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Evaluation of prenylated peptides for use in cellular imaging and biochemical analysis.

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Abstract

Protein prenylation involves the addition of a farnesyl (C15) or geranylgeranyl (C20) isoprenoid moiety onto the C-terminus of approximately 2% of all mammalian proteins. This hydrophobic modification serves to direct membrane association of the protein. Due to the finding that the oncogenic protein Ras is naturally prenylated, several researchers have developed inhibitors of the prenyltransferase enzymes as cancer therapeutics. Despite numerous studies on the enzymology of prenylation *in vitro*, many questions remain about the process of prenylation in living cells. Using a combination of flow cytometry and confocal microscopy, we have shown that synthetic fluorescently-labeled prenylated peptides enter a variety of different cell types. Additionally, using capillary electrophoresis we have shown that these peptides can be detected in minute quantities from lysates of cells treated with these peptides. This method will allow for further study of the enzymology of protein prenylation in living cells.

Keywords

Peptide; lipid modification; post-translational modification; prenylation; farnesyl; cell-penetrating peptide

1. Introduction

An important post-translational modification that occurs on approximately 2% of all mammalian proteins (1) is called protein prenylation. Protein prenylation involves the addition of a hydrophobic isoprenoid moiety (15 carbon farnesyl or 20 carbon geranylgeranyl) to the C-terminal cysteine of proteins that contain a 'CAAX' box motif (2-4). In this case, 'A' represents an aliphatic amino acid and 'X' represents a directing residue that controls whether a protein is farnesylated or geranylgeranylated (5). There is considerable interest in this field due to the finding that several oncogenic proteins,

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including Ras, are naturally prenylated. Many researchers have developed potent inhibitors of the prenyltransferase enzymes as anti-cancer therapeutics (6,7), which have progressed to various stages of clinical trials (8-10). In addition to studying the prenyltransferase enzymes for anti-cancer therapies, new light has recently been shed on the involvement of protein prenylation in several other important diseases such as Alzheimer's and Parkinson's disease (11-13). Eckert and coworkers have shown that the levels of farnesyl and geranylgeranyl diphosphate (the isoprenoid substrates for the protein prenyltransferases) are elevated in the brains of Alzheimer's patients (14). Additionally, Liu *et al.* have shown that inhibition of the farnesylated protein UCH-L1 may be a therapeutic strategy for slowing the progression of Parkinson's disease (15).

While *in vitro* research has contributed significantly to the understanding of protein prenylation, it is clear that an enhanced understanding of the enzymology of prenylation *in vivo* is required to develop enhanced therapeutics. We have synthesized farnesylated peptides that are N-terminally labeled with a fluorescein derivative (5-carboxyfluorescein, 5-Fam), based on the C-terminal sequence of the naturally prenylated protein CDC42 (16,17). A variety of cell types have been shown to take up these synthetic peptides, and thus allow studies in living cells to be performed. In accordance with this finding, we have recently described the synthesis of several peptides, as well as their applications in cellular studies using flow cytometry and confocal microscopy. This paper will describe the methods involved in the use of these farnesylated peptides (Figure 1) to quantify cellular uptake and study localization patterns in living cellular systems, from both immortalized cell lines and primary neuron cells. Additionally, micellar electrokinetic capillary electrophoresis (MEKC) is used to detect subnanomolar levels of the peptides in lysates of cells treated with them.

2. Materials

2.1. Cell Culture

1. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Intergen, Purchase, NY).
2. F12K medium (ATCC, Manassas, VA) supplemented with 10% horse serum (HS) (Fischer Scientific, Hampton, NH) and 5% FBS.
3. Solutions of trypsin (10X, 2.5%) and versene (Invitrogen, Carlsbad, CA) (*See Note 1 and Note 2*).
4. Mouse β -nerve growth factor (β -NGF) (Raybiotech, Norcross, GA) was dissolved at 100 μ g/mL in phosphate buffered saline (PBS, *See Note 3*) and stored in single use aliquots at -80°C . Working solutions are prepared by dilution to 100 ng/mL in F12K medium supplemented with 1% HS.

¹Unless stated otherwise, all solutions should be prepared in water that has a resistivity of at least 15 M Ω -cm. This standard is referred to as "water" in this text.

²The trypsin (10X) and versene were purchased separately from Invitrogen and stored at -20°C and 4°C , respectively. Prior to use, trypsin was added to versene to reach a final concentration of 0.063% trypsin.

³Phosphate buffered saline (PBS) is composed of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM of KH₂PO₄. The solution is pH adjusted to 7.4, autoclaved, and stored at 4°C for future use.

5. Poly-D-lysine hydrobromide (mol. wt. > 300,000, Sigma Aldrich, St. Louis, MO) was dissolved at 0.1 mg/mL in PBS and stored in aliquots at -20°C .

2.2. Primary cell isolation and culture

1. Incubation Medium: Neuronal basal medium (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, HyClone, Ogden, UT), 1% Penicillin/Streptomycin (100 Unit/ml), 2 mM GlutaMax (Gibco) and 10% B-27 (Gibco) or 10% Gem 21 (Gemini Bio Prod).
2. Dissecting medium: Same as item 1, but supplemented with 50 $\mu\text{g/mL}$ Fungin (purchased as a 10 mg/mL solution, InvivoGen, San Diego, CA).
3. 1X TrypLE Express (Invitrogen, Carlsbad, CA).
4. 5-Fluoro-2-deoxyuridine and uridine (Sigma Aldrich, St. Louis, MO).
5. 70 μm cell strainer (BD Biosciences, Franklin Lakes, NJ).
6. 15 mL polypropylene centrifuge tubes with a conical bottom, sterilized (herein referred to as '15 mL centrifuge tube', BD Biosciences, Franklin Lakes, NJ).
7. Dissecting scissors and forceps.
8. C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine).

2.3. Flow cytometry

1. 12 $\times$ 75 mm polypropylene round bottom test tube (BD Biosciences, Franklin Lakes, NJ).
2. 15 mL centrifuge tubes.

2.4. Confocal Microscopy

1. Culture dishes (35 mm) fitted with a glass bottom (14 mm diameter, No. 1.5 coverslip) (MatTek Corporation, Ashland, MA).
2. Wheat germ agglutinin Alexa Fluor 594 conjugate plasma membrane stain (Invitrogen, Carlsbad, CA) was dissolved at 5 mg/mL in PBS and stored in aliquots at -20°C .
3. Hoechst 34850 nuclear stain (Invitrogen, Carlsbad, CA) was dissolved at 5 mg/mL in water and stored in aliquots at -20°C .

2.4. Capillary electrophoresis

1. Fused silica capillary tubing (50 μm ID, 363 μm OD, 20 μm CT) (Polymicro Technologies, Phoenix, AZ).
2. Run buffer composed of 100 mM sodium dodecyl sulfate (SDS, MP Biomedicals, Solon, OH), 25 mM sodium tetraborate (Fisher Chemical, Fair Lawn, NJ), and 3 M urea (Sigma Aldrich, St. Louis, MO) (*See Note 4*).

3. Methods

The peptides used in this study (Figure 1) were previously synthesized as described in ref. (16). These peptides were first evaluated for their ability to be taken up by immortalized HeLa cells using flow cytometry, which was followed by visualizing their localization inside cells using confocal microscopy. Several cell types were tested using these methods to establish the versatility of peptide uptake. Having established that these peptides were naturally cell penetrating, we sought to explore their utility in primary cultured cells using mouse neurons. Neurons were chosen due to the potential relevance of protein prenylation in Alzheimer's Disease (see Introduction). Because these peptides were demonstrated to exhibit uptake in both immortalized and primary cells, we next sought to analyze the peptides from cell lysates using a modification of capillary electrophoresis. Micellar electrokinetic capillary electrophoresis (MEKC) coupled with laser induced fluorescence detection (LIF) is used to separate components in the cell lysate and detect any fluorescent peptide present.

3.1. Preparation of Immortalized Cells

1. HeLa, NIH/3T3, D1TNC1 astrocyte, and PC-12 cells are passaged when nearing complete confluence with trypsin/versene to continue growth of maintenance cultures. All cell lines are maintained in DMEM supplemented with 10% FBS except PC-12, which are maintained in F12K medium supplemented with 10% HS and 5% FBS (*See Note 5*).
2. HeLa, NIH/3T3, and D1TNC1 astrocyte cells are seeded in 35mm glass bottomed culture dishes (*See Note 6*) at 7.8×10^3 cells/cm² and grown to approximately 50% confluence (typically 24 h). PC-12 cells are seeded at 3.9×10^3 cells/cm² and grown to approximately 20% confluence (typically 24 h). Prior to peptide incubation, PC-12 cells are differentiated into neurons (*See Note 7*).

3.2. Isolation and Maintenance of Primary Cells

1. Coat 35 mm glass bottom culture dishes with polylysine (*See Note 5*).
2. Sterilize dissecting scissors, forceps and all metallic equipment with 95% ethanol.
3. 17 to 18 day gestated C57BL/6J mice were asphyxiated with CO₂, cleaned with 95% ethanol, and the stomach cut open with scissors. The uterus was removed and the embryos were transferred into a 10 cm² dish on ice. The head was snipped just below the ear and the opening at the bottom of the head was widened by cutting on

⁴After preparing the capillary electrophoresis run buffer at the specified concentration, the pH is adjusted to 9.3 and the buffer is filtered through a 0.2 µm PTFE acrodisc syringe filter (Sigma Aldrich, St. Louis, MO).

⁵PC-12 cells are maintained and seeded on poly-lysine coated culture dishes. To coat the dishes, a thin layer (enough solution to completely coat the bottom of the plate) of a 0.1 mg/mL poly-D-lysine hydrobromide solution is added to the plates and allowed to sit at RT overnight. The following day, the plates are rinsed twice with PBS and are ready for use.

⁶Glass bottom culture dishes (MatTek) are used if confocal microscopy will be performed. If flow cytometry is desired, regular plastic 35 mm culture dishes are used.

⁷After seeding the PC-12 cells to 20% confluence in 35 mm glass bottom dishes, the cells are serum starved for 24 h with F12K + 1% HS. Addition of F12K medium containing 1% HS and 100 ng/mL of Nerve Growth Factor β (β-NGF) initiated neuron differentiation. Incubation with β-NGF proceeded for 6–8 days until maximum neurite growth was observed; the medium was replaced every 2 days. Once the cells begin differentiating, they are quite fragile and easily disturbed. When giving cells fresh medium, leave a small amount of the previous medium behind so the cells do not become exposed to the air as they will rapidly dry out.

both the left and right side with a small scissors. Carefully apply force with forceps on the frontal portion of the head to remove the brain. The brains were collected in a 15 mL centrifuge tube containing 10 mL dissecting medium on ice.

4. Under the microscope, the cerebellum and brain stem were removed while the cerebral cortices were collected for primary neuronal preparation.
5. Mince the cortices using a small scissor and suspend in TrypLE Express in a 15 mL centrifuge tube. The suspension was defragmented by pipetting up and down (2 to 3 times) using a 1 mL pipetman pressed against the bottom of the tube. The cell suspension was placed in a 10 cm² dish, covered, and placed in an incubator at 37°C with 5% CO₂ for 7 min.
6. Neutralize the suspension with 2 mL FBS and 3 mL neuronal basal medium containing Fungin (50 µg/mL). Pipet the solution repeatedly (5-10 times) to dislodge clumps, filter through a 70 µm cell strainer, and centrifuge in a 15 mL centrifuge tube at 130 x g for 5 min.
7. Aspirate the medium from the pellet and re-suspend in 10 mL neuronal basal medium containing Fungin (50 µg/mL). Seed the cells at 0.5 × 10⁶ cells/cm² and place in an incubator at 37°C with 5% CO₂ and 80% humidity.
8. After 1 h, remove the medium and replace it with freshly warmed incubation medium and place back in the incubator.
9. 48 h later, replace the medium with fresh incubation medium containing 5-fluoro-2-deoxyuridine (0.75 mg/mL) and uridine (1.75 mg/mL) to inhibit any mitotic processes of astrocytes or glia. Incubate with this medium for an additional 48 h.
10. Replace the medium with fresh incubation medium and replace every 48 h until ready to use the primary cells.

3.3. Analyzing Cells Using Flow Cytometry

1. After reaching appropriate confluency, the cells are rinsed with PBS (2x, 1 mL each) and serum free DMEM is added (or F12K with 1% HS in the case of PC-12).
2. The farnesylated peptide is added to the culture medium to reach the desired concentration, generally between 0.3 and 3 µM.
3. Incubate the cells with the peptide for 1h at 37°C and 5% CO₂, then rinse the cells twice with PBS and trypsinize for 15 min (0.2 mL of trypsin/versene solution, *See Note 2*). Trypsinization normally proceeds for 5 min, however, a 15 min time is used in this case to remove any peptide bound to the outer cell membrane and thus allow measurement of only internalized peptide.
4. Add 1.8 mL complete media to the cells and transfer to a 15 mL centrifuge tube. Centrifuge cells at 100 x g for 5 min at room temperature.
5. Aspirate medium from cell pellet and re-suspend cells in 2 mL PBS. Transfer cells to a 12 × 75 mm round bottom test tube for flow cytometry analysis.

6. The mean fluorescence intensity of 10,000 cells is counted using a BD FACS Calibur flow cytometer. Typical results of this analysis are shown in Figure 2.

3.4. Localization Studies with Confocal Microscopy

1. Once the cells have reached confluence in glass bottom dishes, rinse the cells with PBS (2x, 1mL each) and add serum free DMEM (or F12K with 1% HS in the case of PC-12).
2. Add the desired peptide to the culture medium to reach the desired concentration, generally either 1 or 3 μ M, and incubate at 37°C and 5% CO₂ for 2 h (*See Note 8*).
3. During the final 20 min of incubation, add Hoechst 34580 nuclear dye to a final concentration of 1 μ g/mL. Additionally, for the final 10 min of incubation, add wheat germ agglutinin Alexa Fluor 594 plasma membrane stain to a final concentration of 5 μ g/mL.
4. Rinse the cells twice with PBS (1 mL each) and place cells back in serum free DMEM (or F12K with 1% HS in the case of PC-12).
5. The cells are imaged live using an Olympus FluoView 1000 inverted confocal microscope with a 60X objective. Excitation at 405 nm induces fluorescence of Hoechst nuclear dye (emission 461 nm), excitation at 488 nm induces peptide fluorescence (from 5-Fam, emission 519 nm), and excitation at 543 nm induces wheat germ-Alexa Fluor 594 plasma membrane fluorescence (emission 618 nm). Collection of all fluorescence channels occurs simultaneously using the confocal laser scanning microscope (See Figures 3 and 4 for sample pictures).

3.5. Micellar Electrokinetic Capillary Electrophoresis (MEKC)

1. Before performing MEKC, the sample must first be prepared. This sample consists of HeLa cells that have been treated with the **VS** peptide and lysed.
2. Grow and treat HeLa cells with peptide following the steps in section 3.3, 1-6.
3. Aspirate the medium from the pellet and re-suspend the cell pellet in 2 mL PBS and centrifuge at 100g for 5 min again (to rinse the pellet and remove all medium).
4. Add 150 μ L of MEKC run buffer (100 mM SDS, 25 mM borate, 3 M urea) to the cell pellet to immediately lyse the cells. The sample will become thick and viscous. This sample is frozen at -80°C until analysis with MEKC.
5. Cut a 35 cm length of fused silica capillary and burn a detection window approximately 12 cm from one end of the capillary.
6. Feed capillary through the detector end of the capillary cartridge (Beckman) and position the detector window in the opening for the detector; replace white plastic clips with red seals for coolant flow.

⁸We have discovered that longer peptide incubation times lead to increased cellular uptake. In order to study the peptide localization with microscopy, 2 h peptide incubation is used to increase the uptake.

7. Using the metal template (provided with instrument), cut the capillary to the correct length and attach the detector pieces for the laser induced fluorescence (LIF) detector.
8. Insert capillary and cartridge into the instrument and condition the capillary (*See Note 9*).
9. After capillary conditioning, it is ready for use. Set up the sample(s) in the sample tray and run. For each sample, injection occurs at a pressure of 0.3 psi for 5 sec and separation occurs for 12 min at 12.0 kV.
10. The data is exported as an ascii file and graphed using Origin graphing software (see Figure 5 for typical data that is obtained).

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⁹To condition the capillary, rinse at 20 psi for 8 min with the following solutions, in order: 0.1 M HCl, water, 0.1 M NaOH, and run buffer. This will deprotonate the silica in the capillary and replaces the counter ions with ions from the run buffer.

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Farnesylated CAAX
peptides:

1a: (VM)

$A_1=V$, $A_2=I$, $X=M$

1b: (VS)

$A_1=V$, $A_2=L$, $X=S$

Figure 1.

Synthetic, farnesylated, and fluorescently labeled cell penetrating peptides. For detailed synthesis of these peptides, see ref. (16).

Figure 2.

Flow cytometry data for two farnesylated peptides incubated with HeLa cells at 37°C for 1 h at varying concentrations. The 'control' in this case consists of HeLa cells that were not treated with peptide. After peptide incubation, cells were trypsinized 15 min to remove membrane-bound peptide. Each bar represents the geometric mean fluorescence intensity of 10,000 cells. Experiments performed in triplicate with the results expressed as the mean fluorescence intensity $\pm$ standard deviation.

Figure 3.

Confocal microscopy images of different live immortalized cells after incubation with peptide VS at either 1 or 3 μM for 2 h. A) HeLa cells treated at 3 μM . B) NIH/3T3 fibroblasts treated at 1 μM . C) D1TNC1 astrocytes treated at 1 μM . D) PC-12 differentiated neurons treated at 1 μM . Hoechst 34850 was used to stain the nucleus blue and wheat germ agglutinin Alexa Fluor 594 conjugate was used to stain the plasma membrane red. The peptide is visualized as green fluorescence. Size bar represents 20 μm .

Figure 4.

Confocal microscopy images of primary mouse neurons after incubation with peptide VS at 1 μ M for 2 h. The left panel is an overlay of all fluorescence channels, while the right panel represents only the green channel for clarity. Hoechst 34850 was used to stain the nucleus blue and wheat germ agglutinin Alexa Fluor 594 conjugate was used to stain the plasma membrane red. The peptide is visualized as green fluorescence. Size bar represents 20 μ m.

Figure 5.

Micellar electrokinetic capillary electrophoresis (MEKC) chromatograms of HeLa lysate samples. Chromatogram 1 represents peptide VS injected alone at 25 nM as a standard. In chromatogram 2, HeLa cells were treated with peptide VS at 1 μ M for 2h, rinsed, and lysed. The lysate was diluted 1:2 and analyzed with MEKC. Chromatogram 3 represents the same lysate (also diluted 1:2) that has been spiked with the peptide standard (VS) at a concentration of 25 nM. The co-elution of the large peak at approximately 7.5 min verifies the presence of the VS peptide in the cell lysate. The peak from the lysate at 2.5 min in Chromatogram 2 and 4.6 min in Chromatogram 3 represents a possible proteolytic fragment of peptide VS (this peak is not present in a lysate sample not treated with peptide (data not shown)). The average t_R of the peptide peak is as follows: Chromatogram 1: 7.46 ± 0.29 min. Chromatogram 2: 7.22 ± 0.45 min. Chromatogram 3: 7.48 ± 0.46 min (standard deviation represents at least 3 trials).