Identification of G Protein α Subunit-Palmitoylating Enzyme*

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G-protein-coupled receptors (GPCRs) form the largest family of cell surface receptors, consisting of more than 700 members in humans. GPCRs respond to a variety of extracellular signals, including hormones and neurotransmitters, and are involved in various physiological processes, such as smooth muscle contraction and synaptic transmission (20, 25). Heterotrimeric G proteins, composed of α, β, and γ subunits, transduce signals from GPCRs to their effectors and play a central role in the GPCR signaling pathway (13, 21, 24, 32). Although the G protein complex and subsequent lipid palmitoylation and regulation of the signaling pathway. However, the enzymes that add palmitate to proteins (palmitoyl-acyl transferases [PATs]) and those that cleave the thioester bond (palmitoyl-protein thioesterases) were long elusive.

Recent genetic studies in Saccharomyces cerevisiae identified Erf2/Erf4 (1, 40) and Akr1 (29) as PATs for yeast Ras and yeast casein kinase 2, respectively. Erf2 and Akr1 have four- to six-pass transmembrane domains and share a common domain, referred to as a DHHC domain, a cysteine-rich domain with a conserved Asp-His-His-Cys signature motif. Because the DHHC domain is essential for the PAT activity, we isolated 23 mammalian DHHC domain-containing proteins (DHHC proteins) and developed a systematic screening method to identify the specific enzyme-substrate pairs (11, 12): DHHC2, -3, -7, and -15 for PSD-95 (11): DHHC21 for endothelial NO synthase (10); and DHHC3 and -7 for GABA_A receptor γ2 subunit (9). Several other groups also reported that DHHC9 with GCP16 mediates palmitoylation toward H- and N-Ras (36) and that DHHC17, also known as HIP14, palmitoylates several neuronal proteins: huntingtin (14), SNAP-25, and CSP (14, 23, 35). However, the existence of PATs for Gα has been contro-
versial because spontaneous palmitoylation of Gα could occur in vitro (4).

In this study, we screened the 23 DHHC clones to examine which DHHC proteins can palmitoylate Gα. We found that DHHC3 and -7 specifically and robustly palmitoylate Gα at the Golgi apparatus. Inhibition of DHHC3 and -7 reduces Gαq/11 palmitoylation levels and delocalizes it from the PM to the cytoplasm in HeLa cells and primary hippocampal neurons. Also, DHHC3 and -7 are necessary for the continuous Gαq shunting between the Golgi apparatus and the PM. Finally, blocking DHHC3 and -7 inhibits the α1a-adrenergic receptor (Gα1a-R) Gαq-mediated signaling pathway, indicating that DHHC3 and -7 play an essential role in GPCR signaling by regulating Gα localization.

**MATERIALS AND METHODS**

**Cell culture and transfection.** The drugs used were 2-bromohexadecanoic acid (2-bromopalmitate [2-BP]) (Fuka), cycloheximide (CHX; Sigma), phenylephrine (Sigma), and prazosin (Sigma). For transfection of plasmid DNA and small interfering RNA (siRNA) into HeLa or HEK293T cells, Lipofectamine Plus reagent and Lipofectamine 2000 (Invitrogen) were used, respectively. Cultured hippocampal neurons (2.5 × 10^5 cells) were seeded onto 12-mm coverslips in 24-well dishes. Neurons (DIV8) were transfected with pCAGGS-mCherry-miR or mCherry vectors by Lipofectamine 2000. 24-h after transfection, neurons were fixed with 4% paraformaldehyde, permeabilized, and stained with antibodies to mCherry (Santa Cruz Biotechnology) and GODZ/DHHC3 (Abcam); mouse monoclonal antibodies to β-catenin (BD Biosciences); chicken polyclonal antibody to green fluorescent protein (Santa Cruz Biotechnology) and GODZ/DHHC3 (Abcam); mouse monoclonal antibodies to CREB (Cell Signaling) and phospho-CREB Ser133 (pCREB) (Cell Signaling). Rabbit polyclonal antibodies to GFP and moesin were raised against glutathione S-transferase-GFP and glutathione S-transferase-moesin (amino acids 307 to 577), respectively.

**Plasmid constructions.** Gα1q-tagged with the fluorescent protein Dendra2 (Evr10gen) (16) was made by replacing a GFP fragment of Gαq-GFP, which was well characterized (15). Gαq-Dendra2 stimulated phospholipase C in response to α1a-R activation (data not shown); it was palmitoylated by DHHC3 and -7 (data not shown) and was localized at the PM (Fig. 1A) as effectively as Gαq-GFP. We concluded that Gαq-Dendra2 is functional as endogenous Gαq and Gαq-GFP. Gαq-GFP was constructed by inserting enhanced GFP (EGFP) with an SGGGS linker at both the N and C ends between 114A and 115G of Gαq-PEF-BoS-Ha-mouse DHHC (mDHHC) clones were described previously (11). Dendra2-DHHC3 and FLAG-DHHC3 were constructed by subcloning cDNA of DHHC3 into pDendra2 and pCAGGS, respectively. DHHC3 with the mutation C157S (DHHC3(C157S)), DHHC7(C160S), and Gαq/11 IP, followed by fluorography. For hydroxylamine treatment, [3H]palmitate-labeled cells were collected and sonicated in phosphate-buffered saline and then mixed with an equal volume of 1 M hydroxylamine (NH₂OH) (pH 7.0) for 1 min. After a 2-h incubation at room temperature, the cell lysates were subjected to fluorography and Western blotting.

**In vitro PAT assay.** The assay was performed as described previously, with modification (11). Gαq-GFP and FLAG-DHHC3 were immunoprecipitated from transfected HEK293T cells using anti-GFP antibody and protein A-Sepharose and M2 anti-FLAG agarose beads (Sigma), respectively. Eluted FLAG-DHHC3 (50 nM) was added to Gαq/11 immunoprecipitation (IP). Briefly, Gαq-immunocomplexes were washed twice with 1% SDS-IP buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 50 μg/ml phenylmethylsulfonyl fluoride). After a 5-min extraction, 900 μl of SDS-free IP buffer was added. After centrifugation at 10,000 × g for 10 min, the supernatants were treated with 2 μg of protease K for 1 h and then incubated with 30 μl of protein A-Sepharose (GE Healthcare). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were silver stained (Daiichi) for [3H] detection or treated with Amplify (GE Healthcare) for [H] detection. Dried gels were exposed to films at −80°C. Screening of the candidate PATs was performed in HEK293T cells as described previously (11, 12). To detect palmitoylation of endogenous Gαq/11 in siRNA-treated cells, cells were metabolically labeled with 0.5 μCi/ml [3H]palmitate-containing medium for 4 h at 24 h after siRNA transfection. Cells were then lysed and subjected to anti-Gαq/11 IP, followed by fluorography. For hydroxylation treatment, [3H]palmitate-labeled cells were collected and sonicated in phosphate-buffered saline and then mixed with an equal volume of 1 M hydroxylamine (NH₂OH) (pH 7.0) for 1 min. After a 1-h incubation at room temperature, the cell lysates were subjected to fluorography and Western blotting.

**Quantitative PCR.** Total RNA was extracted using TRIzol (Invitrogen), and cDNA was synthesized using a high-capacity cDNA RT kit (Applied Biosciences). Quantitative PCR was performed using an Applied Biosystems 7000 system (Applied Biosciences) and Power SYBR green PCR Master Mix (Applied Biosciences). Primers were used for the following: DHHC3, 5'-CTTGTTGCACTTTGGTCTC-3' and 5'-CGTTGATCTTCTGTTGTTCC-3'; DHHC7, 5'-TGAGACATTCTCTGGTCTAC-3' and 5'-TGACTGAACTGGTGAGATC-3'; and 5'-GTTGGGATCTTGATGCTACCC-3' and 5'-GGTTTACAGGCGACAGAGTGC-3'.

**Metal labeling and pulse-chase assay.** For pulse-chase analysis, HEK293 cells (5 × 10⁵ per six-well plate) were preincubated with 1 μl of serum-, cysteine-, and methionine-free Dulbecco's modified Eagle's medium containing 5 mg/ml fatty acid-free bovine serum albumin for 30 min. The cells were then metabolically labeled for 4 h with a medium containing 50 μCi/ml [3H]methionine-cysteine (GE Healthcare) or 0.5 μCi/ml [3H]palmitate (Perkin Elmer). Cells were washed with PBS and incubated in incubation medium (500 μl) containing 4% paraformaldehyde, permeabilized, and stained with antibodies to GFP, and then incubated with 30 μl of Gαq-GFP-containing beads were washed twice with ice-cold IP buffer. The Gαq-GFP-containing beads were suspended in SDS sample buffer with 10 mm dithiothreitol. Samples were then resolved by SDS-PAGE, followed by fluorography and Western blotting.

**Immunofluorescence analysis.** HEK293 cells were seeded onto poly-l-lysine-coated 12-mm glass slips (Fisher brand). Cells were fixed with 4% paraformaldehyde, 120 mM sucrose, and 100 mM HEPES (pH 7.4) for 10 min and permeabilized with 0.1% Triton X-100 for 10 min. For Gαq/11 staining, the cells were fixed with methanol for 5 min at −20°C. Cells were then stained with indicated antibodies. Fluorescent images were obtained using an LSM 510 EXCITER system (Carl Zeiss) with a PASCAL 63x objective.
Because overexpressed DHHC3 sometimes mislocalizes substrate proteins at the Golgi apparatus, we limited the amount of plasmid to mildly express DHHC3. To quantitate the intensity of \( \text{G}\alpha_{q/11} \) at the PM and the cytosol, 20 GFP-DHHC3-expressing cells were randomly chosen. The regions of the PM and cytosol were traced by polyline drawing, and their mean intensities were measured using Zeiss ZEN software. The relative PM intensities were calculated by the ratio of mean intensities of the PM and cytosolic regions.

Living-cell imaging. HeLa cells were seeded onto a poly- D-lysine-coated 35-mm glass-bottom dish (Iwaki) and observed at 37°C in a CO\( _2 \) chamber (Tokai Hit). For photoconversion analysis, \( \text{G}\alpha_{q/11} \)-Dendra2 at the Golgi apparatus or PM was converted with a 405-nm laser using an LSM5 Exciter system and Zeiss ZEN software. Images were obtained every 5 min. After live imaging, the cells were fixed and stained by anti-GM130 antibody. For fluorescence recovery after photobleaching (FRAP) analysis, \( \text{G}\alpha_{q/11} \)-GFP at the GalT-mCherry-positive region was bleached with a 488-nm laser. Images were acquired every 10 s for 20 min with or without 2-BP, and the average intensities of the regions were plotted in the graph.

Microscope control and all image analysis were performed with Carl Zeiss ZEN software. For total internal reflection fluorescence microscopy

FIG. 1. Dynamic palmitate turnover on \( \text{G}\alpha_{q/11} \). (A) \( \text{G}\alpha_{q/11} \) shuttles between the PM and the Golgi apparatus. When \( \text{G}\alpha_{q/11} \)-Dendra2, \( \text{G}\beta_1 \), and \( \text{G}\gamma_2 \) were coexpressed in HeLa cells, \( \text{G}\alpha_{q/11} \)-Dendra2 (green) was localized at the PM and the endomembrane. \( \text{G}\alpha_{q/11} \)-Dendra2 in the endomembranes (upper) and a part of PM (lower) within the white regions was photoconverted by 405-nm laser. Converted \( \text{G}\alpha_{q/11} \)-Dendra2 (gray scale) was monitored for 20 min. Cells were then immunostained with anti-GM130 (magenta) antibody (right). Scale bar, 10 \( \mu \)m. (B) Inhibition of palmitoylation causes detachment of \( \text{G}\alpha_{q/11} \) from the PM. HeLa cells were treated with 2-BP (100 \( \mu \)M) or CHX (20 \( \mu \)g/ml) for 4 h. The cells were then doubly stained with anti-\( \text{G}\alpha_{q/11} \) (green) and anti-\( \beta \)-catenin (red) antibodies. Scale bar, 20 \( \mu \)m. (C) 2-BP blocks palmitoylation of \( \text{G}\alpha_{q/11} \). HeLa cells were metabolically labeled with [\( ^{3} \)H]palmitate and subjected to fluorography (upper) or Western blotting (lower). IB, immunoblotting. (D) HeLa cells were treated with 2-BP for 4 h. Then, 2-BP was washed out, and protein synthesis was inhibited by 20 \( \mu \)g/ml CHX for 4 h. The cells were stained with anti-\( \text{G}\alpha_{q/11} \) antibody at the indicated times. The inhibition of palmitoylation for 4 h caused delocalization of \( \text{G}\alpha_{q/11} \) from the PM. The \( \text{G}\alpha_{q/11} \) dispersed by 2-BP came back to the PM again within 2 h after removal of 2-BP in the presence of CHX, indicating that the relocation of \( \text{G}\alpha_{q/11} \) depends on palmitoylation and depalmitoylation. Scale bar, 20 \( \mu \)m. (E) Pulse-chase analysis of \( \text{G}\alpha_{q/11} \) palmitoylation. HeLa cells were labeled with [\( ^{3} \)H]palmitate or [\( ^{35} \)S]methionine-cysteine for 4 h. After incubation with chase medium for 0, 1, 2, 4, and 6 h, cells were lysed and subjected to IP with anti-\( \text{G}\alpha_{q/11} \) antibody. Immunoprecipitates were separated by SDS-PAGE, followed by fluorography. The ratio of [\( ^{3} \)H]palmitate to [\( ^{35} \)S]methionine-cysteine-labeled \( \text{G}\alpha_{q/11} \) was plotted in the graph. Error bars show \( \pm SD \) (\( n = 3 \)). IgG, control immunoglobulin G.
(TIRFM) imaging, transfected HeLa cells were observed at 37°C by an IX81 TIRF system (Olympus) with a Plan-Apochromat 100× TIRFM objective. Images were captured using an ImageEM charge-coupled-device camera (C9100-13; Hamamatsu). Fluorescent intensities from epifluorescence and TIRF images were analyzed using MetaMorph software (version 7.1; MDS Analytical Technologies).

\textbf{PAT activity on Gq palmitoylation.} In the PM region diffused throughout the PM, and some population of \( \text{G}_q \)-Dendra2 accumulated in the GM130-labeled endomembranes (i.e., Golgi apparatus) in a retrograde manner. Consistently, similar shuttling between the PM and the Golgi apparatus. Consistently, similar shuttling between the PM and the Golgi apparatus. Consistently, similar shuttling between the PM and the Golgi apparatus.

\section*{RESULTS}

\subsection*{Dynamic palmitate cycling on \textit{G}_q.} Taking advantage of \( \text{G}_q \) tagged with Dendra2, a green-to-red photoconvertible fluorescent protein, we first visualized the dynamic movement of \( \text{G}_q \) in HeLa cells. When \( \text{G}_q \)-Dendra2 was coexpressed with \( \text{G}_i/B_1 \) and \( \text{G}_Y/G_2 \) subunits in HeLa cells, \( \text{G}_q \)-Dendra2 was localized at the PM and weakly distributed in the endomembranes (Fig. 1A). Photoconverted \( \text{G}_q \)-Dendra2 in the endomembranes was rapidly targeted to the PM within 10 min (Fig. 1A, upper panel). In contrast, photoconverted \( \text{G}_q \)-Dendra2 in the PM region diffused throughout the PM, and some population of \( \text{G}_q/G_q \)-Dendra2 accumulated in the GM130-labeled endomembranes (i.e., Golgi apparatus) in a retrograde manner (Fig. 1A, lower panel). These results indicate that \( \text{G}_q \) dynamically shuttles between the PM and the Golgi apparatus. Consistently, similar shuttling between the PM and the Golgi apparatus. Consistently, similar shuttling between the PM and the Golgi apparatus. Consistently, similar shuttling between the PM and the Golgi apparatus. Consistently, similar shuttling between the PM and the Golgi apparatus. Consistently, similar shuttling between the PM and the Golgi apparatus.

A previous study using GFP-tagged \( \text{G}_q \) showed that palmitoylation of \( \text{G}_q \) is essential for its membrane targeting (15). We examined whether palmitoylation of \( \text{G}_q \) is a dynamic process in HeLa cells. Because the antibody against \( \text{G}_q \) cross-reacts with \( \text{G}_{11} \), the closest isoform (90% identity), we describe them as \( \text{G}_{11} \). Treatment of the HeLa cells for 4 h with 2-BP, an inhibitor of protein palmitoylation, relocalized \( \text{G}_{11} \) from the PM at the cell-to-cell contact sites to the cytoplasm (Fig. 1B) and blocked palmitoylation of \( \text{G}_{11} \) (Fig. 1C). This treatment did not affect the PM localization of \( \text{G}_{11} \) because CHX, an inhibitor of protein synthesis, did not affect the \( \text{G}_{11} \) localization at the PM (Fig. 1B). \( \text{G}_{11} \) dispersed by 2-BP came back to the PM again within 2 h after removal of 2-BP in the presence of CHX (Fig. 1D). Furthermore, the pulse-chase experiments with \([^{3}H] \)palmitate and \([^{35}S] \)methionine-cysteine revealed that palmitate on \( \text{G}_{11} \) turns over rapidly (Fig. 1E).

The half-life of palmitate on \( \text{G}_{11} \) is approximately 2 h, whereas the half-life of \( \text{G}_{11} \) protein itself is much longer, about 35 h. These results suggest that the de-/re-palmitoylation cycle on \( \text{G}_q \) dynamically regulates \( \text{G}_q \) subcellular localization.

\subsection*{Screening of \textit{G}_q palmitoylating enzymes.} To understand the molecular mechanisms for dynamic regulation of \( \text{G}_q \) localization, we screened the candidate \( \text{G}_q \)-palmitoylating enzyme. We transfected individually 23 DHHC proteins (11) together with \( \text{G}_q \)-GFP in HEK293T cells and assessed palmitoylation of \( \text{G}_q \)-GFP by metabolic labeling with \([^{3}H] \)palmitate (Fig. 2A). Only DHHC3/GODZ and DHHC7/SERZ-\( \beta \) showed robust PAT activity toward \( \text{G}_q \). Western blotting with anti-HA antibody indicates that all transfected HA-DHHC clones express in HEK293T cells albeit at different levels. Because some DHHC proteins, such as DHHC16, -20, and especially -21 express at much lower levels than DHHC3 and -7, it is possible that the clones with lower levels of expression have PAT activity toward \( \text{G}_q \). To verify the possibility, we limited the amount of transfected DHHC3 and examined the PAT activity. The limited DHHC3, even at a 1:50 transfection ratio, still induced \( \text{G}_q \) palmitoylation (Fig. 2B).

Taking advantage of \( \text{G}_q \)-GFP in HEK293T cells and assessed palmitoylation of \( \text{G}_q \)-GFP by metabolic labeling with \([^{3}H] \)palmitate (Fig. 2A). Only DHHC3/GODZ and DHHC7/SERZ-\( \beta \) showed robust PAT activity toward \( \text{G}_q \). Western blotting with anti-HA antibody indicates that all transfected HA-DHHC clones express in HEK293T cells albeit at different levels. Because some DHHC proteins, such as DHHC16, -20, and especially -21 express at much lower levels than DHHC3 and -7, it is possible that the clones with lower levels of expression have PAT activity toward \( \text{G}_q \). To verify the possibility, we limited the amount of transfected DHHC3 and examined the PAT activity. The limited DHHC3, even at a 1:50 transfection ratio, still induced \( \text{G}_q \) palmitoylation (Fig. 2B).

Under these conditions, DHHC proteins except for DHHC21 were expressed at higher (or equivalent) levels than limited DHHC3 and showed no PAT activity toward \( \text{G}_q \) (Fig. 2B). DHHC21 apparently showed the PAT activity toward Lck in spite of a very low expression level, whereas DHHC3 did not show activity toward Lck (Fig. 2C). These results indicate that the expression level of DHHC clones hardly affects our screening results.

\( \text{G}_q \) palmitoylation induced by DHHC3 and -7 is mediated by a labile thioester bond because the \([^{3}H] \)palmitate incorporated into \( \text{G}_q \) was released with 0.5 M hydroxylamine treatment (Fig. 2D). We also found that the DHHC motif in DHHC3 and -7 was essential for \( \text{G}_q \) palmitoylation because mutating a cysteine residue in the DHHC motif to serine in DHHC3 and -7 blocked their effects on \( \text{G}_q \) palmitoylation (Fig. 2E). Furthermore, palmitoylation by DHHC3 and -7 required cysteines 9 and 10 of \( \text{G}_q \) (Fig. 2E).

We next investigated whether other \( \text{G}_q \) subunits such as \( \text{G}_{12} \) and \( \text{G}_{15} \) are also palmitoylated by DHHC3 and -7. We systematically screened candidate PATs for \( \text{G}_{12} \) and \( \text{G}_{15} \) (data not shown) and found that DHHC proteins showed similar specificity for palmitoylation of \( \text{G}_{12} \) and \( \text{G}_{15} \) (Fig. 2F). However, \( \text{G}_{12} \), which undergoes both myristoylation and palmitoylation, was palmitoylated by DHHC3 and -7 and, to a lesser extent, by DHHC2 and DHHC21 (Fig. 2F). Thus, DHHC3 and -7 are common candidate PATs for \( \text{G}_q \), \( \text{G}_{12} \), and \( \text{G}_{15} \). We noted that DHHC3 and -7 show high conservation (87% identity) in the catalytic DHHC domain and form a subfamily in the phylogenetic tree of DHHC proteins (9, 11).

We next asked whether purified DHHC3 and -7 could directly palmitoylate purified \( \text{G}_q \) in vitro. We immunosolated FLAG-DHHC3 and \( \text{G}_q \)-GFP from transfected HEK293T
FIG. 2. Screening of potential \( \alpha_q \) palmitoylating enzymes. (A) Individual HA-DHHC clones (0.5 µg plasmid) were transfected with \( \alpha_q \)-GFP (0.5 µg) into HEK293T cells. After metabolic labeling with \( ^{3}H \)palmitate, proteins were separated by SDS-PAGE, followed by fluorography and Western blotting with anti-GFP antibody for \( \alpha_q \)-GFP and anti-HA antibody for DHHC proteins. An arrow indicates the position of \( \alpha_q \)-GFP. White asterisks indicate autopalmitoylation of expressed DHHC proteins. Coexpression of DHHC3 or -7 robustly and specifically increased \( \alpha_q \) palmitoylation. Note that several DHHC proteins, such as DHHC16, -20, and -21, express at lower levels than DHHC3 and -7. M, molecular mass. (B) HEK293T cells were transfected with the indicated amount of DHHC proteins with \( \alpha_q \)-GFP and were labeled with \( ^{3}H \)palmitate. DHHC16 and -20 did not increase \( \alpha_q \) palmitoylation, whereas limited DHHC3 expression by 10 ng of plasmids (showing expression levels similar to DHHC16 and -20) still enhanced \( \alpha_q \) palmitoylation. An arrow indicates the position of DHHC21. (C) Although DHHC21 expressed at a lower level than DHHC3, DHHC21 has apparent PAT activity toward Lck. An arrow indicates the position of DHHC21. (D) Treatment of labeled cell lysates with 0.5 M hydroxylamine (NH\(_2\)OH) but not 0.5 M Tris-HCl (−) released DHHC3- or DHHC7-mediated \( ^{3}H \)palmitate incorporated into \( \alpha_q \)-HA, indicating that DHHC3- or DHHC7-induced palmitoylation is mediated by a thioester bond. (E) The DHHC3(C157S) and DHHC7(C160S) mutations (shown as CS) abolished the palmitoylating activity. The mutations cysteines 9 and 10 in \( \alpha_q \) (CS) abolished its palmitoylation. Asterisks indicate the autopalmitoylation of DHHCs. (F) HEK293T cells cotransfected with indicated DHHC clones (2, 3, 7, 21, and 15) and \( \alpha_q \)-GFP subfamily (\( \alpha_q \), \( \alpha_s \), and \( \alpha_i \)) were metabolically labeled with \( ^{3}H \)palmitate. All \( \alpha_q \) members were palmitoylated by DHHC3 and DHHC7. \( \alpha_q \) was also palmitoylated by DHHC2 and DHHC21 to a lesser extent. WT, wild type; IB, immunoblotting.
cells (Fig. 3A) and incubated them with [3H]palmitoyl-CoA. Purified DHHC3 apparently mediated incorporation of radio-

labeled palmitate into wild-type Go_q-GFP (Fig. 3B). Under

these conditions, spontaneous (nonenzymatic) palmitate trans-

fer into Go_q but not Go_q (CS).

Knockdown of DHHC3 and -7 impairs Go_q palmitoylation and PM targeting. To determine whether DHHC3 and -7 are responsible for Go_q palmitoylation in cells, we knocked down DHHC3 and -7. Treatment of HeLa cells with siRNAs di-

rected against human DHHC3 and/or human DHHC7 specifically reduced the expression of DHHC3 and/or -7 (validated

by quantitative RT-PCR) (Fig. 4A). When DHHC3 and/or DHHC7 was knocked down in HEK293T cells, the incorporation of [3H]palmitate into endogenous Go_{q/11} was markedly reduced compared to control siRNA-transfected cells (Fig.

4B). A similar result was obtained in HEK293T cells (data not shown). Because palmitoylation of Go_{q/11} is essential for its

PM localization (15) (Fig. 1B and D), we performed immuno-

fluorescence analysis of endogenous Go_{q/11} in HeLa cells. Knockdown of DHHC3 and/or -7 delocalized Go_{q/11} from the PM to the cytoplasm, whereas the intensity of β-catenin at the cell-to-cell contact sites did not change (Fig.

4C). The mislocalization of Go_{q/11} to the cytoplasm by siRNAs to human DHHC3 and human DHHC7 was rescued by siDHHC3-resistant wild-type mDHHC3 (Fig. 4D). In contrast, the mislocalization of Go_{q/11} was not rescued by the PAT-inactive mDHHC3(C157S) (Fig. 4D). We next examined the effect of

DHHC3 knockdown on the Go_{q/11} localization in primary hippocampal neurons, in which DHHC3 plays a more dominant role than DHHC7 (9). When DHHC3 was knocked down by vector-based RNAi, Go_{q/11} at the PM was significantly reduced and relocalized into the cytoplasm, whereas DHHC2 knock-
down did not affect the PM localization of Go_{q/11} (Fig. 5).

Next, to visualize Go_q at the cytoplasmic face of the PM with a high signal-to-noise ratio, we used TIRFM, which excites the molecules within 100-nm of the cover glass. When Go_q-GFP was expressed in HeLa cells, strong signals throughout the ventral surface of cells were detected by TIRFM (Fig. 6A). In contrast, the TIRFM signals of Go_q-GFP were apparently reduced when the cells were treated with 2-BP. The intensity of the palmitoylation-deficient mutant of Go_q, i.e., Go_q (CS)-GFP, was also lower than that of wild-type Go_q-GFP, suggesting that the signals visualized by TIRFM mainly reflect the membrane-bound palmitoylated Go_q-GFP. Supporting this, the intensity of GFP-K-Ras-CAAX (where A and X represent aliphatic and any residues, respectively), which targets to the PM by polybasic and prenylation sequences, did not change on 2-BP treatment. To examine the role of DHHC3 and/or -7 in the Go_q localization at the PM, we treated cells with siRNAs, observed them by TIRFM and epifluorescence microscopy, and measured the intensity ratio of TIRFM images to epifu-

orescence images. Knockdown of DHHC3 and/or -7 reduced significantly the intensity visualized by TIRFM (Fig. 6B). Under

these conditions, GFP-K-Ras-CAAX intensity by TIRFM was not affected (Fig. 6B). Taken together, these results indi-

cate that DHHC3 and/or -7 are authentic PATs for Go_q and are necessary for PM targeting of Go_q.

DHHC3 palmitoylates Go_q at the Golgi apparatus and drives PM-Golgi apparatus shuttling of Go_q. We next exam-

ined the cellular location of Go_{q/11} palmitoylation. We first confirmed that our anti-DHHC3 antibody is specific because two bands detected by the DHHC3 antibody completely disappeared in the knocked down cell lysate (Fig. 7A). Two these bands may contain (i) splicing variants as previously reported (17); (ii) differentially modified proteins by posttranslational modifications, such as phosphorylation, glycosylation, and palmitoylation; and (iii) degradation products. Consistent with previous observations in neurons (17), when HeLa cells were

stained by this specific DHHC3 antibody, DHHC3 immunore-

activity occurred only at the GM130-labeled Golgi apparatus (Fig. 7B). The staining is specific because this signal disappeared in DHHC3 knocked down cells (Fig. 7B). When mCherry-DHHC3 was coexpressed with Go_q-GFP, some Go_q was colocalized with DHHC3 in the Golgi apparatus (Fig. 7C). Photoconversion analysis by Dendra2-DHHC3 showed that DHHC3 localizes stably at the Golgi apparatus (Fig. 7D), in contrast to Go_q (Fig. 1A). These results strongly suggest that DHHC3 palmitoylates Go_q at the Golgi apparatus. We also found that HA-tagged DHHC3 showed similar distribution to DHHC3 at the Golgi apparatus (data not shown).

To examine the role of DHHC3 and -7 in the dynamic shuttling of Go_q, the Go_q dynamics were assessed by monitor-

ing FRAP. To mark the Golgi apparatus, we coexpressed mCherry-tagged GalT (16, 34) together with Go_q-GFP with or

without the β_1 and γ_2 subunits. After GFP fluorescence at the Golgi apparatus was bleached, Go_q-GFP fluorescence at the Golgi apparatus recovered within 20 min (64.6% ± 14.3%)
This newly arrived G\textsubscript{q}\text{11}q-GFP did not include newly synthesized G\textsubscript{q}\text{11}q-GFP because CHX did not affect the fluorescence recovery (data not shown). Because the fluorescence recovery of G\textsubscript{q}\text{11}q-GFP at the Golgi apparatus was not affected in the presence or absence of \(\beta_1\gamma_2\) subunits (Fig. 8A), we expressed only G\textsubscript{q}\text{11}q-GFP in the following experiments. We next asked whether palmitoylation is involved in the retrograde PM-Golgi trafficking of G\textsubscript{q}\text{11}q-GFP. We found that inhibition of
protein palmitoylation by 2-BP significantly reduced the fluorescence recovery at the GalT-positive Golgi region (33.3% \pm 3.7%; \(P < 0.05\) compared to control) (Fig. 8B), indicating that palmitoylation of \(G_{\alpha_q}\) at the Golgi apparatus is necessary for the retrograde trafficking from the PM to the Golgi apparatus. \(G_{\alpha_q}\)-GFP intensity in the cytoplasm and at the PM did not apparently change during the 20-min observation with 2-BP treatment. In contrast, the apparent delocalization of endoge-

FIG. 5. Depletion of DHHC3 expression impairs the PM targeting of \(G_{\alpha_q}\), in hippocampal neurons. Rat hippocampal neurons (DIV8) were transfected with mCherry-miR RNA (to rat DHHC2 or -3) expression vectors (red). At 7 days after transfection, neurons were fixed and stained with anti-\(G_{\alpha_q}\) antibody (green) and Hoechst (blue). Scale bars, 5 \(\mu\)m (high magnification) and 10 \(\mu\)m (low magnification). Graph shows ratio of fluorescence intensities of the PM to the cytosol. Error bars show \(\pm\)SD (\(n = 12\)). **, \(P < 0.01\). miRNA, microRNA.

FIG. 6. TIRFM imaging of membrane-bound palmitoylated \(G_{\alpha_q}\). (A) HeLa cells were transfected with GFP-tagged \(G_{\alpha_q}\) (WT), palmitoylation-deficient \(G_{\alpha_q}\) (CS), or the C-terminal sequence of K-Ras including polybasic and prenylation sequences (GFP-CAAX). Cells were observed by epifluorescence microscopy (Epi) and TIRFM before and at 4 h after treatment with 2-BP. \(G_{\alpha_q}\)-GFP was clearly detected by TIRFM, and the intensity was reduced on 2-BP treatment. The intensity of \(G_{\alpha_q}\) (CS) was apparently weaker than that of \(G_{\alpha_q}\) (WT). Scale bar, 20 \(\mu\)m. (B) HeLa cells were transfected with control or DHHC3/DHHC7 siRNAs together with \(G_{\alpha_q}\)-GFP or GFP-CAAX. Scale bar, 20 \(\mu\)m. Relative fluorescence intensities of cell images from TIRFM compared to those of epifluorescence microscopy are indicated in the graph. Note that \(G_{\alpha_q}\)-GFP intensity visualized by TIRFM was reduced significantly in DHHC3 and DHHC7 knocked down cells. Error bars show \(\pm\)SD (\(n = 5\)). **, \(P < 0.01\); *, \(P < 0.05\).
trapping of $\alpha_q$ at the Golgi apparatus, leading to the constitutive Golgi compartment-PM shuttling.

**DHHC3 and -7 are involved in the GPCR-mediated signaling pathway.** Next, we investigated whether DHHC3 and -7 are involved in the physiologic GPCR-mediated signal transduction. We selected $\alpha_1A$-AR as the $G_q$/11-coupled receptor and monitored its downstream signaling by pCREB (28) and IP$_3$ production. When HeLa cells transiently transfected with $\alpha_1A$-AR were stimulated with phenylephrine, an $\alpha_1A$-AR agonist, the phosphorylation level of CREB increased (Fig. 9A and D). This increase was completely blocked by the coapplication of prazosin, an $\alpha_1A$-AR antagonist (Fig. 9A). Knockdown of $G_q$ inhibited $\alpha_1A$-AR activation-induced CREB phosphorylation (Fig. 9B), indicating that $\alpha_1A$-AR-mediated CREB phosphorylation is mediated by $G_q$. Palmitoylation of $G_q$ is necessary for the $\alpha_1A$-AR-mediated signaling pathway because wild-type $\alpha_q$ (RNAi-resistant mouse $\alpha_q$), but not the palmitoylation-deficient $G_q$ (CS), rescued $\alpha_1A$-AR-mediated CREB phosphorylation (Fig. 9B). Furthermore, DHHC3 and -7 knockdown significantly blocked $\alpha_1A$-AR-mediated CREB phosphorylation (Fig. 9C and D). We also found that wild-type DHHC3 (RNAi-resistant mDHHC3) but not the PAT-inactive mDHHC3(C157S), rescued $\alpha_1A$-AR-mediated CREB phosphorylation (Fig. 9D). Finally, we examined whether DHHC3 and -7 are involved in the $\alpha_1A$-AR/Gi$_3$-induced phospholipase C activation by measuring IP$_3$ production. When HeLa cells transiently transfected with $\alpha_1A$-AR were stimulated with phenylephrine, the IP$_3$ production significantly increased (Fig. 9E). This increase was significantly blocked by knockdown of DHHC3 and -7 but not by knockdown of DHHC9. Thus, DHHC3 and DHHC7 play an essential role in the $\alpha_1A$-AR-mediated GPCR signaling pathway by $G_q$ palmitoylation.

**DISCUSSION**

This study identified DHHC3 and DHHC7 as $G_q$ palmitoylating enzymes that mediate palmitoyl transfer to $G_q$, $G_s$, and $G_q$. Golgi compartment-resident DHHC3 and -7 play essential roles in the PM-Golgi apparatus shuttling of $G_q$. Furthermore, we showed that DHHC3 and -7 are necessary for $\alpha_1A$-AR-mediated GPCR signaling pathway through $G_q$ targeting to the PM.

Identification of $G_q$ palmitoylating enzymes has long been controversial. Some studies have suggested that $G_q$ palmitoylation in cells might occur nonenzymatically because the formation of palmitoyl thioester linkage on proteins could occur spontaneously in vitro in the presence of a high concentration (10 to 20 $\mu$M) of palmitoyl-CoA (4). Recent systematic proteomic analysis revealed that DHHC family proteins are the main PATs that catalyze most of the protein palmitoylation in yeast because 29 of the 30 surveyed palmitoyl proteins were not palmitoylated in yeast lacking six of seven DHHC genes (30). In addition, our systematic screening analyses using 23 mammalian DHHC clones revealed that the palmitoylation levels of the more than 20 tested substrates were all enhanced by specific DHHC proteins (9, 10, 11, 12; also data not shown). These studies strongly suggest that members of the DHHC protein family mediate $G_q$ palmitoylation. Our analyses showed decisively that DHHC3 and -7 are authentic $G_q$ palmitoylating.
enzymes in vitro and in vivo. It is conceivable that similar approaches will be useful to identify PATs for other subfamilies of Gα/H9251, GPCRs, RGSs, and small GTPases.

By a knockdown approach, we found that DHHC3 and/or DHHC7 knockdown reduces endogenous Gα/H9251 palmitoylation in HEK293T cells (Fig. 4B). This result raised a couple of questions. One may wonder why knockdown of DHHC3 and/or DHHC7 does not completely abolish Gα/H9251 palmitoylation (Fig. 4B). Because our knockdown efficiency was about 85%, judging from our immunofluorescence analysis with anti-DHHC3 antibody, the remaining Gα/H9251 palmitoylation may be largely derived from the nontransfected cells with siRNA or

FIG. 8. Retrograde PM-Golgi trafficking of Gαq depends on Gαq palmitoylation by Golgi apparatus-resident DHHC3 and -7. (A) HeLa cells were transfected with Gαq-GFP (green and white) and GalT-mCherry (red) expression vectors with or without Gβγ2. The Golgi region (white circle) marked by GalT-mCherry was bleached, and then fluorescent recovery of Gαq-GFP was monitored by acquiring images every 10 s. Fluorescence intensities were plotted in graphs (right). (B) HeLa cells expressing Gαq-GFP (green and white) and GalT-mCherry (red) were treated with or without 2-BP for 30 min before FRAP analysis. The region (white circle) identified with GalT-mCherry was bleached, and then fluorescent recovery of Gαq-GFP was monitored. Treatment with 2-BP inhibited the recovery of fluorescence. (C) Knockdown (KD) of DHHC3 and -7 inhibited the Golgi and PM targeting of Gαq-GFP, resulting in the diffuse cytoplasmic localization. (D) Palmitoylation-deficient Gαq (CS)-GFP showed similar cytoplasmic distribution and rapid recovery both around the GalT-positive region (CS region 1) and at cytoplasmic region (CS region 2). The recovery of palmitoylation deficient Gαq (CS)-GFP was as rapid as that of DHHC3 and -7 knocked down cells in panel C. Scale bar, 10 μm. Error bars show ±SD (n = 5). WT, wild type.
the cells in which DHHC3 and/or DHHC7 were partially knocked down. Although the existence of other PATs and nonenzymatic mechanisms (4) cannot be completely ruled out, our results strongly suggest that DHHC3 and DHHC7 represent major Gαq palmitoylating enzymes. Double-knockout experiments of DHHC3 and DHHC7 will decisively demonstrate this issue. On the other hand, others may ask why knockdown of either DHHC3 or DHHC7 reduces Gαq palmitoylation (Fig. 4B) although the redundancy of DHHC3 and DHHC7 is expected. A straightforward possibility is that the total amount of DHHC3 and DHHC7 limits the palmitoylation of Gαq in the intact cells. Acute knockdown of DHHC3 and DHHC7 may not be fully compensated by the rest of DHHC. Alternatively, DHHC3 and DHHC7 may have synergistic effects on their PAT activity. In fact, DHHC3 and DHHC7 form homo-/heteromultimers (9). Further studies using the
DHHC3/DHHC7 knockout cells/animals will be required to reveal the molecular redundancy issue.

In this study, we found that Goq1/GFP shuttles between the PM and Golgi apparatus within 10 min in HeLa cells. In contrast, Chisari and coworkers have recently shown that Goα2-GFP and also the βγ3 subunits cycle in a matter of seconds in CHO cells (2). These differences may depend on the cellular context. In CHO cells, the distance between the PM and the endomembrane looks much shorter than that in HeLa cells. Alternatively, the difference in acylation types of Goα (dual palmitoylation) and Goα3 (myristoylation and adjacent palmitoylation) may contribute to individual kinetics of shuttling. Because 16-carbon palmitate has higher membrane affinity than the 14-carbon myristate, dually palmitoylated Goα3 may be more pronounced in PM localization than myristoylated/monopalmitoylated Goα1, leading to slower retrograde trafficking. In support of this idea, Rocks and colleagues reported that monopalmitoylated N-Ras displays a faster retrograde PM-Golgi apparatus trafficking than dually palmitoylated H-Ras (27). Direct comparison between Goα3 and Goα1 in the same cell type will address this question.

Wedegaertner and Bourne reported that activation of β-AR accelerates the palmitoylation cycle on Goα1 (depalmitoylation and subsequent repalmitoylation of Goα1) and induces PM-to-cytosol translocation of Goα1 (37). In contrast, another report showed that Goα1-GFP at the PM did not change on α1A-AR agonist stimulation in HEK293T cells (15). Our analysis in HeLa cells using FRAP and photoconversion methods showed that Goα3 constitutively cycles between the PM and Golgi apparatus and that this cycling requires the palmitoylation of Goα3 by Golgi apparatus-resident DHHC3 and -7. Although we attempted to decipher whether α1A-AR agonist stimulation affects Goα dynamics relocalization, our imaging resolution could not detect a significant difference (data not shown). These results imply that Goα dynamics depend on the subfamily of Goα and cellular/agonist contexts. Considering that H-Ras and N-Ras shuttle between the PM and Golgi apparatus through de-/repalmitoylation (27), such a constitutive cycling between the PM and intracellular organelles may be a general mechanism that allows cells to rapidly adjust to extracellular stimulation. To understand the whole picture of this shuttling mechanism that allows cells to rapidly adjust to extracellular stimulation, we found that incorporation of [3H]palmitate into Goα3 did not change when the cells were treated with an α1A-AR agonist (data not shown). Also, the localization of DHHC3 at the Golgi apparatus did not change with an α1A-AR agonist (data not shown). In contrast, DHHC21, one of the endothelial NO synthase PATs, was shown to be involved in the release of nitric oxide stimulated by both ionomycin and ATP, suggesting that DHHC21 is regulated downstream of calcium and ATP (10). These results imply that the individual DHHC proteins are regulated differently. Because protein palmitoylation modifies a wide variety of GPCR-related proteins and because GPCR signaling pathways play important roles in physiologic and pathological phenomena, the DHHC enzyme family may become an ideal therapeutic target.

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