Structural Requirements for the Stabilization of Metarhodopsin II by the C Terminus of the α subunit of Transducin*

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The retinal receptor rhodopsin undergoes a conformational change upon light excitation to form metarhodopsin II (Meta II), which allows interaction and activation of its cognate G protein, transducin (Gt). A C-terminal 11-amino acid peptide from transducin, Gt-(340–350), has been shown to both bind and stabilize the Meta II conformation, mimicking heterotrimeric Gt. Using a combinatorial library we identified analogs of Gt-(340–350) that bound light-activated rhodopsin with high affinity (Martin, E. L., Rens-Domiano, S., Schatz, P. J., and Hamm, H. E. (1996) J. Biol. Chem. 271, 361–366). We have made peptides with key substitutions either on the background of the native Gt-(340–350) sequence or on the high affinity sequences and used the stabilization of Meta II as a tool to determine which amino acids are critical in G protein-rhodopsin interaction. Removal of the positive charge at the N termini by acylation or delocalization of the charge by K to R substitution enhances the affinity of the Gt-(340–350) peptides for Meta II, whereas a decrease was observed following C-terminal amidation. Cys-347, a residue conserved in pertussis toxin-sensitive G proteins, was shown to interact with a hydrophobic site in Meta II. These studies provide further insight into the mechanism of interaction between the Gt C terminus and light-activated rhodopsin.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are critical regulatory proteins in a variety of cell signaling pathways. Stimulation of a G protein-coupled receptor by an appropriate agonist results in conformational changes leading to its interaction with a heterotrimeric G protein, catalysis of GDP release, and subsequent G protein activation (for reviews see Refs. 1–3). In the visual system, the retinal light receptor rhodopsin is activated by absorption of a single photon of light followed by interaction with and activation of the G protein transducin (Gt), leading to stimulation of 3′,5′ cyclic GMP phosphodiesterase (for review see Ref. 4). In the absence of additional guanine nucleotides, the G protein enhances agonist binding to a G protein-coupled receptor. Binding of either GDP or GTP to the G protein disrupts the high affinity complex or active state of the G protein-coupled receptor (5, 6).

The receptor-G protein interface has been defined in some detail and involves portions of the intracellular loops and juxtamembrane regions of the G protein-coupled receptor with several regions on heterotrimeric G proteins (for review see Ref. 7). Distinct regions on Gt that are involved in receptor recognition, GTP binding and hydrolysis, guanine nucleotide-induced conformational changes, and effector interaction have been elucidated using diverse studies including disruption by ADP ribosylation (8), binding of antibodies (9–11), proteolytic mapping (12), alanine scanning (13, 14), peptide or minigene studies (15–17), and studies of chimeric Gt proteins (18–21). Researchers have determined that the N terminus, C terminus, and parts of the α5 helix of Gα are important sites for receptor recognition.

For rhodopsin-transducin interactions, all three intracellular loops of rhodopsin have been implicated (22–28) as well as the C-terminal tail (29). The receptor appears to interact with both the α (30, 31) and βγ subunit (32–34) of heterotrimeric Gt. On the α subunit of Gt, multiple sites of contact have been identified (reviewed in Ref. 35). Three regions, the N-terminal 23 residues, an internal sequence (Gt-(311–323)), and the C-terminal 11 amino acids, were identified (reviewed in Ref. 35). Using chimeric proteins, the C terminus and residues 299–314 of Gt were shown to contribute to rhodopsin binding (36–38). Of these sites of interaction, the C terminus of Gt α subunit has been the most extensively investigated. Rhodopsin-Gt interaction can be disrupted by a number of treatments that block the C terminus of Gtα, including pertussis toxin-catalyzed ADP ribosylation (8) and binding by an antibody (39). The C-terminal peptide Gtα-(340–350) has been shown to directly bind to and stabilize Meta II (31), mimicking the entire holo-G protein. Furthermore, selective mutagenesis of this C-terminal Gtα region leads to alterations in G protein function (14, 40, 41).

Important insights into the mechanism of G protein-mediated signal transduction have been provided by the crystallization and high resolution structure determination of G protein α (42–47) and βγ (48) subunits as well as heterotrimeric Gαβγ complexes (49, 50). However, in many of the crystal structures the final residues of the Gt C terminus are disordered and not visible. NMR studies indicate that the C-terminal 11-amino acid peptide of Gtα has no structure in solution, but it takes on significant structure when it is bound to either excited (light...
activated) or unexcited rhodopsin (51–53), suggesting a direct physical interaction between the C-terminal residues of \( G_{\alpha} \) and rhodopsin. However, detailed structures of the \( G_{\alpha} \) peptide-receptor or \( G_{\alpha} \)-peptide-R* complexes are still uncertain because of methodological limitations.

In an alternative approach to defining important determinants in the C terminus, Martin et al. (54) used a combinatorial peptide library to identify high affinity analogs of \( G_{\alpha}-(340–350) \) that bound to light-activated rhodopsin. Sequences derived from panning the biased library demonstrated the presence of certain positions in which amino acids were absolutely conserved (Cys-347 and Gly-348). Based on work by Dratz et al. (51), as well as peptide substitutions, these residues are predicted to be part of a type II β turn, which is thought to be required for establishing Meta II stabilization. Also highlighted by the combinatorial library screening procedure was that the Lys-341 in the native sequence was selected against. In nearly 70% of the sequences obtained from the panning, and in all of the highest affinity sequences, Lys-341 was changed to a noncharged group (54). It was hypothesized that a positive charge at position 341 in \( G_{\alpha} \) might decrease its affinity for rhodopsin, leading to a faster "off" rate and a higher rate of G protein activation. Given that other G proteins do not have a positive charge at this position, the presence of a charged residue in \( G_{\alpha} \) has implications for the evolved properties of the signaling mechanisms.

In this study we have examined the structural basis for the invariance of Gly and Cys at the −3 and −4 positions, respectively, and the loss of a positively charged residue at the −10 position in the high affinity analogs from the combinatorial library. The structural features predicted by NMR were also further explored with analog peptide studies of the functional interaction of these peptides with activated rhodopsin leading to Meta II stabilization.

MATERIALS AND METHODS

Peptide Synthesis—\( G_{\alpha}-(340–350) \) analogs were synthesized by the solid-phase Merrifield method using Fmoc chemistry on Milligen 9050 or Applied Biosystems peptide synthesizers. Peptides were purified by reverse-phase HPLC using C4 or C18 preparative columns and an acetonitrile, 6 mM HCl, water gradient. The purified peptides were subjected to fast-atom bombardment or electrospray mass spectrometry to determine authenticity and analytical reverse-phase HPLC to determine purity. Fmoc-2-amino butyric acid was purchased from Advanced ChemTech (Louisville, KY).

Protein Preparation—Washed bovine rod outer segments-containing rhodopsin were prepared from fresh bovine retinas using sucrose gradient centrifugation and washing of the membranes with EDTA to remove peripherally bound proteins (55). Heterotrimeric \( G_{\alpha} \) was prepared as described previously by Stryer et al. (56).

*Extra Meta II Assay*—The absorbance spectra of washed rod outer segments (5 μM) were measured in the presence of varying concentrations of \( G_{\alpha}-(340–350) \) analog or heterotrimeric \( G_{\alpha} \) using an SLM Amino DW2000 spectrophotometer in decay buffer (10 mM K2PO4, pH 6.5, 0.1 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol) in the presence of either 1 or 2 mM \( G_{\alpha} \)-(340–350) analog peptides. The spectra were measured in the dark, then completely bleached in room light. Spectra for the bleached samples were measured at 30-min intervals over a 6-h time period. Finally, 260 mM HCl was added to protonate the retinal Schiff base in rhodopsin and leave free retinal unprotonated.

Molecular Dynamics—Calculations were carried out on Silicon Graphics workstations using the program DISCOVER (MSI, Inc.) and the CVFF forcefield. After amino acid substitutions the energy was minimized using steepest descents, and then 250 ps of molecular dynamics were run at 300 K, pH 7 with a distance-dependent dielectric constant.

RESULTS AND DISCUSSION

The three-dimensional structures of heterotrimeric G proteins have provided detailed information about their subunit and receptor interactions and have suggested mechanisms for GTP hydrolysis. In most of the crystal structures the final residues of the \( G_{\alpha} \) C terminus are disordered and not visible. However, in one of the crystal structures of \( G_{\alpha} \)-GTPyS (45) the ordered C-terminal residues 343–349 form an α-helix and make van der Waals contacts with residues of the α2/β4 loop. It is not clear whether this is a crystal-packing artifact or an indication of a preferred conformation of the C terminus. Additionally, for both \( G_{\alpha} \)-(47) and the \( G_{\alpha} \)-RGS4 complex (57), the C terminus of \( G_{\alpha} \) is an extension of the α5 helix. In the \( G_{\alpha} \)-RGS4 complex (57) the extended α5 helix was stabilized by the N-terminal helix as well as by crystal contacts, whereas for \( G_{\alpha} \)-(47) the extended α5 helix is in close proximity with the α4–β6 loop. Sunahara et al. (47) suggest that the divergence of the \( G_{\alpha} \) C-terminal structures may contribute to receptor selectivity.

NMR studies indicate that the C-terminal 11-amino acid peptide of \( G_{\alpha} \) has little or no structure in solution, but it takes on a significant structure when it is bound to rhodopsin (51–53), indicating a direct physical interaction between the C terminus of \( G_{\alpha} \) and its receptor. However, detailed structures of the \( G_{\alpha} \) peptide-receptor or \( G_{\alpha} \)-peptide-R* complexes are still uncertain because of methodological limitations. It has not been possible to refine the published bound peptide structures (51, 52) by comparing experimental NMR data to that calculated from the bound structures, indicating that there are significant errors in the NMR distance constraints. In addition, many of the predictions for amino acid substitutions that would be tolerated or favorable for the Kisselev et al. (53) proposed R*-bound structure were not born out by experiment (58). When more accurate NMR data are obtained, it may be possible to refine the protein-bound peptide structures within the uncertainty in 1.6 Å resolution X-ray structure.

For an “alkaline” peptide, we identified potent high affinity sequences related to the C terminus of \( G_{\alpha} \) using a “peptides-on-plasmids” combinatorial technique (59) in which a library with greater than 108 different peptide sequences was tested for binding to light-activated rhodopsin (54). We have now expanded on observations from the combinatorial screening by making specific point mutations within the C-terminal

\[
Y = \text{Bottom} + (\text{Top} – \text{Bottom})/1 + 10^{(\text{EC}_{50} – \text{Hillslope} \times \text{Hillslope})} \quad (\text{Eq. 1})
\]

Bottom is the Y value at the bottom plateau, which was set to 0, and Top is the Y value at the top plateau. LogEC50 is the logarithm of the EC50, the concentration that gives a response halfway between Bottom and Top. The variable Hillslope controls the slope of the curve such that (i) when Hillslope equals 1, the equation generates a standard dose-response curve, (ii) when Hillslope is less than 1.0, the curve is more shallow, and (iii) when Hillslope is more than 1.0, the curve is steeper.
peptide sequence to clarify how the C terminus of G_{ta} interacts with and stabilizes the activated rhodopsin species Meta II.

Rhodopsin can be measured spectrophotometrically in many of its light-induced conformational states (for review see Ref. 4). The binding of G_{i} to light-activated rhodopsin stabilizes an active signaling state of the receptor (R*) that can be measured spectrophotometrically (60, 61). The active state can be stabilized by the R*-catalyzed loss of GDP from G_{i}, leading to an empty guanine nucleotide binding pocket (62). Addition of either GTP or GDP promotes the loss of the active R* state, as measured by the loss of Meta II stabilization (62–64). The biologically active Meta II state can be differentiated from its precursor, Meta I, by the Meta II assay. This assay makes use of the observation that under conditions of slightly alkaline pH and low temperatures Meta I is strongly favored in the absence of G_{i}, Meta II is stabilized in the presence of G_{i} and can be measured spectrophotometrically. We have exploited the ability of G_{i}-(340–350) C-terminal peptide analogs to stabilize Meta II in the same manner as G_{i} to investigate the interface between G proteins and their agonist-activated receptors.

The Role of Free Amino Groups in the Peptide Stabilization of Meta II—The native G_{i}-(340–350) peptide is of relatively low potency in its ability to interact with and stabilize Meta II, with an EC_{50} of 1209 μM (Fig. 1, Table I). Two similar peptide analogs were found to have increased potencies at stabilizing Meta II; G_{i}-(340–350)-K341R, with one fewer amino group, displayed an EC_{50} of 180 μM, and G_{i}-(340–350)-K341R, with two fewer amino groups, had an EC_{50} of 163 μM. The potencies of G_{i}-(340–350)-K341R and G_{i}-(340–350)-K341R are 6.7- and 7.4-fold higher, respectively, than that of native G_{i}-(340–350) peptide (Table I). Substitution of the Lys at position 345 with Arg in the acetylated peptide 23 or amidating the carboxyl group of G_{i}-(340–350)-K341R decreases the EC_{50} only slightly, whereas acylation of peptide 24 decreases its EC_{50} 16-fold (Fig. 2, Table I). Substitution of the Lys at position 345 with Arg in the acetylated peptide 23 or substitutions at positions 342 and 345 in acetylated peptide 24 do not affect the EC_{50} values significantly (Fig. 2, Table I). Taken together, the data in Table I suggest that removal of a localized positive charge by acylation of the N terminus or delocalization of the positive charge that occurs when Lys is replaced with Arg can enhance the affinity of certain G_{i}-(340–350) peptide analogs for their interaction with Meta II.

The Role of the Negatively Charged C Terminus in the Peptide Stabilization of Meta II—NMR studies of the G_{i} peptide analogs bound to rhodopsin indicate substantial structural changes upon light excitation, suggesting that the C-terminal carboxyl group shifts its orientation upon interaction with the activated receptor (51–53). We tested the importance of the free C-terminal negative charge. This resulted in a peptide (Ac-G_{i}-(340–350)-K341R-Amide) that stabilized Meta II but was 2-fold less potent than the same peptide with a free C terminus (Fig. 1, Table I). Screening of the peptide library showed that the C-terminal final seven amino acids were the most invariant, with the fourth round clones having identities at these positions ranging from 72 to 100% (54). These are the same residues that are disordered in most of the crystal structures. It is reasonable to speculate that the C terminus of G_{i} directly contacts the activated receptor, and our data supports the idea that the negative charge at the C terminus may participate in this interaction.

Roles of Cys-347 and Gly-348 in the Peptide Stabilization of Meta II—The Cys residue at position 347 of G_{i} has long been known to play a critical role in the interaction between G_{i} and rhodopsin. It is the site of pertussis toxin-catalyzed ADP ribosylation, which leads to an uncoupling of G_{i} family G proteins from their cognate receptors (8). That Cys-347 and Gly-348 are critical residues in rhodopsin binding was apparent from the combinatorial library study (54), in which all of the Meta II-binding peptide analogs sequenced from the fourth round were conserved at these positions. This phenomenon suggests that other amino acids could not substitute effectively for the Cys-
The dose response for the ability of each of the listed \( G_t \) analogs to stabilize Meta II was measured as described under “Materials and Methods.” Bold letters indicate amino acid residues that differ relative to the native \( G_t \)-(340–350) sequence. Dose-response curves were analyzed by non-linear regression using a sigmoidal dose-response variable slope equation (GraphPad Prism) to obtain the EC\(_{50}\) values, S.E., and slope. The number of independent experiments, done in duplicate, is listed as \( n \). The EC\(_{50}\) obtained for heterotrimeric \( G \) protein was 0.28 ± 1.12 \( \mu \)M, with a slope of 1.19 (\( n = 8 \)).

| Peptide                  | Sequence | EC\(_{50}\) ± S.E. | Slope | \( n \) |
|--------------------------|----------|--------------------|-------|------|
| Role of N terminus       |          |                    |       |      |
| \( G_t \)-(340–350)      | I K E N L K D C G L F | 1209.0 ± 2.7 | 0.82  | 4    |
| \( G_t \)-(340–350)-K341R | I R E N L K D C G L F | 180.4 ± 1.2 | 1.40  | 3    |
| \( G_t \)-K341R          | I R E N L K D C G L F | 162.9 ± 2.0 | 0.78  | 3    |
| \( G_t \)-K341R-C347S    | I R E N L K D C G L F | 167.2 ± 1.3 | 0.89  | 7    |
| Ac-Gt\( _t \)-(340–350)  | I R E N L K D C G L F | 34.8 ± 1.2 | 1.18  | 3    |
| Role of C terminus       |          |                    |       |      |
| \( G_t \)-(340–350)      | I K E N L K D C G L F | 0.7 ± 1.1 | 1.06  | 2    |
| \( G_t \)-K341R          | I K E N L K D C G L F | 0.5 ± 1.7 | 1.27  | 2    |
| \( G_t \)-K341R-C347S    | I K E N L K D C G L F | 1.1 ± 3.6 | 0.78  | 3    |
| Peptide 24               | M L K N L K D C G M F | 152.1 ± 1.8 | 0.89  | 3    |
| Ac-peptide 24            | A c M L K N L K D C G M F | 9.7 ± 1.2 | 0.83  | 4    |
| Ac-peptide 24            | I K E N L K D C G L F | 9.9 ± 1.2 | 0.81  | 3    |
| Ac-peptide 24            | I K E N L K D C G L F | 2.0 ± 1.2 | 1.78  | 2    |
| Role of C terminus       |          |                    |       |      |
| Ac-Gt\( _t \)-(340–350)  | I K E N L K D C G L F | 1.2 ± 1.02 | 1.02  | 6    |

FIG. 2. The role of N termini and random linker sequence in peptide 24-mediated Meta II stabilization. Dose-response curves of Meta II stabilization by the 11-mer and 15-mer high affinity analogs of peptide 24. The resulting dose-response curves are presented for heterotrimeric \( G_{\alpha} \), \( G_t \)-K341R, peptide 24 (+), Ac-peptide 24 (○), Ac-peptide 24 long (□), and Ac-peptide 24-K342R-K345R (■). Data presented are the average of at least three independent experiments ± the standard error of the mean.

FIG. 3. The role of Cys-347 in Meta II stabilization by \( G_{\alpha} \)-(340–350) analogs. Dose-response curves of Meta II stabilization by \( G_{\alpha} \), \( G_t \)-(340–350)-K341R (○), \( G_t \)-(340–350)-K341R-C347A (●), \( G_t \)-(340–350)-K341R-C347D (◆), and \( G_t \)-(340–350)-K341R-C347S (▲) are presented. Data presented are the average of at least four independent experiments ± the standard error of the mean.

347 and Gly-348 residues when the peptides were being selected for binding to activated rhodopsin. To test this idea, substitutions were made at positions 347 and 348, and the peptides were then tested for their ability to stabilize Meta II.

To identify the role of the Cys side chain in its interaction with Meta II, we initially synthesized a Cys to Ala analog, \( G_t \)-(340–350)-K341R-C347A. This analog showed a substantial decrease in its ability to stabilize Meta II. Meta II could be stabilized maximally to 77% of control by 1.8 mM the peptide stabilized only 25% of Meta II, as compared with the control \( G_t \)-(340–350)-K341R peptide (Fig. 3). The EC\(_{50}\) values obtained for both peptides (\( G_t \)-(340–350)-K341R-C347S and \( G_t \)-(340–350)-K341R-C347D) were greater than 1 mM (Table II). To determine whether it is the hydrophobicity of Cys that is important, we substituted Cys-347 with 2-aminoisobutyric acid (Abu), a compound that replaces the sulfhydryl group of the cysteine with a methyl group and approximately mimics cysteine in both size and hydrophobicity. This -SH to -CH\(_2\) peptide (\( G_t \)-(340–350)-K341R-C347Abu) was able to stabilize Meta II with essentially the same potency as the \( G_t \)-(340–350)-K341R parent peptide, with an EC\(_{50}\) of 127 \( \mu \)M versus 163 \( \mu \)M (Fig. 3, Table II). Therefore, the data suggest that the Cys-347 side chain of \( G_{\alpha} \) interacts in a hydrophobic manner with Meta II.

To further test the role of the side chain of Cys-347, we synthesized a Cys to Ser analog, \( G_t \)-(340–350)-K341R-C347S, because Ser has a size similar to the sulfhydryl moiety. To ascertain whether the anionic form of the sulfhydryl moiety is important in the binding pocket environment, a Cys to Asp analog, \( G_{\alpha} \)-(340–350)-K341R-C347D, was synthesized. At 1.8 mM the \( G_{\alpha} \)-(340–350)-K341R-C347S peptide stabilized only 40% of Meta II, and the \( G_{\alpha} \)-(340–350)-K341R-C347D peptide stabilized only 25% of Meta II, as compared with the control \( G_{\alpha} \)-(340–350)-K341R peptide (Fig. 3). The EC\(_{50}\) values obtained for both peptides (\( G_{\alpha} \)-(340–350)-K341R-C347S and \( G_{\alpha} \)-(340–350)-K341R-C347D) were greater than 1 mM (Table II). To determine whether it is the hydrophobicity of Cys that is important, we substituted Cys-347 with 2-aminoisobutyric acid (Abu), a compound that replaces the sulfhydryl group of the cysteine with a methyl group and approximately mimics cysteine in both size and hydrophobicity. This -SH to -CH\(_2\) peptide (\( G_{\alpha} \)-(340–350)-K341R-C347Abu) was able to stabilize Meta II with essentially the same potency as the \( G_{\alpha} \)-(340–350)-K341R parent peptide, with an EC\(_{50}\) of 127 \( \mu \)M versus 163 \( \mu \)M (Fig. 3, Table II). Therefore, the data suggest that the Cys-347 side chain of \( G_{\alpha} \) interacts in a hydrophobic manner with Meta II.
The dose response for the ability of each of the listed $G_{t_{34}}$ analogs to stabilize Meta II was measured as described under "Materials and Methods." Bold letters indicate amino acid residues that differ relative to the native $G_{t_{34}}$-(340–350) sequence. The $\Delta$ symbol represents 2-amino butyric acid (Abu). Dose-response curves were analyzed by non-linear regression using a sigmoidal dose-response variable slope equation (GraphPad Prism) to obtain the EC$_{50}$ values, S.E., and slope. The number of independent experiments, done in duplicate, is listed as $n$.

### Table II

| Peptide          | Sequence          | EC$_{50}$ ± S.E | Slope | $n$ |
|------------------|------------------|-----------------|-------|-----|
| Role of Cys-347  |                  |                 |       |     |
| Ac-$G_{t_{34}}$-(340–350)-K341R | Ac-I RE N L K D C G L F | 162.7 ± 1.3 | 0.89 | 7   |
| Ac-$G_{t_{34}}$-(340–350)-K341R-C347Abu | Ac-I RE N L K D G L F | 126.6 ± 1.2 | 1.42 | 6   |
| Ac-$G_{t_{34}}$-(340–350)-K341R-C347A | Ac-I RE N L K D A G L F | 579.1 ± 1.2 | 3.10 | 5   |
| Ac-$G_{t_{34}}$-(340–350)-K341R-C347D | Ac-I RE N L K D D G L F | >1000 | 0.79 | 4   |
| Ac-$G_{t_{34}}$-(340–350)-K341R-C347S | Ac-I RE N L K D S G L F | >1000 | 1.11 | 4   |
| Ac-peptide 23-K345R | Ac-V L E D L R D C G L F | 1.1 ± 3.6 | 0.78 | 3   |
| Ac-peptide 23-K345R-C347Abu | Ac-V L E D L R D A G L F | 10.6 ± 1.2 | 1.09 | 4   |
| Ac-peptide 23-K345R-C347M | Ac-V L E D L R D M G L F | 11.0 ± 1.4 | 1.12 | 3   |
| Ac-peptide 23-K345R-C347V | Ac-V L E D L R D V G L F | 2.0 ± 1.3 | 0.71 | 3   |
| Role of Gly-348   |                  |                 |       |     |
| Ac-$G_{t_{34}}$-(340–350)-K341R-G348A | Ac-I RE N L K D C A L F | 783.9 ± 1.6 | 1.33 | 3   |

348 in the $n + 1$ position in the dark-bound conformation (51) and the light-bound conformation (52, 53). Consistent with this observation, peptides in which Gly was substituted with $\text{d}$-Leu, which would be predicted to break the $\beta$-turn, lost functional activity (51). Meanwhile, $\text{d}$-Ala, predicted to maintain the peptide structure, was almost as potent as the parent peptide (51). Using molecular dynamics calculations to estimate the relative energy to form the $\beta$-turn, with Gly being taken as 0 kcal mol$^{-1}$, we found that the energy required would be 0 kcal mol$^{-1}$ for a Gly at position 348, 3.2 kcal mol$^{-1}$ for a Gly at position 348, and 88.2 kcal mol$^{-1}$ for $\text{d}$-Ala. We therefore made a synthetic peptide with a Gly to Ala substitution (Ac-$G_{t_{34}}$-(340–350)-K341R-G348A). The $\text{d}$-Ala substitution at this position is much milder than that of $\text{d}$-Leu, and it was of interest to see whether this analog could stabilize Meta II. As predicted by the energy calculation, the peptide with the Gly to Ala substitution at position 348 was only minimally capable of stabilizing Meta II, with an EC$_{50}$ value of nearly 1 mm and maximal stabilization of 15% at 1.8 mm (Fig. 4, Table II). Thus, the Gly at position 348 appears to be essential for the ability of the C-terminal peptide to effectively stabilize Meta II. This result is consistent with the presence of a type II $\beta$-turn at the C terminus of the peptide. Alternatively, the binding pocket on rhodopsin may not be able to tolerate bulky side chains at position 348. This alternative interpretation is not as likely, because substitution of the Gly at this position with $\text{d}$-Ala is equipotent at stabilizing Meta II.

### The Role of Lys-341 in the Peptide Stabilization of Meta II—The six highest affinity peptide analogs from the combinatorial library had all replaced the positive charge at position 341 with a hydrophobic residue (54). To further test the idea that a hydrophobic residue at position 341 increases the affinity for Meta II, we substituted a Leu for Lys at position 341 in the Ac-$G_{t_{34}}$-(340–350)-K341L-C347Abu peptide and tested its ability to stabilize Meta II (Fig. 5). We found that these changes substantially enhanced the EC$_{50}$ such that the EC$_{50}$ of Ac-$G_{t_{34}}$-(340–350)-K341L-C347Abu is 127 $\mu$M, whereas that of Ac-$G_{t_{34}}$-(340–350)-K341L-C347Abu is 44 $\mu$M. When peptides were tested with the K341L substitution that had also been substituted with a hydrophobic residue at position 347 (Met or Val), we found EC$_{50}$ values of 19.6 $\mu$M for Ac-$G_{t_{34}}$-(340–350)-K341L-C347Abu (●). Data presented are the average of at least three independent experiments ± the standard error of the mean, except for Ac-$G_{t_{34}}$-(340–350)-K341L-C347Abu, which was only tested twice.
The dose response for the ability of each of the listed peptide analogs to stabilize Meta II was measured as described under “Materials and Methods.” Bold letters indicate amino acid residues that differ relative to the native Gtα-(340–350) sequence. The Δ symbol represents 2-aminobutyric acid (Abu). Dose-response curves were analyzed by non-linear regression using a sigmoidal dose-response variable slope equation (GraphPad Prism) to obtain the EC50 values, S.E., and slope. The number of independent experiments, done in duplicate, is listed as n.

| Peptide Sequence | EC50 ± S.E. | Slope | n |
|------------------|-------------|-------|---|
| Role of Lys-341  |             |       |   |
| Ac-Gtα-(340–350)-K341L-C347Abu | 43.7 ± 1.3 | 0.67  | 4 |
| Ac-Gtα-(340–350)-K341L-C347M  | 19.6 ± 1.2 | 0.64  | 4 |
| Ac-Gtα-(340–350)-K341L-C347V  | 43.6 ± 2.7 | 0.54  | 2 |

| Peptide Sequence | EC50 ± S.E. | Slope | n |
|------------------|-------------|-------|---|
| Role of random sequences |             |       |   |
| Peptide 23 V L E D L K D C G L F | 0.7 ± 1.1 | 1.07  | 2 |
| Peptide 23 long T G G R V L E D L K D C G L F | 0.4 ± 1.1 | 1.39  | 3 |
| Peptide 24 M L K N L K D C G M F | 152.1 ± 1.8 | 0.89  | 3 |
| Peptide 24 long K G Q A M L K N L K D C G M F | 10.1 ± 1.1 | 0.93  | 4 |

can be substituted with any of those residues, confirming that it interacts in a hydrophobic manner with the receptor. However, the changes did cause a decrease in the potency (11.0 μM EC50 for Ac-peptide 23-C347M, 2.0 μM EC50 for Ac-peptide 23-C347V, and 10.6 μM EC50 for Ac-peptide 23-C347Abu compared with 0.5 μM EC50 for Ac-peptide 23). Therefore, even though these peptides were all more potent than the native peptide at binding Meta II, changes at the critical 347 position diminished the affinity of the Ac-peptide 23 analogs for the receptor. Thus, the detailed fit of many residues within the peptide binding site on rhodopsin appear to be critical for stabilization of Meta II.

Our results corroborate that the Lys at position 341 decreases the affinity of native Gtα for rhodopsin. One might wonder why such a disruptive residue at the site of receptor interaction would be present. Perhaps it is important for the G protein to have a lower affinity for the receptor so that the receptor can be a more efficient catalyst and thus more rapidly catalyze activation of more G proteins following ligand activation. We are currently testing this idea in full-length Gtα by making a K341L point mutation. We predict that a G protein with this single mutation will have a higher affinity for rhodopsin.

The Role of Random Linker in the Peptide Stabilization of Meta II—To examine the effect of the random 4-amino acid linker present on the high affinity peptide analogs identified in the combinatorial screening, we synthesized and tested the full-length 15-mer peptides for clones 23 and 24 (peptides 23 and 24). The 15-mer of peptide 24 long was found to be 15-fold more potent than the corresponding 11-mer peptide (Fig. 2, Table IV). Thus, although no obvious consensus sequences were present in the 4-amino acid linker region of the sequenced clones (54), the region may contribute to the affinity for light-activated rhodopsin in some cases.

The results presented here confirm that the C terminus of Gtα is critical in stabilizing the active conformation of the receptor. Understanding the changes that can be tolerated in this region is essential to furthering our knowledge of how G protein–coupled receptors interact with their cognate G proteins. Previous work from our laboratory indicates that C-terminal peptides can compete with G protein for binding receptor and therefore potentially block signal transduction (16). By screening a receptor of interest, it should be possible to select for peptide sequences with high affinity for the receptor that will competitively inhibit receptor-G protein interaction and potentially block signal transduction through that receptor. Peptide sequences so obtained can then be expressed in cells as minigenes (17, 65), providing a facile approach for studying G protein signaling.

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