Communication

Efficient Interaction with a Receptor Requires a Specific Type of Prenyl Group on the G Protein \(\gamma\) Subunit

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Post-translational prenylation of the carboxyl-terminal cysteine is a characteristic feature of the guanine nucleotide-binding protein (G protein) \(\gamma\) subunits. Recent findings show that the farnesylated COOH-terminal tail of the \(\gamma 1\) subunit is a specific determinant of rhodopsin-transducin coupling. We show here that when synthetic peptides specific to the COOH-terminal tail of \(\gamma 1\) are chemically modified with geranyl, farnesyl, or geranylgeranyl groups and tested for their ability to interact with light activated rhodopsin, the farnesylated peptide is significantly more effective. These results show that an appropriate isoprenoid on the G protein \(\gamma\) subunit serves not only a membrane anchoring function but in combination with the COOH-terminal domain specifies receptor-G protein coupling.

Heterotrimeric (\(\alpha\)\(\beta\)\(\gamma\)) G proteins\(^1\) control key signaling pathways inside a cell by relaying information from activated transmembrane receptors to specific effector molecules (1–3). All known G protein \(\gamma\) subunits are modified by either a 15-carbon farnesyl or by a 20-carbon geranylgeranyl moiety (4, 5). The \(\gamma 1\) subunit, which is associated with the G protein \(GT\), is farnesylated, whereas most other \(\gamma\) subunits are geranylgeranylated. Prenylation occurs on the cysteine in the COOH-terminal CAAX motif (C is a cysteine, A is an aliphatic amino acid, X is any amino acid), and the nature of the last residue is thought to determine farnesylation or geranylgeranylation.

Prenylation of the \(\gamma\) subunits has been shown to be necessary for G protein membrane attachment (6–8). There is also evidence that suggests the involvement of the prenyl group in protein-protein interactions (9–14). However, it is unclear why G protein \(\gamma\) subunits are modified by two different types of isoprenoids. Studies of some prenylated proteins (rhodopsin kinase (10), p21 (11), yeast a-factor (13)) demonstrate that the biological consequences of differential prenylation may be more complex than anticipated. It has also been difficult to examine the role of the different isoprenoids because recombinant proteins with altered CAAX domains seem to be prenylated with a mixture of farnesyl and geranylgeranyl (13, 15). To circumvent this problem, we have used peptides chemically modified with various isoprenoids, since we could confirm appropriate prenylation accurately in each case by mass spectrometry.

To identify the role of the isoprenoid, we have examined peptides chemically modified with farnesyl, geranyl (C-10), and geranylgeranyl, for their relative efficacies at stabilizing light activated rhodopsin. We have recently shown that the farnesylated COOH-terminal domain of the \(\gamma 1\) subunit directly interacts with an activated receptor (light-activated rhodopsin) and is important for the holomeric G protein to effectively couple with rhodopsin (12, 16). Both the farnesyl group and the appropriate primary structure of the COOH-terminal domain of \(\gamma 1\) are essential for effective rhodopsin-Gt interaction. By asaying farnesylated peptides of varying lengths we have identified the portion of the \(\gamma 1\) COOH-terminal region that is necessary for optimal interaction with the receptor. More importantly, we show that a receptor can distinguish between the \(\gamma\) subunit domain modified with different prenyl groups: farnesyl, geranyl, and geranylgeranyl.

MATERIALS AND METHODS

Chemical Synthesis and Purification of Prenylated Peptides—Solid phase peptide synthesis, mass spectrometry, and amino acid analysis were performed at the Protein and Nucleic Acid Chemistry Laboratory, Washington University School of Medicine. Chemical prenylation of peptides was performed essentially as we described before (12). The trans-geranyl bromide and trans,trans-farnesyl bromide were from Aldrich, all-trans-geranylgeranyl bromide was from American Radiolabeled Chemicals, Inc., St. Louis, MO. The peptide thiodiion was generated with carbonate and alkylated with the corresponding isoprenyl bromide. 5 \(\mu\)M peptide was dissolved in 50\% n-propyl alcohol. \(Na_2CO_3\) was added to a final concentration of 20 mM. The reaction was started by adding 30 \(\mu\)M isoprenyl bromide as a 10% solution in n-propyl alcohol. For the geranylgeranylation a mixture of butanol/methanol/water = 1/1/1 (v/v/v) was used as an alternative solvent. The reactions were carried out at room temperature for 24 h in the dark in nitrogen. Preynlated peptides were purified by reverse phase chromatography on a PepRPC FPLC column (Pharmacia Biotech Inc.) using a linear (0–100%) gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. An additional chromatographic step was employed using a linear (70–100%) gradient of acetonitrile in water containing 0.1% trifluoroacetic acid to separate the unreacted geranylgeranyl bromide from the geranylgeranylated peptide. Molecular weights of purified peptides were determined by electrospray mass spectrometry. Concentrations of the peptides in working solutions were estimated by quantitative amino acid analysis. Results were consistent with the predicted values after dilutions of the peptide stock solutions in water 20–100 times.

Preparation of Urea-washed Rod Outer Segment Membranes—Urea-washed rod outer segment membranes were prepared as described before (17).

Stabilization of a Light-activated Metarhodopsin II—Spectral analysis of rhodopsin was performed with a Beckman DU-64 spectrophotometer at 4 °C as we described previously (12). Rhodopsin bleached for 2 h under continuous illumination at room temperature was used as a reference. Measurements were performed in 10 mM Tris-HCl, pH 8.0, 2 mM MgCl\(_2\), 1 mM EDTA, 1 mM diithiothreitol, and 140 mM NaCl using quartz cuvettes with a 10-mm light path. Peptides were added at appropriate concentrations to both the reference and the sample cuvettes containing 4 \(\mu\)M rhodopsin. The sample was exposed to 480-nm light for 20 s and light spectra were recorded a minute later. The amount of metarhodopsin II (MII) formed was measured as an A\(_{395}\)/A\(_{470}\) difference.
RESULTS AND DISCUSSION

The assay used in these studies was based on the quantitative analysis of spectrally different intermediates of rhodopsin, metarhodopsin I (M1), and MII. Absorption of light by dark-adapted rhodopsin triggers the formation of a relatively stable equilibrium of two major rhodopsin intermediates, M1 (A_max = 495 nm) and MII (A_max = 380 nm) (18). Under the experimental conditions used here (4°C, pH 8.0), Gt stabilizes MII and significantly shifts the ratio of MII/M1 toward MII formation (19, 20). Synthetic peptides specific to different domains on Gt, the COOH-terminal regions of αt (21) and γ1 (12), mimic the heterotrimer by stabilizing MII in a dose-dependent fashion.

To define the optimal length of the COOH-terminal domain of γ1 required for interaction and stabilization of MII, we synthesized a set of eight peptides of various lengths (5 amino acid long to 22 amino acid long, all of them ending with the cysteine, Fig. 1). Extending the amino acid sequence at the NH2 terminus farther than 14 amino acids did not produce any significant change in peptide-MII interaction. These results showed that the COOH-terminal 14 residues contain all the information for appropriate recognition of the peptide by rhodopsin. These results also confirmed our original observation that the farnesylated COOH-terminal 12 amino acids of γ1 represent a distinct domain critical for rhodopsin-Gt interaction. To examine the role of the isoprenoid attached to this domain, the 12-amino acid-long peptide, γ1(D60-C71), was modified with different isoprenoids.

The COOH-terminal cysteine in the peptide was modified chemically with geranyl (C-10), farnesyl (C-15), and geranylgeranyl (C-20) moieties (γ1(D60-C71)g, γ1(D60-C71)f, and γ1(D60-C71)gg) (Fig. 2a). Chromatographic behavior of the three peptides on a reverse phase FPLC column was as anticipated, based on their predicted relative hydrophobicities (Fig. 2b). It was notable, however, that the solubility of all three peptides in water was the same as observed empirically and as determined by the quantitative amino acid analysis of the working solutions of these peptides in comparison with stock solutions.

Peptides modified with different isoprenoids were then compared in the MII stabilization assay. All three peptides were able to stabilize MII. The relative efficacies, however, were counter to the hydrophobic properties of the geranyl, farnesyl, and geranylgeranyl groups. The farnesylated peptide was most effective, followed by the geranylgeranylated and geranylated peptides (Fig. 3). The corresponding EC50 (half-maximal effective concentration) values were 40, 200, and 500 μM, assuming that the maximal extent of MII stabilization for the three peptides was the same. Since our original results showed that the interaction of the γ1 tail and rhodopsin is predominantly hydrophobic, the reduced potency of the geranylated peptide in terms of stabilizing MII is not surprising. However, the reduced efficacy of the geranylgeranylated peptide in comparison with the farnesylated peptide was unanticipated, since it is more hydrophobic. If the efficacy of interaction had been in a fashion proportional to the hydrophobicity of a prenyl group (geranylgeranyl > farnesyl > geranyl), it could have been due to the higher effective concentration of more hydrophobic peptides near rhodopsin. In fact, behavior correlating with the relative hydrophobicities of peptides has been noted in the case of geranylgeranylated peptides, which inhibit the interaction of αt and βγt subunits more potently than when farnesylated (22). However, the γ1(D60-C71)gg peptide is five times less effective at MII stabilization than the γ1(D60-C71)f peptide. This effect cannot be explained by poor solubility of γ1(D60-C71)gg. The amounts of prenylated peptides added to rhodopsin are the same as confirmed by the quantitative amino acid analysis of working solutions (see ‘Materials and Methods’). Since the effect we observed is opposite to the hydrophobic nature of the γ1(D60-C71)gg, we conclude that ineffective MII stabilization by the γ1(D60-C71)gg reflects poor interaction of this peptide with rhodopsin. Earlier results have shown that the appropriate primary structure of the COOH-terminal tail of γ1 is important for interaction with rhodopsin (12, 16). In combination with the results here, it can be inferred that rhodopsin contains
a site that specifically recognizes both the COOH-terminal domain of γ₁ and the appropriate isoprenoid. This inference is supported by studies of rhodopsin kinase interaction with rhodopsin. Rhodopsin kinase is normally farnesylated and exhibits light-dependent translocation to the membrane, indicating that it binds to the light-activated rhodopsin rather than to the lipid surface. A geranylgeranylated mutant of rhodopsin kinase was effective at phosphorylating rhodopsin, but it stayed attached to the rhodopsin-containing membranes independent of light. The farnesyl group, thus, appears to be recognized specifically by a site on rhodopsin which binds it with sufficiently low affinity such that the characteristic cycling of transducin between cytosol and membrane during visual transduction is not disrupted.

The γ subunits of G proteins can be divided into two groups based on whether their COOH-terminal cysteine is modified by farnesyl or geranylgeranyl moieties. Several subtypes that are geranylgeranylated and at least three members that are potentially farnesylated have been identified (23, 24).² It is possible that the G protein-linked receptors in general possess sites that specifically recognize both the γ subunit COOH-terminal protein domain and the appropriate isoprenoid.

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² S. Kalyanaraman, G. B. Downes, and N. Gautam, unpublished data.