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Identification of Structural Features in the G-protein Regulatory Motif Required for Regulation of Heterotrimeric G-proteins

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The G-protein regulatory (GPR) motif, a conserved 25–30 amino acid domain found in multiple mammalian proteins, stabilizes the GDP-bound conformation of Gαi, inhibits guanosine 5′-O-(3-thiotriphosphate) (GTPγS) binding to Gαi, and competes for Gβγ binding to Gαi. To define the core GPR motif and key amino acid residues within a GPR peptide (TMGEEDFFDLLAKSKRMDDQVRDLAG), we determined the effect of truncation, insertion, and alanine substitutions on peptide-mediated inhibition of GTPγS binding to purified Gαi1. The bioactive core GPR peptide consists of 17 amino acids (F7-R20). Within this core motif, two hydrophobic sectors (FF8 and LL11) and Q22 are required for bioactivity, whereas M19A and R23A increased IC50 values by 10-fold. Disruption of spatial relationships between the required sectors in the amino and carboxyl regions of the peptide also resulted in a loss of biological activity. Mutation of three charged sectors (EED9, R18, &DD21) within the 28-amino acid GPR decreased peptide affinity by ~10-fold. Alanine substitutions of selected residues within the core GPR peptide differentially influenced peptide inhibition of GTPγS binding to Gαi versus Gαo. These data provide a platform for the development of novel, G-protein-selective therapeutics that inhibit Gαi-mediating signaling, selectively activate Gβγ-sensitive effectors, and/or disrupt specific regulatory input to G-proteins mediated by GPR-containing proteins.

The activation/deactivation cycle of heterotrimeric G-proteins, key players in cell signaling events, involves guanine nucleotide exchange, GTP hydrolysis, and a number of dynamic, conformationally sensitive protein interactions. In addition to the extensively studied activation of G-proteins by the superfamily of G-protein-coupled receptors, the G-protein activation/deactivation cycle is regulated by nonreceptor proteins that influence subunit interactions, GTPase activity, and guanine nucleotide binding properties of Gαi. One signature motif for such regulatory proteins is the regulator of G-protein signaling (RGS)1 domain, a ~120-amino acid motif found in all members of the RGS family (1). Another signature motif (~25–30 amino acids) is defined by the G-protein regulatory (GPR) domain in activator of G-protein signaling (AGS)3 (2–6), which was discovered in a functional screen for receptor-independent activators of G-protein signaling. The GPR motif was also recognized in RGS12 and RGS14 by general sequence analysis/alignment and termed the GoLOCO motif (7, 8).

Surprisingly, interaction of the GPR motif with Gαi stabilizes the GDP bound conformation of Gαi, competes with Gβγ for Gαi binding, and inhibits guanine nucleotide exchange (3–6, 9–12). Thus, the GPR motif acts as a guanine nucleotide dissociation inhibitor of Gαi. The GPR motif is evolutionarily conserved within individual orthologs and among proteins with apparently diverse functions (see S.M.A.R.T. data base at dylan.embl-heidelberg.de/). Four spatially conserved GPR motifs are found in AGS3 (3) and LGN (13), which were isolated as Gαi-regulatory/binding proteins. Recombinant AGS3 constructs with more than one GPR motif actually bind more than one Gαi at the same time (5), suggesting a scaffolding role for such proteins. The AGS3/LGN-related protein PINS, which plays key roles in cell polarity (14–17), possesses a similar domain structure. The interaction of the PINS protein GPR domains with Gαi is involved in the function of PINS in cell polarity and asymmetric cell division (17). AGS3 is also involved in synaptic adaptation in rat models of addiction (22).

Single GPR motifs are found in Rap1GAP, Pcp2, RGS12, and RGS14, which are all implicated as G-protein regulators. Protein interaction studies and/or functional screens in yeast indicate that the AGS3 GPR motif interacts with Gαi1–3, but not Gαo, Gαq, or Gα12 or Gα15 (3, 5, 11). Specific GPR motifs are capable of interacting with Gαo, albeit with apparently lower affinity than observed for Gαi (10). Rap1GAP was actually isolated in yeast two-hybrid screens using Gαo (18) and Gαo (19), whereas Pcp2 was isolated in similar screens using Gαo (20). Thus the GPR motif appears to serve as a discrete motif to anchor a variety of proteins that influence the guanine nucleotide binding/hydrolysis properties of G-proteins.

The existence of such a fairly discrete and highly conserved binding motif that inhibits GDP dissociation is of particular interest. As a first step toward developing a small organic molecule that would mimic the action of a GPR peptide, we defined the key structural features of the GPR motif required for biological activity. These data provide a platform for the development of novel, G-protein-selective therapeutics that target this critically important signaling protein within the cell. Such agents might inhibit Gαi-mediating signaling by G-protein-coupled receptors, selectively activate Gβγ-sensitive effectors, and/or disrupt specific regulatory input to G-proteins.
Identification of Structural Features in the GPR Motif

FIG. 1. Effect of magnesium on inhibition of GTP\(^{35}\)S binding to Go\(_i\). GTP\(^{35}\)S (500 nM) binding (using 0 and 25 mM MgCl\(_2\)) to Go\(_i\) (100 nM) was measured in absence and presence of increasing amounts of GPR peptide as described under “Experimental Procedures.” Data are expressed as the percent of specific binding (1–2 pmol) observed in the absence of added peptide and are expressed as the mean ± S.E. of two experiments with duplicate determinations.

FIG. 2. Identification of the core GPR motif. A: left panel, amino acid sequence of GPR peptide and truncated GPR peptides. Consensus amino acids are depicted in red. The right panel portrays GTP\(^{35}\)S binding (500 nM with 2 mM MgCl\(_2\)) to Go\(_i\) (100 nM) measured in the absence and presence of GPR peptides (100 pmol) as described under “Experimental Procedures.” Data are expressed as the percent of specific binding (1 pmol) observed in the absence of added peptide and are expressed as the mean ± S.E. of two experiments with duplicate determinations. Similar results were obtained in two to five separate experiments. B: left panel, sequence alignment and IC\(_{50}\) values of mutated GPR peptides. Conserved amino acids are depicted in red, and mutated amino acids are colored green. Right panel, effect of increasing concentrations of competing peptides on GTP\(^{35}\)S binding (500 nM with 2 mM MgCl\(_2\)) to Go\(_i\) (100 nM). Data are expressed as the percent of specific binding (1 pmol) observed in the absence of added peptide and are expressed as the mean ± S.E. of two experiments with duplicate determinations. Similar results were obtained in two to five separate experiments. Inactive, inhibition of GTP\(^{35}\)S binding was less than 30% at peptide concentrations of 100 \(\mu\)M.

EXPERIMENTAL PROCEDURES

Materials—Peptides were synthesized and purified by Bio-Synthesis, Inc. (Lewisville, TX) and peptide identity verified by matrix-assisted laser desorption ionization mass spectrometry. Peptides were synthesized with an acetylated amino terminus and an amidated carboxyl terminus. All other materials were obtained as described elsewhere (3–5).

GTP\(^{35}\)S Binding and Protein Interaction Assays—GTP\(^{35}\)S binding assays and protein interaction assays were conducted as described previously (4, 5). The GPR domain of AGS3 (468–550) was generated as a glutathione S-transferase fusion protein in pGEX-4T1. GST-AGS3 was expressed and purified from BL21 bacteria using glutathione-Sepharose 4B. Go\(_i\), and Go\(_s\) were purified in the GDP bound state from Sf9 insect cells infected with recombinant virus as described previously (21). Concentration response curves with GPR peptides were analyzed by PRISM (Graphpad Software, Inc, San Diego, CA) to calculate IC\(_{50}\) values.

RESULTS AND DISCUSSION

Stabilization of the GDP-bound conformation of Go\(_i\) by a 28-amino acid peptide encompassing the GPR motif presents an unexpected aspect of regulation within the G-protein activation/deactivation cycle. This activity of the peptide essentially prevents nucleotide exchange on Go and binding of GTP to G-protein. The GPR-Go\(_i\)-GDP complex is quite stable and is observed in the absence and presence of added magnesium, which is generally required for high affinity binding of GTP\(^{35}\)S (Fig. 1). As part of a broad approach to define the structural properties of this regulation and develop a small molecule ligand that would target this novel regulatory site on Go subunits, we defined the core GPR motif and the key amino acid residues within this core motif. A panel of peptides derived from the GPR motif were characterized in GTP\(^{35}\)S binding assays and protein interaction assays using purified mammalian G-protein \(\alpha\) subunits.

Several conserved features constitute the GPR motif. The invariant Q\(^{15}\) essentially divides the peptide into two general regions of conserved residues (Fig. 2A). Both helical wheel and hydrophobic moment analysis indicate that the area upstream of Q\(^{15}\) likely exists as an alpha helix. We first asked if either of the two general regions of conserved residues (i.e. upstream and downstream of Q\(^{15}\)) were active by themselves. Neither GPR1–15 nor GPR15–28 inhibited GTP\(^{35}\)S binding to Go\(_i\). The region upstream of Q\(^{15}\) is distinguished by the negatively charged EED\(^6\) followed by two hydrophobic sectors (FF\(^{11}\)LL\(^{11}\)) (Fig. 2). The region downstream of the Q\(^{15}\) peptide is characterized by the conserved sequence RMDDQR\(^{23}\). Deletion of the first eight residues at the amino terminal, which removed FF\(^{8}\) or mutation of R\(^{23}\) to phenylalanine (3–5), resulted in an inactive peptide indicating that the minimal sequence for bioactivity is FF–R\(^{23}\) (Fig. 2A). The RMDDDQR\(^{23}\) sequence is one of the most highly conserved regions of the peptide among different species and proteins. To test the importance of the spatial relationship between this region and the remainder of the conserved residues found elsewhere in the peptide, we inserted two and four alanines just upstream of the RMDDDQR\(^{23}\) region. Insertion of the alanines completely abolished bioactivity in GTP\(^{35}\)S binding assays (Fig. 2A). Thus, these data indicated that there is a core GPR motif (residues 7–23) in which a defined spatial relationship among the conserved residues is required for bioactivity.

We then addressed the relative importance of conserved amino acids within the core GPR motif by alanine substitutions and subsequent peptide evaluation in GTP\(^{35}\)S binding assays (Fig. 2B). Peptides with alanine substitutions at FF\(^{11}\), LL\(^{11}\), and Q\(^{22}\) were inactive and thus identify the key residues of the peptide. Both FF\(^{8}\) and LL\(^{11}\) likely constitute the core of the predicted alpha helix in this region, indicating the importance of this structural feature for bioactivity (Fig. 1). The importance of this region is further emphasized by loss of biological activity observed with an F\(^{8}\)R mutation in the context of a GST-AGS3 fusion protein (3, 5, 17). The IC\(_{50}\) values for D9A, K13A, Q15A, and S16A peptides were similar to the consensus GPR peptide (Fig. 2B). Alanine substitutions at the other key residues within the GPR motif indicated that the residues could be grouped as those causing 7–10-fold (EED\(^6\), R\(^{8}\), and DD\(^{21}\)) or 50–70-fold (M\(^{19}\) and R\(^{23}\)) shifts in IC\(_{50}\) values (Fig. 1).
The loss of affinity observed with the alanine substitutions at 4EED is consistent with the retention of bioactivity by the truncation mutant GPR3–24, whereas the loss of activity with the 4FF alanine substitutions was also observed by elimination of these residues in the GPR10–24 truncated peptide. The larger reduction in affinity observed with the M10 and R53 alanine substitutions indicate the importance of these residues within the core GPR peptide. Indeed, reversal of charge or hydrophobicity at these residues (M19D and R23F) essentially rendered these peptides inactive (3).2

The inhibition of GTPγS binding and apparent stabilization of the GDP-bound conformation of Go, by the GPR peptide likely involves two events, the initial binding of the peptide to G-protein, followed by a conformational change in Go itself. To determine whether the two events can be functionally dissociated, we performed two series of experiments. We first asked if mutant peptides that exhibited lower affinity in GTPγS binding assays were also deficient in inhibiting binding of a GPR-containing GST-AGS3 fusion protein to Go. In the second series of experiments, we determined whether truncated GPR peptides could act in a complementary fashion and whether they could antagonize the action of the GPR consensus peptide.

Data generated from this series of experiments indicated that the loss of function in binding studies was also reflected in the protein interaction assays (Fig. 3, A and B). The partial inhibition of GST-AGS3 binding to G-protein in the presence of GPR6–24 indicates that this peptide is not as potent as GPR3–24 or wild type peptide, likely due to the loss of the 4EED acidic cluster (Fig. 2, A and B, and Fig. 3B). These data suggested that G-protein interaction and inhibition of GTPγS binding could not be dissociated from each other. This point is further emphasized by data generated in another series of experiments (Fig. 3C). A combination of the inactive GPR truncation peptides (GPR 1–15 and GPR 15–28) did not inhibit binding of GST-AGS3 to Go. Neither of the peptides antagonized the action of the consensus GPR peptide nor were they able to rescue the activity of nonfunctional, alanine-substituted GPR peptides (Fig. 3). These data confirm the importance of the spatial relationship within the core GPR motif. These data also indicate that the GPR domain must function as an intact unit and that there are multiple points of contact between the GPR domain and Go.

Although the GPR peptide clearly prefers Go over Goi, subtle mutations within the core motif differentially affect peptide interaction with the two G-proteins (Fig. 4A). A screen of the mutant GPR peptides for selectivity indicated that inhibition of GTPγS binding to Go is completely eliminated by alanine substitutions within the core GPR motif that only minimally altered GTPγS binding to Goi (Fig. 4A). The difference in the interaction of the GPR peptide with Go versus Goi is also observed by comparing the relative inhibition of GTPγS binding in the presence and absence of magnesium. The inhibition of GTPγS binding to Goi is much more sensitive to magnesium as compared with the effects of the peptide on Go (Fig. 4B). These data indicate that the GPR peptides can differentially target G-protein α subunits. Amino acids outside the GPR motif may also influence G-protein selectivity or bioactivity (see Discussion in Ref. 4).

The core GPR motif identified in the present study is indicated in Sequence 1.

\[
\text{FF-LL---M-QR}
\]

**Sequence 1**

Within this core, 7FF8, 10LL11, and Q22 are absolutely required for bioactivity. The carboxyl terminus R25 and the M27 residue also play key roles in bioactivity as their mutation results in large shifts in affinity. Within the larger GPR peptide (Sequence 2),

\[
\text{EE-FF-LL---RMDDDQR}
\]

**Sequence 2**

4EE5, 30DD21, and the internal R26 all exert effects on affinity (~7–10-fold). Minimizing the core GPR motif to its essential pharmacophores identified the chemical moieties within the GPR motif that regulate G/Go nucleotide exchange.

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2 Y. K. Peterson and S. M. Lanier, unpublished observations.

3 R. Kimple, J. Sondek, and D. P. Siderovski, personal communication.
of its novel mode of regulation of G-proteins, the core GPR motif provides an unexpected platform for the development of G-protein selective therapeutics.

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