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SNAREs and epithelial cells

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Abstract

SNARE proteins control the membrane fusion events of membrane trafficking pathways. Work in epithelial cells has shown that polarized trafficking to the apical and basolateral plasma membrane domains requires different sets of SNAREs, suggesting a mechanism that contributes to the overall specificity of polarized trafficking and, perhaps, the formation and maintenance of polarity itself. This article describes methods that have been designed and adapted specifically for the investigation of SNAREs in epithelial cells. The knowledge of the subcellular localization of a SNARE of interest is essential to understand its function. Unfortunately, the endogenous expression levels of SNAREs are often low which makes detection challenging. We provide guidelines for determination of the localization of SNAREs by immunofluorescence microscopy including methods for signal amplification, antigen retrieval, and suppression of antibody cross-reactivity. To define which trafficking pathway a SNARE of interest is involved in, one needs to specifically inhibit its function. We provide guidelines for SNARE inhibition by overexpression of the SNARE of interest. An alternative is to introduce inhibitors of SNARE function, such as antibodies or clostridial toxins, into cells. Two methods are presented to make this possible. The first allows the monitoring of effects on trafficking pathways by biochemical assays, and is based on plasma membrane permeabilization using the bacterial toxin streptolysin-O. The second is suitable for single-cell observations and is based on microinjection.
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1. Introduction

The SNARE machinery controls membrane fusion events in all membrane trafficking pathways investigated to date [1,2]. SNAREs are relatively small, membrane-anchored proteins that belong to distinct protein families which are related to each other by the presence of one or two conserved coiled-coil domains [3,4]. These so-called "SNARE motifs" mediate the binding interactions of SNARE proteins to each other. The resulting SNARE complexes generally consist of four coiled-coil domains contributed by SNAREs residing in the target membrane (t-SNAREs) and vesicle membrane (v-SNAREs). Recent experiments have shown that only matching combinations of v- and t-SNAREs lead to successful fusion [5,6], indicating that SNAREs not only mechanistically accomplish membrane fusion but also contribute to the specificity of this process. In support of this, different SNARE proteins are generally localized to specific organelles and are involved only in specific trafficking pathways.

Investigation of SNARE function in epithelial cells has indicated that the polarized distribution of t-SNAREs, most notably the apical-specific syntaxin 3 and the basolateral-specific syntaxin 4, is highly conserved among epithelial cell types and is likely to be crucial for the establishment and maintenance of epithelial polarity [7–11]. This article describes and discusses techniques that have proven highly valuable for the investigation of expression, subcellular localization, and function of SNAREs in epithelial cells. While these methods have been specifically adapted and optimized for this purpose, many of them are applicable to the study of other proteins and cell systems as well.

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2. Immunolocalization of SNAREs

The best initial approach to aid in the identification of the trafficking pathway in which a t-SNARE is involved is to determine its steady-state localization. In general, t-SNAREs appear to mediate fusion events to the organelle/domain in which they reside most of the time. For example, at steady state the majority of syntaxin 3 is found at the apical plasma membrane of epithelial cells [8,9,12]. Consequently, it functions in trafficking pathways that are directed to this domain [10,13]. Although it has not formally been excluded it appears very unlikely that syntaxin 3 (or any other t-SNARE) also functions at another location that is "invisible" at steady state. The case of v-SNAREs is more complicated because they are generally thought to cycle between the donor compartment from which the respective transport vesicles are formed and the target membrane.

The steady-state localization of a SNARE can be determined either by detecting the endogenously expressed protein or after expression of an exogenous protein. The endogenous expression levels of most SNAREs are relatively low (with the exception of neuronal SNAREs in neurons) which can make detection a challenge. Below are descriptions of our methods for enhancing signals in immunofluorescence experiments using cultured cells or tissue sections.

2.1. Immunofluorescence staining with signal amplification by anti-fluorescein tertiary antibody

This method uses a primary rabbit antibody against a SNARE protein, followed by a fluorescein-labeled donkey anti-rabbit IgG secondary antibody. To enhance the signal, a rabbit antibody is then used that recognizes fluorescein and is coupled to the fluorophore Alexa-488 (Molecular Probes, Eugene, OR, USA, Catalog No. A-11090). Since the spectral properties of Alexa-488 are nearly identical to those of fluorescein, the result is an amplification of the fluorescein signal. In our experience, this increases the signal approximately fivefold.

1. Culture MDCK cells on 12-mm Transwell filters in MEM containing 10% FBS. Allow the cells to polarize for at least 3 days, changing the medium every other day.
2. Rinse cells briefly three times with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^+).
3. Fix with 4% paraformaldehyde in PBS^+ for 20 min at room temperature. During fixation, place the cells on an orbital shaker at very low speed (alternatively, cells can be fixed in cold methanol at −20 °C for 10 min).
4. Quench any remaining fixative with 75 mM NH_4Cl and 20 mM glycine (both from a 1 M stock) in PBS at room temperature for 10 min with shaking (in the case of the methanol fixation, omit the quenching step).
5. After two brief rinses in PBS, block the cells and permeabilize in Block Solution (PBS containing 3% BSA and 0.2% Triton X-100) at 37 °C for 30 min.
6. Centrufuge primary antibody diluted in Block Solution at 12,000*g* for 15 min to pellet any aggregates.
7. Carefully cut out the membrane of the Transwell mount and place it on Parafilm on a 30-μl drop of the antibody. Place another 30 μl of the diluted antibody on top of the filter. Incubate in a humid chamber at 37 °C for 2 h.
8. Transfer the membrane back to a 12-well dish and wash 4 × 5 min in Wash Solution (PBS with 0.05% Triton X-100 and 0.7% fish skin gelatin (Catalog No. G-7765, Sigma, St. Louis, MO, USA).
9. Dilute the secondary antibody conjugated with fluorescein in Wash Solution and centrifuge again for 15 min to pellet out any aggregates; apply it to the membrane as described for the primary antibody. Incubate for 1 h at 37 °C in a humid chamber.
10. Wash the membrane 4 × 5 min in Wash Solution. Dilute the Alexa-488-conjugated anti-fluorescein antibody in Wash Solution and apply it to the membrane after centrifugation as described previously. Allow the antibody to incubate for 1 h at 37 °C.
11. Remove excess antibody by washing 4 × 5 min in Wash Solution and 2 × 3 min in PBS containing 0.1% Triton X-100, followed by two rinses in PBS.
12. Mount the membrane cell side up. It is ready for viewing under the microscope.

2.2. Antigen retrieval by pressure cooking

In our experience, immunofluorescence staining of SNAREs in sections of fixed tissues often results in weak signals. This may be due to the fact that SNAREs appear to spend most of the time in complexes with other SNAREs or regulatory proteins. Fixation with protein-crosslinking fixatives, like formaldehyde, may then mask many epitopes. This could theoretically lead to localization artifacts because a subpopulation of a SNARE may be "invisible" by immunostaining. Several methods are used to unmask epitopes in tissue sections, including digestion with proteolytic enzymes; denaturation with urea, SDS, or guanidine hydrochloride; and heat treatment. In our hands, heat treatment using a pressure cooker leads to the most reproducible signal enhancement while preserving tissue morphology. Paraffin sections of tissues from animals perfusion-fixed with 4% paraformaldehyde in PBS^+ have worked best for us.

1. Deparaffinize and rehydrate sections on slides as usual (leave slides wet until ready for pressure cooking).
2. Make 2 liters of 10 mM Na-citrate buffer, pH 6.0, by diluting from a 1 M stock.
3. Put the citrate buffer in a large stainless-steel household pressure cooker (must not be aluminum as it reacts with the citrate). With the lid only loosely on the cooker, heat until boiling.
4. Place slides in a glass or steel slide holder and put into boiling citrate buffer.
5. Close lid tightly. Place the weight on the pressure cooker's valve.
6. Continue to heat until weight starts to wobble.
7. Heat for one more minute.
8. Remove cooker from heater; place under running cold water tap.
9. Once pressure is down, open lid and flood cooker with running cold tap water.
10. Remove slides and proceed with immunostaining as usual.

2.3. Suppression of antibody cross-reactivity

Immunolocalization experiments with endogenous proteins suffer from the inherent problem that usually no negative control is available (in contrast to experiments with transfected cells in which the nontransfected cells can serve as the negative control). Omitting the primary antibody controls only for autofluorescence, background by the secondary antibody, etc., but does not establish the specificity of the primary antibody. Competition with the antigen is better but does still not exclude cross-reactivity of the primary antibody. Polyclonal antibodies have to be affinity-purified against the antigen. Unpurified antisera almost always lead to artifactual staining results.

Many SNAREs are closely related to each other, especially in the "SNARE" motifs [3], which can lead to problems of antibody cross-reactivity. For example, we found that polyclonal antibodies raised against GST fusion proteins of any of the mammalian plasma membrane t-SNAREs syntaxins 2, 3, and 4 cross-react slightly with the other two syntaxins even after affinity purification. A simple method to overcome this problem is competitive inhibition using lysates of bacteria that express the related syntaxin–GST fusion proteins (e.g., immunostaining for syntaxin 2 would be carried out in the presence of syntaxin 3 and 4 lysates). Because the nonspecific antibodies may be against both native and denatured antigen, we add a mixture of denatured and nondenatured lysates to the antibody solution before staining.

1. Grow *Escherichia coli* expressing GST–syntaxins under the appropriate conditions. Prepare a total cell lysate using a standard lysozyme protocol. Bacteria from a 1-liter culture will lead to approximately 25 ml of lysate.
2. Add 250 μl SDS lysis buffer (0.5% SDS, 100 mM NaCl, 50 mM triethanolamine-Cl, pH 8.1, 5 mM EDTA) to 250 μl of bacterial lysate.
3. Boil the above solution for 10 min.
4. Add 250 μl Triton dilution buffer (2.5% Triton X-100, 50 mM triethanolamine-Cl, 100 mM NaCl, 5 mM EDTA).
5. Mix the above solution, add 250 μl of nondenatured bacterial lysate, and store at −80 °C in aliquots.
6. Add the above mixture at 4% to the primary anti-syntaxin antibody dilution during immunofluorescence staining.

3. Inhibition of SNARE function

A major strategy for defining the function of SNAREs is to specifically inhibit the function of an individual SNARE protein and measure the effects on the kinetics or fidelity of membrane trafficking pathways, the targeting of cargo proteins, or parameters of epithelial cell polarity. The difficulty lies in the fact that no ideal and simple method is available to inhibit epithelial SNAREs efficiently and specifically. Nature has provided us with the clostridial neurotoxins—tetanus and botulinum toxins—which are highly specific metalloproteases that cleave and inactivate several SNARE proteins [14]. However, most of these toxins cleave only neuronal SNAREs such as syntaxin 1, SNAP-25, and synaptobrevin, which are not normally expressed in epithelial cells. To make matters worse, clostridial neurotoxins can attach to and enter neurons but not nonneuronal cells. It is therefore necessary to introduce these toxins by other means. The same is the case for other inhibitory reagents such as antibodies and recombinant fragments of SNAREs. Two methods, use of permeabilized cells and microinjection, for introducing these membrane impermeable inhibitors into epithelial cells are described below. An alternative strategy is to express dominant-negative inhibitors by gene transfer.

3.1. Dominant-negative inhibition by overexpression of SNAREs

It has been observed in several systems that the overexpression of a wild-type syntaxin causes inhibition of the trafficking pathway in which the syntaxin is normally involved. Examples are syntaxin 3 in MDCK cells [10], syntaxin 5 in BHK-21 cells [15], and syntaxin 4 in mast cells [16]. It is not clear why the observed inhibition occurs but a plausible hypothesis is that the overexpression of one SNARE results in a stoichiometric imbalance with the other SNAREs involved in the

same pathway. This may lead to the formation of non-productive, incomplete SNARE complexes and may cause one of the other required SNAREs (or a regulatory factor) to become limiting. In any case, the effect appears to be quite specific as other trafficking pathways generally remain unaffected. However, successful inhibition requires relatively high levels of overexpression. For example, overexpression of syntaxin 3 ($\sim 10\times$ over endogenous levels) by stable transfection in MDCK cells resulted in partial inhibition of biosynthetic trafficking to the apical membrane, as well as apical recycling, however, similar overexpression of syntaxin 4 had no measurable effect on any pathway [10].

For this reason, an expression system should be chosen that results in high-level expression but can ideally also be regulated. Dasher and Balch used a vaccinia virus system that allows constitutive high-level expression [15]. However, it is useful only for relatively short-term expression and may therefore be unsuitable to investigate long-term parameters such as development of epithelial cell polarity. The same is the case with the usual transient transfection approaches. A promising alternative is expression by stable transfection using a regulatable system such as those using the tetracycline repressor or transactivator. Also useful are adenoviral vectors that express the gene of interest under tetracycline control. The ability to regulate expression of the SNARE may be essential because inhibition of any trafficking pathway may be potentially toxic.

In all cases it is important to verify that the overexpressed SNARE is still correctly targeted. Mistargeting of the SNARE of interest may compromise the specificity of the desired inhibition. Since too strong overexpression of any protein may result in its mislocalization, it is again desirable to be able to regulate the expression level.

Dominant-negative inhibition can also be achieved by expression of truncated SNAREs. There have been no systematic studies aimed at identifying domains of SNAREs that may be most potent and/or specific inhibitors. But is it generally believed that a non-membrane-anchored truncation mutant will form complexes with its cognate SNAREs that are nonproductive due to the lack of proper membrane attachment. A potential caveat is that non-membrane-anchored SNAREs generally localize throughout the cytoplasm. Since SNARE–SNARE interactions, at least in vitro, are relatively promiscuous [17], the potential exists that a non-membrane-anchored SNARE may be a less specific inhibitor than the same full-length SNARE when overexpressed.

Successful examples are the expression of the cytoplasmic domain of syntaxin 4 in adipocytes (inhibits GLUT4 translocation [18]) and the expression of the cytoplasmic domain of syntaxin 5 in BHK-21 cells (inhibits ER-to-Golgi transport [15]). In both cases, vaccinia virus expression systems were used for relatively short-term experiments ($\sim$3–6 h postinfection). We have found that expression of non-membrane-anchored mutants of syntaxin 2 and endobrevin/VAMP-8 using a replication-deficient, tetracycline-regulatable adenovirus system very effectively inhibited the function of the respective SNAREs in MDCK cells [27]. In this case, the cells could be monitored for periods up to 24 h postinfection. As an important control, the observed inhibitory effects could be eliminated by tetracycline-suppression.

3.2. Introduction of SNARE inhibitors by cell permeabilization using streptolysin-O

Streptolysin-O (SLO) is a bacterial protein toxin that can bind to and integrate into plasma membranes of mammalian cells due to its affinity for cholesterol [19]. Once integrated, SLO oligomerizes and forms large pores of up to 35 nm in diameter. These pores are too small to allow membrane-bound organelles to escape; however, macromolecules can be introduced into these permeabilized cells. To subsequently study membrane trafficking events it is necessary to resupply cytoplasmic proteins and ATP (which are lost after permeabilization). Under appropriate conditions, many cell functions including polarized membrane traffic can be reconstituted. If an inhibitory reagent, such as an antibody, recombinant protein, or peptide, is included, the involvement of the protein of interest can be studied if an appropriate assay is available to monitor the membrane traffic pathway of interest. Such assays are usually pulse–chase experiments. The secretion of a soluble reporter protein into the culture medium can then be monitored. Alternatively, the arrival at the plasma membrane of a reporter membrane protein can be monitored by surface immunoprecipitation or surface biotinylation. Using MDCK cells, several laboratories have successfully employed experimental protocols based on work by Gravotta et al. [20] and Pimplikar et al. [21]. Membrane traffic pathways such as biosynthetic apical and basolateral trafficking, transcytosis, recycling, and endocytosis have been reconstituted [10,13,22–25].

Below is our protocol for the permeabilization of polarized MDCK cells cultured on Transwell filters combined with an assay of the surface delivery of the polymeric immunoglobulin receptor by metabolic labeling and surface immunoprecipitation. This protocol can be modified for other trafficking pathways. This protocol has been used to introduce intact IgG antibodies and recombinant botulinum toxin light chains [10].

The following points should be noted: (1) Permeabilizing the basolateral plasma membrane generally leads to more reproducible results than permeabilizing the

apical membrane. We found that apical SLO permeabilization leads to membrane shedding into the apical medium. (2) In our experience, the source of the SLO is critical. All commercially available SLO preparations that were tested were highly problematic. In contrast, recombinant SLO purchased from the Institute of Medical Microbiology, Mainz University (Dr. S. Bhakdi, sbhakdi@mail.uni-mainz.de), led to efficient permeabilization and highly reproducible results. (3) A reducing environment must be provided for reconstitution of cell functions after permeabilization. This is commonly achieved by inclusion of DTT in all buffers. However, since DTT is membrane permeable it can potentially reduce disulfide bonds present in secretory proteins and luminal domains of membrane proteins, thereby disrupting their secretion or function. A better alternative is reduced gluthathione which we use at 5 mM. Glutatione is also less likely to reduce antibodies that are to be introduced into cells. (4) The amount of SLO required for efficient permeabilization has to be determined empirically. This can be done by performing a mock experiment according to the protocol described below using varying concentrations of SLO. Release of the cytoplasmic protein lactate dehydrogenase (LDH) can then be measured using an enzymatic assay kit (Sigma Diagnostics No. 500). Release of 70–80% of the LDH into the medium indicates efficient permeabilization. (5) The source of cytosol to be used for reconstitution can be critical. We have had the best results with cytosol prepared from HeLa cells grown in suspension culture. Rat liver and rat brain cytosols were somewhat less efficient. However, each membrane trafficking step may have different requirements which should be determined experimentally.

3.2.1. Materials

0.5 M EGTA stock: Suspend 19 g EGTA in ~60 ml water, adjust pH with KOH to 7.4 (during which the EGTA will dissolve), make up volume to 100 ml, filter through disposable cell culture sterile filtration unit, and store at 4 °C.

0.5 M glutathione stock: Dissolve 3.073 g reduced glutathione in ~17 ml water, adjust pH to 7.4, make up volume to 20 ml, filter through 0.45-μm syringe filter, and store 1-ml aliquots at −80 °C.

0.1 M K–Ca–EGTA buffer: Dissolve 1 g $CaCO_3$ and 3.8 g EGTA in ~70 ml water until everything is dissolved (may take up to 1 h), adjust pH to 7.4 with KOH, make up volume to 100 ml, filter through disposable cell culture sterile filtration unit, and store at 4 °C.

KOAc-minus buffer: Combine 115 mM potassium acetate, 20 mM Hepes, and 2.5 mM magnesium acetate, adjust pH to 7.4 with KOH, filter through disposable cell culture sterile filtration unit, and store at 4 °C.

KOAc-plus buffer: Combine 115 mM potassium acetate, 20 mM Hepes, 0.5 mM magnesium acetate, and 0.9 mM $CaCl_2$, adjust pH to 7.4 with KOH, filter through disposable cell culture sterile filtration unit, and store at 4 °C.

KTM buffer: To 50 ml KOAc-minus buffer add 0.5 ml 5 mM glutathione stock (5 mM final), 0.2 ml EGTA stock (2 mM final), 0.1 ml K–Ca–EGTA buffer; use final buffer only fresh.

Starving medium for metabolic labeling with [^{35}S]cysteine or [^{35}S]methionine: Dissolve powdered DME medium (without cysteine and methionine, Sigma No. D-0422) and add 1/100th vol of 100× Pen/Strep (penicillin/streptomycin); 1/50th vol of 1 M Na-Hepes, pH 7.3; 0.35 g/liter $NaHCO_3$; and 0.584 g/liter L-glutamine. For cysteine-deficient medium add 30 mg/liter methionine or for methionine-deficient medium add 48 mg/liter cystine. Filter through disposable cell culture sterile filtration unit, and store at 4 °C, keep sterile.

MEM etc: Dissolve powdered MEM medium (Sigma M-4642) in water and add 1/50th vol of 1 M Hepes–NaOH, pH 7.3; 1/100th vol of 100× Pen/Strep; 6 g/liter bovine serum albumin; and 0.35 g/liter $NaHCO_3$. Adjust pH to 7.35–7.4 with HCl or NaOH, and store frozen at −20 °C.

ATP-regenerating system: Prepare these three stock solutions: (a) 800 mM creatine phosphate in water; (b) 100 mM ATP in 10 mM Hepes pH 7; (c) 5 mg/ml creatine kinase (Roche No. 127566) in water. Store all three solutions in single-use aliquots (e.g., 50 μl) at −80 °C.

3.2.2. Method

All washes are done in 12-well culture plates.

Cells: Seed MDCK type II cells onto 12-mm Transwell filters (0.4 μm) and culture for 3–4 days.

Metabolic labeling: Wash cells twice with prewarmed Starving Medium and starve for 15 min in a humid chamber placed in a 37 °C water bath; metabolically label for 10 min by placing individual Transwell filters onto 25-μl drops of Starving Medium including 40–50 μCi [^{35}S]cysteine or [^{35}S]methionine (specific activity 1000 Ci/mmol) on Parafilm inside a humid chamber at 37 °C.

TGN accumulation of newly synthesized membrane proteins: Wash twice with MEM-etc precooled to 17 °C; chase/accumulate for 2 h at 17 °C with MEM-etc in a humid chamber placed in a 17 °C water bath.

SLO binding: (Everything is done on the surface of a metal plate placed on ice.) Wash both sides of the Transwell filter twice with KOAc-plus buffer and once with KTM buffer; place filter onto a 20-μl drop of SLO (100 μg/ml stock) diluted with KOAc-minus buffer + 5 mM glutathione; incubate 10 min on ice; wash three times with KTM buffer.

Permeabilization: Replace with 0.5 ml fresh KTM buffer precooled to 17 °C; at this point the desired inhibitor can already be included (e.g., antibody or clostridial neurotoxins); incubate 45 min at 17 °C

(during this time cell permeabilization will occur and the cytosol will be washed out of the cells).

Reconstitution of transport: Add to the basolateral side of filter the following (prewarmed to 37 °C): 200 μl HeLa cytosol, 15 μl ATP, 15 μl creatine kinase, 15 μl creatine phosphate, and the desired inhibitor (e.g., antibody or clostridial neurotoxins). Make volume up to 500 μl with KTM buffer. Add to apical side of filter 500 μl of prewarmed KTM buffer; incubate at 37 °C in a humid chamber for desired period. This is followed by the appropriate method for detection of the protein of interest that has reached the apical or basolateral surface such as surface immunoprecipitation or biotinylation. For an example, see Low et al. [10].

3.3. Introduction of SNARE antibodies by microinjection

The SLO permeabilization method described above is useful for biochemical assays of membrane traffic pathways that require that the SNARE inhibitor is introduced into the majority of the cells in a population. If effects on trafficking pathways are to be analyzed microscopically on an individual cell basis, microinjection may be preferable. Several points should be considered. (1) It is helpful to know how much of the targeted protein is present in the cells. This information will guide the initial estimation of the antibody concentration required to inhibit a protein of interest. Otherwise, one can prepare a concentrated antibody stock and titrate down to the lowest concentration needed to detect effects. (2) Cells generally tolerate injection of no more than 10% of the total cell volume. Five to ten percent is generally used. (3) Antibody stocks for microinjection must be prepared in physiological buffers that do not, alone, affect cellular processes. We routinely prepare antibodies for injection in a Hepes–KCl buffer, which also appears to prevent aggregation of antibody during long-term storage. Do not use Tris-based buffers! Tris has been shown to inhibit endocytosis, and microinjection of even the smallest amount usually results in rapid cell death. (4) Cells must be cultured on glass coverslips rather than filters since filters are not transparent to transmitted light and thus cells cannot be visualized during injection. MDCK cells grown on glass form distinct apical (with microvilli) and basolateral membranes, form tight and adherent junctions, and support polarized transport of apical and basolateral markers to the plasma membrane. Thus, like filter-grown cells, MDCK cells cultured on glass can fully polarize and are suitable for studies of polarized trafficking pathways.

3.3.1. Methods

3.3.1.1. Preparing antibodies for microinjection.

1. Both, IgG fractions prepared using standard protein A purification and affinity-purified antibodies are suitable for microinjection. Antibodies should be dialyzed against Microinjection Buffer (10 mM Hepes, 140 mM KCl, pH 7.4). Determine the antibody concentration by measuring the absorbance at 280 nm in a spectrophotometer. An A_{280} of $1.4 = 1$ mg/ml IgG.
2. Concentrate the purified antibody. We have found that 8–12 mg/ml injection stock of syntaxin 3 IgG is effective in inhibiting the appearance of apical membrane proteins at the apical surface of MDCK and FRT epithelial cells. We routinely use vacuum dialysis but other methods such as ultrafiltration work as well. After concentration, determine the antibody concentration by diluting a small aliquot and measuring the absorbance at 280 nm using a microcuvette.
3. Store the antibody in single-use, 5- to 10-μl aliquots at −20 °C (−80 °C for long-term storage). Repeated freezing and thawing of purified antibodies often results in protein aggregation and loss of activity.
4. Just prior to microinjection, thaw antibody stock on ice and spin at 14,000 rpm in a microfuge for 5–10 min at 4 °C to pellet any aggregated protein. Transfer antibody to a new tube and keep on ice.

3.3.1.2. Preparing the cells. Cells are cultured on glass coverslips for microinjection experiments. We routinely place 7–20 coverslips (depending on the size of the glass) into a single 10-cm culture dish before adding the cells. MDCK type II cells are plated at high density, resulting in ~80% confluence after attachment. Cells are subsequently cultured for 3–5 days until a fully polarized monolayer has formed. Medium is changed on the first day after plating.

3.3.1.3. Injection.

1. Transfer coverslips to individual culture dishes with fresh medium.
2. If you are performing a time course, it is important that you rigorously control the amount of time spent injecting cells on each coverslip. We generally limit injection times to 5 min per coverslip and then transfer the cells back to the incubator. Use multiple coverslips to increase the number of injected cells at each time point.

3.3.1.4. Example: effect of microinjected anti-syntaxin 3 IgG on protein delivery to the apical membrane. A reporter protein was expressed in polarized MDCK cells in order to monitor apical trafficking. We used the GFP-tagged apical membrane protein p75-neurotrophin receptor (p75-GFP) which was expressed by nuclear microinjection of the cDNA. Subsequently, either anti-syntaxin 3 IgG or nonspecific IgG was introduced by cytoplasmic microinjection. A simplification is to co-inject both the cDNA and the antibody at the same time, which is possible because nuclear injections always lead to a significant cytoplasmic delivery.

One hour after microinjection, the cells were incubated at 20 °C to accumulate newly synthesized p75-GFP in the Golgi apparatus (see Kreitzer et al. [26] for a more complete description of protein expression using microinjection). Once p75-GFP had accumulated in the Golgi, cells were shifted to 37 °C to permit exit from the Golgi, and were fixed at 30-min intervals. This assay relies on the ability to differentiate between intracellular and surface-associated membrane proteins. Thus, cells were briefly fixed in paraformaldehyde (2 min at room temperature) without subsequent permeabilization so that p75-GFP at the cell surface could be selectively immunolabeled. The appearance of p75-GFP at the apical surface was determined by immunostaining with an anti-p75 ectodomain antibody after fixation. The effects of microinjected antibodies on delivery of p75 to the apical membrane were then determined by measuring the ratio, over time, of surface:total p75-GFP fluorescence (immunostained p75:direct GFP fluorescence) in the presence of control or syntaxin 3 antibodies [28].

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