The EMBO Journal vol.14 no.18 pp.4460–4469, 1995

Transducin-α C-terminal mutations prevent activation by rhodopsin: a new assay using recombinant proteins expressed in cultured cells

Pablo D.Garcia, Rene Onrust, Sean M.Bell, Thomas P.Sakmar[1] and Henry R.Bourne[2]

Department of Cellular and Molecular Pharmacology, Department of Medicine, Program in Cell Biology, and Cardiovascular Research Institute, University of California, San Francisco CA 94143-0450 and [1]Howard Hughes Medical Institute, Laboratory of Molecular Biology and Biochemistry, Rockefeller University, New York, NY 10021, USA

[2]Corresponding author

We have measured the activation by recombinant rhodopsin of the α-subunit (α_t) of retinal transducin (G_t, also recombinant) using a new assay. Cultured cells are transiently transfected with DNAs encoding opsin and the three subunits of G_t (α_t, β_1 and γ_1). In the microsomes of these cells, incubated with 11-*cis*-retinal, light causes the rapid activation of G_t, as measured by the ability of GTPγS to protect α_t fragments from proteolytic degradation. The activation of G_t is also observed when *all-trans*-retinal is added to microsomes under constant illumination. Activation depends on both opsin and retinal. Opsin mutants with known defects in activating G_t show similar defects in this assay. α_t mutations that mimic the corresponding mutations in the α-subunit of G_s also produce qualitatively similar effects in this assay. As a first step in a strategy aimed at exploring the relationships between structure and function in the interactions of receptors with G proteins, we tested mutant α_t proteins with alanine substituted for each of the 10 amino acids at the C-terminus, a region known to be crucial for interactions with rhodopsin. Alanine substitution at four positions moderately (K341) or severely (L344, G348, L349) impairs the susceptibility of α_t to activation by rhodopsin. All four mutants retain their ability to be activated by AlF_4^-. Two other substitutions (N343 and F350) resulted in very mild defects, while substitutions at the remaining four positions (E342, K345, D346 and C347) had no effect. In combination with previous observations, these results constrain models of the interaction of the C-terminus of α_t with rhodopsin.

Keywords: α-subunit/C-terminal mutations/retinal transducin/rhodopsin

Introduction

Heterotrimeric GTP binding proteins (G proteins) transmit signals from cell surface receptors to effector enzymes or ion channels (Freissmuth *et al.*, 1989; Birnbaumer, 1990; Bourne *et al.*, 1990; Kaziro *et al.*, 1991). These effectors regulate the concentrations of intracellular second messengers and/or changes in membrane potential, signals that in turn trigger and modulate cellular responses. G proteins cycle between active and inactive states with respect to their ability to regulate effectors. In the inactive state, the G protein's α-subunit (α) binds GDP and forms a complex with the βγ subunit (βγ), a heterodimer of β and γ polypeptides. In the active state, α binds GTP and cannot bind βγ. The G protein cycle begins when an extracellular ligand activates a cell surface receptor. The activated receptor then binds to the G protein heterotrimer and reduces the affinity of α for GDP. The 'empty' α-subunit quickly binds GTP, which is in excess over GDP in the cell. GTP-induced conformational changes in α activate the G protein, causing α•GTP to dissociate from βγ and both α•GTP and βγ to dissociate from the receptor. Finally, the cycle is completed when the intrinsic GTPase activity of α hydrolyzes GTP to GDP, returning α to its inactive state. Thus, the initiation of the G protein cycle depends upon the ability of activated receptors to catalyze GDP/GTP exchange. Termination of the cycle depends upon the GTPase activity of α.

Two kinds of approach will accelerate the understanding of the molecular mechanisms responsible for the steps in the G protein cycle: (i) analyzing the 3-D structures of G proteins, their receptors and a diverse array of their effectors; and (ii) designing a more useful experimental systems for studying each step in the cycle, in which the functional consequences of structural changes can be rapidly assessed and evaluated in detail. With respect to the first, recent reports have described the 3-D structures of two α-subunits, α_t and α_{i1}, bound to GDP, GTP or AlF_4^- (Noel *et al.*, 1993; Coleman *et al.*, 1994; Lambright *et al.*, 1994; Sondek *et al.*, 1994); the determination of the 3-D structures of βγ complexes is eagerly awaited, while those of their receptors (Baldwin, 1993; Schertler *et al.*, 1993) and effectors will probably take longer to define.

This paper focuses on the second approach, in particular with respect to a model experimental system for analyzing the structure and function in receptor-promoted GDP/GTP exchange, the first step of the G protein cycle. One way to study this step involves assessing the interactions among receptor and G protein components resolved and purified from biological sources and assayed in detergent solutions or reconstituted lipid vesicles. A second method seeks to study the interactions among recombinant receptors and G protein subunits in the potentially more natural environment of a cell membrane. The latter approach can make possible the rapid study of a large number of mutations, if components are expressed after transient transfection, but it has two major disadvantages: (i) results—and therefore inferences from them—are often rather indirect, in that the experimental 'read-out' may depend upon measuring the downstream consequences of effector activation (e.g. cAMP, inositol phosphates or Ca^{2+} release); and (ii) the presence of an endogenous receptor, G

protein subunit or effector often confounds the conclusions with respect to the effect of a mutation in the corresponding transfected recombinant protein. Faced with these difficulties, we have devised a straightforward and reliable experimental system for directly assessing the interactions between the G protein of retinal rod outer segments, transducin (G_t), and the retinal photoreceptor, rhodopsin. The system uses microsome fractions of cultured cells transfected with DNAs encoding rhodopsin and G_t subunits. It does not require purified components and can be used to test mutations in both rhodopsin and α_t.

After validating the new assay by testing the effects of α_t and rhodopsin mutations studied previously in other systems, we applied it to test the effects of alanine substitutions at 10 positions in the C-terminal tail of α_t. Considerable evidence has implicated this region as a key site for Gα interaction with receptors. In combination with previous observations, our results constrain the models of the interaction of the C-terminus of α_t with rhodopsin.

Results

α_t activation assayed by trypsin sensitivity

DNAs for bovine opsin and the three subunits of G_t were cotransfected at high efficiencies (usually 60–80%) into COS-7 cells. After expression of the transfected DNAs for 44–48 h, cells were harvested and a crude microsomal fraction was prepared. β_1 and γ_1 DNAs were included in the transfections because the presence of both (but not of either alone) increased by 2- to 4-fold the amounts of α_t associated with microsomes (results not shown). Immunoassays showed that concentrations of α_t and opsin in microsomal preparations ranged between 0.6 and 4.8 and 0.03 and 0.25 nmol/mg protein, respectively, depending on the transfection efficiencies.

The microsomal fractions were used in an *in vitro* assay for the rhodopsin-dependent activation of α_t. Active α_t was determined by measuring the appearance of trypsin-resistant fragments of α_t produced by conformational changes that follow the binding of GTPγS (Fung and Nash, 1983), as detailed in Materials and methods. Briefly, microsomal membranes were incubated at 20°C for 10 min in the absence or presence of 10 μM 11-*cis*-retinal in the dark, or *all-trans*-retinal in ambient light, before the addition of 10 μM GTPγS. Aliquots of the reaction mixture were removed before and at various times after the addition of GTPγS. The activation reaction was stopped by transferring the aliquots to an ice water bath and quickly mixing with a solution containing detergent, trypsin and excess GDP. Proteolysis proceeded for 1 h at 0°C before the addition of protease inhibitors. Proteolytic products were then resolved by SDS–PAGE and α_t fragments were detected and quantified on Western blots.

α_t activation requirements and kinetics

In microsomal membranes of transfected cells incubated in the dark with 11-*cis*-retinal, no trypsin-resistant fragments of α_t were observed before the addition of GTPγS (Figure 1A and B, lanes labeled –60 s), indicating that α_t was in the inactive GDP-bound form. Trypsin-resistant α_t fragments of ~31 kDa appeared only after the addition of GTPγS (Figure 1A and B, arrows), indicating the formation of active α_t•GTPγS. In time course experiments carried

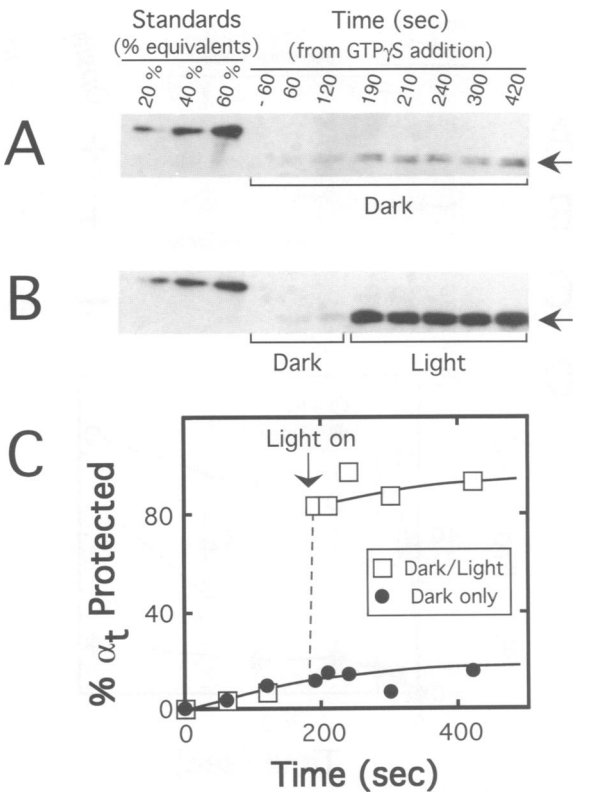

Fig. 1. Light-dependent activation of α_t. Microsomal fractions from COS-7 cells expressing bovine opsin, α_t, β_1 and γ_1 were incubated with 11-*cis*-retinal under a dim-red light. Aliquots were removed at the indicated times before and after the addition of GTPγS and treated with trypsin, as described in Materials and methods. Arrows indicate the 31 kDa trypsin-resistant α_t fragment. (**A**) The experiment was carried out under dim-red light for its entire duration. (**B**) The sample was illuminated with a 250 W lamp 180 s after the addition of GTPγS. (**C**) A quantitative analysis of the appearance of trypsin-resistant α_t fragments determined using standard samples included in each SDS–PAGE, as described in Materials and methods.

out in the dark (Figure 1A), protease-resistant α_t fragments appeared slowly and accumulated in amounts representing only a small fraction (~10–15%; see below) of α_t (Figure 1A and C). In microsomes exposed to light 3 min after the addition of GTPγS (Figure 1B), trypsin-resistant fragments appeared rapidly (detectable in <10 s) and involved most (~80–90%; see below) of the α_t present (Figure 1B and C).

The rapid activation of α_t was also observed when *all-trans*-retinal (the product of 11-*cis*-retinal photoisomerization) was added to the microsomes (Figure 2B) under ambient illumination. In the absence of retinal, the activation of α_t was much slower (Figure 2A), similar to the rate of activation in the dark with 11-*cis*-retinal. The appearance of trypsin-resistant fragments also depended on the presence of opsin in the microsomes; no GTPγS-protected fragments appeared in microsomes incubated with *all-trans*-retinal lacking opsin (Figure 2C). Thus, the slow α_t activation observed in Figure 2A was probably caused by the basal (non-stimulated) activity of opsin in the absence of retinal (Surya *et al.*, 1995). The slow activation of α_t observed in the dark in the presence of 11-*cis*-retinal (Figure 1A) could be caused by the non-stimulated activity of opsin not regenerated with 11-*cis*-retinal or the presence of small quantities of *all-trans*-retinal formed by thermal isomerization.

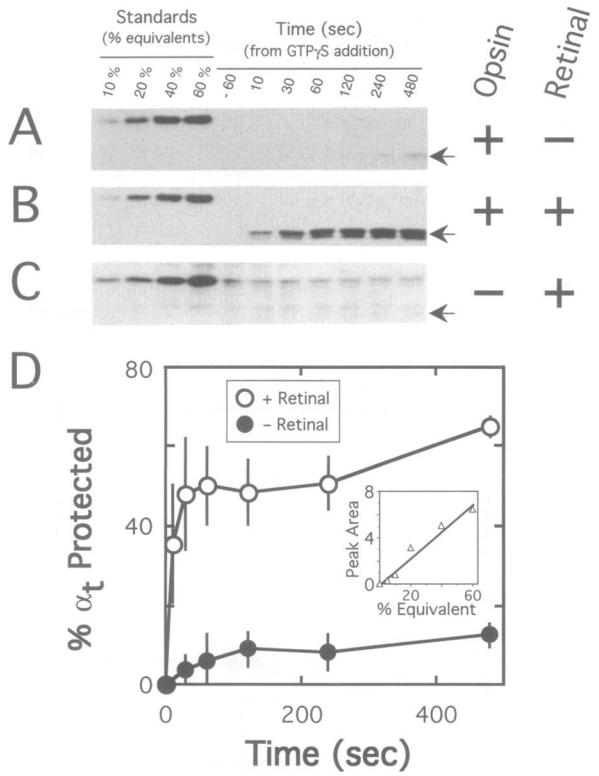

Fig. 2. Opsin- and retinal-dependent activation of α_t. Microsomal fractions from COS-7 cells expressing wild-type bovine opsin, α_t, β_1 and γ_1 were incubated in the absence (**A**) or presence (**B**) of *all-trans*-retinal. Aliquots were removed 60 s before and at the indicated times after the addition of GTPγS and treated with trypsin, as described in Materials and methods. The experiment shown in (**C**) was performed in the presence of retinal using microsomes from cells transfected with α_t, β_1 and γ_1 but without opsin. The quantitative time course (**D**) of the appearance of trypsin-resistant α_t fragments from microsomes containing opsin, α_t, β_1 and γ_1 was determined as described in Materials and methods. They represent the average of eight or six experiments in the presence and absence, respectively, of *all-trans*-retinal. Error bars represent two standard errors of the mean (SEM). The inset in (D) is a representative standard curve and corresponds to the experiment shown in (B). Similar standard curves were determined for each experiment.

The time course of appearance of trypsin-resistant α_t fragments (Figures 1C and 2D) was analyzed by quantitative immunoblotting. Each SDS–PAGE included as standards samples containing varying amounts of undigested reaction mixture (see Materials and methods). Standard curves (e.g. Figure 2D, inset), in which the amount of reaction mixture loaded was directly proportional (by densitometry) to the area of the peak of absorbance, were used to determine the percentage of protected (i.e. activated) α_t at each time point (Figures 1C and 2D). This quantitative analysis shows that in the presence of 11-*cis*-retinal, light promotes the very fast (essentially complete in 10 s) activation of G_t (Figure 1C). G_t activation promoted by *all-trans*-retinal was slower, with more than half of maximal α_t activation completed during the first 30 s after the addition of GTPγS. Although G_t activation was slower, the addition of *all-trans*-retinal was more attractive as the basis of a routine assay because of the difficulty of working in dim red light. Therefore, the protocol using *all-trans*-retinal was used in all experiments described below.

To compare the rates of α_t activation, we determined the slopes of the curves during the linear phase of activation (first 30 s after the addition of GTPγS in the presence of *all-trans*-retinal). For wild-type rhodopsin and α_t the average slope was 1.9%s^{-1} in the presence of *all-trans*-retinal, although the rates varied somewhat (1.4–2.3%s^{-1}) between experiments probably because each determination was based on only two data points. In the absence of *all-trans*-retinal, the rate of appearance of trypsin-resistant fragments was at least 60-fold slower (~0.03%s^{-1}, Figure 2D). The total fraction of α_t eventually activated was also somewhat variable (40–60% in most experiments). Because this variation was seen in multiple experiments with a single microsomal preparation, it presumably represents experimental variability rather than intrinsic differences between microsomal preparations. The maximal extent of α_t activation was independent of the concentration of GTPγS, between 1 and 100 μM (data not shown). In different microsomal preparations, the number of α_t molecules activated (~50% of the total) exceeded the number of rhodopsin molecules present in microsomes by 8- to 19-fold.

Activation of α_t depends on functional rhodopsin

We tested the effects of previously described rhodopsin mutations, including three mutations in intracellular loop 2 (ic2) and one in ic3. The ic2 mutations included E134R/R135E (CD1; Franke *et al.*, 1990), the replacement of residues 140–152 by a different sequence (CD2; Franke *et al.*, 1990) and the deletion of residues 143–150 (ΔCD1; Franke *et al.*, 1992); the ic3 mutation was S240A/T242G/T243G (EF6; Franke *et al.*, 1992). In experiments using pure recombinant rhodopsin and transducin purified from rod outer segments, the CD1, CD2 and ΔCD1 mutants did not activate transducin, while the EF6 mutant activated transducin at only one-tenth the rate observed with wild-type rhodopsin; all four mutants bound retinal normally (Franke *et al.*, 1990, 1992). In close agreement with these results, no GTPγS-protected α_t fragments appeared in retinal-treated microsomes carrying the CD2 mutant (Figure 3A); the same result was seen with microsomes containing the CD1 and ΔCD1 mutants (results not shown). In agreement with its reported quantitative defect (Franke *et al.*, 1992), microsomes containing the EF6 mutant (Figure 3B) showed a very slow rate of α_t activation in the presence of *all-trans*-retinal (Figure 3C). Decreased opsin expression was not responsible for these defects. Quantities of mutant opsins in microsomes (0.04 and 0.10 nmol/mg of protein; see the legend to Figure 3) were comparable with that of wild-type opsin (0.03–0.25 nmol/mg of protein).

α_t mutations cognate to mutations in α_s

In α_t we tested two mutations cognate to mutations first discovered in α_s, the α-subunit of the stimulatory regulator of adenylyl cyclase, G_s. One α_s mutation was identified in cultured cells that had lost their ability to synthesize cAMP in response to a β-adrenergic receptor (β-AR) agonist. This mutant, α_s-G226A, failed to respond to stimulation by β-AR agonists, cholera toxin or AlF_4^-, apparently because the methyl group of the mutated residue impairs the protein's ability to switch into the active conformation upon binding GTP (Miller *et al.*,

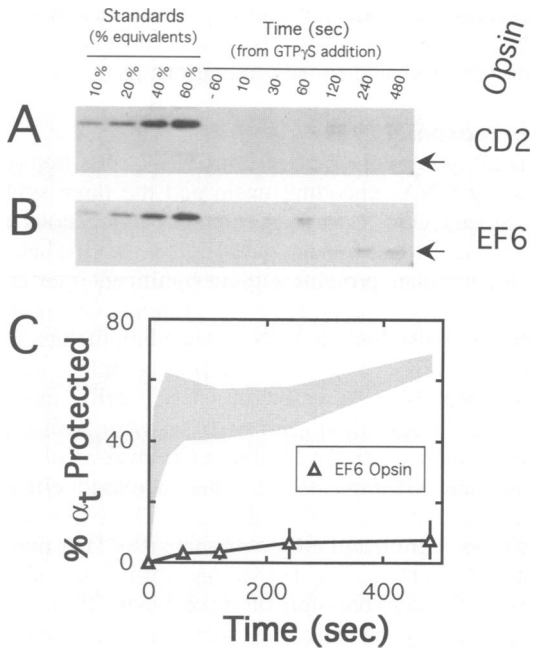

Fig. 3. Effect of opsin mutations on α_t activation. Microsomes from cells transfected with α_t, β_1, γ_1 and opsin mutants CD2 (**A**) or EF6 (**B**) in the presence of *all-trans*-retinal were exposed to GTPγS for the indicated times and tested for the appearance of trypsin-resistant α_t fragments, as in previous figures. Opsin mutants CD1 and ΔCD1 produced results similar to those seen with mutant CD2 (data not shown). (**C**) The quantified time course of the appearance of trypsin-resistant α_t fragments from the EF6 mutant. Data points represent the mean ± two SEM of two separate experiments. For comparison, the shaded area depicts the range of responses (mean ± two SEM; $n = 8$) to wild-type opsin in the presence of *all-trans*-retinal.

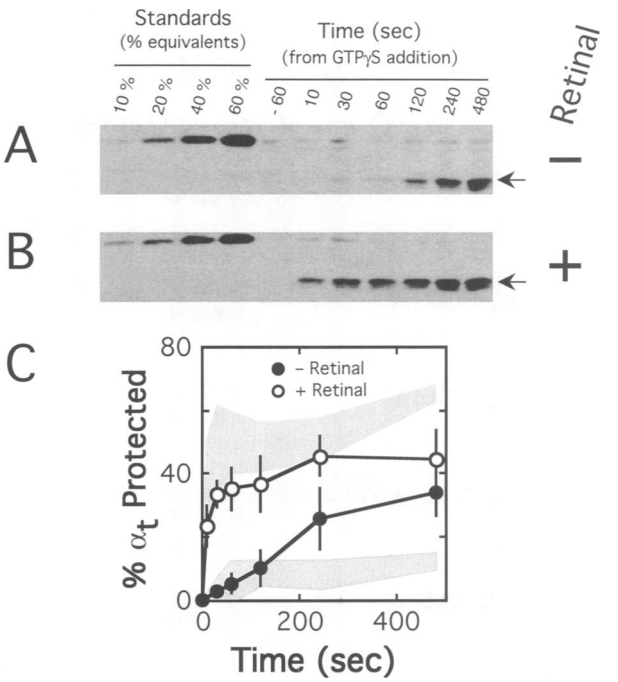

Fig. 4. Effects of the α_t-A322S mutation. α_t activation was assessed in microsomes from cells transfected with wild-type opsin, β_1, γ_1 and α_t-A322S and tested in the absence (**A**) or presence (**B**) of *all-trans*-retinal. The appearance of trypsin-resistant α_t fragments (**C**) was quantitated and compared with wild-type α_t, as in Figure 3C. Data points represent the mean ± two SEM of results in three separate experiments.

1988; Lee *et al.*, 1992). In microsomes of transfected COS-7 cells, the cognate α_t mutant, α_t-G199A, was not activated by wild-type opsin plus retinal (results not shown).

A second α_s mutation, α_s-A366S, was recently discovered in patients suffering from a rare combination of endocrine diseases, type I pseudohypoparathyroidism and testotoxicosis. The α_s-A366S mutation activates the protein by decreasing its affinity for GDP (Iiri *et al.*, 1994), thereby increasing the intrinsic rate of GDP/GTP exchange and allowing the protein to stimulate cAMP synthesis in the absence of receptor stimulation. The cognate mutation in α_t (α_t-A322S) conferred a partial version of the same biochemical phenotype. In the absence of retinal, trypsin-resistant fragments of α_t-A322S appeared at a rate ~3- to 4-fold faster than that observed with wild-type α_t (Figure 4), as expected for an α_t that has a lower affinity for GDP and a higher intrinsic rate of GDP/GTP exchange. In the presence of retinal, trypsin-resistant fragments of α_t-A322S appeared at a rate and to a maximal extent similar to those observed with wild-type α_t (Figure 4). The dramatic thermolability of α_s-A366S (Iiri *et al.*, 1994) was not reproduced in α_t-A322S. In COS-7 cells at 37°C, α_t-A322S was expressed in amounts similar to those observed with wild-type; in contrast, at this temperature α_s-A366S disappeared from cultured cells at a rate of ~50%h^{-1} (Iiri *et al.*, 1994), >20-fold the rate at which wild-type α_s is metabolized.

Mutations in the α_t C-terminus

Several lines of evidence indicate that the receptor activation of G proteins involves direct contacts between the C-termini of G protein α-subunits and cytoplasmic loops of receptors (West *et al.*, 1985; Sullivan *et al.*, 1987; Hamm *et al.*, 1988; Hirsch *et al.*, 1991; Conklin *et al.*, 1993; Dratz *et al.*, 1993). To test the relative importance in this interaction of each side chain in the C-terminal tail of α_t, we individually substituted alanine for the amino acids at each of the last 10 positions. The 10 mutant proteins were not different from wild-type α_t in their ability to switch into the active conformation, as judged by the ability of AlF_4^-, which mimics the γ-phosphate of GTP (Sternweis and Gilman, 1982; Bigay *et al.*, 1985, 1987; Sondek *et al.*, 1994), to protect α_t fragments from trypsin (Figure 5). However, mutations at four positions (K341A, L344A, G348A and L349A) impaired the susceptibility of α_t to activation by rhodopsin in the presence of *all-trans*-retinal (Figures 5 and 6).

Figure 5 shows the results of proteolysis sensitivity experiments of some α_t mutants after treatment for 2 min with either no addition or in the presence of GTPγS alone, *all-trans*-retinal plus GTPγS or AlF_4^-. They include the four mutants (K341A, L344A, G348A and L349A) with defects in α_t activation by rhodopsin, as indicated by the reduced amounts of trypsin-resistant α_t in the presence of *all-trans*-retinal plus GTPγS. Note that one mutant, L349A, was not protected from trypsin at all by retinal plus GTPγS. We also include the results obtained with the D346A mutant, which is indistinguishable from that of wild-type α_t, as a representative of the mutants not shown

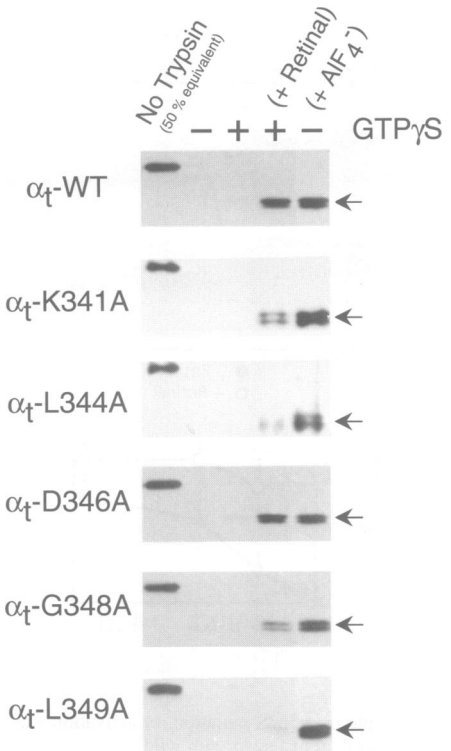

Fig. 5. Trypsin protection by AlF_4^- of α_t C-terminal mutants. α_t activation was assessed in microsomes from cells transfected with wild-type opsin, β_1 and γ_1 plus wild-type and the indicated α_t mutants. Experiments were performed as indicated in the legends of previous figures for at least two independent microsomal preparations, although only the 2 min time points after the addition of GTPγS or AlF_4^- are shown. The α_t fraction protected from trypsin by AlF_4^- reached a maximal level of ~50–60% for all 10 C-terminal mutants.

(E342A, N343A, K345A, C347A and F350A) that appear unaffected in this analysis.

Figure 6A shows the representative results of time courses of activation by *all-trans*-retinal plus GTPγS for five α_t mutants. Figure 6B depicts in graphic form the results of multiple experiments with each of the 10 mutants compared with wild-type α_t. Of these, L349A showed no response, while two others (L344A and G348A) showed detectable but severely reduced responses in comparison with wild-type α_t. For these mutants, both the rate and the maximal extent of activation were reduced. The K341A mutant showed a reduced rate of activation, but after 4 min of incubation the maximal extent of activated α_t approached that of wild-type α_t. Responses of the two other mutants, N343A and F350A, showed consistently somewhat lower maximal levels of activation (perhaps indicating a subtle phenotype), although their initial rates of activation appeared normal (Figure 6B). All of these phenotypes appear to be specific for activation by rhodopsin, because all mutants were activated to about the same maximal level (~50–60%) by AlF_4^- (Figure 5). Responses of the four remaining mutants (E342A, K345A, D346A and C347A) were indistinguishable from wild-type α_t in both rate and maximal level of activation by *all-trans*-retinal plus GTPγS (Figure 6B).

Discussion

Here we report a straightforward biochemical assay designed to detect and quantify the functional interactions of recombinant rhodopsin and α_t. We shall first discuss the aspects of the assay itself and then turn to the phenotypes produced by new mutations in α_t.

The microsomal assay

The assay uses microsomes from COS-7 cells transiently expressing DNAs encoding opsin and the three subunits of G_t. Since COS-7 cells do not express endogenous opsin or α_t, inferences drawn from our results reflect the behavior of the recombinant proteins without significant interference from endogenous receptors or Gα proteins. Our transfection protocol also included DNAs encoding the predominant subunits of G_t, β_1 and γ_1. The $\beta_1\gamma_1$ complex, which is not endogenously expressed in COS-7 cells, has been reported to couple rhodopsin to α_t *in vitro* with greater efficiency than other βγ complexes (Fawzi *et al.*, 1991; Kisselev and Gautam, 1993). This increased efficiency was not seen in the COS cell assay: The expression of $\beta_1\gamma_1$ did not significantly affect the rate (%s^{-1}) or maximal extent (~40–60%) of α_t activation (results not shown). However, the coexpression of recombinant $\beta_1\gamma_1$ with α_t did significantly increase the microsomal concentration (2- to 4-fold) of α_t (compared with microsomes from cells expressing α_t alone). This effect increased the sensitivity of the assay as seen in the higher signal intensity in the immunoblots.

The abundance of α_t (0.6–4.8 nmol/mg, or 2–19% of microsomal protein) in our preparations was very much greater than the abundance of G protein α-subunits in most membranes (0.02–1.00%), but was comparable with that of α_t in retinal rod outer segments. The abundance of opsin (0.03–0.25 nmol/mg, or 0.1–0.8% of microsomal protein) was much lower than in rod outer segments (~70% of protein), although it was greater than that usually seen with G protein-coupled receptors. The variability in expression of both proteins, which reflected varying transfection efficiencies (see Materials and methods), did not appear to affect the rates or extents of α_t activation, even when the ratio of α_t to opsin varied 3- to 4-fold (results not shown). Of the available α_t present in microsomes, 40–60% was maximally activated. This observation, combined with the high ratio of α_t to opsin, indicates that each rhodopsin molecule activated multiple α_t molecules. We do not know why rhodopsin activated only a fraction of microsomal α_t. A portion of microsomal α_t may be located in vesicles that lack rhodopsin, be located inside vesicles and therefore inaccessible to GTPγS, or be structurally impaired so that it is sensitive to trypsin even in the presence of bound GTPγS.

To validate the assay, we tested rhodopsin mutants with known phenotypes. Mutants shown previously to be absolutely or partially defective as activators of G_t in an assay that uses purified components exhibited similarly absolute or partial activation defects, respectively, when assayed in the crude microsomes. Compared with previous strategies, our assay offers several advantages for investigators of the structure and function in receptor–G_t interactions. The assay is relatively simple and requires no protein purification, and its sensitivity makes it possible to perform multiple experiments with the microsomes prepared from a small number of transiently transfected cells. The assay detects the conformational change in α_t that results from rhodopsin-stimulated GDP/GTP exchange

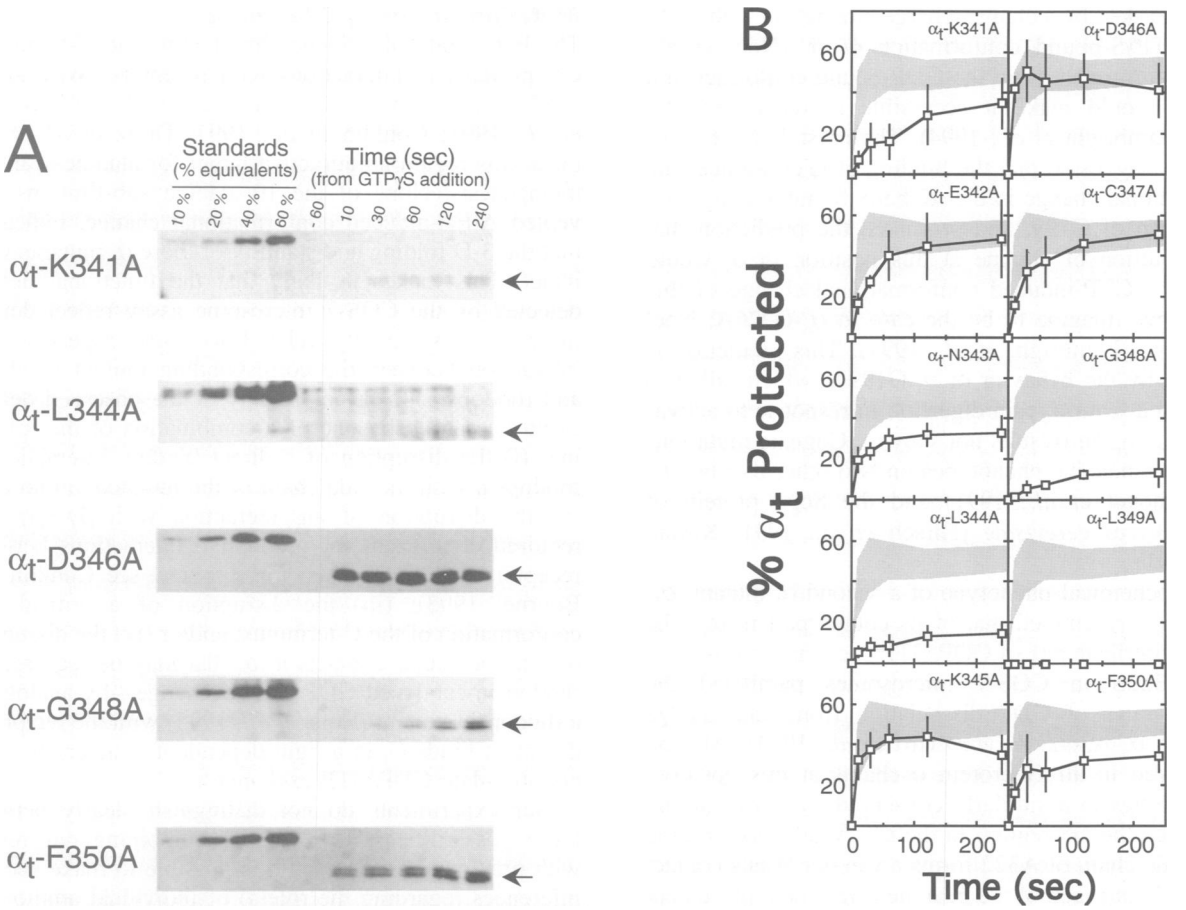

Fig. 6. Time course of the rhodopsin-dependent activation of α_t C-terminal mutants. (**A**) Representative results of time course experiments in the presence of *all-trans*-retinal for five of the α_t C-terminal mutants. They include three moderately/severely impaired mutants (K341A, L344A and G348A), one mildly affected (F350A) and one not affected (D346A). Similar time course experiments with the L349A mutant failed to show any trypsin-resistant α_t fragments (see B, below). Time courses in the absence of *all-trans*-retinal were also performed (results not shown) and showed no activation (for severely impaired mutants) or low rates of activation similar to those observed with wild-type α_t under the same conditions. (**B**) Quantitative analysis of time course experiments for the 10 C-terminal α_t mutants. For each mutant, the data represent the mean ± two SEM of four to nine separate experiments, performed on microsomal membranes derived from two or more independent transfections. For comparison, as in Figures 3C and 4C, the shaded area depicts the range (two SEM above and below the mean) of results with wild-type α_t in the presence of *all-trans*-retinal.

rather than the downstream effects on a complex signaling pathway, thus eliminating the potential effects of α mutations on interactions with effectors. Finally, the same COS-7 assay allows the testing of mutations in both rhodopsin and α_t.

The rate at which G_t is activated by *all-trans*-retinal plus GTPγS in COS-7 membranes is much slower than the reported (Vuong *et al.*, 1984) rate of GDP release (10^3 s^{-1}) from G_t induced by photoactivated rhodopsin in retinal rod outer segments. This discrepancy could be caused by many differences between rod outer segments and COS-7 microsomes, including the ~100-fold difference in opsin concentration (see above). Compared with opsin plus *all-trans*-retinal, photoactivated rhodopsin in COS-7 microsomes activated G_t at a somewhat faster rate (at least 7%s^{-1}, or ~10^{-1} s^{-1}), as indicated by the slope of the dashed line in Figure 1C. The time resolution of our experiments did not allow for the more precise measurement of the rate of G_t activation by photoactivated rhodopsin.

α_t mutations cognate to mutations in α_s

Although the abundance of α_t in rod outer segments has made it an attractive target for biochemical experiments and studies of 3-D structure, α_t has only rarely (Faurobert *et al.*, 1993) been subjected to mutational analysis. Consequently, many inferences about molecular mechanisms that activate or turn off α-subunits have been drawn from mutant phenotypes studied in α_s and interpreted in the context of the α_t 3-D structure and the biochemical behavior of wild-type α_t. The application of the COS-7 assay to two α_t mutants directly supports the idea that basic mechanisms and immediate consequences of GDP/GTP exchange are identical in α_t and α_s.

The α_t-G199A mutation, like its counterpart in α_s (Miller *et al.*, 1988; Lee *et al.*, 1992), prevents the α-subunit from responding to receptor stimulation. In α_s, this mutation produces a protein that binds GTPγS but is not protected from tryptic cleavage at a conserved arginine (R204 in α_t). The binding of GTPγS blocks tryptic cleavage at this arginine in all α-subunits tested so far, a finding that forms the basis of our α_t activation assay. Comparison of the GTPγS- and GDP-bound conformations of α_t (Noel *et al.*, 1993; Lambright *et al.*, 1994) provided a clear explanation for GTPγS-induced protection against the action of trypsin. R204 of α_t is located in the conserved α2 helix, where its guanidino

group is masked by a charge–charge interaction with E241 in the GTPγS-bound conformation of α_t (Noel *et al.*, 1993) but protrudes from the GDP-bound conformer in a way that would make it accessible to recognition by trypsin (Lambright *et al.*, 1994). The crystal structure of α_t•GTPγS suggested that the binding of GTP induces the conformational change in the α2 helix by interacting with the nitrogen of G199, and prompted the prediction that the substitution of alanine at this position in α_t would prevent the GTP-induced conformational change of this helix (as we imagine to be the case in α_s-G226A; Noel *et al.*, 1993; Lambright *et al.*, 1994). This prediction is supported by the behavior of α_t-G199A, which failed to be protected from trypsin digestion in response to activation by rhodopsin (results not shown). Cognate mutations confer unresponsive phenotypes on two other α-subunits, α_{i2} (Hermouet *et al.*, 1991) and the Scg1 protein of *Saccharomyces cerevisiae* (Hirsch *et al.*, 1991; Kurjan *et al.*, 1991).

The biochemical phenotype of a second α_t mutant, α_t-A322S, also resembled that of its counterpart in α_s. The increased intrinsic rate of GDP/GTPγS exchange observed for α_t-A322S in COS-7 microsomes paralleled the increased rates of GDP dissociation from and GTPγS binding to α_s-A366S *in vitro* (Iiri *et al.*, 1994). Alanine is conserved in all G protein α-chains at this position, which belongs to a similarly conserved stretch of amino acids called the G5 region. In the α_t crystal structure, the methyl side chain of A322 forms a van der Waals contact with the N7 nitrogen of the guanine ring. The replacement of A322 by serine would be expected to disrupt the tight fit of the guanine nucleotide into its binding pocket, thereby increasing the rate of GDP dissociation. The biochemical phenotype of α_t-A322S confirms this inference from the 3-D structure and makes it likely that the G5 region of α_s interacts with bound guanine nucleotide in a fashion that closely resembles that observed in the α_t structure.

The functional phenotypes produced by this Ala → Ser mutation in the two α-subunits differed in two subtle ways. The apparent rate constant of GDP dissociation from α_t-A322S was ~300-fold slower than that observed with α_s-A366S (data not shown). Some portion of this difference probably reflects the different experimental conditions, e.g. βγ and other proteins are present in COS-7 microsomes, while the analysis of α_s-A366S was conducted with pure protein. The slower rate in the α_t mutant compared with the α_s mutant may also relate to the 20-fold slower rate of spontaneous dissociation of GDP from wild-type G_t versus wild-type α_s (1.5×10^{-4} versus 3×10^{-3} s^{-1}, respectively; Ramdas *et al.*, 1991; Iiri *et al.*, 1994). The second difference is that α_t-A322S behaved as a stable protein in COS-7 cells at 37°C, while α_s-A366S was quite thermolabile in cultured cells and (apparently) in patients. We suspect that the much greater effect of the mutation on the GDP dissociation rate in the α_s context makes α_s-A366S more thermolabile than α_t-A322S, in keeping with the evidence (Iiri *et al.*, 1994) that the thermal denaturation of α_s-A366S depends upon the formation of the transiently 'empty' guanine nucleotide binding site created by the release of GDP.

Mutations in the α_t C-terminus

The large body of evidence implicating the C-termini of Gα proteins in interactions with receptors (West *et al.*, 1985; Sullivan *et al.*, 1987; Hamm *et al.*, 1988; Hirsch *et al.*, 1991; Conklin *et al.*, 1993; Dratz *et al.*, 1993) made this region an attractive target for alanine-scanning mutagenesis. None of the 10 alanine substitutions prevented AlF_4^--induced conformational change, indicating that the 3-D folding and stability of these α_t mutants were intact. This makes it likely that the functional defects detected by the COS-7 microsome assay reflect defects more or less directly related to some aspect of the interaction between the corresponding mutant α_t chains and rhodopsin. In principle, any of the observed defects could be caused by one or a combination of the following: (i) the disruption of a direct binding interaction of rhodopsin with the side chain of the mutated amino acid; (ii) the disruption of an interaction with βγ (βγ is a required component for productive interactions between receptors and G proteins; for a review see Conklin and Bourne, 1993); (iii) the disruption of a critical 3-D conformation of the C-terminus; and/or (iv) the disruption of intramolecular contacts in α_t that may be required for rhodopsin-catalyzed GDP/GTP exchange—by analogy to a rhodopsin mutant (Ernst *et al.*, 1995) with the reciprocal defect (it binds G_t in a light-dependent manner, but does not stimulate GDP/GTP exchange).

Our experiments do not distinguish clearly between these possibilities. However, by combining our results with previous observations it is possible to make specific inferences regarding the role(s) of individual amino acid side chains in the α_t C-terminus. In this regard, it is important to stress that an 11 residue peptide representing the C-terminal tail of α_t can interact specifically with rhodopsin—stabilizing the metarhodopsin II state (Hamm *et al.*, 1988)—in the complete absence of βγ or holo-α_t. In addition, the same peptide is reported (Dratz *et al.*, 1993) in transfer NOESY experiments to take on quite different 3-D conformations when bound to dark- versus light-activated rhodopsin.

Of the six α_t mutants defective in susceptibility to activation by rhodopsin, we suspect that the hydrophobic side chains of the two leucines (L344 and L349) are especially likely to participate directly in contacts with rhodopsin. The hydrophobic side chain of F350—a position where the mutation produced a mild defect—may also participate in such contacts. This suggestion is based on observations that hydrophobic amino acids often play critical roles in protein–protein contacts (Clackson and Wells, 1995) and that hydrophobic residues in intracellular loops II (Moro *et al.*, 1993) and III (Cheung *et al.*, 1992) of G protein-coupled receptors are essential for effective coupling to G proteins. Note also that leucine residues at positions corresponding to L344 and L349 are absolutely conserved in all mammalian Gα chains, and that a phenylalanine or tyrosine is conserved at the extreme C-terminus of all proteins in the α_i family (Figure 7). We cannot rule out the possibility that any or all of these residues may be involved in binding to βγ and/or in an intramolecular interaction with the Gα N-terminus, which is thought to interact with βγ (for a review see Conklin and Bourne, 1993). It is likely that the N- and C-termini are located close to each other in the 3-D Gα structure (Noel *et al.*,

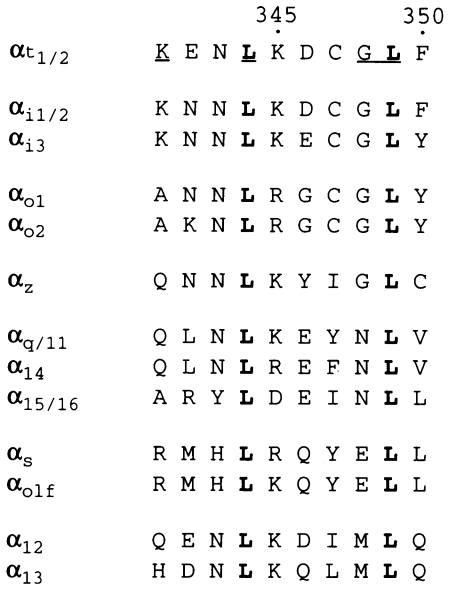

Fig. 7. C-terminal sequences of mammalian G protein α-subunits. Amino acid sequences are shown in the single-letter code. Underlined residues in the $\alpha_{t1/2}$ sequence (top) indicate the positions at which alanine substitutions moderately or severely impaired the susceptibility to activation by rhodopsin. Bold-face type indicates two leucine residues (L) that are conserved in all mammalian G protein α-subunits. Residue numbers refer to those of α_t.

1993; Coleman *et al.*, 1994; Lambright *et al.*, 1994), although much of the N-terminal region is absent or invisible in reported crystal structures.

The detrimental effects of two other mutations in α_t, G348A and K341A, can be interpreted in light of the reported (Dratz *et al.*, 1993) 3-D structure of the analogous C-terminal peptide. In the peptide conformation that was bound to dark rhodopsin, the last four residues formed a type II′ β-turn. Such a turn requires a glycine residue at position $i + 1$ of the turn (G348 in α_t). The replacement of this glycine by any other natural amino acid is incompatible with the formation of a type II′ β-turn, and its replacement by L-leucine produced a peptide that did not stabilize metarhodopsin II (Dratz *et al.*, 1993). The replacement of G348 by D-alanine (Dratz *et al.*, 1993), which is compatible with a type II′ β-turn, did not impair the peptide's ability to stabilize metarhodopsin II. Although the G348A substitution adds only an extra methylene group, such an addition at this position should disrupt the formation of a type II′ β-turn. In our experiments, this mutation caused a >10-fold reduction in the rate of α_t activation (Figures 5 and 6). This result strongly supports the hypothesis that the formation of a type II′ β-turn is required at some point during the rhodopsin-catalyzed activation of G_t.

The functional defect caused by the K341A substitution (Figures 5 and 6) may also indicate a key feature of the dark rhodopsin-bound conformation of the α_t peptide (Dratz *et al.*, 1993). In the peptide, the basic group in the side chain at position 341 (for technical reasons arginine replaced lysine at this position) was located close to the carboxyl group of F350 at the peptide's extreme C-terminus. This apparent salt bridge may have stabilized the peptide's tightly packed conformation, which would not have been possible without the type II′ β-turn centered on G348. In addition, the salt bridge could neutralize the electrical charges that may interfere with the binding to rhodopsin. Although the side chains of lysine and arginine differ by two nitrogen atoms, our results are consistent with the hypothesis that a salt bridge between K341 and the negatively charged C-terminus is somehow important for the functional interaction of α_t with rhodopsin. Alternatively, the four carbon side chains of K341 could be involved in hydrophobic interactions similar to those suggested for L344, L449 and F350.

In a reported individual with Albright's hereditary osteodystrophy, a mutation (R385H) at the cognate position of human α_s prevents G_s from interacting with its receptors and caused a generalized defect in response to hormones that work via G_s (Schwindinger *et al.*, 1994). Although an arginine at this position in α_s is somehow critical for receptor interaction, α_s lacks the key downstream glycine that would be required for a type II′ β-turn. A basic residue (arginine or lysine) is found at this position in all mammalian Gα subfamilies, with a single exception (α_o) (Figure 7).

The two other mutations in our experiments, N343A and F350A, decreased the maximal activation of α_t by rhodopsin (Figure 6). This functional defect was mild in degree and is not to our knowledge correlated with the results of other experiments. Side chains at both positions are highly conserved within each mammalian Gα family (Figure 7).

Two other changes at Gα C-termini should be mentioned. The replacement by proline of an arginine at position 389 of α_s (equivalent to K345 of α_t) uncoupled G_s from activation by receptors (Sullivan *et al.*, 1987). The normal function of the K345A mutant in our experiments (Figures 5 and 6) is consistent with the idea that functional disruption by the α_s-R389P mutation was caused by a proline-induced change in backbone conformation rather than by the loss of a specific side-chain function at this position. Similarly, our experiments suggest that the cysteine side chain at position 347 is not required for normal interaction with receptors, although it is conserved in α_i proteins and is the site of the pertussis toxin-catalyzed ADP ribosylation of α_i proteins (including α_t; Van Dop *et al.*, 1984), a modification that prevents them from interacting with receptors. Thus this modification presumably affects G_i/G_t function by steric hindrance rather than by inactivating a cysteine side chain.

In summary, our experiments strongly support the hypothesis that the rhodopsin-catalyzed activation of α_t requires a conformation of the C-terminal tail similar to that observed when the analogous peptide was bound to dark rhodopsin (Dratz *et al.*, 1993). Our results also indicate that hydrophobic side chains at two positions (L344 and L349) are critical for the activation of α_t by rhodopsin, presumably by virtue of direct interactions with rhodopsin and/or βγ. Finally, it should be noted that the C-terminal peptide showed a quite different conformation when bound to light-activated rhodopsin (Dratz *et al.*, 1993), a conformation that probably reflects a change induced by rhodopsin. Thus, the critical residues we have identified may participate in different interactions (or interactions with other components) at different stages of the activation cycle.

Materials and methods

Materials

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO), except TPCK-trypsin (Worthington Inc., Freehold, NJ), GTPγS (Boehringer Mannheim, Indianapolis, IN), polyvinylidine difluoride (PVDF) membranes and Bradford Reagent (Bio-Rad Laboratories Inc., Hercules, CA) and sheep anti-mouse Ig antibodies and ECL reagent (Amersham Life Science, Arlington Heights, IL). 11-*cis*-retinal was obtained from the National Eye Institute. The monoclonal antibody TF15 (which recognizes α_t; Navon and Fung, 1988) was a generous gift from B.K.K.Fung (UCLA). The ID4 antibody (which recognizes bovine opsin; Hodges *et al.*, 1988) was provided by M.Hirschel (Cell Culture Center, Minneapolis, MN). Samples of known concentration of purified α_t were provided by H.Hamm (University of Illinois at Chicago, IL). Cell culture media were obtained from the UCSF Cell Culture Facility.

Plasmids and mutagenesis

The wild-type and mutant bovine opsin DNAs are contained in the pMT4 expression vector, as described previously (Franke *et al.*, 1990, 1992). The bovine α_t DNA is contained in the expression vector pDNA1 (Invitrogen Co., San Diego, CA), as described previously (Lustig *et al.*, 1993). DNAs for the G protein polypeptides β_1- (in the pCMV5 vector) and γ_1-subunits (in the pDNA1 vector) were kindly provided by Janet Robishaw (Geisinger Clinic, Weis Center for Research, Danville, PA). Site-directed mutagenesis of α_t was performed using the method of Kunkel *et al.* (1987) and mutations were confirmed by dideoxy sequencing.

Transfections

COS-7 cells were transfected at high efficiencies using a recently developed DEAE/adenovirus method (Forsayeth and Garcia, 1994). Briefly, plasmid DNA (expression vectors) encoding opsin, α_t, β_1 and γ_1 (2 μg each) were diluted in 5 ml of DME-H21 culture medium containing 80 μg/ml DEAE-dextran, the appropriate amount (see Forsayeth and Garcia, 1994) of adenovirus strain Dl343 and 3×10^6 cells in suspension. The mixture was then cultured in a 10 cm culture dish at 37°C for 2 h, during which time the cells were transfected and attached to the dish. The transfection mixture was then removed, and cells were washed with PBS containing 10% dimethylsulfoxide and cultured for 20–24 h in DME-H21 containing 10% calf serum. The cells were removed by trypsin treatment, transferred to new dishes and cultured for an additional day before harvesting. The transfection efficiency was monitored by including 1 μg per 3×10^6 cells of an expression vector encoding β-galactosidase in the transfection mixture and by staining a sample of the transfected cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, as described previously (Forsayeth and Garcia, 1994). Usually, 60–80% of the cells were transfected, as judged by β-galactosidase expression, with only occasional transfection with efficiencies <50%. The expression of α_t and opsin was monitored by Western blotting of cell extracts from each transfection.

Microsome preparation

Microsome preparations were made from four transfected 10 cm culture dishes. The culture medium was removed and cells were washed once with 1 ml PBS containing 10 mM EDTA, 4 mM EGTA, 40 μg/ml bacitracin, 20 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Next, 2 ml per dish of the same solution were added and incubated until the cells began to detach. Cells were then resuspended by pipetting up and down several times and were collected by centrifugation at 1000 r.p.m. The cell pellet was resuspended in 1 ml of ice-cold lysis buffer [50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), 20 μg/ml aprotinin and 0.5 mM PMSF] and homogenized by passing the suspension 20 times through a 27-G needle. A post-nuclear supernatant fraction was obtained by spinning twice at 3000 r.p.m. for 10 min in an Eppendorf microcentrifuge at 4°C. The post-nuclear supernatant fraction was then overlaid onto 2 ml of 250 mM sucrose, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM DTT in a TL100.3 ultracentrifuge tube. The microsomes were then pelleted by centrifugation at 60 000 r.p.m. for 30 min at 4°C in a Beckman fixed angle TL100.3 ultracentrifuge rotor. Finally, the microsomes were resuspended in 400 μl of 250 mM sucrose, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM DTT by passing the solution 10 times through a 27-G needle. The microsomes were then quickly frozen in liquid nitrogen and stored at –70°C. The microsomes were slowly thawed on ice when needed and could be stored again by quick-freezing in liquid nitrogen. Under these conditions, no loss of activity was observed over at least 10 freeze–thaw cycles. Protein concentrations in the microsomal preparation were determined with the Bio-Rad Bradford reagent using the manufacturer's recommended procedure.

Determination of microsomal concentrations of opsin and α_t

Concentrations of opsin and α_t in microsomes were determined by immunoassay using as standards purified protein samples of known concentration. Briefly, microsomes were dissolved by 10-fold dilution in a solution containing 100 mM NaCl, 50 mM Tris–HCl, pH 6.8 and 0.1% *n*-dodecyl-β-D-maltoside. A 1:2 serial dilution of the solubilized proteins was then prepared and samples of each were blotted onto PVDF membranes using a Bio-Rad Bio-Dot blotting apparatus. Samples of several concentrations of the standard proteins were included in the blots to obtain standard curves. Opsin and α_t were detected and quantified in these membranes using the procedure described below for quantitative Western blots.

Protease protection assay

The activation of α_t was measured by the appearance of trypsin-resistant fragments as a result of conformational changes associated with GTPγS binding (Fung and Nash, 1983). A reaction mixture was prepared by mixing 8 μl of 50 mM Tris–HCl, pH 7.5, 1.5 M NaCl, 20 mM $MgCl_2$ and 10 mM DTT, 52 μl of distilled water and 20 μl of microsomes. Before the addition of GTPγS, the sample was incubated for 10 min at 20°C in the absence or presence of 1 μl of 800 μM 11-*cis*-retinal under dim-red light or *all-trans*-retinal in ambient light (both prepared fresh by diluting in water from a 10 mM solution in ethanol). A no-trypsin control sample was obtained by removing 16 μl of the reaction mixture 2 min before the addition of GTPγS. Another 8 μl sample, collected 1 min before the addition of GTPγS, was treated with trypsin, as indicated for the following samples. The exchange reaction was started by the addition of 1 μl 560 μM GTPγS and was terminated at the indicated times by transferring 8 μl aliquots to an ice/water bath and quickly mixing with 1.5 μl of a solution containing 1 mg/ml TPCK-trypsin, 2 mM GDP and 5% Lubrol. Trypsin treatment was continued in the ice/water bath for 1 h and was terminated by the addition of 2 μl 10 mg/ml aprotinin and 10 mM PMSF. The no-trypsin control sample was subjected to identical treatment: 3 μl of a solution containing GDP and Lubrol without trypsin were added, then it was incubated in an ice/water bath for 1 h and finally 4 μl of the aprotinin/PMSF solution were added. The AlF_4^- trypsin protection assays were carried out by incubating 10 μl reactions at 20°C for 2 min in the presence of either freshly mixed NaF (30 mM final) and $AlCl_3$ (150 μM final) or NaF (10 mM final) and $AlCl_3$ (50 μM final) added stepwise. No extra GDP was necessary in these experiments, indicating that enough of the nucleotide is present in the microsomal preparations. The reaction mixtures were then transferred to an ice/water bath and treated with trypsin as indicated above. All samples were prepared for SDS–PAGE by the addition of 15 μl loading buffer (or 30 μl for the no-trypsin control sample) and by boiling for 3 min before loading onto 12% polyacrylamide gels.

Quantitative Western blots

The no-trypsin control sample was diluted with SDS–PAGE loading buffer to obtain several percentage equivalents (5, 10, 20, 40 and 60%) of the concentration of each of the trypsin-treated samples. These samples were loaded with the trypsin-treated samples in the same SDS–PAGE, for use as standards to determine the percentage of trypsin-resistant α_t. After SDS–PAGE, the samples were transferred to PVDF membranes in a semi-dry blotting apparatus (E&K Scientific Products, Saratoga, CA). After transfer, the blots were incubated for 12–16 h in PBS containing 1% skimmed milk, 0.2% Tween-20 and 0.02% NaN_3. The blots were incubated with 5 μg/ml of the TF15 monoclonal antibody in the same solution for 2 h at room temperature. They were then washed three times for 15 min each with PBS containing 0.2% Tween-20 and then incubated for 2 h with 5 μg/ml of HRP-conjugated sheep anti-mouse Ig antibodies in PBS containing 0.2% Tween-20. The blots were washed three times for 15 min each with PBS containing 0.2% Tween-20 and once for 30 min with PBS. The blots were developed with the ECL reagent and several exposures were made using Kodak X-OMAT AR film. Images of the films were captured with a high-resolution CCD video camera using the NIH-Image (version 1.54) program. The area of the peak of absorbance for each protein band was measured using the same program. Standard curves of the no-trypsin control samples were fitted using the regression function in the Microsoft Excel (version 4.0) package, with the constraint that they intersect the origin. *R*-factors for these regressions varied between 0.95 and 1.00.

Acknowledgements

We thank Shaun Coughlin, Olivier Lichtarge and the members of our laboratories for helpful discussions and reading of the manuscript. We also thank Paul Herzmark and Ronco Moledor for technical assistance, Helen Czerwonka for preparing the manuscript and support, H.Hamm for the sample of pure α_t protein, B.K.K.Fung for the TF15 monoclonal antibody and M.Hirschel for the ID4 monoclonal antibody. P.D.G. was supported by NIH Training Grant HL07731. R.O. is a Helen Hey Whitney fellow. This work was supported by the National Institutes of Health grants GM-27800 and CA-54427 (to H.R.B.) and a grant from the March of Dimes (to H.R.B.). T.P.S. is supported by the Howard Hughes Medical Institute.

References

Baldwin,J.M. (1993) *EMBO J.*, **12**, 1693–1703.
Bigay,J., Deterre,P., Pfister,C. and Chabre,M. (1985) *FEBS Lett.*, **191**, 181–185.
Bigay,J., Deterre,P., Pfister,C. and Chabre,M. (1987) *EMBO J.*, **6**, 2907–2913.
Birnbaumer,L. (1990) *Annu. Rev. Pharmacol. Toxicol.*, **30**, 675–705.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1990) *Nature*, **348**, 125–132.
Cheung,A.H., Huang,R.R. and Strader,C.D. (1992) *Mol. Pharmacol.*, **41**, 1061–1065.
Clackson,T. and Wells,J.A. (1995) *Science*, **267**, 383–386.
Coleman,D.E., Berghuis,A.M., Lee,E., Linder,M.E., Gilman,A.G. and Sprang,S.R. (1994) *Science*, **265**, 1405–1412.
Conklin,B.R. and Bourne,H.R. (1993) *Cell*, **73**, 631–641.
Conklin,B.R., Farfel,Z., Lustig,K.D., Julius,D. and Bourne,H.R. (1993) *Nature*, **363**, 274–276.
Dratz,E.A., Furstenau,J.E., Lambert,C.G., Thireault,D.L., Rarick,H., Schepers,T., Pakhlevaniants,S. and Hamm,H.E. (1993) *Nature*, **363**, 276–281.
Ernst,O.P., Hofmann,K.P. and Sakmar,T.P. (1995) *J. Biol. Chem.*, **270**, 10580–10586.
Faurobert,E., Otto,B.A., Chardin,P. and Chabre,M. (1993) *EMBO J.*, **12**, 4191–4198.
Fawzi,A.B., Fay,D.S., Murphy,E.A., Tamir,H., Erdos,J.J. and Northup,J.K. (1991) *J. Biol. Chem.*, **266**, 12194–12200.
Forsayeth,J.R. and Garcia,P.D. (1994) *Biotechniques*, **17**, 354–358.
Franke,R.R., Konig,B., Sakmar,T.P., Khorana,H.G. and Hofmann,K.P. (1990) *Science*, **250**, 123–125.
Franke,R.R., Sakmar,T.P., Graham,R.M. and Khorana,H.G. (1992) *J. Biol. Chem.*, **267**, 14767–14774.
Freissmuth,M., Casey,P.J. and Gilman,A.G. (1989) *FASEB J.*, **3**, 2125–2131.
Fung,B.K. and Nash,C.R. (1983) *J. Biol. Chem.*, **258**, 10503–10510.
Hamm,H.E., Deretic,D., Arendt,A., Hargrave,P.A., Koenig,B. and Hofmann,K.P. (1988) *Science*, **241**, 832–835.
Hermouet,S., Merendino,J.J., Gutkind,J.S. and Spiegel,A.M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 10455–10459.
Hirsch,J.P., Dietzel,C. and Kurjan,J. (1991) *Genes Dev.*, **5**, 467–474.
Hodges,R.S., Heaton,R.J., Parker,J.M., Molday,L. and Molday,R.S. (1988) *J. Biol. Chem.*, **263**, 11768–11775.
Iiri,T., Herzmark,P., Nakamoto,J.M., van,D.C. and Bourne,H.R. (1994) *Nature*, **371**, 164–168.
Kaziro,Y., Itoh,H., Kozasa,T., Nakafuku,M. and Satoh,T. (1991) *Annu. Rev. Biochem.*, **60**, 349–400.
Kisselev,O. and Gautam,N. (1993) *J. Biol. Chem.*, **268**, 24519–24522.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Kurjan,J., Hirsch,J.P. and Dietzel,C. (1991) *Genes Dev.*, **5**, 475–483.
Lambright,D.G., Noel,J.P., Hamm,H.E. and Sigler,P.B. (1994) *Nature*, **369**, 621–628.
Lee,E., Taussig,R. and Gilman,A.G. (1992) *J. Biol. Chem.*, **267**, 1212–1218.
Lustig,K.D., Conklin,B.R., Herzmark,P., Taussig,R. and Bourne,H.R. (1993) *J. Biol. Chem.*, **268**, 13900–13905.
Miller,R.T., Masters,S.B., Sullivan,K.A., Beiderman,B. and Bourne,H.R. (1988) *Nature*, **334**, 712–715.
Moro,O., Lameh,J., Hogger,P. and Sadee,W. (1993) *J. Biol. Chem.*, **268**, 22273–22276.
Navon,S.E. and Fung,B.K. (1988) *J. Biol. Chem.*, **263**, 489–496.
Noel,J.P., Hamm,H.E. and Sigler,P.B. (1993) *Nature*, **366**, 654–663.
Ramdas,L., Disher,R.M. and Wensel,T.G. (1991) *Biochemistry*, **30**, 11637–11645.
Schertler,G.F., Villa,C. and Henderson,R. (1993) *Nature*, **362**, 770–772.
Schwindinger,W.F., Miric,A., Zimmerman,D. and Levine,M.A. (1994) *J. Biol. Chem.*, **269**, 25387–25391.
Sondek,J., Lambright,D.G., Noel,J.P., Hamm,H.E. and Sigler,P.B. (1994) *Nature*, **372**, 276–279.
Sternweis,P.C. and Gilman,A.G. (1982) *Proc. Natl Acad. Sci. USA*, **79**, 4888–4891.
Sullivan,K.A., Miller,R.T., Masters,S.B., Beiderman,B., Heideman,W. and Bourne,H.R. (1987) *Nature*, **330**, 758–760.
Surya,A., Foster,K.W. and Knox,B.E. (1995) *J. Biol. Chem.*, **270**, 5024–5031.
Van Dop,C., Yamanaka,G., Steinberg,F., Sekura,R.D., Manclark,C.R., Stryer,L. and Bourne,H.R. (1984) *J. Biol. Chem.*, **259**, 23–26.
Vuong,T.M., Chabre,M. and Stryer,L. (1984) *Nature*, **311**, 659–661.
West,R.J., Moss,J., Vaughan,M., Liu,T. and Liu,T.Y. (1985) *J. Biol. Chem.*, **260**, 14428–14430.

Received on June 13, 1995