Abstract

Plasma membrane proteins are a large, diverse group of proteins comprised of receptors, ion channels, transporters and pumps. Activity of these proteins is responsible for a variety of key cellular events, including nutrient delivery, cellular excitability, and chemical signaling. Many plasma membrane proteins are dynamically regulated by endocytic trafficking, which modulates protein function by altering protein surface expression. The mechanisms that facilitate protein endocytosis are complex and are not fully understood for many membrane proteins. In order to fully understand the mechanisms that control the endocytic trafficking of a given protein, it is critical that the protein’s endocytic rate be precisely measured. For many receptors, direct endocytic rate measurements are frequently achieved utilizing labeled receptor ligands. However, for many classes of membrane proteins, such as transporters, pumps and ion channels, there is no convenient ligand that can be used to measure the endocytic rate. In the present report, we describe a reversible biotinylation method that we employ to measure the dopamine transporter (DAT) endocytic rate. This method provides a straightforward approach to measuring internalization rates, and can be easily employed for trafficking studies of most membrane proteins.

Protocol

Procedure Overview:

Using this approach, cell surface proteins are covalently labeled with biotin on available extracellular lysine residues using a membrane impermeant, disulfide-coupled biotinylation reagent (sulfo-NHS-SS-biotin) under trafficking restrictive conditions (i.e. low temperature) (see Fig. 1 for illustration). One set of cells is shifted to trafficking permissive conditions (37°C) and biotinylated proteins internalize. The other set of cells are kept at low temperature as controls for 1) the total surface protein at time=0, and 2) stripping control. Following a short period of internalization, cells are shifted back to low temperature to stop internalization, and any residual surface biotin is stripped off by treating cells with a reducing agent, which cleaves the disulfide-coupled biotin. Biotinylated proteins that arose from the cell surface and were internalized are protected from the stripping step, and will be the only biotinylated proteins that remain. Following cell lysis, biotinylated proteins are isolated by streptavidin affinity chromatography and the protein of interest is detected by quantitative immunoblotting. To determine the endocytic rate, the amount of internalized protein is compared to the total surface control labeled at time=0. We have successfully used this approach to measure the internalization rate of the neuronal norepinephrine\(^{1}\) and dopamine\(^{1-4}\) transporters.

Detailed Protocol:

Day 1:

1. Plate cells in 6 well plates such that they will be ~80% confluent on Day 2. Alternatively, if transfected cells are being used, transfect at a density such that they will be ~80% confluent at the time the internalization rate will be measured. If cells are not strongly adherent, tissue cultureware should be treated with a cell adhesion substrate (e.g. poly-D-lysine) to prevent cell loss during the extensive wash steps.

2. For each protein being tested, plate 2 wells on one plate to be used as the total surface protein (t=0) and stripping controls. On a second plate, plate one well for each internalization condition being tested (i.e. basal endocytic rate vs. drug-treated).

3. Prepare the following solutions and store at the indicated temperatures for use on Day 2:
   - PBS\(^{2+}\): Phosphate buffered saline (pH 7.4) supplemented with 1.5 mM MgCl\(_2\), 0.2 mM CaCl\(_2\), (4°C)
   - Biotinylation Quench Solution: PBS\(^{2+}\) supplemented with 100 mM glycine, (4°C)
   - NT buffer: 150 mM NaCl, 1.0 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6, (4°C)
   - RIPAbuffer: 10 mM Tris, pH 7.4, 150 mM NaCl, 1.0 mM EDTA, 0.1% SDS, 1.0% Triton X 100, 1.0% sodium deoxycholate, (4°C)
   - Sulfo-NHS-SS-biotin stock solution: Dissolve in dimethylsulfoxide (DMSO) to 200 mg/ml, (-20°C)
   - Tris(2-Carboxyethyl)phosphine Hydrochloride (TCEP) stock solution: 500 mM in H\(_2\)O, (-20°C, covered in foil to block light)

Day 2:

1. Prepare PBS\(^{2+}\) supplemented with 0.18g/ml glucose, 0.2% IgG/protease-free bovine serum albumin (PBS\(^{2+}\)/g/BSA). Pre-warm this solution to 37°C in water bath.

2. Thaw out the sulfo-NHS-SS-biotin stock solution on the bench top to melt the DMSO. Immediately prior to use, prepare fresh sulfo-NHS-SS-biotin stock solution (2.5 mg/ml in ice cold PBS\(^{2+}\), sufficient for 0.75 ml/well). Vortex the solution vigorously to solubilize the DMSO. Note that the NHS-biotin reagent is easily hydrolyzed in aqueous solution. Therefore, all solutions should be prepared immediately prior to use.

3. Biotinylation: Place plates on an ice bath in the cold room and rinse 3 x 2 ml with ice cold PBS\(^{2+}\). Be certain that plates are slightly angled to allow for complete drainage and removal of the buffer solution. Add 0.75 ml/well of the fresh sulfo-NHS-SS-biotin solution to each well. Incubate x 15’, 4°C on the ice bath with vigorous shaking. After the incubation is complete, prepare another fresh sulfo-NHS-SS-biotin solution. Replace the old solution with the fresh solution and incubate x 15’, 4°C.
Representative Results:

A representative immunoblot result is shown in Figure 2A. The strongest signal is in the "total" lane (T), which is total amount of surface protein prior to internalization. The "strip" control (S) should ideally be close to blank, which demonstrates that the strip was efficient for the experiment. The strip efficiency is calculated by comparing the density of the "strip" lane to that of the "total" lane (e.g. protein that was biotinylated in parallel with the strip, but was neither warmed to 37°C nor exposed to stripping solution). The following formula is used:

\[
\text{[1-(strip/total)]*100}
\]

Using this formula, the strip in Figure 2 was 99.8% efficient. Finally, you will see bands of lesser intensity than the total in the internalization lane(s) (I). In the example (Figure 2), cells treated with either vehicle or 1μM phorbol myristate acetate (PMA) during a 10' internalization and dopamine transporter internalization rates were measured for a 10' initial internalization period. The internalization rate is calculated as follows:

\[
\frac{\text{internalized}}{\text{total}}*100
\]

As seen in Figure 2B, 10.4% surface DAT internalized over 10' under vehicle-treated conditions. PMA treatment increased DAT internalization rates to 23.2% of total surface DAT.
Figure 1. Protocol illustration. Cells are biotinylated at 4°C to exclusively label the surface population, and are shifted to 37°C to initiate internalization. Following internalization, cells are rapidly chilled to stop endocytic processes and residual surface biotin is stripped by treating cells with a reducing agent. The only biotinylated proteins that remain are those that arose from the surface at t=0 and were internalized, thus protecting them from the stripping treatment. Biotinylated proteins are isolated by batch affinity chromatography with streptavidin beads and the protein of interest is detected by immunoblotting.

Figure 2. PKC activation increases the DAT endocytic rate. Internalization assay. PC12 cells stably expressing DAT were biotinylated, 4°C as described in "Detailed Protocol". Cells were rapidly warmed to 37°C ±1 μM PMA and incubated 10’, 37°C. Residual biotin was stripped by...
reducing, cells were lysed and biotinylated proteins were isolated by streptavidin affinity chromatography. (A) Representative immunoblot showing total surface DAT at t=0 (T), strip control (S), and internalized DAT (I) under the indicated conditions. (B) Bands were captured with a CCD camera and quantified with Quantity Data software (Bio-Rad). Data are expressed as % total DAT internalized/10 min.

Figure 3. Example immunoblot depicting a poor biotin strip. Internalization assay. PC12 cells stably expressing DAT were biotinylated, 4°C as described in "Detailed Protocol". Cells were rapidly warmed to 37°C and incubated 10', 37°C. Residual biotin was stripped by reducing, cells were lysed and biotinylated proteins were isolated by streptavidin affinity chromatography. The immunoblot shows total surface DAT at t=0 (T), strip control (S), and internalized DAT (I). Note the visible band in the strip control lane, indicative of poor strip efficiency.

Discussion

Common problems: The most common problem that arises in these experiments is poor strip efficiency. The efficiency of the strip is critical in being able to interpret the results. Unless the strip was highly efficient, it is not possible to conclude that any biotinylated proteins in the internalization lanes were, in fact, internalized from the surface. Strips ≥90% efficiency are optimal, and we discard any results if the strip falls below this level. An example of a poor strip is shown in Figure 3. Note that the band in the strip lane is quite visible, and corresponds to a strip efficiency of 34%. If poor strip efficiencies occur, fresh TCEP may not have been made immediately prior to adding to the cells. Alternatively, the TCEP may be degraded and new reagent will need to be purchased. Use of TCEP powder, rather than solution, may circumvent this problem.

Possible modifications: In the current report, we measured a relative DAT internalization rate, taken over the course of the initial 10 minutes of internalization, and compared it to the DAT internalization rate during PKC activation. Alternatively, an absolute rate could be measured by warming cells for increasing time points. While this may be possible, our experience suggests that the low signal levels at early time-points, coupled with inter-experimental variability, do not yield highly reproducible results at very early time-points. Also, although we used adherent cells, the assay can be modified for cell suspensions. Tissue suspensions, such as synaptosomes, can also be used. However, it is imperative that the integrity of the membranes is first established. If the preparation has a significant percentage of "leaky" membranes, the biotinylation reagent will label intracellular proteins and it will not be possible to reliably determine the endocytic rate. In this situation, immunoblots should also be probed for an intracellular marker protein, such as actin, to test whether the intracellular protein pool was exposed to the biotinylation reagent.

Acknowledgements

This work was funded by NIH grant #DA15169 to H.E.M.

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