

Monitoring Wnt Protein Acylation Using an In Vitro Cyclo-Addition Reaction

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Abstract

We describe here a technique for visualizing the lipidation status of Wnt proteins using azide-alkyne cycloaddition chemistry (click chemistry) and SDS-PAGE. This protocol incorporates in vivo labeling of a Wnt-IgG Fc fusion protein using an alkynylated palmitate probe but departs from a traditional approach by incorporating a secondary cycloaddition reaction performed on single-step purified Wnt protein immobilized on protein A resin. This approach mitigates experimental noise by decreasing the contribution of labeling from other palmitoylated proteins and by providing a robust method for normalizing labeling efficiency based on protein abundance.

Key words Wnt acylation, Click chemistry, Biotinylation, Palmitoleate, IgG fusion protein

1 Introduction

The advent of chemical probes that mimic endogenous metabolites has enabled the systematic quantification of biochemical events that interlink cellular metabolic and signaling machinery [1]. This effort has so far yielded a plethora of novel therapeutic intervention entry points. Whereas many of these posttranslational modifications can be readily inferred from mass spectrometric analysis of proteins isolated using the covalently attached probe as a handle, efforts focused on specific protein targets can be challenging due to limitations imposed by their abundance and their amenability to labeling [2]. In particular, secreted fatty acylated proteins such as the Wnt and Hedgehog proteins represent atypical subjects for this technique given their localization to the outer plasma membrane and the necessity of the fatty acyl donor (and thus the fatty acid probe) to traverse the plasma membrane twice prior to immobilization onto the substrate—once to be affixed to a CoA moiety and again to access the extracellular polypeptide likely by exiting a channel created by the Porcn acyltransferase [3].

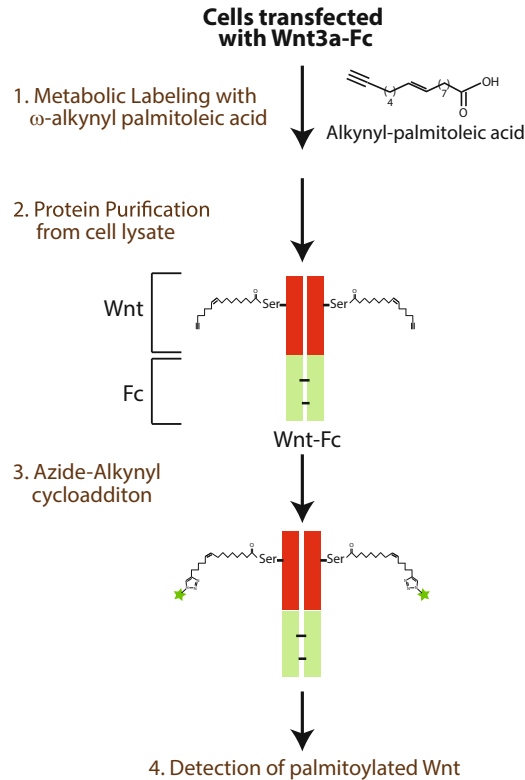


Fig. 1 Overview of modified Wnt labeling protocol using an alkynylated fatty acyl probe and copper-catalyzed alkyne-azide cycloaddition

To increase the efficiency of using fatty acid-like probes for labeling Wnt proteins, we have leveraged the amenability of secreted proteins to single-step purification using the IgG Fc fusion-protein A system and a modified *in vitro* secondary cycloaddition reaction for incorporating either a fluorescent or a biotin conjugate (Fig. 1). These fusion proteins serve as faithful substrates of the native Wnt production machinery given that (a) lipidation of Wnt-Fc fusion proteins *in vivo* is sensitive to inhibitors of the Wnt acyltransferase Porcupine [4], and (b) their lipidation status correlates with their ability to interact with the Wnt chaperone Wntless (WLS) [5]. This technique enables the *in vivo* evaluation of synthetic small molecules on Wnt lipidation, for example, and serves as a template for achieving similar experimental robustness for the study of other acylated secreted proteins.

2 Materials

2.1 Cell Labeling Components

1. Tissue cultureware: 60 mm² and 10 cm² tissue culture plates.
2. HEK293 cells.

3. DMEM culture medium: DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin.
4. Serum-free DMEM: DMEM with no additives.
5. Cell trypsinization solution (0.25%).
6. Liposomal transfection reagent (i.e., Effectene, Qiagen).
7. Alkynyl palmitoleic acid: Prepare 50 mM stock solution in DMSO.

2.2 Protein Purification Reagents

1. Lysis buffer: 1% NP40 in PBS with protease inhibitors.
2. Protein A sepharose: Wash with 1% NP40/PBS, store as a slurry (1 volume beads: volume 1% NP40) at 4 °C until required.

2.3 Cycloaddition Reaction Reagents

1. Azide-biotin: Dissolve in DMSO to prepare 5 mM stock solution.
2. TCEP (Tris-2-carboxyethyl phosphine hydrochloride): Prepare a fresh 50 mM stock solution by dissolving in H₂O.
3. TBTA (tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine): Dissolve in DMSO:t-butanol (1:4) to prepare 1.7 mM stock solution.
4. CuSO₄: Dissolve in H₂O to prepare 50 mM stock solution.

2.4 SDS-PAGE and Immunoblotting Components

1. 7.5% Acrylamide/bis-acrylamide gels: This can either be purchased from any number of vendors (Criterion Gels, Biorad) or prepared as described in [6].
2. SDS-PAGE running buffer: 0.025 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% SDS.
3. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, and 20% methanol.
4. Nitrocellulose membrane, 0.45 μm.
5. Diluent solution: PBS containing 0.05% Tween-20 (PBST).
6. Blocking solution: 5% Nonfat powdered milk dissolved in PBST.
7. Streptavidin cross-linked to infrared dye (Streptavidin IRDye).
8. Gel-imaging system or radiographic film.

3 Methods

3.1 Labeling Wnt Protein

1. Cell trypsinization: Wash 75% confluent HEK293 cells cultured in a 10 cm² culture dish with PBS and trypsinize the cells by adding 2 ml of trypsinization solution. Let cells incubate at 37 °C for 2 min. Neutralize the trypsin with DMEM culture medium and spin cells at 200 xg for 5 min. Resuspend the cells with DMEM culture medium and seed 1 x 10⁶ cells in each 60 mm² culture dish.

2. Transiently transfect 60 mm plates of HEK293 cells with Wnt3a-Fc DNA before cells become adherent using a liposomal transfection reagent. Wait for 24 h before proceeding to the click chemistry reaction.
3. Dilute alkynyl palmitoleic acid in serum-free DMEM to the final concentration of 100 μM (*see Note 1*).
4. Sonicate the alkynyl palmitoleic acid containing DMEM for 15 min at room temperature.
5. Incubate the medium for 15 min at room temperature. This promotes formation of pre-complexes between alkynyl palmitoleic acid and medium.
6. Aspirate off the medium from cultured HEK293 cells.
7. Wash with PBS.
8. Add alkynyl palmitoleic acid containing medium to the cells.
9. Incubate for 6 h at 37 °C/5 % CO₂ (*see Note 2*).

3.2 Purifying Wnt Protein

1. Aspirate the alkynyl palmitoleic acid-containing media from the cells and wash with PBS. Repeat two times.
2. Lyse the cells using 500 μl of 1 % NP40 lysis buffer on ice.
3. Centrifuge at 16,000 $\times g$ for 10 min at 4 °C. Transfer the cell lysate to an eppendorf tube without disturbing the cell pellet.
4. Add 50 μl of protein A sepharose bead slurry to the cell lysate (*see Note 3*).
5. Rotate the samples minimally for 2 h at 4 °C. The Wnt-Fc protein in the cell lysis conditions described here appears to be stable with overnight incubations at 4 °C.
6. Centrifuge at 1000 $\times g$ for 5 min. Discard the supernatant.
7. Wash the beads by adding 1 % NP40 and rotating for 10 min at 4 °C.
8. Centrifuge at 1000 $\times g$ for 5 min. Discard the supernatant.
9. Repeat three times.

3.3 Azide Attack

1. Dilute azide-biotin in 100 μl of 1 \times PBS to the final concentration of 50 μM . A total volume of 90 μl of the azide-biotin-containing reaction buffer is added for each click chemistry reaction (*see Note 4*).
2. Add the azide-biotin mixture to Wnt3a-Fc protein immobilized on protein-A beads from Subheading 3.2 and mix gently.
3. Add 2 μl of TCEP (*see Note 5*).
4. Add 6 μl of TBTA solution and mix gently.
5. Add 2 μl of CuSO₄ solution. Mix gently.
6. Rotate the sample for 1 h at room temperature protected from light.

7. Centrifuge at $1000 \times g$ for 5 min and aspirate the solution. Wash with cold lysis buffer. Repeat three times.
8. Add 60 μ l sample-loading buffer to the protein-A bead slurry and heat at 95 °C for 2 min.
9. Centrifuge at $1000 \times g$ again for 2 min to let the beads settle at the bottom and run the supernatant on SDS-PAGE.

3.4 SDS-PAGE and Immunoblotting

1. Prepare 7.5% acrylamide/bis-acrylamide gel by rinsing wells with running buffer and securing in gel-running chamber. Add running buffer to top and bottom chambers.
2. Load 30 μ l of protein sample with gel-loading tips.
3. Run the gel for approximately 45 min at 250 mA.
4. Immediately following SDS-PAGE, transfer the gel to a nitrocellulose membrane for 1.5 h.
5. After the transfer is completed, cut excess membrane to smoothen edges.
6. Block the membrane with 5% milk solution for an hour at room temperature on a platform shaker.
7. Wash the membrane with PBST for 5 min. Repeat two times.
8. Incubate with Streptavidin IRDye for an hour (1:1000 dilution) at room temperature on a platform shaker (*see Note 6*).
9. Wash with PBST for 10 min. Repeat three times.

3.5 Detection

Scan the membrane with an Odyssey Fc imaging system using the 800 nm detection channel (*see Note 7*).

4 Notes

1. Instead of using alkynyl palmitoleic acid, you can label Wnt3a-Fc with azide palmitoleic acid.
2. Incubation of alkynyl palmitoleic acid for more than 6 h might be toxic to cells.
3. Protein A sepharose is supplied as a slurry that contains ethanol and/or sodium azide for preservation. It is important to wash the protein A sepharose with 1% NP40 several times before using for Wnt3a-Fc purification.
4. If azide palmitoleic acid is used for labeling Wnt3a-Fc (instead of alkynyl palmitoleic acid), you can use various alkyne-containing reagents for detection purposes.
5. A commercially available cycloaddition reaction kit can also be substituted starting from this point (for example, Click-iT Cell Protein Reaction Buffer Kit, Invitrogen).

6. If you prefer using chemiluminescent Western blotting detection system instead of infrared system, you can replace the Streptavidin IRDye with HRP-Streptavidin and use an ECL Western blotting substrate to detect the acylated Wnt3a-Fc proteins.
7. If the Streptavidin IRDye was substituted with a horseradish peroxidase (HRP)-coupled streptavidin protein, then expose the nitrocellulose film to chemiluminescence-compatible film.

Acknowledgement

This work was supported by the NIH (R01 CA168761, R01CA196851), CPRIT (RP130212), and the Welch Foundation (I-1665).

References

1. Speers AE, Adam GC, Cravatt BF (2003) Activity-based protein profiling in vivo using a copper(i)-catalyzed azide-alkyne [3+2] cycloaddition. *J Am Chem Soc* 125: 4686–4687
2. Peng T, Thinon E, Hang HC (2016) Proteomic analysis of fatty-acylated proteins. *Curr Opin Chem Biol* 30:77–86
3. Tuladhar R, Lum L (2015) Fatty acyl donor selectivity in membrane bound O-acyltransferases and communal cell fate decision-making. *Biochem Soc Trans* 43: 235–239
4. Dodge ME, Moon J, Tuladhar R, Lu J, Jacob LS, Zhang LS, Shi H, Wang X, Moro E, Mongera A, Argenton F, Karner CM, Carroll TJ, Chen C, Amatruda JF, Lum L (2012) Diverse chemical scaffolds support direct inhibition of the membrane-bound O-acyltransferase porcupine. *J Biol Chem* 287:23246–23254
5. Herr P, Basler K (2012) Porcupine-mediated lipidation is required for Wnt recognition by Wls. *Dev Biol* 361:392–402
6. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685