Biotin Labeling and Quantitation of Cell-Surface Proteins

Major histocompatibility complex (MHC) antigens, along with a variety of other cell-surface receptors, have been shown to enter the endocytic pathway through the invagination of clathrin-coated pits. Previous assays used to measure endocytosis and recycling were cumbersome and often required radioactive reagents. Biotin labeling of cell-surface antigens is an established methodology for labeling all cell-surface proteins to study endocytosis without perturbing surface expression or endocytic transport, and for subsequently monitoring the proteins as they recycle back to the cell surface. Sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (sulfo-NHS-S-S-biotin) can be used to covalently label cell-surface proteins on viable cells with biotin. N-Hydroxysuccinimide (NHS) is a highly reactive ester group that covalently links to any polypeptide that contains either an unblocked terminal amine or an exposed amino group of a reactive lysine residue. Biotinylation of surface proteins provides a rapid and safe alternative to radioactive labeling.

The technique of protein biotinylation of viable nonadherent cells (see Basic Protocol) or adherent cells (see Alternate Protocol) allows direct, accurate quantitation of surface expression, endocytosis, and recycling for a variety of plasma-membrane receptors and antigens. The amount of biotin bound to a specific receptor or antigen can be quantitated using an antibody-capture enzyme-linked immunosorbent assay (ELISA; see Support Protocol 1) with horseradish peroxidase (HRP)–avidin detection. Utilization of this methodology provides a noninvasive technique to chemically tag cell-surface proteins and allows rapid comparison of the endocytosis pathways for a wide variety of cell-surface molecules. One is only limited by the availability of monoclonal antibody (mAb) to the specific cell-surface molecule in question. Two alternate methods can be used to measure protein biotinylation and endocytosis. These include an immunoblotting method (see Support Protocol 2), which is useful in confirming the specificity of the antibodies used in the capture ELISA, and a protocol for quantifying the surface half-life of biotinylated proteins (see Support Protocol 3).

**NOTE:** Carry out all steps, other than the warming for endocytosis, at 4°C or on ice. All centrifugations should be done at 4°C. This is essential, as even slight warming of cells or buffers permits protein endocytosis.

**DETECTION OF ENDOCYTOSIS IN NONADHERENT CELL LINES**

In this protocol, surface proteins on nonadherent cells are biotinylated using sulfo-NHS-S-S-biotin and allowed to endocytose during a 37°C incubation period, which ranges from 5 to 60 min. Cells are treated with a solution containing glutathione to remove biotin from proteins remaining at the cell surface, and are then lysed with Triton X-100. After intact nuclei are removed by centrifugation, lysates containing biotin-labeled proteins can be detected by capture ELISA (see Support Protocol 1) or one of the other techniques described (see Support Protocols 2 and 3) to quantitate endocytosis of cell-surface proteins and MHC antigens.

**Materials**

- Sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (sulfo-NHS-S-S-biotin; Pierce)
- HBSS (*APPENDIX 2*)
- Nonadherent cells

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HBSS with 5 mM Tris, pH 7.4
0.45 M sucrose in HBSS (optional)
0.3 mM primaquine solution in complete RPMI-1 (APPENDIX 2) with 5 mM HEPES
Glutathione stripping solution (see recipe)
Lysis buffer (see recipe)
0.5 M Tris·Cl, pH 7.4 (APPENDIX 2)

Additional reagents and equipment for quantitation of biotin-labeled protein (see Support Protocols 1 to 3)

Biotin label cell-surface antigens
1. Dissolve sulfo-NHS-S-S-biotin in HBSS at 10 mg/ml and store on ice for use the same day.

*Sulfo-NHS-S-S-biotin has an extender spacer arm, which reduces steric hindrance and allows the biotin group access to avidin. Sulfo-NHS-S-S-biotin is also water soluble, allowing the biotinylation to be carried out in the absence of organic solvents, thus avoiding protein denaturation. Buffers containing amines (such as Tris or glycine) should be avoided, since these compete with protein biotinylation reactions. Sulfo-NHS-S-S-biotin powder should be stored desiccated at −20°C and made fresh for each use.*

2. For each sample, wash 1–3 × 10⁷ nonadherent cells three times with 10 ml ice-cold HBSS.

*Separate samples are required for determining total cell surface levels and for measuring endocytosis. An extra control sample should be prepared in which endocytosis will be prevented (step 8).*

3. For each sample, resuspend cells in 1 ml ice-cold HBSS and add 50 µl of 10 mg/ml sulfo-NHS-S-S-biotin solution (final 0.5 mg/ml).

*The reaction is carried out in a balanced salt solution, like HBSS, to maintain cell viability and membrane integrity during biotin labeling.*

4. Gently rotate cells for 15 min at 4°C to promote biotin labeling of proteins.

5. To quench excess sulfo-NHS-S-S-biotin, wash cells three times by centrifuging 5 min at 200 × g, 4°C, and resuspending in 10 ml ice-cold HBSS with 5 mM Tris, pH 7.4.

*Removal of any free sulfo-NHS-S-S-biotin is essential prior to cell lysis.*

6. Aliquot samples for further analysis:
   a. For endocytosis: Aliquot ~10⁷ cells for each time point to be tested, centrifuge cells to remove HBSS, and proceed with steps 7 to 17. To determine efficiency of glutathione stripping of biotin-labeled surface proteins, resuspend an aliquot from the control sample in 2 ml ice-cold HBSS and keep on ice (see step 8).
   b. For biotin labeling: Aliquot ~10⁷ cells, centrifuge to remove HBSS, and proceed with step 18.

*Quantitation of the percent protein endocytosed requires measurements of both total cell-surface expression and endocytosis for proteins.*

Allow biotin-labeled surface proteins to undergo endocytosis
7. Resuspend biotinylated cells in 2 ml HBSS, prewarmed to 37°C.

8. Incubate in a 37°C water bath for varying lengths of time (5 to 60 min) to allow endocytosis. As a control, keep the control sample of biotinylated cells on ice to prevent endocytosis.
A glutathione solution will be used to remove or strip biotin from proteins remaining at the cell surface (steps 11 to 17). Endocytosed proteins labeled with biotin are resistant to this procedure.

The efficiency of the glutathione stripping varies between experiments and with each receptor tested within an experiment. To confirm the efficiency of stripping, cells that have been labeled with biotin at 4°C but have not undergone endocytosis are treated with glutathione solution. Typically 95% to 100% of the biotin label can be stripped off the proteins or antigens remaining at the cell surface using the glutathione solution.

9. **Optional:** Treat cells with 0.45 M sucrose in HBSS for 10 min at 37°C prior to biotinylation of surface proteins to block endocytosis of proteins or antigens through clathrin-coated pits. Then biotin label cells on ice in HBSS as normal (step 3). Incubate cells in this hypertonic sucrose solution during any subsequent 37°C incubations (e.g., steps 8, 16) to continually block endocytosis.

   Exposure of cells to hypertonic media blocks receptor-mediated endocytosis by preventing the formation of clathrin-coated pits.

   NOTE: Sucrose treatment may be toxic to cells, especially for extended periods of time. The toxicity of each treatment should be tested for each cell line prior to use, by trypan blue exclusion (APPENDIX 3B).

10. **Optional:** Treat cells with 0.3 mM primaquine solution during the incubation at 37°C (step 8) to allow protein internalization into the endocytic pathway without recycling back to the cell surface.

   Primaquine treatment, which nonspecifically disrupts endosomal/lysosomal function, can be used as a probe to establish recycling of biotin-labeled proteins. Primaquine has been shown to slow the rate of recycling while allowing internalization into the endosomes, and thereby increases the amount of biotin-labeled proteins within endosomes using this assay.

   NOTE: Primaquine treatment may be toxic to cells, especially for extended periods of time. The toxicity of each treatment should be tested for each cell line prior to use, by trypan blue exclusion (APPENDIX 3B).

**Strip biotin label from cell-surface proteins using glutathione**

11. Resuspend biotinylated samples in 5 ml ice-cold glutathione stripping solution to release biotin label from proteins at the cell surface.

12. Rotate cells for 25 min at 4°C.

13. Pellet the cells by centrifugation for 5 min at 200 × g, 4°C, and resuspend in 5 ml fresh ice-cold glutathione stripping solution.

14. Rotate cells for 30 min at 4°C.

15. Wash cells three times by centrifugation and resuspension in ice-cold HBSS. Proceed to step 18 (cell lysis) to quantitate biotinylation of proteins. Alternatively, perform the following steps to analyze recycling of endocytosed proteins.

16. **Optional:** To demonstrate the continuous cycling of endocytosed proteins back to the cell surface, rewarm glutathione-stripped cells to 37°C for varying lengths of time (5 to 60 min; step 8) to permit endocytosis and recycling.

17. **Optional:** Perform a second glutathione treatment as described in steps 12 to 15 to remove any biotin from biotinylated receptors or antigens that have recycled back to the plasma membrane during the second 37°C incubation (step 16).

**Prepare cell lysates for quantitation of biotin-labeling and antigen endocytosis**

18. Lyse cells 10 min at 4°C (on ice) in 1 ml lysis buffer.

   The final concentration of cells must be ≥10⁷ cells/ml to permit detection of labeled proteins.
19. Centrifuge lysates 5 min at 200 \( \times \) g, 4\(^\circ\)C, to remove intact nuclei. Proceed to quantitation of biotin-labeled proteins (see Support Protocols 1 to 3).

20. As a control in which both surface and intracellular proteins are labeled with biotin, lyse cells as described in steps 18 to 19, but add 0.5 mg/ml sulfo-NHS-S-S-biotin to the post-nuclear cell supernatant. Incubate 15 min on ice, then quench any unreacted sulfo-NHS-S-S-biotin by adding 0.5 M Tris \( \cdot \) Cl (pH 7.4) to a final concentration of 5 mM. Quantitate total biotin labeling of specific proteins (see Support Protocols 1 to 3).

**DETECTION OF ENDOCYTOSIS IN ADHERENT CELL LINES**

The Basic Protocol can be modified, as described here, for use with adherent cells.

**Additional Materials** *(also see Basic Protocol)*

150 \( \times \) 25–mm tissue culture dishes

1. Plate 1 \( \times \) 10\(^7\) adherent cells on a 150 \( \times \) 25–mm plate (1 plate/sample) and allow cells to attach overnight.

   *Separate plates are required for biotin labeling and for measuring endocytosis.*

2. Label cells as described (see Basic Protocol, steps 1 to 5), but perform washes without centrifugation, and perform biotinylation using 5 ml of 0.5 mg/ml sulfo-NHS-S-S-biotin solution per sample.

3. Place 5 ml prewarmed (37\(^\circ\)C) HBSS on the cells and incubate for the appropriate time (see Basic Protocol, step 8) in a CO\(_2\) incubator at 37\(^\circ\)C.

4. *Optional:* Block endocytosis or recycling (see Basic Protocol, steps 9 and 10).

5. Strip biotin from cell-surface proteins using glutathione as described (see Basic Protocol, steps 11 to 15), again performing washes without centrifugation. Perform optional endocytosis/stripping steps as necessary (steps 16 and 17).

6. To lyse cells, add \( \geq \)2 ml lysis buffer to each plate and incubate on ice for 10 min. Remove the cells using a cell scraper and transfer the lysates to a 15-ml conical centrifuge tube. Allow the lysis suspension to incubate for an additional 5 min on ice.

7. Centrifuge out the intact nuclei (see Basic Protocol, step 19).

8. Perform the ELISA with cell lysates as described (see Support Protocol 1), but use 200\(\mu\)l lysate per well, rather than 100\(\mu\)l. Alternatively, quantitate by immunoblotting (see Support Protocol 2).

**ELISA FOR QUANTITATING BIOTIN-LABELED PROTEINS**

The ELISA described in this protocol is used to quantitate the endocytosed biotin-labeled surface proteins. These proteins can include surface receptors or other antigens of interest. In all experiments, it is important to include a cell-surface protein endocytosis positive control. The transferrin receptor serves as an excellent positive control since it is known to rapidly internalize and recycle through the endocytic pathway. The cell surface biotin-labeling method described in the Basic Protocol allows for simultaneous tagging and monitoring of multiple surface receptors.

**Materials**

Purified monoclonal antibodies (mAbs) specific for the receptors or antigens of interest

Anti-transferrin receptor mAb (Pharmingen; Boehringer Mannheim)
PBS (APPENDIX 2)
5% (v/v) FBS in PBS
Irrelevant isotype-matched antibody (optional)
PBS-T: PBS containing 0.05% Tween 20
Biotin-labeled cell lysate (see Basic Protocol or Alternate Protocol)
2.5 µg/ml HRP-avidin solution: horseradish peroxidase–conjugated avidin in PBS containing 10% (v/v) heat-inactivated FBS
2,2′-Azino-di(3-ethylbenzthiazoline sulfonate) (ABTS; BioFX Laboratories)
96-well EIA/RIA high protein–binding plates
Microtiter plate reader with 405-nm filter

Prepare antibody-coated plates
1. Dilute each antibody to be used (purified mAbs of interest and anti-transferrin receptor mAb) to 1 to 10 µg/ml in PBS.

   Purified mAbs should be used, since they are more efficient at capturing receptors and other antigens than crude preparations such as antibody-containing supernatants from B cell hybridomas.

   The transferrin receptor serves as an excellent positive control for endocytosis of cell-surface proteins. This receptor is known to rapidly internalize and recycle through the endocytic pathway. Since the biotin-labeling technique allows for simultaneous tagging and monitoring of multiple surface receptors, the authors recommend that transport of the transferrin receptor be followed as a positive control in each experiment.

2. For each biotinylated sample to be tested, add 100 µl of the appropriate diluted antibody to triplicate wells in a 96-well EIA/RIA plate.
3. For each sample to be tested, add 100 µl diluted anti-transferrin receptor mAb to another triplicate set of wells as a positive control for endocytosis.
4. For each sample to be tested, add 100 µl of 5% FBS in PBS or an irrelevant isotype-matched antibody to another triplicate set of wells to use as a negative control for nonspecific (background) binding of proteins to the EIA/RIA plates.
5. As a final negative control, add 100 µl of the specific antibody of interest to one triplicate set of wells that will contain lysis buffer alone instead of sample lysate.
6. Incubate overnight at 4°C to allow antibody binding.
7. Wash all coated wells three times with 300 µl/well PBS-T.
8. Add 300 µl/well of 5% FBS in PBS and incubate 10 min at room temperature. Repeat two more times to block the wells.
9. Wash the wells three times with PBS-T.

Bind lysates to plates
10. Add 100 µl/well biotin-labeled cell lysates to the appropriately coated sample and control wells (steps 2 to 4). Add 100 µl/well lysis buffer to the sample-free negative control (step 5).
11. Incubate 2 hr at 4°C to allow antigen-antibody binding.
12. Wash plates five times with PBS-T.

Label with HRP-avidin
13. Add 100 µl/well of 2.5 µg/ml HRP-avidin solution.
14. Incubate 30 min at room temperature.

15. Wash five to eight times with PBS-T.

16. Add 100 µl/well ABTS.

    ABTS is the chromogenic substrate for the peroxidase.

17. Read the color reaction in a microtiter plate reader at 405 nm.

Perform statistical analysis

18. Use triplicate sets of ELISA data to calculate standard deviations for each measurement. To calculate biotin labeling of a protein at the cell surface or following endocytosis, subtract the average value absorbance at 405 nm for the control sample (step 4) from the average absorbance of the test sample.

    This will yield the adjusted mean absorbance value for each test sample. For example, if the mean absorbance for control or FBS sample was 0.063 and mean absorbance for cell surface biotin-labeled antigen was 1.064, then the adjusted mean absorbance for the cell surface would be 1.064 − 0.063 = 1.001. Similarly, if antigen internalized via endocytosis gives a measured value of 0.694, the adjusted mean absorbance value is 0.694 − 0.063 = 0.631.

19. Set the amount of cell surface antigen labeled with biotin at 4°C to 100% to facilitate calculations of relative endocytosis. Calculate the percent endocytosis for each antigen or receptor being studied by dividing the adjusted mean absorbance value for each endocytosis sample by the adjusted mean absorbance for the cell surface sample, and multiplying by 100.

    Thus, in the example above, the adjusted mean absorbance for endocytosis (0.631) is divided by cell surface adjusted mean absorbance (1.001) to give a final percent endocytosis of 63%.

20. Compare absorbance values using one-way analysis of variance (ANOVA; Ryder and Robakiewicz, 2000). Use the Tukey HSD procedure to define differences when ANOVA results are significant (Milliken and Johnson, 1984).

SUPPORT PROTOCOL 2

IMMUNOBLOTTING FOR DETECTION OF BIOTINYLATED PROTEINS

The techniques of immunoprecipitation and immunoblotting can be used as an alternative to ELISA (Support Protocol 1) to follow protein biotinylation and endocytosis. This approach is useful in confirming the specificity of antibodies used in the capture ELISA. A rough quantitation of endocytosis and glutathione stripping efficiency can be determined by densitometric scanning of biotin-labeled proteins after immunoprecipitation and immunoblotting. However, this method of quantitation is considerably more time consuming and less accurate than the capture ELISA.

Materials

    Biotin-labeled cell lysate (see Basic Protocol or Alternate Protocol)
    Mouse antibodies specific for cell-surface receptors or antigens of interest
    Protein A–Sepharose or protein G–Sepharose beads precoated with rabbit anti–mouse IgG (UNIT 8.3)
    TBS (see recipe)
    2× reducing SDS-PAGE sample buffer (see recipe)
    1% (w/v) BSA/0.05% (v/v) Tween 20/PBS (see APPENDIX 2 for PBS)
    0.5 µg/ml horseradish peroxidase (HRP)–streptavidin in PBS
    Enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech)
    Electroblotting apparatus
Additional reagents and equipment for immunoprecipitation (UNIT 8.3), SDS PAGE (UNIT 8.4), and electroblotting (UNIT 8.10)

1. Immunoprecipitate 1-ml samples of biotin-labeled cell lysates with antibodies specific for the receptors or antigens of interest by rotating samples for 2 hr at 4°C.

   *The final antibody concentration should be 1 to 5 µg/ml. See UNIT 8.3 for additional details on immunoprecipitation.*

2. Add 50 µl protein A–or protein G–Sepharose beads precoated with rabbit anti–mouse IgG (UNIT 8.3).

3. Rotate 30 min at 4°C.

4. Wash three to five times with TBS.

5. Elute the antigen-antibody complexes by boiling 5 min in 50 µl of 1× reducing SDS PAGE sample buffer.

6. Run samples on a 10% (w/v) SDS polyacrylamide gel according to standard protocol (UNIT 8.4).

7. Transfer to nitrocellulose using an electroblotting apparatus.

   *See UNIT 8.10 for additional details on electroblotting.*

8. Block the nitrocellulose overnight in 1% BSA/0.05% Tween 20/PBS at 4°C.

9. Probe the blot for 30 min with 0.5 µg/ml HRP-streptavidin in PBS at 4°C.

10. Develop blot with enhanced chemiluminescence reagents (ECL) according to manufacturer's instructions.

11. Expose to X-ray film for ~1 min at room temperature.

**QUANTITATING SURFACE HALF-LIFE OF PROTEINS USING BIOTIN LABELING**

A modification of the capture ELISA procedure described in Support Protocol 1 can be used to determine the surface half-life of a variety of cell-surface receptors and antigens. This procedure starts with a biotinylation procedure that is a modification of the one presented in the Basic Protocol for nonadherent cells.

**Additional Materials (also see Basic Protocol)**

   Sulfo-N-hydroxysuccinimido-biotin (Sulfo-NHS-biotin; Pierce) 0.45-µm filter unit

1. Dissolve sulfo-NHS-biotin in HBSS at 10 mg/ml, filter sterilize with 0.45-µm filter unit, and store on ice for use the same day.

   *Sulfo-NHS-biotin should be stored desiccated at −20°C and made fresh for each use.*

2. Wash and biotinylate cells as described (see Basic Protocol, steps 2 to 4) using sterile sulfo-NHS-biotin.

3. Wash biotinylated cells three times with 10 ml sterile HBSS plus 5 mM Tris, pH 7.4.

4. Centrifuge to remove HBSS/Tris and resuspend at 0.5–1 × 10^6 cells/ml in the appropriate medium for the cells being tested.
5. Incubate cells at 37°C. Remove equal-volume aliquots of these cells at various time intervals (e.g., 0 to 24 hr) starting with a zero time point.

6. Wash the aliquots three times with HBSS by centrifuging 5 min at 200 × g, 4°C. Freeze the cell pellets at −80°C until use.

7. Thaw the pellets and lyse them by resuspending in lysis buffer to a final concentration of 1 × 10^7 cells/ml.

8. Use the lysates in the ELISA as described (see Support Protocol 1).

9. Calculate the percentage of each protein remaining in the cell at each time point by dividing the measured absorbance at each time by the absorbance reading at zero time. All absorbance readings should first be corrected by subtracting the absorbance of control samples.

   For example, if the FBS control values are 0.268 and 0.260 at 0 and 4 hr, respectively, and transferrin receptor sample values are 0.733 and 0.581 at 0 and 4 hr, respectively, then the percentage of surface transferrin receptors remaining on or in the cells after 4 hr is (0.581 − 0.260)/(0.733 − 0.268) × 100 = 69.0%.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5.

**Glutathione stripping solution**
Dissolve in 18 ml water:
- 310 mg glutathione (0.05 M final)
- 0.30 ml 5 M NaCl (0.075 M final)
- 0.20 ml 0.1 M EDTA (0.001 M final)
Just prior to use add:
- 0.150 ml 10 M NaOH (0.075 M final)
- 2 ml FBS (10% final)
Prepare fresh each day
Keep on ice and use cold

The final pH should be ∼8.6 and should not be adjusted.

**Lysis buffer**
HBSS (APPENDIX 2)
1% (v/v) Triton-X 100
Store up to 6 months at 4°C
Immediately before use add the protease inhibitors:
- 0.02 mM phenylmethylsulfonyl fluoride (PMSF)
- 0.01 mM N-tosyl-lysine chloromethyl ketone (TLCK)

**Reducing SDS-PAGE sample buffer, 2×**
- 20 ml glycerol
- 2 ml 2-mercaptoethanol
- 4 g SDS
- 1.52 g (0.13 M) Tris
- 1 mg bromphenol blue
- 78 ml water
Adjust pH to 6.8 with HCl
Store up to 6 months at 4°C
Tris-buffered saline (TBS)
10 mM Tris-Cl, pH 7.4 (APPENDIX 2)
150 mM NaCl
0.1% (v/v) Triton X-100
Store up to 6 months at 4°C

COMMENTARY

Background Information

Receptor-mediated endocytosis enables eukaryotic cells to continuously internalize receptor-bound ligands and other plasma-membrane proteins and lipids, as well as extracellular solutes. A variety of cell-surface receptors and major histocompatibility complex (MHC) antigens have been shown to enter the endocytic pathway of cells. Extensive studies have demonstrated the rapid endocytosis and recycling of transferrin receptors (TfR; Ajioka and Kaplan, 1986). TfR is endocytosed into clathrin-coated pits, which pinch off from the plasma membrane and form clathrin-coated vesicles. The vesicles rapidly uncoat and fuse together or with pre-existing vesicular structures called early endosomes, where the acidic environment of endosomes mediates the dissociation of Fe³⁺ from transferrin (Tfn), while Tfn remains bound to its receptor. The Tfn-TfR complex then recycles back to the cell surface via carrier or recycling vesicles, allowing cells to reuse these receptors to take up additional iron from the extracellular environment (Geuze et al., 1987; Gruenberg and Howell, 1989; Pearse and Robinson, 1990). More recently, MHC class I and II antigens have been shown to endocytose and recycle, although far less is known about this process and its regulation (Tse and Pernis, 1984; Reid and Watts, 1990). The recycling of MHC antigens is thought to play an important role in antigen presentation. Studies of this process for plasma-membrane receptors and antigens has been dependent upon the availability of radiolabeled ligands or affinity reagents for individual proteins (Bretscher and Lutter, 1988; Pinet et al., 1995). Assays to measure the endocytosis and recycling of MHC antigens have been cumbersome, involving iodinated antibodies, cleavable radiolabeling reagents (Bretscher and Lutter, 1988), or biotinylated Fab fragments detected with radiolabeled avidin (Pinet et al., 1995). To facilitate studies of cell-surface receptors and MHC antigens, a nonradioactive methodology was devised to quantitate surface expression and endocytosis. Biotin-labeling of cell-surface antigens has been established as a methodology for monitoring transport to the cell surface (Brachet et al., 1997), polarized sorting (Marmorstein et al., 1996), and endocytosis (Volz et al., 1995). For receptors such as TfR and MHC class II antigens, the addition of a biotin tag has been shown not to perturb surface expression or endocytic transport (Pinet et al., 1995; Volz et al., 1995). Yet, direct quantitation of the cell surface and endocytosed biotin-tagged proteins has been cumbersome and has relied upon PAGE followed by densitometry. Here the techniques of protein biotinylation in viable cells has been extended to permit direct, accurate quantitation of surface expression, endocytosis, and recycling for a wide variety of plasma-membrane proteins, including receptors and MHC antigens. This assay detects even low levels of endocytosis for specific cell-surface molecules, but does not induce aberrant internalization of proteins from the plasma membrane, as demonstrated in control studies using the Fc receptor in B cells. Fc receptors expressed by B cells lack the appropriate signal sequences for endocytosis and were found not to internalize following biotinylation. Confirmation that the biotin label tags only cell-surface proteins was obtained via studies of an endosomal/lysosomal protease, cathepsin D. This intracellular protease could be readily biotin labeled in detergent-lysed cells, but was not tagged by cell-surface biotinylation (Turvy and Blum, 1998).

Biotinylation of surface proteins provides a rapid and safe alternative to radioactive labeling that is independent of ligand binding. N-Hydroxysuccinimide (NHS) is a highly reactive ester group that introduces a biotin tag covalently into any polypeptide that contains either an unblocked terminal amine or an exposed amino group of a reactive lysine residue (Anderson et al., 1964). Since lysine residues account for 7% of the total amino acid residues in an average protein, the probability that biotinylation of any single protein will occur is extremely high (Luna, 2000). Sulfo-NHS-S-S-biotin is advantageous due to its water solubility and membrane impermeability, which allow only cell-surface proteins to be labeled. Endo-

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cytosis of the biotin-tagged proteins can be detected following incubation of cells at 37°C. Treatment of these cells with a reducing reagent releases biotin from cell-surface proteins, while endocytosed molecules are protected and retain their biotin label. The amount of biotin bound to a specific receptor or antigen can be quantitated using an antibody-capture technique and HRP-avidin detection. Extensive studies with this assay demonstrated that the basal levels of TIR or MHC class II antigen endocytosis measured were comparable to results obtained with more complex radioactive assays. Biotin labeling was specific for cell-surface proteins, and this modification did not promote the endocytosis of proteins lacking internalization signals, such as the B lymphocyte Fc receptor (Amigorena et al., 1995). Furthermore, the assay allows rapid comparison of the endocytosis of a wide variety of cell-surface molecules on immune cells.

The recycling of internalized biotin-labeled molecules from endosomes back to the plasma membrane can be demonstrated in two ways. Primaquine nonspecifically disrupts endosomal/lysosomal function and has been shown to slow the rate of recycling of TIR (Schwartz et al., 1984; Stoovogel et al., 1996) and MHC class II (Reid and Watts, 1990). Therefore, primaquine treatment of cells increases the amount of biotin-labeled proteins within endosomes. The mechanism by which primaquine blocks receptor recycling has not been fully elucidated, but most likely involves dissipation of endosomal pH gradients and osmolarity. Alternatively, the recycling of molecules can be demonstrated by permitting cells to endocytose biotin-labeled proteins followed by removal of any remaining surface biotin by glutathione cleavage. These cells are then allowed to resume metabolism to permit re-expression of the internalized biotin-labeled molecules. A second glutathione cleavage results in a loss of biotin label from proteins that recycled to the plasma membrane during the second warming.

Exposure of cells to hypertonic media blocks receptor-mediated internalization by preventing the formation of clathrin-coated pits (Daukas and Zigmond, 1985). Microscopy studies have revealed that hypertonic media have the same effect as K⁺ depletion in triggering cells to display empty clathrin “microcages” at the cell surface (Larkin et al., 1983). Abnormal formation of microcages inhibits internalization by rendering clathrin unavailable for assembly into normal coated pits (Heuser and Anderson, 1989). The addition of hypertonic sucrose solutions reduces the internalization of both MHC class II proteins and TIR (Bos et al., 1995).

Critical Parameters and Troubleshooting

Sulfo-NHS-S-S-biotin should be stored desiccated at −20°C and made fresh for each use. The glutathione solution also needs to be made fresh for each experiment. It is important to keep the cell samples and all buffers cold because even slight warming may allow endocytosis to occur. Because this method allows multiple receptors to be monitored simultaneously, the authors recommend that the TIR be analyzed as a positive control. In addition, to confirm the membrane impermeability of individual cell lines, an intracellular protein such as a cytoplasmic or lysosomal resident protein should also be monitored. To determine the background absorbance values for the ELISA, wells should be coated overnight with FBS or an irrelevant control antibody. Each sample of cell lysates should be added to these wells in triplicate to establish the background for each condition.

Purified antibodies are more efficient at capturing antigens than crude preparations of tissue culture supernatant containing antibodies. The authors suggest using only purified antibodies for this assay. Not all antibodies will work well in a capture ELISA, and the optimal concentration for each antibody must be empirically determined. It is important to test each antibody at a range of concentrations. A sample of cells should be surface biotinylated, and an ELISA should be performed to determine the ability of each antibody to capture its antigen and to select an optimal concentration for antibody use.

Anticipated Results

Results in human B cell lines demonstrated that ∼60% to 70% of biotinylated TIR molecules and 17% to 22% of biotinylated MHC class II DR molecules are internalized following a 30-min incubation at 37°C. Only ∼3% to 6% of MHC class I molecules are internalized during this time. The glutathione stripping efficiency varies between receptors within an experiment and between experiments, and therefore needs to be measured for each molecule in each experiment. However, ∼95% to 100% of biotin moieties should be cleaved following glutathione stripping of biotinylated cells that are kept on ice. Primaquine results in
an increased accumulation of intracellular biotinylated receptors if these receptors normally recycle to the plasma membrane. If no increased accumulation is detected, it may suggest that these molecules internalize, but do not recycle to the plasma membrane. If recycling is measured by multiple rounds of warming and glutathione stripping, a decrease in intracellular biotinylated molecules will be detected for recycling receptors and antigens. The endocytosis of cell-surface receptors and antigens that enter the cell through clathrin-dependent mechanisms should be blocked by sucrose treatment, resulting in a decrease in the amount of intracellular biotinylated molecules detected.

**Time Considerations**

This assay takes ~7 to 10 hr depending on whether endocytosis alone is measured or recycling of molecules is also quantitated. The start of each sample should be staggered so that each sample reaches the lysis step at approximately the same time. Alternatively, each sample can be pelleted and frozen at ~80°C before the lysis step. The ELISA can then be performed the following day.

**Literature Cited**


Volz, B., Orberger, G., Porwoll, S., Hauri, H.P., and Tauber, R. 1995. Selective reentry of recycling cell surface glycoproteins to the biosynthetic

**Key References**


*Overview of membrane protein recycling in lymphocytes, including the first description of recycling MHC molecules in lymphocytes.*

Reid and Watts, 1990. See above.

*First description of MHC class II endocytosis and recycling.*

Turvy and Blum, 1998. See above.

*First publication of this methodology, describing the use of biotin labeling to quantitate cell-surface protein endocytosis.*

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