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Carboxyl methylation and farnesylation of transducin γ-subunit synergistically enhance its coupling with metarhodopsin II

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A heterotrimeric G-protein in vertebrate photoreceptor cells is called transducin (Tαβγ), whose γ-subunit is a mixture of two components, Tγ-1 and Tγ-2. Tγ-2 is S-farnesylated and partly carboxyl methylated at the C-terminal cysteine residue, whereas Tγ-1 lacks the modified cysteine residue. To elucidate the physiological significance of the double modifications in Tγ, we established a simple chromatographic procedure to isolate Tγ-1, methylated Tγ-2 and non-methylated Tγ-2 on a reversed phase column. Taking advantage of the high and reproducible yield of Tγ from the column, we analyzed the composition of Tγ subspecies in the Tα–Tβγ complex which did not bind with transducin-depleted rod outer segment membranes containing metarhodopsin II. The binding of Tα–Tβγ with the membranes was shown to require the S-farnesylated cysteine residue of Tγ, whose methylation further enhanced the binding. This synergistic effect was not evident when Tα was either absent or converted to the GTP-bound form which is known to dissociate from Tβγ. Thus we concluded that a formation of the ternary complex, Tα–Tβγ–metarhodopsin II, is enhanced by the farnesylation and methylation of Tγ. This suggests that the double modifications provide most efficient signal transduction in photoreceptor cells.

Key words: carboxyl methylation/farnesylation/GTP-binding protein/transducin/visual transduction

Introduction

GTP-binding regulatory proteins (G-proteins) are involved in a variety of biological signal transduction processes (Stryer and Bourne, 1986). In rod outer segments (ROS), a heterotrimeric G-protein called transducin couples with a photobleaching intermediate of rhodopsin, metarhodopsin II (Fukada and Yoshizawa, 1981), and activates cGMP-phosphodiesterase (Yee and Liebman, 1979; Fung and Stryer, 1980). Transducin (T) is composed of two functional subunits, Tα (39 kDa) and Tβγ (Tβ: 36 kDa and Tγ: 8 kDa). Tα has a guanine nucleotide-binding site and Tβγ regulates an interaction between Tα and metarhodopsin II (Kühn, 1980; Fung, 1983).

We have found that bovine Tβγ can be separated into two components, Tβγ-1 and Tβγ-2, each of which has its own γ-subunit, Tγ-1 and Tγ-2, respectively. Tβγ-2 is ~30 times more effective than Tβγ-1 in enhancing the binding of GppNHp to Tα in the presence of metarhodopsin II (Fukada *et al.*, 1989). Our previous study on the structure of Tγ subspecies revealed that the active Tγ-2 is S-farnesylated and partly carboxyl methylated at the C-terminal cysteine residue which is missing in the inactive Tγ-1 (Fukada *et al.*, 1990). In this paper, the complete structure of the inactive Tγ-1 was determined to be a mixture of peptides, Pro1–Gly68 and Pro1–Gly69, having identical sequence with Tγ-2 (Pro1–Cys70). Since the structural difference between Tγ-1 and Tγ-2 is restricted to the C-terminal part, it is clear that the modified cysteine residue in Tγ-2 influences the binding of GTP to Tα.

Recently it has been shown that two γ-subunits (γ_5 and γ_6) of bovine brain G-protein are modified with a geranylgeranyl isoprenoid moiety at their C-terminal cysteine residue which is simultaneously carboxyl methylated (Mumby *et al.*, 1990; Yamane *et al.*, 1990). It is also known that $p21^{ras}$ and its related small G-proteins are similarly isoprenylated and carboxyl methylated at their C-terminal cysteine residue (Clarke *et al.*, 1988; Casey *et al.*, 1989; Hancock *et al.*, 1989; Schafer *et al.*, 1989; Kawata *et al.*, 1990). These observations raise an important question about the physiological significance of the double modifications, isoprenylation and carboxyl methylation, commonly found in the G-proteins. So far, it has been postulated that the hydrophobicity of the proteins increased by the modifications might be essential for their membrane attachment. In fact, Hancock *et al.* (1991) have demonstrated recently that farnesylation-dependent binding of $p21^{Kras(B)}$ to plasma membranes is further strengthened by the carboxyl methylation. As they suggested, however, a putative receptor molecule in the membranes might specifically bind farnesylated and methylated $p21^{ras}$. This hypothesis predicts that these modifications might be involved in a specific protein–protein interaction. This idea has presumably come from a finding that a membrane-bound receptor for the N-terminal part of N-myristoylated $p60^{v\text{-}src}$ is essential for its membrane binding (Resh and Ling, 1990). Consistent with this hypothesis, we have proposed that the farnesyl group of Tγ would interact with Tα for efficient formation of the Tα–Tβγ complex (Fukada *et al.*, 1990) based on the observation that Tα (in its GDP-bound form) has a much higher affinity for Tβγ-2 than Tβγ-1 (Ohguro *et al.*, 1990).

Now, it is interesting to ask whether the carboxyl methylation of Tγ might also affect protein–protein interaction. Although we have not yet succeeded in isolating methylated and non-methylated Tβγ (in S-farnesylated forms) under non-denaturing conditions, we have established a simple chromatographic procedure by which the amount of methylated and non-methylated Tγ in the Tα–Tβγ complex can be quantified. By measuring the composition of the species remaining unbound to metarhodopsin II in transducin-depleted ROS membranes, we now show that

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carboxyl methylation, in addition to S-farnesylation, of Tγ significantly enhances formation of a Tα−Tβγ−metarhodopsin II complex. It should be emphasized that these effects are most evident in the presence of Tα in GDP-bound form, suggesting that the enhanced complex formation due to the modifications could be ascribed to the efficient coupling between Tα and Tβγ, or between Tα−Tβγ and metarhodopsin II. In either case, our observation strongly supports the hypothesis that both the methyl and isoprenyl groups might provide specific protein−protein interaction.

Results

Isolation of Tγ subspecies and their structures

When purified Tβγ-1 was directly injected into the Cosmosil $5C_{18}$-P300 reversed phase column, a single peak was observed (Figure 1, upper trace; retention time = 51 min) due to the elution of Tγ-1 (Figure 1, inset, lane 1). On the other hand, injection of Tβγ-2 onto the column resulted in separation of Tγ-2 into two peaks (Figure 1, lower trace) designated as Tγ-2a and Tγ-2b (retention time = 56 and 57 min, respectively). Their apparent molecular weight estimated by urea/SDS−PAGE was 6000 (Figure 1, inset, lanes 2a and b), which coincided with that of Tγ-2 (Fukada *et al.*, 1989).

Accurate molecular weights of Tγ-2a (8315.7) and Tγ-2b (8330.2) measured by an ion-spray mass spectrometry (Figure 2) were in good agreement with the theoretical masses of non-methylated (8315.7) and carboxyl methylated forms (8329.7) of Tγ-2 [Pro1−Cys70(S-farnesyl)] (Fukada *et al.*, 1990), respectively. To confirm the whole sequences, the Tγ subspecies (Tγ-1, Tγ-2a and Tγ-2b) isolated above were digested with *Staphylococcus aureus* protease V8 and the proteolytic fragments were separated from each other using a reversed phase column as shown in Figure 3. Elution positions of nine fragments (designated fragments 1−9 in Figure 3) derived from the three Tγ subspecies were identical with each other, whereas fragments 10 and 11 were characteristic of Tγ-2a and Tγ-2b, respectively. Sequence analyses and FAB mass spectra of each fragment indicated no difference in structure from Pro1 to Glu65 among the subspecies. Eventually, fragments 10 and 11 were identified with non-methylated and carboxyl methylated C-terminal fragments [Leu66−Cys70(S-farnesyl)], respectively, whose structures had already been determined (Fukada *et al.*, 1990). Thus, the structures of Tγ-2a (non-methylated Tγ-2) and Tγ-2b (carboxyl methylated Tγ-2) were determined as shown in Figure 4.

On the other hand, the C-terminal proteolytic fragment of Tγ-1 seemed to flow through the reversed phase column (Figure 3, top trace). This hydrophilic nature of the C-terminal fragment can be ascribed to the absence of the

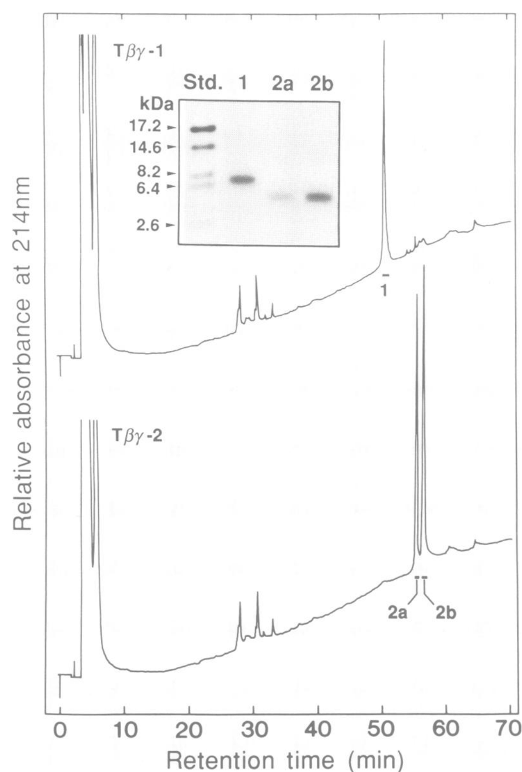

Fig. 1. Isolation of Tγ using reversed phase HPLC. Purified Tβγ-1 (upper trace) or Tβγ-2 (lower) was injected onto a reversed phase column (Cosmosil $5C_{18}$-P300, 4.6 × 150 mm^2) and then eluted with a linear gradient of acetonitrile (5−75%, 1%/min) in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The absorbance at 214 nm of the eluate was continuously monitored. Three fractions (1 = Tγ-1, 2a = Tγ-2a, 2b = Tγ-2b) were collected, lyophilized and subjected to urea/SDS−PAGE (inset) and sequence analyses. Several tens of amino acid sequences of Tγ-1, Tγ-2a and Tγ-2b from the N-terminus were identical with that of Tγ previously reported (Ovchinnikov *et al.*, 1985).

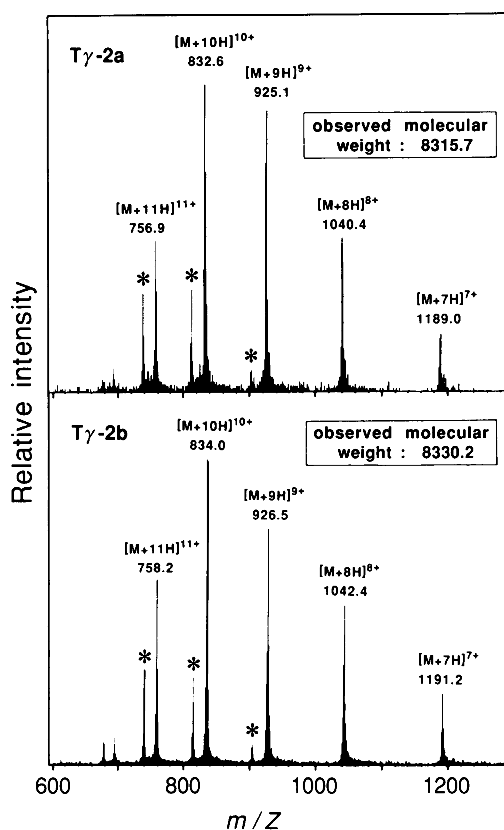

Fig. 2. Ion spray mass spectra of intact Tγ-2a and Tγ-2b. Tγ-2a (**upper panel**) and Tγ-2b (**lower panel**) isolated by reversed phase HPLC (shown in Figure 1) were directly subjected to an ion spray mass spectrometry. The molecular weight (M) of Tγ-2a (8315.7) or Tγ-2b (8330.2) was calculated from the observed mass values of the multiply charged ion signals ($[M + nH]^{n+}$). The signals marked by asterisks arose from the fragment ions produced by the loss of a farnesyl group during the measurements.

modified cysteine residue in Tγ-1 (Fukada *et al.*, 1990). To determine its complete C-terminal structure, a longer C-terminal fragment of Tγ-1 was obtained by treatment with formic acid (Piszkiewicz *et al.*, 1970) which cleaved Tγ-1 between Asp50 and Pro51. Then the C-terminal fragment was revealed to be identical with the corresponding part of Tγ-2 (Pro51–Gly69) by a sequence analysis, where the amount of the last glycine residue (Gly69) was extremely low (data not shown). This observation is consistent with the following mass spectrometry data indicating that some Tγ-1 lacks Gly69: a FAB mass spectrum of the fragment displayed two major signals at *m/z* 2008.9 and 2066.0. The former value coincides with a calculated mass of a peptide Pro51–Gly68 (theoretical: 2009.1) and the latter corresponds to a peptide Pro51–Gly69 (theoretical: 2066.2). Thus it was concluded that Tγ-1 is heterogeneous in peptide length as illustrated in Figure 4.

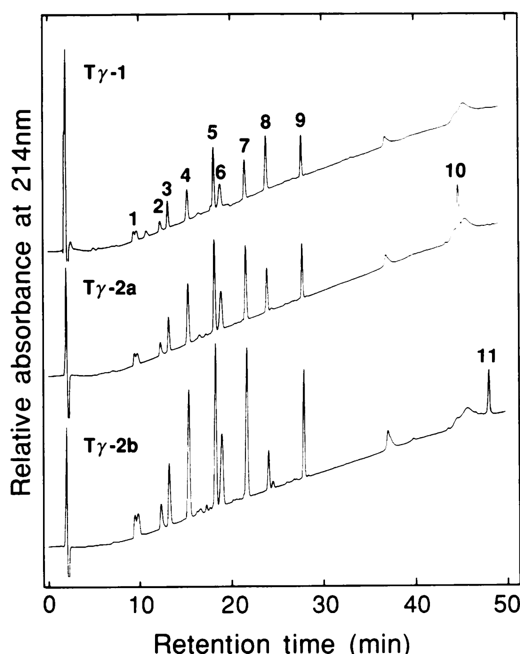

Fig. 3. Elution profiles of the proteolytic fragments derived from the Tγ subspecies. Tγ-1 (top trace), Tγ-2a (middle) or Tγ-2b (bottom; 1–2 nmol each) was incubated for digestion with 1 μg of *S.aureus* protease V8 in 50 μl of 12.5 mM NH_4HCO_3 (pH 7.8) at 37°C for 6 h. Then the fragments were separated from each other by the reversed phase HPLC as described in the legend to Figure 1. By sequence analyses and mass spectra, each fragment was identified as follows: fragment 1: Val18–Glu24; 2: Val25–Glu28; 3: Lys11–Glu17; 4: Asp59–Glu65; 5: Phe39–Glu45; 6: Arg29–Glu38; 7: Arg46–Glu58; 8: Arg46–Glu65; 9: Pro1–Glu10; 10: Leu66–Cys70(S-farnesyl)-OH; 11: Leu66–Cys70(S-farnesyl)-OCH_3. Observed mass value (1195.2–1195.5) of fragment 6 indicated that the neighboring cysteine residues Cys35 and Cys36 in every subspecies formed a disulfide bond (theoretical mass: 1195.5).

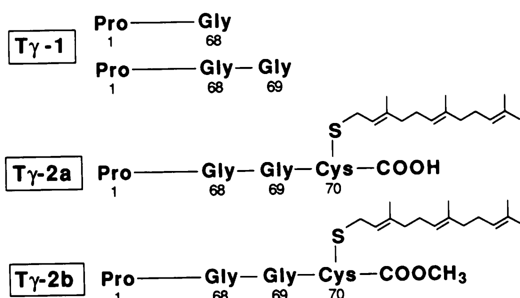

Fig. 4. Structures of the Tγ subspecies.

Binding of Tβγ with the stripped ROS membranes

When either Tβγ alone or Tα/Tβγ was injected onto the Cosmosil $5C_{18}$-P300 column, ~90% (quantified by amino acid analysis) of injected Tγ was reproducibly recovered, whereas most Tβ was adsorbed to the column. The recovery of Tγ subspecies from the column was not affected by the presence of Tα, which eluted much later than Tγ. We can now quantify the composition of Tγ subspecies in Tβγ or in the Tα–Tβγ complex. As Tα and Tβγ are known to bind to metarhodopsin II in the absence of GTP, we examined whether the carboxyl methylation of Tγ can affect the receptor–G-protein coupling. In the following experiment, a mixture of Tβγ-1 and Tβγ-2 was used to compare their affinities at the same time. The composition of Tβγ-1, Tβγ-2a and Tβγ-2b in the Tβγ preparation was calculated to be 8%, 44% and 48%, respectively, from their peak areas in the chromatogram of the reversed phase HPLC.

Transducin-depleted 'stripped ROS membranes' were kept in the dark or irradiated at 4°C with orange light (>540 nm) to convert rhodopsin into metarhodopsin II. Immediately after the irradiation, Tα and Tβγ were mixed with the membranes (final concentration: [Tα] = [Tβγ] = [Rh] = 1.0 μM) in the dark, and the suspension was centrifuged (430 000 *g* for 30 min at 2°C). The composition of Tγ subspecies remaining in the resultant supernatant was analyzed by reversed phase HPLC. In the absence of the membranes, we observed no appreciable change in the amount of Tγ subspecies before and after centrifugation (data not shown). Furthermore, the composition of Tγ

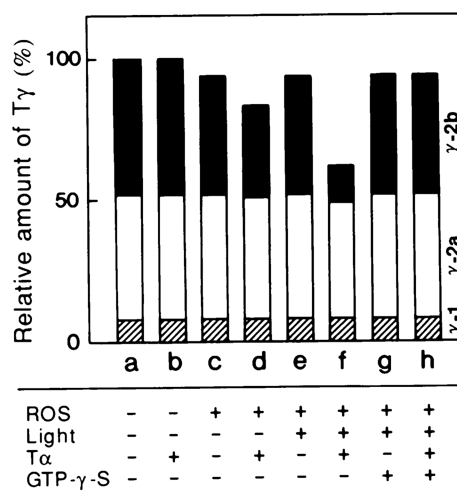

Fig. 5. Compositions of the Tγ subspecies in Tβγ complex not bound to the stripped ROS membranes. The stripped ROS membranes were kept in the dark (c and d) or irradiated (e–h) with orange light (>540 nm) at 0°C for 1 min to produce metarhodopsin II. Immediately, Tβγ (Tβγ-1:Tβγ-2a:Tβγ-2b = 8:44:48) was mixed in the dark with Tα (b, d, f and h), GTP-γ-S (g and h) and the stripped ROS membranes (c–h) in a total volume of 1.05 ml (buffer A) at the final concentrations of 1.0 μM (Tα, Tβγ and rhodopsin in the membranes) and 50 μM (GTP-γ-S). The mixtures were centrifuged (430 000 *g*; Beckman TL-100 Ultracentrifuge) at 2°C for 30 min, and the resultant supernatant (1.0 ml) was directly injected onto the reversed phase column to analyze the composition of Tγ subspecies. The data shown are representative of three independent experiments. The variation of the data was <3% within an experiment.

subspecies in the supernatant was not affected by the addition of $T\alpha$ in the absence of the membranes (Figure 5a and b). These observations indicate that no measurable aggregation of $T\beta\gamma$ occurred during the experiments. On the other hand, even in the absence of $T\alpha$, addition of the non-irradiated membranes induced a slight decrease (6%) in the amount of total $T\gamma$ remaining in the supernatant (Figure 5, compare c with a). The slight decrease was mainly due to sedimentation of $T\beta\gamma$-2b with the membranes, and the sedimentation was not affected by the irradiation of the membranes (compare e with c), suggesting that $T\beta\gamma$-2b has some affinity for the lipid bilayer without $T\alpha$ and metarhodopsin II. In the presence of $T\alpha$, $T\beta\gamma$-2 in the supernatant decreased further without irradiation (compare d with c). This sedimentation of the $T\alpha-T\beta\gamma$-2b complex with non-irradiated membranes would partly reflect the localization of transducin *in vivo* at the membrane surface. The amount of $T\beta\gamma$-2b in the supernatant decreased most remarkably when the membranes were irradiated (Figure 5f). This was ascribed to formation of a $T\alpha-T\beta\gamma$-2b$-$metarhodopsin II complex in the membranes, because a significant amount of $T\beta\gamma$-2b was again recovered in the supernatant by the addition of guanosine 5′-O-(3-thio-triphosphate) (GTP-γ-S) (compare h with f) which is known to dissociate the complex. The most noticeable feature in this experiment is that the amounts of $T\gamma$-1 and $T\gamma$-2a remained almost constant in the supernatant under various conditions, suggesting strongly that the carboxyl methylation of $T\gamma$ is indispensable for efficient formation of the ternary complex, $T\alpha-T\beta\gamma-$metarhodopsin II.

This was clearly shown by increasing the amount of irradiated membranes in the mixtures in which the concentrations of $T\alpha$ and $T\beta\gamma$ were kept constant at 1.0 μM (Figure 6). In this experiment, an aliquot of the supernatant obtained by the centrifugation was subjected to SDS$-$PAGE to quantify $T\alpha$ and $T\beta$, and the remainder of the supernatant was injected onto the reversed phase HPLC to analyze $T\gamma$ composition. As shown in Figure 6A, the total amount of $T\gamma$ ($T\gamma$-1 + $T\gamma$-2a + $T\gamma$-2b) calculated from the peak area of the chromatogram decreased by the addition of increasing amount of irradiated membranes. The profile in the decrease of $T\gamma$ was in good agreement with that of $T\alpha$ and $T\beta$ measured by densitometric scanning of the gel (Figure 6A, inset), indicating that $T\gamma$ sedimented as a complex of $T\alpha-T\beta\gamma-$metarhodopsin II in the membranes. Accordingly, the individual profile in the decrease of $T\gamma$ subspecies estimated by HPLC analysis was considered to reflect their efficiencies to form $T\alpha-T\beta\gamma-$metarhodopsin II complex. To compare them more clearly, the individual amount of $T\gamma$ subspecies presented in Figure 6A was plotted as a function of concentration of irradiated rhodopsin in Figure 6B, where each content of $T\gamma$ subspecies in the absence of membranes was normalized to 100%. It is evident that metarhodopsin II bound with $T\gamma$-2b (●) in preference to $T\gamma$-2a (○) whereas $T\gamma$-1 (Δ) decreased only slightly under these experimental conditions. These results indicate that the carboxyl methylation at the C-terminal cysteine residue significantly enhances the $T\alpha-T\beta\gamma-$metarhodopsin II complex formation, which entirely depends on the presence of an S-farnesylated cysteine residue in $T\gamma$.

As shown in Figure 6B, the irradiated rhodopsin concentration that induces half maximal binding of $T\gamma$-2b (0.65 μM) was about four times smaller than that of $T\gamma$-2a

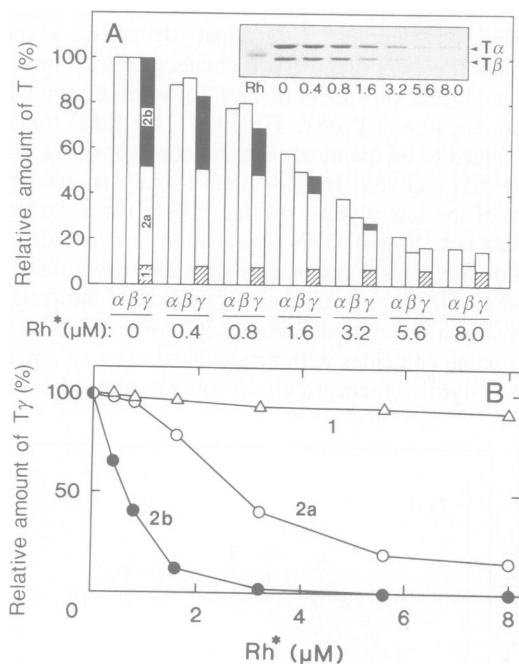

Fig. 6. Analysis of transducin subunits not bound with metarhodopsin II. $T\alpha$ and $T\beta\gamma$ (1.0 μM final concentration) were mixed in buffer A with various amounts of the stripped membranes which had been irradiated as in Figure 5, and the mixture (1.05 ml) was immediately centrifuged (430 000 *g*) at 2°C for 30 min. The resultant supernatant (1.0 ml) was saved, from which 30 μl was used for SDS$-$PAGE analysis (panel A, inset) to estimate the amount of $T\alpha$ and $T\beta$. The remainder of the supernatant (0.97 ml) was directly injected onto the reversed phase column to analyze the composition of $T\gamma$ subspecies. **A: inset**, SDS$-$PAGE analysis of the supernatant. The irradiated rhodopsin concentrations in the mixtures before the centrifugation are shown at the bottom of the gel. The stripped ROS membranes (34 pmol rhodopsin) were electrophoresed in the lane 'Rh' to demonstrate the absence of $T\alpha-T\beta$ in the membranes. The relative amounts of $T\alpha$ or $T\beta$, estimated from the densitometric scanning of the gel, were shown by normalizing their densities in the absence of the membranes to 100%. The amount of each $T\gamma$ subspecies ($T\gamma$-1, shaded bars; $T\gamma$-2a, open bars; $T\gamma$-2b, black bars) was estimated from the peak areas in the HPLC analysis. The total amount of $T\gamma$ recovered in the absence of the membranes was normalized to 100%. **B**: Relative amount of the $T\gamma$ subspecies in the supernatant was plotted as a function of concentration of irradiated rhodopsin. The amount of each $T\gamma$ subspecies recovered from the mixture in the absence of the membranes was normalized to 100%. The data shown are representative of three independent experiments. The variation of the data was <3% within an experiment.

(2.8 μM). However, this ratio does not always reflect the ratio in their affinities for metarhodopsin II, because $T\alpha-T\beta\gamma$-2a did not bind with the membranes until most $T\alpha-T\beta\gamma$-2b disappeared from the supernatant (Figure 6B). The binding of $T\alpha-T\beta\gamma$-2a seems to be competitively inhibited by $T\alpha-T\beta\gamma$-2b having higher affinity than the former when the concentration of metarhodopsin II is lower than that of $T\alpha-T\beta\gamma$ (1.0 μM). Accordingly, the ratio in affinity between $T\beta\gamma$-2a and $T\beta\gamma$-2b would be smaller than that observed in Figure 6B. On the other hand, $T\beta\gamma$-1 displayed almost no affinity for the membranes under any experimental conditions (Figures 5 and 6). This result agrees with our initial observation (Fukada *et al.*, 1989), but is somewhat curious because $T\beta\gamma$-1 was purified from a $T\alpha-T\beta\gamma$ preparation which had been extracted from ROS membranes by the addition of GTP. At the present time,

we cannot entirely exclude the possibility that Tγ-1 might have been produced from Tγ-2 after the extraction.

Discussion

All the deduced amino acid sequences of known G-protein γ-subunits (Hurley *et al.*, 1984; Yatsunami *et al.*, 1985; Gautam *et al.*, 1989; Robishaw *et al.*, 1989; Whiteway *et al.*, 1989) possess a C-terminal Cys-Aaa-Aaa-Xaa motif, where Aaa and Xaa are an aliphatic and any amino acid, respectively (Clarke *et al.*, 1988). This motif is a signal for isoprenylation of the sulfydryl group of the cysteine residue (Reiss *et al.*, 1990). The γ-subunit of transducin is S-farnesylated (Fukada *et al.*, 1990; Lai *et al.*, 1990), while that of brain G-protein is S-geranylgeranylated (Mumby *et al.*, 1990; Yamane *et al.*, 1990). After isoprenylation, the three terminal residues are removed, followed by methylation of the newly exposed α-carboxyl group of the cysteine residue (Fukada *et al.*, 1990; Fung *et al.*, 1990; Yamane *et al.*, 1990). This is the first study demonstrating the physiological significance of the methylation of a heterotrimeric G-protein γ-subunit: the farnesylated and methylated Tγ is the form most capable of coupling Tα to metarhodopsin II.

We considered three possible mechanisms by which the farnesylation and methylation of Tγ affect the formation of Tα−Tβγ−metarhodopsin II: (i) the modifications of Tγ are essential for the efficient coupling between Tα and Tβγ; (ii) the modifications are involved in the interaction between metarhodopsin II and Tβγ when the latter is complexed with Tα; and (iii) the hydrophobic modifications stabilize the membrane association of Tβγ, which enables Tα to couple efficiently with metarhodopsin II in the membranes. As expected from mechanism (iii), we observed that Tα−Tβγ-2b partly sedimented with the non-irradiated membranes, whereas almost all Tα−Tβγ-2a remained in the supernatant (Figure 5d). The result suggests that the farnesylation is not enough to associate Tβγ with the membranes. This contrasts with more hydrophobic modification, geranylgeranylation of γ-subunits of brain G-proteins: the geranylgeranylation leads to complete membrane association of the βγ subunit (Simonds *et al.*, 1991), though the effect of the methylation has not been evaluated exclusively. In the dark, both the methyl and farnesyl groups in Tγ could play a role *in vivo* in the localization of Tα−Tβγ at the membrane surface where the receptor−G-protein coupling occurs. In the light, however, the double modifications had the most remarkable effect on the coupling: the binding of Tβγ-2b, unlike Tβγ-2a or Tβγ-1, to the membranes was increased 2- to 3-fold by irradiation of the membranes (compare f with d in Figure 5). Mechanism (iii) does not fully account for the increase in binding caused by the irradiation, because the effect was not evident when Tα was either absent (compare e with f) or converted to the GTP-γ-S bound form (compare h with f) which is known to dissociate from Tβγ. If the modifications contributed solely to the membrane attachment of Tβγ [mechanism (iii)], Tβγ-2b once attached to the irradiated membranes would not be recovered again in the supernatant after the conformational change of Tα induced by GTP-γ-S. We therefore concluded that the membrane attachment of Tα−Tβγ-2b enhanced by the irradiation should be ascribed to an effect of the modifications on protein−protein interaction between Tα and Tβγ [mechanism (i)] or between Tα−Tβγ and metarhodopsin II [mechanism (ii)].

It is difficult to distinguish between mechanisms (i) and (ii) on the basis of the experimental data presented here. However, our previous observation that Tα in its GDP-bound form has a much higher affinity for Tβγ-2 (a mixture of methylated and non-methylated forms) than Tβγ-1 in the absence of the membranes (Ohguro *et al.*, 1990) indicates that either or both of the modifications is involved in an efficient coupling between the α and βγ subunits [mechanism (i)]. Taken together, we suppose that both the farnesylation and methylation of the C terminus of Tγ are involved in the most efficient formation of Tα−Tβγ complex that facilitates coupling with metarhodopsin II in the light. Some conformational change(s) of Tα and/or Tβγ due to their coupling might also strengthen their membrane association in the dark (compare d with c in Figure 5). It is interesting to speculate that the methylation/demethylation of Tγ (Perez-Sala *et al.*, 1991) might regulate the visual transduction process by changing the coupling efficiencies.

Materials and methods

Isolation and digestion of Tγ subspecies

Tα, Tβγ-1 and Tβγ-2 were purified from freshly dissected bovine retinas as described previously (Fukada *et al.*, 1989). Tβγ-1 or Tβγ-2 thus isolated was directly injected onto a reversed phase Cosmosil $5C_{18}$-P300 column (4.6 × 150 mm^2; Nacalai Tesque) equipped with an HPLC system (Model 600E; Waters). The γ-subunits were eluted from the column with a linear gradient of acetonitrile (5−70%) in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min (Figure 1).

Isolated Tγ subspecies (1−2 nmol) were lyophilized, dissolved in 50 μl of 12.5 mM NH_4HCO_3 (pH 7.8) and incubated with 1 μg of *S.aureus* protease V8 (Miles Laboratories) at 37°C for 6 h. Alternatively, purified Tγ-1 (1 nmol) was incubated at 37°C for 48 h in 50 μl of 70% formic acid to cleave between Asp50 and Pro51 (Piszkiewicz *et al.*, 1970). The fragments thus produced were separated from each other on the reversed phase column with a linear gradient of acetonitrile (5−60%) in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min.

Sequence analysis

The purified Tγ subspecies and their digested fragments were subjected to automated Edman degradation on a gas phase amino acid sequencer (Model 477A, Applied Biosystems) equipped with an on-line HPLC system (Model 120A, Applied Biosystems). For sequencing peptides containing cysteine residues, the residues were pyridylethylated according to the method of Hermodson *et al.* (1973).

Measurement of FAB and ion spray mass spectra

The fast atom bombardment (FAB) mass spectra were measured by a double-focusing mass spectrometer (JEOL JMS-HX100) equipped with a FAB ion source. The isolated peptides (0.1−0.5 nmol) derived from the Tγ subspecies were lyophilized, dissolved in 50% aqueous acetonitrile and mixed with glycerol:1-thioglycerol (1:2, v/v) on the target. The ion spray mass spectra (Fenn *et al.*, 1989) were measured by a triple quadrupole mass spectrometer (SCIEX API III) equipped with the standard atmospheric pressure ionization source. Tγ-2a and Tγ-2b (0.1 nmol) isolated by reversed phase HPLC (Figure 1) were dissolved in 20 μl of 0.1% aqueous trifluoroacetic acid containing 50% acetonitrile and infused into the ion source at a flow rate of 10 μl/min.

Preparation of stripped ROS membranes

Bleached ROS membranes (from 330 bovine retinas), from which Tα−Tβγ had been extracted by the addition of GTP (Fukada *et al.*, 1989), were washed five times with 200 ml of a hypotonic buffer (5 mM Tris−HCl, 0.5 mM $MgCl_2$, 5 mM EDTA, 1 mM dithiothreitol, pH 7.2) including 2 μM GTP-γ-S to remove transducin completely. After the washing, the membrane suspension in 200 ml of the hypotonic buffer was mixed with 11-*cis*-retinal (an equimolar amount to the opsin) dissolved in ethanol (0.13 ml) and then incubated at 25°C for 2.5 h in the dark to regenerate

rhodopsin. This regenerated membranes which were further washed four times with the hypotonic buffer in the dark, and suspended finally in buffer A (10 mM MOPS−NaOH, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethane sulphonyl fluoride, 4 μg/ml leupeptin, 50 kallikrein inhibitor units/ml aprotinin, pH 7.5). The suspension (25 μM rhodopsin) was designated as 'stripped ROS membranes' and stored at −80°C in the dark until use.

Other analytical methods

Urea/SDS−PAGE was performed by the method of Swank and Munkres (1971) modified slightly (Fukada *et al.*, 1989). Protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin as a standard. Small peptide concentrations were estimated by the amount of individual amino acid residues quantified by amino acid analysis after HCl hydrolysis. Amino acid analysis was performed using an automated amino acid analyzer (Model 835, Hitachi). The rhodopsin concentration in the stripped ROS was determined from the absorption spectra by assuming a molar extinction coefficient of 40 600/M/cm (Wald and Brown, 1953).

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