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Addressing Membrane Protein Topology Using the Fluorescence Protease Protection (FPP) Assay

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Summary Determining a protein's correct topological distribution within the cell is essential for understanding the proper functioning of many proteins. Here, we describe a fluorescence-based technique, termed FPP for fluorescence protease protection, to determine protein topology in living cells. The FPP assay uses the restricted proteolytic digestibility of green fluorescent protein-tagged membrane proteins to reveal their intramembrane orientation. Membrane protein topology can be assessed using this technique for proteins residing in organelles as diverse as the Golgi apparatus, the endoplasmic reticulum (ER), peroxisomes, mitochondria, and autophagosomes. To illustrate the technique, we describe its use for deciphering the topology of a membrane protein in the ER.

Keywords Digitonin permeabilization; fluorescence assay; membrane protein; topology.

1 Introduction

Cellular membranes act as barriers and scaffolds, separating different cellular environments. Within such membranes, proteins adopt specific topologies to achieve their proper cellular function. Because the way a transmembrane protein distributes across a membrane bilayer dictates which of its domains (i.e., cytoplasmic, transmembrane, extracellular, or intralumenal) is accessible to modifying enzymes and interacting partners, understanding a protein's topological distribution is essential for understanding its function.

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Both experimental and theoretical approaches have been employed to determine the topology of membrane proteins. Many of these approaches, including biochemical modification and antibody labeling, are demanding with respect to time, effort, and expense of materials and do not always provide congruent data *(1,2)*. Here, we describe an alternative method to obtain information regarding membrane protein topology using GFP (green fluorescent protein)-tagged fusion proteins.

The approach, referred to as the fluorescence protease protection (FPP) assay, provides basic information about the position of the GFP tag relative to the membrane *(3)*. It requires no additional design beyond the construction of a GFP fusion protein expressed in either tissue culture or primary cells. The assay is principally based on the inaccessibility of proteases to protected intracellular regions of permeabilized cells *(4)*. The protease-induced destruction of GFP attached to a protein of interest after plasma membrane permeabilization reveals whether the GFP tag is oriented facing the cytosol or facing a protected intracellular environment.

The cholesterol-binding drug digitonin is used to selectively permeabilize the plasma membrane in the FPP assay. Digitonin permeabilization has been successfully used in yeast and numerous mammalian cells *(5–8)*. The extent of permeabilization is sufficient to allow cytosolic contents to diffuse across the plasma membrane, while intracellular organelles and the cytoskeletal system are retained *(7,8)*. After digitonin permeabilization, the protease trypsin or proteinase K is added to the medium. These proteases readily enter the cytoplasm from the extracellular environment but cannot cross the intact membranes of intracellular organelles. Any GFP molecule attached to a protein of interest facing the cytosol, therefore, will be destroyed and lose its fluorescence. It thus becomes possible to determine whether an organellar protein is membrane associated or lumenal, which portion of a membrane protein faces the lumen (or cell exterior) and cytoplasm, and whether a protein associated with nonmembranous structures is protected from molecules in the surrounding nucleoplasm or cytoplasm.

2 Materials

1. GFP expression vector (Clontech, Mountain View, CA) containing DNA encoding the protein of interest.
2. Mammalian cells to be transfected with GFP fusion protein.
3. Transfection reagents for mammalian cells (e.g., FuGENE 6 transfection reagent, Roche, Indianapolis, IN; or Lipofectamine, Invitrogen, Carlsbad, CA).
4. Standard cell culture medium for cells of interest.
5. KHM buffer: 110 m*M* potassium acetate, 20 m*M* HEPES, 2 m*M* $MgCl_2$.
6. Digitonin.
7. Trypsin or an alternative protease, such as proteinase K, reconstituted in KHM buffer.
8. Lab-Tek chambered cover glass (Nalge Nunc International, Rochester, NY).
9. Fluorescence microscope with image acquisition-and-capture system.

3 Methods

The FPP assay described outlines (Subheading 3.1.) the principle construction of the expression plasmid, (Subheading 3.2.) the expression of a GFP-tagged fusion protein in mammalian cells, (Subheading 3.3.) the selective permeabilization of the plasma membrane, (Subheading 3.4.) the degradation of exposed peptides on protease application, and (Subheading 3.5.) the determination of membrane protein topology by fluorescence microscopy.

3.1 Expression Plasmid Construction

GFP or any other fluorescent protein variant such as cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) can be fused to the deoxyribonucleic acid (DNA) coding for the membrane protein of interest. For single-spanning membrane proteins, two versions of the protein should be created: an amino- and a carboxy-terminal fusion protein with GFP. For multispanning membrane proteins, either a fusion protein with GFP inserted within the protein of interest or a truncated fusion protein should be made. It is important to note the exact position of GFP within the fusion protein's amino acid sequence (*see* **Note 1**). As a control, transfect cells with DNA encoding GFP alone. DNA cloning and modification can be performed by standard recombinant DNA methods *(9)*.

3.2 Preparation and Transfection of Sample Cells

1. Passage the cells of interest into cell chambers (e.g., Lab-Tek chambered cover glass). Because the cells in the chamber will be both transfected with DNA and monitored under the microscope, plate enough cells so that at the time of the FPP assay the cells are about 60–90% confluent.
2. At 16 to 24 h postpassage, transfect cells with the expression plasmid encoding the GFP-tagged fusion protein using a standard transfection protocol. Culture cells at the appropriate conditions until fluorescent signals are detectable (6–20 h posttransfection).

3.3 Selective Permeabilization of the Plasma Membrane

1. Remove cell culture medium and wash cells three times for 1 min each in KHM buffer at a temperature that is appropriate for the experiment. Temperatures ranging from 20 °C to 37 °C are suitable for the FPP protocol.

2. Place chamber containing cells on the fluorescence microscope stage. Set up microscope for fluorescence imaging and record first images, which represent the prepermeabilization situation.
3. To permeabilize the plasma membrane, add digitonin-containing KHM buffer to the cells. Determine the effective digitonin concentration by applying increasing concentrations of digitonin to cells that express only GFP. GFP diffuses freely in the cytosol and nucleoplasm. Effective permeabilization of the plasma membrane by digitonin results in the disappearance of the GFP signal within 10–60 s. A good starting concentration for most cell lines tested (COS-7, NRK, HeLa, BHK, N2a) is 20 µ*M* digitonin. If 20 µ*M* digitonin is insufficient to permeabilize the cells, increase the digitonin concentration in 20-µ*M* increments. Use the lowest possible digitonin concentration that provides efficient plasma membrane permeabilization (*see* **Note 2**).
4. Incubate cells that express the protein of interest tagged with GFP in KHM buffer containing the (previously determined) effective digitonin concentration. Efficient permeabilization of most cell lines occurs within 1 min after digitonin application.
5. Take images of the cells after digitonin application to capture the postpermeabilization situation (*see* **Notes 3 and 4**).

3.4 Degradation of Exposed Amino Acids on Protease Application

1. Wash permeabilized cells quickly but thoroughly in KHM buffer.
2. Add 4–8 m*M* of the protease trypsin (in KHM buffer) directly onto the cells. Immediately begin taking images on the fluorescence microscope to record whether fluorescent signals persist or disappear. Alternatively, 50 µg mL^{-1} proteinase K can be used for the FPP assay (*see* **Note 3**).

3.5 Determination of Protein Topology by Fluorescence Microscopy

Assess recorded images and quantify signal intensities to determine the subcellular localization and topology of the protein. Freely available image analysis software (i.e., NIH Image, Image J) or software on existing microscope platforms (e.g., Zeiss LSM Image Examiner, Zeiss Microimaging, Thornwood, NY) can be used to measure fluorescence intensities.

4 Notes

1. Incubation of permeabilized cells with proteases *(see* Subheading 3.4.) provides more detailed information about the protein's subcellular localization and topology. At this point, knowing the exact position of the GFP tag within the fusion protein sequence becomes important for topology determinations (*see* Subheading 3.1.). If a protein is contained within a protected subcellular environment, such as the lumen of intracellular organelles, the GFP signal will be unaffected by the addition of protease regardless of the specific placement of the GFP tag within the fusion protein (i.e., N- or C-terminal or central). In contrast, if the protein of interest spans the membrane of an intracellular organelle such that some domains are facing the organelle's lumen and other domains are exposed to the cytosol, the placement of the GFP tag will dictate whether its fluorescence is resistant to protease addition (Figs. 1 and 2, middle and bottom panels).
2. Regarding potential pitfalls, the most crucial step in the FPP protocol is the selective permeabilization of the plasma membrane by digitonin (*see* Subheading 3.3.). For each particular experiment, it is highly recommended to adjust this step depending on the cells and particular conditions used. The best way to establish appropriate conditions is to express GFP alone in the cells of interest.

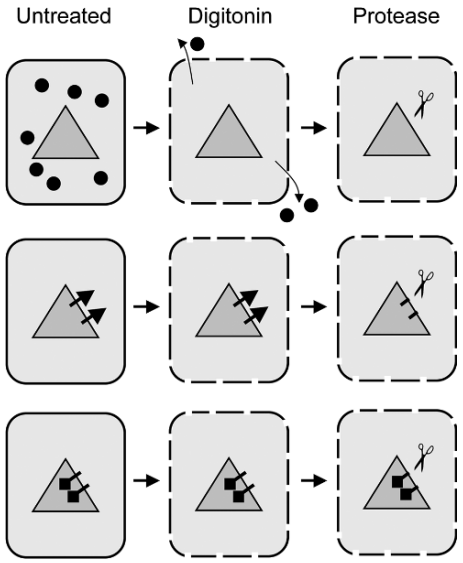

Fig. 1 Schematic presentation of the FPP assay illustrating cells before (left column) and after digitonin (middle column) and trypsin (right column) application. The filled circle symbols (top panel) represent unbound cytosolic molecules. The arrow symbols with the pointed (middle panel) and square ends (bottom panel) represent transmembrane proteins bound to a membrane (big triangles) with the fluorescent tag facing the cytoplasm or the organelle's lumen, respectively. The pre- (left column) and postpermeabilization (middle column, digitonin) conditions and the resulting phenotypes after protease (scissor symbol, right column) treatment are shown.

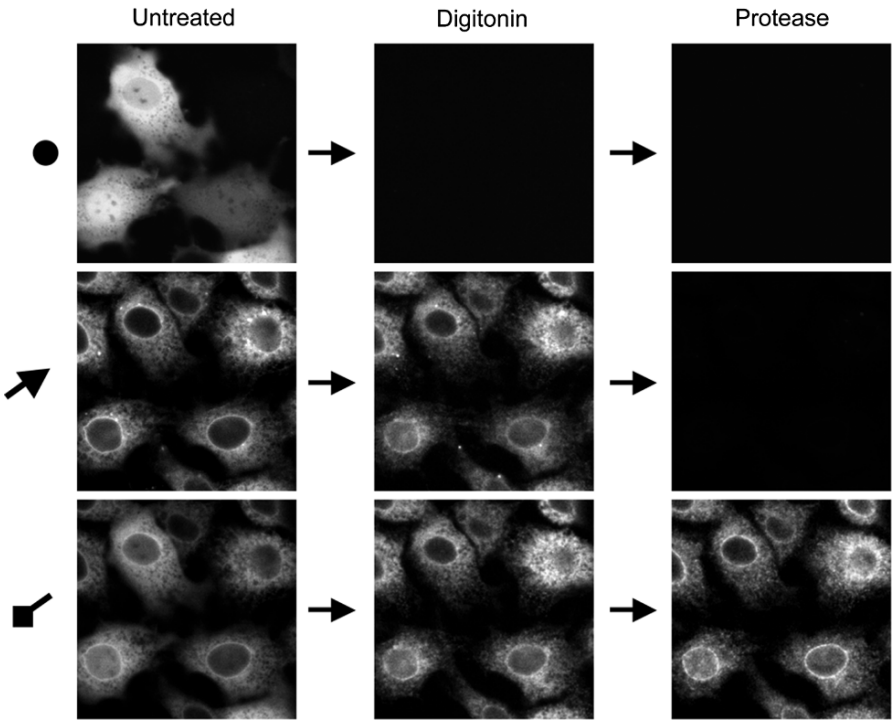

Fig. 2 Protein topology determination by FPP. Micrographs show NRK cells expressing GFP alone (top panel) and either the type I membrane protein CD3δ ***(10)*** tagged C-terminally to YFP (middle panel) or CD3δ tagged N-terminally to CFP (bottom panel). Performing the FPP assay with these cells reveals that CD3δ is membrane bound, unlike GFP alone (compare postdigitonin treatment), and that only the N-terminal portion of CD3δ is protected from protease (compare postprotease treatment). Therefore, the type I membrane orientation of CD3δ could be derived from the FPP assay. Individual images were taken before (left panel, untreated) and after treatment with 20 μ*M* digitonin (middle panel) and 4 m*M* trypsin (right panel). The filled circle and arrow symbols on the left of each panel correspond to the schematic presentation of the FPP assay in Fig. 1.

GFP easily diffuses out of the cytoplasm and across the plasma membrane on efficient digitonin permeabilization. Therefore, by using GFP, the lowest efficient digitonin concentration can be determined. This will significantly reduce any risks of affecting intracellular membranes. After successful permeabilization, it is recommended that the digitonin-containing KHM buffer be washed off the cells prior to protease application.

3. On permeabilization of the plasma membrane by digitonin (see Subheading 3.3.), the FPP assay will immediately provide data regarding a protein's localization. The disappearance of intracellular fluorescent signals indicates that the protein of interest is freely diffusing in the cytosol or nucleoplasm and is neither membrane associated nor localized in the lumen of an intracellular organelle (Figs. 1 and 2, top panels).
4. Fluorescent signals that are retained on cell permeabilization indicate that the protein of interest is not freely diffusing in the cytosol and nucleoplasm.

References

1. Traxler, B., Boyd, D., and Beckwith, J. (1993) The topological analysis of integral cytoplasmic membrane proteins. *J. Membr. Biol*. **132,** 1–11.
2. Ott, C.M., and Lingappa, V.R. (2002) Integral membrane protein biosynthesis: why topology is hard to predict. *J. Cell Sci*. **115,** 2003–2009.
3. Lorenz, H., Hailey, D.W., and Lippincott-Schwartz, J. (2006) Fluorescence protease protection of GFP chimeras to reveal protein topology and subcellular localization. *Nature Methods* **3,** 205–210.
4. Lorenz, H., Hailey, D.W., Wunder, C., and Lippincott-Schwartz, J. (2006) The fluorescence protease protection (FPP) assay to determine protein localization and membrane topology. *Nature Prot*. **1,** 276–279.
5. Cordeiro, C., and Freire, A.P. (1995) Digitonin permeabilization of *Saccharomyces cerevisiae* cells for in situ enzyme assay. *Anal. Biochem*. **229,** 145–148.
6. Gowda, L.R., Joshi, M.S., and Bhat, S.G. (1988) In situ assay of intracellular enzymes of yeast (*Kluyveromyces fragilis*) by digitonin permeabilization of cell membrane. *Anal. Biochem*. **175,** 531–536.
7. Plutner, H., Davidson, H.W., Saraste, J., and Balch, W.E. (1992) Morphological analysis of protein transport from the ER to Golgi membranes in digitonin-permeabilized cells: role of the P58 containing compartment. *J. Cell Biol*. **119,** 1097–1116.
8. Wilson, R., Allen, A.J., Oliver, J., Brookman, J.L., High, S., and Bulleid, N.J. (1995) The translocation, folding, assembly and redox-dependent degradation of secretory and membrane proteins in semi-permeabilized mammalian cells. *Biochem. J*. **307,** 679–687.
9. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
10. Yang, M., Omura, S., Bonifacino, J.S., and Weissman, A. M. (1998) Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. *J. Exp. Med*. **187,** 835–846.