Potent Peptide Analogues of a G Protein Receptor-binding Region Obtained with a Combinatorial Library*

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The C terminus of the G protein α subunit represents an important site of interaction between heterotrimeric G proteins and their cognate receptors. We have screened a combinatorial peptide library based on the C terminus of the α subunit of G, (340–350) and have identified unique sequences that bind rhodopsin with high affinity. Six of these sequences, as both fusion proteins and synthetic peptides, were significantly more potent than the parent sequence in binding to and stabilization of metarhodopsin II. These sequences provide information about which residues are required for appropriate receptor interaction. We observed that in all the high affinity sequences, a positively charged residue at position 341 was changed to a neutral one. Thus, it appears that the receptor-G protein interaction was designed to be low affinity to ensure efficient catalysis of G protein activation. We also observed Cys-347 and Gly-348 to be invariant, and hydrophobic residues were always located at positions 340, 344, 349, and 350, demonstrating the critical nature of these residues. A composite of the structures of the high affinity sequences was modeled based upon the structure of rhodopsin-bound trNOESY NMR of this region of Gαi (Dratz, E. D., Fursteneau, J. E., Lambert, C. G., Thireault, D. L., Rarick, H., Schepers, T., Pakhlevaniants, S., and Hamm, H. E. (1993) Nature 363, 276–280) and provides insight into the complementary G protein-binding surface of the receptor.

Understanding the structural basis of receptor-G protein interaction is essential to defining a molecular mechanism of signal transduction. Activated receptors display a high affinity binding site for heterotrimeric G proteins, which, depending on the receptor type, appears to consist of one or more of the cytoplasmic regions of the receptor. Interestingly, however, comparison of the primary sequences of receptors that are known to interact with the same G protein has not revealed the underlying consensus sequence. High affinity interaction of heterotrimeric G proteins with activated receptors requires the presence of both the G protein α and βγ subunits. Three regions on the α subunit are known to be important for receptor interaction, the N-terminal 23 residues, an internal sequence that includes the TCAT region which contacts the guanine ring, and the C terminus (1). The extreme C terminus of G protein α subunits is one of the critical points of contact with activated receptors (1–7). This C-terminal region of G protein α subunits is also important in determining the specificity of receptor-G protein interaction (9), and other regions of α subunits are also involved (10). Synthetic peptides corresponding to the last 11 amino acids of α subunits have been shown to mimic the conformational effect of heterotrimeric G proteins on receptors by stabilizing the active, agonist-bound form of the receptor, although with low potency (1, 7, 8). Additionally, C-terminal peptides from Gαi subunits also can competitively block receptor-G protein interactions (1, 8).

The advent of random peptide libraries displayed on bacteriophage (11–13) or bacterial plasmids (14) has brought the screening power of very large combinatorial libraries to the search for potent peptide agonists and antagonists, as well as to provide detailed structural analysis of the requirements for peptide-receptor interactions. In this study, we have used a random “peptides-on-plasmids” library approach (14) to identify potent rhodopsin-binding peptide sequences related to a C-terminal Gαi peptide, Gαi(340–350) (1, 7) from a library of greater than 109 peptides. Using this method, we have developed several high affinity analogues of Gαi(340–350) and have gained insight into the structural requirements for peptide binding and stabilization of the activated conformation of rhodopsin, metarhodopsin II (Meta II).

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Bacterial strains, plasmids, and library construction are essentially as described (15). Escherichia coli ARI BL4 (Δsrl-recA) endAI nupG lon-11 sulA1 hisdR17 (ompT-tetP) ΔpDA319::kan Δlac lacZU118) was used for all experiments. Plasmid pSL142 was used for library construction. A second plasmid, pELM3, a pMal-c2 derivative with a modified polylinker (New England Biolabs), was used for expression of maltose-binding protein (MBP) fusion proteins. This isopropyl β-D-thiogalactopyranoside-inducible vector contains the E. coli malE gene with a deleted leader sequence, leading to cytoplasmic expression of the fusion proteins.

Library Construction—An oligonucleotide, ON-2333, was synthesized to encode a mutagenesis library based on the K341R derivative of the native 340–350 C-terminal sequence of Gαi, IKENLKDCGLF (5'-GAG GTG GGT NNK NNK NNK NK att ctg gaa aac tta aaa gat tgt ggt dtg ttc TAA CTA AGT AAA GC). Uppercase letters denote positions synthesized with pure phosphoramidites or with equimolar mixtures (N = A, C, G, and T). Lowercase letters denote bases synthesized with 70% of the indicated base and 10% of each of the other bases. This mutagenesis rate leads to approximately a 50% chance that a codon will be mutated to encode another amino acid. Additionally, four random NNK codons were synthesized on the 5'-end of the sequence to make a total of 15 randomized codons.

Panning—Buffers and general methods are described elsewhere (15). In the dark, EDTA-washed rod outer segment (ROS) membrane frag-
exceptions that the EDTA-washed rhodopsin (16) were directly immobilized on Immulon 4 (Dynatech) microvials and an acetonitrile, 6 mM HCl gradient. The purified peptides were subjected to fast atom bombardment mass spectrometry and analytical reverse-phase HPLC to determine purity and authenticity. Peptides 18S, 18L, 23S, and 23L were synthesized by SynPep Corporation and were further purified and characterized using similar methods.

Peptide Synthesis—Gt

"Extra" Meta II Assay—Stabilization of Meta II was measured in an ELISA format (15). Microtiter wells were coated with 0.5 µg of protein/well in cold 35 mM Hepes, pH 7.5, buffer containing 100 mM NaCl, 1 mM MgCl2, and 1 µM dithiothreitol (HEK/DTT). The samples were maintained at 5.3°C by a water-cooled thermal jacketed cuvette holder. The resulting dose-response curves were analyzed by the non-linear regression program yielding a positive signal in the MBP ELISA (Fig. 1A). The signal intensities from these positive clones are stronger than that of the positive control clone, pELM17, which encodes the Gt

α-K341R-340–350 peptide analog (K341R). pELM6, which expresses a MBP fusion protein, was added. The incubation was continued for 30 min to allow the competition of the two protein-fusion complexes. Chasing low affinity complexes off of the immobilized receptor. After the final round, the enriched population of peptide coding sequences was transferred to the vector pELM3 so that they were fused in frame with the E. coli MBP. Peptides fused to MBP can be analyzed in a competition ELISA where the resulting signal is a rough correlate of their affinity for the rhodopsin (15, 17). Of 28 individually selected clones, 24 yielded a positive signal in the MBP ELISA (Fig. 1A). The signal intensities from these positive clones are stronger than that of the positive control clone, pELM17, which encodes the Gt

α-K341R-340–350 undecamer. DNA sequencing of these MBP ELISA-positive clones yielded 18 unique, readable sequences. The positive sequences are shown (Fig. 2B), as well as the C-terminal amino acids of the known mammalian G protein α2 subunits (Fig. 2A) for comparison. Also shown are the rhodopsin-specific binding sequences from the second round of panning, which were presumably lower affinity, on average, than those present after later rounds of panning. The resulting percent identity and similarity (homology) with Gt

α for each residue is shown in Fig. 2C. Interestingly, the four upstream amino acid linkers do not share an obvious consensusequence.

Sequences derived from the fourth round of panning suggest that 2 of the 11 residues, Cys-347 and Gly-348, are absolutely required for binding to rhodopsin (Fig. 2). These two amino acids are conserved among all of the members of the Gt

α G protein subfamilies (Fig. 2A), and Cys-347 is the site of pertussis toxin modification in this subfamily (5). These 2 residues are also part of a type II” β turn, which is required for Meta II stabilization (7). Other residues conserved within the Gt

α subfamily could be replaced only with amino acids having similar properties. Ile-340 is strictly conserved in this subfamily, and throughout G proteins it is generally only restricted to alanine, valine, or leucine.

RESULTS AND DISCUSSION

Library Construction and Panning—To explore the sequence requirements for binding of the C terminus of Gt

α to activated rhodopsin and to search for potent peptide analogs, we constructed a mutagenesis library based on a 15-mer protein sequence consisting of a 4-amino acid linker and 11 amino acids from the C-terminal analog, Gt

α-K341R-340–350 (7). To construct the library, we used the “peptides-on-plasmids” vector pSI42 (14, 15), which resulted in a library containing approximately 2 × 109 independent recombinants. The library peptides were fused to the C terminus of the DNA binding protein LacI. Each plasmid vector from which the fusion proteins are expressed contains two lacO DNA sequences to which the LacI fusion protein binds with high affinity, leading to the formation of peptide-LacI-plasmid complexes in each transformed cell in the library population. After cell lysis, the library complexes expressing peptides of interest were affinity purified by "panning" on immobilized rhodopsin. Plasmids recovered after each round of panning were amplified for additional rounds of panning after transformation of E. coli (14, 15). To select for the highest affinity peptides in the population, we carried out the last two of the four total rounds of panning in the presence of a Gt

α-K341R-340–350 peptide analog as a competitor in a wash step to "chase" low affinity complexes off of the immobilized receptor. After the final round, the enriched population of peptide coding sequences was transferred to the vector pELM3 so that they were fused in frame with the E. coli MBP. Peptides fused to MBP can be analyzed in a competition ELISA where the resulting signal is a rough correlate of their affinity for the rhodopsin (15, 17). Of 28 individually selected clones, 24 yielded a positive signal in the MBP ELISA (Fig. 1A). The signal intensities from these positive clones are stronger than that of the positive control clone, pELM17, which encodes the Gt

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α subfamily could be replaced only with amino acids having similar properties. Ile-340 is strictly conserved in this subfamily, and throughout G proteins it is generally only replaced with Leu (Fig. 2). Even among the most potent of the peptides (see below) Val, Leu, and Met substitution occurred, suggesting that several hydrophobic residues are acceptable. The large hydrophobic residues Phe or Tyr are found at the large hydrophobic residues Phe or Tyr are found at the
although Gsα and Gα15/16 are more heterogeneous than the others (Fig. 2A). Two Leu residues, Leu-344 and Leu-349, are absolutely conserved in all G proteins, yet in our peptides conservative substitutions were possible. Among the high affinity peptides, Leu-344 was always present, but Leu-349 was switched to Met in sequences 8 and 24. The conservation of hydrophobic residues at certain positions (340, 344, 349, and 350) suggests a hydrophobic component to the conserved binding pocket on the receptor. This, in fact, has been suggested by mutagenesis studies on muscarinic (18, 19) and β-adrenergic (20) receptors.

Rhodopsin Binding and Meta II Stabilization by the Fusion Proteins and Peptides—As a rough indicator of potency, we initially examined the ability of the MBP fusion proteins to be competed off of light-activated rhodopsin by a single high concentration of Gtα-Ac-K341R-340–350. Presumably, those that were not well competed would be significantly more potent than the native peptide. Six of the least strongly competed MBP-peptide fusion proteins were then chosen for further study. The affinities of these MBP fusion proteins for light-activated rhodopsin were estimated using a competition ELISA. The IC50 values of these six MBP-peptide fusions range from 3.8 to 42 nM, up to 3 orders of magnitude more potent than the 6 μM IC50 of MBP-Gαs-K341R-340–350 (encoded by plasmid pELM17) (Table I). Gα, a much stronger competitor than its C-terminal peptide, was tested for its ability to compete with the purified MBP fusion proteins for binding to rhodopsin in the MBP ELISA. Gα, at 0.5 μM, inhibited binding of all six of the MBP fusion proteins, decreasing the signal by an average of 50% (Fig. 1B), indicating that these peptide sequences share the binding site on rhodopsin with Gα.

C-terminal peptides of Gα subunits cannot only bind to their cognate receptors but can mimic the heterotrimeric G proteins’ ability to affect receptor conformation and stabilize the active state (1, 7, 8). The fusion protein, MBP-Gαs-K341R-340–350,
TABLE I

Binding to rhodopsin and Meta II stabilization by MBP fusion proteins and synthetic peptides

The dose response for the ability of each of the six high affinity MBP fusion proteins to compete with the LacI-Gt-K341R-340-350 fusion protein for binding to light-activated rhodopsin was measured in an ELISA format. Microtiter wells were coated with 0.5 μg of ROS/well and blocked with BSA in the dark. Serial dilutions of purified MBP fusion proteins were added after light activation of the rhodopsin, incubated for 45 min at 4°C. The LacI-Gt-K341R-340-350 fusion protein was added, after 30 min the amount of bound LacI was detected by an anti-LacI primary antibody and AP-conjugated goat anti-rabbit secondary antibody, and the assay was developed with p-nitrophenyl phosphate. Meta II stabilization was measured as described under “Materials and Methods.”

| MBP fusion protein | Meta II binding IC50 (μM) | Meta II stabilization EC50 (μM) | Synthetic peptides | Meta II binding IC50 (μM) | Meta II stabilization EC50 (μM) |
|--------------------|---------------------------|-------------------------------|--------------------|---------------------------|-------------------------------|
| MBP8               | 0.0078 ± 0.0003 (0.57–1.56) | 0.94 (5.7–15.6)              | 8S                 | 16 ± 5                    | 8.3 (5.4–13)                 |
| MBP9               | 0.010 ± 0.003 (0.44–2.5)       | 9S                             | 15 ± 4             | 12.0                      | 7.8 (7.8–18.3)               |
| MBP10              | 0.042 ± 0.02 (2.3–12.8)        | 10S                            | 34 ± 20            | 24.0                      | 10.6 (10.6–54)               |
| MBP18              | 0.0038 ± 0.002 (0.74–2.0)      | 18S                            | 10 ± 1             | 15.7                      | 8.3 (8.3–29)                 |
| MBP23              | 0.020 ± 0.004 (1.66–7.3)       | 23S                            | 0.64 ± 0.08 (0.74–1.15) | 0.92                      | 0.74 (0.74–1.15)             |
| MBP24              | 0.0066 ± 0.002 (0.36–0.66)     | 24S                            | 110 ± 40           | 152                       | 62 (62–376)                  |
| MBP-K341R-340-350  | 6.0a,b                        | >100                           | K341R-340-350      | 200 ± 50                   | 223 (155–321)                |

| Gt                  | 0.084b                        |                                |                    | 0.32                      | (0.26–0.39)                  |

a Error not accurately determined since curve does not saturate.
b Experiment only conducted once.

was able to stabilize Meta II with an EC50 of greater than 100 μM. At similar concentrations, the MBP protein alone (encoded by plasmid pELM6) had no effect. The high affinity fusion proteins derived from the combinatorial library were significantly more potent than MBP-Gt-K341R-340-350 in their ability to stabilize Meta II (Fig. 3A). Their EC50 values ranged from 0.49 to 5.4 μM (Table I).

To examine the sequences directly, in the absence of the 43-kDa MBP fusion partner, we chemically synthesized the six 11-mer peptides (denoted “S”) and compared them with the Gt-K341R-340-350 11-mer peptide. Dose-response studies in the competition ELISA revealed that the 11-mer peptides displayed higher binding affinity than the parent peptide by 2 to 30-fold (23S) (Table I). The synthetic peptides were then tested for their ability to stabilize Meta II. The newly identified peptides were all significantly more potent than Gt-K341R-340-350 in their ability to stabilize Meta II (Fig. 3B). Their EC50 values ranged from 0.76 (23S) to 152 (24S) μM, which is up to 290 times higher affinity than the parent sequence (Table I). Additionally, these peptide sequences also appear to be potent antagonists of receptor activation of G proteins, since the peptides competitively inhibit the ability of light-activated rhodopsin to stimulate GTPyS binding to Goα in a dose-dependent fashion.2

It is interesting to note that the relative potency of the 24S peptide decreased substantially in the absence of the fusion protein. In general, the MBP fusion proteins all appear to be of higher affinity than the peptides in both the binding and Meta II assays. This is probably due to the constraining effect on the N terminus of the peptide, which would lead to a decrease in conformational freedom. This difference between the fusion proteins and peptides was not as great in the Meta II assay, probably due to the higher sensitivity of the ELISA assay.

“Extra” Meta II is measured as a tiny absorbance change (0.02 absorbance unit), and to detect such a small change the concentration of rhodopsin must be high (>0.05 μM), thus limiting the dynamic range of the assay.

To examine the effect of the added four random N-terminal amino acids, the full-length 15-mer peptides were also synthesized for clones 18 and 23 (peptides 18L and 23L) (Table I). Although no obvious consensus sequences were present in the four random residues extended upstream from the core library, this region contributes to the increase in affinity in these two peptides. Peptide 18L is approximately 70- and 16-fold more potent than the corresponding 18S 11-mer peptide in the binding and Meta II assays, respectively, whereas this difference is only 3-fold for peptides 23L and 23S.

By surveying a large number of peptide sequence possibilities unattainable by standard peptide synthetic techniques, we found several sequences that bind to rhodopsin with substantially higher affinity than the native sequence. Presumably, these analogues bind more tightly and dissociate at a slower rate. It is interesting that the screening procedure selected against a positive charge at position 341 but not at position 345 (Fig. 2B). In approximately 70% of the high affinity peptides from panning round 4 and all of the highest affinity peptides we fully characterized, the Lys-341/Arg-341 was changed to a non-charged group. This positive to neutral change correlates well with higher affinity. Such a change could have implications for the evolved functional properties of signaling pathways. In order to permit rapid and amplified information transfer, the receptor-G protein interaction must allow for rapid dissociation of the activated G protein. We described an activation-dependent conformational change of Gt-Ac-K341R-340-350 in its rhodopsin-bound conformation by trNOESY and speculated that this conformational change is important for the highly amplified Gt activation process (7). Especially marked changes occur in a helix-like tight turn at the N terminus involving

2 C. E. Ford and H. E. Hamm, unpublished observation.
Ile-340, Arg-341, Glu-342, and Asn-343, with formation of a salt bridge between Arg-341 and the free carboxyl of the C terminus in the inactivereceptor-bound form and relaxation of this interaction in the activated form (7). The hypothesis that the presence of a positive charge at position 341 might decrease the affinity of binding to receptor and increase the receptor’s catalytic rate is directly testable using site-directed mutagenesis. Interestingly, a mutation that removes this positive charge (Arg-341 to His) has been reported to functionally uncouple Gs from its receptor in a patient with Albright hereditary osteodystrophy (21). It would be of interest to examine whether this mutation leads to a higher affinity for receptor. Other G proteins do not have a positive charge at this position (Fig. 2A), and we predict that these G proteins may have higher receptor affinity and lower activation rates.

The crystal structure of Gtα has provided much information on its overall three-dimensional conformation (22–25). However, in the crystal structure of Gtα-GDP (which in combination with the βγ subunit is the rhodopsin binding conformation) the C-terminal region was disordered and not visible (23). Only in the GTPγS-bound form of Gtα could any structural features of the C terminus be discerned (22). Three possibilities are that 1) it is disordered and extended in the GDP-bound conformation in order to “sense” the activated receptor and conform to it by induced fit, 2) the disorder is an artifact of the crystal packing, or 3) it is disordered because it is missing an organizing site contributed by the N terminus, which was deleted from the Gtα used for crystal formation.

In the absence of good crystallographic data on the C terminus, our data can be used to suggest an approximation of the tolerances of rhodopsin’s binding site for the Gtα-340–350 region by utilizing a combination of information on the peptide sidechains that can be accommodated in each position and the structural information obtained by trNOESY (7). The resulting models were constructed using INSIGHT and GRASP (developed by A. Nicholls and B. Honig, Columbia University). The amino acids are labeled based on their sequence position (1–11) and the orientation similar to that previously shown (2). We have not accounted for the possibility that the lack of a positively charged side chain at position 341, which interacts with the free C terminus, may cause global changes in the backbone structure.
and their identification would greatly facilitate our understanding of the critical point-to-point interactions required for receptor-G protein interaction.

This paper has demonstrated the power of using a combinatorial library to develop potent peptide analogues useful in furthering our understanding of rhodopsin-Gt interactions, as well as having broader implications in the study of signal transduction systems. Additionally, this work will lay the structural framework by which therapeutic agents can be developed to interfere with signal transduction mediated via G proteins.

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