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Production of *N*-Lauroylated G Protein α-Subunit in Sf9 Insect Cells: The Type of *N*-Acyl Group of Gα Influences G Protein–Mediated Signal Transduction

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The α-subunit of rod photoreceptor G protein transducin ($G_{t1}α$) is heterogeneously modified at the N-terminus by a mixture of acyl groups, laurate (C12:0), myristate (C14:0), and two unsaturated fatty acids (C14:1 and C14:2). Although the *N*-fatty acylation of $G_{t1}α$ plays important roles in protein-protein and protein-membrane interactions in light signaling, the biological significance of the heterogeneous acylation remains unclear due to the difficulty in isolating each $G_{t1}α$ isoform from the retinal rod cells. Here we found that $G_{t1}α/G_{i1}α$ chimera ($G_{t/i}α$) expressed in Sf9 cells is also heterogeneously modified by myristate (~90%) and laurate (~10%), raising the possibility that the *N*-acyl group of recombinant $G_{t/i}α$ may be manipulated by modifying culture media. In fact, addition of myristic acid to the medium decreased the relative content of lauroylated $G_{t/i}α$ to an undetectable level, whereas exogenously added lauric acid significantly increased the relative content of lauroylated $G_{t/i}α$ in a concentration-dependent manner. By culturing the $G_{t/i}α$-virus infected Sf9 cells with fatty acids, we obtained four different preparations of *N*-acylated $G_{t/i}α$, in which the relative abundance of lauroylated isoform was 0%, 20%, 33% and ~70%, respectively. Functional analysis of these proteins showed that an increase in the relative content of the lauroylated isoform remarkably slowed down the steady-state GTP hydrolysis rate of $G_{t/i}α$; the steady-state GTPase activity of the lauroylated isoform was estimated to be one order of magnitude lower than that of the myristoylated isoform. These results suggest that the retinal $G_{t1}α$ is composed of isoforms having functionally heterogeneous signaling properties.

Key words: heteroacylation, *N*-myristoylation, phototransduction, rod photoreceptor, transducin.

Abbreviations: API, *Achromobacter lyticus* protease I; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FPLC, fast protein liquid chromatography; $Gβ_1γ_1$, βγ complex of retinal transducin; G protein, guanine nucleotide-binding regulatory protein; $G_{t1}α$, α-subunit of retinal rod transducin; GTPγS, guanosine 5′-*O*-(thiotriphosphate); MALDI-TOF/MS, matrix-assisted laser desorption ionization-time of flight/mass spectrometry; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMT, myristoyl-CoA: protein *N*-myristoyltransferase; PC, L-α-phosphatidylcholine from egg yolk; ROS, rod outer segment; TFA, trifluoroacetic acid.

Upon light absorption, the photoreceptor rhodopsin in vertebrate rod cell is converted into a physiologically active intermediate, metarhodopsin II, which binds to transducin, a heterotrimeric guanine nucleotide-binding regulatory protein (G protein) composed of GDP-bound α ($G_{t1}α$) and βγ ($Gβ_1γ_1$) subunits. This association promotes GDP release and subsequent GTP-binding to $G_{t1}α$, allowing dissociation of GTP-bound $G_{t1}α$ and $Gβ_1γ_1$ from metarhodopsin II. One molecule of metarhodopsin II catalyzes formation of several hundred molecules of GTP-bound $G_{t1}α$, an activator of cyclic GMP-phosphodiesterase. Thus, a photon-signal is highly amplified through the rapid association/dissociation cycles of $G_{t1}α$, $Gβ_1γ_1$ and metarhodopsin II in rod cells (*1*).

$G_{t1}α$ is a member of the G_i family of G-protein α-subunits, which includes $G_iα$, $G_oα$ and $G_zα$. Primary structures of the α-subunits in this family contain a consensus sequence for *N*-myristoylation at their N-termini. In fact, native $G_iα$, $G_oα$ and $G_zα$ proteins are fully and uniformly acylated with C14:0 at their N-terminal glycine residues (*2*, *3*). On the other hand, the N-terminal glycine of the α-subunit of transducin ($G_{t1}α$) is modified by a mixture of fatty acids, laurate (C12:0), 5-*cis*-tetradecenoic acid (C14:1), 5-*cis*, 8-*cis*-tetradecadienoic acid (C14:2), and a small amount of myristate (C14:0) (*4*, *5*). Thus, $G_{t1}α$ is a mixture of four isoforms modified by four different fatty acids. The relative contents of these four isoforms (C12:0-$G_{t1}α$, C14:1-$G_{t1}α$, C14:2-$G_{t1}α$ and C14:0-$G_{t1}α$) in purified

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bovine $G_{t1}\alpha$ were ~23, ~30, ~35 and ~5%, respectively (*4*). Heterogeneous *N*-acylation has been also observed in other retina-specific proteins, such as recoverin (*6*, *7*) and guanylyl cyclase-activating protein (GCAP) (*8*). This unique modification was also detected in the catalytic subunit of cAMP-dependent protein kinase purified from bovine retina, while the same protein purified from bovine heart or brain was uniformly myristoylated (*9*). Therefore, it appears that heteroacylation is a retina-specific variation of protein co-translational *N*-myristoylation and that the sequence of the modified protein is not a determinant for heteroacylation.

In general, the roles of protein *N*-myristoylation are not fully understand, but it seems to promote directly or indirectly protein-protein and/or protein-membrane interactions. As examples of its active role in protein function, *N*-acylation of Gα is indispensable for its functional coupling with Gβγ complex (*4*, *10*) and effectors (*11*), as well as for its attachment to cell membranes (*2*, *3*, *12*). Previously, we synthesized C12:0-, C14:0-, C14:1-, and C14:2-nonapeptide corresponding to the N-terminal region of bovine $G_{t1}\alpha$, and found that the peptides differed in the magnitude of the inhibitory effect on the $G_{t1}\alpha$-$G\beta_1\gamma_1$ interaction [C14:0- > C14:1- > C14:2- ≈ C12:0-peptide; Refs. *4*, *13*)]. However, no direct experimental data showing the functional difference among $G_{t1}\alpha$ isoforms have yet been presented, due to the difficulty in separating individual isoforms of heterogeneously *N*-acylated $G_{t1}\alpha$ under non-denaturing conditions.

In this study, in order to determine if the type of *N*-acyl group does influence the function of transducin, we tried to produce a myristoylated form of the α-subunit of transducin by using baculovirus-Sf9 insect cell system (*14*). For stable expression of transducin, a part of $G_{t1}\alpha$ (216–294; amino acid number of bovine $G_{t1}\alpha$) was replaced by the corresponding region of bovine $G_{i1}\alpha$ (220–298) as described originally by Skiba *et al.* (*15*). We obtained a recombinant chimera of $G_{t1}\alpha/G_{i1}\alpha$ ($G_{t/i}\alpha$) in a complex with $G\beta_1\gamma_1$, and found that the expressed $G_{t/i}\alpha$ was modified by a mixture of fatty acids, myristate and a small amount of laurate. By extending this, we found that myristic acid exogenously added to the culture medium decreased the relative content of lauroylated $G_{t/i}\alpha$ to an undetectable level, whereas exogenously added lauric acid increased the relative content of lauroylated $G_{t/i}\alpha$ in Sf9 cells. Based on these results, we produced and purified uniformly myristoylated $G_{t/i}\alpha$ (C12:0-$G_{t/i}\alpha$, 0%) and heterogeneously acylated $G_{t/i}\alpha$ (C12:0-$G_{t/i}\alpha$: 20%, 33% and ~70%). By comparing the function of these proteins, we present evidence for the functional difference between C14:0-modified Gα and C12:0-modified Gα.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Baculoviruses—The full-length cDNA encoding bovine $G_{t1}\alpha$ (*16*) digested with *Nhe*I was ligated into the *Xba*I site of the baculovirus transfer vector, pVL1393. Then the insert was excised with *Acc*65I and *Hin*dIII, and ligated into the *Acc*65I/*Hin*dIII site of pUC19 to produce a vector termed pUC19-$G_{t1}\alpha$. To replace an internal region (Cys^{216}-Asp^{294}) of $G_{t1}\alpha$ with the corresponding region of $G_{i1}\alpha$ (Ala^{220}-Glu^{298}), polymerase chain reaction (PCR) was performed with a template of pGα28 (*17*), which contained a cDNA of bovine $G_{i1}\alpha$, with the following primers: 5′-CGGGA TCCAT TGCTT CGAAG GAGTG ACC-3′ and 5′-GAAGC CGGCC TCTTC ATATG TGTTT GAGCC-3′. These primers were used to add a *Bam*HI site and an *Ngo*MI site at their terminus, respectively. The amplified product (~270 bp) was digested with *Bam*HI and *Ngo*MI, and ligated into the pUC19-$G_{t1}\alpha$ that had been digested with *Bam*HI and *Ngo*MI. The resulting plasmid was digested with *Eco*RI and *Pst*I, and ligated into the *Eco*RI/*Pst*I site of pVL1393. This construct expressing $G_{t1}\alpha/G_{i1}\alpha$ chimera ($G_{t/i}\alpha$) was termed pVL1393-$G_{t/i}\alpha$, and the nucleotide sequence was confirmed by DNA sequencing. The pVL1393-$G_{t/i}\alpha$ and BaculoGold viral DNA (Pharmingen) were co-transfected into *Spodoptera frugiperda* (Sf9) cells using Lipofectin (Invitrogen). Recombinant baculoviruses thus obtained were plaque-purified and amplified. The recombinant baculoviruses encoding bovine $G\beta_1$ and bovine $G\gamma_1$ were obtained as described previously (*18*).

Expression of $G_{t/i}\alpha$ in Sf9 Cells—Sf9 cells were maintained in 75 cm^2 culture flasks with Tc100 medium (Invitrogen or SIGMA) supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) fetal bovine serum (Invitrogen), 0.26% (v/v) tryptose phosphate broth (Difco) and 100 μg/ml kanamycin (Nacalai Tesque, Kyoto).

For large-scale production of recombinant $G_{t/i}\alpha$, Sf9 cells were grown to a cell density of 2×10^6 cells/ml at 27°C in Tc100 medium supplemented with 10% (v/v) heat inactivated (56°C, 30 min) fetal bovine serum, 0.26% (v/v) tryptose phosphate broth, kanamycin (100 μg/ml) and 0.1% (v/v) of pluronic F68 (Invitrogen).

For expression of $G_{t/i}\alpha/G\beta_1\gamma_1$ complex in Sf9 cells, the cells grown to 2×10^6 cells/ml were sedimented by centrifugation (80 ×*g*, 5 min, at room temperature) and resuspended in fresh medium. The cells were co-infected with $G_{t/i}\alpha$, $G\beta_1$ and $G\gamma_1$ recombinant baculoviruses at a multiplicity of infection (moi) of 3 for each virus in Erlenmeyer flasks (1-liter). Each flask contained 300 ml of Tc100 medium, and myristic acid (SIGMA) or lauric acid (Nacalai Tesque, Kyoto) dissolved in ethyl alcohol was added to the media immediately after virus infection to give the final concentration of 100 μM or 500 μM, respectively. The final concentration of ethyl alcohol was lower than 0.1%(v/v). The cells in the flasks were then cultured for 48 h at 27°C on an orbital shaker platform. The rotating speed was set at 125–130 rpm for the culture with 100 μM myristic acid or without added fatty acid, or at 90 rpm for the culture with 500 μM lauric acid.

When the cells were cultured with final 1 mM of lauric acid, the cells were infected with recombinant baculoviruses as described above in Erlenmeyer flasks (200 ml) each containing 60 ml of complete Tc100 medium. After addition of final 1 mM lauric acid, the cell culture was incubated at 27°C for 48 h on an orbital shaker platform. The rotating speed was set at 130 rpm.

The cells were harvested by centrifugation, washed twice with PBS (10 mM Na-phosphate, 140 mM NaCl, 1 mM $MgCl_2$; pH 7.4), snap-frozen in liquid nitrogen, and stored at –80°C until use. In large-scale procedures, the frozen pellets were thawed quickly in 30 ml of buffer C1 (20 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 4 μg/ml aprotinin, 4 μg/ml leupeptin; pH 8.0 at 4°C), and for the lysis of the

cells, the cell suspension was transferred to a nitrogen bomb (Parr) and subjected to a charge of 600 psi for 30 min at 4°C using nitrogen gas. After the pressure had been slowly reduced, the lysate was recovered and centrifuged at 100,000 ×*g* for 1 h at 4°C to separate the membrane fraction from the cytosol. The membrane fraction was stirred at 4°C for 1 h in 100 ml of buffer C2 (buffer C1 supplemented with 1% [w/v] CHAPS) for solubilization, and then centrifuged at 100,000 ×*g* for 1 h at 4°C to separate the CHAPS extract and the insoluble fraction.

For small-scale preparation (*e.g.*, for optimization of experimental conditions), the cultured cells were lysed by drawing/ejecting through a 25-gauge needle. The membrane fraction obtained by centrifugation of the lysate was solubilized with 1% CHAPS (buffer C2) by drawing/ejecting through a 25-gauge needle, then centrifuged to isolate the CHAPS extract. The centrifugation conditions were identical to those described in the large-scale preparation.

For expression of $G_{t/i}\alpha$ (without $G\beta_1\gamma_1$), the Sf9 cells grown as described above were infected with the $G_{t/i}\alpha$ recombinant baculovirus at moi of 3, and the culture was started at a cell density of 1.0×10^6 cells/ml at 27°C. After 48 h of culture, the cells were harvested by centrifugation, washed twice with PBS, snap-frozen in liquid nitrogen, and stored at –80°C until use. The frozen harvested cells (stocks from 3–4 liter culture of Sf9 cells) were thawed quickly in 30 ml of buffer C1 and burst by N_2 cavitation using the nitrogen bomb (described above). The cell lysate was centrifuged at 100,000 ×*g* for 1 h at 4°C to separate the cytosol and membrane fractions.

Protein Purification—All the purification procedures were carried out at 4°C unless otherwise stated. Recombinant $G_{t/i}\alpha/G\beta_1\gamma_1$ was purified from the CHAPS extract of Sf9 cells expressing $G_{t/i}\alpha/G\beta_1\gamma_1$. The extract (~100 ml prepared from 2 liters of culture) was applied to a DEAE-Sepharose (Amersham Biosciences) column (diameter 14.5 × 60 mm) preequilibrated with buffer C2. The column was then washed with ~30 ml of buffer C2 and subsequently with ~30 ml of buffer C3 (buffer C1 supplemented with 0.7% [w/v] CHAPS), and proteins were eluted with a linear gradient of NaCl (0–500 mM) formed in 60 ml of buffer C3 at a flow rate of 50 ml/h. Fractions containing $G_{t/i}\alpha/G\beta_1\gamma_1$ were pooled (~12 ml) and applied to a gel filtration Superdex 200-pg column (diameter 26 × 600 mm, Amersham Biosciences) preequilibrated with buffer C4 (buffer C3 supplemented with 100 mM NaCl). Using a FPLC system (Amersham Biosciences), $G_{t/i}\alpha/G\beta_1\gamma_1$ was eluted from the column with buffer C4 at a flow rate of 2.5 ml/min. The $G_{t/i}\alpha/G\beta_1\gamma_1$-enriched fractions were pooled (~10 ml), applied to a Q-Sepharose column (HiTrap Q, 1 ml, Amersham Biosciences) equilibrated with buffer C5 (buffer C3 without aprotinin and leupeptin), and eluted with a linear gradient of NaCl (50–500 mM) formed in 23 ml of buffer C5 at a flow rate of 1 ml/min using the FPLC system. The peak of elution of $G_{t/i}\alpha/G\beta_1\gamma_1$ was centered at ~250 mM NaCl. Purified proteins were divided into aliquots and stored at –80°C. Approximately 3–4 nmol of $G_{t/i}\alpha/G\beta_1\gamma_1$ (>80% of purity; estimated from Coomassie Blue-stained gels) was obtained from 3 liters of Sf9 cell culture (at a cell density of 1.0×10^6 cells/ml) in the presence of myristic acid (final 100 μM) or lauric acid (final 500 μM or lower) added to the medium.

$G_{t/i}\alpha$ expressed in the absence of $G\beta_1\gamma_1$ was enriched in the cytosol fraction of Sf9 cells, and therefore the cytosolic fraction was subjected to the purification procedures as described above except that all the buffers used for the chromatography did not contain CHAPS. The protein purity of the $G_{t/i}\alpha$-rich fractions eluted from the last Q-Sepharose column was unsatisfactory, so the fractions were combined, dialyzed against buffer HA (10 mM Tris-KOH, 1 mM DTT; pH 8.0) containing 10 mM potassium phosphate, and applied to a hydroxyapatite (Bio-Rad, Macro-Prep Ceramic Hydroxyapatite) column (HR 5/5, Amersham Biosciences). $G_{t/i}\alpha$ was eluted with a linear gradient of potassium phosphate (10–500 mM) in buffer HA at a flow rate of 1 ml/min using the FPLC system. Fractions enriched with $G_{t/i}\alpha$ were dialyzed against buffer C1 and concentrated by using Q-Sepharose column as described above. Purified proteins were divided into aliquots and stored at –80°C. Approximately 6 nmol of cytosolic $G_{t/i}\alpha$ (>80% of purity; estimated from Coomassie Blue-stained gels) was obtained from 4 liters of Sf9 cell culture (at a cell density of 1.0×10^6 cells/ml).

Retinal transducin ($G_{t1}\alpha/G\beta_1\gamma_1$) was purified from the dark-adapted bovine retinas as described previously (*19*). Bovine rhodopsin was purified and reconstituted in phosphatidylcholine (PC) liposomes as described (*20*).

Analyses of N-Terminal Lipid Structure of $G_{t/i}\alpha$—A purified preparation of $G_{t/i}\alpha/G\beta_1\gamma_1$ (approx. 1 nmol) was concentrated to ~50 μl by using a SpeedVac concentrator (SVC100H, Savant), and dialyzed against buffer AP (50 mM Tris-HCl, 0.01% [w/v] CHAPS; pH 8.5 at room temperature) containing 8 M urea. After mixing with the same volume of buffer AP, the protein sample was supplemented with 1 μg of *Achromobacter lyticus* protease I (API; Wako, Osaka), and then incubated at 37°C for 30 h. After 8 h and 24 h of incubation, 1 μg each of API was added to the reaction mixture for complete digestion. The digests were loaded onto a reverse phase HPLC column (Develosil ODS-T-5; 4.6 × 150 mm, Nomura Chemical, Aichi) equipped with a HPLC system (model 600E; Waters, Tokyo). Fragments were eluted at a flow rate of 1 ml/min with H_2O-acetonitrile gradient in the presence of 0.1% trifluoroacetic acid (TFA). The gradient program started with 2% acetonitrile constant over the first 5 min, and then the concentration of acetonitrile was increased at 1%/min up to 80%. The absorbance of the eluate at 214 nm was continuously monitored, and each peak fraction was collected, dried and stored at –20°C until use.

A peptide corresponding to the N-terminal nonapeptide of $G_{t1}\alpha$ (Gly^2-Lys^{10}: one of the API-digestion products) was synthesized, and the N-terminus was either myristoylated or lauroylated by chemical reaction (*4*). The concentrations of the modified peptides were determined by the amino-acid analysis (*4*), and then they were also loaded onto a Develosil ODS-T-5 column and eluted with the same gradient as described above. The peak areas were used as calibration standards for estimating the yield of API proteolytic fragments derived from the N-terminus of $G_{t/i}\alpha$. The yield of recovery from the column of the total amount of *N*-acylated fragments (C12:0-plus C14:0-modified peptide derived from the expressed protein) was almost constant (70–80% in molar ratio of $G_{t/i}\alpha$ subjected to the API-digestion) among different preparations.

The proteolytic peptides eluted from the reverse phase HPLC were lyophilized, resuspended in 8 μl of 60% acetonitrile containing 0.12% TFA, and subjected to the matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass/spectrometric (MS) analysis. Briefly, an aliquot (1 μl) of the sample was spotted onto the sample plate of the mass spectrometer and mixed with 1 μl of matrix, α-cyano-4-hydroxycinamic acid that had been saturated in 60% acetonitrile and 0.1% TFA. These samples were air-dried and analyzed by a Voyager-DE mass spectrometer (Applied Biosystems) with two-point external calibration by using des-Arg^1-Bradykinin ([M+H]$^+$= 904.5) and human angiotensin I ([M+H]$^+$= 1296.7) as standards.

Analysis of N-Terminal Structure of Cytosolic $G_{t/i}\alpha$—A purified preparation of cytosolic $G_{t/i}\alpha$ (~600 pmol) was dialyzed against 12.5 mM NH_4HCO_3 (pH 8.0), and then incubated at 37°C for 1 h with 0.48 μg endoproteinase Asp-N (Asp-N; Boehringer Mannheim). The digested mixture was subjected to the reverse phase HPLC with the Develosil ODS-T-5 column, and the peptides were eluted under the same condition as described for the analysis of $G_{t/i}\alpha/G\beta_1\gamma_1$. Each peak fraction was subjected to MALDI-TOF mass spectrometry, in which adenocorticotropic hormone clip 18–39 ([M+H]$^+$= 2465.2) was used as a standard instead of des-Arg^1-Bradykinin. The N-terminal fragment composed of 20 amino acid residues (Gly^2-Glu^{21}) was quantitated by the amino-acid analysis. The amount of non-acylated form of the N-terminal peptide was 85% (in average of two independent determinations) in molar ratio of the amount of $G_{t/i}\alpha$ subjected to this analysis.

Electrophoresis and Immunoblotting—SDS-PAGE was carried out as described (*21*), and the gel was stained with Coomassie Brilliant Blue. For immunoblotting, proteins resolved by SDS-PAGE were transferred to a PVDF membrane, FineTrap NT-31 (Nihon-eido, Tokyo), and probed with a mouse monoclonal antibody (LA4) raised against *N*-lauroylated form of bovine $G_{t1}\alpha$ (*13*) or rabbit antiserum (anti-p$T_r\alpha$) raised against bovine $G_{t1}\alpha$ (*22*). Bound antibodies were detected with goat anti-mouse or anti-rabbit IgG, both conjugated to horseradish peroxidase, and visualized by using Renaissance (NEN Life Science Products). For reprobing, the membrane was submerged in stripping buffer (62.5 mM Tris-HCl; pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) for incubation at 50°C for 30 min with agitation to remove the first antibody, then subjected to the immunostaining procedure with a different antibody.

Other Methods—The concentrations of purified proteins were estimated by the method of Bradford (*23*) using bovine serum albumin as a standard. Alternatively, the amount of $G_{t/i}\alpha$ protein was estimated by immunoblot analysis with antibody (anti-p$T_r\alpha$) against bovine $G_{t1}\alpha$, and the active $G_{t/i}\alpha$ level was determined by the maximal incorporation of [^{35}S]GTPγS catalyzed by metarhodopsin II (*18*). The level of cytosolic $G_{t/i}\alpha$ was evaluated by the protein concentration, instead of GTPγS-binding, which proceeded only very slowly. Rhodopsin concentration was calculated from the change in absorbance at 500 nm before and after complete bleaching of rhodopsin in the presence of 100 mM NH_2OH (pH 7.0).

RESULTS

Expression of $G_{t/i}\alpha$ Chimeric Protein in Sf9 Cells—For functional studies on the *N*-acylated form of transducin α-subunit ($G_{t1}\alpha$), a bacurovirus-Sf9 cell system was utilized to produce a mutant $G_{t1}\alpha$ in which the residues (216–294) of bovine $G_{t1}\alpha$ were replaced with the corresponding residues (220–298) of bovine $G_{i1}\alpha$. This chimeric protein, termed $G_{t/i}\alpha$, was originally developed by Skiba *et al*. (*15*), and it is 94% identical to wild-type $G_{t1}\alpha$ at the amino acid level. This chimera in "non-acylated form" was expressed successfully in *Escherichia coli* and used for functional (*24*) and structural analyses (*25–27*), instead of wild-type $G_{t1}\alpha$.

When $G_{t/i}\alpha$ was expressed in Sf9 cells using the recombinant baculovirus, approximately 12% of expressed $G_{t/i}\alpha$ was recovered in the cytosol fraction, and the rest was found in the membrane fraction, from which only a part (~10%) of $G_{t/i}\alpha$ was solubilized with CHAPS and the rest remained in the detergent-insoluble fraction. We tried to purify $G_{t/i}\alpha$ from both cytosol and CHAPS-extract fractions. As shown in Fig. 1A, $G_{t/i}\alpha$ in the cytosol fraction was purified to near homogeneity through five steps of column chromatography. On the other hand, a large amount of $G_{t/i}\alpha$ in the CHAPS-extract appeared aggregated, and we were unable to purify it (data not shown).

The purified preparation of cytosolic $G_{t/i}\alpha$ was subjected to the peptide sequence analysis, and we found that a large fraction of the protein had the N-terminal sequence Gly^2-Ala^3-Gly^4- without fatty-acylation, indicating that the initiator Met was removed in Sf9 cells. To examine if a subset of cytosolic $G_{t/i}\alpha$ might be *N*-acylated, the protein was completely digested with endoproteinase Asp-N and subjected to reverse phase HPLC (Fig. 2A). MALDI-TOF/MS analysis identified a fragment with a mass value in good agreement with the non-acylated N-terminal fragment of $G_{t/i}\alpha$ (20 amino acid residues without initiator Met, Fig. 2A, peak p1). On the other hand, we were unable to detect a peak with a mass value corresponding to the C14:0-modified peptide or a related fatty acid-modified peptide. Similarly, no evidence was obtained for *N*-fatty acylation of cytosolic $G_{t/i}\alpha$ after digestion with lysyl endopeptidase API (data not shown), and hence we concluded that cytosolic $G_{t/i}\alpha$ has unmodified Gly at the N-terminus.

Co-Expression of $G_{t/i}\alpha$ with $G\beta_1\gamma_1$ in Sf9 Cells—To obtain an *N*-fatty acylated form of $G_{t/i}\alpha$, we tried to reduce aggregation of $G_{t/i}\alpha$ in the CHAPS-extract. Because co-expression of Gβγ has been shown to facilitate solubilization and purification of $G_q\alpha$, $G_{11}\alpha$, $G_{14}\alpha$ and $G_{16}\alpha$ (*28–30*), $G_{t/i}\alpha$ was co-expressed with $G\beta_1\gamma_1$ in Sf9 cells by co-infection with $G_{t/i}\alpha$-, $G\beta_1$-, and $G\gamma_1$-recombinant viruses at a moi ratio of 1:1:1, which we found to be the best combination of those tested for expression and solubilization of a larger amount of $G_{t/i}\alpha/G\beta_1\gamma_1$ complex. Under these conditions, more than ~80% of expressed $G_{t/i}\alpha$ was recovered with $G\beta_1\gamma_1$ in the membrane fraction, from which 20–30% of $G_{t/i}\alpha$ was solubilized with CHAPS. $G_{t/i}\alpha$ in the CHAPS-extract of the membrane fraction (termed "membrane $G_{t/i}\alpha$") was co-purified with $G\beta_1\gamma_1$ to near homogeneity by three steps of column chromatography (Fig. 1B). Membrane $G_{t/i}\alpha$ was co-eluted with $G\beta_1\gamma_1$ from a Superdex 200 gel filtration column (data not

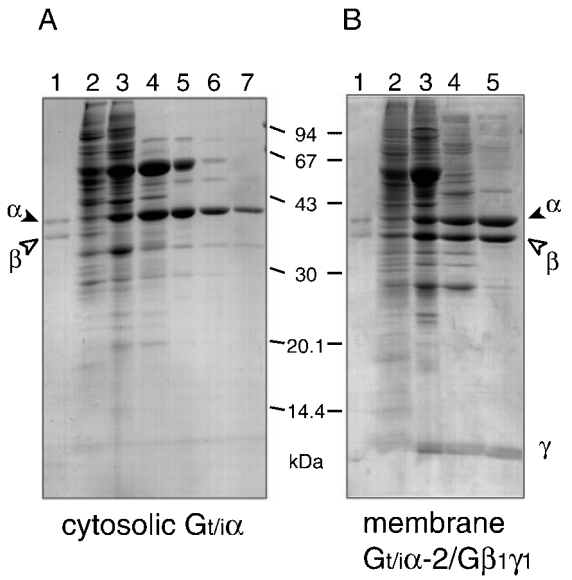

Fig. 1. **Purification of recombinant $G_{t/i}\alpha$ from the lysate of baculovirus-infected Sf9 cells.** (A) Purification of $G_{t/i}\alpha$ from the cytosolic fraction of Sf9 cells expressing $G_{t/i}\alpha$ alone. (B) Purification of $G_{t/i}\alpha$ as a complex with $G\beta_1\gamma_1$ from the membrane fraction of Sf9 cells co-expressing $G_{t/i}\alpha$ and $G\beta_1\gamma_1$. Aliquots obtained in the purification steps were subjected to SDS-PAGE (acrylamide, 13%) and stained with Coomassie Blue. Lane 1: purified retinal transducin. Lane 2: cytosolic fraction of Sf9 cells expressing $G_{t/i}\alpha$ (panel A) or CHAPS-extract fraction of Sf9 cells expressing $G_{t/i}\alpha$-2/$G\beta_1\gamma_1$ (panel B). Lane 3: eluate from the DEAE-Sepharose column. Lane 4: eluate from the gel filtration Superdex 200-pg column. Lane 5: eluate from the Q-Sepharose column. Lane 6: eluate from the hydroxyapatite column. Lane 7: eluate from the Q-Sepharose column. The numbers between Panel A and Panel B are the molecular weights of the standard proteins.

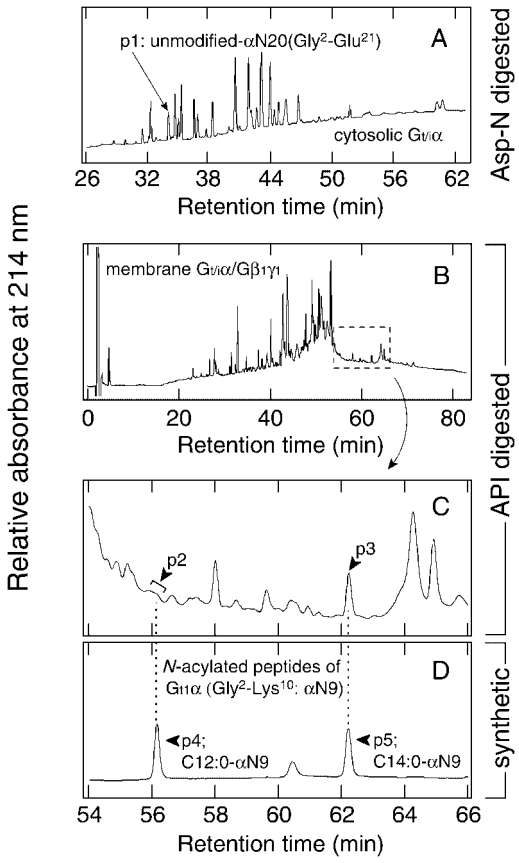

Fig. 2. **Analysis of the N-terminal peptides of $G_{t/i}\alpha$ expressed in Sf9 cells cultured without the addition of fatty acids.** (A) Purified cytosolic $G_{t/i}\alpha$ was digested with endoproteinase Asp-N (Asp-N) and loaded onto the reverse phase HPLC column. The N-terminal fragment (Gly^2–Glu^{21}: αN20) of cytosolic $G_{t/i}\alpha$ was identified by peptide-sequencing and MALDI-TOF/MS analysis (p1, indicated by an arrow). The observed mass ($[M+H]^+$) of the fragment p1 (2197.3) agreed well with the calculated mass (2,197.2) of unmodified-αN20. (B) Purified membrane $G_{t/i}\alpha/G\beta_1\gamma_1$ trimer was completely digested with lysyl endopeptidase API and loaded onto the reverse phase HPLC column. (C) An expanded display of a part of the elution profile enclosed by the broken line in Panel B. (D) The synthetic peptide corresponding to the N-terminal region of $G_{t/i}\alpha$ (Gly^2–Lys^{10} of $G_{t1}\alpha$: αN9) was *N*-lauroylated (C12:0-αN9; p4) or *N*-myristoylated (C14:0-αN9; p5), and they were loaded onto the reverse phase HPLC column as standards. The observed masses ($[M+H]^+$) of the fragments p2 (1,001.6) and p3 (1,029.7) agreed well with the calculated masses of C12:0-αN9 (1,001.6) and C14:0-αN9 (1,029.6), respectively.

shown), and the intensity of the Coomasie Blue-stained band of $G_{t/i}\alpha$ was nearly the same as that of co-purified $G\beta_1$, indicating that membrane $G_{t/i}\alpha$ was purified as a heterotrimeric complex of $G_{t/i}\alpha/G\beta_1\gamma_1$.

The purified preparation of membrane $G_{t/i}\alpha$ was refractory to Edman degradation, suggesting that the N-terminus is acylated. To determine the N-terminal structure, membrane $G_{t/i}\alpha/G\beta_1\gamma_1$ was completely digested with lysyl endopeptidase API, and the proteolytic fragments were separated by reverse phase HPLC (Fig. 2B). To identify the N-terminal peptide of $G_{t/i}\alpha$, all the peak fractions were subjected to MALDI-TOF/MS analysis, and their retention times were compared to those of the synthetic *N*-myristoylated and *N*-lauroylated peptides (Fig. 2D). We found a fragment (Fig. 2C, p3) whose retention time and mass value were in good agreement respectively with those of the *N*-myristoylated (C14:0-) nonapeptide (Gly^2–Lys^{10}) of $G_{t/i}\alpha$ (Fig. 2D, p5), indicating that $G_{t/i}\alpha$ expressed in Sf9 cells is myristoylated at its N-terminus. We also detected a smaller peak (Fig. 2C, p2) whose retention time and mass value respectively agreed well with those of the *N*-lauroylated (C12:0-) nonapeptide corresponding to the N-terminus of $G_{t/i}\alpha$ (Fig. 2D, p4). The presence of C12:0-modified $G_{t/i}\alpha$ in membrane $G_{t/i}\alpha$ was further confirmed by using LA4 monoclonal antibody (Fig. 3A, lane 1; for details, see below). LA4 antibody specifically recognizes the C12:0-modified N-terminal structure of $G_{t1}\alpha$ with no significant cross-reaction to the C14:0-modified isoform (*13*). These results indicate that a small population of membrane $G_{t/i}\alpha$ is modified by laurate. We did not detect any peptide peak with a mass value corresponding to the C14:1- or C14:2-modified N-terminal peptide that were observed in native $G_{t1}\alpha$ purified from bovine retina (*4*, *5*). Quantification of the peptides in the two peaks p2 and p3 (Fig. 2C) revealed that the molar ratio of p3 (C14:0-peptide) to p2 (C12:0-peptide) was approximately 9, and that 70–80% of the N-terminal peptide of $G_{t/i}\alpha$ subjected to the API-digestion was recovered in this analysis. Considering the efficiency of the API-digestion and the

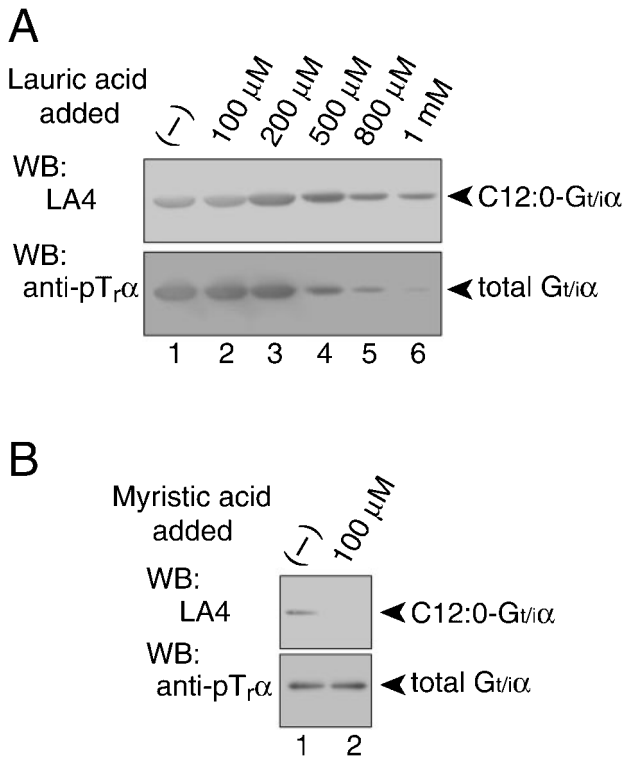

Fig. 3. **Exogenously supplied fatty acids alter the relative amount of *N*-lauroylated isoform of $G_{t/i}\alpha$.** Sf9 cells co-infected with baculoviruses encoding $G_{t/i}\alpha$, $G\beta_1$ and $G\gamma_1$ were cultured for 48 h in the medium supplemented with various concentrations of lauric acid (panel A), or myristic acid (panel B). The membrane fractions of harvested cells were solubilized with 1% (w/v) CHAPS. Aliquots (5 μl each) were electrophoresed on SDS-polyacrylamide (13%) gels, transferred to PVDF membranes, and probed with LA4 monoclonal antibody, which specifically recognizes an *N*-lauroylated form of $G_{t/i}\alpha$. The sheet was then reprobed with anti-$pT_r\alpha$, which recognizes the internal region of $G_{t/i}\alpha$. Arrowheads indicate the position of an *N*-lauroylated form of $G_{t/i}\alpha$ (upper panels in A and B) or total $G_{t/i}\alpha$ (lower panels in A and B). Final concentrations of lauric acid and myristic acid added to the medium are indicated on the top of each figure.

recovery yield in the HPLC analysis, we estimated that membrane $G_{t/i}\alpha$ is composed of ~90% C14:0-modified isoform and ~10% C12:0-modified isoform.

Alteration of the Composition of Fatty Acids Attached to $G_{t/i}\alpha$—Heterogeneous fatty acylation of $G_{t/i}\alpha$ in Sf9 cells suggests the presence of pools of lauroyl-coenzyme A (C12:0-CoA) and myristoyl-coenzyme A (C14:0-CoA) that are available as substrates of endogenous NMT. The cellular pool sizes of C12:0-CoA and C14:0-CoA (*31*) may be one of the possible determinants of the relative contents of C12:0-$G_{t/i}\alpha$ and C14:0-$G_{t/i}\alpha$ (*9*). Then we attempted to alter the relative content of C12:0-$G_{t/i}\alpha$ by changing the pool size of C12:0-CoA or C14:0-CoA in Sf9 cells. For this purpose, Sf9 cells co-infected with $G_{t/i}\alpha/G\beta_1/G\gamma_1$-recombinant viruses were cultured in the presence of various concentrations of exogenously supplied lauric acid or myristic acid, which may affect the (relative) abundance of C12:0-CoA and C14:0-CoA pools in the cells. Then, after harvesting the cells, the relative content of C12:0-$G_{t/i}\alpha$ in total membrane $G_{t/i}\alpha$ (in the CHAPS-extract) was estimated by immunoblotting with LA4 monoclonal antibody specific for C12:0-modified isoform of bovine $G_{t1}\alpha$ (*13*). As described in the previous section, LA4 recognized membrane $G_{t/i}\alpha$ expressed in Sf9 cells cultured without the addition of fatty acids (Fig. 3A, lane 1). To evaluate the total amount of expressed $G_{t/i}\alpha$, the blot was probed in parallel with an antiserum (anti-$pT_r\alpha$) that recognizes the internal region of bovine $G_{t1}\alpha$ (*22*). When lauric acid was added at a lower concentration (final 100 μM or 200 μM) to the culture medium, the relative amount of C12:0-$G_{t/i}\alpha$ in membrane $G_{t/i}\alpha$ (CHAPS extracted) increased in a concentration-dependent manner (Fig. 3A, lanes 2 and 3). At a higher concentration of added lauric acid (>500 μM), however, the expression level of total membrane $G_{t/i}\alpha$ markedly decreased with increasing concentration of lauric acid (Fig. 3A, lower panel, lanes 4–6), and concomitantly the level of C12:0-$G_{t/i}\alpha$ decreased (Fig. 3A, upper panel). In contrast, the addition of myristic acid (final 100 μM) decreased the amount of C12:0-$G_{t/i}\alpha$ to an undetectable level (Fig. 3B), suggesting that membrane $G_{t/i}\alpha$ expressed under this condition is modified almost exclusively by C14:0. These results demonstrate that the relative amount of C12:0-modified $G_{t/i}\alpha$ can be manipulated by adding lauric acid or myristic acid to the cell culture medium.

To purify several batches of membrane $G_{t/i}\alpha$ with various contents of C12:0-$G_{t/i}\alpha$, Sf9 cells co-expressing $G_{t/i}\alpha$, $G\beta_1$ and $G\gamma_1$ were cultured on a large scale (2–6 liters) in the presence of final 100 μM myristic acid, 500 μM lauric acid or 1 mM lauric acid, and four types of membrane $G_{t/i}\alpha$ were purified, each as a complex with $G\beta_1\gamma_1$. These α-subunits were termed $G_{t/i}\alpha$-1 (from cell culture with 100 μM myristic acid), $G_{t/i}\alpha$-2 and –3 (both from culture with 500 μM lauric acid) and $G_{t/i}\alpha$-4 (from culture with 1 mM lauric acid). The final yields of membrane $G_{t/i}\alpha$-1/$G\beta_1\gamma_1$, $G_{t/i}\alpha$-2/$G\beta_1\gamma_1$ and $G_{t/i}\alpha$-3/$G\beta_1\gamma_1$ were 0.8–1.7 nmol from 1 liter of culture, and purities were >80%. On the other hand, the expression level of $G_{t/i}\alpha$-4/$G\beta_1\gamma_1$ was very low, probably due to the toxicity of a high concentration of added lauric acid (see Fig. 3A), and the final yield of $G_{t/i}\alpha$-4/$G\beta_1\gamma_1$ from 2 liters of culture was less than 25 pmol (determined by the maximal incorporation of [^{35}S]GTPγS catalyzed by metarhodopsin II) with purity of ~20% (estimated from SDS-PAGE; data not shown).

Next, relative contents of C12:0-$G_{t/i}\alpha$ in these four types of membrane $G_{t/i}\alpha/G\beta_1\gamma_1$ were estimated by immunoblot analysis with LA4 antibody (Fig. 4A). As expected, LA4 did not recognize membrane $G_{t/i}\alpha$-1 expressed in the C14:0-enriched medium (Fig. 4A, lane 2), and similarly cytosolic (unmodified) $G_{t/i}\alpha$ was not recognized by LA4 (Fig. 4A, lane 1). On the other hand, immuno-positive bands were detected in membrane $G_{t/i}\alpha$-2, -3, -4 which were expressed in the C12:0-enriched media (Fig. 4A, lanes 3, 4 and 5). The strongest signal was detected in membrane $G_{t/i}\alpha$-4 expressed with the highest concentration (1 mM) of lauric acid (Fig. 4A, lane 5).

In order to determine the relative contents of C14:0-$G_{t/i}\alpha$ and C12:0-$G_{t/i}\alpha$ in membrane $G_{t/i}\alpha$-1/$G\beta_1\gamma_1$, $G_{t/i}\alpha$-2/$G\beta_1\gamma_1$ and $G_{t/i}\alpha$-3/$G\beta_1\gamma_1$, these preparations were completely digested with lysyl endopeptidase API and subjected to reverse phase HPLC. As shown in Fig. 4C, C14:0-modified fragment (p6) was detected in the API-digest of membrane $G_{t/i}\alpha$-1/$G\beta_1\gamma_1$, but we did not detect C12:0-modified fragment or other related fragments (C14:1-

and C14:2-modified fragments) as judged from the elution positions and the mass values of the eluted peptides. Therefore, we concluded that membrane $G_{t/i}\alpha$-1/$G\beta_1\gamma_1$ is modified uniformly by C14:0 (see Table 1). On the other hand, the API-digestion of membrane $G_{t/i}\alpha$-2/$G\beta_1\gamma_1$ (Fig. 4D) and $G_{t/i}\alpha$-3/$G\beta_1\gamma_1$ (Fig. 4E) yielded C12:0-modified fragments (p7 and p9) in addition to C14:0-modified fragments (p8 and p10). Again, neither C14:1- nor C14:2-modified fragments were detected, and the calculation of the molar ratio of the two peaks indicated that the ratio of C12:0-$G_{t/i}\alpha$ to C14:0-$G_{t/i}\alpha$ in membrane $G_{t/i}\alpha$-2 and –3 was 20:80 and 33:67, respectively (Table 1). Because of the low yield of $G_{t/i}\alpha$-4/$G\beta_1\gamma_1$, the relative amount of C12:0-$G_{t/i}\alpha$ in this preparation was estimated by immunoblot analysis, in which LA4-immunoreactivity of $G_{t/i}\alpha$-4 was compared with those of known amounts of the purified preparation of membrane $G_{t/i}\alpha$-3 as a standard. The relative amount of C12:0-$G_{t/i}\alpha$ in membrane $G_{t/i}\alpha$-4 was estimated ~70% (Table 1).

Effect of N-Terminal Fatty Acylation on the Function of $G_{t/i}\alpha$—As summarized in Table 1, we obtained five batches of purified $G_{t/i}\alpha/G\beta_1\gamma_1$, in which the α-subunits have diverged and defined compositions in terms of the N-terminal structure. Then we examined how the type of fatty acid (C14:0 vs. C12:0) attached to the N-terminus affects the function of $G_{t/i}\alpha$. For this purpose, five batches of $G_{t/i}\alpha/G\beta_1\gamma_1$ were reconstituted with light-activated rhodopsin (metarhodopsin II) in PC liposomes, and the reaction rates of steady-state GTP-hydrolysis (GTP-turnover rates) were measured (Fig. 5). In this experiment, cytosolic $G_{t/i}\alpha$ (purified as a monomer) was mixed with an equal amount of retinal transducin βγ (=$G\beta_1\gamma_1$), the γ-subunit of which was fully farnesylated and carboxyl methylated at the C-terminus as judged by reverse phase HPLC analysis (*19*). Similarly, more than 90% of recombinant $G\beta_1\gamma_1$ each in a complex with $G_{t/i}\alpha$-1, $G_{t/i}\alpha$-2 and $G_{t/i}\alpha$-3 had the fully modified γ-subunit (data not shown; $G_{t/i}\alpha$-4/$G\beta_1\gamma_1$ was not analyzed due to its low yield), and this result is consistent with the previous report on the expression of G-protein in Sf9 cells (*32*).

As shown in Fig. 5A, cytosolic $G_{t/i}\alpha$ hydrolyzed GTP very poorly even in the presence of retinal $G\beta_1\gamma_1$ and metarhodopsin II. On the other hand, one molecule of membrane $G_{t/i}\alpha$-1, -2, -3 and -4 as a complex with $G\beta_1\gamma_1$ hydrolyzed multiple molecules of GTP within 20 min of incubation in the presence of metarhodopsin II. Among these *N*-acylated $G_{t/i}\alpha$s, $G_{t/i}\alpha$-1 exhibited the highest GTP-turnover rate, followed by $G_{t/i}\alpha$-2, $G_{t/i}\alpha$-3 and $G_{t/i}\alpha$-4, in this order. This rank order correlated well with the relative contents of C14:0- and C12:0-modified species, and

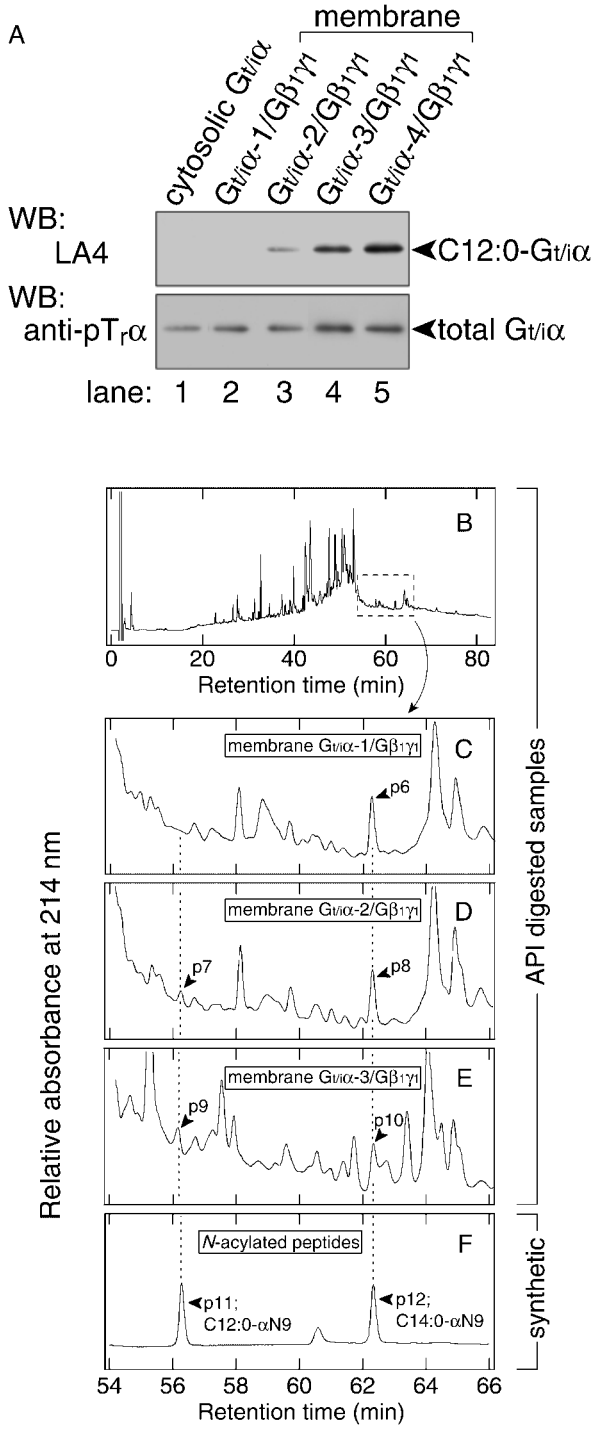

Fig. 4. **Analysis of purified membrane $G_{t/i}\alpha$ expressed in Sf9 cells cultured in the presence of exogenously added fatty acids.** (A) Immunoblot analysis of purified cytosolic $G_{t/i}\alpha$ (lane 1) and membrane $G_{t/i}\alpha$ in a complex with $G\beta_1\gamma_1$ (lanes 2–5). Membrane $G_{t/i}\alpha$s were purified each as a complex with $G\beta_1\gamma_1$ from membrane fractions of virus-infected Sf9 cells cultured with 100 μM myristic acid ($G_{t/i}\alpha$-1/$G\beta_1\gamma_1$, lane 2), 500 μM lauric acid ($G_{t/i}\alpha$-2/$G\beta_1\gamma_1$, lane 3; $G_{t/i}\alpha$-3/$G\beta_1\gamma_1$, lane 4) or 1 mM lauric acid ($G_{t/i}\alpha$-4/$G\beta_1\gamma_1$, lane 5). Purified proteins were subjected to SDS-PAGE (13% acrylamide), transferred to PVDF membrane, and probed with antibodies, anti-$pT_r\alpha$ and LA4. (B–F) Separation of N-terminal proteolytic fragments derived from purified membrane $G_{t/i}\alpha$ by the reverse phase HPLC. Panel B is a representative elution profile of proteolytic fragments derived from membrane $G_{t/i}\alpha$-1/$G\beta_1\gamma_1$ after complete digestion with lysyl endopeptidase API. Panels C ($G_{t/i}\alpha$-1/$G\beta_1\gamma_1$), D ($G_{t/i}\alpha$-2/$G\beta_1\gamma_1$) and E ($G_{t/i}\alpha$-3/$G\beta_1\gamma_1$) are expanded displays of parts of the elution profiles. The synthetic N-terminal peptides of $G_{t/i}\alpha$, C12:0-αN9 (p11) and C14:0-αN9 (p12) were loaded onto the reverse phase HPLC column as standards (panel F). The observed masses ($[M+H]^+$) of proteolytic fragments designated p6, p7, p8, p9, p10, p11 and p12 were 1029.7, 1001.8, 1029.6, 1001.7, 1029.9, 1001.8 and 1029.7, respectively. These values agreed well with either of the calculated masses of C12:0-αN9 (1001.6) or C14:0-αN9 (1029.6).

Table 1. **N-terminal structures of $G_{t/i}\alpha$ expressed in Sf9 cells.** $G_{t/i}\alpha$-1, -2, -3 and -4 were purified as a complex with $G\beta_1\gamma_1$, whereas cytosolic $G_{t/i}\alpha$ was purified as a monomer from Sf9 cells cultured with or without exogenous myristic acid (Myr) or lauric acid (Lau). Relative contents of C12:0-modified, C14:0-modified and unmodified forms were estimated as described in the text. A variation in content of C12:0-modified form between $G_{t/i}\alpha$-2 and $G_{t/i}\alpha$-3, both of which were expressed in Sf9 cells cultured under the same condition (in the presence of 500 μM of lauric acid) might be due to the difference of the lot of fetal bovine serum (FBS) used for cell culture.

Preparations	Membrane $G_{t/i}\alpha$-1	Membrane $G_{t/i}\alpha$-2	Membrane $G_{t/i}\alpha$-3	Membrane $G_{t/i}\alpha$-4	Cytosolic $G_{t/i}\alpha$
Fatty acid supplied (final concentration)	Myr (100 μM)	Lau (500 μM)	Lau (500 μM)	Lau (1 mM)	none
N-terminal structure					
C12:0-modified	–[a]	20%	33%	~70%	–[a]
C14:0-modified	100%	80%	67%	N.D.[b]	–[a]
unmodified	–[a]	–[a]	–[a]	N.D.[b]	100%

[a]Not detected. [b]Not determined.

the steady-state GTP-hydrolysis rate decreased with increasing content of C12:0-$G_{t/i}\alpha$ (Fig. 5B). To investigate whether the difference of the GTP-turnover rate is attributable to the rate of GDP/GTP-exchange reaction on $G_{t/i}\alpha$, the time courses of GTPγS-binding reaction to $G_{t/i}\alpha$ were measured using membrane $G_{t/i}\alpha$-1 and $G_{t/i}\alpha$-3 (Fig. 6). We observed little difference, if any, in the initial rate of GTPγS-binding reaction to $G_{t/i}\alpha$ between $G_{t/i}\alpha$-1 (100% myristoylated) and $G_{t/i}\alpha$-3 (67% myristoylated, 33% lauroylated), suggesting that the *N*-acyl group of $G_{t/i}\alpha$ has a marginal effect on the nucleotide-exchange reaction on $G_{t/i}\alpha$.

DISCUSSION

Before producing the chimeric form of $G_{t/i}\alpha$, we attempted to express wild type $G_{t1}\alpha$ in Sf9 cells. When Sf9 cells were infected with the $G_{t1}\alpha$-baculovirus, however, more than 99% of expressed $G_{t1}\alpha$ was accumulated in the detergent-insoluble fraction, as reported by other groups (*15*, *33*). We also tried to co-express $G_{t1}\alpha$ with $G\beta_1\gamma_1$, human NMT and/or bovine opsin in Sf9 cells, but these trials were unsuccessful. Recently, Min *et al*. (*34*) reported purification of "functional" $G_{t1}\alpha$ that was expressed in baculovirus-infected Sf9 cells, but we could not find out the reason and key conditions important for the functional expression of wild-type $G_{t1}\alpha$. We then made use of a recombinant chimera, $G_{t/i}\alpha$, instead of wild-type $G_{t1}\alpha$. Skiba *et al*. (*15*) expressed N-terminally His_6-tagged (and hence non-acylated) $G_{t/i}\alpha$ chimera in *Escherichia coli* and demonstrated that the initial rate of GTPγS-binding reaction to $G_{t/i}\alpha$ was 6-fold lower than that of retinal $G_{t1}\alpha$ (*15*) in the presence of light-activated rhodopsin and $G\beta_1\gamma_1$. This result strongly suggests that the *N*-acylation of $G_{t/i}\alpha$ is crucial for its function. Based on this idea, we established the system to produce a relatively large amount of N-terminally acylated isoforms of $G_{t/i}\alpha$ in Sf9 cells.

Heterogeneous N-Acylation in Sf9 Cells—By careful mass spectrometric analysis of the proteolytic fragments of purified proteins, we found that a small population of $G_{t/i}\alpha$ expressed as a complex with $G\beta_1\gamma_1$ was modified by C12:0, and the rest was modified by C14:0 (Fig. 2, B and C). To our knowledge, this is the first report of *N*-lauroylation of G protein α-subunit expressed in non-retinal cells. It has been thought that G protein α-subunits having an *N*-myristoylation signal sequence are modified with C14:0 in insect cells, based on the previous reports (*2*, *3*, *14*) in which *N*-acylation was determined solely by radiolabeled fatty acid incorporation and/or analyses of

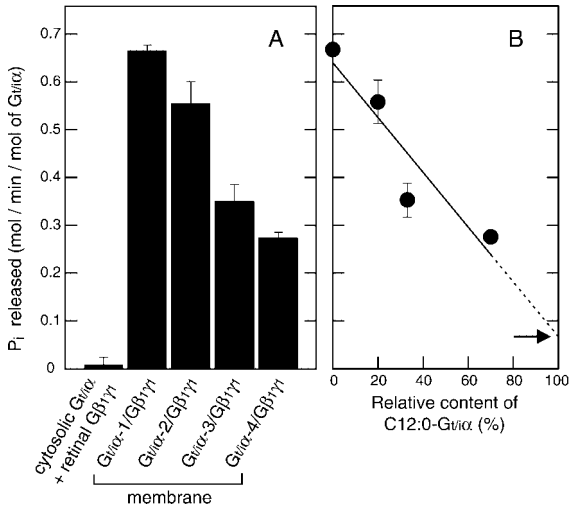

Fig. 5. **Effect of N-terminal fatty acylation on the reaction rate of the steady-state GTP-hydrolysis of $G_{t/i}\alpha$.** (A) Bovine rhodopsin was reconstituted in PC liposomes (*20*) in buffer B1(10 mM MOPS-NaOH, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin; pH 7.5), and irradiated with an orange light (>540 nm) for 1 min at 4°C to convert rhodopsin into metarhodopsin II. Then, metarhodopsin II in the liposome (12.5 nM) was mixed with recombinant membrane $G_{t/i}\alpha/G\beta_1\gamma_1$ heterotrimer (6.25 nM) or recombinant cytosolic $G_{t/i}\alpha$ plus retinal $G\beta_1\gamma_1$ (6.25 nM each), [γ-^{32}P]GTP (10 μM, 1 Ci/mmol), CHAPS (0.07% [w/v]), soybean trypsin inhibitor (0.8 mg/ml), and 145 mM NaCl in 50 μl of the mixture (35 μl of buffer B1 plus 15 μl of buffer C1). The reaction was started by the addition of a mixture of [γ-^{32}P]GTP and metarhodopsin II, and after 20 min of incubation at 25°C, the reaction mixture was mixed with 250 μl of 5% charcoal (in 50 mM Na-phosphate buffer; pH 6.5), and centrifuged to obtain a supernatant containing $^{32}P_i$ produced by the hydrolysis of [γ-^{32}P]GTP. The GTP-hydrolysis rate was estimated from the amount of released $^{32}P_i$. Under the experimental condition, the GTP-hydrolysis rate was almost linear up to 20 min. The data shown are average ± SD of triplicate determinations from a single experiment, and this is a representative set out of four independent experiments with similar results. (B) The data in Panel (A) were plotted against the relative content of C12:0-$G_{t/i}\alpha$ in total amount of $G_{t/i}\alpha$ in the preparation. The steady-state GTPase activity of C12:0-$G_{t/i}\alpha$ was estimated to be 0.067 mol of P_i released per min per mol of $G_{t/i}\alpha$ (arrow) by extrapolating the activities of the mixture of C12:0-$G_{t/i}\alpha$ and C14:0-$G_{t/i}\alpha$.

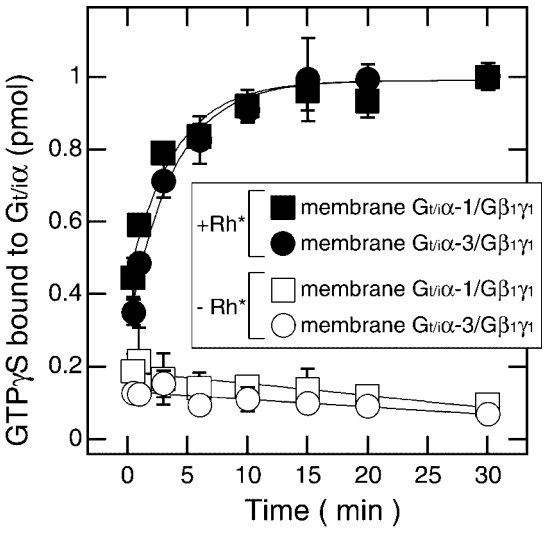

Fig. 6. **Effect of N-terminal fatty acylation on the GTPγS-binding reaction to $G_{t/i}α$ catalyzed by metarhodopsin II.** Bovine rhodopsin reconstituted in PC liposomes was light-activated (as described in Fig. 5) and mixed with [^{35}S]GTPγS. The time course of the GTPγS-binding to $G_{t/i}α$ catalyzed by metarhodopsin II (Rh*) was measured in a reaction mixture (100 μl: 80 μl of buffer B1 plus 20 μl of buffer C1) composed of recombinant membrane $G_{t/i}α/Gβ_1γ_1$ heterotrimer (0.1 μM), ovalbumin (1 mg/ml), GDP (0.5 μM), [^{35}S]GTPγS (5 μM, 10 Ci/mmol), CHAPS [0.07%(w/v)], 150 mM NaCl, with or without metarhodopsin II-containing liposomes (30 nM; +Rh*: closed symbols, -Rh*: open symbols). The reaction was started by the addition of a mixture of [^{35}S]GTPγS and metarhodopsin II (solid symbols) or a mixture of [^{35}S]GTPγS and buffer B1 (open symbols). The reaction was carried out at 4°C, and terminated by diluting the 10-μl aliquots with 180 μl of ice-cold buffer W (100 mM Tris-HCl, 1 mM $MgCl_2$; pH 7.5 at 4°C) supplemented with 2 mM GTP and 50 μM GTPγS. $G_{t/i}α$-bound [^{35}S]GTPγS was isolated by filtrating the samples over 0.45-μm cellulose membranes (type HATF; Millipore) fitted with MultiScreen Assay System (Millipore). After filtration, the membranes were washed four times with 0.2 ml of buffer W, and the radioactivities of the membranes were determined by liquid scintillation counting. The data shown are average ± SD of triplicate determinations from a single experiment, and this is a representative set out of three independent experiments with similar results.

the fatty acids using HPLC without complementary mass spectrometric analysis. *N*-lauroylation of Gα might have been overlooked in these analyses, taking no account of incorporation of C12:0.

Although the molecular mechanism underlying the heterogeneous fatty acylation in Sf9 cells and in the retina is not fully understood, it is likely that the (relative) levels of fatty acyl-CoA pools available to NMT may be an important determinant of the fatty acyl groups to be linked to protein N-terminus. Consistent with this idea, myristic acid exogenously added to the culture media decreased the relative content of C12:0-$G_{t/i}α$, while exogenously supplied lauric acid was capable of increasing the relative abundance of C12:0-$G_{t/i}α$ in a concentration-dependent manner (Fig. 4A). As human NMT can use not only C14:0-CoA but also C12:0-CoA as a substrate *in vitro* (*35*), C12:0-CoA may serve as a substrate for Sf9 NMT as well. It should be noted, however, that the pool composition of acyl-CoAs (C12:0-CoA, C14:1-CoA, C14:2-CoA and C14:0-CoA) in the total retinal extract was similar to those in the other tissues such as heart and liver (*31*). Another mechanism may also contribute to the heterogeneous fatty acylation in the retina.

Functional Difference between C14:0-Gα and C12:0-Gα—Using several isoforms of $G_{t/i}α$ with or without *N*-acylation (C12:0/C14:0), we showed that 1) *N*-fatty acylation of $G_{t/i}α$ is absolutely required for the light-signal transduction (Fig. 5), and 2) the structure of the *N*-acyl group linked to $G_{t/i}α$ influences the steady-state GTP-hydrolysis (GTP-turnover) rate of $G_{t/i}α$ in the presence of $Gβ_1γ_1$ and metarhodopsin II (Fig. 5). As the complex formation among $G_{t/i}α$, $Gβ_1γ_1$ and metarhodopsin II is the critical rate-limiting step of the steady-state GTP-hydrolysis reaction, it is most likely that the type of the *N*-acyl group linked to $G_{t/i}α$ determines the efficiency of the ternary complex formation ($G_{t/i}α/Gβ_1γ_1$/metarhodopsin II) on the membrane. However, our data do not exclude the possibility that the *N*-acyl group also influences other steps such as GTP-hydrolysis reaction of $G_{t/i}α$ itself. In this case, the *N*-acyl group may serve as an allosteric regulator of $G_{t/i}α$.

Since the extreme N-terminal region of G protein α-subunit plays an essential role in the interaction with the βγ complex (*36–39*), it is conceivable that C14:0-$G_{t/i}α$ has higher affinity for $Gβ_1γ_1$ and forms a Gα/Gβγ heterotrimer more efficiently than C12:0-$G_{t/i}α$. This idea is based on our previous study showing that the C14:0-nonapeptide corresponding to the N-terminus of $G_{t1}α$ inhibits the $G_{t1}α$-$Gβ_1γ_1$ interaction more efficiently than the C12:0-peptide (*4*, *13*). It is possible that Gβγ may recognize the *N*-fatty acyl group of Gα, and that the structure of the fatty acyl group may determine the affinity between Gα and Gβγ. Another possible explanation is that C14:0-$G_{t/i}α$ may have a higher affinity than C12:0-$G_{t/i}α$ for liposomes containing metarhodopsin II. The structure and/or hydrophobicity of the *N*-fatty acyl group of Gα may determine the affinity between Gα and lipid membranes.

Previously, Neubert and Hurley (*40*) reported that C12:0-$G_{t1}α$ and C14:2-$G_{t1}α$ were preferentially eluted from the light-irradiated bovine ROS membranes containing the ternary complex of $G_{t1}α$(GDP)/$Gβ_1γ_1$/metarhodopsin II, when the membranes were incubated with a low concentration (1 or 3 μM) of GTP to allow only a few cycles of GTP hydrolysis. On the other hand, C14:0-$G_{t1}α$ was efficiently eluted when an excess amount of GTP (400 μM) or GTPγS (50 nM-30 μM) was used. These observations may be consistent with our conclusion that C12:0-$G_{t1}α$ forms the ternary complex less efficiently than C14:0-$G_{t1}α$ during the receptor-mediated activation cycles of transducin.

Physiological Significance of Heterogeneous N-Acylation in the Light-Signal Transduction—Several proteins involved in the light-signal transduction in rod cells are specifically modified by heterogeneous fatty acid, and hence it is reasonable to speculate that the heterogeneous fatty acylation contributes to the function of rod cells. It is unlikely that the four isoforms of transducin are distributed differently among rod cells (*13*), so that these isoforms should operate together in a single rod cell. Heterogeneous *N*-acylation similar to $G_{t1}α$ is also found in recoverin, a retinal Ca^{2+}-binding regulatory protein (*41*, *42*) of which the acyl group serves as a Ca^{2+}-dependent membrane anchor (*43*, *44*). Interestingly, the recoverin

isoforms with distinct *N*-acyl groups show functional divergence which appears to correlate with the hydrophobicities of the acyl chains (*7*). In the present study, we demonstrated the functional difference between C14:0-$G_{t1}\alpha$ and C12:0-$G_{t1}\alpha$ (Fig. 5). Assuming that C14:0- and C12:0-$G_{t/i}\alpha$ do not interact with each other, the GTPase activity of C12:0-$G_{t/i}\alpha$ is calculated to be 0.067 (mol of P_i released/min/mol of $G_{t/i}\alpha$) by extrapolating the activities of the mixture (Fig. 5B, arrow). Although the GTPase activities of C14:1- and C14:2-modified species are not known, it should be emphasized that the GTPase activity of C12:0 isoform is likely one order of magnitude lower than that of C14:0 isoform (Fig. 5B). Considering the intermediate hydrophobicities of C14:1- and C14:2-modified isoforms (*4*, *5*, *7*), these species may exhibit intermediate activities between C14:0 and C12:0 isoforms. Then, if $G_{t1}\alpha$ were modified exclusively by myristate, the turn-off process mediated by the GTPase activity of transducin would be much faster, and the duration of the activated state of its target enzyme, cGMP-phosphodiesterase, would become shorter. Therefore, the N-terminal modification of $G_{t1}\alpha$ by fatty acids other than myristate may help to prolong the activation of cGMP-hydrolysis for an extremely high amplification of the light signal. It is still an open question as to whether C14:1- and C14:2-modified isoforms of transducin may have specific roles, but we speculate that these isoforms modified by unsaturated fatty acids may be akin to C12:0-modified $G_{t1}\alpha$ and that these three isoforms occupying ~90% in total of retinal $G_{t1}\alpha$ would have low GTPase activities and would be similarly important for the rod visual function, in which myristoylated $G_{t1}\alpha$ (~5% of total $G_{t1}\alpha$) could be of less importance.

Recently, a "lipid raft" was identified in bovine rod photoreceptor outer segments, and several proteins including transducin were shown to translocate to the raft upon light illumination (*45*, *46*). Heterogeneous *N*-acylation might also contribute to the light-dependent translocation of $G_{t1}\alpha$ between the raft and non-raft membrane.

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