A Peptide Core Motif for Binding to Heterotrimeric G Protein α Subunits*

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William W. Ja1, Anirban Adhikari1, Ryan J. Austin1, Stephen R. Sprang3, and Richard W. Roberts1,2
From the 1Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125 and the 2Department of Biochemistry and Molecular Biophysics Graduate Program and 3The Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Recently, in vitro selection using mRNA display was used to identify a novel peptide sequence that binds with high affinity to Gαi1. The peptide was minimized to a 9-residue sequence (R6A-1) that retains high affinity and specificity for the GDP-bound state of Gαi1, and acts as a guanine nucleotide dissociation inhibitor (GDI). Here, we demonstrate that the R6A-1 peptide interacts with Gα subunits representing all four G protein classes, acting as a core motif for Gαi interaction. This contrasts with the consensus G protein regulatory (GPR) sequence, a 28-mer peptide GDI derived from the GoLoco (Gα, -Loco interaction)/GPR motif that shares no homology with R6A-1 and binds only to Gαi3.1. In this assay, Binding of R6A-1 is generally specific to the GDP-bound state of each Gα subunit and excludes association with Gβγ. R6A–Gαi complexes are resistant to trypsin digestion and exhibit distinct stability in the presence of Mg2+, suggesting that the R6A and GPR peptides exert their activities using different mechanisms. Studies using Gαi1/Gαi3 chimeras identify two regions of Gαi1 (residues 1–35 and 57–88) as determinants for strong R6A-Gαi1 interaction. Residues flanking the R6A-1 peptide confer unique binding properties, indicating that the core motif could be used as a starting point for the development of peptides exhibiting novel activities and/or specificity for particular G protein subclasses or nucleotide-bound states.

Heterotrimeric guanine nucleotide-binding proteins (G proteins), composed of α, β, and γ subunits, mediate signaling from transmembrane receptors (GPCRs)4 to a wide variety of effectors (1, 2). In the inactive state, intra-cellular Gα-GDP is tightly bound to Goβγ to form a Goβγ heterotrimer. Activation of a GPCR results in GDP exchange with GTP in the Gα subunit, concomitant dissociation of Goβγ, and subsequent signal transduction through Goβγ and/or Goβγ. The inherent guanosine triphosphatase (GTPase) activity of Gα is accelerated by various GTPase-activating proteins, returning the protein to the GDP-bound state, resulting in reassociation with Goβγ and termination of signaling.

Approximately 50% of currently marketed drugs target cell surface-accessible GPCRs (3, 4). Drug discovery targeting intracellular G proteins directly is inherently difficult due to (i) the broad spectrum of signaling events mediated at the G protein level, (ii) the requirement that drugs must cross the cell membrane to reach intracellular G proteins, and (iii) the high sequence and structural similarities between G protein classes (5, 6). Nevertheless, a number of diseases have been attributed to aberrant G protein activity (7, 8), and direct G protein ligands will provide new approaches and selectivities for drug treatment (5, 6, 9).

Selection methodologies facilitate the isolation of rare molecules with unique properties from large libraries (10, 11). Selections with combinatorial libraries have already been used to identify peptides that bind to various proteins in the G protein signaling cycle (9). We recently demonstrated that mRNA display, a selection technique where peptides are covalently attached to their encoded RNA, could be used to isolate Goαi1-binding sequences (12). The dominant peptide from the selection, as well as a minimized, active 9-mer sequence (R6A-1), acts as guanine nucleotide dissociation inhibitors (GDIs) and compete with Goβγ for binding to Goαi1 (12).

Here, we examine the specificity of R6A peptides for binding to various in vitro translated Gα subunits. Surprisingly, the R6A-1 core motif interacts with Gα subunits representing all four G protein families. Binding of R6A-1 is generally specific for the GDP-bound state of each Gα subunit and appears to exclude heterotrimer formation with Goβγ. Our findings suggest that the R6A-1 core motif peptide will be useful for the molecular design of Gα ligands with unique specificities.

EXPERIMENTAL PROCEDURES

Materials—Human cDNA clones encoding various G proteins were obtained from the UMR cDNA Resource Center (www.cdna.org) in the pCDNA3 (Invitrogen) vector. The Gα subunits used were, 11, 12, 13, α, γ, s (short form), 12, and 15. All in vitro translated Gβ and Gγ subunits refer to Gβ1 and G-terminal hemagglutinin (HA)-tagged Gγ, respectively. Rat Gαi1, bovine short form Gαs, and their chimeric constructs were generously provided by Prof. N. Artemyev (University of Iowa). DNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Peptide/Protein Preparation—The C-terminal amidated peptides GPR-biotin (TMGEEDFDLD LAKSQKRLDQRV DQLGRLNSYK, K = biotin), L19 GPR (TMGEEDFDDLLAKSQKRLDQVRDLAGYK), R6A-1-biotin (DQLYWWEYVQWRNSYK), R6A-1 (DQLYWWEYVL) and R6A-4 (SQTKRLDLQDYWWEYL) were synthesized and purified as described previously (12). R6A-4 lacks an N-terminal methionine that the originally studied “full-length” R6A peptide contained. Biotinylated peptides were immobilized using streptavidin-agarose (immobilized NeutrAvidin on agarose, Pierce). Approximately 500–800 pmol of biotinylated peptide were used per 10 μl of agarose.

Full-length R6A (MSQTKRLDLQDLYWEYL) was expressed as a fusion to maltose-binding protein (MBP) using an in vitro biotinylation system (13). Cloning, expression, and purification were performed as described previously (12). R6A-MBP or MBP was immobilized by random aniline coupling on CNBr-Sepharose 4B (Amersham Biosciences) as per the manufacturer’s instructions at a concentration of ~1 mg/ml hydrated matrix. N-terminal biotinylated Gαi1 (NB-Gαi1) was expressed and purified as described previously (12). A vector for producing biotinylated Nb-Gαi1 (short form) was constructed analogously, except that an N-terminal His tag was also incorporated to facilitate a primary purification by metal chelation chromatography.

In Vitro Translation—All G protein subunits were translated separately in coupled transcription/translation reactions using the TNT reticulocyte lysate system (Promega, Madison, WI). Typically, 0.3–1.0 μg of plasmid DNA and 25 μC of [35S]methionine (MP Biomedicals, Irvine, CA) were used in a 25-μl reaction. Translation efficiency of Gα subunits was quantitated by trichloroacetic acid precipitation of a 2-μl aliquot of each reaction, as per the manufacturer’s instructions. Gγ reactions were supplemented with 10 μM mevalonic acid lactone (Sigma) to ensure complete polyisoprenylation (14). To make Goβγ heterodimers, independently translated subunits were mixed together (3:1 by volume, GβGγ) and incubated at 37 °C for 30 min. To reconstitute Goβγ heterotrimers, equal volumes of Gαi and preformed Goβγ were mixed and incubated at 37 °C for an additional 30 min. For the heterotrimer immunoprecipitation assays, Gγ was translated without radioactive labeling due to possible interference in the resolution of Gα subunits by SDS-PAGE. These unlabeled reactions were supplemented with l-methionine (40 μM final).

Gα Interaction Assay—Gα translation reactions were desalted and exchanged using MicroSpin G-25 columns (Amersham Biosciences) into buffer (50 mM HEPES-KOH at pH 7.5, 6 mM MgCl2, 75 mM sucrose, 1 mM EDTA, 1 mM GDP, and 0.05% (v/v) Tween 20 (Bio-Rad)). Equivalent aliquots (2–6 μl) of the desalted Gα subunits were used for the in vitro binding assays.

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5 To whom correspondence should be addressed. Tel: 626-395-2321; Fax: 626-568-9430; E-mail: roberts@caltech.edu.

6 The abbreviations used are: GPCR, G protein-coupled receptor; GDI, guanine nucleotide dissociation inhibitor; GPR, G protein regulatory; HA, hemagglutinin; MBP, maltose-binding protein; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)methyl)glycine; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; GTPγS, guanosine 5′-O- (3-thiotriphosphate).
Gα was added to 0.6 ml of binding buffer (25 mM HEPES-KOH at pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 150 mM NaCl, 0.05% Tween 20, 0.05% (w/v) bovine serum albumin, 1 mM β-mercaptoethanol, and 10 μM GDP) containing ~10-μl matrix with or without immobilized target. After rotating at 4°C for 1 h, samples were briefly centrifuged and the supernatant was removed. The matrix was transferred to a 0.45-μm cellulose acetate spin filter (Costar Spin-X, Corning, Inc., Corning, NY) and washed with 3 × 0.6 ml of binding buffer at 4°C (1500 × g, ~40 s). The washed matrix was then removed from the spin filter for scintillation counting or analysis by SDS-PAGE. An approximation of the fraction of bound G protein was calculated (bound cpm divided by input protein counts, as determined by trichloroacetic acid precipitation). Assays with aluminum fluoride were performed identically, except that the binding buffer was supplemented with 10 mM NaF and 25 μM AlCl₃.

**RESULTS AND DISCUSSION**

**R6A-1 Is a Core Motif for Gα Binding**—Previously, in vitro selection with an mRNA display library was used to isolate novel peptide sequences that act as GDIIs for Gαs (12). The minimal 9-mer peptide, R6A-1, retained high affinity and competed with Gβγ for binding to Gαs. To investigate the binding specificity of the R6A-1 peptide, a pull-down assay was developed using radiolabeled G protein subunits (Fig. 1A). Cell-free coupled transcription/translation reactions were performed and analyzed by Tricine SDS-PAGE. Gels were fixed, dried in vacuo, and imaged by autoradiography (Storm Phosphorimag, Amersham Biosciences).

**Trypsin Protection Assay—Gαs (~25 μM) in digest buffer (50 mM EPPS at pH 8.0, 10 mM MgCl₂, 2 mM dithiothreitol, 1 mM EDTA, and 10 μM GDP or GTPyS) was incubated with and without 1.5-fold excess peptide (R6A-1, R6A-MBP, or R6A-MBP), identical to R6A-1 with the addition of two C-terminal lysines to enhance solubility, and GPR1, the first GPR motif of AGS3 (15)) and digested with trypsin (1:50 trypsin/Gαs) at 4°C for the indicated time. Reactions were analyzed by SDS-PAGE.

**Gαβγ Heterotrimer Immunoprecipitation**—Equivalent aliquots (10 μl) of reconstituted Gαβγ heterotrimer were added to 0.6 ml of binding buffer containing ~10-μl matrix or 1-μl anti-HA monoclonal antibody (Sigma, clone HA-7). After rotating at 4°C for 1 h, ~10 μl of protein G-Sepharose 4B Fast Flow (Sigma) was added to the monoclonal antibody-containing samples. After an additional 30 min of rotating at 4°C, immobilization matrices were washed in 0.45-μm spin filters (3 × 0.6 ml of binding buffer) as described above. A fourth wash was performed in batch, after transferring the matrices to new tubes, to prevent contamination from the spin filter membrane. The samples were resuspended in 2× SDS-loading buffer, incubated at 90°C for 5 min, and analyzed by Tricine SDS-PAGE. Gels were fixed, dried in vacuo, and imaged by autoradiography (Storm Phosphorimag, Amersham Biosciences).

**Binding of various in vitro translated G proteins to L19 GPR (A), R6A-1 (B), or R6A-MBP (C).** Binding has been scaled relative to the interaction to Gαs. For comparison, Kᵦ values of Gαs binding to L19 GPR, R6A-1, and R6A are 82, 200, and 60 nM, respectively (12). The negative control matrices used in the assay were streptavidin-agarose (A and B) and MBP-Sepharose (C). Gαs consistently exhibited higher nonspecific binding which was especially noticeable on the MBP-Sepharose in C, which was background subtracted to more clearly show the binding activity. In B and C, binding assays were also performed in the presence of aluminum fluoride (AIF) except for Gα₁₅ (C) and H-Ras (B and C).

**FIGURE 2.** Binding of various in vitro translated G proteins to L19 GPR (A), R6A-1 (B), or R6A-MBP (C). Binding has been scaled relative to the interaction to Gαs. For comparison, Kᵦ values of Gαs binding to L19 GPR, R6A-1, and R6A are 82, 200, and 60 nM, respectively (12). The negative control matrices used in the assay were streptavidin-agarose (A and B) and MBP-Sepharose (C). Gαs consistently exhibited higher nonspecific binding which was especially noticeable on the MBP-Sepharose in C, which was background subtracted to more clearly show the binding activity. In B and C, binding assays were also performed in the presence of aluminum fluoride (AIF) except for Gα₁₅ (C) and H-Ras (B and C).

**FIGURE 3.** R6A-MBP and L19 GPR compete with Gβγ for binding to Gα subunits. A, in vitro translated, [35S]methionine-labeled Gβ₁₂ and HA-tagged Gγ₂ subunits. B, reconstituted Gαβγ heterotrimers, with the indicated Gα subunit, were pulled down with an anti-HA antibody. R6A-MBP, or L19 GPR. Only the Gα and Gγ₂ subunits were radiolabeled. Immunoprecipitation with anti-HA confirmed the presence of reconstituted heterotrimers in the reaction mix. Gγ was not coprecipitated when Gα subunits were pulled down by R6A-MBP or L19 GPR (B), suggesting that binding of Gα-GDP to Gβγ and R6A are exclusive events (C). Results were similar for Gα₁₅ (data not shown).

**FIGURE 1.** Binding analysis with in vitro translated G proteins. A, autoradiograph of indicated Gα subunits or H-Ras, directly translated from human cDNA vectors in coupled transcription/translation reactions with [35S]methionine labeling. A blank reaction (~) did not contain vector. The slightly lower molecular weight bands (seen clearly for Gα₁₅ and Gα₁₅) correspond to translation initiation at alternate methionine codons. B, pull down of radiolabeled Gαs, on matrix with (+) or without (−) the indicated peptide (L19 GPR or full-length R6A).

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**RESULTS AND DISCUSSION**

**R6A-1 Is a Core Motif for Gα Binding**—Previously, in vitro selection with an mRNA display library was used to isolate novel peptide sequences that act as GDIIs for Gαs (12). The minimal 9-mer peptide, R6A-1, retained high affinity and competed with Gβγ for binding to Gαs. To investigate the binding specificity of the R6A-1 peptide, a pull-down assay was developed using radiolaabeled, in vitro translated G protein subunits (Fig. 1A). Cell-free coupled transcription/translation offered a rapid method for screening multiple G proteins (16–18). R6A-1 and L19 GPR peptides were synthesized with a C-terminal biotin-containing linker peptide derived from the constant region used in the original selection (12). The full-length R6A peptide was also expressed as an N-terminal fusion to MBP, which was subsequently immobilized by random amine coupling. [35S]methionine-labeled Gαs was first tested against immobilized L19 GPR and full-length R6A, demonstrating specific pull-down of full-length Gα₁₅, as well as a slightly higher mobility band that corresponds to an alternate translation initiation site (Fig. 1B).

The R6A-1 minimal peptide exhibited strong pull-down for all Gα subunits tested (Fig. 2B). There was negligible interaction with H-Ras (a member of the monomeric or small G protein family), suggesting that the R6A-1 peptide is a core motif for binding to Gα subunits in the heterotrimeric G protein family. Conversely, the L19 GPR peptide was highly specific for Gα₁₅ and showed negligible interaction with other Gα subunits (Fig. 2A). This contrasts with previous results that show the GPR consensus exhibits significantly lower, but detectable, affinity and GDI activity for Gαs compared with Gα₁₅ (19, 20). The determined affinities of R6A-1 to Gαs and Gα₁₅ (Kᵦ = ~200 nM and ~50 μM, respectively; data not shown) suggest that the sensitivity of the binding assay was sufficient to detect weak interactions.
The nucletotide state specificity of R6A-derived peptides to various Gs subunits, radiolabeled G proteins were assayed for binding to immobilized R6A-1 and R6A-MBP in the presence and absence of aluminum fluoride. The minimal peptide was specific for the GDP state of all G proteins except for Gαs and Gαq, where binding was observed in the presence of aluminum fluoride (Fig. 2B). Full-length R6A-MBP demonstrated strong specificity for the GDP state of all G proteins, including Gαs, confirming the weak but specific interaction (Fig. 2C). These findings suggest that residues flanking the 9-mer core motif play a strong role in modulating the binding properties of the peptide, modifying Gα class as well as nucleotide state specificity.

**R6A Competes with Gβy for Binding to Gα Subunits**—Previously, GPR and R6A-derived peptides had been shown to compete with Gβy heterodimers for binding to Gαs (12, 21–24). To determine whether R6A would exclude Gβy binding for other Gα subunits, in vitro translated Gβi and HA-tagged Gγi, (Fig. 3A) were reconstituted with various Gα subunits and pulled down by immobilized L19 GPR, R6A-MBP, or an anti-HA monoclonal antibody. The tested Gα subunits all appeared to couple to Gβy heterodimers (Fig. 3B). Co-precipitation of Gγ subunits was not seen when heterotrimeric peptides were pulled down by L19 GPR or R6A-MBP (Fig. 3B), clearly indicating that binding to these motifs excludes Gβy interaction (Fig. 3C). Results for R6A-MBP with reconstituted Gαs, Gαβγ heterotrimer were similar (data not shown).

**R6A and GPR Peptides Exert Distinct Modes of Activity**—Previous analyses of the R6A and GPR peptides suggested that their binding modes and mechanisms of action differ (12). Here, additional assays suggest that R6A binds and stabilizes a GDP-bound conformation of Gαi, unlike the conformation of the inactive Gαi-GDP subunit. Mg2+ binds with high affinity and stabilizes the GTP-bound state of Gαi subunits (21). In contrast, Mg2+ binds Gαi-GDP with weak affinity (25). Measurements of L19 GPR and R6A binding to Gαs-GDP as a function of Mg2+ concentration revealed distinct trends (Fig. 4A). While R6A showed higher relative binding with increasing Mg2+ concentrations, L19 GPR exhibited the highest binding in the absence of Mg2+. The isolated original of the R6A peptide, from a doped library based on the GPR motif, most likely resulted from the presence of Mg2+ in the selection buffer (12). Crystalllographic structures of a Gαi-GDP-Mg2+ complex indicate that binding of Mg2+ induces switch I on Gαi, to assume a conformation similar to that observed in the active Gαi-GTPyS-Mg2+ complex (26). Hence, the R6A peptide may stabilize a conformation of Gαi-GDP that is similar to the structure of an active, GTP-bound subunit.

Trypsin digestions were performed to further probe the peptide-bound Gαi complex. GTP-bound Gαs is highly resistant to protease digestion, compared with the GDP-bound subunit (27, 28). R6A peptides protected Gαi-GDP from trypsin similarly to Gαi-GTP (Fig. 4B). Conversely, a GPR peptide-Gαi complex showed similar proteolysis as Gαi-GDP (Fig. 4B). The R6A and GPR peptides did not appear to inhibit trypsin directly (data not shown). These results suggest that the R6A-bound complex is structurally analogous to Gαi-GTP and further support distinct binding modes and mechanisms of activity for the R6A and GPR peptides.

**Mapping of the R6A-Gαi Interaction Surface**—A series of Gαi/Gαi chimerae, C1–C8 (Fig. 5A), was used to determine the binding site of the R6A-1 peptide. The chimerae were previously shown to be correctly folded and functional in GTPyS binding assays (29). Based on the binding seen for chimeras C4, C5, C6, C7, and C8 for immobilized R6A-1, two regions of Gαi were required for strong interaction (Fig. 5B). Substitution of residues Gαi58–88 for the corresponding residues in Gαs (comparing chimeras C5 and C6) resulted in substantially reduced binding. This region, which includes the linker between the Ras-like and helical domains of Gαi, might be involved in determining the binding specificity of the R6A peptide. The N-terminal 35 residues of Gαi also play an important role (comparing chimeras C2 and C5), most likely by affecting global protein stability and/or conformation.

The full-length R6A peptide, compared with the minimal R6A-1 sequence, demonstrated significantly less binding to Gαs and Gαq, and negligible interaction with Gαo (Fig. 2C). It is not clear whether the differences between R6A and R6A-1 in relative binding to the various G proteins was due to the N-terminal flanking region of the full-length R6A sequence, the altered immobilization scheme (amine coupling versus C-terminal biotinylation on the R6A-1 peptide), or steric effects from the comparatively large MBP fusion. However, assuming that the various G proteins are structurally homologous and that the R6A and R6A-1 peptides bind to Gαs subunits analogously, the differences in relative binding would most likely be a direct result of the R6A flanking residues.

Previously, it was shown that R6A was highly specific for the GDP state of Gαi and did not bind to Gαs-GDP-ATF or Gαq-GTPyS (12). To establish...
Pcp2, a protein containing a single GPR motif, exhibited binding to the G protein chimeras C4 and C7 (data not shown), agreeing with previous studies (29). In contrast, the consensus GPR sequence appears to be highly optimized for specific interaction with Goa, as none of the chimeras showed substantial binding to the L19 GPR peptide (Fig. 5B). Previous studies of different GPR motif-containing proteins have mapped key binding interactions that promote class specificity to regions in the Gα helical domain (29–31). The GPR consensus region itself, however, binds primarily to the Gα Ras-like domain (30). Hence, it appears that the determinants for Gα class specificity can be contained within the Ras-like domain, despite the high sequence and structural conservation of the GPR binding site on Goa subunits of different classes (9).

The GPR consensus peptide may be highly sensitive to the global conformation of Goa subunits rather than specific for particular amino acid contacts, perhaps making the motif easily adaptable for binding to specific Gα classes (e.g. by the addition of flanking residues, as in Pcp2 (30)).

We have shown that the R6A-1 peptide, previously identified by in vitro selection against Gαi1, acts as a core motif for interaction with Goa subunits and appears to compete with Gβγ for binding. It will be of interest to determine whether R6A-1 also retains its GDI activities for different Gα family members. The R6A-1 core motif may serve as a template for the molecular design of Gα ligands with unique class and/or nucleotide state specificities. Additionally, as the core motif appears to stabilize a distinct conformation of Goa-GDP, it may be possible to develop peptides with unique effects on G protein-effector interaction, GDP exchange, and GTP hydrolysis.

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