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Analysis of Protein Palmitoylation by Metabolic Radiolabeling Methods

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1. Introduction

Many eukaryotic signaling proteins are modified by the covalent attachment of long-chain lipids. These highly hydrophobic molecules bind target proteins and facilitate interaction with cellular membranes, lipid molecules and other proteins. Generally, there are three classes of lipids that modify target proteins: myristate, isoprenoids and palmitate (for reviews, *see* ***refs. 1,2***). Myristate, a 14-carbon saturated fatty acid, binds proteins at N-terminal glycine residues via an amide linkage. This usually occurs cotranslationally after removal of the initiating methionine, although it also can occur after proteolytic cleavage and exposure of an internal glycine. Long-chain isoprenoid lipids, including farnesyl and geranylgeranyl groups, modify proteins posttranslationally and attach via a thioether linkage to C-terminal cysteine residues. Palmitate is a 16-carbon saturated fatty acid that modifies proteins posttranslationally through thioester incorporation at cysteines. Palmitate also can modify proteins at additional sites and by alternative mechanisms, as in proteins palmitoylated at serine and threonine residues via oxyester linkages and by amide-linked N-terminal cysteines and glycines. In addition, other fatty acid species form thioester linkages with proteins. For these reasons, the general term for protein lipid modification by thioester attachment is *thioacylation*, or *S-acylation*, and the term *palmitoylation* refers specifically to protein modification by palmitate. Proteins that undergo palmitoylation by thioester attachment at cysteine residues, or *S-palmitoylation*, represent the majority of protein targets of palmitoylation. This chapter will focus on methods for identifying protein substrates for thioester incorporation of palmitate.

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A wide range of proteins become palmitoylated, including intracellular signaling proteins, transmembrane enzymes, sreted oncophogens and cell surface receptors. Although the precise role of palmitoylation has proven difficult to discern, the modification has well-described pleiotropic effects on protein function. The common feature shared by all palmitoylated proteins is the capacity for interaction with cellular membranes. The extended hydrocarbon chain of the palmitate group acts as a stable membrane attachment point. This is particularly important for peripheral membrane proteins that otherwise are inherently soluble. The palmitate groups have high affinity for the membrane microdomain structures called lipid rafts in particular, and modification by palmitate is thought to target proteins to these membrane regions for selective protein interactions and signaling purposes. Preventing protein palmitoylation by point mutation or pharmacologic inhibition can exclude proteins from lipid rafts, impair biological activity and has effects on protein-protein interactions, protein stability and subcellular trafficking. For comprehensive reviews of protein palmitoylation, *see* ***refs. 3–5***.

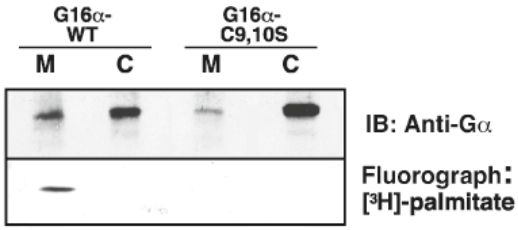
Protein palmitoylation is an enzyme-regulated process that differs from other types of lipid modifications by its reversible nature. Both myristoylation and prenylation are considered stable modifications for the lifetime of the protein, but some palmitoylated proteins exhibit measurable half-lives for the lipid modification. Furthermore, intracellular signaling proteins can undergo multiple cycles of depalmitoylation followed by reloading with fresh palmitate. Guanine nucleotide-regulated proteins like Ras and heterotrimeric Gα protein subunits are examples of palmitoylated proteins that dynamically incorporate palmitate during signaling events. The rate of palmitate turnover on these proteins is enhanced dramatically by pharmacologic stimulation of cell surface receptors or direct protein activation (reviewed in ***ref. 6***). For this reason, reversible attachment of palmitate to signaling proteins is thought to be a regulatory mechanism for controlling biological activity of the targeted proteins. Two classes of enzymes that regulate this process have been identified: protein acyltransferases (PATs) catalyze the transfer of palmitate from an intracellular donor such as palmitoyl-coenzyme A to a protein substrate, and protein thioesterases (PTEs) control removal of the lipid. For detailed information on these enzymes and the molecular mechanisms that underlie protein palmitoylation, *see* ***ref. 7***.

The protocols presented in this chapter outline how to assess if a protein is palmitoylated in living cells. The general approach is to incubate cells expressing the protein of interest with [^{3}H]palmitate, isolate the protein from the cells by immunoprecipitation, and determine whether it incorporated radioactive palmitate using SDS-PAGE and fluorography. In some cases, palmitate may be incorporated into serine or threonine residues through an oxyester linkage, thus it is necessary to verify that a labeled protein is a result of authentic, thioester-linked lipid.

This is accomplished by treatment of the gel containing the labeled proteins with neutral hydroxylamine, which hydrolyzes only thioester-linked lipids. This method also is useful for analysis of proteins that have an N-terminal glycine to discern [^{3}H]palmitate incorporation from incorporated [^{3}H]myristate metabolically converted from the [^{3}H]palmitate. Identity of the attached fatty acid is confirmed by cleaving thioester-linked fatty acids with base. The fatty acids are then extracted from the hydrolysate and analyzed by HPLC or TLC.

The methods described here for palmitoylation analysis by metabolic radiolabeling and fluorography have many advantages. The general experimental techniques are basic and commonly employed in labs utilizing cell culture systems. The specific methods are straightforward and require reagents and only standard laboratory equipment. The primary limitation of this approach is that it only provides a qualitative measure of palmitoylation. The stoichiometry of palmitate incorporation in a protein substrate cannot be determined using these methods. Other methods are available for quantitative analysis of protein palmitoylation. A newly developed technique called fatty acyl exchange labeling is a sensitive and quantitative approach to evaluating protein palmitoylation. In this method, palmitate molecules are cleaved from the modified protein by treatment with neutral hydroxylamine, and the resulting free sulfhydryl groups are coordinated with radiolabeled -specific reagents like [^{3}H]N-ethylmaleimide (NEM) ***(8)***. This approach also can be adapted for use with non-radioactive (e.g. biotin-conjugated) reagents to bind available cysteine residues. Other methods for analyzing protein palmitoylation include mass spectrometry and in vitro palmitoylation assays with purified and partially purified PATs. These methods are described in more detail with good discussion on advantages and limitations of the techniques, particularly for fatty acyl exchange labeling, in reference ***(8)***.

Once it is established that a protein is palmitoylated, the site(s) for palmitate attachment can be determined by mutation of cysteines to alanine or serine and the mutant protein assayed for loss of palmitoylation. There is no known consensus sequence for palmitate incorporation, so every cysteine on the surface of a protein should be considered candidate site for palmitoylation. For a review of some shared sequence motifs in palmitoylated proteins, see reference ***(6)***. Proteins that incorporate palmitate at more than one site may contain a primary cysteine, where palmitoylation is required prior to incorporation at other sites within the protein. For this reason, it is important to design both individual and multi-site cysteine mutants to properly map the sites and/or sequence requirements for palmitate incorporation. For our studies, we use the PCR-based QuikChange® Site-Directed Mutagenesis Kit from Stratagene for creating individual and combination cysteine-to-serine point mutations in our proteins of interest (*see* **Fig. 1**). It is important to note that site-directed mutagenesis is an effective way to establish the dependence of the modification on a specific amino acid



G16α: MARSLTWR**CC**PW**C**LTEDEKAAARVDQEINRILLEQKKQDRG

G16α-C9,10S: MARSLTWR**SS**PW**C**LTEDEKAAARVDQEINRILLEQKKQDRG

Fig. 1. Palmitoylation of G16 -terminal cysteines determined by metabolic radiolabeling analysis combined with site-directed mutagenesis. Top, upper panel: Immunoblot analysis of G16 -EE wild type (WT) and C9,10S protein immunoprecipitated (anti-EE antibody) from membrane (M) and cytosol (C) fractions of transfected HEK293 cells. Immunoblot analysis was performed with a Gq-family-specific antibody (Z811) kindly provided by Dr. Paul Sternweis (UT Southwestern Medical Center, Dallas, TX). Top, lower panel: Fluorography of G16 -EE WT and C9,10S protein immunoprecipitated from fractionated cells as described above. Exposure time was 56 days at −80°C. Bottom: Sequence alignments of the first 41 amino acids of G16 WT and C9,10S. Cysteine residues for putative palmitoylation and selected cysteine-to-serine mutations are indicated in bold type. Cysteine-to-serine mutations were generated using QuikChange® Site-Directed Mutagenesis kit from Stratagene (cat. no. 200518). (Reproduced from ref. 14 with permission of American Society for Biochemistry and Molecular Biology).

residue. However, definitive proof ires isolation and identification of the acylated peptide by mass spectrometry.

To successfully perform the techniques described in this chapter, several general considerations should be made. First, specific antibodies are required in order to immunoprecipitate the expressed protein. Since it often is difficult to produce antibodies against proteins directly, epitope-tagged versions of proteins can be engineered. In this chapter, we describe palmitoylation analysis of a G protein, G16α, that contains an internal glutamate-glutamate (EE) epitope tag. Other convenient epitopes can be used instead.

Sonicient quantities of protein need to be expressed in order to produce a [^{3}H]-labeled signal. Sf9 ins have provided an excellent expression system for palmitoylation studies, particularly for heterotrimeric G protein alpha subunits (Gα) ***(9)***. The primary advantage of ins s is that infection with recombinant baculovirus produces relatively high levels of heterologous protein expression. Because they are eukaryotic cells, Sf9 contain the cellular enzyme machinery to modify expressed proteins. Detailed protocols for radiolabeling of Sf9 cells and for analysis of lipid modifications can be found in ***ref. 10***.

Although Sf9 cells have proven to be a good model system for palmitoylation studies of Gα proteins, some mammalian proteins may be processed or regulated inappropriately in an insect expression system. Subtle processing differences can have significant effects on palmitate incorporation into the protein. While it is desirable to analyze palmitoylation of any protein in a native system, often proteins are difficult to detect in the endogenous cells or tissues. In this chapter, we describe methods for analyzing palmitoylation of recombinant proteins heterologously expressed in a mammalian cell line. These methods have been used regularly by our labs to evaluate palmitoylation of various G protein subunits and protein Regulators of G Protein Signaling (RGS proteins) ***(9,11–14)***. In this chapter, our example protein is G16α, a G protein alpha subunit from the Gq family of heterotrimeric G proteins. We recently showed that G16α is posttranslationally modified by palmitate and that at least two of three cysteine residues in the N-terminus of the protein are essential for palmitoylation **(Fig. 1)** ***(14)***. We used HEK293 cells for our studies, although COS-7, CHO, or other readily transfectable adherent cell line may be substituted (*see* **Note 1**). Most of the methods described here for mammalian cells are the same or similar to those for Sf9 cells ***(10)***. The differences between the two expression systems are the methods for introduction of the construct into the cells and cell lysis. Since many laboratories are equipped for mammalian cell culture, these protocols are likely to be straightforward to adopt.

2. Materials

2.1. Measuring Palmitate Incorporation into Recombinant EE-Tagged G16α Protein in Mammalian Cell Culture Systems

2.1.1. Transfection and Metabolic Labeling of EE-Tagged G16α in Mammalian Cells

1. HEK293 cells (American Type Culture Collection, cat. no. CRL-1573)
2. Transfection-quality G16α-EE plasmid DNA (*see* **Note 2**)
3. Lipofectamine™ 2000 Transfection Reagent (Invitrogen, cat. no. 11668-019)
4. Opti-MEM® I reduced-serum media (Invitrogen, cat. no. 31985)
5. Complete HEK293 medium: Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, cat. no. 10-013-CV), 10% fetal bovine serum (Atlanta Biologicals, cat. no. S11150), penicillin/streptomycin (CellGro, cat. no. 30-002-CI)
6. [^{35}S]methionine wash medium: 95% methionine-free DMEM (Invitrogen, cat. no. 21013-024), 5% DMEM, 2.5% dialyzed fetal bovine serum (Invitrogen, cat. no. 26400-036)
7. [^{35}S]methionine labeling medium: 95% methionine-free DMEM, 5% DMEM, 2.5% dialyzed fetal bovine serum, 1 mM sodium pyruvate (Invitrogen, cat. no. 25-000-CI), 100 Ci/mL L-[^{35}S]methionine (>1000Ci/mmol) (Amersham Biosciences, cat. no. SJ1015). Aliquot the [^{35}S]methionine and store at −80°C. Make media fresh before use.

8. [^{3}H]palmitate labeling medium: 1 mCi/mL [^{3}H]palmitate ([9,10-^{3}H]-Palmitic Acid (31 Ci/mmol), PerkinElmer Life and Analytical Sciences, cat. no. NET043), 1% DMSO, 2.5% fetal bovine serum, 1 mM [illegible]m pyruvate, 1 mM non-essential amino acids (Invitrogen, cat. no. 25-025-CI). Make fresh before use (see **Note 3**).
9. Nitrogen gas

2.1.2. Subcellular Fractionation

1. Potter-Elvehjem tissue grinders with polytetrafluoroethylene (PTFE)-coated pestle, 2 mL capacity, 0.1–0.15 mm clearance (Wheaton Potter-Elvehjem Tissue Grinder, Thermo Fisher Scientific, cat. no. 22-290-066)
2. Ice-cold phosphate-buffered saline (PBS)
3. Hypotonic lysis buffer: 50 m*M* HEPES, pH 8.0, 1 m*M* EDTA, 1 mM [illegible], protease inhibitors (2 g/mL aprotinin, 1 g/mL leupeptin, 100 M phenylmethylsulfonyl fluoride (PMSF)). Add DTT and protease inhibitors just before use.
4. 2X isotonic buffer: 50 m*M* HEPES, pH8.0, 10 m*M* $MgCl_2$, 300 m*M* NaCl, 1 m*M* EDTA, 500 m*M* sucrose, protease inhibitors (4 g/mL aprotinin, 2 g/mL leupeptin, 200 M [illegible]F). Add protease inhibitors just before use.
5. 1.5 mL capacity microfuge tubes for high-speed centrifugation (Microfuge Tube Polyallomer, Beckman, cat. no. 357448)
6. 2 mL capacity microfuge tubes
7. 1X Immunoprecipitation (IP) buffer: 50 m*M* HEPES, pH8.0, 5 m*M* $MgCl_2$, 150 m*M* NaCl, 1 m*M* EDTA, 250 m*M* sucrose, protease inhibitors (2 g/mL aprotinin, 1 g/mL leupeptin, 100 M [illegible]F). Add protease inhibitors just before use.
8. IP buffer with 4% DβM: 4% *n*-Dodecyl-β-D-maltoside (DβM; Calbiochem, cat. no. 324355) in 1X IP buffer. DβM is used at a final concentration of 2% for extraction of membrane proteins.

2.1.3. Immunoprecipitation of labeled protein, SDS-PAGE and fluorography

1. Anti-GLU-GLU (EE) monoclonal antibody (Covance, cat. no. MMS-115R)
2. Protein A Sepharose (nProtein A Sepharose® 4 Fast Flow, GE Healthcare, cat. no. 17-5280-01)
3. Bovine serum albumin (Sigma, cat. no. A3059)
4. Coomassie blue stain: 30% isopropanol, 10% acetic acid, 0.25% Coomassie Brilliant Blue R-250
5. Destain solution: 30% methanol, 10% acetic acid
6. En^{3}Hance autoradiography enhancer solution (En^{3}Hance™, PerkinElmer Life and Analytical Sciences, cat. no. 6NE9701) (*see* **Note 4**)
7. 2X Laemmli sample buffer: 100 m*M* Tris pH6.8, 0.5% SDS, 20% glycerol, 0.5% β-mercaptoethanol, 0.004% bromophenol blue. Prepare stocks of 2X Laemmli sample buffer with and without 20 m*M* DTT.

2.2. Hydroxylamine Sensitivity by Thin Layer Chromatography

1. Hydroxylamine solution: 0.5 *M* hydroxylamine hydrochloride (Sigma-Aldrich, cat. no. 431362), pH7.0, in 50% isopropanol. Use concentrated NaOH to pH the solution.
2. 0.5 *M* Tris, pH7.0, in 50% isopropanol

2.3. Identification of Fatty Acids on Labeled Protein

1. 13 × 100 mm glass tubes with Teflon-lined caps (Corning, cat. no. 9826-13)
2. HPLC grade methanol, chloroform, acetonitrile, acetic acid
3. [^{3}H]-fatty acid standards: [^{3}H]palmitate, [^{3}H]myristate, [^{3}H]stearate (Perkin Elmer Life and Analytical Sciences)
4. Nitrogen gas
5. NaOH
6. 6N HCl, high grade (Pierce Chemicals)
7. Palmitic acid (Sigma, cat. no. P5585)
8. Trifluoroacetic acid
9. C:18 reversed-phase thin-layer chromatography plates (Whatman, cat. no. 4800-620)
10. En^{3}Hance™ Spray Surface Autoradiography Enhancer (Perkin Elmer Life and Analytical Sciences, cat. no. 6NE970C)

3. Methods

3.1. Measuring palmitate incorporation into recombinant G16α-EE protein in mammalian cells

3.1.1. Transfection and metabolic labeling of G16α-EE protein in mammalian cells

This protocol calls for metabolically labeling parallel dishes of transfected cells: one with [^{3}H]palmitate, the counterpart with [^{35}S]methionine. The purpose of labeling cells with [^{35}S]methionine is to control for expression and recovery of the recombinant protein of interest. Alternatively, immunoblot analysis of the protein of interest immunoprecipitated from unlabeled cells may be substituted for radiolabeling with [^{35}S]methionine **(Fig. 1)**.

1. Plate 2–10 cm^2 dishes of HEK293 cells so that they will be 85–90% confluent the next day.
2. Transfect HEK293 cells with G16α-EE DNA construct using Lipofectamine™ 2000 Transfection Reagent according to manufacturer's instructions (*see* **Note 5**). Incubate 5 h, 37°C, 5% CO_2/95% air.
3. Replace transfection media with complete media, and allow cells to recover in 37°C incubator overnight (*see* **Note 6**).
4. Approximately 36 h after beginning the transfection, label one plate with [^{35}S] methionine: wash once in [^{35}S]methionine wash media, then add labeling media. Incubate overnight in the 37°C incubator.

5. The following day, label the sonicate with [^{3}H]palmitate: wash cells once with pre-warmed DMEM, then incubate for 1–2 h in [^{3}H]palmitate labeling medium (*see* **Note 7**).
6. Harvest each plate 48 h post-transfection: using a clean razor blade or rubber policeman, scrape cells into separate conical tubes on ice.
7. Collect cells by centrifugation at 500 *g*, 4°C for 4 min.
8. Aspirate media into a receptacle for radioactive liquids. Resuspend cells in 5 mL ice-cold PBS to wash away residual media.
9. Collect cells by centrifugation at 500 *g*, 4°C for 4 min.

3.1.2. Subcellular Fractionation (*see* **Note 8**)

1. Aspirate PBS, and resuspend each cell pellet in 0.5 mL hypotonic lysis buffer. Transfer cells to separate Potter-Elvehjem tissue grinders on ice.
2. Homogenize cells with 30–50 strokes to break open cells, and transfer lysates to separate 1.5 mL microfuge tubes on ice (*see* **Note 9**).
3. Add 0.5 mL 2X isotonic buffer to each tube to restore normal osmotic pressure.
4. Centrifuge lysates at 600 *g*, 4°C for 10 min to collect nuclei, unbroken cells, and plasma membrane sheets.
5. Transfer the supernatants to Beckman polyallomer microfuge tubes. Spin in TLA100.3 rotor with adaptors, 100,000 *g*, 4°C for 30 min.
6. Remove supernatants from the high-speed spin (cytosol) to clean 2 mL microfuge tubes. Add 1 mL IP buffer with 4% DβM to each for a final detergent concentration of 2% DβM.
7. Resuspend pellets (membranes) in 0.5 mL IP buffer (without detergent) in the polyallomer tubes, and transfer to Potter-Elvehjem tissue grinders on ice. Lightly homogenize the membrane pellets (15 strokes).
8. Add 0.5 mL IP buffer with 4% DβM to each membrane sample for a final detergent concentration of 2% DβM.
9. Solublize membranes by incubating at 4°C for 3 h on an end-over-end rocker (*see* **Note 10**). Incubate cytosol samples similarly for the same duration.
10. Pellet the insoluble material from membrane samples by high-speed centrifugation at 100,000 *g*, 4°C for 30 min.
11. Remove the supernatants (membrane extract) to clean microfuge tubes (*see* **Note 11**).

3.1.3. Immunoprecipitation of Labeled Protein, SDS-PAGE, and Fluorography (*see* **Note 12**)

1. Preblock membrane extracts and cytosol samples in 0.7 mg/mL BSA solution and pre-absorb with 50 L Protein A Sepharose. Incubate with end-over-end rotation, 4°C from 30 min to 2 h.
2. Add 6–7 L anti-EE antibody to each sample of membrane extracts and cytosol. Incubate at 4°C at least 3 h, or overnight if convenient, rotating end-over-end.
3. Collect sepharose beads and immunoprecipitated protein complexes by centrifugation: 200 *g*, 4°C for 1 min.
4. Aspirate supernatant, and resuspend sepharose beads in 1 mL IP buffer containing 0.2% DβM.

5. Repeat spin: 200 *g*, 4°C for 1 min. Wash sepharose beads two more times in the same fashion.
6. Suspend the washed beads in 60 L 2X Laemmli sample buffer without DTT (*see* **Note 13**).
7. Boil samples 1 min, then spin 1 min in microcentrifuge at maximal speed. Load the supernatant samples on SDS-PAGE gels and run (*see* **Note 14**).
8. Stain gels with Coomassie blue stain, rocking, 30 min.
9. Incubate in several changes of destain solution, rocking, until proteins are visualized on the gel (*see* **Note 15**).
10. Soak gels in En^{3}Hance™ autoradiography enhancer solution, 60 min, with gentle rocking.
11. Wash gels thoroughly in a large volume of cold water for 30 min with gentle rocking.
12. Dry down the gels to completion on a vacuum dryer, 60°C.
13. Expose the gels to film in a cassette. Store the cassette at −80°C for at least 3 days to visualize the [^{35}S]-labeled bands. Replace with new film and expose for 1–4 weeks to visualize [^{3}H]-labeled bands **(See Note 16)**.

3.2. Hydroxylamine Sensitivity of [^{3}H]palmitate-Labeled Proteins (*see* Note 17; Fig. 2).

1. Transfect cells, label and fractionate cells as in **steps 3.1.1** and **3.1.2 (See Note 18)**.
2. Immunoprecipitate EE-tagged protein from cells as outlined in 3.1.3 steps 1–6 above.
3. Boil samples 1 min, then spin 1 min in microcentrifuge at maximal speed. Divide each sample in half, and run duplicate gels of each.
4. Fix gels by incubating in destain solution for 30 min, rocking.

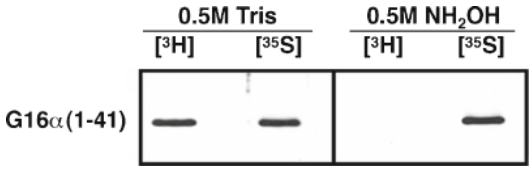


Fig. 2. Confirmation of thioester linkage of [3H]palmitate by hydroxylamine cleavage. Fluorography of the radiolabeled G16 N-terminus with and without neutral hydroxylamine treatment. A peptide representing the N-terminal domain of G16 (amino acids 1–41) was expressed in HEK293 cells as a FLAG-tagged GFP fusion protein. The fusion protein was immunoprecipitated (Anti-FLAG M2 Affinity Gel, Sigma, cat. no. A2220) from transfected membranes of HEK293 cells metabolically radiolabeled with either [35S]methionine or [3H] palmitate. Immunoprecipitated proteins were treated with 0.5 M Tris, pH7.0 (left panel) or 0.5 M hydroxylamine, pH7.0 (NH2OH, right panel) and prepared for fluorographic analysis. Exposure time was 2 days at −80°C for [35S]-signals, 5 days for [3H]-signals. (Reproduced from ref. 14 with permission of American Society for Biochemistry and Molecular Biology)

5. Soak one gel in fresh 0.5 *M* hydroxylamine solution (pH7.0) for 12–18 h (overnight). As a control, soak the other gel in 0.5 *M* Tris (pH7.0) in 50% isopropanol. Replace the solutions at least once after approximately one h gels will shrink as they are dehydrated by the isopropanol.
6. Wash each gel 4 times in 50% isopropanol for a of 48 h.
7. Soak gels in Coomassie stain, then destain solutions as above to visualize precipitated proteins. The gels will rehydrate and return to original size during this step.
8. Soak gels in En^{3}Hance™ autoradiography enhancer solution for 60 min with gentle rocking.
9. Dry down the gels on a vacuum dryer, 60°C xpose to film at −80°C.

3.3. Identification of Fatty Acids on Labeled Protein

3.3.1. Base Hydrolysis and Extraction of Thioester-Linked Fatty Acids

1. Transfect cells, label, and fractionate cells as in **steps 3.1.1** and **3.1.2**.
2. Immunoprecipitate protein from cells as outlined in **Subheading 3.1.3, steps 1–6**.
3. Boil samples 1 min, then pellet for 1 min in microcentrifuge at maximal speed. Divide each sample in half and run duplicate SDS-PAGE gels.
4. Stain gels with Coomassie blue stain, rocking, 30 min.
5. Incubate in several changes of destain solution until proteins are visualized on the gel.
6. Excise the immunoprecipitated protein bands from membranes and cytosol with a razor blade or scalpel, keeping gel slices equal in size. Also excise three gel slices from unused region e gel to run with standards.
7. Place gel slices in ai screw-cap 13 × 100 mm glass tubes. Wash gel slices overnight with five changes of 50% methanol.
8. Remove methanol with Pasteur pipet. To the blank gel slices, add [^{3}H]myristate, [^{3}H]stearate, or [^{3}H]palmitate standards, 25,000–50,000 dpm per sample. Dry gel slices under a nitrogen stream.
9. To hydrolyze thioester-linked lipids, resuspend gel slices in 0.7 mL 1 NaOH. Flush tubes with nitrogen and cap. Incubate 3 h, 37°C, mixing periodically.
10. Acidify the solutions to pH1-2 by adding 6N (high-grade) HCl. This typically requires 100–200 L of acid.
11. To extract the released fatty acids, add 3.75 volumes of chloroform:methanol (1:2 v/v) in a fume hood. This results in chloroform:methanol:aqueous 1.25:2.5:1 v/v/v. Incubate 10 min, room temperature with occasional mixing.
12. To separate the fatty acids from water-soluble components and gel slices, add 1.25 volumes (of original aqueous volume) of chloroform and 1.25 volumes of water.
13. Vortex tubes, then centrifuge at 1,000 rpm at room temperature. This will separate the mixture into an upper aqueous phase and a lower organic phase, with the gel slice in between.
14. Remove the bottom phase containing fatty acids by bubbling a Pasteur pipette through the aqueous phase to the bottom of the tube. Transfer to new screw-cap tubes.
15. To extract remaining fatty acids, add the same amount of chloroform as in **step 12**. Incubate 5 min, room temperature with occasional mixing. Centrifuge 1,000 rpm and remove bottom phase as before. Combine with first extracts.

16. Dry down extracts under nitrogen.
17. Add 0.5 mL chloroform and 40 g cold palmitic acid as a carrier. Flush tubes with nitrogen and store at −20°C.

3.3.2. Thin-Layer Chromatographic Analysis of Hydrolyzed Lipids

1. Count a small volume of each sample (50 L) in scintillation vials to determine how much sample to load. Let the chloroform dry before counting or it will quench.
2. Spot equivalent amounts of each sample (25,000–50,000 dpm) on a reverse-phase C:18 TLC plate. Also spot equivalent standards.
3. Resolve the extracts using 90% acetonitrile/10% acetic acid in a chromatography tank. This should take 20–30 min.
4. After the solvent has migrated to the top, remove plates and allow them to dry thoroughly in the hood.
5. Spray plates with En^{3}Hance™ spray. Let plates dry slightly and expose to film at −80°C for 5–10 h. Re-expose for 24–48 h if signal is undetectable.

4. Notes

1. The protocols for heterologous protein expression, cell lysis and fractionation (**Subheadings 3.1.1** and **3.1.2**) are described here specifically for mammalian cells. The remainder of the methods are performed the same independently of eukaryotic cell type used.
2. We use Midi- or Maxi-prep kits from Qiagen to produce DNA for transfections. Follow the manufacturer's protocols. Our G16α-EE DNA plasmid came from UMR cDNA Resource Center (www.cdna.org; cat. no. GNA1500000). Many other DNA constructs for mediators of G protein signaling (GPCRs, G proteins, RGS proteins) also are available from UMR in epitope-tagged and untagged forms.
3. [^{3}H]palmitate is supplied as a solution in ethanol and cannot be added directly to media. First, measure the appropriate amount into a glass tube and evaporate the ethanol under a nitrogen stream. When the palmitate is dry, it will appear as a light film on the bottom of the tube. Resuspend the palmitate first in DMSO (for final concentration of 1% in palmitate labeling media; volume will depend on final volume of labeling media), then add the fetal bovine serum (final concentration 2.5%). Add the remaining reagents to the dissolved [^{3}H]palmitate.
4. We have successfully used a homemade version of fluorographic reagent: 1 sodium salicylate in 15% methanol. Store in a dark bottle.
5. Detailed instructions for optimizing transfection conditions with Lipofectamine™ 2000 Transfection Reagent are provided by the manufacturer. The amount of DNA and lipofectamine used per dish can vary depending on the protein to be expressed.
6. The optimal time to harvest transfected cells for peak protein expression may vary depending on the protein. In this protocol, cells are collected 48 h post-transfection. For shorter post-transfection incubations, labeling should be started at the appropriate time so that [^{35}S]methionine labeling media is incubated overnight and [^{3}H] palmitate media is incubated for 1–2 h.

7. Some proteins undergo turnover of the palmitate modification. For example, the half-life for palmitate on H-Ras has been measured as approximately 2.4 h; however, the half-life decreases to 90 min for ectopically-expressed ated H-Ras, suggesting that G protein activation is linked to palmitate turnover ***(15)***. The propensity for palmitate turnover should be considered when establishing a time period for metabolic labeling with [^{3}H]palmitate. A 1–2 h labeling period with high concentrations of [^{3}H]palmitate as suggested in this protocol is a good starting point. G protein alpha subunit palmitoylation has been detected with labeling periods as short as 15 min ***(16)***.
8. All solutions should be kept cold (4°C), and all samples should be kept on ice during the following steps in order to minimize protein degradation.
9. If using the same Potter-Elvehjem tissue grinder for both [^{35}S]- and [^{3}H]-labeled cells during an experiment, pass the [^{3}H]-labeled samples first to avoid accidental transfer of the higher energy [^{35}S]-signal into the [^{3}H]-labeled samples. Wash out the tissue grinder thoroughly with ethanol to remove trace [^{3}H]-residue. Alternatively, cells may be lysed by another convenient method (e.g. repeated cycles of snap-freezing and quick-thawing).
10. Other buffers may be used to solublize proteins successfully. We found this HEPES-based buffer formulation with 2% DβM detergent to work well for extraction and immunoprecipitation of our Gα proteins with the anti-EE antibody. If a different antibody is used, the buffer may need to be adjusted. Also, a shorter incubation period for membrane extraction may be sufficient for other proteins. Generally, a 1-h extraction period is sufficient to release proteins from membranes prepared at 1 mg/mL membrane protein concentration.
11. At this step, the membrane extracts and cytosol samples may be snap-frozen in liquid nitrogen and stored at −80°C until later use, as limited by the half-life of the [^{35}S]-signal. Frozen samples should be thawed quickly and kept on ice.
12. Immunoprecipitation protocols vary widely. For a review and guide to optimization of immunoprecipitation using different types of antibodies and solid support matrices, see reference ***(17)***. Other protocols may be easily substituted.
13. The thioester linkage for S-palmitoylation is vulnerable to reducing agents such as DTT. For this reason, we exclude DTT from our Laemmli sample buffer for [^{3}H] palmitate-labeled samples. This does not affect migration of our G proteins on SDS-polyacrylamide gel, but the same may not be true for other proteins. 20 mM DTT may be included in [^{3}H]palmitate-labeled samples if needed, but the lability of the thioester linkage should be considered. Laemmli sample buffer containing 20 mM DTT should be used for all other protein samples prepared for SDS-PAGE.
14. Avoid loading sepharose beads onto the SDS-PAGE gel. Thioester-linked fatty acid also is susceptible to prolonged heating. We have found that excluding DTT from the sample buffer and heating for 1 min is sufficient to obtain good resolution of Gα proteins on gels without a substantial loss of radioactive palmitate on the protein.
15. The gel can be left in destain solution overnight if desired.

16. Do not use plastic wrap to cover the gel during exposure as it will block the [^{3}H]-signal. Storage of the cassette at −80°C is essential for the En^{3}Hance™ fluorography reagent to work. Do not use a screen. The [^{35}S]-labeled samples may be visualized within a few days. The [^{3}H]-labeled bands may be detected after [illegible] exposure, but it can take one month or longer, depending on the amount of protein on the gel. Exposure time must be determined empirically.
17. If the membrane-bound protein of interest incorporates [^{3}H]palmitate, further analysis should be done to confirm authentic thioester palmitate attachment. Treatment of the gel containing the [^{3}H]-labeled sample with neutral hydroxylamine should result in the reduction or disappearance of the labeled band. As a control, a duplicate gel is soaked in Tris buffer at the same pH. The [^{3}H]-labeled sample should be visible in this gel. Finally, [^{35}S]methionine-labeled proteins should be run in parallel to control for the loss of protein from the hydroxylamine- and Tris-treated gels during processing. A hydroxylamine-insensitive label may be analyzed as a potential amide-linkage by treating the gels with acid instead. Amide-linked fatty acids are insensitive to hydroxylamine treatment but sensitive to acid hydrolysis.
18. The number of cells required for these experiments may vary depending on protein expression and stoichiometry of palmitoylation. Adjust the size and/or number of dishes transfected based on the autoradiograph in **Subheading 3.1**.

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