SNAP-23 Requirement for Transferrin Recycling in Streptolysin-O-permeabilized Madin-Darby Canine Kidney Cells*

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Fusion of recycling and transcytotic vesicles with the apical and basolateral plasma membrane domains of Madin-Darby canine kidney (MDCK) cells requires the N-ethylmaleimide-sensitive factor and is sensitive to botulinum neurotoxin serotype E (BoNT/E). BoNT/E is thought to selectively proteolyze the 25,000-dalton synaptosomal associated protein (SNAP-25), a protein found in neurons or cells of neuroendocrine origin. However, SNAP-25 is not found in MDCK cells. One possible target for BoNT/E in MDCK cells is SNAP-23, a newly described SNAP-25 homolog that is found in several organs including kidney. Currently, the function of SNAP-25 is unknown. We have reconstituted transferrin recycling in permeabilized MDCK cells to assess the role of SNAP-23 in the endocytic traffic of this protein. We find that: (i) SNAP-23 is expressed in MDCK cells and is found both at the basolateral plasma membrane and associated with apical and basolateral vesicles, (ii) canine SNAP-23 is cleaved by BoNT/E, (iii) transferrin recycling is N-ethylmaleimide-sensitive factor-dependent and BoNT/E-sensitive, and (iv) addition of either exogenous SNAP-23 or anti-SNAP-23 antibodies inhibits ligand recycling. Our observations suggest that SNAP-23 may be required for fusion of recycling vesicles with the basolateral membrane of polarized MDCK cells.

Membrane trafficking is mediated by transport vesicles that bud from a donor membrane and then fuse with an acceptor membrane (1). An important regulatory step in this process is controlling the vesicle target specificity and subsequent fusion event. In many systems, the vesicle targeting/fusion machinery is composed of both cytosolic and membrane bound proteins (2). The cytosolic components are the N-ethylmaleimide-sensitive factor (NSF)1 (3, 4), and the soluble NSF attachment protein (SNAP) that binds NSF to the fusion complex (5, 6). The membrane bound components, the SNAP receptors (SNAREs), are divided into two types: vesicle membrane-associated (v-SNAREs) and target membrane-associated (t-SNAREs). The v-SNAREs are thought to dock vesicles to the appropriate target membranes via interactions with cognate t-SNAREs (7). Recent evidence suggests that the v-SNAREs/t-SNARE complex may also be the minimal machinery needed for vesicle fusion to occur with the target membrane (8). Interactions between v-SNAREs/t-SNAREs are regulated by Rab proteins, N-sec-1/Munc-18 homologs (9–12), as well as hydrolysis of ATP by NSF, which may act to dissociate v-SNARE/t-SNARE complexes priming them for another round of fusion (13–15).

In neurons, SNAREs are thought to control synaptic vesicle docking and fusion. One v-SNARE, vesicle-associated membrane protein (VAMP, also known as synaptobrevin), and two t-SNAREs, syntaxin and SNAP-25 (not related to the SNAP of the cytosolic component), have been isolated and characterized from synaptosomal membranes (reviewed in Ref. 16). Clostridial neurotoxins inhibit synaptic vesicle fusion by acting as specific proteinases of a single SNAP component without cleaving the others (reviewed in Ref. 17). For example, VAMP is cleaved by tetanus toxin, BoNT/B, D, F, and G, while syntaxin is cleaved by BoNT/C1, and SNAP-25 is cleaved by BoNT/A and E. It has now been shown that many trafficking reactions require SNARE-mediated targeting and fusion including endoplasmic reticulum to Golgi transport and trans-Golgi to basolateral plasma membrane transport (18–20). In other cases, SNAREs have been identified but their exact role in trafficking is unknown (for example, see Refs. 21–23).

Previously, we analyzed the role of the vesicle fusion machinery in the exit of IgA from the apical recycling endosome (ARE) and fusion with the apical and basolateral plasma membrane in streptolysin-O (SLO)-permeabilized MDCK cells expressing the polymeric Ig receptor (pIgR) (24). The ARE is a tubulovesicular compartment that receives molecules transcytosing in the basolateral to apical direction and those recycling from the apical and basolateral poles of the cell (25–27). The pIgR protein transports polymeric Igs (IgA and IgM) across epithelial cells and into secretions. Both transcytosis in the basolateral to apical direction and recycling of pIgR ligand is dependent on NSF and inhibited by BoNT/E (which cleaves SNAP-25) (24). Surprisingly, BoNT/A has no apparent effect on either pathway. In contrast, transport of a newly synthesized protein from...
the trans-Golgi network to the apical surface is apparently independent of NSF/SNAP/SNAREs while transport from the trans-Golgi network to the basolateral surface requires these complexes (19). The above observations suggest that fusion with the apical plasma membrane of MDCK cells may occur by multiple mechanisms, one of which may require NSF/SNAP/SNAREs (basal to apical transcytosis) and one which may not (trans-Golgi network to apical cell surface delivery). While studies have found several isoforms of VAMP and syntaxin are expressed within MDCK cells (19, 23), attempts to identify SNAP-25 in MDCK cells have failed (24). This led us to hypothesize that a BoNT/E-sensitive SNAP-25 homolog(s) may exist in MDCK cells and may be required for transcytosis and recycling (24).

A SNAP-25 homolog, called SNAP-23, has recently been cloned and characterized from human B lymphocytes (28). Moreover, a mouse isofrom of SNAP-23 (also known as syndet) has been cloned from 3T3 L1 adipocytes and is 98.6% similar and 86.7% identical to human SNAP-23 (29, 30). Unlike SNAP-25, which is distributed along axons, SNAP-23 is concentrated on nerve cell bodies with only limited staining of the axons and synaptosomes. In addition, SNAP-23 is expressed in several non-neuronal tissues including heart, lung, liver, muscle, pancreas, and kidney (where it is expressed at high levels) (28, 31). It has been shown to bind various syntaxins (isoforms 1, 2, and 3 with high affinity to 4) and VAMP (28, 30). While SNAP-23 is localized to the plasma membrane and in vesicular structures, little is known presently about its role in membrane trafficking. Our goal was to determine if SNAP-23 was required for endocytic traffic in polarized epithelial cells.

We find that SNAP-23 is expressed in MDCK cells, both at the basolateral plasma membrane and in apical and basolateral vesicles. TF recycling is NSF-dependent and BoNT/E-sensitive, and addition of either exogenous SNAP-23 or anti-SNAP-23 antibodies inhibits ligand recycling. Our observations suggest that SNAP-23 may regulate fusion of recycling vesicles with the basolateral domain of polarized MDCK cells. These findings further our understanding of some of the underlying fusion mechanisms used in polarized MDCK cells and may ultimately lead to a better understanding of how polarized cells achieve and maintain their distinct plasma membrane domains.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Proteins, and Other Reagents—Canine apo-Tf (Sigma) was loaded with Fe**⁺³ as described (32). Rat monoclonal antibody ascites to ZO-1, a protein associated with tight junctions, was obtained from Chemicon (Temecula, CA) and was used at 1:100 dilution. Mouse monoclonal antibody 38B10 (BD Biosciences—PharMingen, San Diego, CA) was used at 1:50 dilution. Mouse monoclonal antibody 2H8 (BD Biosciences—PharMingen, San Diego, CA) was used at 1:200 dilution. Rabbit polyclonal antibody to human E-cadherin (hybridoma rr1) (33) was used at 1:1 dilution. Horseradish peroxidase-conjugated goat anti-rabbit IgG, or goat anti-mouse IgG, or goat anti-mouse IgM, and goat anti-mouse IgA were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and fluorescent labeled secondary antibodies used at 5–10 μg/ml.

Preparation of affinity purified antiseraum to SNAP-23 was as described (28, 30). 2 A human SNAP-23 cDNA was obtained by polymerase chain reaction from a human platelet cDNA library using primers (gggatccATGGATAATCTGTCATCAG and cccaagcttTTAGCTGTCAATGGTGG ATTTC) based on the human B cell SNAP-23 sequence (28). The human SNAP-23 cDNA was inserted into pQE-9 vector using human platelet cDNA encoding SNAP-23 is identical to that reported previously (28, 30). 2 The His₆-SNAP-25 construct was a generous gift of Dr. Thomas Sollner. The flow-through antibody, termed affinity purified anti-SNAP-23, showed no detectable cross reaction to His₁₅-SNAP-25 (25).

**Cell Culture—** MDCK strain II cells expressing the wild-type rabbit plgR have been described (34). Cells were maintained in minimal essential medium (MEM; CellTek, Fisher, Pittsburgh, PA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂, 95% air. In order to maintain a high level of receptor expression, and to obtain reproducible results, only cells were thawed every 1–2 weeks. For all experiments, cells were cultured on 12-mm diameter, 0.4-μm pore size Transwells (Costar, Cambridge, MA) as described (34). Following the second day of plating, cells were fed every fresh medium (1.0 ml apical and 1.5 ml basal) and were used 3–4 days after plating.

**Fixation, Fluorescent Labeling, and Scanning Laser Confocal Analysis of Labeled Cells—** Samples were fixed with paraformaldehyde using a pH-shift protocol, quenched, blocked with 5% (v/v) goat serum, stained, mounted, and stored as described previously (25). The samples were analyzed using an krypton-argon laser coupled to a Molecular Dynamics (Mountain View, CA) Multiprobe 2001 confocal, attached to a Diaphot microscope (Nikon, Melville, NY) with a Plan Apo 60X 1.4 N.A. objective lens (Nikon). The images were converted to tag-information-file-format and the contrast levels of the images adjusted in the Photoshop program (Adobe Co., Mountain View, CA) on a Power PC Macintosh 9500 (Apple, Cupertino, CA). The contrast-corrected images were imported into Freehand (Macromedia, San Francisco, CA) and printed from a Kodak (Rochester, NY) 8650PS dye sublimation printer.
transport buffer (standard assay conditions/buffer are given below). All of these observations confirm that the apical membrane remained unpermeabilized in these preparations and there was little leakage or transcytosis of unbound \[^{125}\text{I}\]Tf from one chamber of the Transwell to the opposite in the time frame of the experiments presented in this work.

Preparation of Rat Liver Cytosol and Reconstitution of \[^{125}\text{I}\]Tf Recycling in Permeabilized MDCK Cells—Preparation of rat liver cytosol has been described previously (24). Reconstituting traffic in the permeabilized cells requires an exogenous source of both energy and cytosol, hence 0.5 ml of KTM containing 8–10 mg/ml rat liver cytosol, 1 mM ATP, 80 mM creatine phosphate, and 50 \(\mu\)g/ml creatine kinase (transport buffer) was added to the basolateral surface of the cells as described previously (24), and 0.5 ml of KTM alone was added to the apical cell surface. The cells were then incubated for periods up to 90 min at 37 °C. At the end of the incubation the amount of \[^{125}\text{I}\]Tf released basolaterally (recycled), released apically (transcytosed), or that remained bound associated was quantified in a Cobra II Auto-Gamma counter from Packard (Downers Grove, IL). Cytosol was not added in some experiments, and in other initial experiments the ATP regenerating system was replaced with 40 units/ml of the ATP depleting enzyme apyrase (grade VI, Sigma). However, we later found that in this permeabilized cell system there is no difference between adding apyrase or not adding it, therefore, the addition of this enzyme was omitted in later experiments.

BoNT/E Treatment of Permeabilized MDCK Cells—The 150-kDa single-chain BoNT/E was produced and purified as described (36). BoNT/E was treated with trypsin to convert the single-chain protein into the 150-kDa di-chain BoNT/E as described previously (37). Neurotoxins (1 mg/ml stocks) were diluted 1:10 in KTM buffer containing 10 mM DTT (freshly dissolved in degassed buffer), and incubated at room temperature for 60 min. The neurotoxins were further diluted in KTM containing 1 mM DTT and then added to the cells during their permeabilization at 37 °C. One difference from the normal assay condition described above was that following SLO binding the permeabilization step was extended to 10 min at 37 °C to allow for maximal proteolytic action of the neurotoxins on SNAP-23. Additional inhibition was observed in this permeabilized cell system when neurotoxin was included in the 90-min transport reaction at 37 °C so this step was omitted.

In one set of experiments (see Fig. 7) single chain or di-chain BoNT/E was reduced with DTT as described above or sham treated. Following SLO binding, cells were washed with DTT-free KTM and then warmed up for 10 min at 37 °C in the presence of neurotoxin reduced or unreduced with DTT. The cells were then washed once with ice-cold DTT-free KTM and then incubated 30 min in ice-cold DTT-free KTM. Transcytosis and recycling of ligand in these neurotoxin-treated cells was assayed as described above except the transport buffer did not contain DTT.

Western Blot Analysis—Immunoblotting was performed as follows. Transwell filters with BoNT/E-treated or sham-treated cells were boiled in 100 mM triethanolamine, pH 8.6, 5 mM EDTA, 0.5% SDS, vortex-shaken for 15 min at 4 °C in a vortex mixer (Model 5432 Eppendorf), and aliquots resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (15% gels). Rainbow markers (Amersham, Arlington Heights, IL) were loaded in adjacent lanes as molecular weight standards. The gels were then equilibrated in 10 mM CAPS-NaOH buffer, pH 11.0 (CAPS buffer), for 10 min at room temperature before the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), for 75 min at 375 mA constant current in CAPS buffer. The Immobilon-P membrane was blocked overnight in 5% BSA (diluted in KTM containing 1 mM DTT and then added to the cells during their permeabilization at 37 °C). One difference from the normal assay condition described above was that following SLO binding the permeabilization step was extended to 10 min at 37 °C to allow for maximal proteolytic action of the neurotoxins on SNAP-23. Additional inhibition was observed in this permeabilized cell system when neurotoxin was included in the 90-min transport reaction at 37 °C so this step was omitted.

NEM Treatment of Permeabilized MDCK Cells and NSF, SNAP-23, and Anti-SNAP-23 Addition—NEM treatment and addition of recombinant NSF were performed as described (24). In some experiments 0.5–0.05 \(\mu\)M recombinant SNAP-23 was added to the transport buffer during the 90-min reaction at 37 °C. In other experiments affinity purified rabbit anti-SNAP-23 or affinity purified anti-human IgA antibodies were added (following the SLO binding step) in the warm-up step at 37 °C, during the cytosol wash-out at 4 °C and in the transport buffer during the 90-min reaction at 37 °C.

RESULTS

MDCK Cells Express SNAP-23—We previously hypothesized that a SNAP-25 homolog exists in polarized MDCK cells (24). One possible candidate is SNAP-23, a widely distributed SNAP-25 homolog that has recently been found in several organs including kidney (28–31). Initially, we determined if SNAP-23 was expressed in MDCK cell by probing Western blots of lysates from SLO-permeabilized MDCK cells with an affinity-purified rabbit polyclonal antibody against SNAP-23. A major protein band was observed at approximately 28 kDa (Fig. 1, left lane), and is slightly larger than the predicted molecular mass of 23 kDa. The slower than expected mobility has been reported previously (31). There were additional protein bands at 60 and 90 kDa. The 60-kDa protein was susceptible to BoNT/E treatment (see below; Fig. 1, right lane, +). The cells were lysed in detergent, the proteins resolved by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon P membranes. The membranes were reacted sequentially with affinity purified anti-SNAP-23 antibodies and goat anti-rabbit horseradish peroxidase, and SNAP-23 (small arrow) was detected by chemiluminescence. Molecular weight markers are shown to the left and the asterisk to the right indicates the 25-kDa cleavage product of SNAP-23 found upon BoNT/E treatment.

SNAP-25 is specifically cleaved by BoNT/E at the Arg 180-Ile181 peptide bond (38), and SNAP-23 was predicted to be cleaved at this site. We previously hypothesized that canine SNAP-23 is susceptible to BoNT/E, we treated SLO-permeabilized MDCK cells with BoNT/E and analyzed the effects of toxin treatment on SNAP-23 immunoreactivity. In cells treated with 650 nM of reduced di-chain BoNT/E there was a large reduction (approximately a 70% loss) in the amount of the 28-kDa band (Fig. 1, right lane) and the appearance of an additional faint band at 25 kDa (marked with an asterisk in Fig. 1). These results are consistent with earlier observations during the 90-min reaction at 37 °C. In other experiments affinity purified rabbit anti-SNAP-23 or affinity purified anti-human IgA antibodies were added (following the SLO binding step) in the warm-up step at 37 °C, during the cytosol wash-out at 4 °C and in the transport buffer during the 90-min reaction at 37 °C.
that BoNT/E cleavage of SNAP-25 removes an approximately 3-kDa fragment from the C terminus of this protein (38) and is predicted to remove a similar size fragment from SNAP-23 (31). This cleavage is consistent with a recent report that canine SNAP-23, unlike human SNAP-23, is susceptible to BoNT/E cleavage (39).

The amount of cleaved fragment and uncleaved SNAP-23 following toxin treatment did not add up to the amount of SNAP-23 found in untreated cells. One possible explanation is that the cleaved fragment is only inefficiently recognized by our polyclonal antibody (i.e. as a result of loss of epitopes). Another possibility is that the uncleaved SNAP-23 fragment is susceptible to other proteinases that rapidly degrade it into small peptides. The significant fraction of uncleaved SNAP-23, following BoNT/E treatment most likely represents SNAP-23 bound in SNARE complexes since, as previously demonstrated, BoNT/E is only weakly effective on assembled fusion complexes (41). 650 nM BoNT/E causes a significant inhibition of recycling in our permeabilized cell system (see below), and we have previously shown that this concentration of toxin gives maximal inhibition of IgA transcytosis and recycling (24).

SNAP-23 Is Found at the Basolateral Cell Surface and in Small Intracellular Vesicles—The distribution of SNAP-23 has been analyzed in several non-polarized cell lines and is found at the plasma membrane and in intracellular vesicles (29, 31). In addition, SNAP-23 has recently been localized to the basolateral surface of pancreatic acinar cells and when human SNAP-23 is overexpressed in MDCK cells it is found at both the apical and basolateral cell surfaces (39, 42). We localized endogenous canine SNAP-23 within MDCK cells using confocal microscopy which allows visualization of individual optical planes through the three-dimensional MDCK monolayer. Our goal was to identify if endogenous SNAP-23 was associated with the plasma membrane and might therefore act (in a manner analogous to SNAP-25) as a t-SNARE for endocytic traffic. The cells were co-stained with a monoclonal antibody that recognizes ZO-1, a marker for the tight junction which separates the apical and basolateral plasma membrane (Fig. 2B). At the level of the brightest ZO-1 staining, SNAP-23 was found in small vesicles across the apical pole of the cell (Fig. 2D). It was not possible to determine whether SNAP-23 was present at the apical plasma membrane because the signal was low and at the light level it is hard to discriminate microvillar staining (which also gives a punctate staining pattern) from SNAP-23 found in subapical vesicles. The brightest SNAP-23 staining was observed in small vesicles along the lateral and basal surfaces and also on what appeared to be the basolateral plasma membrane (Fig. 2, F and H). The specificity of the antibody labeling was confirmed by the addition of exogenous SNAP-23 protein which competitively inhibited binding of SNAP-23 antibodies to the cells and completely eliminated the signal (data not shown).

In order to confirm the localization of SNAP-23 at the basolateral plasma membrane, we performed double labeling with an antibody against E-cadherin (Fig. 3, A, D, G, and J) and anti-SNAP-23 (Fig. 3, B, E, H, and K). At steady-state E-cadherin was found predominantly at the lateral plasma membrane. Fig. 3, G, H, and I, demonstrate that SNAP-23 and E-cadherin were colocalized along the lateral membrane surfaces, but there were distinct, SNAP-23-containing vesicles that lacked E-cadherin. The presence of SNAP-23 on the plasma membrane and intracellularly is consistent with previous observations on the localization of this protein in other cell types (29, 31); however, in MDCK cells there seemed to be an extensive amount of vesicular staining.

Tf Recycling in Permeabilized MDCK Cells Requires ATP,

Cytosol, and NSF—The exit of pIgR-IgA complexes from the ARE and fusion with the apical and basolateral plasma membrane were previously shown to require NSF and were BoNT/E sensitive (24). However, basolateral recycling of IgA ligand was more sensitive to BoNT/E than transcytosis (50% inhibition of recycling versus 30% inhibition of transcytosis). Coupled with the above observations that SNAP-23 was found predominantly on the basolateral plasma membrane and in basolateral vesicles, we determined whether basolateral recycling of Tf (the classical basolateral recycling marker) could be reconstituted.
FIG. 3

SNAP-23 Required for Transferrin Recycling

E-cadherin  SNAP-23  merge

A  B  C

D  E  F

G  H  I

J  K  L

FIG. 3
in a permeabilized cell system. Our objective was to use this system to determine if Tf recycling was susceptible to BoNT/E and required SNAP-23. In addition, we hoped to confirm and extend our original observations and gain insight into the molecular requirements for the basolateral recycling pathway.

Because MDCK cells do not express the appropriate receptors for BoNT/E binding, we have reconstituted Tf recycling traffic in SLO-permeabilized MDCK cells. SLO forms pores in the plasma membrane which are sufficiently large enough to allow BoNT/E to enter the cells. In this assay [125I]Tf was internalized from the basolateral surface for 45 min at 37 °C (upon which all the Tf containing compartments were maximally loaded with ligand). The cells were washed at 4 °C to remove nonspecifically bound ligand and then they were incubated for 2.5 min at 37 °C to allow for cell surface-bound Tf to be internalized, as described previously (25, 32). Under these internalization conditions Tf was found in small vesicles in the basolateral region of the cell and underlying the lateral borders (data not shown). In addition, Tf was found at the apical pole of the cell, both at and above the level of the tight junctions, in a vesicular compartment that has previously been characterized as a subdomain of the AEE (25).

Following [125I]Tf internalization, the cells were rapidly cooled and SLO bound to the basolateral surface. Free SLO was washed from the cell surface, the cells were warmed up to 37 °C to allow permeabilization to occur and then re-cooled to 4 °C for extended cytosol washout. When permeabilized cells were incubated in the presence of an ATP regenerating system and exogenous rat liver cytosol, approximately 45–50% of the pre-internalized ligand was released basolaterally (recycled) over a 90-min incubation period at 37 °C (Fig. 4). This is an efficiency of 50–55% (approximately 90% of ligand is recycled basolaterally in non-permeabilized cells during the same time course).

Basolateral recycling of [125I]Tf required both ATP and cytosol (Fig. 4). These observations are consistent with a previous analysis of the energy and cytosol requirements for Tf recycling in mechanically perforated MDCK cells (32). In the absence of ATP there was a 4–5-fold reduction in recycling and cytosol depletion resulted in a 3-fold reduction.

In this cell system, there was a fraction of Tf (approximately 18–19%) that was released apically (transcytosed). This is more than has been observed in nonpermeabilized cells (typically 5–8%) and may reflect some loss of cytosolic machinery that controls the amount of Tf transcytosis. Because this signal is relatively small, and because Tf is generally considered a marker of the basolateral recycling pathway we have not included the apical release of this marker in the subsequent figures. However, for the sake of completeness the amount of Tf released apically is included in each of the subsequent figure legends. In general, all treatments affected Tf recycling and transcytosis in a similar manner.

As an additional control, we also determined if Tf recycling requires NSF. If so, it would suggest that a bona fide membrane fusion event(s) was occurring. In fact, NEM treatment (which inactivates NSF) reduced recycling by 82% (Fig. 5). No inhibition was observed if both NEM and DTT were added simultaneously, confirming that NEM treatment is the cause of the inhibition. More importantly, exogenous NSF added subsequent to NEM treatment restored much of the recycling activity (72% of the control value) (Fig. 5). This observation confirmed that Tf recycling is NSF-dependent and likely requires use of an NSF-SNAP-SNARE complex. The inability of NSF to completely restore activity has been reported previously and probably reflects the inactivation of other NEM-sensitive proteins other than NSF (24, 43).
The primarily basolateral release, high efficiency, and the requirements for energy, cytosol, and NSF confirm that we have faithfully reconstituted the Tf basolateral recycling pathway in MDCK cells. Previous investigators have permeabilized the plasma membrane domain opposite of the cell surface from which trafficking will be explored (19, 44). However, we find that basolateral recycling in apically permeabilized cells is inefficient and the large amount of SLO toxin required is cost prohibitive. Permeabilizing the same membrane that is the chief target for fusion is not inherently different from other SLO-permeabilized cell systems involving nonpolarized cells in which there is only one plasma membrane domain that is both permeabilized and the target of fusion (45–47).

**Basolateral Recycling of Tf Is BoNT/E-sensitive**—The dependence of Tf recycling on NSF and the expression of SNAP-23 at the basolateral plasma membrane suggests that SNAP-23 may be a t-SNARE involved in Tf delivery to the basolateral cell surface. In order to determine the involvement of SNAP-23 in Tf traffic, SLO-permeabilized cells were treated with reduced di-chain BoNT/E. Tf recycling was inhibited by reduced di-chain BoNT/E in a concentration-dependent fashion (Fig. 6). Maximal inhibition was observed at 650 nM which inhibited ~80% of the cytosol-dependent basolateral release. The cytosol-dependent release was calculated by subtracting the percent release that occurred in the absence of cytosol from the percent release that occurred in the presence of cytosol. This is similar to the reporting technique used by Simons and co-workers (19, 44). BoNT/E had little effect on the fraction of basolateral recycling that occurred in the absence of cytosol (Fig. 6). These cytosol-independent reactions may represent SNAP-23 that is already bound in fusion complexes. As described above, these complexes are thought to be toxin-insensitive. These results support the conclusion that the majority of the cytosol-dependent reaction is sensitive to BoNT/E and therefore may require SNAP-23.

The neurotoxicity of single chain neurotoxins is enhanced by proteolytic cleavage (nicking) into a di-chain form (48), and the inhibitory activity of the di-chain form is further enhanced after reduction of its disulfide bond (49, 50). For example, proteolysis of synthetic peptides of SNAP-25 and VAMP-2 by BoNT/E, BoNT/A, and BoNT/B are stimulated by reduction with DTT. Following the cytosol wash-out, the percent of total ligand released basolaterally (recycled) during a 90-min incubation at 37 °C in the presence of an ATP-regenerating system (ATPRS) and in the presence or absence of rat liver cytosol (RLC) was quantified. The percent of total ligand released apically was as follows: ATPRS + RLC, 19.3 ± 0.2%; ATPRS + RLC + 65 nM toxin, 18.9 ± 1.9%; ATPRS + RLC + 130 nM toxin, 13.5 ± 1.5%; ATPRS + RLC + 650 nM toxin, 4.3 ± 3.4%; ATPRS – RLC, 5.5 ± 0.5%; ATPRS – RLC + 650 nM toxin 8.5 ± 1.0%. Mean and S.D. of values from a representative experiment are given (n = 3).

**Fig. 6. Effect of reduced di-chain BoNT/E on recycling of Tf.** Ligand was internalized, SLO bound to the basolateral cell surfaces, and cells permeabilized for 10 min at 37 °C in the presence of 0 (control), 65, 130, or 650 nM di-chain BoNT/E reduced with DTT. Following the cytosol wash-out, the percent of total ligand released basolaterally (recycled) during a 90-min incubation at 37 °C in the presence of an ATP-regenerating system (ATPRS) and in the presence or absence of rat liver cytosol (RLC) was quantified. The percent of total ligand released apically was as follows: ATPRS + RLC, 19.3 ± 0.2%; ATPRS + RLC + 65 nM toxin, 18.9 ± 1.9%; ATPRS + RLC + 130 nM toxin, 13.5 ± 1.5%; ATPRS + RLC + 650 nM toxin, 4.3 ± 3.4%; ATPRS – RLC, 5.5 ± 0.5%; ATPRS – RLC + 650 nM toxin 8.5 ± 1.0%. Mean and S.D. of values from a representative experiment are given (n = 3).

**Fig. 7. Inhibitory activity of di-chain and single chain BoNT/E (ζ reduction) on Tf release.** Ligand was internalized, SLO bound to the basolateral cell surfaces, and cells permeabilized for 10 min at 37 °C in the absence or presence of 300 nM di-chain (panel A) or 600 nM single chain toxin (panel B). BoNT/E that was either sham-treated or reduced with DTT. Following a cytosol washout in the absence of DTT, the percent of ligand recycled during a 90-min incubation at 37 °C was determined. Neurotoxin and DTT were not included in the final incubation at 37 °C. The percent of total ligand released apically was as follows: – di-chain toxin + DTT, 17.9 ± 1.0%; – di-chain toxin – DTT, 18.0 ± 1.3%; + di-chain toxin + DTT, 17.9 ± 1.0%; + di-chain toxin – DTT, 17.5 ± 1.0%; – single chain toxin + DTT, 18.2 ± 0.3%; – single chain toxin – DTT, 16.5 ± 1.6%; + single chain toxin + DTT, 19.4 ± 1.1%; + single chain toxin – DTT, 19.2 ± 2.2%; The mean and S.D. of values are given (n = 3).
be proteolyzing some unknown substrate that is required for Tf recycling. Since endogenous SNAP-23 is believed to be membrane-bound (probably via palmitoylation of its internal cysteine residues) (28, 29) and part of the SNARE complex for vesicle fusion, addition of exogenous SNAP-23 might act as a competitive inhibitor of basolateral recycling by forming non-productive interactions between exogenous SNAP-23 and endogenous syntaxin or VAMP. Earlier studies have shown that a 20-amino acid peptide derived from the C terminus of SNAP-25 can inhibit SNAP-25-dependent exocytosis in chromaffin cells (53). In this system, addition of recombinant SNAP-23 inhibited approximately 45% of cytosol-dependent Tf recycling (Fig. 8A), but had no effect on the cytosol-independent reactions.

As additional evidence, we tested the effect of anti-SNAP-23 antibody addition on the assay (Fig. 8B). This is expected to inhibit endocytic traffic by binding SNAP-23 and sterically hindering its ability to interact with other proteins. Antibody treatment resulted in an approximately 35% inhibition of the cytosol-dependent recycling reaction (Fig. 8B). A nonspecific antibody against human IgA (used as a control) had no effect on Tf recycling. These results confirm that SNAP-23 is required for a fraction of Tf recycling.

**DISCUSSION**

The SNARE hypothesis predicts that the specificity of vesicle targeting and fusion is mediated through specific interactions between v-SNAREs and t-SNAREs (7). If true, then each membrane trafficking event would require distinct isoforms or homologs of the various SNARE components. Until recently, however, only isoforms of syntaxin and VAMP, but no mammalian homologs of SNAP-25 have been identified (54, 55). In the last 2 years, Ravichandran et al. (28) identified a SNAP-25 homolog with a predicted molecular mass of 23 kDa called SNAP-23. This protein, expressed in several tissues, is able to bind various syntaxins and VAMPs. A mouse isoform of SNAP-23, also known as syndet, has recently been cloned from an adipocyte 3T3 L1 cDNA library (29, 30), and has a similar cellular distribution to human SNAP-23. Munc 18c, an N-sec-1 homolog and putative regulator of the NSF/SNAP/SNARE complex, has been shown to inhibit SNAP-23 and syntaxin 4 binding in vitro (30). While these observations identify some of the potential binding partners of SNAP-23, the role of SNAP-23 in membrane trafficking remains largely uncharacterized.

Previous work is consistent with the hypothesis that SNAP-23 plays a role in exocytosis. Banerjee et al. (56, 57) have shown that exocytosis of large core granules in permeabilized PC12 cells is inhibited completely by BoNT/E but not BoNT/A, even though SNAP-25 is efficiently cleaved by both toxins. However, when PC12 cells are treated with nerve growth factor, release of granules became BoNT/A-sensitive. It is now known that these cells express both SNAP-25 and SNAP-23 (31). It is therefore possible that SNAP-23, which is sensitive to E-toxin and predicted to be insensitive to A-toxin, may be required for release of PC12 storage granules in untreated cells, while in nerve growth factor-treated cells SNAP-25 (and possibly SNAP-23) is required. These observations also suggest that usage and expression of SNARE components may change with the development of any particular cell or tissue.

In the present study we have demonstrated that SNAP-23 is not only present in MDCK cells, and cleaved by BoNT/E, but is required for basolateral recycling of Tf. Our observation that canine SNAP-23 is susceptible to toxin cleavage is consistent with work by Low et al. (39) which have shown that the canine isoform of SNAP-23 is sensitive to BoNT/E cleavage while the human isoform of SNAP-23 is insensitive. Even though BoNT/E is thought to specifically cleave SNAP-25 and SNAP-25 homologs, other proteins in the cell may be susceptible. In order to examine SNAP-23 involvement more directly, we added either exogenous SNAP-23 or anti-SNAP-23 antibodies to the assay. Addition of either resulted in an inhibition of recycling; however, the level of inhibition was less than that attained with BoNT/E treatment. The added SNAP-23 and anti-SNAP-23 antibodies might only compete at steps prior to SNARE complex formation and only ineffectively compete or

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**Fig. 8. Effect of SNAP-23 and anti-SNAP-23 antibodies on Tf recycling in permeabilized MDCK cells.** A, SNAP-23 (0.05–0.5 μg/ml) was included during the 90-min transport reaction at 37 °C. The percent of total ligand released basolaterally in the presence of an ATP-regenerating system (ATPRS) and in the presence or absence of rat liver cytosol (RLC) was quantified. The percent of total ligand released apically was as follows: ATPRS + RLC, 19.9 ± 1.9%; ATPRS + RLC + 0.05 μg SNAP-23, 18.9 ± 1.0%; ATPRS + RLC + 0.125 μg SNAP-23, 13.6 ± 1.8%; ATPRS + RLC + 0.5 μg SNAP-23, 11.8 ± 3.3%; ATPRS – RLC, 6.9 ± 1.3%; ATPRS – RLC + 0.5 μg SNAP-23, 7.5 ± 1.4%. Mean and S.D. of values from a representative experiment are given (n = 3). B, ligand was internalized, SLO bound to the basolateral cell surfaces, and cells permeabilized for 10 min at 37 °C in the absence (control) or presence of anti-SNAP-23 or anti-human IgA (hIgA) antibodies. Antibodies were included during the cytosol wash-out and during the 90-min transport reaction at 37 °C. The percent of ligand released basolaterally during a 90-min incubation at 37 °C in the presence of an ATP-regenerating system (ATPRS) and in the presence or absence of rat liver cytosol (RLC) was quantified. The percent of total ligand released apically was as follows: ATPRS + RLC, 13.7 ± 0.6%; ATPRS + RLC + 20 μg/ml anti-SNAP-23, 10.4 ± 1.0%; ATPRS + RLC + 4 μg/ml anti-SNAP-23, 10.5 ± 0.6%; ATPRS + RLC + 20 μg/ml anti-hIgA, 15.1 ± 1.6%; ATPRS – RLC, 10.0 ± 0.5%; ATPRS – RLC + 0.5 μg/ml anti-SNAP-23, 0.3 ± 0.9%. Mean and S.D. of values from a representative experiment are given (n = 3).
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sterically hinder already formed SNARE complexes. This possibility would be consistent with recent observations by Weber et al. (8) who have reconstituted v- and t-SNAREs into separate lipid bilayer vesicles which fuse with one another in a SNARE-dependent reaction. This fusion reaction can be inhibited when soluble VAMP or t-SNAREs are included in a preincubation reaction (during which fusion complex formation is occurring). However, they have no effect when added to reactions in which fusion complexes have already formed. Another possibility is that there are additional BoNT/E-sensitive SNAP-23/SNAP-25 homologs in MDCK cells. These unidentified homologs could account for the higher level of BoNT/E inhibition observed than when SNAP-23 or anti-SNAP-23 antibodies were added exogenously to the permeabilized cell system.

Localization of SNAP-23 reveals that it is present along the basolateral plasma membrane. It was not possible to determine unequivocally if SNAP-23 was present at the apical cell surface. Ultrastructural localization of SNAP-23 is currently unequivocally if SNAP-23 was present at the apical cell surface. There was extensive localization of endogenous face. Ultrastructural localization of SNAP-23 is currently unequivocally if SNAP-23 was present at the apical cell surface. It was not possible to determine when SNAP-23 or anti-SNAP-23 antibodies were added exogenously to permeabilized cell system.

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