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Optimization of Metabolic Labeling with Alkyne-Containing Isoprenoid Probes

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Abstract

Protein prenylation, found in eukaryotes, is a post translational modification in which one or two isoprenoid groups are added to the C terminus of selected proteins using either a farnesyl group or a geranylgeranyl group. Prenylation facilitates protein localization mainly to the plasma membrane where the prenylated proteins, including small GTPases, mediate signal transduction pathways. Changes in the level of prenylated proteins may serve a critical function in a variety of diseases. Metabolic labeling using modified isoprenoid probes followed by enrichment and proteomic analysis allows the identities and levels of prenylated proteins to be investigated. In this protocol, we illustrate how the conditions for metabolic labeling are optimized to maximize probe incorporation in HeLa cells through a combination of in-gel fluorescence and densitometric analysis.

Keywords

Protein prenylation; farnesylation; geranylgeranylation; metabolic labeling; isoprenoid analogue; in-gel fluorescence; lovastatin

1. Introduction

Protein prenylation, found in eukaryotes [1] is a post translational modification in which one or two isoprenoid groups are added to the C terminus of selected proteins using either a farnesyl group (C15) or a geranylgeranyl group (C20) [2]. This irreversible covalent modification is catalyzed by three prenyltransferase enzymes including farnesyl transferase (FTase) and geranylgeranyl transferase type I (GGTase-I) and geranylgeranyl transferase type II (GGTase-II) [3–5]. FTase and GGTase-I catalyze the attachment of a single farnesyl group or geranylgeranyl group to a cysteine residue located within a CaaX motif, respectively in which “C” is cysteine, “a” is an aliphatic amino acid and the “X” residue is critical for determining which isoprenoid group is attached and hence which enzyme is involved [2]. In contrast, GGTase-II catalyzes the addition of two geranylgeranyl groups to two cysteine residues in sequences including CXC and CC. In addition to the known canonical CaaX motif that can be recognized by FTase, recent studies have showed that a longer C(x)₃X motif can also be recognized by both yeast and mammalian FTases [6]. Protein prenylation by FTase and GGTase-I typically is followed by the removal of the aaX residues

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promoted by Ras converting enzyme (Rce1) or Ste24 [7,8]. In the final step, the mature protein is generated by the action of carboxylmethyltransferase (Ste14), which catalyzes the transfer of a methyl group from S-adenosyl methionine to the carboxylate of the C-terminal prenyl cysteine residue to yield a C-terminal methyl ester [9]. Prenylation facilitates protein localization mainly to the plasma membrane where the prenylated proteins including small GTPases mediate signal transduction pathways [10]. Changes in the level of prenylated proteins may be critical in a variety of diseases including Parkinson's disease [11], Alzheimer's disease [12], neurodegeneration [13], viral infections [14], and some type of cancers [15].

To improve understanding of the process of protein prenylation, as well as identify protein substrates that are lipidated through this process, synthetic small molecule analogues of the isoprenoids are being used. Photoactivatable [16,17] and fluorescent [18] isoprenoid analogues have been used to examine protein-protein interactions of the prenylated proteins and to develop convenient assays for prenyltransferase activity, respectively. In addition, there has been significant interest in identifying prenylated proteins via metabolic labeling using modified isoprenoid probes followed by enrichment and proteomic analysis. In this approach, the isoprenoid probe is functionalized with a bioorthogonal functional group (azide or alkyne) that allows conjugation of a fluorophore or an affinity handle for pulldown and enrichment of labeled proteins for subsequent identification [19]. Several strategies using this approach have emerged and been used to identify the prenylome in mammalian [20] and parasitic systems [21], as well prenylated proteins with antiviral activity [14]. We have previously demonstrated that isoprenoid analogues (Fig. 1) C15AlkOP and C15AlkOPP served as good substrates for metabolic labeling in COS-7 cells whereas C15AlkOH was incorporated with much lower efficiency [22]. In that study, we showed how labeling efficiency can be optimized by varying the probe concentration. While some incorporation can be observed using 1 μ M alkyne-functionalized-probe, 10 μ M probe gives significantly higher labeling without significantly raising the level of background labeling which occurs at higher probe concentrations [23]. Importantly, probe incorporation can also be enhanced by inhibition of the synthesis of the endogenous isoprenoid substrates farnesyl and geranylgeranyl diphosphate. Hence, a common strategy to accomplish this is to employ statins that inhibit HMG-CoA reductase upstream in the FPP biosynthetic pathway [24]. In this protocol, we illustrate how the concentration of lovastatin can be optimized to maximize probe incorporation (but avoid toxicity) in HeLa cells through a combination of in-gel fluorescence and densitometric analysis.

2. Materials

2.1 Cell Culture

1. Dulbecco's Modified Eagle's Medium (DMEM)
2. Fetal bovine serum (FBS)
3. Penicillin/streptomycin (Pen/strep): 10,000 U penicillin, 10 mg/mL streptomycin (10 $\times$ solution)
4. DMEM culture medium: DMEM, 10% FBS, 1 $\times$ Pen/strep

5. Phosphate-buffered saline: 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl
6. 2.5% trypsin media (10×), no EDTA (Invitrogen, product number 15090–046)
7. Versene: 1X PBS + 0.6 mM Na₂EDTA
8. 100 mm culture dish

2.2 Metabolic labeling

1. Isoprenoid probe analogues: C15AlkOH, C15AlkOP, and C15AlkOPP [22,25]
2. Lovastatin (Cayman Chemical)

2.3 Cell Harvest, lysis, Protein Assay

1. 1X PBS
2. Cell scraper
3. Lysis buffer: 1× PBS, 1% sodium dodecyl sulfate (SDS), 2.4 μM phenylmethylsulfonyl fluoride (PMSF), 85 kU/mL benzonase nuclease, 1.5% (v/v) protease inhibitor cocktail
4. BCA protein assay kit (Thermo Scientific)
5. Absorbance plate reader

2.4 Click reaction on labeled lysates

1. 1 mM TAMRA-PEG3-azide in DMSO (Broadpharm)
2. 50 mM Tris(2-carboxyethyl)phosphine (TCEP) in DMSO
3. 10 mM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO
4. 50 mM Copper (II) sulfate (CuSO₄) in water.
5. ProteoExtract® protein precipitation kit (Calbiochem)

2.5 Gel electrophoresis and in-gel fluorescence

1. Laemmli buffer (1×): 125 mM dithiothreitol (DTT), 2% SDS, 10% glycerol, 0.02% bromophenol blue, 50 mM Tris-HCl, pH 6.8
2. 12% SDS-PAGE gel (3 in × 4 in)
3. Fluorescence scanner (Typhoon FLA 9500)
4. Coomassie Blue staining solution: 0.2% (w/v) Coomassie Brilliant Blue R250, 10% (v/v) acetic acid, 45% (v/v) methanol, 45% water
5. Gel destaining solution: 10% (v/v) acetic acid, 30% (v/v) methanol, 60% (v/v) water

3. Method

3.1 Cell Culture and Metabolic Labeling

1. Maintain HeLa cells in DMEM culture medium and incubate at 37 °C. under 5% CO₂.
2. When cells near confluence, passage cells (1:10 split). Wash cells with PBS and detach from the dish using 1 mL of 0.25% trypsin in Versene. Incubate for 5 minutes, collect the cells and dilute with DMEM culture medium.
3. For metabolic labeling, seed 9×10^5 cells in 100-mm culture dishes in 10 mL of DMEM culture medium.
4. After 24 hours (40–60% confluency), replace the medium in each plate with 5 mL of fresh culture medium containing the desired concentration of lovastatin (0, 0.1, 1.0, 10, or 25 μ M, *see* NOTE 1).
5. After 6 hrs of lovastatin pre-treatment, replace the medium with 5mL of fresh medium in the presence or absence of lovastatin. Add the isoprenoid probes according to the desired final concentrations. In this protocol, a final concentration of 10 μ M was used for all three probes. Incubate cells at 37°C under 5% CO₂ for 24 h.

3.2 Cell Harvest, Lysis, Protein Assay

1. Harvest cells 24 h after the addition of the isoprenoid probes. Place the cell culture dishes on ice. Remove the medium by suction and wash adherent cells with 5 mL of cold 1 $\times$ PBS twice. Gently scrape the cells into 1 mL ice-cold PBS using a cell scraper. Collect the cells in 1.5 Eppendorf tubes using a 1-mL pipette and pellet by centrifugation for 5 minutes at $180 \times g$. Remove the supernatant by suction. Store the cell pellets at -80°C .
2. Add Lysis buffer (240 to 300 μ L, *see* NOTE 2) to each cell pellet. Lyse the cells by sonication for 6 to 8 times for 2 seconds at 10-minute intervals.
3. Determine the protein concentration in the cell lysates using BCA protein assay kit and following the manufacturer's protocol.

3.3 Click Reaction with TAMRA-PEG3-N₃

Perform reactions in 1.5 mL Eppendorf tubes and keep covered to avoid exposure to light.

1. Dilute aliquots of protein lysates (100 μ g) with 1 $\times$ PBS containing 1 % SDS to final volume of 92.5 μ L. Add 2.5 μ L of 1 mM TAMRA-PEG3-N₃ (final concentration, 25 μ M), 2 μ L of 50 mM TCEP (final concentration, 1mM) and 1 μ L of 10 mM TBTA (final concentration, 100 μ M) to each reaction tube. Vortex each sample briefly prior to adding 2 μ L of 50 mM CuSO₄ (final concentration, 1 mM). Incubate samples at room temperature in the dark (covered with aluminum foil) on a rotor mixer for 1 hour

2. After the reaction, spin down sample tubes to capture the reaction mixtures at the bottom of the tube. Precipitate protein using a ProteoExtract precipitation kit following the manufacturer's protocol. Recover proteins as white pellets at the bottom of the tube and allow to dry (*see* NOTE 3).

3.4 Gel Electrophoresis and Densitometry Analysis

1. Dissolve protein pellets in 45 μ L of 1 $\times$ Laemmli loading buffer and boil at 95 $^{\circ}$ C for 5–10 minutes. Apply each sample (15 μ L) to a 12% SDS-PAGE gel and electrophorese at 120 V.
2. Analyze gels for TAMRA fluorescence (542/568 nm excitation/emission).
3. Stain gels using Coomassie Blue staining solution for 30 minutes, followed by incubation with destaining solution for 2 to 3 hours. Scan gels using white light transillumination to image total proteins in the gel.
4. Quantify isoprenoid analogue labeling of proteins using ImageJ, 32-bit type images with brightness/contrast adjusted accordingly; in some cases, such as when C15AlkOH is used, it is necessary to increase the brightness/contrast to allow fainter bands to be visualized. For each lane, the area of interest is selected using a rectangular selection tool. In Fig. 2, a rectangle including the 25 kDa region (where most small GTPases appear) was used. To normalize the fluorescence intensity to the total protein in each lane, the intensity from the fluorescence image is divided by the intensity of the same region of the Coomassie Blue-stained image (lower panels). For each gel, the normalized data is transformed to numerical values of 0–1, by dividing all data by the maximum value in each set.

Data from different gels have variable intensities due to brightness and contrast settings to best visualize the labeled protein bands. The graphical representations of the data are plotted using the graphing program Origin® 2016 version 93E (Fig. 3).

4. Notes

1. Lovastatin is toxic at some concentrations to sensitive cell lines, *e.g.* COS-7. Hence it is necessary to optimize the concentration used. No significant toxicity was observed at 25 μ M lovastatin on HeLa cells.
2. The volume of the lysis buffer can be adjusted depending on the amount of cells collected. For example, lower amounts of cells were recovered when using COS-7 cells where lovastatin toxicity was observed. Hence, a lower volume of the lysis buffer was used (200 μ L). The target protein concentration should be between 1.5 to 2 mg/mL to be consistent with the protocol suggested for the click reaction.
3. Protein pellets should not be allowed to overdry as this results in difficulty in redissolving them in 1 $\times$ Laemmli loading buffer. To avoid this difficulty, after decanting the wash buffer, any remaining wash buffer is aspirated using suction

through a glass pipette. The protein pellet should then immediately be dissolved in loading buffer.

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Figure 1. Isoprenoid phosphate analogues functionalized with alkyne moieties for metabolic labeling of prenylated proteins.

Figure 2. In-gel fluorescence analysis on HeLa cells treated with varying lovastatin concentrations and metabolically labeled with isoprenoid phosphate analogues C15AlkOH (A), C15AlkOP (B), C15AlkOPP (C), and the comparison of probe labeling at 10 μM with lovastatin at 10 μM . The lovastatin is either removed (*e.g.* 10R) or retained in the media. Quantification through densitometry is performed on the bands in the 25 kDa region (indicated by the dashed rectangle) where many different prenylated small GTPases migrate.

Figure 3.

Densitometric analysis on the fluorescence intensities of the labeled proteins in the presence of varying lovastatin concentrations from HeLa cells metabolically labeled with isoprenoid analogues C15AlkOH (A), C15AlkOP (B), C15AlkOPP (C), and the comparison of probe labeling at 10 μ M with lovastatin at 10 μ M (D). Fluorescence intensities are evaluated using the 25 kDa region where many prenylated proteins reside. The optimal concentration of lovastatin for treatment is 10 μ M.