Video Article

Determining Cell-surface Expression and Endocytic Rate of Proteins in Primary Astrocyte Cultures Using Biotinylation

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Abstract

Cell-surface proteins mediate a wide array of functions. In many cases, their activity is regulated by endocytic processes that modulate their levels at the plasma membrane. Here, we present detailed protocols for 2 methods that facilitate the study of such processes, both of which are based on the principle of the biotinylation of cell-surface proteins. The first is designed to allow for the semi-quantitative determination of the relative levels of a particular protein at the cell-surface. In it, the lysine residues of the plasma membrane proteins of cells are first labeled with a biotin moiety. Once the cells are lysed, these proteins may then be specifically precipitated via the use of agarose-immobilized streptavidin by exploiting the natural affinity of the latter for biotin. The proteins isolated in such a manner may then be analyzed via a standard western blotting approach. The second method provides a means of determining the endocytic rate of a particular cell-surface target over a period of time. Cell-surface proteins are first modified with a biotin derivative containing a cleavable disulfide bond. The cells are then shifted back to normal culture conditions, which causes the endocytic uptake of a proportion of biotinylated proteins. Next, the disulfide bonds of non-internalized biotin groups are reduced using the membrane-impermeable reducing agent glutathione. Via this approach, endocytosed proteins may thus be isolated and quantified with a high degree of specificity.

Video Link

The video component of this article can be found at https://www.jove.com/video/55974/

Introduction

Proteins at the cell surface play a variety of roles central to maintaining cell function. In numerous instances, their activity is dependent on, or modulated by endocytic processes that either temporarily sequester them in intracellular sites, or that direct them towards degradative pathways[1,2,3,4,5]. Here, we highlight 2 biotinylation-based approaches designed to allow the user to specifically tag and isolate proteins expressed at the plasma membrane, and those newly internalized. Via these methods, the cell-surface expression and endocytic rate of any protein of interest may be quantified, thus allowing a clearer assessment of its regulation to be achieved.

Determining relative cell-surface protein expression by biotinylation

Biotin, or vitamin B7, formerly known as vitamin H[6], is a small water soluble molecule that can be used to chemically modify reactive amine, sulfhydryl, and carboxyl groups of biological molecules. The current crop of cell-surface biotinylation reagents consist primarily of membrane-impermeant sulfonated N-hydroxysuccinimide (sulfo-NHS) esters of biotin or its derivatives, designed to react with the amines present on the side-chains of lysine residues of proteins expressed at the cell surface when these become deprotonated under basic conditions, resulting in the latter forming an amide bond with the biotin moiety[7]. Thusly modified, cell-surface proteins may then be isolated via the use of avidin, a 66 - 69 kDa tetrameric protein possessing great affinity for biotin, binding to the latter with a dissociation constant of approximately 10^{-15}, marking it as one of the strongest noncovalent interactions known[8,9].

A number of alternative methods of quantifying protein expression at the cell surface have been used in previous studies. The labeling of unpermeabilized cells using fluorescently tagged antibodies specific for the protein of interest, followed by visualization via fluorescence microscopy, for instance, is a commonly employed approach, but is heavily reliant on the availability of antibodies that can bind to extracellular epitopes. More recently, methods involving the use of chimeric proteins bearing pH-sensitive fluorophores that react to being exposed to acidic media have also been successfully employed[10]. However, such assays usually involve the exogenous expression of these constructs in cell lines in which the protein of interest is not natively found. These approaches are nonetheless able to provide valuable information regarding the subcellular localization and exocytic itinerary of the target protein, and should therefore be used in conjunction with the biotinylation-based approaches described here if the tools are available.

In a typical biotinylation assay, the cells are first washed thoroughly in 4 °C PBS. This removes any traces of serum proteins introduced by the culture medium, thus ensuring that these will not consume excess amounts of biotin in the next step. More importantly, the reduction in temperature causes endocytosis to decelerate significantly. The biotinylation reagent is then added. Next, the cells are washed again, and then incubated with a quenching buffer containing either glycine or NH_4Cl, the purpose of which is to inactivate all remaining traces of unreacted

biotin. The cells are then lysed, following which agarose-immobilized streptavidin is added to precipitate the biotinylated proteins. Analysis is commonly performed via western blotting, allowing the relative cell-surface expression of various proteins to be quantified.

Due to the basis of this assay, it is suitable for use only with proteins possessing portions exposed to the extracellular environment. Multipass transmembrane proteins, which likely possess a number of reactive lysines within their loop regions, are the most amenable to this method, while single-pass proteins tend to be less susceptible to being biotinylated. Even in these cases, there remains a possibility that conformational changes or intermolecular interactions may occlude certain reactive sites, resulting in a lower-than-expected biotinylation yield.

Determining internalization rate of cell-surface proteins by biotinylation

The principles of this assay are largely similar to those of cell-surface biotinylation, with a number of exceptions, the most important of which is the use of reversible biotinylation reagents. The biotin groups (of these) possess disulfide bonds, within their structures, that are susceptible to reducing agents; this is exploited to ensure that only cell-surface proteins taken into intracellular sites during the assay period will be left biotinylated. An assay generally takes place in the following manner. The cells are first washed and biotinylated with cold reagents, then cell culture medium at 37 °C is re-introduced, and the cells are returned to the incubator; this causes the labeled cell-surface proteins to undergo endocytosis. The reducing agent glutathione - which cannot penetrate the membrane - is then added to break the disulfide bonds of the biotin moieties attached to proteins remaining on the cell surface. Finally, the broken disulfide bonds are reacted with iodoacetamide, consuming the labile thiol groups and preventing the bonds from reforming. As before, the cells are then lysed, and the labeled proteins are precipitated using streptavidin-agarose.

The limitations discussed in the previous section also apply here due to the similarities shared between the methods. In addition, it is worth bearing in mind that the temperature shifts involved in this assay preclude the exact determination of how much protein is endocytosed for each increment of time, particularly in the case of rapidly internalized or rapidly recycling proteins. The assay therefore only provides a semiquantitative estimation of endocytic rates. Total internal reflection fluorescence microscopy can be used to track the uptake of each loaded vesicle and provide a more precise measurement of the kinetics of endocytosis. It can therefore provide a very useful complement to this assay, assuming that a fluorescently tagged chimeric construct of the protein of interest is available[11].

Protocol

1. Determining Relative Cell-surface Protein Expression in Astrocytes by Biotinylation

NOTE: Here, we illustrate the application of this biotinylation technique to the study of the effects of the extracellular matrix molecule laminin on the cell-surface localization of the water-permeable channel aquaporin-4 (AQP4). Specialized materials required for this assay include sulfo-NHS-LC-biotin and streptavidin-agarose resin (see **Table of Materials**).

1. Using the method outlined in Noel *et al.*[12], prepare cultures of cortical astrocytes approximately 2 weeks in advance of the assay, and grow them in 75 cm^2 vented culture flasks. When astrocytes are 80 - 90% confluent, detach them from the culture surface using 0.05% trypsin, and then passage them 1:3.
2. At 48 h prior to the assay, when cells are again 80 - 90% confluent, passage astrocytes 1:3 (accounting for the change in the culture format) onto 60 mm cell culture dishes so that they are >70% confluent on the day the experiment is to take place. Ensure that cells are evenly distributed between dishes.
3. At 16 h prior to assay, pipette laminin into culture medium to a final concentration of 24 nM, and incubate at 37 °C.
4. Immediately prior to assay, prepare the following, and then place on ice or refrigerate: CM-PBS (100 mg/L $MgCl_2 \cdot 6H_2O$ and 100 mg/L $CaCl_2$ in 1X PBS, pH 7.4), biotin buffer (0.5 mg/mL sulfo-NHS-LC-biotin in CM-PBS), quenching buffer (50 mM NH_4Cl in CM-PBS), lysis buffer (25 mM Tris, pH 7.4, 25 mM glycine, 150 mM NaCl and 5 mM EDTA, 1% triton X-100, 1X protease inhibitor cocktail), 3X loading buffer (150 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 300 mM DTT and 0.01% bromophenol blue), and wash buffer (10 mM Tris (pH 7.4), 1.5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1X protease inhibitor cocktail).
5. Remove dishes holding the astrocyte cultures from the incubator, and discard the medium.
6. Wash cells thrice with 4 mL chilled CM-PBS, and place the dishes on crushed ice.
7. Pipette 2 mL biotin buffer into each well, and gently tilt dishes back and forth a few times to ensure complete coverage. Leave on ice for 30 min.
8. Remove the biotin buffer using an aspirator, and replace with 4 mL quenching buffer. Leave on ice for 10 min.
9. Aspirate the quenching buffer, and replace with an equivalent volume of the same. Again, leave on ice for 10 min.
10. Discard the quenching buffer, and wash cells thrice with 4 mL chilled CM-PBS.
11. Scrape cells into 1 mL chilled CM-PBS using a cell lifter, and transfer the suspension to microcentrifuge tube.
12. Pellet cells by centrifugation at 100 x g for 3 min. Discard the supernatant, and re-suspend cells in 500 µL of lysis buffer.
13. Leave samples on ice for 30 min, vortexing every 5 min, or place them on an end-over-end rotator at 4 °C.
14. Centrifuge the lysate at 14,000 x g for 10 min at 4 °C to pellet any detergent-insoluble materials. Transfer the supernatant into a new microcentrifuge tube.
    1. Save 50 µL of this lysate and add loading buffer to it. Then denature it by heating at 95 °C in a dry bath; this is the "input" fraction, containing both biotinylated cell-surface proteins, as well as non-biotinylated cytosolic proteins.
15. Widen the opening of a pipette tip by cutting off approximately 0.5 cm of material from its end using a pair of sharp scissors. Using this pipette tip, transfer 75 µL of streptavidin-agarose beads (normally stored at 4 °C) to the lysate, and incubate at 4 °C for 3 h on shaker/rocker.
    1. As streptavidin-agarose is frequently sold as a slurry containing 50% beads by volume, suspended in an antimicrobial solution, triturate the slurry to ensure that the beads are evenly suspended, and then pipette 150 µL of the suspension into each sample.
16. Pellet streptavidin-agarose beads by centrifugation at 1500 x g for 30 s at 4 °C.

1. Save 50 µL of the supernatant (add loading buffer and denature it at 95 °C in a water bath or heating block); this represents the "intracellular" fraction, and is comprised primarily of non-biotinylated cytosolic proteins.

17. Resuspend the pelleted beads in 1 mL wash buffer, and rock this for 3 min at 4 °C. Pellet beads (as in step 1.16), and discard the supernatant. Repeat this process 4x to minimize the nonspecific binding of nonbiotinylated cytosolic proteins.
18. Pellet the beads by centrifugation (1500 x g for 30 s at 4 °C), and discard the overlying wash buffer. Add 50 µL of 1X loading buffer (diluted using lysis buffer). Release biotin and streptavidin from beads by denaturing this at 95 °C; this fraction should contain biotinylated cell-surface proteins only ("cell-surface" fraction).
    1. Separate input, cell-surface, and intracellular fractions by SDS-PAGE [13], and analyze by western blotting [14].
    NOTE: While we used a 4 - 20% precast gradient gel in our experiments, a 12 - 14% separating gel with a 4% stacking layer (each containing 0.1% SDS) should suffice for the proteins of interest in this study. A molecular weight standard of the appropriate size range should also be used. Note that there can sometimes be an observable upshift in the apparent molecular masses of biotinylated proteins.

2. Determining Internalization Rate of Cell-surface Proteins in Astrocytes by Biotinylation

NOTE: In the following, we describe a typical pulse-chase biotinylation experiment used in this instance to track the endocytosis of AQP4 in astrocytes. This method is based on that used by Madrid *et al.*[15]. Specialized materials required include sulfo-NHS-SS-biotin, streptavidin-agarose resin, reduced glutathione, and iodoacetamide (see the **Table of Materials**).

1. Prepare cultures of mouse cortical astrocytes in 60 mm dishes using the methods outlined in the previous section. Ensure that cells are approximately 70% confluent on the day of the assay, and that each dish contains an equivalent number of cells.
2. Immediately prior to assay, prepare the following, and place on ice or refrigerate: CM-PBS (100 mg/L $MgCl_2 \cdot 6H_2O$ and 100 mg/L $CaCl_2$ in 1X PBS, pH7.4), biotin buffer (0.5 mg/mL sulfo-NHS-SS-biotin in CM-PBS), reducing buffer (50 mM reduced glutathione, 75 mM NaCl and 75 mM NaOH), quenching buffer (50 mM iodoacetamide, 1% BSA, in CM-PBS), lysis buffer (25 mM Tris, pH 7.4, 25 mM glycine, 150 mM NaCl and 5 mM EDTA, 1% triton X-100, 1X protease inhibitor cocktail), 3X loading buffer (150 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 300 mM DTT and 0.01% bromophenol blue), and wash buffer (10 mM Tris, pH 7.4, 1.5 mM EDTA, 150 mM NaCl, 1% triton X-100, 1X protease inhibitor cocktail).
3. Prepare fresh cell culture medium (DMEM supplemented with 10% Fetal Bbovine Serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine), and place it in a 37 °C water bath.
4. Remove astrocyte cultures from the incubator, and aspirate the medium using an aspirator.
5. Wash cells thrice with 4 mL chilled CM-PBS, and place the dishes on crushed ice.
6. Pipette 3 mL biotin buffer into each dish, tilt dishes back and forth a few times to ensure that buffer is well-distributed, and leave this on ice for 30 min.
7. Aspirate biotin buffer, and replace it with 5 mL warm medium. Incubate a culture dish at 37 °C for 15 min, and a second dish at the same temperature for 30 min. Leave another dish at 4 °C as the 0 min sample.
8. At the end of the incubation period, discard medium, and wash cells thrice with 4 mL chilled CM-PBS. Pipette 6 mL reducing buffer over cells, and leave on ice for 15 min.
9. Remove reducing buffer, and then replace with 6 mL fresh reducing buffer. Place on ice for an additional 15 min.
10. Remove reducing solution and replace with 6 mL quenching buffer. Leave on ice for 15 min.
11. Repeat quenching step once more.
12. Discard quenching buffer, and wash cells thrice with 4 mL chilled PBS.
13. Scrape cells into 1 mL chilled PBS using a cell lifter, and transfer the suspension into a microcentrifuge tube.
14. Pellet cells by centrifugation at 100 x g for 3 min. Discard the supernatant, and resuspend cells in 500 µL lysis buffer.
15. Leave this on ice for 30 min and vortex every 5 min. Alternatively, place the samples on an end-over-end rotator at 4 °C for this duration.
16. Centrifuge the lysate at 14,000 x g for 10 min at 4 °C to pellet the detergent-insoluble materials, then transfer supernatant to a new microcentrifuge tube. Save 50 µL of this lysate, add loading buffer to it and denature at 95 °C in a dry bath; this is the "input" fraction, containing both biotinylated endocytosed proteins, as well as nonbiotinylated proteins.
17. Using a cut pipette tip, add 150 µL of the streptavidin-agarose slurry to the lysate, and incubate at 4 °C for 3 h on shaker/rocker. See 1.15 for additional details on this step.
18. Pellet streptavidin-agarose beads by centrifugation at 1,500 x g for 30 s at 4 °C.
19. Resuspend beads in 1 mL wash buffer, and rock for 3 min at 4 °C. Pellet beads (as per step 2.18), and discard the supernatant. Repeat this process 4x to minimize the nonspecific binding of nonbiotinylated cytosolic proteins.
20. Pellet beads by centrifugation at 1,500 x g for 30 s, at 4 °C, and discard overlying wash buffer. Add 50 µL 1X loading buffer (diluted using lysis buffer). Release biotin and streptavidin from beads by denaturing at 95 °C; this fraction should contain internalized cell-surface proteins only ("endocytosed" fraction).
21. Separate input, cell-surface, and unbound fractions by SDS-PAGE[13], and analyze by western blotting[14].
NOTE: While we used a 4 - 20% precast gradient gel in our experiments, a 12 - 14% separating gel with a 4% stacking layer (each containing 0.1% SDS) should suffice for the proteins of interest in this study. A molecular weight standard of the appropriate size range should also be used. Note that there can sometimes be an observable upshift in the apparent molecular masses of biotinylated proteins.

Representative Results

Using cell-surface biotinylation to assess the plasma membrane expression of AQP4 in astrocytes
Laminin-treated astrocyte cultures and untreated control cells were subject to cell-surface biotinylation using the methods described. Biotinylated proteins were precipitated with agarose-conjugated streptavidin, and then separated via SDS-PAGE. Cell-surface fractions were probed for AQP4 and β-dystroglycan (β-DG) as a cell-surface loading control, while the input and intracellular fractions were blotted for AQP4 and β-actin (**Figure 1A**) as a loading control. Band intensities for the cell-surface fraction were quantified via densitometry, and AQP4 levels for each set of cells were first normalized against β-DG values, and these ratios were then normalized against those for the control cells. The histogram (**Figure 1B**) represents the mean values ± SEM for three independent experiments. Laminin treatment, on average, causes a near 2X increase in AQP4 expressed at the cell surface.

Investigating the endocytic rate of AQP4 using biotinylation
AQP4 endocytosis in astrocytes was tracked over a 30 min period. Briefly, a cleavable biotin analog was first used to label surface proteins in 3 dishes of astrocytes. The cells were shifted to 37 °C for 0, 15, and 30 min, respectively, during which the labeled proteins were internalized. Labeling that remained on the surface was then removed using a reducing agent, and endocytosed proteins were precipitated with agarose-conjugated streptavidin. Following separation via SDS-PAGE, these were then probed for AQP4 (**Figure 2A**). AQP4 levels were quantified using densitometry, and the values corresponding at the 15 & 30 min time points were normalized to those for the 0 min sample. The histogram (**Figure 2B**) represents the averaged values ±SEM for four such experiments.

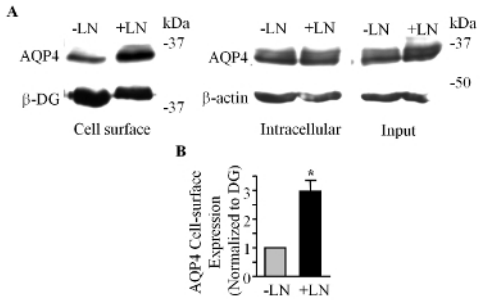

Figure 1. AQP4 Expression at the Cell-surface in the Presence of Laminin. (A) The cell-surface, intracellular, and total (Input) fractions from untreated astrocytes (-LN) and astrocytes treated with 24 nM laminin-111 (+LN) were probed for AQP4 and β-DG. **(B)** Histogram illustrating the differences in the cell-surface levels of AQP4 untreated and laminin-treated astrocytes, normalized against β-DG levels. Values represent normalized mean pixel intensities ±SEM, expressed relative to the values for the control cells. The asterisk indicates a statistically significant increase in AQP4, as determined by a two-tailed Student's *t*-test (n = 3, $*p = 0.033$). This figure has been modified from Tham *et al.*[16] Please click here to view a larger version of this figure.

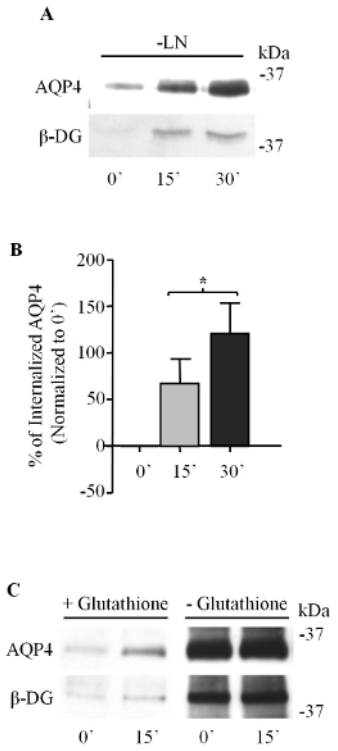

Figure 2. AQP4 Internalization in Astrocytes.(A) Proteins internalized by astrocyte cultures at 0, 15, and 30 min were probed for AQP4. **(B)** Histogram summarizing the internalization rates of AQP4 for 4 independent experiments, normalized against values for the 0 min time-point. The asterisk indicates a statistically significant increase in the amount of AQP4 endocytosed between 15 & 30 min as determined by a two-tailed Student's *t*-test (n = 3, *p = 0.017). **(C)** When glutathione is used to break the disulfide bond in the cleavable biotin analog, the contribution of cell-surface AQP4 to the biotinylated fraction is sharply reduced, resulting in a clear difference between the 0 & 15 min samples **(C, top left)**. When this step is omitted, the signal originating from the cell-surface pool renders the change between the 2 time-points undetectable **(C, top right)**. This figure has been modified from Tham *et al.*[16] Please click here to view a larger version of this figure.

Discussion

Modifications:
As these methods were designed for use with adherent cells, we have specified the use of PBS containing 100 mg/L $MgCl_2 \cdot 6H_2O$ and

100 mg/L $CaCl_2$ (CM-PBS) for the washing steps and as the base of certain buffers so as to ensure that the cells remain attached to the culture surface and that cell-cell junctions are not disrupted. However, the protocols may also be applied to nonadherent cell types if the cells are pelleted in between each step of the procedure. In these instances, CM-PBS may be replaced with PBS.

Additionally, it is important to note that each of the conditions mentioned here are specific to this protocol, and should only be considered as rough guidelines if the methods are to be employed for other applications. Particularly, one should independently verify that the detergent extraction procedure is appropriate for their cell type, and that the centrifuge settings are suitable for the cell type, and for the streptavidin-agarose beads being used.

Finally, if there are concerns regarding non-specific binding, and/or excessive background, one can substitute streptavidin with the various other avidin forms currently available on the market. Deglycosylated avidin, for instance, has significantly lower affinity for lectins, and for negatively charged molecules such as DNA.

Troubleshooting and controls:
While the procedures reviewed in this report are fairly straightforward, one should be mindful of a number of critical issues that can potentially affect the outcome of the experiment. Firstly, while both assays depend heavily on the membrane-impermeant nature of the sulfated biotinylation reagents, certain conditions could cause the plasma membrane of the cells to become disrupted, thereby resulting in certain intracellular proteins being biotinylated. To control for this possibility, it is suggested that biotinylated fractions should be probed for targets known to not be expressed at the plasma membrane, such as β-actin.

One should be careful to adhere to the proper storage conditions for the biotinylation reagents (typically 4 or -20 °C, with some form of desiccation), as these may fail otherwise, due to their highly sensitive nature. Nevertheless, it is good practice to probe the input, biotinylated, and nonbiotinylated fractions with streptavidin-HRP to ascertain that biotinylation has indeed occurred, and that the biotinylated proteins have been efficiently precipitated (which will be evident from the absence of a signal in the non-biotinylated fraction). Doing so should allow for the elimination of the possibility of defective reagents.

Disulfide bond reduction is a crucial step in the endocytic biotinylation procedure, as it ensures that only internalized proteins will be isolated in the biotinylated fraction. Incorporating controls in which the reducing reagent is omitted (**Figure 1C**) allows one to gain an impression of the efficacy of the treatment.

Disclosures

The authors declare no conflict of interest.

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