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Protein Lipidation Protocols

Edited by

Michael H. Gelb

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Preface

It is hard to think of a protein in eukaryotic cells that does not undergo some type of posttranslational modification. The covalent attachment of lipids to proteins, protein lipidation, occurs for a few thousand proteins. Several functions for protein lipidation are known. Protein lipids may target proteins to specific cellular membranes, they may serve as molecular switches that allow cytosol-to-membrane transfer, they may direct protein–protein complexation, and they may stabilize protein structure. In cases such as the fatty acylation of intracellular loops of transmembrane proteins, the functions of the protein lipidations are not well understood.

This volume—*Protein Lipidation Protocols*—provides detailed methodologies for the study of these processes. Since this is a rapidly growing field, many new experimental techniques have been developing over the past few years. All the experimental techniques described in this volume have emerged during this time. The editor has made a special effort to include only those techniques not previously described in a "hands-on" format.

Three areas of protein lipidation are included. The first section deals with glycosyl phosphatidylinositol (GPI)-containing proteins. Protein prenylation is covered next, followed by protein fatty acylation. Very recently, mammalian cell mutants defective in GPI biosynthesis have been obtained, and such cell lines will undoubtedly lead to a more complete understanding of the role of GPI groups in protein function. This work is described in the first two chapters. There is accumulating evidence that GPI groups target proteins to specific sites on cellular membranes such as caveolae, and new methods for visualizing the location of GPI-anchored proteins on cell membranes are described in the next four chapters. Finally, Chapters 7 and 8 describe the very recent addition of techniques for determining the chemical structure of GPI-anchors.

The middle portion of this volume is concerned with protein prenylation, the attachment of 15-carbon farnesyl and 2-carbon geranylgeranyl groups to proteins. Interest in protein prenylation has escalated in the last five years because of its medicinal impact. The cancer-causing protein Ras is farnesylated, and this lipid is required for the ability of Ras to switch cells into a proliferating mode. Approximately 30% of human tumor cell lines contain activated Ras proteins, and there is a massive, worldwide effort to develop inhibitors of

the protein farnesyltransferase that attaches farnesyl groups to proteins, including Ras. Chapters 9 and 10 describe a novel technique for radiolabeling the prenyl groups of proteins in eukaryotic cells. Chapter 11 describes a recent development in the manipulation of yeast protein farnesyltransferase. Chapter 12 describes a whole-cell system for studying the functions of protein prenyl groups. Finally, Chapter 13 describes ways to quantify the binding of prenylated peptides to membranes.

The last section of the book is focused on protein fatty acylation. Chapter 14 describes the assay and purification of the mammalian enzyme that attaches myristoyl groups to the N-terminus of specific proteins. Although the yeast protein myristoyltranfserase has been purified, the mammalian homolog has been difficult to obtain. The volume ends with a chapter on an exciting lysosomal enzyme that cleaves fatty acyl groups from proteins. This enzyme is the first such protein fatty acylase to have been discovered.

All the chapters of *Protein Lipidation Protocols* contain a brief introduction, followed by detailed methodological descriptions, notes on optimizing the protocols for use in other systems, and some concluding remarks. Each chapter can be read on its own.

The editor is particularly grateful for the cooperation of all the chapter authors. The Series Editor, John Walker, and the production team at Humana Press, headed by Tom Lanigan, have provided invaluable assistance toward the publication of this important volume. The editor is grateful to Ms. Kathlene Bennett for excellent assistance in the process of organizing this volume.

Michael H. Gelb

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Contents

1

In Vitro Analysis of GPI Biosynthesis in Mammalian Cells

Victoria L. Stevens

1. Introduction

1.1. Background

The basic strategy used in most assays of activities involved in the biosynthesis of glycosylphosphatidylinositol (GPI) in mammalian cells is the same as is employed for other lipid biosynthetic pathways. That is, radioactivity is transferred from a water-soluble substrate into a lipophilic product. After the reaction is complete, the differential solubility of the substrate and product(s) is exploited to separate these radiolabeled compounds. In GPI biosynthesis, at least one of the substrates in each step and all of the enzymes in the pathway are membrane-associated and localized to the endoplasmic reticulum. Therefore, multiple GPI biosynthetic activities, as well as some of the substrates for later steps in the pathway, are present in the cellular preparations used in the assays. For this reason, multiple intermediates in GPI biosynthesis are usually generated in a single reaction. Although it is possible to optimize the assay conditions for one step, it is usually impossible to study one reaction independently with this type of cell-free system.

Assays for individual reactions in GPI biosynthesis are possible if synthetic GPI intermediates are available. To date, the second and third reactions in the pathway have been measured in mammalian cells with exogenously supplied GlcNAc-PI *(1)* and GlcN-PI *(2)*, respectively. In the latter case, a short-chain (dioctanoyl) analog of the GPI intermediate was used. Because the short-chain analogs are more water-soluble, they are much easier to deliver to the membranes used as a source of the GPI biosynthetic activities.

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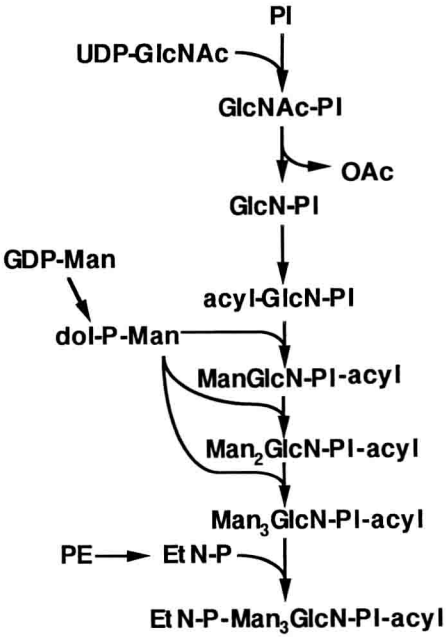

Fig. 1. GPI biosynthesis in mammals and yeast.

1.1.1. Pathway for GPI Biosynthesis in Mammalian Cells

The biosynthesis of GPI proceeds by the sequential addition of carbohydrates to phosphatidylinositol (PI), as is shown in **Fig. 1** (reviewed in **refs. *3*** and ***4***) . In the first step, N-acetylglucosamine (GlcNAc) is transferred from UDP-GlcNAc to PI ***(5,6)***. The resulting product, GlcNAc-PI, is then deacetylated to glucosamine-PI (GlcN-PI) in the second step. Next, in a reaction that only occurs at this step in mammals and yeast, an acyl chain is added to the inositol ring to form GlcN-PI(acyl) ***(7,8)***. Mannoses are then sequentially transferred to the growing GPI core. The endogenous source of all three mannoses is dolichol-phospho-mannose ***(9)***, which is made from GDP-mannose and dolichol-phosphate. Finally, a phosphoethanolamine residue is transferred from phosphatidylethanolamine to the third mannose to complete the GPI core ***(10,11)***. Analysis of the structures of GPI precursors from normal and Thy-1-deficient murine lymphoma cell lines suggests that there may be one or two extra phosphoethanolamines added to the first and second mannoses before addition of the final phosphoethanolamine ***(12–16)***. However, the exact sequence of steps leading to these precursors, and whether they are really intermediates in the synthesis of the GPI anchor in all cases, is not known.

All the intermediates in GPI biosynthesis should be detected if UDP-[6-^{3}H]GlcNAc is used in the assay. However, it is really only practical to use this radiolabeled sugar nucleotide to assay the first three steps in the pathway. Conditions to measure at least the first two mannose addition reactions using GDP-[2-^{3}H]mannose have been described ***(17)***. To date, no in vitro assays for the addition of the third mannose, the terminal phosphoethanolamine, or the extra phosphoethanolamines which extend from the GPI core, have been developed.

1.2. Sources of GPI Biosynthetic Enzymes

The enzymatic activities necessary for GPI biosynthesis are localized to the endoplasmic reticulum ***(18)***. Therefore, cell lysates, permeabilized cells, microsomal preparations, and isolated endoplasmic reticulum all will contain these enzymes and can be used for the assays described here. All of these preparations also contain phosphatidylinositol in sufficient quantities so that detection of the initial GPI intermediates upon labeling with UDP-[6-^{3}H]GlcNAc should be possible. However, the levels of later intermediates in the pathway are much lower in any of these membranes, which may explain why detection of intermediates with GDP-[2-^{3}H]mannose is so difficult.

2. Materials

2.1. Cell Lysis

1. Phosphate buffered saline (PBS).
2. Lysis buffer: 10 m*M* HEPES, pH 7.5, 1 µg/mL leupeptin, 0.1 m*M* N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK). Add fresh protease inhibitors to cold lysis buffer.
3. Bath sonicator.

2.2. Permeabilization of Cells

1. Streptolysin O (Gibco BRL).
2. Dithiothreitol (DTT): supplied in the 10X activating solution from Gibco BRL.
3. PBS (Ca^{2+}- and Mg^{2+}-free).
4. Lysis buffer: 10 m*M* HEPES, pH 7.5, 1 µg/mL leupeptin, 0.1 m*M* N^{α}-TLCK.

2.3. Cellular Fractionation

1. PBS.
2. Fractionation buffer: 0.25 *M* sucrose, 0.5 m*M* DTT, 0.1 m*M* TLCK, 1 µg/mL leupeptin.
3. Cell disruption bomb, nitrogen gas.
4. High-speed centrifuge.
5. Ultracentrifuge.
6. Microsome buffer: 10 m*M* HEPES, pH 7.5, 0.5 m*M* DTT, 0.1 m*M* TLCK, 1 µg/mL leupeptin.

7. Sucrose solutions of 38, 30, and 20% sucrose in 10 m*M* HEPES, pH 7.5, 1 m*M* DTT.
8. Glycerol.
9. Swing bucket rotor.

2.4. Labeling with UDP-[6-^{3}H]GlcNAc

1. Incubation buffer: 60 m*M* HEPES, pH 7.5, 30 m*M* $MgCl_2$, 3 m*M* DTT, 0.6 μg/mL leupeptin, 1.2 μ*M* tunicamycin.
2. 50 m*M* ATP.
3. 50 m*M* GTP.
4. UDP-[6-^{3}H]GlcNAc (5–15 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO).
5. 50 m*M* dimercaptopropanol.
6. 50 m*M* EDTA.
7. Water bath at 37°C.
8. 13 × 100 glass screw top tubes with teflon-coated caps.
9. Chloroform–methanol–0.1 *M* HCl, 1:2:0.5 (v/v).

2.5. Labeling with GDP-[2-^{3}H]mannose

1. Incubation buffer (-tunicamycin): 60 m*M* HEPES, pH 7.5, 30 m*M* $MgCl_2$, 3 m*M* DTT, 0.6 μg/mL leupeptin.
2. 50 m*M* ATP.
3. 50 m*M* GTP.
4. UDP-GlcNAc.
5. GDP-[1-^{3}H]mannose (5–15 Ci/mmol, American Radiolabeled Chemicals).
6. 50 m*M* dimercaptopropanol.
7. 50 m*M* EDTA.
7. Water bath at 37°C.
8. 13 × 100 glass screw-top tubes with teflon-coated caps.
9. Chloroform–methanol, 1:1 (v/v).

2.6. Synthesis and Purification of [6-^{3}H]GlcNAc-PI

1. Chloroform–methanol–H_2O, 2:3:1 (v/v).
2. UDP-[6-^{3}H]GlcNAc.
3. 50 m*M* ammonium acetate.
4. Pasteur pipet.
5. Glass wool.
6. DEAE cellulose pre-equilibrated with chloroform–methanol–H_2O, 2:3:1 (v/v).
7. Chloroform–methanol–50 m*M* ammonium acetate, 2:3:1 (v/v).
8. Speed-Vac concentrator.
9. Scintillation vials.
10. Ethanol.

2.7. Extraction of [6-^{3}H]GlcNAc-Labeled Products

1. Chloroform.
2. H_2O.

3. Tabletop centrifuge.
4. Pre-equilibrated acidic upper phase: Prepare by mixing chloroform–methanol–0.1 *M* HCl, 2:2:1.5 (v/v), in a separatory funnel. Let layers separate completely. Collect upper phase.
5. Speed-Vac concentrator.

2.8. Extraction of [1-^{3}H]-Mannose-Labeled Products

1. Tabletop centrifuge.
2. Chloroform–methanol–H_2O, 1:1:0.3 (v/v).
3. Speed-Vac concentrator.
4. H_2O-saturated butanol.
5. H_2O.

2.9. Thin Layer Chromatography (TLC) of Products

1. TLC tank.
2. Silica gel 60 (20 × 20 cm) TLC plates (E. Merck, VWR Scientific, Atlanta, GA).
3. Chloroform–methanol–1 *M* ammonium hydroxide, 10:10:3 (v/v).
4. Imaging scanner capable of detecting ^{3}H or En^{3}Hance spray (NEN/Dupont) and Kodak XAR-5 film.

3. Methods

These methods have been developed for use with cultured cells. In some cases, the procedure may have to be modified slightly to optimize conditions for different types of cells or tissues.

3.1. Preparation of Membranes for Analysis of GPI Biosynthesis

Each of these methods will yield preparations that can be used in each of the assays described in **Subheading 3.2.**

3.1.1. Cell Lysates

1. Wash cells with PBS by centrifugation (5 min at 800*g*).
2. Resuspend the cells in lysis buffer at a density of approximately 1.2×10^8 cells/mL.
3. Disrupt cells by three cycles of sonic irradiation (10 s each).

3.1.2. Permeabilized Cells

1. Solubilize the streptolysin O by adding distilled water to generate a stock solution of 1000 U/mL.
2. Activate as much of the stock solution as needed by incubating the streptolysin O with 2 m*M* DTT for 15 min at 37°C. If using Streptolysin O obtained from Gibco-BRL, this activation is accomplished by adding one part of the 10X activating solution per nine parts of the streptolysin O stock solution.
3. Wash cells twice with PBS by centrifugation (5 min at 800*g*).
4. Resuspend cells in cold streptolysin O solution at a density of 50–100 U/10^7 cells. Incubate on ice for 20 min to allow the toxin to insert into the membrane.

5. Pellet the cells by centrifugation (5 min at 800*g* at 4°C). Wash cells once with cold PBS.
6. Resuspend in lysis buffer at a concentration of approx 10^8 cells/mL.

3.1.3. Microsomes

1. Wash cells twice with PBS by centrifugation (5 min at 800*g*).
2. Resuspend the cells in fractionation buffer at a density of 0.5–1 × 10^8 cells/mL.
3. Lyse the cells by nitrogen cavitation using 450 psi for 15–30 min.
4. Centrifuge at 10,000*g* for 5 min to remove unbroken cells and nuclei.
5. Centrifuge the resulting supernatant (18,000*g*, 15 min) to remove mitochondria.
6. Centrifuge the supernatant at 100,000*g* for 1 h, to pellet the microsomes.
7. Resuspend this pellet in microsome buffer. Recentrifuge at 100,000*g* for 1 h, to wash the microsomes.
8. Resuspend the final microsomal pellet microsome buffer containing 10% glycerol at a protein concentration of approx 70 mg/mL.

3.1.4. Endoplasmic Reticulum

1. Wash cells twice with PBS by centrifugation (5 min at 800*g*).
2. Resuspend the cells in fractionation buffer at a density of 0.5–1 × 10^8 cells/mL.
3. Lyse the cells by nitrogen cavitation using 450 psi for 15–30 min.
4. Centrifuge at 10,000*g* for 15 min at 4°C, to pellet unbroken cells and nuclei.
5. Layer the 4.06 mL of the resulting postnuclear supernatant onto a preformed sucrose gradient consisting of 2.52 mL 38% sucrose, 1.26 mL 30% sucrose, and 1.26 mL 20% sucrose.
6. Centrifuge this gradient 2 h at 28,000*g* in a Sorvall TH-641 rotor.
7. Collect four fractions of 1.96 (1), 2.1 (2), 2.38 (3), and 2.66 (4) mL from the top of the tube. Resuspend the pellet in 1 mL of microsome buffer to make fraction 5. The endoplasmic reticulum will be enriched in fractions 4 and 5.

3.2. In Vitro Biosynthesis of GPI Intermediates from Radiolabeled Precursors

At least the first five steps in GPI biosynthesis can be assayed in vitro with various membrane preparations. The choice of radiolabeled precursor for the assay will depend on which reaction or reactions the investigator wants to measure.

3.2.1. UDP-N-Acetylglucosamine

1. Mix incubation components in a 13 × 100 glass screw-top tube in a total volume of 300 µL. Components should include 50 to 100 µL of the appropriate membrane preparation (about 300 µg protein, measured using the bicinchoninic acid assay of Smith *[19]*), 50 µL incubation buffer, and 1 m*M* ATP. The following effectors should be added to optimize synthesis of various intermediates: for GlcNAc-PI, no additions; for GlcN-PI, 0.1–1 m*M* GTP; for GlcN-PI(acyl),