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The Effect of Carboxyl-terminal Mutagenesis of $G_t\alpha$ on Rhodopsin and Guanine Nucleotide Binding*

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The carboxyl terminus of G protein α subunits plays an important role in receptor recognition. To identify the amino acids that participate in this interaction, COOH-terminal mutants of α_t (the transducin α subunit) were expressed *in vitro* and analyzed for their ability to interact with rhodopsin and to bind guanine nucleotide. Gly-348, the reported site of a β turn, was replaced with other neutral amino acids without severely affecting rhodopsin binding. However, proline substitution abolished rhodopsin interaction, suggesting that flexibility is important at this site. A comparison between C347Y, which lost both rhodopsin and guanine nucleotide binding, and a mutant substituted with α_q sequence (D346E/C347Y/G348N/F350V), in which guanine nucleotide binding was restored, implies that distinct motifs maintain the structure of the α subunit and are necessary for selective interaction with receptors. Surprisingly, mutants L344A, L349A, F350stop, and stop351A demonstrated a parallel loss of rhodopsin and guanine nucleotide binding. Altered profiles of L344A and F350stop on sucrose density gradients indicate that these mutants may undergo denaturation. The equivalent of α_tL344A generated in α_s and α_i did not show such a severe loss of guanine nucleotide binding, revealing that the α_t carboxyl terminus is unique in its susceptibility to changes in amino acid sequence.

Transducin (G_t),[1] a heterotrimeric G protein activated by rhodopsin in response to light, regulates visual signal transduction pathways in the vertebrate rod cell. Because of their relative abundance, the G_t α, β, and γ subunits and rhodopsin have been used extensively as structural models for the study of receptor-G protein interaction. Several investigations have indicated that the COOH terminus of the α subunit plays an important role in receptor recognition. For example, the interaction of α_t with rhodopsin can be disrupted by ADP-ribosylation of the COOH-terminal cysteine (Cys-347) with pertussis toxin (1). A synthetic peptide corresponding to the COOH-terminal 11 amino acids of the α subunit can also block the binding of α_t to rhodopsin. When added alone, this peptide can stabilize the active form of rhodopsin, metarhodopsin II, a property of the G protein heterotrimer (2). NMR studies have demonstrated that this peptide exhibits dramatic conformational changes when incubated with light-activated rhodopsin, strongly suggesting that the COOH terminus of α_t binds to rhodopsin and undergoes conformational changes necessary for the promotion of guanine nucleotide exchange (3). A number of studies (for review, see Ref. 4) have verified the participation of the α subunit COOH terminus in receptor coupling for other G proteins (5–12). Despite the extensive literature on this subject, the mechanism by which these amino acids interact with G protein-coupled receptors is not well understood, although it has been proposed that a conserved glycine in the $\alpha_{i/o/t}$ family plays a central role in regulating the conformation of the COOH terminus (3, 11).

In this report, we undertake a mutational strategy to define the COOH-terminal amino acids of α_t that participate in receptor interaction. Efforts to express the full-length α_t polypeptide have so far been unsuccessful using either bacterial or baculovirus expression, although recently an amino-terminal truncation of α_t has been expressed (13). We have chosen the method of *in vitro* translation, which has allowed us to generate full-length, radiolabeled α_t for rhodopsin interaction and guanine nucleotide binding studies. This system has been used successfully by several laboratories to investigate both the receptor and effector coupling properties of α_s (14, 15) and guanine nucleotide binding properties of α_o (16). Our studies have revealed the selective importance of specific amino acids in the interaction between the α_t and rhodopsin. We have also observed that certain mutations in the COOH terminus destroy both rhodopsin binding and guanine nucleotide binding, perhaps by significantly altering their tertiary structure. Equivalent mutants in α_i and α_s were not as severely affected, suggesting that the α_t COOH terminus is unique in its structural properties compared to other G protein α subunits.

EXPERIMENTAL PROCEDURES

Isolation of Rod Outer Segments—Urea-stripped rod outer segments (ROS) containing rhodopsin were isolated in the dark from frozen, dark-adapted retina (W. H. Lawson, Lincoln, NE) on 25/30% (w/w) sucrose gradients as described (17, 18). The concentration of functional rhodopsin, quantified by absorbance at 500 nm and using an extinction coefficient of 42,700 M^{-1} cm^{-1} (19), was 4 $\mu g/\mu l$. The purity was approximately 90%, estimated by Coomassie Blue staining on SDS-polyacrylamide gels. ROS membranes were analyzed for the presence of α_t and $\beta\gamma$ by immunoblot analysis (20). The membranes were chromatographed on 10% polyacrylamide gels, transferred to nitrocellulose, and incubated with AS/7, an anti-COOH-terminal antibody that recognizes both α_t and α_i (DuPont NEN), and with an antibody that recognizes $G_t\beta\gamma$ (a gift from Dr. Gary L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). The binding of the antibodies was visualized by incubation with ^{125}I-protein A and

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[1] The abbreviations used are: G_t, transducin; ROS, rod outer segment; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

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autoradiography.

Mutagenesis of α_t—The *Eco*RI-*Pst*I fragment from the α_t cDNA (a gift from Dr. Henry Bourne, University of California at San Francisco) (21) was inserted into pSelect (Promega) at the *Hin*dIII site by the addition of *Hin*dIII linkers. Site-directed mutagenesis was performed using the "Altered Sites" mutagenesis system according to the manufacturer's protocols (Promega).

Preparation of in Vitro Translated Subunits—The double-stranded pSelect-α_t and mutant constructs were linearized with *Eco*RV and incubated with T7 polymerase to synthesize sense RNA (22). The RNAs were incubated at 30 °C for 1 h with rabbit reticulocyte lysate (Promega) in the presence of 2 μl of [^{35}S]methionine (1200 Ci/mmol; Amersham Life Sciences) in a final volume of 25 μl according to the manufacturer's directions. The radioactivity incorporated into protein was measured as the insoluble fraction from a hot trichloroacetic acid precipitation (23). The mutant and wild-type α_s and α_{i2} cDNAs (24) were inserted into the *Hin*dIII site of pSP72 (Promega). All procedures for *in vitro* transcription and translation were carried out as described for α_t.

Rhodopsin Binding Assay—The *in vitro* translation products were diluted 2-fold and centrifuged through Bio-Spin P-6 columns (Bio-Rad) to remove salts and free nucleotides. The eluates were equilibrated in buffer A (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol). Urea-stripped ROS membranes containing 7 μg (0.18 nmol) of rhodopsin were mixed with approximately 50,000 cpm (0.02 pmol) of *in vitro* translated mutant or wild-type α_t in buffer A and incubated for 30 min at 4 °C. Experiments were performed in the light or the dark and in the presence or absence of 200 μM GTPγS. To terminate the assay, the reaction mixtures were centrifuged in a TLA45 rotor (Optima TLX centrifuge, Beckman) at 45,000 rpm through a cushion of 0.2 M sucrose in buffer A. After rinsing with buffer A, the pellets were resuspended in SDS-Laemmli sample buffer (25) and chromatographed on 10% SDS-polyacrylamide gels. The gels were treated with Amplify (Amersham) and autoradiographed. The amount of mutant or wild-type α_t bound to ROS membranes was quantified by phosphorimage analysis (Molecular Dynamics). The specific binding of α_t to rhodopsin was calculated as the amount bound in the absence of GTPγS minus the amount bound in its presence. For each construct, an aliquot of the eluate from the Bio-Spin P-6 columns was chromatographed on SDS-polyacrylamide gels and quantified by phosphorimage analysis to determine the amount of radioactivity specifically incorporated into the α_t band. These values were used to correct for differences in expression of the various *in vitro* translated α_t subunits in the rhodopsin binding assay. Under these conditions, approximately 15% of the added wild-type α_t binds to ROS membranes.

Trypsin Resistance Assay—This assay was used to measure GTPγS binding to the G protein α subunits. Approximately 30,000 cpm (0.012 pmol) of the *in vitro* translation reaction were diluted into 25 μl of buffer B (50 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol) and incubated for 2 h at 30 °C with 100 μM GTPγS. The trypsin resistance assay was initiated by incubation with 0.3–0.6 μg of trypsin (sequencing grade modified trypsin, Promega) for 30 min at 30 °C. The reaction was terminated by the addition of SDS-Laemmli sample buffer (25). The samples were boiled and chromatographed on 10% SDS-polyacrylamide gels. The gels were treated with Amplify and analyzed as described above. For each construct, an aliquot of the *in vitro* translation product was chromatographed on SDS-polyacrylamide gels and quantified by phosphorimage analysis to correct for differences in expression levels as described above.

Sucrose Density Gradient Sedimentation—In vitro translated products of α_t and α_t mutants (80,000 cpm, 0.032 pmol) were mixed with 100 μl of buffer A and overlaid on a 5–20% linear sucrose gradient in buffer A (16). After centrifugation at 4 °C for 16 h at 55,000 rpm in an SW55Ti rotor, the gradients were fractionated into 250-μl aliquots per tube and chromatographed on SDS-polyacrylamide gels followed by phosphorimage analysis. The two peak fractions from each sample were selected and reapplied to SDS-polyacrylamide gels, followed again by phosphorimage analysis for estimation of the sizes of the different peaks.

RESULTS

Fig. 1 shows the COOH-terminal 11 amino acids of α_t and 5 other members of the G protein α subunit family. Two of these amino acids, Leu-344 and Leu-349 in α_t, are conserved among all G protein α subunits. A positively charged amino acid (Lys-345 in α_t) and a hydrophobic amino acid at the COOH terminus is conserved in most α subunits. Gly-348, previously shown in α_t to be critical for the interaction of a synthetic peptide corresponding to this sequence with rhodopsin (3), is preserved in α_i and α_o but is replaced by other amino acids outside the $\alpha_{i/o/t}$ family.

	340					345					350
α_t (340-350)	I	K	E	N	L	K	D	C	G	L	F
α_{i2} (345-355)	I	K	N	N	L	K	D	C	G	L	F
α_{oA} (344-354)	I	A	N	N	L	R	G	C	G	L	Y
α_q (343-353)	L	Q	L	N	L	K	E	Y	N	L	V
α_s (384-394)	Q	R	M	H	L	R	Q	Y	E	L	L
α_{12} (369-379)	L	Q	E	N	L	K	D	I	M	L	Q

FIG. 1. **The COOH-terminal amino acid sequence of six representative G protein α subunits.** The *numbers in parentheses* correspond to the amino acids of each α subunit displayed in the figure. The *boxes* outline the two leucine residues conserved among all of the α subunits. The *underlined amino acids* in α_t are those chosen for mutagenesis in this study.

A binding assay for measuring the interaction of α_t with rhodopsin-containing membranes was developed using ^{35}S-labeled, *in vitro* translated protein. This assay is based on the formation of a stable complex between rhodopsin and G_t in the absence of guanine nucleotide (26–28). Fig. 2*A* shows the binding of *in vitro* translated α_t and α_s to bovine ROS membranes. The binding of α_t to rhodopsin in the light is approximately twice the level of binding in the dark. The addition of GTPγS, a nonhydrolyzable GTP analog, releases α_t from the membranes, reflecting the promotion of guanine nucleotide exchange by rhodopsin in this system. In contrast, α_s, which does not interact with rhodopsin, showed no significant increase in binding in the light compared to samples kept in the dark and was not released by incubation with GTPγS. The observed GTPγS-dependent release of α_t from ROS membranes incubated in the dark suggests that some of the rhodopsin in these preparations is already bleached. For this reason, GTPγS-dependent binding (the difference between the binding of α_t to photolyzed rhodopsin in the presence and absence of GTPγS) rather than light-dependent binding (the difference between α_t binding to rhodopsin in the light and in the dark) was used in all subsequent experiments as a measure of specific interaction with rhodopsin. It has been established by several laboratories that α_t requires the presence of $\beta\gamma$ to form a stable complex with rhodopsin (29–31), suggesting that $\beta\gamma$ is present in our assay system. Immunoblots (Fig. 2*B*) performed with subunit-specific antibodies (32) confirmed the presence of low levels of α_t, β, and γ (data not shown) in these ROS preparations. Therefore, the *in vitro* translated α_t and ROS membranes were incubated together without further addition of $\beta\gamma$ to the rhodopsin binding assay.

The COOH-terminal seven amino acids of α_t were mutated individually to alanines, expressed by *in vitro* translation, and assayed for rhodopsin binding. Fig. 3*A* shows an autoradiogram of the rhodopsin binding assay. The results were quantified by phosphorimage analysis and are shown in Fig. 3*B*. Mutants L344A and L349A demonstrated the most severe (96 and 93%, respectively) loss of binding. C347A, the site of ADP-ribosylation by pertussis toxin (33), G348A, the site of a proposed β turn in the COOH terminus (3), and F350A were moderately affected, demonstrating a 32, 36, and 30% loss of binding to rhodopsin, respectively. All of the alanine mutants that bound to rhodopsin (K345A, D346A, C347A, G348A, and F350A) were released by the addition of GTPγS, indicating that they are able to undergo receptor-mediated guanine nucleotide exchange.

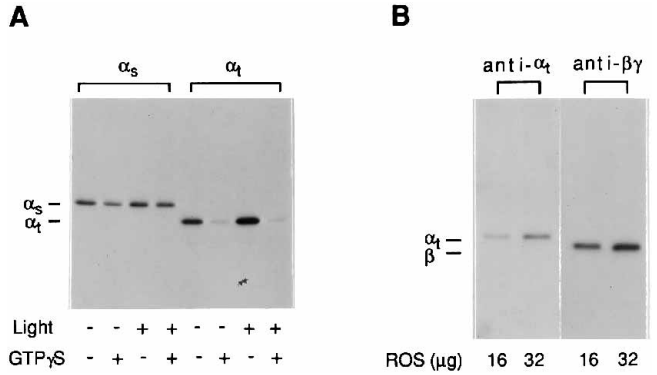


FIG. 2. **The binding of α_t and α_s to ROS membranes.** *A*, [^{35}S]methionine-labeled, *in vitro* translated α_t and α_s were incubated with ROS membranes in the dark or in the light, with or without GTPγS and analyzed as described under "Experimental Procedures." *B*, Western blot analysis of ROS membranes. Urea-stripped ROS membranes were immunoblotted with antibodies against α_t and $\beta\gamma$ antibodies and visualized as described under "Experimental Procedures."

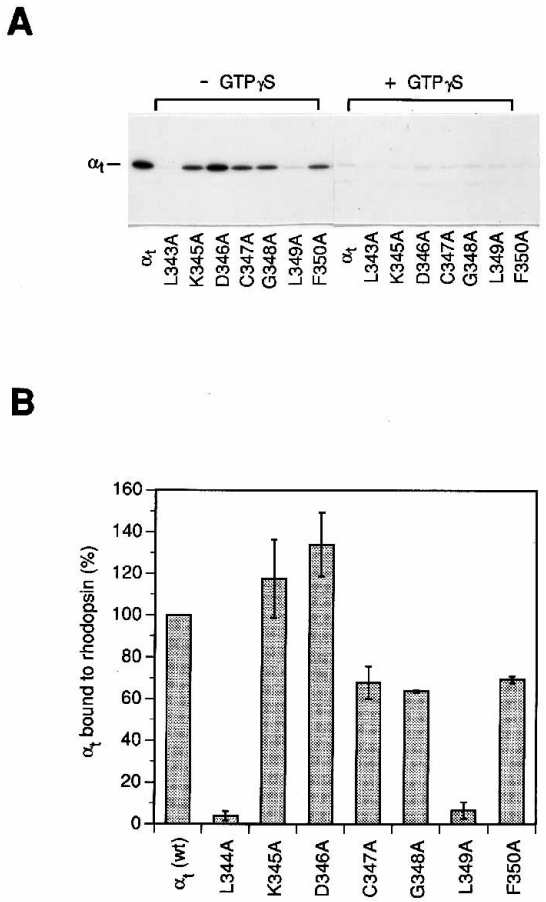


FIG. 3. **The binding of α_t COOH-terminal alanine mutants to rhodopsin.** *A*, mutations generated in the COOH terminus of α_t were expressed by *in vitro* translation in the presence of [^{35}S]methionine and incubated with ROS membranes in the light, in the presence or absence of GTPγS. The samples were visualized by SDS-PAGE and autoradiography as described under "Experimental Procedures." *B*, phosphorimage analysis of the results shown in *A*. The results represent the binding of the α_t mutants to rhodopsin as a percent of the binding of wild-type α_t. The *bars* represent the averages of at least three independent experiments. The *error bars* represent S.E.

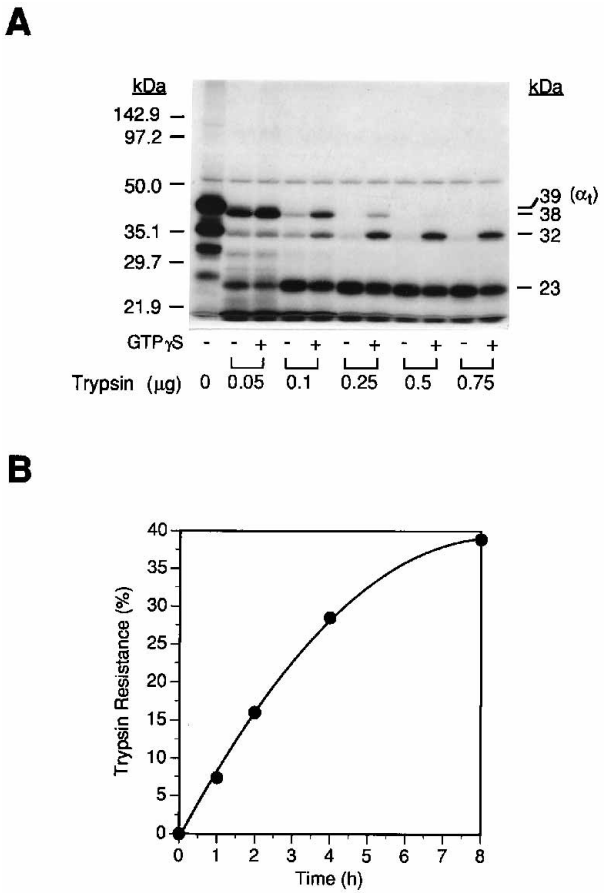


FIG. 4. **Trypsin resistance of *in vitro* translated α_t as a measure of guanine nucleotide binding.** *A*, concentration dependence of GTPγS-dependent trypsin resistance. [^{35}S]Methionine-labeled, *in vitro* translated α_t was preincubated with or without 100 μM GTPγS for 2 h, followed by the addition of the indicated concentrations of trypsin and incubation for 30 min. The reaction was terminated by the addition of Laemmli sample buffer (25) and analyzed by SDS-PAGE as described under "Experimental Procedures." In addition to the full-length 39-kDa (wild-type α_t) band, other bands appear in the absence of trypsin and GTPγS that represent translation initiation at methionines downstream from the primary initiation codon. A 38-kDa band, appearing at lower concentrations of trypsin, is a partial digest of α_t, described previously (37). The 32- and 23-kDa bands are GTPγS- and GDP-protected fragments of α_t, respectively. *Numbers* on the *left side* of the gel indicate approximate molecular weights of prestained molecular markers (Bio-Rad). *B*, time course of GTPγS binding to α_t using the trypsin resistance assay. [^{35}S]Methionine-labeled α_t was incubated with 100 μM GTPγS for the indicated duration, followed by incubation with 0.5 μg of trypsin for 30 min. The samples were subjected to SDS-PAGE followed by phosphorimage analysis. The results are presented as a percent of trypsin-resistant 32-kDa band compared to undigested α_t.

The sensitivity of the two leucines to disruption by alanine mutagenesis raised questions concerning the structural integrity of these mutants. G protein-coupled receptors function as ligand exchange enzymes, meaning that they promote the exchange of a ligand, in this case guanine nucleotide bound to the G protein α subunit (34). Basal guanine nucleotide exchange occurs in α_t at a slow but measurable rate that is enhanced approximately 70-fold in the presence of photolyzed rhodopsin (32). The loss of basal GTPγS binding in the α_t mutants would suggest more extensive changes in the overall structure of the subunit. The binding of guanine nucleotide can be measured by examining the resistance of α_t to digestion by trypsin in the presence of GTPγS (35–37). Fig. 4*A* demonstrates that *in vitro* translated α_t is digested almost entirely by high concentrations of trypsin in the absence of guanine nucleotide. In contrast, a 32-kDa band is protected from digestion in the presence of GTPγS. A 23-kDa band observed in the trypsin-digested samples is due to protection by GDP present in the *in vitro* translation mixture (16, 37). The amount of the trypsin-resistant 32-kDa band increased to 40% of the total *in vitro* translated α_t

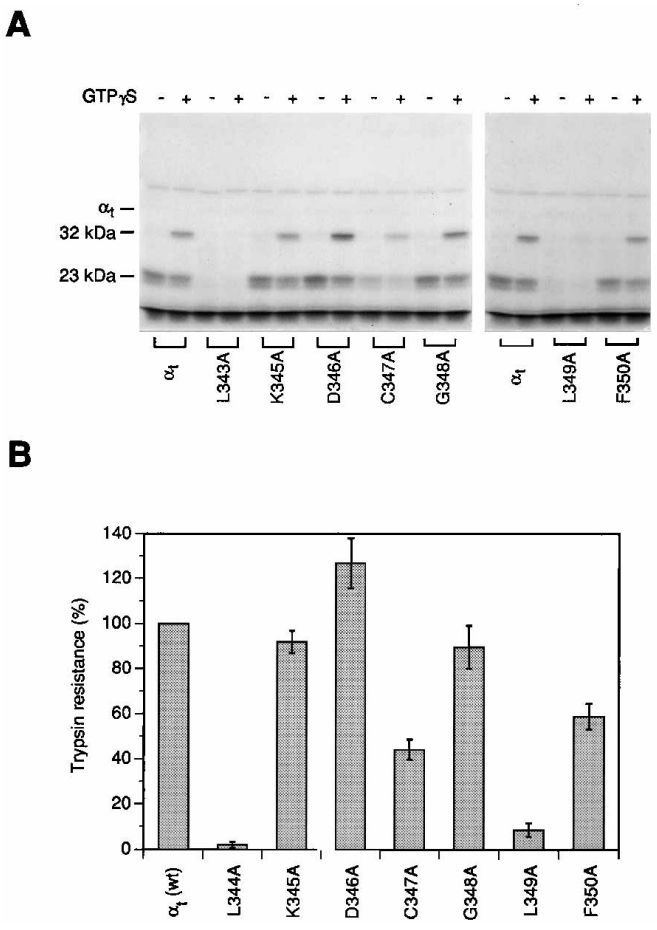


FIG. 5. **Trypsin resistance of α_t COOH-terminal alanine mutants in the presence of GTPγS.** *A*, *in vitro* translated [^{35}S]-labeled α_t mutants were incubated in the presence and absence of GTPγS and subjected to trypsin digestion followed by SDS-PAGE and autoradiography as described under "Experimental Procedures." *B*, phosphorimage analysis of the results shown in *A*. The amount of 32-kDa band (GTPγS bound fragment) was quantified by phosphorimage analysis. For each mutant, the results represent the amount of 32-kDa trypsin-resistant fragment as a percent of the amount of wild-type α_t 32-kDa fragment after trypsin digestion. The *numbers* represent the average of four to six samples. The *error bars* represent S.E.

during 8 h of incubation with GTPγS (Fig. 4*B*). In contrast, more than 80% of α_s and α_i is protected after only a 2-h incubation in GTPγS (data not shown), suggesting that these α subunits undergo basal guanine nucleotide exchange at a much higher rate than α_t in our assay mixture, which contains approximately 6 mM Mg^{2+} ion.

The COOH-terminal alanine mutants were tested for their ability to resist digestion by trypsin in the presence of GTPγS (Fig. 5). C347A and F350A showed a partial decrease in trypsin resistance of 56 and 41%, respectively. The most dramatically affected mutants were L344A and L349A. Very little of the protected 32-kDa band was detected in these samples. Therefore, these mutants appear to be unable to bind GTPγS. Neither L344A nor L349A showed significant amounts of the 23-kDa protected fragment, indicating that they may not bind GDP. Incubation with very high concentrations (2 mM) of either GTP or GDP also did not protect these mutants from trypsin digestion, although both the 32- and the 23-kDa fragments were apparent in samples of wild-type α_t (data not shown). Therefore, these mutants are incapable of binding any form of guanine nucleotide. These results demonstrate a parallel loss of both rhodopsin binding and guanine nucleotide binding for L344A and L349A.

To investigate in more detail the influence of mutation on the properties of α_t, the seven COOH-terminal residues were replaced with different amino acids and assayed for both rhodopsin binding and trypsin resistance (Fig. 6). When isoleucine, a hydrophobic amino acid, was introduced at Leu-344 and Leu-349, the resulting proteins demonstrated trypsin resistance at 69 and 50% of wild-type levels, respectively, and rhodopsin binding at 43 and 27% of wild-type levels, respectively, suggesting that these sites are more critical for rhodopsin binding than for guanine nucleotide binding. Interestingly, mutation to phenylalanine at these positions resulted in significantly reduced activity in both assays, particularly for L344F, perhaps due to steric hindrance caused by the larger size of this amino acid. Rhodopsin binding and guanine nucleotide binding are both abolished by the substitution of proline for Lys-345 or Asp-346. These data suggest that fixing the angle of the COOH terminus with a proline at residue 345 or 346 disrupts both activities of the protein. In contrast, the introduction of a proline at Gly-348 dramatically inhibited rhodopsin interaction but caused only a 39% decrease in intrinsic guanine nucleotide binding. Mutation of Gly-348 to asparagine (G348N) or leucine (G348L) caused significant (53 and 57%, respectively) losses in rhodopsin binding but only small (18 and 27%, respectively) losses in guanine nucleotide binding. Mutation of Cys-347, also conserved in the $\alpha_{i/o/t}$ family, to alanine, caused a partial decrease in both rhodopsin binding (to 68%) and guanine nucleotide binding (to 44%). In contrast, both receptor binding and guanine nucleotide binding were lost when this residue was mutated to tyrosine, the amino acid found at the equivalent position in both α_s and α_q. The COOH-terminal residue, Phe-350, is tolerant to substitutions, retaining activity when changed to alanine (weakly hydrophobic) and glutamine (hydrophilic), and exhibiting significantly reduced activity (a 72 and a 65% loss in rhodopsin binding and trypsin resistance, respectively) only when mutated to glutamic acid, a negatively charged amino acid.

Additional point mutations and deletions at the amino and COOH termini of α_t were compared for rhodopsin and guanine nucleotide binding activities (Fig. 7). The double mutation K345A/D346A, in which two charged residues are eliminated, and a mutation equivalent to the α_o sequence, D346G/F350Y, bound guanine nucleotide and interacted with rhodopsin as well as wild-type α_t. In contrast, the mutant D346E/C347Y/G348N/F350V, which represents the COOH-terminal sequence of α_q, could not bind rhodopsin but bound guanine nucleotide normally. Interestingly, removal of a single amino acid from the COOH terminus (F350stop) was sufficient to destroy both rhodopsin and guanine nucleotide binding, despite the tolerance of Phe-350 to point mutagenesis shown in Fig. 6. Extension of the COOH terminus by a single alanine (stop351A) also caused a complete loss of both rhodopsin and guanine nucleotide binding. Mutations at the amino terminus, such as G2A (in which the myristoylation site is removed), and the deletions delN6/A7M and delN10/H11M resulted in a loss of receptor binding (data not shown) but no loss of guanine nucleotide binding, suggesting that the loss of guanine nucleotide binding and rhodopsin interaction simultaneously is specific to the COOH terminus.

This parallel loss of rhodopsin and guanine nucleotide exhibited by the COOH-terminal mutants could be due to denaturation of the polypeptides. To investigate this possibility, the wild-type protein and mutants L344A, G348A, and F350stop were analyzed by sucrose density gradient sedimentation (Fig. 8). The peak fractions of all of these proteins were at the same position in the gradient. Similar amounts of G348A were recovered from the gradients compared to wild-type α_t. However,

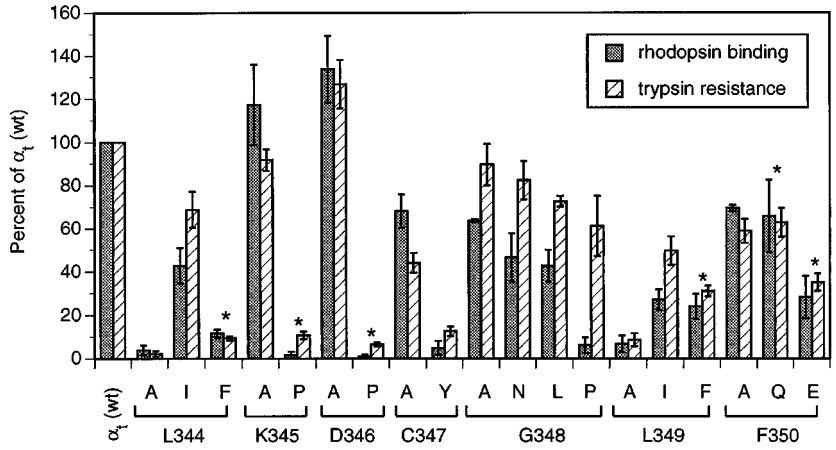


FIG. 6. **Rhodopsin binding and trypsin resistance for additional α_t COOH-terminal point mutants.** The seven COOH-terminal amino acid residues of α_t were subjected to further mutagenesis. These mutants were expressed by *in vitro* translation, radiolabeled with [^{35}S]methionine, and used for rhodopsin binding and trypsin resistance assays as described under "Experimental Procedures" and the legends to Figs. 3 and 5. *, the *error bars* represent the ranges of two samples for both assays. In all other samples, the *error bars* represent S.E. determined from at least three samples. For comparison, the results shown in Figs. 3 and 5 are included in this figure.

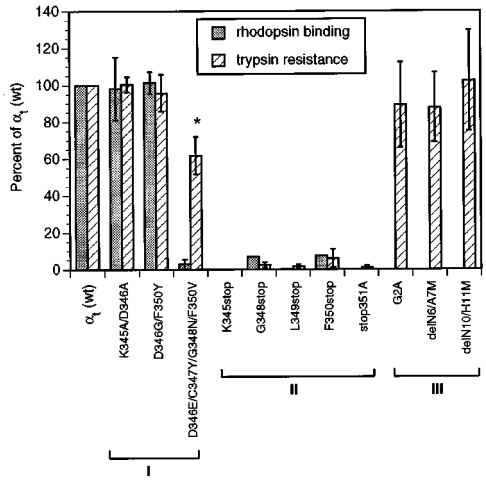
substantially less L344A and F350stop were recovered, despite equal loading of these proteins on the gradients. No peaks were observed at any other position on the sucrose gradients, which could account for the lost radioactivity. A similar result was observed by Denker *et al.* (16) with a 14-amino acid COOH-terminal truncation of α_o, which, when stripped of guanine nucleotide, formed no discrete peaks on sucrose density gradients. These data imply that L344A and F350stop are less stable than wild-type α_t and are likely to be denatured in our assays.

There have been no reports in the literature of such sensitivity to mutagenesis in the COOH terminus of other G proteins, raising the prospect that our observations are specific for α_t. To test this possibility, the equivalent of the mutant α_tL344A was also generated in α_s (L388A) and α_{i2} (L349A). Neither α_sL388A nor α_{i2}L349A demonstrated a severe decrease in GTPγS-dependent trypsin resistance compared to their respective wild-type proteins, indicating that these mutants are better able to bind guanine nucleotide than the equivalent α_t mutant (Figs. 9 and 10). Because α_i is closely related in sequence to α_t and can be activated by rhodopsin (38), the ability of α_iL349A to bind rhodopsin was measured (Fig. 10). This mutant showed only a modest decrease in rhodopsin binding (35%) compared to wild-type α_i. Therefore, the extreme sensitivity of the α_t COOH terminus to mutagenesis does not appear to be shared with other members of the G protein α subunit family.

DISCUSSION

A number of laboratories have suggested that information for receptor interaction resides in the COOH terminus of G protein α subunits (1–3, 5–12). Using NMR, Dratz *et al.* (3) described the structure of a synthetic peptide corresponding to the COOH-terminal 11 amino acids of α_t (amino acids 340–350) bound to rhodopsin. Gly-348 was shown to be essential to the formation of a β turn, which was necessary for the binding of the COOH-terminal peptide to rhodopsin. In these studies, substitution of this glycine with leucine abolished the binding of the synthetic peptide. In our experiments, replacement of Gly-348 with alanine, leucine, or asparagine partially reduced rhodopsin binding, whereas substitution with proline abolished this interaction. All of these mutations had a less severe effect on guanine nucleotide binding, suggesting that this residue is critical for the interaction of α_t with rhodopsin. However, the effect of mutation of Gly-348 on rhodopsin binding was less severe than would be predicted from the synthetic peptide studies described above. Perhaps other regions in the full-length protein also interact with rhodopsin and partially compensate for the loss of this COOH-terminal residue. For example, residues 311–329 have been proposed to interact with rhodopsin and to participate in receptor-catalyzed guanine nu-

A



B

I. C-terminal multiple point mutants

	340					345					350	
α_t(wt)	I	K	E	N	L	K	D	C	G	L	F	
K345A/D346A	-	-	-	-	-	A	A	-	-	-	-	
D346G/F350Y	-	-	-	-	-	-	G	-	-	-	Y	(=α_o)
D346E/C347Y/G348N/F350V	-	-	-	-	-	-	E	Y	N	-	V	(=α_q)

II. C-terminal truncation and addition mutants

	340					345					350
α_t(wt)	I	K	E	N	L	K	D	C	G	L	F
K345stop	-	-	-	-	-	*					
G348stop	-	-	-	-	-	-	-	-	*		
L349stop	-	-	-	-	-	-	-	-	-	*	
F350stop	-	-	-	-	-	-	-	-	-	-	*
stop351A	-	-	-	-	-	-	-	-	-	-	-	A

III. N-terminal deletion and point mutants

	1				5					10				15
α_t(wt)	M	G	A	G	A	S	A	E	E	K	H	S	R	E	L
G2A	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-
delN6/A7M							M	-	-	-	-	-	-	-	-
delN10/H11M											M	-	-	-	-

FIG. 7. **Rhodopsin binding and trypsin resistance of amino- and COOH-terminal mutants of α_t.** *A*, amino- and COOH-terminal mutants of α_t were assayed for their ability to bind rhodopsin and for trypsin resistance in the presence of GTPγS, as described in the legends to Figs. 3 and 5 and under "Experimental Procedures." Group I, rhodopsin binding was determined from duplicate samples; group II, rhodopsin binding was determined from a single sample. *, trypsin resistance was determined from four samples for D346E/C347Y/G348N/F350V. All *error bars* represent the range of duplicate samples except D346E/C347Y/G348N/F350V, which is shown as S.E. The groups I, II, and III are described in Fig. 7*B*. *B*, sequences of the mutations used for rhodopsin binding and trypsin resistance assays shown in *A*.

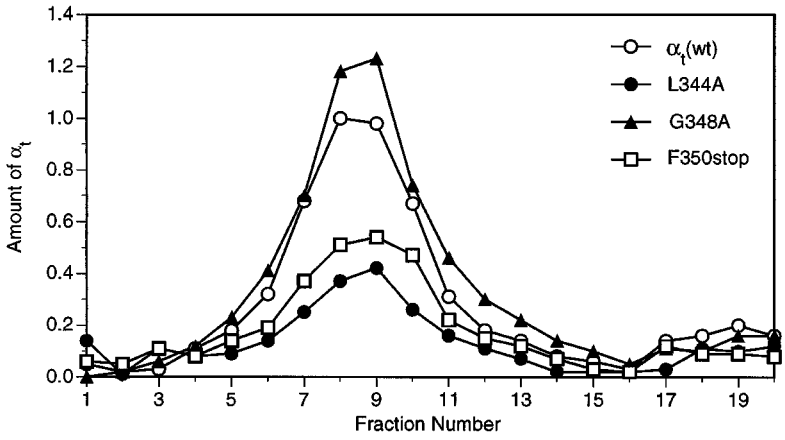


FIG. 8. **Sedimentation profiles of α_t and α_t mutants on sucrose density gradients.** 80,000 cpm of *in vitro* translated products were overlaid on 5–20% linear sucrose gradients and centrifuged for 16 h at 55,000 rpm in an SW55Ti rotor at 4 °C. After centrifugation, the gradients were fractionated into 250-μl aliquots per tube and chromatographed by SDS-PAGE followed by phosphorimage analysis as described under "Experimental Procedures." The data are representative of three independent experiments.

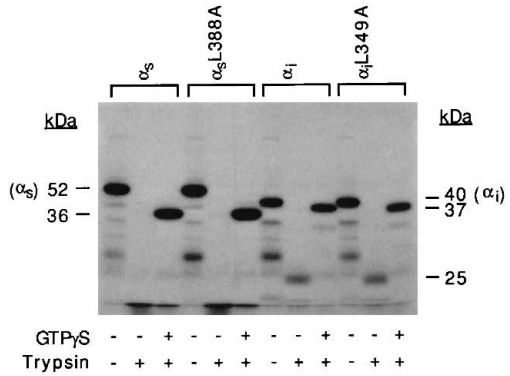


FIG. 9. **Trypsin resistance of α_s and α_i mutants.** Mutants at the equivalent position with α_tL344A in α_s (L388A) and α_i (L349A) were assayed for resistance to trypsin in the presence of GTPγS. The α_sL388A and α_iL349A mutants produced trypsin-resistant fragments of 36 and 37 kDa, respectively. Several bands that appear below the major *in vitro* translation product in the lanes without trypsin are peptides translated from ATG sites downstream from the ATG initiation site.

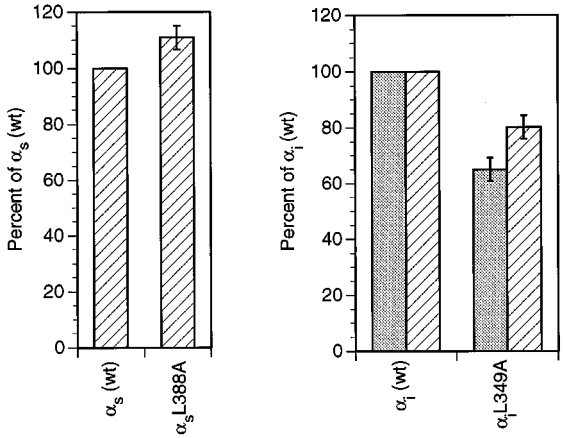


FIG. 10. **Rhodopsin binding and trypsin resistance in α_s and α_i mutants.** Rhodopsin binding and trypsin resistance assays were performed for α_s L388A and α_i L349A, as described under "Experimental Procedures." *Error bars* represent the range of duplicate samples. *Shaded bars*, rhodopsin binding; *lined bars*, trypsin resistance.

cleotide exchange (2, 39). Dratz *et al.* (3) also reported that the β turn in the rhodopsin-bound conformation of the peptide disappeared when rhodopsin was converted to metarhodopsin II by exposure to light. From this result, a requirement for flexibility at Gly-348 for binding to light-exposed rhodopsin may be inferred, which is consistent with our observation that proline is more effective at disrupting rhodopsin binding than other amino acids at this position. Proline substitutions at the neighboring residues, Lys-345 and Asp-346, abolished both rhodopsin binding and guanine nucleotide binding. Therefore, these two residues are more critical for guanine nucleotide binding than is Gly-348.

Conklin *et al.* (11) reported that replacement of the last three amino acids of α_q with the sequence found in α_i (amino acids NLV to GLF) was sufficient to allow the activation of this chimera by the D_2 dopamine receptor, a G_i-coupled receptor. Therefore, not only is the COOH terminus responsible for binding to receptor, it also contains information that determines the selectivity between G proteins and their receptors. In our experiments, C347Y, a substitution in α_t with the α_q amino acid, exhibited a dramatic loss of both rhodopsin and guanine nucleotide binding. Substitution of this amino acid along with other amino acids corresponding to α_q sequence (D346E/C347Y/G348N/F350V) resulted in a rescue of guanine nucleotide binding but no restoration of rhodopsin binding. These data indicate that the tertiary structure of the surrounding domains may be drastically affected by a single mutation but is preserved when the sequence of another α subunit representing a complete structural motif is introduced. Understanding the role of C347Y and its surrounding structural motif in receptor interaction will require further study.

The removal or addition of a single amino acid at Phe-350 but not its substitution resulted in a loss of both receptor coupling and guanine nucleotide binding, suggesting that the length of the COOH terminus is critical for the function of α_t. A dramatic loss of both guanine nucleotide binding and rhodopsin binding was observed for the COOH-terminal mutants L344A and L349A as well. Mutations in Lys-345 and Asp-346 (to proline), Cys-347 (to tyrosine), and Phe-350 (to glutamic acid) also exhibited a parallel loss, either partial or complete, of both rhodopsin and guanine nucleotide binding. In contrast, the amino-terminal mutants (G2A, delN6/A7M, and delN10/H11M) showed only a loss of rhodopsin binding. This unexpected sensitivity of the COOH terminus for changes in both activities was shown to be specific for α_t. For example, mutations equivalent to L344A in α_s (L388A) and α_i (L349A) do not exhibit a large decrease in guanine nucleotide binding. Moreover, α_iL349A showed only a small decrease in rhodopsin binding compared to the wild-type protein. In α_o, removal of 5, 10, or even 14 amino acids from the COOH terminus was reported to have no effect on the binding of GTPγS, although the 14-amino acid deletion mutant lost affinity for GDP (16). Therefore, α_s, α_i, and α_o are clearly less susceptible than α_t to alteration of the COOH terminus. The reduced recovery of L344A and F350stop from sucrose density gradients suggest that the α_t COOH-terminal mutants are unstable and susceptible to denaturation, which may account for the loss of both rhodopsin and guanine nucleotide binding.

The α_t subunit is known to possess several structural and functional properties that are distinct from other closely related G protein α subunits. Unlike α_i or α_o (40, 41), the rate of receptor-independent guanine nucleotide exchange in α_t is not affected by magnesium ion (32). X-ray crystallographic analysis also shows differences between α_t and α_i at the sites of contact with guanine nucleotide (42). Other differences are apparent in the amino terminus. When counted from the common β1 strand, α_t has the shortest amino terminus and is not modified by palmitic acid like other α subunits of this family (α_i, α_o). In addition, α_t is heterogeneously acylated with myristate or one of three other fatty acids (43, 44), whereas α_i is acylated only with myristic acid. This heterogeneous modification is so far unique to retinal photoreceptor cells (45). Therefore, it is presumed that *in vitro* translated α_t (synthesized in a rabbit reticulocyte lysate) is predominantly myristoylated and similar in modification to α_i, ruling out differences in myristoylation, at least, as an explanation for our observations. Many laboratories using a variety of techniques have concluded that the amino and COOH termini are closely associated, allowing for coordinate regulation of $\beta\gamma$ and receptor binding, as well as control of guanine nucleotide binding (37, 46–48). At present, the possibility that the unique properties of the α_t COOH terminus are due to association with a unique amino terminus is speculative because the extreme amino termini of α_t (amino acids 1–26) and α_i (amino acids 1–32) have not been resolved by x-ray crystallography. However, the orientation of the adjacent regions is consistent with their close approximation (39, 42, 49, 50). Future studies in which the amino terminus from α_i is substituted for that of α_t in the L344A, L349A, and F350stop mutants, for example, will help to clarify the relationship between these two domains and their role in rhodopsin and guanine nucleotide binding.

The present report describes the use of mutagenesis to understand the role of the COOH-terminal amino acids of α_t in rhodopsin binding. Of the alanine mutants, L344A and L349A showed the most dramatic decrease in rhodopsin binding and guanine nucleotide binding. Alternative substitutions suggest a preference for hydrophobic amino acids at these positions. Mutations in Gly-348 demonstrated that this residue is more important for rhodopsin binding than for guanine nucleotide binding and that flexibility at this position is critical for the interaction of the COOH terminus with rhodopsin. A comparison between C347Y and a mutant substituted with the α_q sequence (including C347Y) implies that structural motifs exist in the COOH terminus that maintain the structure of the α subunit and are necessary for selective interaction with G-protein-coupled receptors. Mutations at nearly every position in the COOH terminus of α_t but not in the amino terminus caused an unexpected parallel loss of rhodopsin binding and guanine nucleotide binding. Since mutations equivalent to α_tL344A in α_s and α_i did not exhibit a dramatic decrease in these activities, we conclude that the α_t COOH terminus is uniquely susceptible to amino acid alterations compared to other closely related G-protein α subunits, perhaps reflecting distinct biochemical properties necessary for function of this rod cell-specific G protein.

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Note Added in Proof—During the review of this manuscript, Garcia *et al.* (51) reported the effects of mutagenesis of the α_t COOH terminus on rhodopsin binding. Their results are partly similar to our own.

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