the other three classes of G α and even discriminating between the long and short isoforms of Gas. These peptides indicate that it is possible to modulate the SII/ $\alpha 3$ site of G α with class-specific ligands and illustrate a selection-based evolution of peptide binding specificity at a convergent binding site.

RESULTS AND DISCUSSION

The immediate goal of our present work has been to select specific peptide ligands for the therapeutically relevant Gas(s) protein target. More broadly, we are interested in exploring the nature and evolvability of molecular recognition between nominally structured peptide sequences and protein-binding surfaces. To this end, we have chosen the core peptide R6A-1, which binds the SII/ $\alpha 3$ site of G α with a 250-fold preference for the Gai1 target over Gas(s)^{29; 30}, as a starting point for our present selection. Based on previous work demonstrating that the specificity of R6A-1 could be altered by the addition of flanking residues, we synthesized DNA oligonucleotides coding for an R6A-1 based library of peptides. This R6A-1-library was designed to incorporate a 40–50% doped (50–60% mutation rate per amino acid) R6A-1 peptide core flanked by random amino acid hexamers (Fig. 2b)²⁰. We expressed the library as a pool of mRNA-peptide fusions containing 1.6×10^{13} independent peptide sequences in pool 0 (See Materials and Methods).

Positive Selection of the Gas(s)-binding Peptide GSP

To isolate Gas(s)-binding peptide sequences within the R6A-1-library, a positive selection of the library was performed on neutravidin beads coated with N-terminally biotinylated Gas(s) (Gas(s)-beads). Here the positive selective pressure was binding to Gas(s). mRNA-peptide fusions from the pool that were retained on the Gas(s)-beads were amplified by PCR and again expressed as mRNA-peptide fusions for subsequent rounds of selection. Enrichment of Gas(s)-binding sequences was observable after 4 rounds and plateaued after 8 rounds of the positive selection, as measured by pull-down of [³⁵S]Met labeled peptide fusions on Gas(s)-beads (Fig. 2b). Discrete peptide sequences from pool 8 were cloned and expressed as biotinylated peptide-maltose-binding protein fusions. These peptide-MBP fusions were individually immobilized on neutravidin beads (peptide-beads) and screened for binding to soluble [³⁵S]Met labeled Gai1 and Gas(s) using a previously developed *in vitro* binding assay²⁹.

The majority of pool 8 peptide sequences screened retain affinity for Gai1 equal to or greater than their affinity for Gas(s) in the G α -binding screen. However, one Gas(s)-binding peptide

(GSP), representing 5% of the pool 8 sequences screened, binds preferentially to Gas(s). In evaluating the selected round 8 sequences, we find only a fraction of the R6A-1 core residues to be conserved. A hydrophobic leucine or valine residue is conserved at position 3 of the selected clones, along with an EFL-motif at positions 7–9 of the core.

Many of the enriched peptide sequences contain 3–4 non-conservative mutations in their core. The selected GSP sequence, for instance, contains 5 mutations within the 9 residue core, 3 of which are of low likelihood (D1K [0.7%]; Y4T [1.0%]; W5V[1.0%]) based on the theoretical complexity of our pool. The mutagenic probability of finding the GSP core in the R6A-1-library is 1.9×10^{-10} , meaning that in our 16 trillion molecule pool 0, roughly 800 full-length copies of the GSP core were present. This copy number is too low to effectively sample the flanking sequences of the R6A-1-library, which may explain why a separate selection of the R6A-1-library in the presence of Gai1 competitor was unsuccessful (data not shown).

Maturation Selection of Gas(s)-specific mGSP Peptides

To explore GSP variants with increased Gas(s)-binding specificity, a GSP-library based on a 50% doped GSP sequence was constructed and subjected to a maturation selection (Fig. 2c). This maturation selection was designed to incorporate negative selective pressures against peptides that bind to Gai1. GSP variants were selected for retention on Gas(s)-beads in the presence of a molar excess of soluble Gai1 competitor, with the concentration of Gai1 competitor and other negative selective pressures increasing over the course of the maturation selection (See Materials and Methods). After 6 rounds of selection, the majority of GSP variants sequenced bound exclusively to Gas(s) in the G α -binding screen (Fig. 2c).

These matured GSP peptide variants (mGSPs) retain the EFL-motif, along with the GSP core residues K1, T4, and V5. As in the positive selection, we again observe significant mutations in the core sequence. In particular, peptides that display the greatest Gas(s)-selectivity in the G α -binding screen have an arginine residue at position 6 and either a methionine residue at position 3 (in mGSP-1) or a leucine residue at position 2 (in mGSP-2) of the core (Fig. 2c). The average number of GSP-mutations in these mGSP variants is 5.7 ± 1.3 residues; a mutational distance that is covered by only 1–4 copies of each variant in the GSP-library pool 0. This significant mutational distance suggests that additional selections could generate peptides with even greater Gas(s)-binding specificities. Representative peptide variants mGSP-1 (mut: L3M) and mGSP-2 (mut: R2L) along with the GSP peptide were synthesized for further analysis.

8000-fold Specificity Change of mGSP-1 and mGSP-2 Compared to R6A-1

We have used surface plasmon resonance (SPR) to measure dissociation constant (K_D) values for a matrix of peptide-G α complexes between GSP, mGSP-1, and mGSP-2 peptides and G α i1 and G α s(s) proteins (Fig. 3, Table 1). The K_D matrix charts the course of our two-selection step walk. 1) In the positive selection we evolved peptides with increased Gas(s)-affinity, walking through sequence space from the Gai1-specific R6A-1 peptide to GSP, which exhibits affinity for both Gai1 and Gas(s) proteins. 2) In the maturation selection we applied a negative Gai1 selective pressure to the GSP-library, evolving GSP variants that retained affinity for Gas(s), but no longer bound Gai1. Gas(s)-binding specificities are quantified in Table 1 as the relative free energy stability of the peptide-Gas(s) complex vs. the peptide-Gai1 complex (Gas(s)- $\Delta\Delta G$). In sum, the mGSP-1 and mGSP-2 peptides exhibit a $5.4 \text{ kcal mol}^{-1}$ increase in Gas(s)-binding specificity over R6A-1, equivalent to a greater than 8,000 fold inversion in Gai1/Gas(s) target discrimination.

Gα-class Recognition by GSP, mGSP-1 and mGSP-2 Peptides

We were expressly interested in evaluating the binding specificity of GSP and mGSP variants across the spectrum of Gα subunits. To address this question, a palette of 11 different [³⁵S]-Met labeled Gα subunits and isoforms (Gα: i1, i2, i3, oA, q, 11, 15, s(s), s(l), Olf, and 12) representing the four classes of Gα (i/o, q/11, s, 12/13) as well as the small GTPase H-Ras were expressed *in vitro* and assayed for pull-down on peptide-beads. The result was a Gα-class specificity profile for each peptide tested (Fig. 4). Here 3 experimental controls are worth noting. Firstly, pull-down reactions were performed in the presence of reticulocyte lysate, which acts as a general binding competitor, mimicking the cytosolic environment of the cell. Secondly, the presence of aluminum fluoride (AlF₄) in control pull-down experiments abrogated all binding between Gα subunits and the GSP, mGSP-1 and mGSP-2 peptides. This finding is consistent with state-specific binding of peptides to the GDP-conformation of Gα as previously reported for R6A-1¹⁸. Finally, the ratio of Gαi1 to Gαs(s) binding in the specificity profile of GSP was consistent with the SPR data for the peptide (Fig. 4a).

The specificity profiles of GSP, mGSP-1 and mGSP-2 peptides, illustrate novel recognition properties of these peptides that have resulted from the two-step directed evolution strategy of our selection. GSP, isolated from the initial positive selection, has a surprisingly focused binding profile, recognizing both i/o and s subunit classes, with only nominal affinity towards q/11 and 12/13 (Fig. 4a). This specificity is further pronounced in the mGSP-1 and mGSP-2 variants, isolated from the subsequent maturation selection step, which bind only the s class of Gα subunits. Our results demonstrate that it is possible to evolve new Gα-binding specificity in a peptide sequence without increasing the binding promiscuity of the peptide for other Gα-classes. Indeed, even class-subspecificity is apparent in selected peptides. GSP, mGSP-1 and mGSP-2 all show reduced binding to the long isoform of Gαs (Gαs(l)) and minimal binding to the GαOlf subunit, which shares 77% amino acid sequence identity with Gαs(s).

Directed evolution experiments that evolve binding promiscuity or non-specificity in an initial step before honing specificity in a subsequent step, are often employed in cases where investigators cannot effectively sample the sequence space of large protein libraries³¹. However, the number of possible amino acid combinations in a 21 residue peptide ($20^{21} = 2.1 \times 10^{27}$) is much smaller than for a larger 100 residue protein ($20^{100} = 1.3 \times 10^{130}$), allowing for a more comprehensive search of amino-acid sequence space in peptide directed evolution experiments. In our case, by taking the R6A-1 peptide as our starting point and constructing doped peptide libraries, we have targeted our selection to the SII/α3 site of Gα facilitating an even denser interrogation of peptide sequence space than afforded by a random 'naïve' peptide selection.

Sequence conservation between R6A-1 and the GSP and mGSP variants indicates that the selected peptides bind in a similar fashion to the SII/α3 site of Gα. This binding site redundancy is supported by our finding that R6A-1 directly competes with GSP for binding to Gαi1 (data not shown). Additionally, the GSP peptide, like R6A-1, inhibits formation of the Gαβγ heterotrimer reiterating our previous finding that peptides targeting the SII/α3 site can disrupt large protein-protein interactions²⁹. [³⁵S]Gβγ pull-down on both Gαi1 and Gαs(s) beads is inhibited by the presence of GSP at IC₅₀ values of 550 nM and 80 nM, respectively (Supplemental Fig. A).

GSP and mGSP-1 Discriminate the Effector-Binding Region of Gas(s)

We designed our selection to target Gαs(s) at the SII/α3 site, which is a convergent protein interaction surface for a number of natural Gα-binding partners. Presently, four models of Gα-binding specificity have been developed from structural characterization of Gα complexes that involve critical contacts within SII/α3 site. These models are delineated by the

classification of $G\alpha$ -binding partner: 1) RGS proteins 32; 33, 2) the GoLoco/GPR motif 34, 3) effector proteins 10; 11, and 4) $G\beta\gamma$ heterodimers 35. A general theme to emerge from the models is that molecular recognition of $G\alpha$ is bipartite, involving conserved contacts along the SII/ $\alpha 3$ -binding surface that are complemented by specific contacts outside of the SII/ $\alpha 3$ site. RGS proteins provide a notable exception to this theme, making highly specific contacts at a nonconserved residue (Gai1-Ser206) within switch-II. To compare the binding specificity of GSP and mGSP variants with structurally characterized models, we have expressed a series of previously studied Gai1/Gas(s) reciprocal mutants 36; 37 and chimeras 38 and tested them for binding to peptide-beads. $G\alpha$ -binding footprints for RGS4, GoLoco, and the effector protein adenylyl cyclase (AC) are shown in figure 5a. The $G\beta\gamma$ binding model, however, is not considered in our analysis as relevant mutants and chimeras have not been characterized.

We considered the 3 remaining models in turn: 1) In the RGS binding model, polar residues from RGS proteins discriminate the primary structure of $G\alpha$ switches at 5 positions 32; 33. Reciprocal substitution at one of these positions, Ser206 in Gai1, with the corresponding residue, Asp229 from Gas(s), abrogates binding of Gai/o-specific RGS proteins 33; 36; 37. Similarly, the Asp229 position of Gas(s) has been implicated in the Gas-specific binding of RGS-PX1 39. Binding of GSP and mGSP-1 peptides is not, however, affected by G protein reciprocal substitutions at this position (Fig. 5b), indicating that the mechanism of peptide specificity is distinct from RGS binding. 2) In the GoLoco/GPR binding model the GoLoco peptide interacts with both GTPase and helical domains of $G\alpha$, docking its N-terminus within the SII/ $\alpha 3$ site 34. The C-terminus of GoLoco/GPR makes discriminate contacts with the helical domain of $G\alpha$ in the crystal structure and functional studies using a series of domain chimeras have demonstrated that these contacts are isoform-specific among Gai subunits 40. However, the Gai1/Gas(s) chimera C3, containing a Gas(s) helical domain and Gai1 GTPase domain, shows no pull-down on GSP or mGSP-1, indicating that our peptides are not recognizing the Gas(s) helical domain. 3) In the effector binding model, non-polar effector-residues dock within the hydrophobic pocket formed between the N-termini of SII($\alpha 2$) and $\alpha 3$ helices (see asterisk in Fig. 1b). Specificity determining contacts are made with the C-termini of these helices and the $\alpha 2$ - $\beta 4$ and $\alpha 3$ - $\beta 5$ effector loops of $G\alpha$ 10; 11. The Gai1/Gas(s) chimera C4, containing the $\alpha 3$ and $\alpha 3$ - $\beta 5$ and $\alpha 4$ - $\beta 6$ effector-binding loops of Gas(s) fully recapitulates Gas(s) pulldown on GSP and recovers mGSP-1 binding. This result suggests that the GSP and mGSP-1 peptides discriminate $G\alpha$ targets in an effector-like binding mode via contacts with $\alpha 3$ and/or the $\alpha 3$ - $\beta 5$ effector loop.

GSP and mGSP Variants Inhibit Nucleotide Exchange in Gas(s); GSP Accelerates Nucleotide Exchange in Gai1

Finally, we wished to examine the effect of our selected peptides on nucleotide exchange. To do this, we have measured the GDP exchange rate in the presence and absence of GSP, mGSP-1, and mGSP-2 for both Gai1 and Gas(s) using two different assays (Fig. 6). The first assay measured GDP dissociation by monitoring [^{35}S]GTP γ S binding to $G\alpha$ under conditions where label binding is limited by GDP release. The second assay, employing [$\gamma^{32}\text{P}$]GTP, measured the steady-state GTP hydrolysis rate of $G\alpha$ under multiple turnover conditions where the rate of GTP hydrolysis is also limited by GDP release.

Both assays demonstrate that GSP, mGSP-1 and mGSP-2 inhibit GDP exchange on Gas(s) (Fig. 6a,c). Incubating increasing amounts of GSP with Gas(s) inhibits [^{35}S]GTP γ S binding with an IC_{50} of 157 nM, consistent with the 100 nM GSP-Gas(s) binding constant determined by SPR under similar, but not identical conditions (Table 1). Under saturating GSP concentrations (10 μM) the peptide slows GDP exchange by 3-fold while similar kinetics are observed for mGSP-1 and mGSP-2 (Supplemental Fig. B). Thus GSP, mGSP-1, and mGSP-2 have guanine dissociation inhibitor-like (GDI) activity against Gas(s).

To our surprise, the peptide GSP accelerates the rate of nucleotide exchange in G*α*i1. Under saturating GSP concentrations (10 μ M) the association rate of [³⁵S]GTP γ S on G*α*i1 is markedly increased compared to the protein alone (Fig. 6b). [³⁵S]GTP γ S binding measurements show GSP acts as a guanine nucleotide exchange factor (GEF) for G*α*i1, accelerating the release of GDP 4-fold. The effective GSP concentration required for 50% maximal GEF activity is 290 nM, consistent with the dissociation constant of GSP-G*α*i1 (K_D = 280 nM). Similar exchange measurements could not be made for mGSP-1 or mGSP-2, possibly due to the weak affinity of the peptides towards G*α*i1. The results demonstrate that GSP is bifunctional (has two activities)—with GDI-like activity for G*α*s(s) and GEF activity for G*α*i1.

Interestingly, GSP is not alone in its bifunctional activity. The bee venom peptide melittin 41 as well as the non-specific peptide KB-752, previously isolated by phage-display⁴², have been shown to have similar activity toward G*α*i1 and G*α*s(s). While melittin likely has a distinct functional mechanism, sequence conservation between GSP and KB-752 suggest that the two peptides bind G*α*i1/G*α*s(s) in a similar fashion. KB-752 shares a consensus EFL-motif ('DFL') with selected peptides (Fig. 7a) and like GSP, accelerates nucleotide exchange in G*α*i1 while inhibiting exchange in G*α*s(s), albeit at higher effective concentrations of 4–5 μ M⁴².

Johnston and coworkers have solved a crystal structure of the KB-752-G*α*i1 complex, revealing peptide binding within the SII/ α 3 site of G*α*i1 (Fig. 7b)¹⁹. The F8 and L9 residues of KB-752 bury themselves within an invariant hydrophobic binding pocket composed of conserved residues R208, W211, I212, F215, L249, and I253 in G*α*i1. The EFL-motifs of GSP and the mGSP variants presumably dock in a similar manner within the SII/ α 3 site. In the case of mGSP-1, the peptide C-terminus (residues 10–15) which is conserved among mGSP variants (Fig. 1c), is positioned to make discriminate contacts near the α 3/ β 5 loop of G*α*s(s) in a cooperative fashion with α 3-binding mGSP-1 core residues.

Interestingly, the effector-binding model of peptide recognition presents a mechanistic rationale for the bifunctional activity of the GSP peptide. Johnston *et al.* have proposed a mechanism for the G*α*i1 GEF activity of KB-752 wherein peptide-binding contacts peel back the switch-II lip of G*α*i1 facilitating nucleotide escape (Fig. 7b). This proposal is consistent with the G*β* γ lever model of GPCR GEF activity⁴³, and provided GSP and KB-752 bind the SII-lip in a similar manner, accounts for the G*α*i1 GEF activity of GSP. However, chimera discrimination experiments suggest that GSP binds the G*α*s(s) target at a shifted orientation within the SII/ α 3 site, accommodating specific contacts along the α 3 helix and α 3/ β 5 loop. Such a shift would disrupt contacts between GSP and the SII-lip, blocking nucleotide release, which is consistent with the observed inhibition of GDP exchange in the GSP-G*α*s(s) complex.

CONCLUSIONS

We have reported the directed evolution of subclass-specific peptides targeting the highly conserved SII/ α 3 convergent binding surface of G*α*. A remarkable aspect of this work is our finding that the SII/ α 3 site, which is composed of nearly identical aminoacids in the G*α*i1 and G*α*s(s) subunits, elicits an 8000-fold range of discrimination by related peptide ligands. This significant range of specificity argues that the surface conformation of the SII/ α 3 site, rather than its amino-acid identity, mediates ligand recognition. Such conformation-specific class discrimination at the SII/ α 3 site has been noted in the effector-binding specificity of G*α*s(s)^{10; 11}, and may also contribute to the specific binding of AC isoforms I-C₁, V-C₁, and VI-C₁ within the SII/ α 3 site of G*α*i1⁴⁴. The work with G*α* suggests that it may be possible to discriminate binding hot spots on other highly conserved proteins in a similar conformation-specific manner, even in the case where crystallographic data indicates structural homology.

It was unclear prior to selection whether specific, high-affinity targeting of $G\alpha$ subunit classes could be achieved using short peptide ligands. Our results demonstrate that linear peptides are well suited to the discrimination of $G\alpha$, exhibiting 10- to 100-fold selectivities with affinity levels between 60 and 300 nanomolar. This finding should open the door to a number of applications. Fluorescent and luminescent biosensors have proven to be valuable molecules for the real-time tracking and visualization of G protein signal transduction^{45; 46; 47} and selected natural peptides could be employed in the development of these tools. Additionally, peptides with 10- to 100-fold binding specificities are viable capture reagents for chip-based proteomic analyses⁴⁸.

Our selected peptides are also capable of modulating $G\alpha$ protein function. The mGSP peptides, for instance, specifically target and inhibit $G\alpha(s)$ at the convergent SII/ $\alpha 3$ binding site of the subunit. It may, therefore, be possible to generate therapeutics targeted to the SII/ $\alpha 3$ site for the treatment of various diseases caused by $G\alpha(s)$ activation^{49; 50}. Constitutive activation of $G\alpha(s)$ by cholera toxin causes the pathophysiological symptoms of the disease. Separately, hyperactivating mutations of $G\alpha(s)$ can result in McCune Albright Syndrome (MAS) and are oncogenic in various endocrine cancers^{4; 50}. $G\alpha(s)$ oncogenes have been shown to increase tumorigenicity and metastasis^{51; 52}, and recent identification of $G\alpha(s)$ -hyperactivating mutations in kidney cancer indicate that the subunit could be a therapeutic target in developed tumors⁵³.

MATERIALS AND METHODS

Materials

The *Escherichia coli* strains BL21, BL21-(DE3), and BL21-gold were from Novagen (Madison, WI). The G protein expression vector, NpT7-5-H6-TEV-G α 1, was generously provided by Prof. Roger K. Sunahara (University of Michigan). The *in vivo* biotinylation vector, pDW363, was kindly supplied by Dr. David S. Waugh (National Cancer Institute., Frederick MD). Human cDNA clones encoding G proteins were obtained from the UMR cDNA Resource Center (www.cdna.org) in the pcDNA3.1+ vector (*Invitrogen*). G α 1-rat, $G\alpha(s)$ -bovine short form chimera constructs were generously provided by Prof. N. Artemyev (University of Iowa). The $G\alpha$ subunits used for the specificity profiles were i1, i2, i3, oA, q, 11, 15, s(s, short isoform), s(l, long isoform), Olf, and 12. $G\alpha(s)$ residues are referred to in the text with the $G\alpha(s)$ numbering convention. DNA oligonucleotides were synthesized by *Integrated DNA Technologies, Inc.* (Coralville, IA). Modified and doped oligonucleotides including pF30P were synthesized at *Keck Oligonucleotide Synthesis* (New Haven, CT). DNA sequencing was performed by *Laragen* (Los Angeles, CA). L-[³⁵S]-methionine (1175 Ci/mmol), [³⁵S]GTP γ S (1050 Ci/mmol), and [³²P]GTP (6000 Ci/mmol) were purchased from *MP Biomedicals* (Irvine, CA). Restriction enzymes and T4 DNA ligase were from *New England Biolabs, Inc.* (Beverly, MA). GSP, mGSP-1, mGSP-2, and R6A-1 peptides were purchased from *BioSynthesis Inc.* (Lewisville, TX).

G α -subunit Cloning and Expression

Cloning pDW363-H6- $G\alpha(s)$: pDW363- $G\alpha(s)$ was modified with an amino-terminal hexahistidine tag by QuikChange (*Stratagene*) PCR using primers pDW363-H6-Top (5' CTT TAA GAA GGA GAT ATA CAT ATG CAC CAC CAT CAC CAT CAC GCT GGA GGC CTG AAC GAT ATT TTC 3') and pDW363-H6-Bottom (5' GAA AAT ATC GTT CAG GCC TCC AGC GTG ATG GTG ATG GTG GTG CAT ATG TAT ATC TCC TTC TTA AAG 3'). A two-stage PCR protocol was adopted to mitigate the effects of primer-dimer formation (150 ng template; 50 °C annealing temperature with a 12 minute extension time at 68 °C; 3 rounds of amplification with primers separated, 19 rounds with pooled reaction)⁵⁴. The pDW363-H6-

Gas(s) sequence encodes H6-Nb-Gas(s); the Gas(s) protein with an N-terminal H6 hexahistidine tag followed by a peptide tag that is biotinylated *in vivo*.

Cloning reciprocal mutants: pcDNA3.1+ Gai1 was mutated at residue 206 (Ser206Asp) by QuikChange (*Stratagene*) PCR using primers S206D-Top (5' AAT GTT TGA TGT GGG AGG TCA GAG AGA TGA GCG GAA GAA G 3') and S206D-Bottom (5' CTT CTT CCG CTC ATC TCT CTG ACC TCC CAC ATC AAA CAT T 3'). pcDNA3.1+ Gas(s) was mutated at residue 229 (Asp229Ser) by QuikChange (*Stratagene*) PCR using primers D229S-Top (5' GGG TGG CCA GCG CTC TGA ACG CCG CAA G 3') and D229S-Bottom (5' CTT GCG GCG TTC AGA GCG CTG GCC ACC C 3').

Expression of Gas(s): Gas(s) was recombinantly expressed with some modifications to previously published protocols⁵⁵. A 100 ml Enriched Media culture [2% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.2% (v/v) glycerol, and 50 mM H₂PO₄ at pH 7.2, supplemented with 50 µg/ml ampicillin and 50 µM D-biotin] of *E. Coli* BL21(DE3) cells harboring pDW363-H6-Gas(s) was induced with 0.3 mM IPTG at OD₆₀₀ = 0.4, grown at 30 °C for 9 hrs, and pelleted by centrifugation. Pellets were rinsed with ddH₂O, snap-frozen in dry ice/ethanol, and stored at -80 °C overnight. Cell pellets were resuspended in 15 ml T₅₀β₂₀P_{0.1} buffer [50 mM Tris-Cl at pH 8.0, 20 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride], lysed by Emulsiflex at 5000 psi for 10 min, and centrifuged. Cleared lysate supernatant was applied to a 0.3 ml bed volume Ni-NTA column (*Qiagen*), pre-equilibrated with T₅₀β₂₀P_{0.1}/(100 mM NaCl). The column was washed with 3 × 2 mL of T₅₀β₂₀P_{0.1}/(500 mM NaCl, 10 mM imidazole). Fractions were eluted into T₅₀β₂₀P_{0.1}/(50 mM imidazole, 10% glycerol (v/v)), concentrated and exchanged into HGD buffer [50 mM HEPES at pH 7.5, 10% glycerol (v/v), 1 mM DTT] using a Centriprep YM-10 concentrator, and stored at -80 °C. A 100 mL culture yielded 0.1 mg of N-terminally biotinylated Gas(s) (Nb-Gas(s)).

Expression of G(i1: Recombinant rat H6-TEV-G(i1 (N-terminal hexahistidine tag followed by a TEV protease cut site) was expressed as previously described¹⁸. G(i1 was also expressed with an N-terminal peptide tag that is biotinylated *in vivo* (Nb-G(i1). A 120 ml LB culture/[50 µg/ml ampicillin, 50 µM D-biotin] of *E. coli* BL21 cells harboring pDW363-G(i1 was induced with 1 mM IPTG at OD₆₀₀ = 0.6, grown at 30 °C for 6 hrs, and pelleted by centrifugation. Cell pellets were rinsed with ddH₂O, snap-frozen in dry ice/ethanol, and stored at -80 °C overnight. A 30 mL cell pellet was resuspended in 3 mL BPER cell lysis reagent (*Pierce*) at room temperature. The lysate was cleared by centrifugation and incubated with 0.4 ml neutravidin-agarose at 4 °C for 1 hr. The beads were washed 5× with Wash Buffer [1× PBS, 3 µM GDP, 2 mM DTT, 0.5% Tween-20 (v/v)] to generate Gai1-beads.

mRNA Display: Template Construction and Selection

Construction of the core-motif R6A-1-library has been described previously²⁰. The GSP-library was designed in a similar fashion to incorporate roughly 50% degeneracy per amino-acid residue⁵⁶. The antisense DNA oligo 115.2 (5' AGC AGA CAG ACT AGT GTA ACC GCC 624 621 621 622 612 623 612 211 543 531 613 624 612 632 544 244 632 243 621 514 623 CAT TGT AAT TGT AAA TAG TAA TTG TCC C 3'; numbers denote dNTP mixtures; 1: 70% A, 10% G, 10% C, 10% T; 2: 70% G, 10% A, 10% C, 10% T; 3: 70% C, 10% A, 10% G, 10% T; 4: 70% T, 10% A, 10% G, 10% C; 5: 90% C, 10% G; 6: 50% G, 50% C) was synthesized by Keck Oligonucleotide Synthesis. This oligo was PCR amplified with the forward primer 47T7FP (5' GGA TTC TAA TAC GAC TCA CTA TAG GGA CAA TTA CTA TTT ACA ATT AC 3') and reverse primer 22.9 (5' AGC AGA CAG ACT AGT GTA ACC G 3') to generate the doped GSP-library. The purified dsDNA construct contained a T7 promoter, an untranslated region, and an ORF containing a 3' constant sequence encoding the peptide QLRNSCA. Sequencing of pool 0 demonstrated ~50% degeneracy of the library per doped

amino-acid position. The expected amino acid distribution of the GSP-library is included in the supplemental material.

All steps in the mRNA display selection cycle, with the exception of the $G\alpha$ binding step, were performed as previously described¹⁸. The $G\alpha$ (s)-beads were prepared immediately prior to use in selections. Nb- $G\alpha$ (s) (15–30 μ g) was rotated with 30 μ l bed volume of neutravidin-agarose (*Pierce*) in 0.5 mL wash buffer [1 $\times$ PBS, 3 μ M GDP, 2 mM DTT, 0.5% Tween-20 (v/v)] at 4 °C for 1 h. Beads were washed with wash buffer and resuspended in Binding Buffer [25 mM HEPES-KOH at pH 7.5, 150 mM NaCl, 1 mM β -mercaptoethanol, 10 μ M GDP, 1 mM EDTA, 5 mM MgCl₂, 0.05% (v/v) Tween-20] supplemented with 1 mM D-biotin [0.1 mM] and rotated for an additional 10 minutes to block biotin-binding sites. Beads were then washed thoroughly with Selection Buffer [25 mM HEPES-KOH at pH 7.5, 150 mM NaCl, 1 mM β -mercaptoethanol, 10 μ M GDP, 1 mM EDTA, 5 mM MgCl₂, 0.05% (v/v) Tween-20, 0.05% (w/v) BSA (electrophoresis-grade, *Sigma*), 1 μ g/mL yeast tRNA (*Roche Diagnostics Corp.*, Indianapolis, IN)] and rotated with RT-fusions in 1 mL Selection Buffer, at 4 °C for 1 h. The matrix was then washed with 4 $\times$ 0.5 mL of Selection Buffer followed by 2 $\times$ 0.5 mL of Binding Buffer. Bound fusions were eluted with 2 $\times$ 0.1 mL of 0.15% (w/v) SDS using a 0.45- μ m centrifuge tube filter. SDS was removed using SDS-OUT (*Pierce*) following manufacturers specifications, and cDNA was ethanol-precipitated with linear acrylamide (*Ambion*). PCR amplification of the cDNA with primers 47T7FP and 22.9 generated the dsDNA template for the next round of selection. DNA templates could also be cloned into pDW363C for sequencing.

Additional negative $G\alpha$ i1 selective pressures were applied during the maturation selection. RT-fusions were sieved through a column of $G\alpha$ i1-beads (0.3 mL bed volume equilibrated in Selection Buffer) prior to incubation with $G\alpha$ (s)-beads. Incubation of the pre-sieved RT-fusion and $G\alpha$ (s)-beads was performed in Selection Buffer supplemented with soluble $G\alpha$ i1 competitor at a concentration ranging from 10 μ g/mL in round 1 (R1) of selection, to 20 μ g/mL (R2), to 40 μ g/mL (R3-R6). Washes of the $G\alpha$ (s)-beads after incubation with pre-sieved RT-fusions were conducted in Selection Buffer supplemented with 20 μ g/mL soluble $G\alpha$ i1 competitor (R3-R6). Binding assays between [³⁵S]Met-peptide fusions and $G\alpha$ i1-beads or $G\alpha$ (s)-beads were performed as previously described¹⁸.

Cloning and Expression of Selected Peptides

Selected pools were cloned into the biotinylation vector pDW363C²⁹ for sequencing and expression. Pool dsDNA was PCR-amplified using universal primer 29.4 (5' TGA AGT CTG GAG TAT TTA CAA TTA CAA TG 3') and reverse primer 22.9 (5' AGC AGA CAG ACT AGT GTA ACC G 3'), digested with *BpmI/SpeI*, and ligated to pDW363C (digested with *BseRI/SpeI*). Ligations were digested with *KpnI* to reduce vector-only contaminant, transformed into the BL21 gold (*Stratagene*) *E. coli* cell line, and plated on LB-Amp. Individual colonies were picked for sequencing.

Selected peptides were expressed as maltose-binding protein (MBP) fusions using the *in vivo* biotinylation system pDW363C (Nb-(Factor Xa site)-peptide-MBP). Expression and cell lysate preparation of pDW363C clones, as well as MBP and R6A-MBP controls, was performed as described above. Nb-peptide-MBP was purified directly onto neutravidin-agarose to generate peptide-beads for the $G\alpha$ binding screen and $G\alpha$ specificity profiles. For applications requiring removal of the biotin tag (Nb) from the peptide, Factor Xa was used following previously published protocols¹⁸. Briefly, cleared lysate was purified on streptavidin sepharose (High Performance, *Amersham*) and washed 5 $\times$ with pDW buffer [50 mM HEPES-KOH at pH 7.5, 200 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100] followed by a 2 $\times$ wash with Xa buffer [50 mM HEPES-KOH at pH 7.5, 150 mM NaCl, and 1 mM CaCl₂]. Protein was incubated overnight with Factor Xa (20 units, *Amersham*) in Xa buffer at room temperature

and eluted with pDW buffer. Factor Xa was removed with p-aminobenzamidase agarose (*Sigma*). Purified proteins were desalted and concentrated in a Centrprep YM-30 into 1× PBS.

Library Complexity and Selected Peptide Sequence Probabilities

The complexity of R6A-1- and GSP-libraries was determined to be 1.6×10^{13} and 2.5×10^{12} peptide sequences in their respective pool 0's. This value was calculated by dividing the radioactive counts (cpm) of dT-purified mRNA-peptide fusions by the cpm/molecule of the incorporated [³⁵S]Methionine and corrected for the incidence of methionine in each peptide (R6A-1-library: 1.48 Met/peptide; GSP-library: 1.66 Met/peptide)⁵⁶. 60 discrete peptide-MBP clones were screened from both round 8 of the positive selection and round 6 of the maturation selection.

Analysis of the GSP sequence: The likelihood of finding the GSP core sequence in our R6A-1-library was calculated from the amino-acid doping probability at each residue in the peptide. The GSP core sequence (K1[0.7%], R2[7.9%], L3[52.5%], T4[1.0%], V5[1.0%], W6[44.1%], E7[44.1%], F8[6.3%], L9[52.5%]) has an incidence of 1.9×10^{-10} . Correcting for the occurrence of stop codons, there should be 773 full-length copies of the GSP core sequence in the R6A-1-library pool 0.

Analysis of mGSP sequences: Sequence analysis was performed on a sample of 11 mGSP sequences shown to have the greatest Gas(s) binding specificity in the Gα-binding screen. These mGSP sequences have an average mutational distance of 5.7 ± 1.3 mutated residues from GSP. Using equations developed by Rob Knight to evaluate doped library sampling probabilities, we calculate that in the GSP-library pool 0, which has a complexity of 2.5 trillion peptides, a mutational distance of 5.7 residues is covered at a copy number of 1.0 copies.⁵⁷ This value, is however, based on the inaccurate assumption that each conserved amino-acid residue is capable of being mutated to each of the 19 other amino-acids at the same frequency. Based on the doping scheme of the GSP-1-library, the probability of generating a discrete mutation at a doped residue ranges from 0.5–7%⁵⁶. If we subtract out the 10 most unlikely mutations, comprising a 5% probability, the copy number increases to 4.4 copies.

Gα Binding Screen and Specificity Profile Assay

[³⁵S]Met-labeled G protein subunits were translated discretely in coupled transcription/translation reactions using the TNT reticulocyte lysate system (T7 promoter, *Promega*, Madison, WI). Typically 0.3–1.0 μg of plasmid DNA and 25 μCi of L-[³⁵S]-methionine were used per 25 μl reaction. Reactions were desalted and exchanged using MicroSpin G-25 columns (*GE Healthcare*) into buffer [50 mM HEPES-KOH at pH 7.5, 6 mM MgCl₂, 75 mM sucrose, 1 mM EDTA, 1 μM GDP, and 0.5% (v/v) Tween-20] and reaction yields were quantitated by trichloroacetic acid precipitation of a 2-μl aliquot of each reaction.

The Gα interaction assay was performed as described previously²⁹. Individual binding reactions were assembled with equivalent aliquots of desalted Gα subunits in 0.5 mL Binding buffer/(0.5% (w/v) BSA) containing 10 μl of peptide-beads. After rotating at 4 °C for 1 h, beads were washed 3× with 0.5 mL binding buffer using a 0.45-μm spin filter tube, and transferred to a vial for scintillation counting. Assays with aluminum fluoride were performed similarly, except that binding buffer was supplemented with 10 mM NaF and 25 μM AlCl₃.

Binding Analysis by Surface Plasmon Resonance

Kinetic measurements were conducted at 25 °C on a Biacore 2000 instrument (*Biacore, Inc.*, Piscataway, NJ) as described previously¹⁸. Briefly, Nb-Gas(s) and Nb-Gai1 were immobilized on research-grade SA (streptavidin) sensor chips at a surface density of ~ 1000 response units. A concentration series (0, 10, 30, 90, 270, 810, and 2430 nM) of each peptide

analyte in modified HBS-EP running buffer [10 mM HEPES at pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween-20, 2 mM MgCl₂, 30 μM GDP, 0.05% (w/v) BSA and 0–0.5% DMSO] was injected across the chip for 2 min at 100 μl/min, followed by a 6 min dissociation period. A negative control surface without immobilized Gα was used to monitor background binding of the analyte. K_D values for peptides were calculated from rates determined by CLAMP. Additionally, K_D values were determined from equilibrium binding responses using Scrubber. These equilibrium fits produced similar results, with K_D values within 50% of those shown.

Gα nucleotide Cycle Assays

GTPγS exchange assays were performed using a nitrocellulose filter binding method⁵⁸ at either 20 °C (Gas(s)) or 30 °C (Gai1). Briefly, Gα was diluted into HEDT buffer [50 mM HEPES-NaOH at pH 7.6, 1 mM EDTA, 1 mM DTT, 0.01% Tween-20] to a final concentration of 250 nM (1 pmol/10 μl assay) on ice. The reaction was started by adding 4 volumes (40 μl/assay) of reaction buffer [50 mM HEPES-NaOH at pH 7.6, 1 mM EDTA, 1 mM DTT, 12.5 mM MgSO₄, 0.2–1.2 μM [³⁵S]GTPγS (50–200 cpm/fmol), 0.01% Tween-20] with or without test peptide. The reactions were stopped by withdrawing duplicate aliquots (50 μl/assay), diluting these into 10 mL ice-cold Stop Buffer [Tris-HCl at pH 8.0, 100 mM NaCl, 25 mM MgCl₂, 100 μM GTP], and immediately filtering over HA-85 nitrocellulose membranes (Whatman). Equilibrium experiments were conducted similarly, with the exception that Gα subunits were pre-incubated for 10 min with or without peptide on ice prior to initiating reactions. Data from kinetic experiments were processed by non-linear, least squares curve fitting to a pseudo first-order association rate. Concentration response curves were fit to a 3 parameter logistic equation. All measurements were performed multiple times.

Steady-State [³²P]GTP assays were undertaken using a charcoal precipitation based method⁵⁹ at either 20 °C (Gas(s)) or 30 °C (Gai1). Gα proteins were diluted on ice to 200 nM (2× desired concentration) in assay buffer [20 mM NaHEPES pH 8.0; 100mM NaCl; 1 mM EDTA; 2 mM MgCl₂; 1 mM DTT; 0.05% Tween-20]. GTPase reactions were initiated by addition of an equal volume of assay buffer containing a 2× concentration of [³²P]GTP 0.3–1 μM (1000–3000 cpm/fmol); ± peptide. Duplicate aliquots (50 μl) were removed at timed intervals and quenched with 900 μL of ice-cold 5% (w/v) activated charcoal in 50 mM NaH₂PO₄. Quenched reactions were centrifuged for 10 min at 8000 × g and duplicate 100 μl aliquots of the resultant supernatant were subjected to scintillation counting to quantify released [³²Pi].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Abbreviations: AC, Adenylyl Cyclase; Fmoc, fluorenylmethoxycarbonyl; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; GoLoco, Gα (i/o)-Loco interaction; GPCR, G protein-coupled receptor; GPR, G protein regulatory; GSP, Gas(s)-binding peptide; GTPγS, guanosine 5'-O-(3-thiotriphosphate); MBP, maltose binding protein; mGSP, matured GSP; RGS, regulator of G protein signaling; RT-PCR, reverse transcription polymerase chain reaction; SPR, surface plasmon resonance.

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Figure 1. Targeting the *Ga* SII/ $\alpha 3$ site

(a) Ribbon structure representation of the *Gas*(s) subunit. Malleable switch regions (SI, SII, SIII) are depicted in black with the bound nucleotide and 3-helix 3 ($\alpha 3$) in slate. The SII/ $\alpha 3$ site of the subunit presents a convergent protein-binding surface that participates in a number of different protein-binding interactions. (b) Molecular surface representation of *Ga* protein sequence homology superimposed on the *Gas*(s) •GTP γ S crystal structure. A sequence alignment of *Ga* proteins (i1, i2, i3, oA, q, 11, 15, s(s), Olf, and 12) was performed by ClustalW⁶⁰ generating a list of variable (near white), similar (light gray), conserved (gray), and identical (slate) *Ga* residues, which were grafted onto the *Gas*(s) •GTP γ S crystal structure. The asterisk denotes an invariant hydrophobic binding pocket within the SII/ $\alpha 3$ cleft. Structural images were made from the Protein Data Bank file 1AZT 15 using PyMOL (<http://www.pymol.org>).

Figure 2. Directed Evolution Selection Scheme

(a) mRNA-display selection cycle. A dsDNA library (pool 0) was synthesized containing an invariant T7 promoter, untranslated region (UTR) and open reading frame (ORF). Template DNA was transcribed (1), ligated to puromycin via a 3'-DNA-tether (2) and translated (3) to generate mRNA-peptide fusions, individually composed of a peptide (red) linked to its corresponding mRNA genotype (black) via puromycin (blue). Purified fusions are reverse transcribed (4) prior to selection on a solid phase target (Gas(s)-beads). PCR amplification of cDNA retained on the target (5) produces dsDNA template (pool 1) for the subsequent round of selection. (b) Scheme for positive selection (R6A-1-library:Gas(s)-beads): The R6A-1-library sequence is listed; residues in bold were doped with a 50% mutation rate and random residues are represented as X's. At left is a plot of pool enrichment for the positive selection:

RNase treated [^{35}S]Met-peptide fusions from iterative rounds of selection were assayed for pull-down on G α s(s)-beads (black vertical bars) with background binding to neutravidin-beads <0.5%. At right, individual peptide sequences from pool 8 were screened for their ability to bind soluble G α i1 (gray horizontal bars) and G α s(s) (black horizontal bars). Conserved residues in selected peptides are boxed in gray. (c) Scheme for maturation selection (GSP-library:G α s(s)-beads with G α i1 competitor): At left, enrichment of pool binding to G α s(s)-beads (black vertical bars) is contrasted with binding to G α i1-beads (gray vertical bars). At right, selected peptides from pool 6 bind specifically to G α s(s) in the G α -binding screen.

Figure 3. SPR Binding Data for the GSP-Gas(s) Complex

A concentration series of GSP peptide was injected (200 μ L at 0s, with a 100 μ L/min flow rate) across ~1000 RU of Gas(s) immobilized on a streptavidin biosensor chip. Global kinetic fits (black) are overlaid on the sensogram data (gray). Two sensogram plots at 90 nM GSP and three plots at 0 nM demonstrate data precision. The derived binding constant is shown in Table 1.

Figure 4. Gα-binding Specificity Profiles

(a) GSP and mGSP-1 peptides were assayed for binding to a profile of 11 Gα proteins and the small GTPase H-Ras, boxed by class along the x-axis of the plot. Binding is a measure of [³⁵S]Met-Gα protein pull-down on peptide-beads performed at 4 °C. Control pull-down on MBP-neut beads showed no binding (<0.01) and presence of aluminum fluoride abrogated pull-down for both peptides. Error bars represent S.D. for multiple experiments. (b) A specificity profile similar to mGSP-1 is shown for the mGSP-2 peptide along with measurements performed in the presence of aluminum fluoride (AlF₄).

Figure 5. Gα-class recognition models

(a) Superimposition of (1) RGS (RGS4 32: dotted black line), (2) GoLoco (GoLoco/GPR 34: black line) and (3) effector (AC VC₁/IIC₂ 15: white line) binding footprints on Gα. The footprints overlap at the conserved SII/α3 site of Gα. Gas(s) residue Asp229 implicated in RGS binding specificity is highlighted in gray. Binding surfaces were grafted onto the Gas(s) structure, pdb 1AZT⁵; the image was generated by PyMol (<http://www.pymol.org>). (b) Mutational analysis: Previously characterized Gai1/Gas(s) reciprocal mutants and chimeras were assayed for pull-down on GSP and mGSP-1 peptide-beads. GSP and mGSP-1 exhibit increased binding to the C4 chimera containing the 3 and 3-β5 effector loop of Gas(s).

Chimera constructs are depicted at left, and denoted by respective binding models 1–3³⁸: Un the constructs Gai1 sequence is shown in light gray and Gas(s) sequence, numbered in the Gas(l) convention, is shown in dark gray.

Figure 6. GSP Nucleotide Exchange Assays

(a) Time course of [^{35}S]GTP γ S binding to 50 nM Gas(s) at 20 °C in the presence of 10 μM GSP or 10 μM suramin. Data are fit to a single exponential association curve to give apparent rates of GTP γ S association: Gas(s) $0.034 \pm 0.003 \text{ min}^{-1}$; + GSP $0.011 \pm 0.004 \text{ min}^{-1}$; + suramin $0.018 \pm 0.003 \text{ min}^{-1}$. Plots are normalized for maximum binding values with error bars representing S.D. of two measurements acquired during the same experiment. A dose response curve is shown in the inset plot for 50 nM Gas(s) incubated with the indicated concentration of GSP for 2.5 min. Logistic fitting of the data gives an IC₅₀ value of $157 \pm 4 \text{ nM}$. Arrows indicate the concentration of peptide used in the time course measurements. (b) Time course of [^{35}S]GTP γ S binding to 50 nM Gai1 at 30 °C in the presence of 10 μM GSP or 10 μM suramin. GTP γ S association rates: Gai1 $0.050 \pm 0.005 \text{ min}^{-1}$; + GSP $0.214 \pm 0.007 \text{ min}^{-1}$; +

suramin $0.034 \pm 0.002 \text{ min}^{-1}$. The inset GSP-G α i1 dose response curve gives an IC₅₀ value of $290 \pm 8 \text{ nM}$. **(c)** Steady-state hydrolysis is measured by inorganic phosphate [γ ³²P]_i released from the reaction of 100 nM G α , with [γ ³²P]GTP in the presence of 10 μ M GSP or 10 μ M suramin. Error bars indicate S.D. of multiple measurements.

a **b** **Figure 7. Gα-binding 'EFL' peptides**

(a) Sequence alignment of R6A-1, GSP, mGSP-1, mGSP-2, and KB-752: identical GSP core residues are highlighted in gray boxes and conserved residues in light gray; residues in the KB-752-Gαi1 crystal structure are underlined in red. (b) Ribbon diagram of KB-752 (red) binding within the SII/α3 site of Gαi1 (slate). The asterisk denotes an invariant hydrophobic binding pocket composed of Gαi1 residues R208, W211, I212, F215, L49, and I253 10. Structural image was made from Protein Data Bank file 1Y3A 19. KB-752 consensus motif residues DFL are labeled (red) along with structural elements of Gαi1 (slate).

Table 1

Peptide-Gα dissociation constants (K_D) and Gas(s) binding specificity (Gas(s)- $\Delta\Delta G$) values.

peptide	K_D (nM)		Gas(s)- $\Delta\Delta G$ (kcal mol ⁻¹)
	Gai1	Gas(s)	
R6A ^a	60		NA
R6A-1 ^b	200	50,000	-3.3
GSP	280±40	100±15	0.5
mGSP-1	10,300	300	2.1
mGSP-2	4,400±220	130	2.1

K_D values, calculated from kinetic parameters (k_d/k_a), are given ± S.D. when more than one independent measurement has been made. Gas(s)- $\Delta\Delta G$ values are calculated $\text{Gas(s)-}\Delta\Delta G = [\Delta G^\circ(\text{peptide-Gai1}) - \Delta G^\circ(\text{peptide-Gas(s)})]$, where $\Delta G^\circ = -RT \ln K_{obs}$

^aR6A and

^bR6A-1 binding constants are previously published values ^{18,29}. The R6A peptide sequence is MSQTKRLD DQLYWWEYL.