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High-Throughput Analysis of the Dynamics of Recycling Cell Surface Proteins

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Summary Recycling via the plasma membrane is a key feature that is shared by many membrane proteins. Using a combination of indirect immunofluorescence labeling and fluorescence detection using a fluorescence multiwell plate reader, we exploited the possibilities of quantitatively measuring the trafficking kinetics of transmembrane proteins. Parameters that can be studied include dynamic appearance/presence at the cell surface, recycling via the cell surface, and internalization. For the insulin-responsive glucose transporter GLUT4 (glucose transporter number 4), details are presented on how to quantitatively measure insulin-induced GLUT4 translocation toward the plasma membrane (transition state) and to analyze cell surface recycling of GLUT4 in basal and insulin-stimulated cells (steady state).

Keywords Cell surface recycling; GLUT4; high throughput; immunofluorescence; intracellular trafficking.

1 Introduction

Many transmembrane proteins fulfill their normal function at the plasma membrane. This includes receptors, channels, or transporter proteins; each communicates with the external environment of the cell in some way to aid

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the regulation of cell growth and maintenance. Such proteins are synthesized in the endoplasmic reticulum (ER) but are equipped with targeting signals to enable them to navigate throughout the biosynthetic track to the plasma membrane. Although some membrane proteins are virtually excluded from the plasma membrane (e.g., nuclear and ER proteins), others are resident plasma membrane proteins, continually recycle between the cell surface and intracellular membranes, or even appear at the plasma membrane in a highly regulated fashion so that their specific function can be called on only when required. An example of the last is the insulin-responsive glucose transporter GLUT4 (glucose transporter number 4); its cell surface appearance is regulated by insulin to aid in the absorption of nutrients after a meal *(1)*.

Although it can be readily established via various methods whether a transmembrane protein trafficks via the cell surface, it remains a challenge to quantitatively analyze this feature. Cell surface iodination and biotinylation have been used as well as immunofluorescence microscopy in a qualitative or semiquantitative manner, but these techniques are laborious and not highly quantitative (*see* **Note 1**). In addition, the trafficking of cell surface proteins has been studied using labeled ligands, but obviously this method is limited to receptors only, while the binding of the ligand to its receptor may very well modify its trafficking, thereby only allowing the analysis of the activated receptor.

We have developed a novel, straightforward, high-throughput 96-well assay to measure cell surface recycling in a highly quantitative fashion based on indirect immunofluorescence labeling and detection using a multiwell fluorescence plate reader *(2,3)* (*see* **Note 2**). This assay is not limited to receptor studies but allows for the analysis of trafficking of all cell surface transmembrane proteins in both stimulated and nonstimulated cells. In the current report, using GLUT4 as an example, we describe in detail two different aspects of protein trafficking that can be analyzed with this technique.

First, in transition experiments it can be readily and quantitatively determined what percentage of the protein resides at the plasma membrane before and after stimulation, at what speed the transition takes place, and whether a quantal mechanism or a mechanism of constant turnover underlies this process. In the case of GLUT4, the last point addresses the question whether insulin dose dependently increases the trafficking speed of all GLUT4 molecules in the cell or whether insulin dose dependently recruits quantal amounts of GLUT4 toward the cell surface *(3)*. Second, steady-state experiments determine the relative amount of the protein that participates in cell surface recycling (which does not necessarily need to be 100%, as is the case for GLUT4), the time that it takes for all participating molecules to reach the plasma membrane, and exocytosis and endocytosis rates. Taken together, the technique described here readily determines many variables of cell surface protein trafficking in a highly quantitative manner. As this is a high-throughput technique, it may also very well be useful for drug-screening studies.

2 Materials

2.1 Cell Culture of BOSC23 Cells and Production of Retroviral Particles

1. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (Invitrogen, Paisley, UK), supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin (200 units/L), and streptomycin (200 g/L; Invitrogen).
2. DMEM with 4.5 g/L glucose (Invitrogen), supplemented with 10% FBS (Invitrogen) but without penicillin and streptomycin.
3. Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Invitrogen).
4. Solution of trypsin (0.5 g/L) and ethylenediaminetetraacetic acid (EDTA; 0.2 g/L) in Hanks' balanced salt solution without $CaCl_2$ and $MgCl_2$ (Invitrogen).
5. 0.2% gelatin (Merck/VWR, Strasbourg, France) (*see* **Note 3**).
6. 0.5% glutaraldehyde (Sigma-Aldrich, Lyon, France).
7. 50 m*M* glycine (Sigma).
8. 25 *M* chloroquine (Sigma) in DMEM, with 4.5 g/L glucose (Invitrogen), supplemented with 10% FBS (Invitrogen), without penicillin and streptomycin. The chloroquine solution should be prepared just before use.
9. HBS solution (2X): 50 m*M* HEPES, 10 m*M* KCl, 12 m*M* D-glucose, 280 m*M* NaCl, 1.5 m*M* Na_2HPO4, pH 7.05.
10. $CaCl_2$ (2*M*).
11. Deoxyribonucleic acid (DNA): Complementary DNA (cDNA) encoding hemagglutinin (HA)-tagged GLUT4 in retroviral pBABE vector *(4)*.

2.2 Cell Culture of 3T3-L1 Cells and Viral Infection

1. 3T3-L1 preadipocytes were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France).
2. 3T3-L1 preadipocyte propagation medium: DMEM with 4.5 g/L glucose (DMEM) (Invitrogen), supplemented with 10% newborn calf serum (NBS) (PAA, Pasching, Austria), penicillin (200 units/L), and streptomycin (200 g/L; Invitrogen).
3. 700 *M* human insulin (Lilly, Suresnes, France), stored at 4 °C.
4. 500 m*M* 3-isobutyl-1-methylxanthine (IBMX, Sigma) in dimethylsulfoxide (DMSO); aliquots are stored at −20 °C and are not refrozen after thawing. Final concentration in medium: 0.5 m*M*.
5. Biotin (Sigma), dissolved at a concentration of 0.1 mg/mL in water; aliquots are stored at 4 °C. Final concentration in medium: 0.1 g/mL.

6. Dexamethasone (Sigma), dissolved at a concentration of 1 mg/mL in ethanol; aliquots are stored at –20 °C. Final concentration in medium: 0.1 g/mL.
7. 3T3-L1 preadipocyte differentiation medium A: DMEM with 4.5 g/L glucose (DMEM) (Invitrogen), supplemented with 10% FBS (PAA), penicillin (200 units/L), streptomycin (200 g/L; Invitrogen), 860 n*M* insulin, 0.5 m*M* IBMX, 0.1 g/mL biotin, 0.1 g/mL dexamethasone.
8. 3T3-L1 preadipocyte differentiation medium B: DMEM with 4.5 g/L glucose (DMEM) (Invitrogen), supplemented with 10% FBS (PAA), penicillin (200 units/L), streptomycin (200 g/L; Invitrogen), 350 n*M* insulin.
9. Polybrene hexadimethrine bromide (Sigma), at a concentration of 1 mg/mL in water. Aliquots are stored at −20 °C and not refrozen on thawing.
10. Puromycin (Sigma), dissolved at a concentration of 10 mg/mL in water. Aliquots are stored at −20 °C and not refrozen on thawing. For selection medium, 100 L stock are added to 500 mL DMEM supplemented with 10% NBS, penicillin, and streptomycin (final concentration puromycin is 2 g/mL).

2.3 *Fluorescence Assays*

1. Black clear-bottom 96-well plates (Greiner, Poitiers, France).
2. Electronic multichannel pipetor (Impact 2, Matrix, Cheshire, UK) (*see* **Note 4**).
3. 700 *M* human insulin (Lilly), stored at 4 °C.
4. Paraformaldehyde (Fischer Scientific, Illkirch, France), which is dissolved in sodium phosphate buffer at a concentration of 16% (w/v), final pH 7.4. Aliquots are stored at −20 °C and not refrozen after thawing.
5. 50 m*M* glycine (Sigma).
6. Saponin (Sigma), 10% (w/v) in PBS containing 0.92 m*M* $CaCl_2$ and 0.50 m*M* $MgCl_2$. Solution is filtered through a 0.22- m filter and stored at room temperature.
7. Normal swine serum (Dako Corp., Trappes, France), stored at 4 °C.
8. Murine anti-HA ascites (Covance, Berkeley, CA), stored at −20 °C.
9. Mouse gamma globulin (Jackson ImmunoResearch, West Grove, PA), stored at −20 °C.
10. Alexa 488-conjugated goat-antimouse antibody (Molecular Probes/Invitrogen).
11. Fluorescence multiwell plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany).

3 Methods

For the ectopic expression of HA-GLUT4 in 3T3-L1 adipocytes, 3T3-L1 preadipocytes are infected with retrovirus that is generated using the packaging cell line BOSC23. After infection and selection of the 3T3-L1 preadipoctes, their differentiation into adipocytes is induced.

The described assays are based on indirect immunofluorescence detection. The principle of transition-state experiments is schematically represented in Fig. 1. The cells, expressing an HA epitope tag in an extracellular domain of GLUT4, are chemically fixed and either left untreated or permeabilized with saponin, after which the tag is immunolabeled. As can be seen from Fig. 2, the choice and concentration of the permeabilizing agent is important (see also *Ref. 5*). In

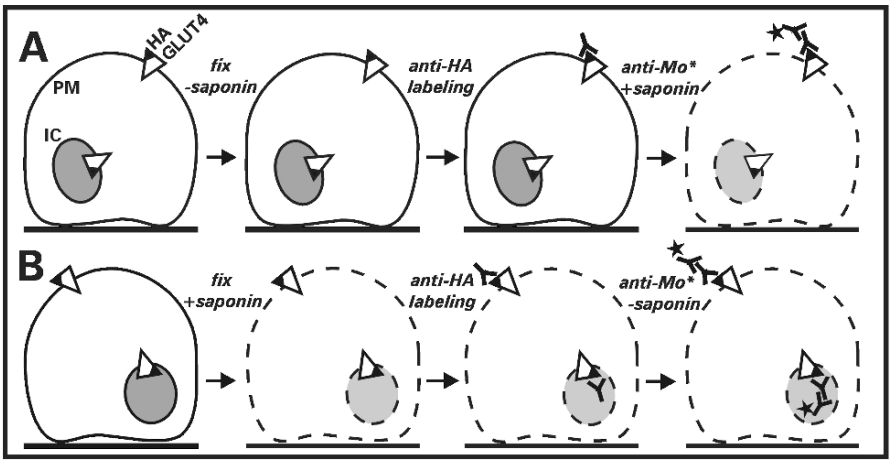

Fig. 1 Experimental setup of transition state assay. Cells are grown in 96-well plates, incubated with or without insulin, fixed, and left untreated **(A)** or permeabilized **(B)**. Then, all cells are incubated with primary murine anti-HA antibody and fluorescent antimouse secondary antibody subsequently. Fluorescence is measured using a multiwell plate reader. Ratio A:B × 100 gives percentage HA-GLUT4 at the cell surface. HA, influenza hemagglutinin epitope tag; IC, intracellular compartment; PM, plasma membrane.

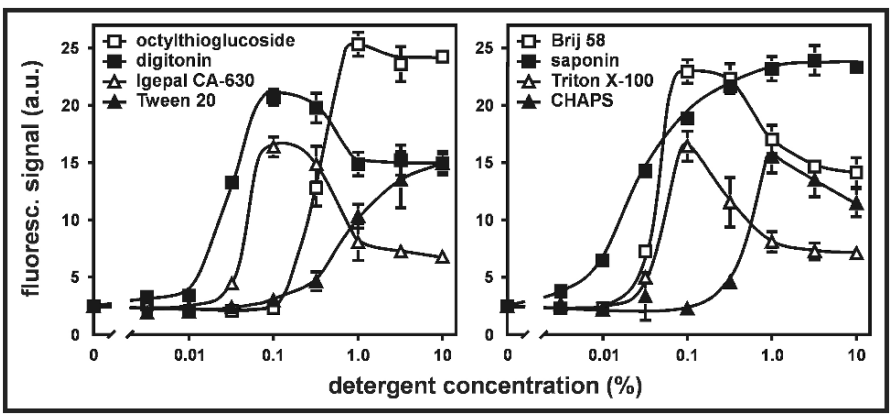

Fig. 2 Effect of detergents on the detection of total cellular HA-GLUT4. Cells are chemically fixed and treated for 20 min with the indicated permeabilizing agents at various concentrations. Cells are labeled with murine anti-HA antibody and fluorescent antimouse antibody subsequently. Note that not all permeabilizing agents allow the detection of the same amount of HA-GLUT4.

addition, for quantitative analysis it is essential to establish the level of nonspecific binding of the antibodies and to correct for this background (Fig. 3).

For steady-state experiments, live cells are incubated with anti-tag antibody, followed by chemical fixation and immunofluorescent detection of the anti-tag antibody. The level of fluorescence when the cells are permeabilized with saponin and incubated with primary antibody after fixation provides the normalizing measure of the total cellular HA-GLUT4. The steady-state experiments determine the dynamics of cell surface GLUT4 recycling in basal and insulin-stimulated cells and the percentage of GLUT4 that is involved in this recycling pathway. Examples of transition and steady-state assays are shown in Fig. 4.

3.1 Propagation of BOSC23 Cells

1. BOSC23 cells are routinely grown in 6-cm Petri dishes at 37 °C and 5% CO_2.
2. The medium is replaced every 2 or 3 days with fresh medium (DMEM with 4.5 g/L glucose and FBS, penicillin, and streptomycin).
3. Once the cells are almost confluent, the medium is aspirated, 1.5 mL of trypsin/EDTA solution are added (*see* **Note 5**), and the cells are incubated for 1 min at 37 °C.
4. Using an inverted light microscope, the cells are examined to ensure that they are detached, and medium is added. The cells are then transferred to a tube and centrifugated for 5 min at 300 *g* in a tabletop centrifuge.
5. Medium/trypsin is aspirated, and the cells are resuspended in fresh medium and seeded in a 1:5 dilution (cells from one dish are seeded into five new dishes) (*see* **Note 6**).

3.2 Production of Retroviral Particles

1. One day before infection, 10-cm tissue culture Petri dishes are coated with 0.2% gelatin for 1 h.
2. Gelatin is discarded, and dishes are rinsed once with PBS.
3. The Petri dish is incubated for 10 min with 0.5% glutaraldehyde in PBS.
4. Glutaraldehyde is discarded, and the dish is rinsed once with PBS.
5. The remaining glutaraldehyde is quenched by incubating the Petri dish for 5 min with 50 m*M* glycine in PBS and two times for 5 min with DMEM/FBS (discarding medium in between).
6. The BOSC23 cells are trypsinized (as in Subheading 3.1., steps 3–5) and seeded into the gelatin-coated Petri dish in such a way that the next day the confluence will be 80% (in practice, this means that the cells from two nearly confluent 10-cm dishes are seeded into three gelatin-coated dishes).

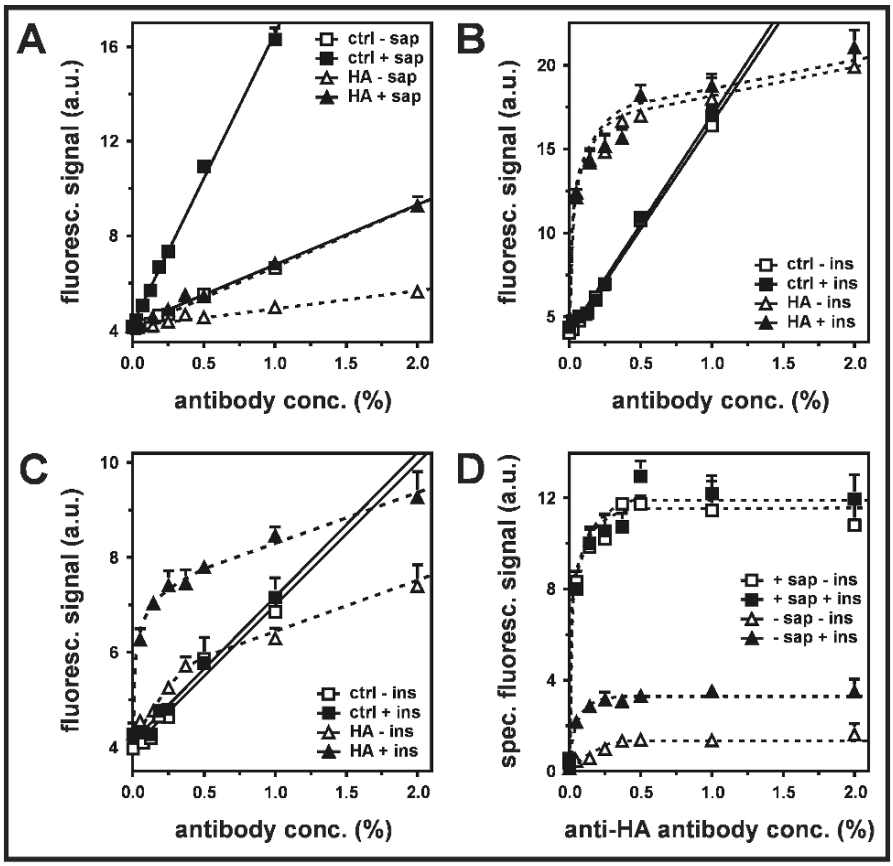

Fig. 3 Analysis of nonspecific background of anti-HA antibody and determination of the concentration to use in transition assay. **(A)** Noninfected cells were fixed and left untreated (−sap) or permeabilized using saponin (+sap). Then, the cells were incubated with various concentrations (v/v) of anti-HA tag antibody (HA) or nonspecific control antibody (ctrl) and subsequently with fluorescent secondary antibody. In this way, the relative concentration of the nonspecific antibody that needs to be used in the assay was determined in relation to the concentration of anti-HA antibody (giving similar background fluorescence). **(B)** Basal (−ins) and insulin-stimulated (+ins) HA-GLUT4-expressing cells were fixed and permeabilized, followed by labeling with nonspecific control (ctrl) or anti-HA antibody. Note that at concentrations of anti-HA higher than 0.5% (v/v) the increase in fluorescence signal is linear (representing nonspecific signal), indicating that from this concentration all HA epitopes are saturated with antibody. **(C)** Basal (−ins) and insulin-stimulated (+ins) HA-GLUT4-expressing cells were fixed without permeabilization, followed by labeling with nonspecific control (ctrl) or anti-HA antibody. Note that also, at concentrations of anti-HA higher than 0.5% (v/v), the increase in fluorescence signal is linear. **(D)** Basal (−ins) and insulin-stimulated (+ins) HA-GLUT4-expressing cells were fixed and left intact (−sap) or permeabilized (+sap), followed by labeling with nonspecific control (ctrl) or anti-HA antibody. At each concentration of anti-HA antibody, the background fluorescence was calculated (by means of nonspecific antibody, corrected for the difference in background fluorescence as determined in **(A)**, and subtracted. Note that concentrations of anti-HA antibody higher than 0.5% (v/v) did not result in more specific fluorescence, indicating that this concentration could be used for transition experiments.

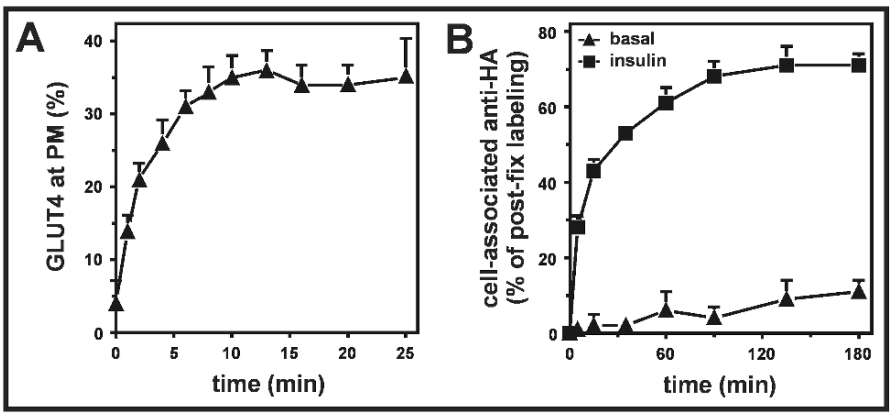

Fig. 4 Examples of transition-state and steady-state experiments. **(A)** Transition-state experiment: Insulin (200 n*M*) is added to the cells, and the increase in cell surface GLUT4 levels is measured **(B)** Steady-state experiment: Cells are incubated for 20 min in the absence or presence of 200 n*M* insulin (resulting in a steady-state GLUT4 distribution), followed by the addition of anti-HA antibody. Amounts of antibody taken up by the cell are determined and expressed as percentage of the total amount of antibody that can bind to fixed permeabilized cells. (Reproduced from ***Ref. 4*** with permission from the American Society for Microbiology.).

7. One day after seeding of the cells into the gelatin-coated dishes, just prior to transfection of the cells, the medium is replaced with 4 mL DMEM/FBS without penicillin/streptomycin, supplemented with 25 *M* chloroquine.
8. For each dish, 10 g DNA and H_2O are combined to a total volume of 438 L, and 62 L of 2 *M* $CaCl_2$ are added. The solution is vortexed, and dropwise 500 L of the 2X HBS solution is added while keeping the tube on the vortex.
9. The DNA/calcium phosphate solution is directly added to the chloroquine-containing medium that is covering the cells.
10. After 10 h, the medium is replaced with 5 mL DMEM/FBS without penicillin/streptomycin.
11. Virus-containing medium is collected at 48 h after transfection of the cells. In case the cells become fully confluent at 24 h after transfection, the virus is collected at this time-point.
12. The medium is centrifugated for 5 min at 300 *g* in a tabletop centrifuge.
13. The virus is aliquoted and stored at −80 °C (or used directly) (*see* **Note 7**).

3.3 Propagation of 3T3-L1 Preadipocytes

1. The cells are cultured at 37 °C and 10% CO_2.
2. The medium is replaced every 2 or 3 d with fresh medium (DMEM with 4.5 g/L glucose, NBS, penicillin, and streptomycin).

3. Once the cells are 60–80% confluent, medium is aspirated, cells are washed once with PBS, and trypsin/EDTA solution is added (5 mL for a 175-cm^2 cell culture flask) (*see* **Note 8**).
4. After 5–15 s, trypsin/EDTA is aspirated, and cells are incubated for 3.5 to 4 min at 37 °C.
5. The cells are examined for detachment using an inverted light microscope, resuspended in propagation medium, and seeded onto new culture plastics (dilution 1:10 to 1:40).

3.4 Retroviral Infection of 3T3-L1 Preadipocytes (see **Note 9***)*

1. One day before infection, the preadipocytes are trypsinized and seeded into 10-cm Petri dishes so that the next day they will be 30% confluent (in practice, this means diluting a 50% confluent cell culture 1:2.5).
2. The next day, 2 mL virus suspension is added to 2 mL DMEM/10% FBS without penicillin/streptomycin and 12 L 1 mg/mL polybrene (final concentration 30 g/mL).
3. The medium of the dish is replaced with the virus/polybrene solution.
4. The cells are incubated for 5 h at 37 °C.
5. The virus solution is replaced with DMEM/10% FBS without penicillin/streptomycin.
6. After 24 h, the cells should be 40–50% confluent; they are trypsinized and seeded into two new 10-cm Petri dishes in DMEM medium with 10% NBS, penicillin, streptomycin, and 2 g/mL puromycin.
7. Over the next few days, the noninfected cells will start to die.
8. The cells are cultured similarly as before infection except that puromycin is included in predifferentiation (NBS) and postdifferentiation (FBS) medium.
9. To quantitatively measure cell surface appearance and recycling of GLUT4, the (retrovirally infected) 3T3-L1 preadipocytes are seeded into black clear-bottom 96-well plates that have a coating of glutaraldehyde-fixed gelatin (as in Subheading 3.2., steps 1–5).

3.5 Differentiation of 3T3-L1 Preadipocytes into Adipocytes (see **Note 10***)*

1. Once the preadipocytes are 100% confluent, the culture medium is refreshed, and the cells are cultured for another 48 h.
2. The cells are cultured for 3 d in differentiation medium A (DMEM containing FBS, penicillin, streptomycin, insulin, IBMX, biotin, and dexamethasone) and

for another 3 d in differentiation medium B (DMEM containing FBS, penicillin, streptomycin, and 350 n*M* insulin) (*see* **Note 11**).
3. Differentiation medium B is replaced with DMEM containing 4.5 g/L glucose, FBS, penicillin, streptomycin, and puromycin.
4. The cells are used for experiments between 8 and 11 d after the addition of differentiation medium A (onset of differentiation).

3.6 Transition State Assay

1. The cells are cultured in black clear-bottom 96-well plates.
2. Cells are serum starved for 2 h in serum-free DMEM containing 0.2% bovine serum albumin (Sigma).
3. Medium is replaced with serum- and bicarbonate-free DMEM supplemented with 20 m*M* HEPES (pH 7.4) and 0.2% bovine serum albumin (100 L/well), and the plate is transferred to a 37 °C water bath. The cells are maintained in this medium throughout the experiments (*see* **Note 12**).
4. Insulin (200 n*M* final concentration; *see* **Note 13**) is added to the cells at different time-points.
5. The plate is transferred to ice and 300 L ice-cold PBS is added to each well.
6. PBS is replaced with 4% paraformaldehyde in PBS (100 L/well). Cells are fixed for 15 min on ice and 15 min at room temperature (*see* **Note 14**).
7. Cells are washed once with PBS, incubated for 5 min with 50 m*M* glycine, and washed once more with PBS.
8. Cells are incubated for 20 min with 5% normal swine serum (NSS) in the absence or presence of 0.5% saponin (to label cell surface GLUT4 or total cellular GLUT4, respectively) (Fig. 1).
9. Cells are incubated for a further 60 min with murine anti-HA antibody or nonspecific murine antibody (total serum gamma globulin) in PBS containing 2% NSS, after which they are washed three times for 5 min with PBS.
10. Cells are incubated for 20 min with 5% normal swine serum in the absence or presence of 0.5% saponin to permeabilize all cells so that the background labeling of the secondary antibody is similar for all cells.
11. Cells are incubated for 60 min with a saturating concentration of Alexa 488-conjugated goat-anti-mouse antibody, followed by three washes of 5 min with PBS.
12. Fluorescence is measured using the bottom reading mode of a fluorescence microtiter plate reader (485-nm excitation wavelength; 520-nm emission wavelength) (*see* **Note 15**).
13. The percentage of GLUT4 at the plasma membrane is calculated by subtracting the fluorescence values of the nonspecific antibody from those of the anti-HA antibody and subsequently by dividing the resulting fluorescence of nonpermeabilized cells by that of permeabilized cells (*see* Fig. 1 and **Note 16**).

3.7 Steady-State Assay

1. Cells are cultured and serum starved as described in Subheading 3.6., steps 1–3.
2. For the insulin-stimulated state, the cells are incubated for 20 min with 200 n*M* insulin (*see* **Note 13**).
3. At certain time-points, medium is replaced by fresh BSA- and HEPES-supplemented DMEM containing murine anti-HA antibody or murine nonspecific antibody (total serum gamma globulin) with or without insulin. Cells are incubated with antibody for different time-periods (up to 3 h).
4. The plate is transferred to ice and 300 L ice-cold PBS are added to each well.
5. PBS is replaced with 4% paraformaldehyde in PBS (100 L/well). Cells are fixed for 15 min on ice and 15 min at room temperature (*see* **Note 14**).
6. The cells are washed once with PBS, incubated for 5 min with 50 m*M* glycine, and washed once more with PBS.
7. All cells are incubated for 20 min with 5% NSS in the presence of 0.5% saponin.
8. The wells that did not receive anti-HA before fixation and are used to measure the total cellular GLUT4 amount are incubated for 60 min with murine anti-HA antibody or nonspecific murine antibody (total serum gamma globulin) in PBS containing 2% NSS, after which they are washed three times for 5 min with PBS.
9. All cells are incubated for a further 60 min with a saturating concentration of Alexa 488-conjugated goat-antimouse antibody, followed by three washes of 5 min with PBS.
10. Fluorescence is measured using the bottom reading mode of a fluorescence microtiter plate reader.
11. Percentage of GLUT4 that recycles via the plasma membrane is calculated by subtracting the fluorescence values of the nonspecific antibody from those of the anti-HA antibody and subsequently by dividing the resulting fluorescence by the specific fluorescence of the cells that have been incubated with primary antibody after fixation.

3.8 Interpreting the Data: Experimental Design, Modeling Pitfalls and Tricks

Interpretation of data obtained from this experimental system requires consideration of a number of variables. For example, the protein under investigation will likely be moving between cellular compartments constantly. The conformation of the protein may change under certain conditions, or the expression of the protein may be modified either transcriptionally or at the level of protein turnover. Each of these variables must be carefully considered in formulating a model.

Changes in experimental variables could include changes in

- the amount of antibody binding
- the amount of antigen-binding sites

Changes in intrinsic cellular variables may include changes in

- the amount of the protein at a particular location like the plasma membrane
- the rate of exocytosis of the protein
- the rate of endocytosis of the protein
- the total amount of protein recycling

There may be changes in more than one of these variables at any given time. Considering the two experimental data sets shown in Fig. 4A,B, both are well fitted by the exponential rise:

$$y = A–B\ exp(–t/\tau),$$

as shown in Fig. 5A,B, respectively.

The parameters A, B, and τ represent the following: A is the long-term (as $t \rightarrow \infty$) level $y = y_{\infty} = A$. At $t = 0$, $y = y_0 = A - B$, or $B = y_{\infty} - y_0$, the difference in the initial and long-term levels. τ is the tim ange from the initial level to the long-term level. The smaller the τ is, the more rapid the rise.

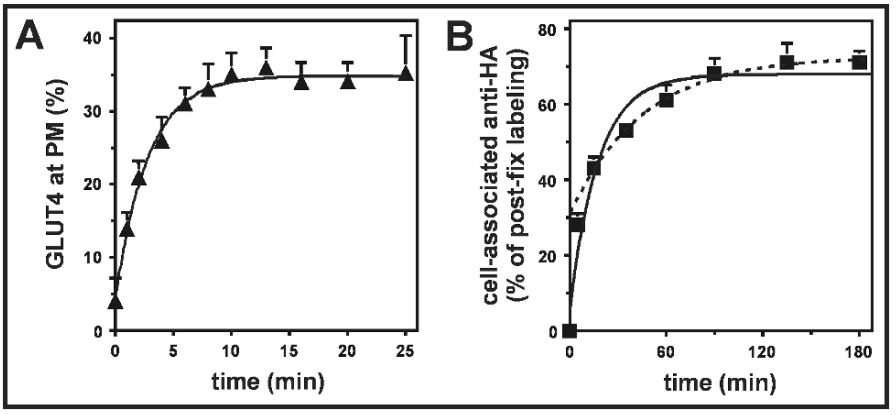

Fig. 5 Least-squares fits to the data for transition-state and steady-state experiments of Fig. 4 to $y = A - B\ \exp(-t / \tau)$. **(A)** Transition-state experiment in which 200 nM insulin is added at $t = 0$, with coefficients (with 95% confidence bounds) of A =34.88 (33.78, 35.97), B = 30.43 (28.2, 32.66), and τ = 2.81 (2.38, 3.43); the goodness of fit had SSE 8.338 R^2 0.9924; adjusted R^2 0.9904; and RMSE 1.021. **(B)** Steady-state experiment of cells preequilibrated in the presence of 200 nM insulin. Two fits are shown. The solid curve includes all data in the fit, with coefficients (with 95% confidence bounds) of A = 67.72 (59.77, 75.67), B = 62.55 (48.03, 77.06), and τ = 17.44 (6.292, 28.58); the goodness of fit had SSE 166.4; R^2 0.9619; adjusted R^2 0.9466; and RMSE 5.769. The dashed curve excludes both the $t = 0$ and $t = 5$ data points, with coefficients (with 95% confidence bounds) of A = 72.56 (69.45, 75.66), B = 41.7 (35.89, 47.51), and τ = 44.87 (29.88, 59.86); the goodness of fit had SSE 2.656; R^2 0.9958; adjusted R^2 0.9931; and RMSE 0.9408.

In the two experiments, however, the experimental interpretation of the parameters *A, B*, and τ is very different.

Figure 6 shows a model for the recycling of the protein. It undergoes exocytosis at a rate K_1 and endocytosis at a rate K_2, so that there is an amount p at the plasma membrane and an amount r in the rest of the recycling pathway. Depending on the situation, any or all of these variables can change with time t.

It is important to emphasize that under a specific experimental condition the distribution of a particular protein within the cell will likely reach a steady state such that the total amount of that protein at various cellular locations will remain constant. However, this steady state is a function of the integrated equilibrium between multiple rate constants that govern the movement of that protein between each of its itinerant stopping points within the cell. In many cases, it is desirable to challenge the cell in some way, in the case of GLUT4 with insulin, in which case the cell will move to a new steady state. The challenge then is to be able to measure as many of the rate constants as possible that depict this dynamic process because this provides invaluable information about the process. For example, in the case of insulin-regulated GLUT4 trafficking it is important to determine if insulin stimulates exocytosis or decreases endocytosis.

To achieve this end point, there are two kinds of experiments that can be performed. The first is a transition kind of experiment in which one studies the system as it changes from one steady state to another. The second is the steady-state experiment in which one analyses the system only after it has reached a new equilibrium. Both of these experiments have their pros and cons as described below.

3.8.1 Transition Kinetics

In Fig. 5A, the data were derived from a transition assay. At $t = 0$, the cells, which were in the basal state, were exposed to 200 nM insulin. It has been suggested that this exposure changes the exocytosis and to some extent also the endocytosis rates,

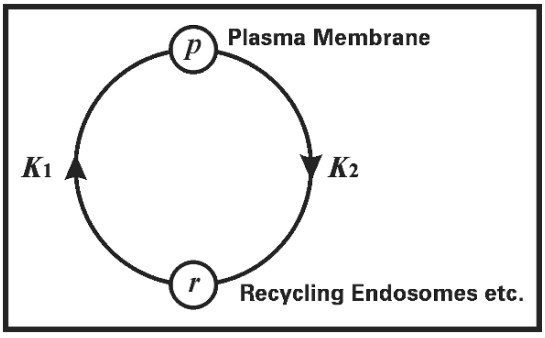

Fig. 6 Hypothesized model for the experiments in Fig. 4. K_1 is the exocytosis rate, and K_2 is the endocytosis rate. The amount of protein at the plasma membrane is p and in the rest of the recycling pathway is r. All these intrinsic variables can be functions of time t.

thereby increasing the amount of GLUT4 at the plasma membrane of the cells. Thus, in this scenario, intrinsic variables of our model are changing with time. The data y display the surface levels of HA-tagged GLUT4 at different stages during the exposure. It is assumed that the proportion of HA-GLUT4 remains constant and evenly distributed compared to endogenous GLUT4 during the process.

At time $t = 0$, we can evaluate the *surface level* of HA-GLUT4 as $y = y_0 = A - B$. This is the basal level at the plasma membrane, before the transition starts. The exocytosis and endocytosis rates may equilibrate very rapidly to new levels in response to the stimulation, but the amount of time taken to redistribute the protein, and the amount of the reporter shuttling between various locations throughout the cell according to these new rates (the new steady state determined by the insulin level) may take a very long time. If the experiment was left to proceed for a sufficient length of time, the system will eventually proceed to this new steady state, but naturally the experiment cannot be performed for an infinite time, and other considerations such as the stability of the cells need to be considered. The fit to the data, however, can be extrapolated to predict the long-term surface level of HA-GLUT4. Letting $t \to \infty$, we have $y = y_\infty = A$, the final *surface level* in the presence of insulin.

In the case of transport of HA-GLUT4 (both exocytosis and endocytosis) at constant rates, then the time constant τ is the inverse of the exocytosis rate, as is the case in the steady-state assay in Fig. 5B. However, we hypothesized that the change in the surface level was possibly caused by a change in the exocytosis rate. Thus, τ cannot be interpreted as a direct measure of the exocytosis rate but rather as a measure of the speed of the process of transition of the cell from the basal steady state to a steady state in the presence of insulin.

3.8.2 Steady-State Kinetics

In Fig. 5B, the data were derived from a steady-state assay. In this case, the cells are allowed to equilibrate in the presence of 200 nM insulin. Then, at $t = 0$, the anti-tag antibody is added to the extracellular medium, and the total amount of antibody taken up by the cells is measured as a function of time. This amount increases in time as HA-GLUT4 molecules arrive at the surface of the cell, where they are exposed to the anti-HA antibody.

In this experiment, the intrinsic cellular variables, like exocytosis and endocytosis rates, are constant, as are the *total* amounts of GLUT4 at the plasma membrane and the rest of the recycling pathway. The experimental variables, such as the amount of antibody-bound HA-GLUT4 (and thus fluorescing) are, however, changing. The variable y here is the total amount of fluorescence as a function of time t. Using the fit to the data and extrapolating the fit back to $t = 0$, $y = y_0 = A - B = p$ is a measure of the total amount of HA-GLUT4 at the *plasma membrane* in the presence of insulin.

Actually, the process of antibody binding takes a finite amount of time, so by excluding early data points from the fit a better estimate can be obtained for the surface level of HA-GLUT4 in the presence of insulin. A comparison of fits to the data excluding early data points is shown in Fig. 5B.

In this assay, the exo- and endocytosis rates are constant. In this case, the time constant τ reflects the rate at which new, unbound HA-GLUT4 is brought to the surface of the cell. This is, of course, the inverse of the exocytosis rate K_1.

In addition, as the cell is in a steady state, the ratio of the amount at the plasma membrane, $p = (A - B)$ to that in the rest of the recycling pathway r is equal to the ratio of the endocytosis K_2 and exocytosis rates K_1:

$$\frac{p}{r} = \frac{K_2}{K_1}$$

Extrapolating the fit to predict the long-term level of HA-GLUT4, we let $t \to \infty$, giving $y = y_\infty = A = r + p$, the total amount of HA-GLUT in the recycling pathway in the presence of insulin. Putting all these together gives a complete set of values for the intrinsic variables of the model shown in Fig. 6.

The assumptions one makes in a model also affect the results. For example, in these assays, the amounts of HA-GLUT4 are measured relative to the total cellular amount observed in a permeabilized cell. If it is assumed that after a sufficient length of time, *all* the cellular HA-GLUT4 has entered the recycling pathway at one point, then this would mean that A is set to 100%. Free parameter fits to the steady-state assay data, as in Fig. 5b, however, show that this level is better fit by a value of around 70% for a 200 nM insulin stimulation and less for lower insulin concentrations *(3)*, meaning that not all cellular HA-GLUT4 enters the recycling pathway. Using a value of $A = 100$ may also produce fits that have reasonable statistical measures and fit with the hypothesis. However, this may not reflect the true underlying process.

3.8.3 Comparing the Experiments

Using different types of assays can also be a useful tool to provide independent estimates of the intrinsic variables. For instance, we can obtain an estimate for the long-term surface level of HA-GLUT4 in the presence of insulin p from both transition and steady-state experiments. For the transition assay, this was determined to be parameter A. The fit to the data found this value to be approx 35 ± 1%. For the steady-state assay, the surface level was related to two of the fitted parameters, $p = A - B$.

Two different fits to the steady-state assay data are shown in Fig. 5b. At $t = 0$, the antibody was applied, so we could hypothesize that this will instantly bind with all HA-GLUT4 at the plasma membrane. In this case, the data point at $t = 0$ (and thus the fit at $t = 0$) should be the surface level of HA-GLUT4 in the presence of insulin. The fit to the steady-state assay data, including all the data points, is shown as a solid curve in Fig. 5b, which gives an estimate for this of approx 5 ± 11%. The second fit, shown as a dashed curve in Fig. 5b, excludes the data at $t = 0$ and at $t = 5$. From this fit, we obtain an estimate of approx 31 ± 5%.

So, we have good correlation between the transition assay result and that from the fit to the steady-state assay data that excluded the data at $t = 0$ and at $t = 5$. This would indicate that the time taken for the antibody to bind affects the data for up to 15 min (the next data point) after application. Of course, this example only

compares single experiments, and better statistical comparisons can be made if repeated trials are made of both assays.

By using models for the system, experimental investigations can be designed to best test the hypotheses and assumptions that are made. In this way, the data fitting and modeling is not just a final stage of the process but can guide and shortcut what can be a long and intensive experimental study.

4 Notes

1. The advantage of the technique described here over the most recently (and currently most frequently) used technique *(6)* is that the assays described here are high throughput (one does not need to analyze individual cells by microscopy), and this method establishes the exact relative amount of GLUT4 at the cell surface rather than a ratio between two fluorophores (fluorescent Cy5 label at the cell surface vs intensity of the green fluorescent protein [GFP] label that is attached to GLUT4) *(6)*. The technique described here is obviously also more quantitative than the so-called GLUT4 rim assay, in which the relative amount of cells are scored (by microscopy) in which endogenous GLUT4 appears to be present at or nearby the plasma membrane *(7)*.
2. A provisional patent has been filed for this technique (PCT/AU2004/001057; D.E. James and R. Govers).
3. The source of the gelatin is essential. The gelatin that is used for the production of retroviral particles and fluorescence assays is from Merck (cat. no. 1.04078.1000). Note that the gelatin works very well for some cells but not for others. For example, assays with HeLa cells work well without coating but not when the microtiter plates are coated with gelatin. Gelatin coating of the cell culture plastics can be done 1 d before seeding of the cells; in this case, the final DMEM wash remains in the wells just until the cells are seeded.
4. All handling of the 96-well plates (including gelatin coating) are performed using a cordless electronic multichannel pipetor in combination with extended 1250- L tips. This is quite essential for easy handling.
5. Note that while cells in culture usually require rinsing with PBS before trypsin/EDTA treatment, the BOSC23 are not washed before trypsin/EDTA is added. This is not required and only leads to cell loss as the BOSC23 cells do not adhere to culture plastics very strongly. It is also for this reason that they are grown on gelatin for the generation of retrovirus.
6. With passaging, the BOSC23 cells are not diluted more than five times; otherwise, these cells form clumps.
7. Once the retrovirus particles are generated, they are frozen at −80 °C and thawed just before use but never refrozen. Refreezing strongly reduces infection efficiency.
8. When propagating 3T3-L1 preadipocytes, they are never allowed to grow to confluence as this negatively affects their subsequent differentiation into adipocytes.

9. The use of retrovirus as described here does not result in extreme overexpression as is the case when, for example, using electroporation-mediated transfer of cDNA. However, after 1 wk of puromycin selection nearly all cells (>95%) express HA-GLUT4.
10. The cell type used in the assays described here (3T3-L1 preadipocytes) do not always differentiate very efficiently. There are batches of these cells on the market that differ in their differentiation efficiency, and not all types and batches of FBS allow good differentiation.
11. Puromycin is not included in the medium during differentiation as this inhibits the differentiation process. It is added to the cells, however, when the differentiation process is completed.
12. In the assays described here, it is essential to use DMEM instead of, for example, Krebs–Ringer phosphate (KRP) solution because of the presence of amino acids in DMEM *(8)*.
13. In the experiments shown in this chapter (Figs. 3 and 4), insulin is used at a concentration of 200 n*M*. At this concentration, insulin has its maximum effect in transition- and steady-state experiments. At lower doses, insulin displays submaximal effects *(5)*.
14. Note that all wells are fixed at the same time. This means that the cells receive insulin (and antibody for the steady-state assays) at different time-points.
15. Choice of wavelength is important. For this technique, fluorescence can easily be measured for green (Alexa 488, FITC) or far-red fluorophores (Alexa 647, Cy5). However, this is much more difficult for red fluorophores (Alexa 594, TRITC, Cy3) as the cells display considerable fluorescence background in the spectra of these fluorophores.
16. For the determination of the binding of anti-HA or nonspecific antibody at least four wells are used for every trial of condition or time-point (quadruplicate).

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