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Gammaglutamyl Transpeptidase Assay: An Example of a Protocol for Determining the Sidedness or Asymmetrical Expression of a Membrane Protein, Enzyme or Transport Activity in an Epithelial or Other Cell Type

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Introduction

Many differentiated cell types exhibit a polarized distribution of structural and functional membrane proteins. For example, epithelial cells express distinct apical (facing the organ lumen) and basolateral (facing other cells and the interstitial space) membrane domains. These membrane domains can be distinguished on the basis of different protein profiles as well as different functional activities, both enzymatic and transport. Much work has been devoted to determining which membrane proteins/functions are expressed at which membrane domain, and how the cell sorts specific proteins to these distinct domains. A large portion of this work has been performed using cell culture, either primary cells or continuous cell lines, cultured on permeable membrane supports which allow easy access to both membrane domains. Described herein is an example of an experimental protocol which was used to localize the membrane-bound enzyme, gammaglutamyl transpeptidase, which is expressed at the apical surface of differentiated renal proximal tubular cells. The procedure for measuring gammaglutamyl transpeptidase enzymatic activity is adapted from the method described by Naftalin, et al. (1969).[1]

Materials and Methods

The following supplies and solutions are required to perform the described experimental protocol.

Supplies for Cell Culture

- Stock culture vessel: BD Falcon™ 75 cm² flasks (Cat. No. 353024)
- Pipets: BD Falcon 1 ml, 5 ml, and 10 ml sterile, plastic, disposable pipets (Cat. Nos. 357520, 357543, and 357551)
- Conical tubes: BD Falcon 15 ml Conical Tubes, sterile, plastic (Cat. No. 352097)
- Insert: BD Falcon Cell Culture Inserts (Cat. No. 353090)
- 6-well plate: BD Falcon 6-well plates (Cat. No. 353046)

Solutions for Cell Culture

- Cell culture medium: Eagles' Minimal Essential Medium
- Fetal bovine serum
- Calcium and magnesium-free phosphate buffered saline solution (PBS)
- Trypsin/EDTA solution: 0.05% trypsin plus 0.02% EDTA in calcium and magnesium-free phosphate buffered saline solution

Solutions for Gammaglutamyl Transpeptidase Assay

- Assay solution: 150 mM NaCl plus 10 mM Tris-HCL (pH 8.5)
- Substrate solution: 5 nM L-glutamyl-p-nitroanilide plus 100 mM glycylglycine in assay solution
- Stop solution: 10% acetic acid
- Standard solution: 1 mM p-nitroanilide in assay solution
- Rinse solution: (for cells, following assay) 150 mM NaCl plus 10 mM Tris-HCL (pH 7.4)

1. Cells are propagated at subconfluent density in stock culture. Culture medium is dependent on cell type and the specific experiment, but EMEM + 10% fetal bovine serum is a common culture medium that performs well with many cell types.
2. Detach cells from stock culture vessels using a standard trypsin/EDTA solution (e.g., 0.05% trypsin, 0.02% EDTA in Ca-Mg-free phosphate-buffered saline solution). Add culture medium containing serum (to terminate the protease digestion), and transfer cells to a sterile plastic centrifuge tube. Pellet cells by centrifugation at 500 g for five to ten minutes. (Centrifugation can be performed at room temperature, although centrifugation at 4°C is desirable.)
3. Resuspend cells in a small volume of culture medium and measure cell number using a hemacytometer or electronic cell counter. Dilute cells with culture medium to achieve a final concentration of 10^6 cells/ml.

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4. Seed 2 x 10^6 cells (2 ml) per BD Falcon™ Cell Culture Insert, 25 mm diameter (Cat. No. 353090), and place insert in well of a sterile BD Falcon 6-well plate (Cat. No. 353046). Add 2-3 ml culture medium to the well, outside the insert, and incubate at 37°C in a 5% CO_2 atmosphere. (If desired, inserts may be pre-coated with an appropriate material to enhance cell attachment, spreading, growth and/or differentiation using standard procedures). Cells should become confluent within two to four days. (Confluency can be confirmed by phase contrast microscopic examination utilizing the excellent optical characteristics of the transparent membrane.)

5. Using these confluent cultures, standard procedures can be used to label proteins, measure enzymatic activity, or measure transport activity expressed at one or the other membrane surface (i.e., at the cell surface facing the medium inside the insert, or at the cell surface in apposition to the membrane). Protein labeling procedures include lactoperoxidase-catalyzed radioiodination of externally exposed tyrosines, and radiolabeling of exposed sialic acid residues with tritiated sodium borohydride. Enzyme and transport activity measurements can be performed as usual for the measurement of surface activity (i.e., using an isoosmotic incubation solution that will not permeabilize the cell membrane.) Described below is an assay for the measurement of exposed cell-surface gammaglutamyl transpeptidase activity.

Transpeptidase Assay

6. Aspirate culture medium and rinse monolayer three times by adding assay solution (room temperature) to both well and insert. (Assay solution - 150 mM NaCl + 10 mM tris - HCL, pH 8.5.)

7. To measure apical activity (transpeptidase facing the medium), add 0.5 ml assay solution to the insert and 2 ml assay solution to the well. Preincubate at room temperature for two to five minutes. To initiate reaction, add 0.5 ml assay solution supplemented with 5 mM L-glutamyl-p-nitroanilide and 100 mM glycylglycine to insert (final concentrations: 2.5 mM L-glutamyl-p-nitroanilide and 50 mM glycylglycine).

   To measure basolateral activity (transpeptidase facing filter), add 1 ml assay solution to insert and 1 ml assay solution to well. Preincubate at room temperature for two to five minutes. To start reaction, add 1 ml assay solution plus 5 mM L-glutamyl-p-nitroanilide and 100 mM glycylglycine to well.

8. Incubate at room temperature for up to 20 minutes, depending on level of enzyme activity present at membrane.

9. To measure amount of product, remove 1 ml of appropriate solution (solution containing substrates) with a pipet, add to 2 ml 10% acetic acid and vortex. Measure A_{405} of solution using a standard spectrophotometer, and calculate quantity of product produced using a standard curve of A_{405} versus known quantities of p-nitroanilide.

10. Aspirate remaining solution in well and insert and rinse several times in saline solution (e.g., PBS). Solubilize cells and measure protein content using a standard assay such as the Lowry or Bradford methods.

Results and Discussion

Using the procedure described above, the level of gamma-glutamyl transpeptidase activity expressed at the apical (facing the medium) versus basolateral (facing the filter) membranes of polarized epithelial cells can be quantitated. This is a single example of a usage for BD Falcon Cell Culture Inserts. The potential applications are limited only by the imagination and creativity of the user in designing the experimental protocols.

Troubleshooting

1. **No enzymatic activity:**
   Test the substrate solution, which can degrade spontaneously during storage at 4°C. To avoid this problem, make substrate solution fresh or weekly.

2. **Indentical or unexpectedly high enzymatic activity when measured from either side:**
   The most likely possibility for this result is that the cell monolayer has not covered all available filter surface area. In this case, substrate will diffuse across the filter if it is not covered with the cell layer, providing substrate at both surfaces. This can be easily checked for cells cultured on the BD Falcon Cell Culture Inserts since the filter material possesses excellent optical characteristics. Microscopic observation of samples prior to assay will permit assessment of the level of confluence and avoid wasting precious cultures and expensive materials.

3. **Cells do not attach or proliferate on inserts:**
   This may indicate the cells being used require a specific surface component for attachment/proliferation. BD Falcon Cell Culture Inserts can be easily coated with a desired matrix material, such as collagen or fibronectin, prior to seeding of cells to avoid such a problem.

Reference

1. Naftalin, et al., Clin. Chem. Acta **26**:293 (1969).

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