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Acyl-PEGyl Exchange Gel Shift Assay for Quantitative Determination of Palmitoylation of Brain Membrane Proteins

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Journal of Visualized Experiments (JoVE)
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Cambridge, MA 02140

Re: Submission of revised manuscript JoVE61018 for Methods Collection focusing on S-palmitoylation

Dear Drs. Myers and Chowdhury,

Enclosed please find a revised manuscript entitled "Acyl-PEGyl exchange gel shift assay for quantitative determination of palmitoylation of brain membrane proteins" that is submitted as part of the Methods Collection focusing on S-palmitoylation. We appreciate and have carefully considered the comments raised by each of the reviewers as well as the editorial comments of our original manuscript and we have outlined in the rebuttal document our response to address these comments. The manuscript is much improved based on the revisions made in response to the reviewer and editorial comments.

Thank you for your consideration.

Sincerely,

Elva D. Diaz, Ph.D.
Professor of Pharmacology
UC Davis School of Medicine

TITLE:

Acyl-PEGyl Exchange Gel Shift Assay for Quantitative Determination of Palmitoylation of Brain Membrane Proteins

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KEYWORDS:

brain, synapse, palmitoylation, AMPA receptor, SynDIG1, SynDIG4, Prrt1, Prrt2, APEGS assay

SUMMARY:

Palmitoylation entails the incorporation of a 16-carbon palmitate moiety to cysteine residues of target proteins in a reversible manner. Here, we describe a biochemical approach, the acyl-PEGyl exchange gel shift (APEGS) assay, to investigate the palmitoylation state of any protein of interest in mouse brain lysates.

ABSTRACT:

Activity-dependent alterations in the levels of synaptic AMPA receptors (AMPARs) within the postsynaptic density (PSD) is thought to represent a cellular mechanism for learning and memory. Palmitoylation regulates localization and function of many synaptic proteins including AMPA-Rs, auxiliary factors and synaptic scaffolds in an activity-dependent manner. We identified the synapse differentiation induced gene (SynDIG) family of four genes (SynDIG1-4) encoding brain-specific transmembrane proteins that associate with AMPARs and regulate synapse strength. SynDIG1 is palmitoylated at two cysteine residues located at positions 191 and 192 in the juxta-transmembrane region important for activity-dependent excitatory synapse development. Here, we describe an innovative biochemical approach, the acyl-PEGyl exchange gel shift (APEGS) assay, to investigate the palmitoylation state of any protein of interest and demonstrate its utility with the SynDIG family of proteins in mouse brain lysates.

INTRODUCTION:

S-palmitoylation is a reversible post-translational modification of target proteins that regulates stable membrane association, protein trafficking, and protein–protein interactions[1]. It involves addition of a 16-carbon palmitate moiety to cysteine residues via thioester linkage catalyzed by palmitoyl acyltransferase (PAT) enzymes**.** Many synaptic proteins in the brain are palmitoylated, including AMPA-Rs and PSD-95, in an activity-dependent manner to regulate stability, localization, and function[2-4]. Alterations in the levels of synaptic AMPARs in the PSD via

interaction of auxiliary factors with synaptic scaffolds such as PSD-95 underlies synaptic plasticity; thus, methods to determine the palmitoylation state of synaptic proteins provides important insight into mechanisms of synaptic plasticity.

Previously, we identified the SynDIG family of four genes (SynDIG1-4) encoding brain-specific transmembrane proteins that associate with AMPARs[5]. Overexpression or knock-down of SynDIG1 in dissociated rat hippocampal neurons increases or decreases, respectively, AMPA-R synapse size and number by ~50% as detected using immunocytochemistry and electrophysiology[5]. We utilized the acyl-biotin exchange (ABE) assay to demonstrate that SynDIG1 is palmitoylated at two conserved juxta-transmembrane Cys residues (found in all SynDIG proteins) in an activity-dependent manner to regulate stability, localization, and function[6]. The ABE assay relies on exchange of biotin on cysteines protected by modification and subsequent affinity purification[7]. Here, we describe an innovative biochemical approach, the acyl-PEGyl exchange gel shift (APEGS) assay[8-12], which does not require affinity purification and instead utilizes changes in gel mobility to determine the number of modifications for a protein of interest. The protocol is described for investigation of endogenous membrane proteins from mouse brain for which suitable antibodies are available.

PROTOCOL:

All animal procedures followed guidelines set forth by the National Institutes of Health (NIH) and have been approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

1. Preparation of mouse brain membranes

1.1. Decapitate mouse using a guillotine apparatus and dissect brain out rapidly (in <1 min, if possible, to minimize palmitoylation changes that may occur during dissection procedure). Homogenize immediately in 10 mL of homogenization buffer (**Table 1**) in a glass homogenizer (~12 strokes) on ice.

1.2. Centrifuge lysates for 15 min at 1,400 x *g* at 4 °C. Transfer the supernatant to a new tube on ice. Resuspend the pellet (~6 strokes) in an equal volume of homogenization buffer and centrifuge at 710 x *g* for 10 min at 4 °C.

1.3. Combine the supernatants from step 1.2 and centrifuge at 40,000 x *g* for 20 min at 4 °C.

1.4. Discard the supernatant (cytosolic fraction) and resuspend the pelleted membrane (P2) fraction in homogenization buffer.

NOTE: Samples can be flash frozen and stored at -80 °C for later use.

1.5. Perform a bicinchoninic acid (BCA) assay to quantitate protein levels. For the APEGS assay, begin with ~100−200 mg protein (maximum 250 mg) in a volume of 470 µL of buffer A (**Table 1**)

in a 1.5 mL tube. Sonicate and centrifuge at >13,000 x *g* for 10 min at 25 °C to remove insoluble protein. Transfer solubilized protein to a new 1.5 mL tube.

2. Acyl-PEGyl exchange gel-shift (APEGS) assay

2.1. Disrupt disulfide bonds and block free cysteines.

2.1.1. Disrupt disulfide bonds by incubating in 25 mM tris(2-carboxyethyl)phosphine (TCEP; 25 µL, added from a stock solution; **Table 1**) for 60 min at 55 °C.

2.1.2. Block free cysteines with 50 mM N-ethylmaleimide (NEM; 12.5 µL, added from a stock solution; **Table 1**) at room temperature for 3 h.

NOTE: The reaction can be extended overnight.

2.2. Perform chloroform methanol (CM) precipitation.

2.2.1. Transfer the protein solution from 1.5 mL tube to a polypropylene or glass tube that can be centrifuged in a swinging bucket rotor at modest speed. A 5 mL tube is ideal. Add four volumes (2 mL) of methanol (MeOH) and vortex briefly.

2.2.2. Add two volumes (1 mL) of chloroform and vortex briefly.

2.2.3. Add three volumes (1.5 mL) of dH_2O and vortex briefly.

2.2.4. Centrifuge the samples at 3,000 x *g* for 30 min at 25 °C in a swinging bucket rotor.

2.2.5. Carefully remove and discard the upper phase.

2.2.6. Add 3 volumes (1.5 mL) of MeOH and mix gently but thoroughly, being careful not to fragment the opaque protein pancake.

2.2.7. Centrifuge at 3,000 x *g* for 10 min at 25 °C in a swinging bucket rotor.

2.2.8. Using a glass serological pipet, remove as much of the top phase as possible without disturbing the protein pancake.

2.2.9. Carefully rinse the pellet with 1 mL of MeOH.

2.2.10. Air dry the pellet for at least 10 min.

NOTE: Dried pellet can be stored at -20 °C.

2.3. Perform cleavage of palmitoyl-thioester linkages with hydroxylamine (NH_2OH, HAM).

2.3.1. Resuspend protein precipitate in 125 µL of buffer A and sonicate briefly. Centrifuge at >13,000 x *g* for 10 min at 25 °C to remove insoluble material. Transfer solubilized protein to a new 1.5 mL tube.

2.3.2. Add 375 µL of buffer H (HAM+; **Table 1**) or buffer T (HAM-; **Table 1**) and incubate samples for 60 min at 25 °C.

2.4. Repeat the CM precipitation described in step 2.2.

NOTE: Dried pellet can be stored at -20 °C.

2.5. Add mPEG to unprotected cysteines.

2.5.1. Resuspend pellet in 100 µL of buffer A containing 10 mM TCEP. Transfer to a 1.5 mL tube.

NOTE: It is normal for significant loss of protein to have occurred during CM precipitation steps. All subsequent steps will be performed in a 1.5 mL tube, constraining the volume of protein to a maximum of ~120−130 µL. This will reduce protein loss during the final CM precipitation.

2.5.2. Add 20 mM mPEG-5k (25 µL, added from a stock solution; **Table 1**) to protein sample and mix by pipetting. Incubate for 60 min at 25 °C with end-over-end rotation.

2.5.3. To remove unincorporated mPEG-5k, perform CM precipitation as described in step 2.2 using these adjusted volumes: 4 volumes of MeOH (500 µL), 2 volumes of chloroform (250 µL), and 3 volumes of dH_2O (375 µL). Centrifuge at >13,000 x *g* for 10 min at 25 °C.

2.5.4. Carefully remove the upper phase as before, avoiding the thick, flocculent pancake. Add 1 mL of MeOH and mix gently but thoroughly. Centrifuge at >13,000 x *g* for 10 min at 25 °C.

2.5.5. Carefully remove the supernatant and rinse the pellet with 1 mL of MeOH. Centrifuge at >13,000 x *g* for 10 min at 25 °C. Air dry pellet.

NOTE: Dried pellet can be stored at -20 °C.

2.5.6. Resuspend the dried pellet in 50 µL of buffer A (without TCEP or any reducing agent). Reserve 5 µL for BCA protein quantitation. After quantitation, add an appropriate amount of 4x Laemmli sample buffer and, depending on the protein of interest, potentially heat the sample to 70−100 °C for 10 min.

NOTE: Boiling may cause aggregation of some membrane proteins.

3. Western blot analysis of PEGylated proteins

3.1. Load the sample onto an sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel[13].

NOTE: In this protocol 10% polyacrylamide was used.

3.2. Use standard separation, transfer and detection protocols for Western blotting[14].

3.3. Use densitometry[14] to determine the relative amounts of PEGylated versus nonPEGylated proteins.

REPRESENTATIVE RESULTS:

Immunoblotting with antibodies against the protein of interest reveals the palmitoylated state (non, singly, doubly, etc.) in mouse brain lysates as determined by mobility shift compared with samples in which HAM was not included. Previously, we had demonstrated that SynDIG1 was palmitoylated at two sites using the ABE assay[6]; however, we could not determine whether both sites were modified in brain tissue. Here we show that SynDIG1, SynDIG4/Prrt1, and Prrt2 are palmitoylated in mouse brain and that they have two potential palmitoylation sites (**Figure 1**). Interestingly, each protein has a different palmitoylation state in mouse brain based on the signal for the non, singly and doubly palmitoylated protein. Densitometry analysis of blots provides quantitative information about the palmitoylated state.

FIGURE AND TABLE LEGENDS:

Figure 1: Detection of PEGylated protein species in mouse membrane preparations. Mouse brains were dissected rapidly and homogenized immediately. P2 membrane fractions were subjected to the APEGs assay as described in this protocol. In this experiment, postnatal day 18 (P18) were separated using a 10% SDS-PAGE gel and immunoblotted and probed with antibodies against SynDIG1 (SD1), Prrt2, and SynDIG4 (SD4). Symbols indicate protein that is not palmitoylated (•), palmitoylated singly (*), or doubly (**). The presence of faint bands in the HAM (-) conditions indicates either an incomplete reduction of disulfide bonds or incomplete blockage of free cysteines by NEM.

Table 1: Solutions used for APEGS assay. Homogenization buffer and buffer A can be prepared ahead of time; however, add protease inhibitors and phenylmethylsulfonyl fluoride (PMSF) immediately before use. All other solutions should be prepared fresh.

DISCUSSION:

In our previous work, we utilized the ABE assay to demonstrate that SynDIG1 is palmitoylated at two conserved juxta-transmembrane Cys residues (found in all SynDIG proteins) in an activity-dependent manner to regulate stability, localization, and function[6]. A limitation is that the ABE assay requires affinity purification with agarose resins conjugated to avidin moieties as the final step in the procedure, resulting in significant loss of signal that complicate quantitative analysis. Furthermore, the ABE assay cannot distinguish if a protein is palmitoylated once or at multiple sites.

Here we use the APEGS assay[8-12] to determine the palmitoylation state of the AMPAR auxiliary transmembrane proteins SynDIG1 and the related proteins SynDIG4 (also known as Prrt1) and Prrt2 from mouse brain extracts; however, the assay can be applied to any membrane protein for which a high-quality and specific antibody suitable for western blotting is available. Use of brain membranes from wild type (WT) and knockout (KO) mice provides an ideal control for antibody specificity as we have demonstrated for antibodies against SynDIG1[15] and SynDIG4[16]. Performing the APEGS assay with brain lysates from WT and KO mice provides an important control for antibody specificity in this method. This method could also be applied to different membrane fractions obtained via differential centrifugation followed by the APEGS assay to investigate the subcellular localization of the protein of interest.

One critical step of the APEGS assay is CM precipitations, the purpose of which is to remove chemicals (NEM and HAM) from interfering with subsequent downstream reactions. Although it is possible to perform CM precipitations in a 1.5 mL tube, the use of larger tubes and volumes should produce more optimal results. At the same time, in part because of the multiple CM precipitations, one should expect a substantial loss of protein from beginning to end. This might result in apparent variability in total protein levels in the minus and plus HAM conditions.

As illustrated in **Figure 1**, the addition of mPEG-5k (5 kDa) does not necessarily produce a linear shift on an immunoblot. This can have advantages in resolving proteins which are palmitoylated at multiple sites. Furthermore, the presence of bands at mobility shift locations in the HAM negative conditions indicates either an incomplete reduction of disulfide bonds or incomplete blockage of free cysteines by NEM. Alternatively, apparent shifted bands in the HAM negative condition might indicate that free cysteines within a particular protein are difficult to block. Thus, additional optimization of the blocking step might be required if only a small percentage of total protein is palmitoylated in contrast to SynDIG1, SynDIG4, and Prrt2 in which the majority of the protein is either singly or doubly palmitoylated (**Figure 1**).

This method is not limited to mouse brain lysates. For example, this method has been used to screen for depalmitoylating enzymes for PSD-95 expressed in heterologous cells[9]. Expression of proteins in heterologous cells also allows for determination of the exact site of modification via site-directed mutagenesis. This method also informed the role of the synaptic scaffold AKAP150 in synaptic plasticity[17,18], demonstrating a broad application in neuroscience.

It is important to note that the APEGS assay is not exclusive to 16-carbon palmitate moieties as the assay will detect other long chain fatty acyl linkages that are subject to cleavage and replacement by mPEG. To establish palmitate modification conclusively requires additional experimentation such as metabolic labeling with tritiated palmitate.

ACKNOWLEDGMENTS:
The authors thank K. Woolfrey for advice and input on the APEGS assay. These studies were funded by research grants to E.D. from the Whitehall Foundation and the NIH-NIMH (1R01MH119347).

DISCLOSURES:

The authors have nothing to disclose.

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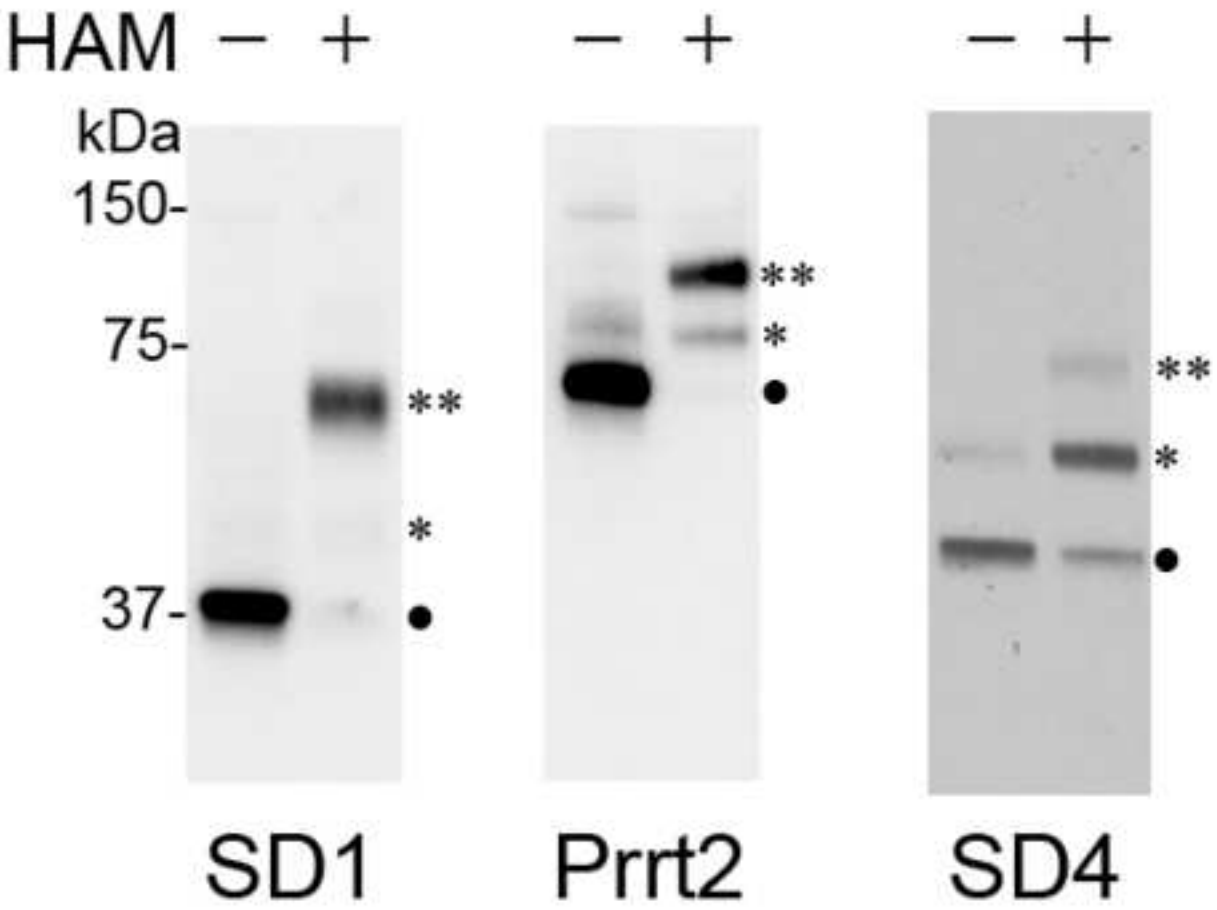
Figure1

Click here to access/download;Figure;Figure1.psd



Buffer	**Components**	**Working conc.**
Homogenization	0.32 M sucrose, 1 mM Tris pH 7.2, 1 mM $MgCl_2$	
Buffer A	PBS pH 7.2, 5 mM EDTA, 4% SDS (w/v)	
TCEP solution	500 mM TCEP in ddH_2O (pH 7.2)	25 mM
NEM solution	2.0 M NEM in 100% ethanol	50 mM
Buffer H (HAM+)	1.33 M HAM, pH 7.0, 5 mM EDTA, 0.2% Triton X-100 (w/v)	1.0 M
Buffer T (HAM-)	1.33 M Tris-HCl, pH 7.0, 5 mM EDTA, 0.2% Triton X-100 (w/v)	
mPEG-5k	100 mM mPEG-5k in ddH_2O	20 mM

Comments

Add PMSF and protease inhibitors immediately before use.

Add PMSF and protease inhibitors immediately before use.

Add 10 N NaOH to increase pH.

Prepare fresh.

Prepare fresh.

Mix well. Highly viscous.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Hydroxylamine (HAM)	ThermoFisher	26103	
Methoxy-PEG-$(CH_2)_3NHCO(CH_2)_2$-MAL (mPEG)	NOF	ME-050MA	MW ~5000 kDa
Microfuge	Eppendorf	5415R	or equivalent equipment
N-ethylmalemide (NEM)	Calbiochem	34115	Highly toxic.
Optical imager for densitometry	Azure Biosystems	Sapphire Biomolecular Imager	or equivalent equipment
Polypropylene tubes with cap	Fisher Scientific	14-956-1D	
Serological pipets (glass)	Fisher Scientific	13-678-27D	
Table top centrifuge	Beckman	Allegra X-15R	or equivalent equipment
Tris(2-carboxyethyl) phosphine-hydrochloride (TCEP)	EMD Millipore	580560	

We appreciate the comments raised by each of the reviewers as well as the editorial comments of our original manuscript. Outlined below are the revisions made to the manuscript to address the comments raised. The revised manuscript is strengthened and improved and we thank the reviewers for their input.

Editorial comments:

We have made all changes (points 1-13) requested by the editorial review.

Reviewers' comments:

Reviewer #1:

We thank the reviewer for stating that, 'As data and protocol presented here are clear and convincing, I think their work is potentially acceptable for publication in the JoVE in the current style.' The changes that we have made in response to the other reviewers and the editorial comments have further strengthened the manuscript.

Reviewer #2:

We thank the reviewer for acknowledging that, 'The method described is of general interest to those studying lipid modifications and...should certainly form a valuable addition to the JoVE video library.' Please see below for our responses to the technical considerations raised.

1) Presence of 'shifted' bands in the no HAM condition: We have further optimized the protocol to substantially minimize such bands and the figure has been updated accordingly. We have expanded the discussion to address this potential issue that might arise for others using the protocol.
2) Variability in total SD1 levels: The revised figure of optimized results shows more consistent levels of protein in the minus and plus HAM conditions. It is possible that protein is lost or possibly degraded in a differential manner during the procedure that results in variable levels. We have noted this limitation in the Discussion.
3) We have indicated in the Discussion that a limitation of the APEGS assay is that it is not exclusive to palmitate as suggested.

4) We have revised the manuscript to address the minor (stylistic) concerns raised (points i-vii).

Reviewer #3:

We thank the reviewer for acknowledging that, 'The protocol is very well structured and easy to follow…the manuscript will be useful for any researcher interested in palmitoylation.' Please see below for our responses to the minor comments raised.

1) Buffers in Table 1: Buffer B (PBS, 4%SDS, 8.9 M urea) was indicated in the referenced protocol (Kanadome et al., 2019) for tissue lysates but we did not find this was necessary in our hands; therefore, we have eliminated this buffer in Table 1. Similarly, protease inhibitors were indicated in the same protocol but in this case we have not compared results in the presence or absence of protease inhibitors.
2) We have expanded the discussion as suggested.