Post-translational Modification of Proteins by Lipids

A Laboratory Manual

Edited by
Urs Brodbeck · Clément Bordier

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With 17 Figures

Springer-Verlag
Berlin Heidelberg New York
London Paris Tokyo

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ISBN 3-540-50215-7 Springer-Verlag Berlin Heidelberg New York
ISBN 0-387-50215-7 Springer-Verlag New York Berlin Heidelberg

Library of Congress Cataloging-in-Publication Data. Post-translational modification of proteins by lipids : a laboratory manuals / edited by Urs Brodbeck, Clement Bordier. p. cm. Includes index. 1. Proteins–Post-translational modification–Laboratory manuals. 2. Lipids–Physiological effect–Laboratory manuals. I. Brodbeck, U. (Urs), 1938-. II. Bordier, Clement. [DNLM: 1. Lipids–laboratory manuals. 2. Membrane Proteins–biosynthesis–laboratory manuals. 3. Translation, Genetic–laboratory manuals. QU 25 P857] QH450.6.P67 1988 574.19'245–dc 19 DNLM/DLC 88-29440

Printed in Germany

Printing and binding: Druckhaus Beltz, Hemsbach/Bergstr.
2131/3130-543210 – Printed on acid-free paper

Preface

The growing interest in recent years in the anchoring to membranes of proteins by post-translational modification is documented by the large number of publications which appeared in this field. In September 1987, scientists from 10 countries from all over the world met in the resort village of Les Diablerets, Switzerland, to discuss the most recent advances made in this field. The sessions were devoted to the anchoring of membrane proteins by covalent attachment of fatty acids and of glycophospholipids. The workshop brought together many scientists working on vastly different proteins such as the variant surface glycoprotein of Trypanosomes and antigens of the mammalian cells. The subject of the workshop unified many scientists who had not met before and thus greatly stimulated interdisciplinary work.

In addition to the lectures, each participant was provided with a collection of **Methods** currently in use in the study of membrane proteins anchored by post-translational modification. An updated version of this collection is now presented as a **Laboratory Manual**, and the techniques described therein will give researchers easy and practical access to the investigation of post-translationally modified proteins. The publication of the present book by **Springer** follows an established tradition of previously published manuals on the handling of membrane proteins.

Our thanks go to the authors who made the essential contribution in writing and adapting the experimental protocols, to Mrs. R. Schneider for her diligence and effort in preparing the camera-ready version of this book, to Dr. S. Stieger for her careful proofreading, and to Mrs. B. Brodbeck for help in creating the titlepage.

Bern/Lausanne, July 1988

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You will find the addresses at the beginning of the respective contribution.

Identification of Glycosyl-Phosphatidylinositol Membrane Anchors by Fatty Acid Labeling

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INTRODUCTION

Glycosyl-phosphatidylinositol (G-PI) membrane anchors are employed by a number of eukaryotic plasma membrane glycoproteins. Examples include protozoal antigens e.g., the variant surface glycoprotein (VSG) of *Trypanosoma brucei*; enzymes e.g., alkaline phosphatase and acetylcholinesterase; T-cell components e.g., Thy-1 and T-cell activating protein; complement regulating proteins e.g., decay accelerating factor; and cell-adhesion molecules e.g., N-CAM_{120}. The occurance and biochemistry of G-PI anchored proteins has been reviewed in detail (Low et al. 1986; Cross 1987; Low 1987).

The physiological significance of using a G-PI anchor in place of a conventional transmembrane polypeptide domain is still unclear, however, possible advantages are:

a) a higher degree of lateral mobility in the membrane bilayer (Woda and Gilman 1983; Ishihara et al. 1987),
b) conservation of space in the membrane, and
c) the potential to release proteins from membranes by stimulating endogenous G-PI-specific phospholipase C (Bulow and Overath 1986; Fox et al. 1986; Hereld et al. 1986).

The best characterized G-PI anchor is that of *T. brucei* VSG (Ferguson et al. 1985a; Ferguson et al. 1985b). The complete structure of this G-PI anchor has recently been completed (Ferguson et al. 1988) and is shown schematically in Figure 1.

IDENTIFICATION OF G-PI ANCHORS

PI-PLC release

Many G-PI anchors have been identified by the use of bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) from either *Staphylococcus aureus* or *Bacillus thuringiensis* (Low

1987). In this case, the PI-PLC cleaves the diacyl-glycerol moiety from the anchor and thereby liberates the attached protein from the membrane. The release may be monitored by immuno-precipitation of the supernatant or by FACS analysis of cells before and after PI-PLC treatment using an antibody directed against the candidate protein (Low and Kincade 1985; Davitz et al. 1986; Koch et al. 1986).

This approach is limited by the need for a probe or assay for the candidate protein and the fact that some G-PI anchors are PI-PLC resistant (Low 1987).

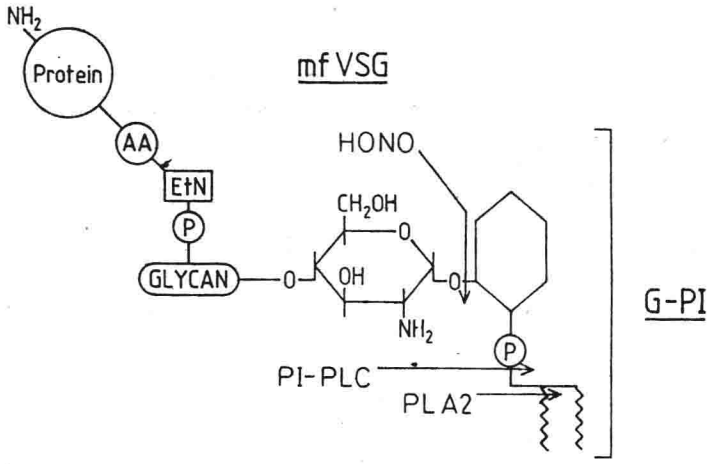

Fig. 1

CRD blotting

Rabbit antisera raised against soluble form VSGs (sVSG) which lacks the diacyl-glycerol moiety of the G-PI anchor, contain a population of antibodies directed against an epitope in the carbohydrate of the G-PI. This fraction of antibodies will cross-react with all VSGs exclusively in this cross-racting determinant or CRD (Holder 1985). Anti-CRD antibody has been used to detect the CRD epitope in the soluble forms of Leishmania GP-63 protease, *Torpedo* and human erythrocyte acetylcholinesterase, and human DAF and alkaline phosphatase (Bordier et al. 1986; Stieger et al. 1986; Davitz et al. 1987).

This approach is limited by the need to generate the soluble form of the protein by PI-PLC action. The CRD epitope is cryptic in the intact membrane form of the proteins

Biosynthetic labeling

Labeling with G-PI-specific precursors such as [^{3}H]ethanolamine and [^{3}H]*myo*-inositol strongly suggest the presence of a G-PI anchor (reviewed in Low 1987). Fatty acid labeling is initially more ambiguous as other types of protein acylation are common. However, subsequent enzymatic and chemical treatments of [^{3}H]fatty acid labeled proteins will quickly provide quite detailed information on the nature of the fatty acid linkage and will give unambiguous information on the presence or absence of a G-PI anchor.

These experiments assume that a protein of interest has been labeled with [^{3}H]fatty acid (Ferguson and Cross 1984) e.g., [^{3}H]myristate or [^{3}H]palmitate, and purified by RP-HPLC (Clarke et al. 1985), preparative SDS-PAGE, and electroelution (Haldar et al. 1985), or other means such as affinity chromatography. The examples given are for [^{3}H]myristic acid labeled membrane form VSG (mfVSG), but any [^{3}H]fatty acid labeled protein can be used.

EXPERIMENTAL PROCEDURES

Base hydrolysis

The first question to ask is whether or not the fatty acid is base-stable (and, therefore, probably amide linked to the N-terminus of the protein) or base-labile, in which case ester linkages are involved (Ferguson and Cross 1984).

Suspend 10,000 cpm aliquots of [^{3}H]mfVSG in 200 μl 90% ethanol (control) and 200 μl 90% ethanol containing 50 mM NaOH. After 40 min at room temperature, add 800 μl of 10% (v/v) acetic acid to acidify the mixture and vortex with 1 ml of toluene. Centrifuge to separate the phases and count 0.4 ml of the upper toluene phase in 10 ml of scintillation fluid. Record the % of [^{3}H]fatty acid released by base hydrolysis:

$$\% \text{ released} = \frac{(+\text{NaOH counts}) - (-\text{NaOH counts})}{10{,}000 - (-\text{NaOH counts})} \times 2.5 \times 100$$

To check the nature of the released product (i.e. free-fatty acid), dry a further 0.5 ml of the toluene phase, redissolve in about 10 μl of "Standard A1" and apply to a TLC-plate (System A).

If all or most of the [^{3}H]fatty acid is released under these conditions as free fatty acid, then an ester linkage can be assumed. This could mean either ester linkage to serine/threonine, thioester linkage to cysteine, or ester linkage to glycerol in a G-PI anchor. The next ex-

periments are specific for G-PI anchor identification.

Acetolysis

Acetolysis may remove [^{3}H]fatty acid from other acylated proteins as free fatty acid, but acetolysis of G-PI anchors will produce a diacylglycerol acetate which can be identified by TLC.

a) Dry 10,000 cpm of [^{3}H]mfVSG in a reaction vial,
b) add 200 μl acetic acid - acetic anhydride (3:2) and place in a heating block at 105^{o}C for 3 h.
c) Dry the reaction mixture under a stream of N_2, add 160 μl $CHCl_3$, 80 μl MeOH, and 60 μl H_2O and vortex.
d) Recover the lower chloroform rich phase with a pasteur pipette (with care!) and transfer to a preweighed (weight A) 1 ml screw cap vial (use a 4 or 5 place balance). Re-extract the upper aqueous phase with 200 μl of pre-equilibrated lower phase. Transfer the lower phase to the same 1 ml vial. Weigh the vial plus pooled lower phases (weight B), then transfer a 40 μl sample to a scintillation vial. Allow the chloroform to evaporate, then add scintillation fluid and count. Weigh the vial again (weight C). Calculate the % of [^{3}H]-fatty acid released from the protein as follows:

$$\% \text{ released} = \frac{(B - A) \times \text{cpm counted}}{(B - C) \times 10{,}000} \times 100$$

Take the remainder of the sample in the 1 ml vial and dry it under a stream of N_2, redissolve it in 10 μl of "Standard A2" and apply to a TLC-plate for product analysis (System A).

If all or most of the [^{3}H]fatty acid is released as diglyceride acetate under these conditions, then it can be assumed that the fatty acids were originally present as diglyceride, most likely as part of a phospholipid structure (Ferguson et al. 1985a).

PI-PLC treatment

Resuspend two 10,000 cpm aliquots of [^{3}H]mfVSG in 500 μl 25 mM HEPES-NaOH buffer, pH 7.4, containing 0.1% (w/v) sodium deoxycholate. To one sample, add 5 μl of PI-PLC stock solution (about 0.5 μg of enzyme) and incubate at 37^{o}C for 1 h. Add 1 ml of toluene, vortex, and centrifuge to separate the phases. Take 0.4 ml of the upper toluene phase and add it to 10 ml of scintillation fluid in a scintillation vial and count. Calculate the % [^{3}H]fatty acid

released as follows:

$$\% \text{ released} = \frac{(+\text{PI-PLC counts}) - (-\text{PI-PLC counts})}{10{,}000 - (-\text{PI-PLC counts})} \times 2.5 \times 100$$

Take a further 500 μl of the +PI-PLC toluene phase, dry, redissolve in 10 μl "Standard A1", and analyse the nature of the products by TLC (System A). If all or most of the [^{3}H]-fatty acid is released as diglyceride, it can be assumed that the fatty acid is present as a phosphoinositide (i.e. G-PI).

PLA_2 treatment

Resuspend two 10,000 cpm aliquots of [^{3}H]mfVSG in 0.5 ml of 0.5 M Tris-HCl, pH 7.5, 15 mM $CaCl_2$ containing 0.05% NP-40. To one, add 50 μl of 1 mg/ml PLA_2 solution and incubate for 1 h at 37°C. Acidify with 50 μl $CHCl_3$ and add 1 ml toluene, vortex, and centrifuge. Count 0.4 ml of the upper toluene phase. Calculate the % [^{3}H]fatty acid released as follows:

$$\% \text{ released} = \frac{(+\text{PLA}_2 \text{ counts}) - (-\text{PLA}_2 \text{ counts})}{10{,}000 - (-\text{PLA}_2 \text{ counts})} \times 2.5 \times 100$$

Take a further 0.5 ml of the PLA_2 toluene phase, dry, redissolve in "Standard A1", and analyse the products by TLC (System A). If about 50% of the [^{3}H]fatty acids are released by PLA_2 as free fatty acid, then a phospholipid structure may be assumed. This enzyme digest may work even when PI-PLC does not.

Nitrous acid deamination

Resuspend two 10,000 cpm aliquots of [^{3}H]mfVSG in 75 μl of 100 mM sodium acetate/acetic acid buffer, pH 4.0. To one, add 75 μl of freshly prepared 0.5 M $NaNO_2$ (sodium nitrite), and to the other 75 μl of 0.5 M NaCl. Incubate at room temperature for 3 h. Add 15 μl of 1 M HCL, 400 μl of $CHCl_3$, and 200 μl MeOH. Vortex and separate the phases by centrifugation. Recover the lower $CHCl_3$ phase with a pasteur pipette and transfer it to a preweighed 3.5 ml glass vial (weight A). Re-extract the aqueous phase with 0.5 ml of pre-equilibrated lower phase. Weigh the vial plus the pooled lower phases (weight B). Take a 200 μl sample and dry it in a scintillation vial for counting. Reweigh the sample vial (weight C). Calculate the % [^{3}H]fatty acid released as follows:

$$\% \text{ released} = \frac{(+NaNO_2 \text{ counts}) - (-NaNO_2 \text{ counts})}{10{,}000 - (-NaNO_2 \text{ counts})} \times \frac{(B - A)}{(B - A)} \times 100$$

Dry the rest of the pooled lower phases, redissolve the products in 10 μl "Standard B1", and apply to a TLC-plate (System B). If most of the [^{3}H]fatty acid is released as phosphatidylinositol, it can be assumed that a G-PI anchor is present where the PI-group is substituted by a non-N-acetylated hexosamine residue (Ferguson et al. 1985b).

TLC SYSTEMS

System A

Using Silica gel G or Si 60 TLC-plates activated at 125^{0}C for 1 h before use.

Solvent:

Petroleum ether - diethylether - acetic acid (80:20:1)

Standard A1:

Use 10 μl of 10 mg/ml (each in chloroform):

monomyristin
1,2 dimyristin
1,3 dimyristin
myristic acid
trimyristin
methylmyristate

Standard A2:

Use 10 μl of 10 mg/ml (each in chloroform):

monomyristin-diacetate
1,2 dimyristin-acetate
1,3 dimyristin-acetate

Prepared by acetylating monomyristin, 1,2 and 1,3 dimyristin in acetic anhydride - pyridine (1:1) for 12 h at room temperature. Dry the products under a stream of N_2 and redissolve in $CHCl_3$.

System B

Using Si 60 TLC-plates activated at 125°C for 1 h before use.

Solvent:

Develop once with acetone - petroleum ether (1:3), dry, then develop with $CHCl_3$ - MeOH - H_2O - acetic acid (25:15:4:2).

Standard B:

Use 20 μl of 10 mg/ml (each in chloroform - methanol (2:1):

phosphatidic acid

phosphatidylcholine

phosphatidylethanolamine

phosphatidylglycerol

phosphatidylinositol

Detection

Visualize the standards with I_2 vapour. Detect the ^3H-labeled products with a TLC-scanner (linear analyser), or by scraping 0.5 cm strips into scintillation fluid.

REFERENCES

Bordier C, Etges RJ, Ward J, Turner MJ, Cardoso de Almeida ML (1986) Proc Natl Acad Sci USA 83:5988-5991
Bulow R, Overath P (1986) J Biol Chem 261:11918-11923
Clarke MW, Olafson RW, Pearson TW (1985) Mol Biochem Parasitol 17:19-34
Cross GAM (1987) Cell 48:179-181
Davitz MA, Low MG, Nussenzweig V (1986) J Exp Med 163:1150-1161
Davitz MA, Gurnett AM, Low MG, Turner MJ, Nussenzweig V (1987) J Immunol 138:520-523
Ferguson MAJ, Cross GAM (1984) J Biol Chem 259:3011-3015
Ferguson MAJ, Haldar K, Cross GAM (1985a) J Biol Chem 260:4963-4968
Ferguson MAJ, Low MG, Cross GAM (1985b) J Biol Chem 260:14547-14555
Ferguson MAJ, Homans SW, Dwek RA, Rademacher TW (1988) Science 239:753-759
Fox JA, Duszenko M, Ferguson MAJ, Low MG, Cross GAM (1986) J Biol Chem 261:15167-15171
Haldar K, Ferguson MAJ, Cross GAM (1985) J Biol Chem 260:4963-4968
Hereld D, Krakow JL, Bangs JD, Hart GW, Englund PT (1986) J Biol Chem 261:13813-13819
Holder AA (1985) Curr Top Microbiol Immunol 117:57-74
Ishihara A, Hou Y, Jacobson K (1987) Proc Natl Acad Sci USA 84:1290-1293
Koch F, Thiele HG, Low MG (1986) J Exp Med 164:1338-1343
Low MG, Kincade PW (1985) Nature 318:62-64

Low MG, Ferguson MAJ, Futerman AH, Silman I (1986) TIBS 11:212-215
Low MG (1987) Biochem J 244:1-13
Stieger A, Cardoso de Almeida ML, Blatter MC, Brodbeck U, Bordier C (1986) FEBS Lett 199: 182-186
Woda BA, Gilman SC (1983) Cell Biol Int Rep 7:2037-2039