

Influenza M2 Proton Channel Activity Selectively Inhibits Trans-Golgi Network Release of Apical Membrane and Secreted Proteins in Polarized Madin-Darby Canine Kidney Cells

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Abstract. The function of acidification in protein sorting along the biosynthetic pathway has been difficult to elucidate, in part because reagents used to alter organelle pH affect all acidified compartments and are poorly reversible. We have used a novel approach to examine the role of acidification in protein sorting in polarized Madin-Darby canine kidney (MDCK) cells. We expressed the influenza virus M2 protein, an acid-activated ion channel that equilibrates luminal and cytosolic pH, in polarized MDCK cells and examined the consequences on the targeting and delivery of apical and basolateral proteins. M2 activity affects the pH of only a subset of acidified organelles, and its activity can be rapidly reversed using ion channel blockers (Henkel, J.R., G. Apodaca, Y. Altschuler, S. Hardy, and O.A. Weisz. 1998. *Mol. Biol. Cell.* 8:2477–2490; Henkel, J.R., J.L. Popovich, G.A. Gibson, S.C. Watkins, and O.A. Weisz. 1999. *J. Biol. Chem.* 274:9854–9860). M2 expres-

sion significantly decreased the kinetics of cell surface delivery of the apical membrane protein influenza hemagglutinin, but not of the basolaterally delivered polymeric immunoglobulin receptor. Similarly, the kinetics of apical secretion of a soluble form of γ -glutamyl-transpeptidase were reduced with no effect on the basolaterally secreted fraction. Interestingly, M2 activity had no effect on the rate of secretion of a nonglycosylated protein (human growth hormone [hGH]) that was secreted equally from both surfaces. However, M2 slowed apical secretion of a glycosylated mutant of hGH that was secreted predominantly apically. Our results suggest a role for acidic trans-Golgi network pH in signal-mediated loading of apical cargo into forming vesicles.

Key words: acidification • polarity • Madin-Darby canine kidney • influenza M2 • apical

Introduction

Although the pH of organelles along the secretory pathway is known to be important for proper processing and sorting of proteins and lipids, the function of acidification in protein trafficking remains poorly understood. Most studies examining the role of pH have demonstrated decreased rates of protein trafficking, both along the biosynthetic and postendocytic pathways; however, the compartments affected are in dispute. In part, this is due to difficulties with current methods used to perturb organelle pH. These reagents include treatment with weak bases such as chloroquine, primaquine, and ammonium chloride (for review see Dean et al., 1984) that must be used at relatively high concentrations (10–50 mM), and cause os-

motonic swelling of many intracellular compartments. In addition, these reagents transiently raise the pH of the cytosol as they alkalinize acidic organelles (Dean et al., 1984; Ilundain, 1992; Seagrave et al., 1992), which may contribute to some of the phenotypes observed in weak base-treated cells (Davoust et al., 1987; Parton et al., 1991). Indeed, some effects of chloroquine on membrane traffic have been demonstrated to be independent of its effects on organelle pH (Romanek et al., 1993). More recent studies have used inhibitors of vacuolar type H⁺ ATPases (V-ATPases),¹ such as concanamycins A and B and the

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¹Abbreviations used in this paper: AMT, amantadine; AV, adenovirus; AV-TA, adenovirus encoding tTA; BafA₁, bafilomycin A₁; endo H, endoglycosidase H; γ GT, γ -glutamyltranspeptidase; ghGH, glycosylated hGH; HA, hemagglutinin; hGH, human growth hormone; pIgR, polymeric Ig receptor; PM, plasma membrane; V-ATPase, vacuolar H⁺-ATPase.

more specific bafilomycin A₁ (BafA₁) to perturb the pH of intracellular compartments. These inhibitors are relatively specific, effective at low concentrations, and alter the pH of all compartments that are acidified by V-ATPases. However, BafA₁ at least is only poorly reversible, and causes swelling of even nonacidic compartments (Palokangas et al., 1994). Moreover, some cells can become resistant to these treatments and are able to reacidify their organelles after even short incubations in drug (Temesvari et al., 1996). Finally, other proton transporters may also play a role in acidification of some compartments (Okhuma et al., 1982). These factors may account for the vastly different effects of these inhibitors on biosynthetic and postendocytic protein traffic reported by different investigators.

Few studies have focused on the function of acidification in protein sorting in polarized cells. Matlin (1986) showed that ammonium chloride slows the rate of apical delivery of newly synthesized influenza hemagglutinin (HA) to the cell surface, but does not appear to affect its polarity of delivery. By contrast, in another study, treatment with monensin or chloroquine resulted in a twofold increase in the ratio of apical to basolateral secretion of both an endogenous protein complex (gp80) that is directed apically, and an exogenous marker (lysozyme) that is secreted equally from both plasma membrane (PM) domains (Parczyk and Kondor-Koch, 1989). Finally, Caplan et al. (1987) observed that polarized MDCK cells treated with the weak base ammonium chloride misdirect about half of the newly synthesized soluble basolateral markers laminin and heparan sulphate proteoglycan to the apical medium. In this study, the polarity of secretion of apical markers was unaffected by ammonium chloride treatment, as were delivery of apical and basolateral membrane proteins; however, the rates of transport were not measured. Thus, the role of TGN pH in polarized protein sorting remains unclear.

We have developed a novel method to alter intraorganellar pH that overcomes many of these difficulties. This involves expression of the acid-activated ion channel M2, a component of influenza virus that allows protons to efflux from acidic compartments. Unlike weak bases or V-ATPase inhibitors, which globally affect the pH of all acidic organelles, M2 should alter the pH of only those compartments in which it is present. We demonstrated previously that M2 expressed in MDCK cells localizes to the PM, where it should be inactive under normal conditions, as well as to the TGN and to the apical recycling endosome (Henkel et al., 1998). M2 expression affected the rate of basolateral-to-apical transcytosis and apical recycling in polarized MDCK cells, but had no effect on the rate of basolateral recycling or on protein degradation. Thus, M2 expression is a useful tool with which to selectively perturb a subset of acidified compartments in the cell. Therefore, we examined the effect of M2 expression on the delivery of newly synthesized proteins in polarized MDCK cells. Interestingly, our data suggest that delivery of apical proteins is preferentially inhibited by M2 activity. Our results are most consistent with a function for TGN acidification in loading presorted apical cargo into forming vesicles.

Materials and Methods

DNA Constructs

A secreted form of γ -glutamyltranspeptidase (gD- γ GT) was generated by substituting the cleavable signal sequence of the herpes gD surface glycoprotein for the signal/anchor of γ GT. The cDNA corresponding to gD residues 1–35 and γ GT residues 28–568 were amplified from cloned cDNAs by PCR using specific primers and cloned separately into pCR2 (Invitrogen). The gD (EcoRI-BssHI) and γ GT (BssHI-XhoI) fragments were subcloned into pREP4 (Invitrogen) to generate the final construct, which contains two additional residues (alanine and arginine) between the gD sequence and γ GT. The chimera was subsequently cloned into pCDNA 3 (Invitrogen) and a stable MDCK cell line generated (see below). The sss/human growth hormone (hGH)-mycHis chimera encodes a secreted version of the hGH with COOH-terminal myc and His tags. To generate this construct, the signal/anchor of rat γ GT was shortened to create a cleavable signal sequence (sss) by removal of nucleotides encoding residues 16–23. This fragment of γ GT (36 residues total) and residues 32–217 from hGH (pOGH; Nichols Institute) were amplified by PCR with specific primers and cloned into pCR2. The chimera was prepared by subcloning the sss (EcoRI-BamHI) and hGH (BamHI-HindIII) fragments first into pBluescript-SK(–) (Stratagene), and a myc-His-tagged version was generated by subsequent cloning into pCDNA3.1mycHisA(–) (Invitrogen). The glycosylated version of sss/hGH-mycHis (ghGH) was prepared by site-directed mutagenesis of sss/hGH-mycHis based on the Morph™ procedure (5 Prime, Inc.). Specific primers were used to convert Met 40 to Thr, and Leu 118 to Asn to generate two consensus sites for N-linked glycosylation. Selection of these sites was based on those used previously to generate glycosylated rat growth hormone (Scheiffele et al., 1995).

Cell Lines

Low passage MDCK cells (type II) were maintained in minimal essential medium (Cellgro; Fisher Scientific) supplemented with 10% FBS (Atlanta Biologicals), streptomycin (100 μ g/ml), and penicillin (100 U/ml). Generation and characterization of the MDCK T23 cell line, which stably expresses the tetracycline-repressible transactivator tTA (Gossen and Bujard, 1992), is described in Barth et al. (1997). Stable lines of MDCK expressing gD- γ GT were generated using Lipofectamine-mediated transfection, and individual clones were selected in 0.5 mg/ml G418 (Life Technologies, Inc.). Stable transfectants of MDCK expressing hGH or ghGH were generated using the methods described in Weisz et al. (1992), and mixed drug-resistant populations were maintained in 0.5 mg/ml G418. Cell lines expressing gD- γ GT, hGH, and ghGH were induced with 2–10 mM butyrate immediately after adenoviral infection. Because these cell lines do not stably express tTA, recombinant adenovirus (AV) encoding tTA (AV-TA) was included in all infections with these cells (see below). Unless indicated otherwise, cells were seeded at high density ($\sim 2 \times 10^5$ cells/well) on 12-mm Transwells (0.4- μ m pore; Costar) for 2–3 d before infection with recombinant AV. Experiments were performed the following day.

Recombinant AVs and Adenoviral Infection

Construction and purification of E1 substituted recombinant AVs encoding M2 in the correct and reverse orientations (AV-M2 and M2-rev, respectively), AV-TA, and influenza HA (AV-HA) are described in Henkel et al. (1998). cDNA encoding rabbit polymeric Ig receptor (pIgR) (provided by Dr. Gerard Apodaca, University of Pittsburgh, Pittsburgh, PA) was subcloned into the pAdlox vector, and a recombinant AV (AV-pIgR) generated as described in Hardy et al. (1997). In some experiments, we measured the effect of M2 activity on trafficking of pIgR stably expressed in MDCK T23 cells. Results from these experiments were similar to those in which pIgR was expressed in MDCK cells using AV. Filter-grown MDCK or MDCK T23 cells were washed by adding 3 ml calcium-free PBS containing 1 mM MgCl₂ (PBS-M) to the apical chamber and allowing it to spill over into the basolateral compartment. After 3–5 min at room temperature, the PBS-M was removed and 150 μ l PBS-M containing recombinant AV was added to the apical compartment (multiplicity of infection [m.o.i.] of 25 for AV-HA or AV-pIgR, 250 for AV-M2 or AV-M2rev, and 125 for AV-TA where appropriate). The medium in the basolateral compartment was replaced with 0.5 ml PBS-M. The dishes were rocked briefly by hand and the cells were returned to an incubator for 1–2 h. Mock-infected cells were treated identically, except that virus was omitted dur-

ing the incubation period. Dishes were then rinsed with 2 ml PBS-M, and cells were incubated overnight in growth medium (1 ml apical, 1.5 ml basolateral). Doxycycline (20 ng/ml; Sigma Chemical Co.) was added as a 1,000-fold concentrated stock prepared in 95% ethanol at this step to inhibit M2 expression.

Cell Surface Delivery of HA

Cell surface delivery of HA was measured essentially as described in Henkel and Weisz (1998). Infected, filter-grown cells were rinsed once with PBS, starved for 30 min in medium A, and pulse labeled for 15 min with 0.5–1 mCi/ml [³⁵S]methionine (in vitro labeling mix; NEN). Unless indicated, cells were then incubated for 2 h at 19°C in medium B to accumulate newly synthesized HA in the TGN. Where indicated, the M2 ion channel inhibitors amantadine (5 μM; AMT; Sigma Chemical Co.) or BL-1743 (5 μM) (gift of Dr. Mark Krystal, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT) were included in the starve medium and during subsequent steps. In the experiment shown in Fig. 3 A, some samples were removed at this time, immunoprecipitated, and treated with endoglycosidase H (endo H) as described in Weisz et al. (1992). The medium was replaced with prewarmed medium B and the cells were transferred to 37°C. At the indicated times, cells were rapidly chilled to 0°C by rinsing with ice-cold PBS, and the apical surface trypsinized for 30 min on ice with medium B containing 100 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin (Sigma Chemical Co.). In some experiments, a lower concentration of trypsin (10 μg/ml) was added directly to the chase medium at 37°C instead. Results using either of these protocols were very similar. The trypsin was quenched by two 10-min incubations in medium B containing 200 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.). The filters were rinsed with PBS, solubilized in detergent solution (50 mM Tris-HCl, 2% NP-40, 0.4% deoxycholate, 62.5 mM EDTA, pH 8.0) supplemented with 1 μg/ml aprotinin and 200 μg/ml soybean trypsin inhibitor, and HA was immunoprecipitated using mAb Fc125. This antibody quantitatively immunoprecipitates HA and its cleavage products (HA1 and HA2, which remain associated via disulfide bonds). After collection of antibody-antigen complexes using fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Novabiochem Corp.), samples were washed three times with RIPA buffer (10 mM Tris-HCl, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 1% NP-40, 0.1% SDS, pH 7.4). Samples were electrophoresed on 10% SDS-polyacrylamide gels and the percentage of cleaved HA was determined using a PhosphorImager with Quantity One software (Personal Molecular Imager FX; Bio-Rad).

Cell Surface Delivery of pIgR

To quantitate cell surface delivery of pIgR, cells were radiolabeled for 15 min in 0.5 ml medium A containing 50 μCi/ml [³⁵S]methionine, then incubated in chase medium at 37°C. TPCK trypsin (25 μg/ml) was added to the basolateral chamber of some filters, while another set was untreated. At the indicated times, the apical and basolateral media from untreated filters were collected and combined, a fivefold concentrated stock of detergent solution was added, and the filters were solubilized in the same tube. For trypsin-treated samples, the basolateral medium was discarded, the filters were quenched twice for 10 min on ice with 100 μg/ml soybean trypsin inhibitor, and the filters and apical medium were solubilized together in detergent solution. pIgR was immunoprecipitated using a sheep antibody raised against secretory component (gift of Dr. Keith Mostov, University of California at San Francisco, San Francisco, CA) conjugated to protein G Sepharose. Samples were electrophoresed on 10% SDS-PAGE gels, and the rate of pIgR delivery to the basolateral cell surface was determined by calculating the amount of pIgR recovered in trypsinized samples compared with untreated filters.

Kinetics of Secretion of Soluble Proteins

Infected, filter-grown cells were rinsed once with PBS, starved for 30 min in medium A, and pulse labeled for the indicated time with [³⁵S]methionine. Cells were chased for 2 h at 19°C in medium B, the medium was replaced with fresh prewarmed medium B, and the cells were transferred to 37°C. The 19°C chase prevented subsequent efficient release of ghGH, so this step was omitted for these experiments. At the indicated chase times, apical and basolateral media were collected separately and replaced with fresh prewarmed medium. At the end of the experiment, filters were solubilized with detergent solution (for hGH and ghGH) or octylglucoside buffer (for gD-γGT; 10 mM Hepes, 150 mM NaCl, 60 mM octylglucoside,

0.1% SDS, pH 7.4), and a fivefold concentrated stock of the lysis solution was added to the media. Samples were immunoprecipitated using a goat polyclonal antibody against γ-glutamyltranspeptidase (Hughes et al., 1986) or mAb 9E10 against the myc tag of hGH and ghGH (provided by Dr. Gerard Apodaca).

Mechanical Perforation of Polarized MDCK T23 Cells

MDCK T23 cells grown on 75-mm diameter filter inserts were infected with AV-HA and either AV-M2rev or AV-M2. MDCK cells stably expressing hGH were similarly cultured and infected with AV-TA and either AV-M2rev or AV-M2. The following day, cells were starved, radiolabeled, and chased at 19°C as described above. Where indicated, AMT was included during all steps. Perforation was performed at 19°C essentially as described by Bennett et al. (1988) with minor modifications. Filters were washed twice with GGA buffer (25 mM Hepes, 38 mM potassium gluconate, 38 mM potassium glutamate, 38 mM potassium aspartate, 25 mM magnesium chloride, 1 mM dithiothreitol, 2 mM EGTA, pH 7.4), then cut from their holders. A prewetted sheet of nitrocellulose was carefully laid on top of the cells, covered with filter paper, carefully stroked using a bent glass pipette, and the filter paper removed. After 90 s, the nitrocellulose was rewetted by addition of KOAc⁻ buffer (115 mM potassium acetate, 25 mM Hepes, 2.5 mM magnesium acetate, pH 7.4), and the excess buffer removed. The filter was cut into six wedges and the nitrocellulose was slowly peeled from the cells with tweezers. Filters were placed cell-side down into 6-well dishes containing KOAc⁻ and incubated for 10 min, then transferred to prewarmed dishes containing GGA with (three or four wedges) or without (two or three wedges) an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, 50 μg/ml creatine kinase). After 60 min at 37°C, the medium was collected, centrifuged for 5 min at maximal speed in a microfuge, and fivefold concentrated detergent solution containing aprotinin was added. Cells were solubilized in detergent solution, and HA or hGH was immunoprecipitated from all samples as described above.

Indirect Immunofluorescence

Indirect immunofluorescence staining of filter-grown MDCK cells expressing M2 and hGH was performed as described previously (Henkel et al., 1998). M2 and hGH were detected using monoclonal anti-M2 and anti-myc antibodies, respectively. Cells were viewed using a Nikon Optiphot microscope (Fryer Company, Inc.) and images were acquired in Canvas (Deneba Software) using a Hamamatsu C5985 chilled CCD camera (8 bit, 756 × 483 pixels).

Results

M2 Slows Apical but Not Basolateral Biosynthetic Delivery

Our earlier studies in nonpolarized cells suggested that M2 activity slowed biosynthetic delivery of newly synthesized proteins to the PM, and that delivery from the TGN to the cell surface was directly affected by M2 activity (Henkel and Weisz, 1998; Henkel et al., 1999). Therefore, we tested whether M2 activity affected apical or basolateral biosynthetic delivery in polarized cells. Initially, we measured the effect of M2 on the rate of transport of newly synthesized HA or pIgR throughout the entire secretory pathway (Fig. 1). Cells expressing HA or pIgR and either M2rev or M2 were radiolabeled, then chased and the kinetics of HA and pIgR cell surface delivery were quantitated as described in Materials and Methods. M2 slowed the rate of HA delivery to the apical surface (Fig. 1, a and b), and the effect of M2 was abolished by inclusion of the M2 ion channel inhibitor AMT. By contrast, M2 activity expression had no effect on the rate of pIgR delivery to the basolateral surface (Fig. 1, c and d). M2 had a very small effect on the rate of acquisition of endo H-resistant oligosaccharides on HA (data not shown); however, this may be due to indi-

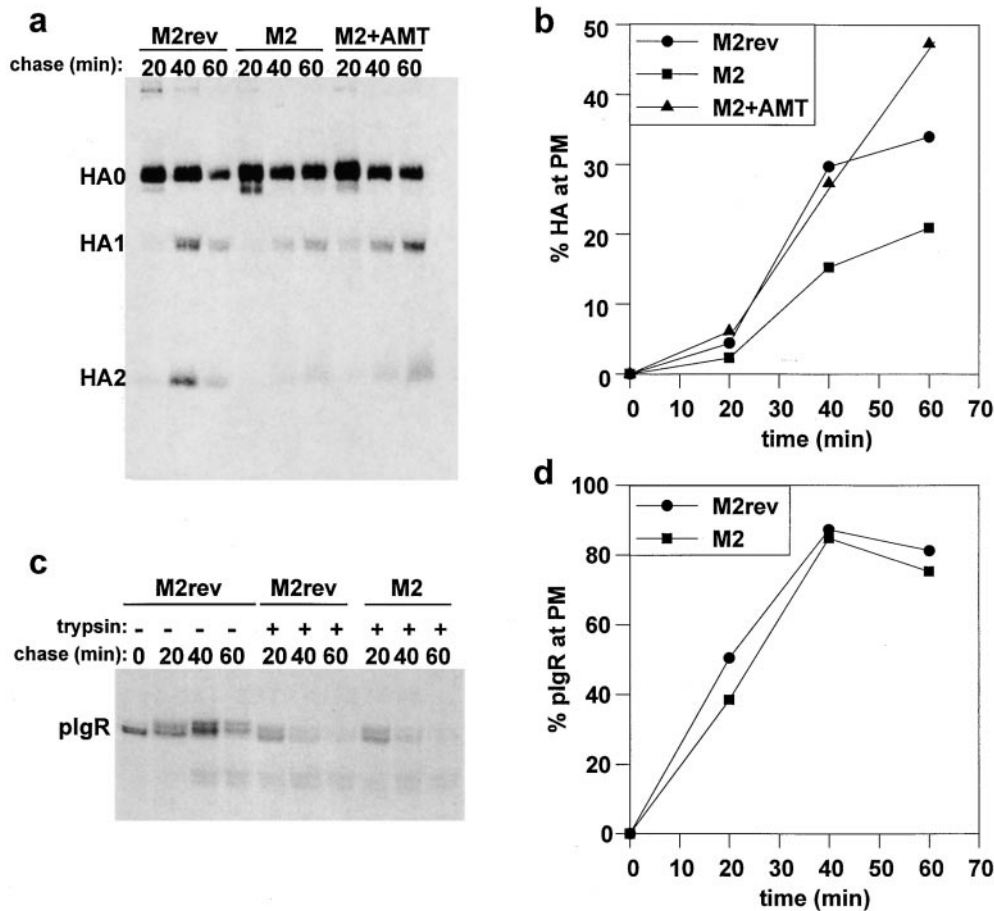


Figure 1. M2 activity slows delivery of proteins to the apical but not the basolateral surface of MDCK cells. Filter-grown MDCK cells were infected with AVs encoding M2rev or M2 and either HA (a and b) or pIgR (c and d). Cells were starved, radiolabeled for 15 min, chased at 37°C for the indicated times, and the kinetics of protein delivery to the PM quantitated using surface trypsinization assays as described in Materials and Methods. (a and b) Kinetics of HA delivery to the apical surface of polarized MDCKs. Quantitation of the gel in a is shown in b. In this experiment, HA2 migrated as a diffuse band in some samples. This was not typically observed. M2 slows cell surface delivery of HA, and the effect of M2 on HA delivery is abolished by inclusion of AMT throughout the experiment. (c and d) Kinetics of pIgR delivery to the basolateral surface. Quantitation of the gel in c is shown in d. Delivery of pIgR to the basolateral surface is unaffected by

M2 expression. The more rapidly migrating doublet represents pIgR cleaved at the apical surface after transcytosis. All experiments were repeated at least three times with similar results.

rect effects of M2 on early secretory traffic (Henkel and Weisz, 1998).

To confirm that TGN-to-apical PM delivery is the predominant step affected by M2, we staged HA in the TGN by chasing for 2 h at 19°C. Under these conditions, HA co-expressed with either M2rev or M2 was equally resistant to treatment with endo H (>80% resistant; Fig. 2 a), suggesting that the majority of newly synthesized HA had transited the medial Golgi complex by this time. The cells were then warmed and HA delivery to the apical and basolateral surfaces was measured as described above (Fig. 2 b). As predicted, M2 slowed delivery of HA to the apical surface, and the effect of M2 was abolished when the M2 inhibitor BL-1743 was included in the medium during the experiment. Very little HA was delivered to the basolateral cell surface, and we did not find any significant effect of M2 expression on the kinetics of HA basolateral delivery, although the low levels of basolateral HA did not allow us to investigate this in detail. Surprisingly, we observed no effect of M2 activity on the ultimate polarity of HA delivery to the cell surface; at long chase times, ~85% of HA was delivered to the apical surface under all conditions (data not shown). Thus, M2 activity appears to delay apical delivery of HA but does not interfere with its ultimate destination. Moreover, the effect of M2 occurs after the protein has reached the TGN.

Several previous studies have suggested that delivery of

membrane and soluble proteins are subject to different constraints and may traffic to the PM in different vesicles (Boll et al., 1991; de Almeida and Stow, 1991; Saucan and Palade, 1994). Therefore, we asked whether M2 affects the rate of secretion of soluble proteins to the apical and basolateral medium. For this purpose, we used a mutant soluble form of γ -glutamyltranspeptidase (gD- γ GT) that is secreted predominantly apically (~70:30, apical:basolateral). The polarity of secretion of this mutant is similar to the polarity of delivery of wild-type, membrane-bound γ GT. Stable cell lines expressing gD- γ GT were mock-infected or infected with AV-TA and AV-M2rev or AV-M2, and the kinetics of TGN-to-apical and -basolateral secretion of gD- γ GT quantitated (Fig. 3). The kinetics of apical secretion of gD- γ GT were slowed in cells expressing M2, whereas basolateral secretion was unaffected. Thus, M2 selectively affects the rate of apical delivery.

M2 Expression Affects Release of Apical Proteins from the TGN

The effect of M2 on apical delivery could be due to a defect in cargo incorporation into apical vesicles, to defective vesicle release from the TGN, or to inhibition of vesicle fusion with the apical cell surface. To distinguish between the latter two possibilities, we tested whether M2 expression interfered with release of apical cargo from the TGN.

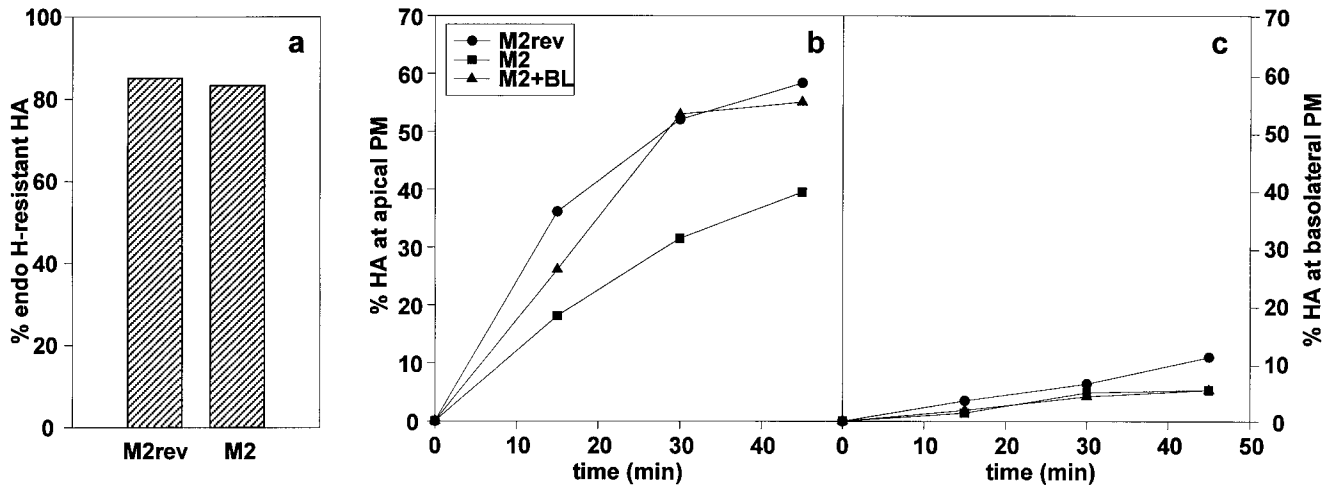


Figure 2. M2 slows delivery of HA from the TGN to the apical surface. (a) MDCK cells expressing HA and M2 or M2rev were pulse labeled for 15 min and chased at 19°C for 2 h. Cells were solubilized, and HA was immunoprecipitated and treated with endo H. M2 activity has no effect on the endo H resistance of HA isolated under these conditions. (b and c) Cells labeled and chased as above were then warmed to 37°C and the kinetics of TGN-to-apical (b) and -basolateral (c) delivery were quantitated. The M2 ion channel inhibitor BL-1743 was included in the indicated samples (M2 + BL) to block M2 activity. Active M2 consistently slowed delivery of HA from the TGN to the apical but not the basolateral cell surface.

MDCK T23 cells expressing HA and either M2rev or M2 were radiolabeled, then chased at 19°C. The apical membranes were then mechanically perforated using nitrocellulose, the cells were warmed to 37°C in the presence or absence of an ATP-regenerating system, and the release of mature HA into the medium was quantitated (Fig. 4). This assay measures the release of HA from the TGN; inhibition of HA release could indicate a defect in vesicle formation or in cargo incorporation into vesicles. Less than 50% as much HA was released from cells expressing M2 compared with M2rev, and inclusion of AMT during the experiment blocked the effect of M2 on HA release. This suggests that the effect of M2 on biosynthetic transport occurs at the level of exit from the TGN.

Mechanism of M2 Effect on Apical Delivery

The effect of M2 on release of apical cargo from the TGN could occur at several steps. M2 could affect the recognition of apical sorting signals in the TGN, although this is unlikely given that the overall polarity of delivery is unaffected by M2 expression. Alternatively, M2 could affect loading of presorted cargo into apical vesicles at the TGN. Finally, M2 could disrupt the regulation or mechanics of apical vesicle formation or budding. To determine whether M2 activity affects the rate of formation or number of apically derived vesicles formed at the TGN, we asked whether M2 affects apical delivery of hGH, a non-polarized secreted protein that does not contain intrinsic apical sorting signals. If M2 activity causes a general defect

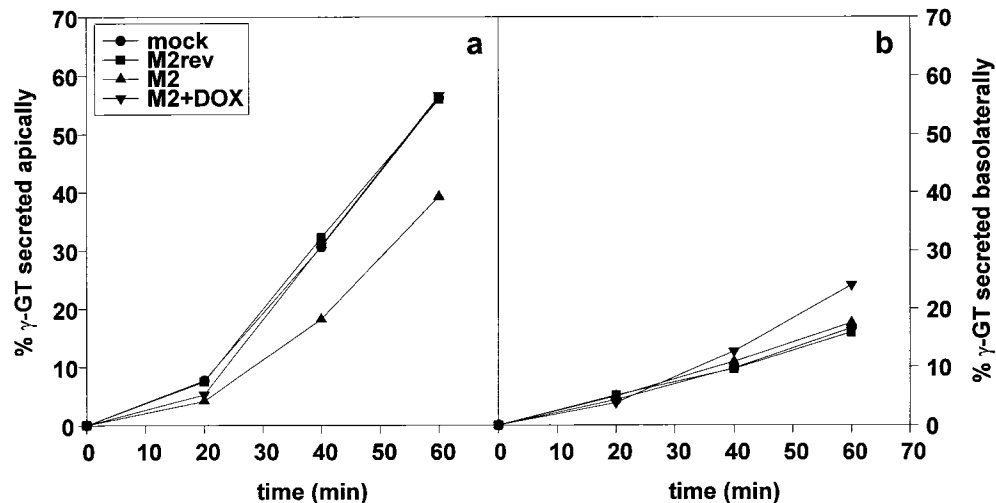


Figure 3. M2 slows apical but not basolateral delivery of a secreted form of γ -glutamyl-transpeptidase. MDCK cells stably expressing gD γ -GT were mock-infected or infected with AV-TA and either AV-M2rev or AV-M2. Indicated filters were treated with doxycycline (DOX) to block M2 expression. The following day, cells were radiolabeled, chased for 2 h at 19°C, then warmed to 37°C, and the release of γ -GT into the apical (a) and basolateral (b) medium was quantitated as described in Materials and Methods. Similar results were obtained in three experiments.

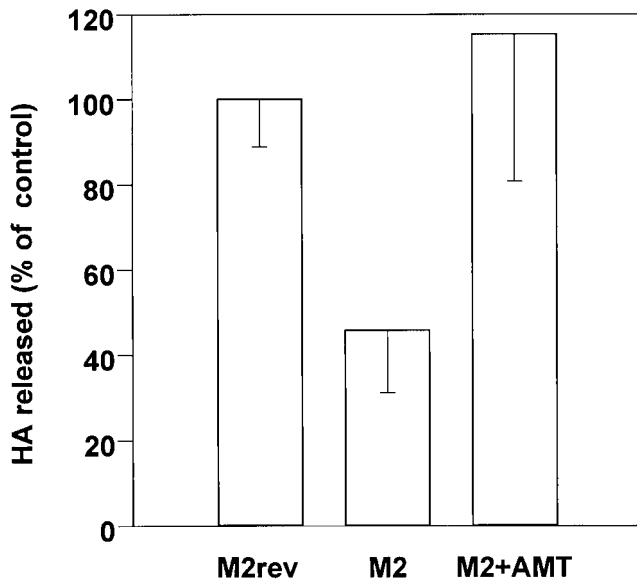


Figure 4. The effect of M2 on HA delivery occurs at the level of TGN budding. Polarized MDCK T23 cells were infected with AV-HA and either AV-M2rev or AV-M2. The following day, cells were starved, pulse-labeled, and chased for 2 h at 19°C. The cells were mechanically perforated as described in Materials and Methods, then returned to 37°C for 60 min in the presence or absence of an ATP-regenerating system. In some experiments, an M2-expressing sample treated with AMT was also included. The supernatant and cells were collected separately, HA was immunoprecipitated, and the amount of mature HA released from cells under each condition was quantitated and normalized to control (M2rev-expressing cells). HA release from samples incubated in the absence of an ATP-regenerating system was typically 10–25% of control. The data represent the mean \pm SEM from six experiments for M2 and M2rev, and three experiments in which AMT was added to M2-expressing cells.

in apical vesicle formation, we would expect to observe an inhibition in apical secretion of hGH. By contrast, if M2 activity interferes with the loading of apically sorted cargo but does not affect vesicle formation, no effect on hGH secretion should be observed. Stable MDCK cell lines expressing myc-tagged hGH were generated and screened for expression and polarity of hGH secretion (Fig. 5). Intracellular hGH was localized to the ER and Golgi complex, as predicted for a secreted protein (Fig. 5 a). Infection of these cells with AV-TA and AV-M2 resulted in M2 expression in a large percentage of the cells (Fig. 5 b). Previous studies using MDCK cells have shown that the effect of M2 on other transport steps (basolateral-to-apical transcytosis and apical recycling) are maximal at similar levels of M2 expression, and that increasing M2 expression 10-fold has no further effects (Henkel et al., 1998). Secretion of the newly synthesized hGH was essentially nonpolarized (~55% apical on average), as reported previously (Scheiffele et al., 1995). We then tested whether M2 activity altered the rate or polarity of hGH secretion (Fig. 6). Virally infected cells were starved, radiolabeled, then chased for 2 h at 19°C. No radiolabeled hGH was released into the medium during this incubation (data not shown). The cells were then incubated with prewarmed medium

and hGH secretion quantitated. Interestingly, we observed no effect of M2 activity on either the rate or polarity of hGH secretion. This result suggests that M2 expression does not affect the rate of formation of apically destined vesicles at the TGN.

To confirm this possibility, we converted hGH to an apically sorted protein and asked whether its delivery was now affected. Scheiffele et al. (1995) have shown previously that N-linked glycosylation of rat growth hormone confers apical sorting information to this normally unglycosylated protein. Thus, we used site-directed mutagenesis to create two N-glycan consensus addition sites in hGH, and tested the polarity of secretion of the resultant mutant (ghGH) expressed in MDCK cells. Initial experiments suggested that chasing radiolabeled cells at 19°C significantly inhibited secretion of ghGH upon subsequent warming to 37°C; thus, the 19°C chase was omitted from these experiments (control experiments confirmed that omitting the 19°C chase had no effect on the release of nonglycosylated hGH; data not shown). As expected, ghGH was secreted predominantly apically (~85%; Fig. 7 a), suggesting that N-glycosylation conferred apical sorting information to this protein. In addition, the rate of apical but not basolateral secretion of ghGH was significantly decreased when it was coexpressed with active M2 (Fig. 7, b and c). The effect of M2 on apical secretion of ghGH was blocked by inclusion of AMT, suggesting that M2 ion channel activity was responsible for the observed inhibition. Thus, our data are consistent with an effect of M2 activity on loading of apically sorted cargo into vesicles at the TGN.

Discussion

We have examined the effects of M2 expression on TGN-to-cell surface delivery of newly synthesized proteins in polarized MDCK cells. M2 expression slowed apical cell surface delivery of HA and delayed the apical release of glycosylated soluble proteins; however, transport of basolateral proteins was unaffected. The delay in transport was due to a block in apical protein export from the TGN, and was not observed when M2 ion channel activity was inhibited. Interestingly, M2 expression had no effect on the apical release of a nonglycosylated soluble protein whose nonpolarized secretion is unlikely to be signal mediated. Together, our results suggest a role for TGN pH in the efficient delivery of apical proteins.

Although we did not directly confirm that M2 expression alters TGN pH, there is considerable indirect evidence to support this. M2 has been demonstrated to function as an acid-activated proton channel *in vivo* and *in vitro*, and to alter the pH of a subset of acidified compartments in cells (Pinto et al., 1992; Schroeder et al., 1994; Wang et al., 1994; Chizhnikov et al., 1996; Shimbo et al., 1996; Henkel et al., 1999). M2 slows TGN-to-PM delivery of newly synthesized proteins when expressed in nonpolarized cells, and the effects are blocked by the M2 ion channel inhibitor AMT (Henkel and Weisz, 1998; Henkel et al., 1999). In addition, M2 activity is normally required to prevent acid-dependent aggregation of the pH-sensitive fowl plague virus HA in the TGN; however, in the absence of active M2, acidotropic agents such as chloroquine or

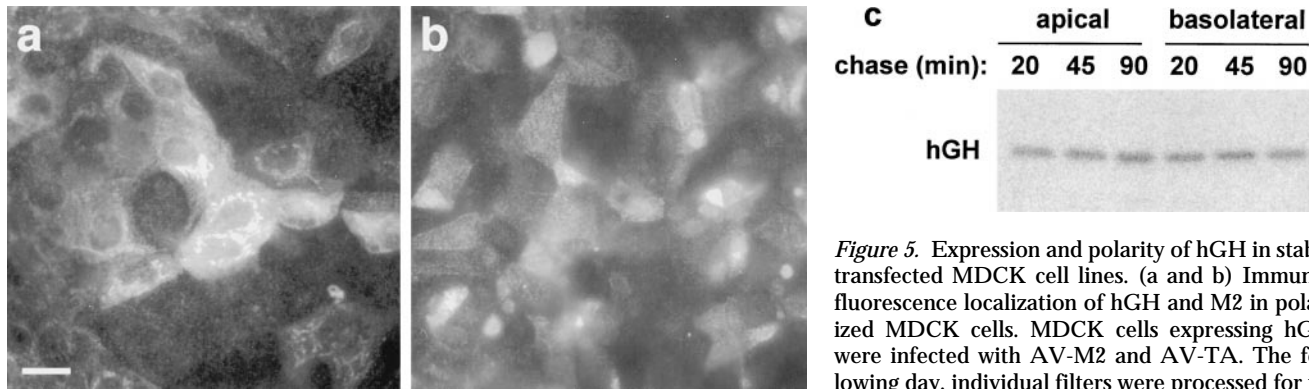


Figure 5. Expression and polarity of hGH in stably transfected MDCK cell lines. (a and b) Immunofluorescence localization of hGH and M2 in polarized MDCK cells. MDCK cells expressing hGH were infected with AV-M2 and AV-TA. The following day, individual filters were processed for indirect immunofluorescence using anti-myc (a) or anti-M2 (b) mAbs. Most of the cells expressed detectable levels of hGH and M2. Bar, 10 μm . Similar results were obtained with ghGH-expressing cells (data not shown). (c) MDCK cells expressing hGH were infected with AV-M2rev and AV-TA and induced with 10 mM butyrate. The following day, cells were starved and pulse labeled for 30 min. Cells expressing hGH were chased for 2 h at 19°C, then warmed for the indicated periods at 37°C. The apical and basolateral media were replaced at each time point and immunoprecipitated using anti-myc antibody.

anti-M2 (b) mAbs. Most of the cells expressed detectable levels of hGH and M2. Bar, 10 μm . Similar results were obtained with ghGH-expressing cells (data not shown). (c) MDCK cells expressing hGH were infected with AV-M2rev and AV-TA and induced with 10 mM butyrate. The following day, cells were starved and pulse labeled for 30 min. Cells expressing hGH were chased for 2 h at 19°C, then warmed for the indicated periods at 37°C. The apical and basolateral media were replaced at each time point and immunoprecipitated using anti-myc antibody.

ammonium chloride can substitute (Ciampor et al., 1992; Ohuchi et al., 1994; Takeuchi and Lamb, 1994). Finally, Grambas and Hay (1992) used conformation-sensitive antibodies to estimate the pH encountered by fowl plague virus HA during biosynthetic transport in MDCK cells; the pH of the Golgi complex in control cells was estimated to be ~ 5.6 , and increased to >6.0 in cells expressing active M2. However, because M2 has been demonstrated to transport other cations besides protons *in vitro*, albeit at very low efficiency (e.g., 10^5 – 10^7 -fold lower efficiency for sodium than for protons) (Chizhnikov et al., 1996; Shimbo et al., 1996), we cannot rule out the possibility that the effects we observed are due to altered Golgi ion composition rather than pH.

Our observations are consistent with earlier findings by Matlin (1986) and Caplan et al. (1987), who observed decreased rates of delivery of apical membrane and secreted proteins, respectively, with no effect on their ultimate polarity in ammonium chloride-treated MDCK cells. We also compared the effect of M2 expression and of pH disruption using various global pH perturbants on the polarity of secretion of gp80, but found no effect of these treatments (data not shown). However, in our MDCK T23 cells, gp80 secretion was $>95\%$ apical under all conditions tested; thus, the twofold increase in the ratio of apical to basolateral secretion in chloroquine-treated cells reported by Parczyk and Kondor-Koch (1989) would be difficult to detect. Interestingly, however, treatment with the V-ATPase inhibitor BafA₁ resulted in general inhibition of apical and basolateral delivery of all proteins tested (data not shown). Together, the disparate observations using global pH perturbants reinforce the need to use selective pH perturbants, such as M2, in order to examine the role of pH in individual trafficking and sorting events.

Apical sorting has been proposed to occur by the recruitment of proteins into glycolipid-rich domains that are subsequently incorporated into apically destined vesicles. A lectin-like protein resident in these rafts, VIP36, has been proposed to function as a sorting receptor for both membrane and soluble proteins in the TGN (Fiedler et al.,

1994; Fiedler and Simons, 1996); however, this protein was recently shown to be localized instead to earlier compartments of the secretory pathway (Fullekrug et al., 1999). Acidic TGN pH could be important for various steps in apical protein delivery, including recognition of apical sorting signals, recruitment of proteins into glycolipid rafts, incorporation of proteins or rafts into apically destined vesicles, vesicle formation or budding, and vesicle fusion. We considered the possibility that M2-mediated alkalization of the TGN affects terminal glycosylation of itinerant proteins and thus disrupts carbohydrate-mediated recognition by a VIP36-like lectin. However, we could not detect any difference in the electrophoretic migration of HA or other proteins in M2-expressing cells compared with control cells, suggesting there was no gross alteration in glycosylation. Moreover, M2 expression had no effect on the mobility of the heavily glycosylated mucin-like protein MUC1 expressed in CHO cells (data not shown). In addition, the role for glycosylation in apical sorting appears to involve core residues as opposed to terminal residues that are added during intra-Golgi transport (Parczyk and Koch-Brandt, 1991; Wagner et al., 1995; Fiedler and Simons, 1996). Finally, M2 expression had no effect on the ultimate polarity of apical protein delivery to the apical surface, suggesting that recognition of sorting signals by the apical sorting machinery was not compromised.

Incorporation of proteins into glycolipid rafts is typically assessed by detergent insolubility, and has been demonstrated for both membrane and soluble apical proteins (Scheiffele et al., 1997; Keller and Simons, 1998). Cholesterol depletion or knockout of the proteolipid VIP17/MAL disrupts lipid rafts, increases Triton X-100 solubility of HA, and results in decreased polarity of HA delivery to the apical surface (Keller and Simons, 1998; Cheong et al., 1999; Puertollano et al., 1999). By contrast, M2 expression did not affect Triton X-100 solubility or apical polarity of HA, suggesting that formation of glycolipid rafts is not inhibited by M2. This is consistent with the observation that apical proteins are incorporated into glycolipid rafts rela-

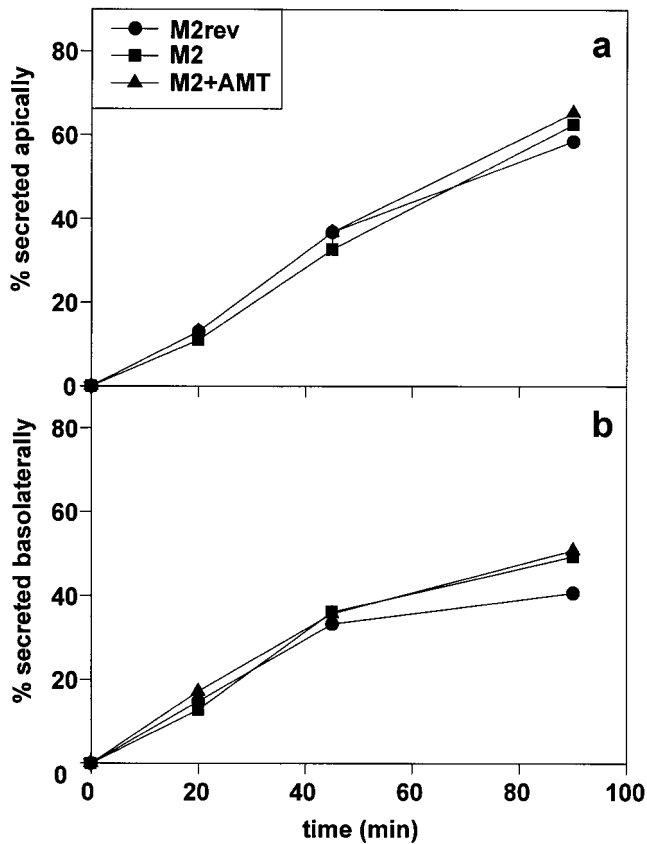


Figure 6. M2 does not affect apical secretion of hGH. MDCK cells stably expressing hGH were infected with AV-M2rev or AV-M2 and induced with 10 mM butyrate. Cells were starved, pulse labeled for 30 min, chased for 2 h at 19°C, then transferred to 37°C. AMT was included in all steps where indicated. The medium was collected and replaced at the indicated time points. At the end of the experiment, filters were solubilized, hGH was immunoprecipitated from all samples, and the kinetics of apical (a) and basolateral (b) secretion were quantitated. The total amount of hGH secreted by control filters (infected with AV-M2rev) at the end of the time course was normalized to 100% to allow comparison between experiments; average secretion at the end of the chase period was $46.1 \pm 9.8\%$ of total hGH. The mean of six independent experiments is plotted. M2 expression had no effect on either apical or basolateral secretion of hGH.

tively early during transit through the Golgi complex (Brown and Rose, 1992; Danielsen, 1995), i.e., during passage through nonacidified compartments that should be unaffected by M2 activity. Moreover, M2 itself is completely soluble in cold Triton X-100, suggesting that it is not incorporated into rafts.

The amount of HA released from the TGN in mechanically perforated cells was decreased in cells expressing active M2, suggesting that acidification is required for efficient apical protein export from the TGN. However, this result does not rule out additional functions for acidification in vesicle targeting to or fusion with the apical PM. Moreover, it cannot be determined from this assay whether the number of apically destined vesicles is affected by M2, or whether there is less apical cargo per vesicle. However, our observation that apical secretion of

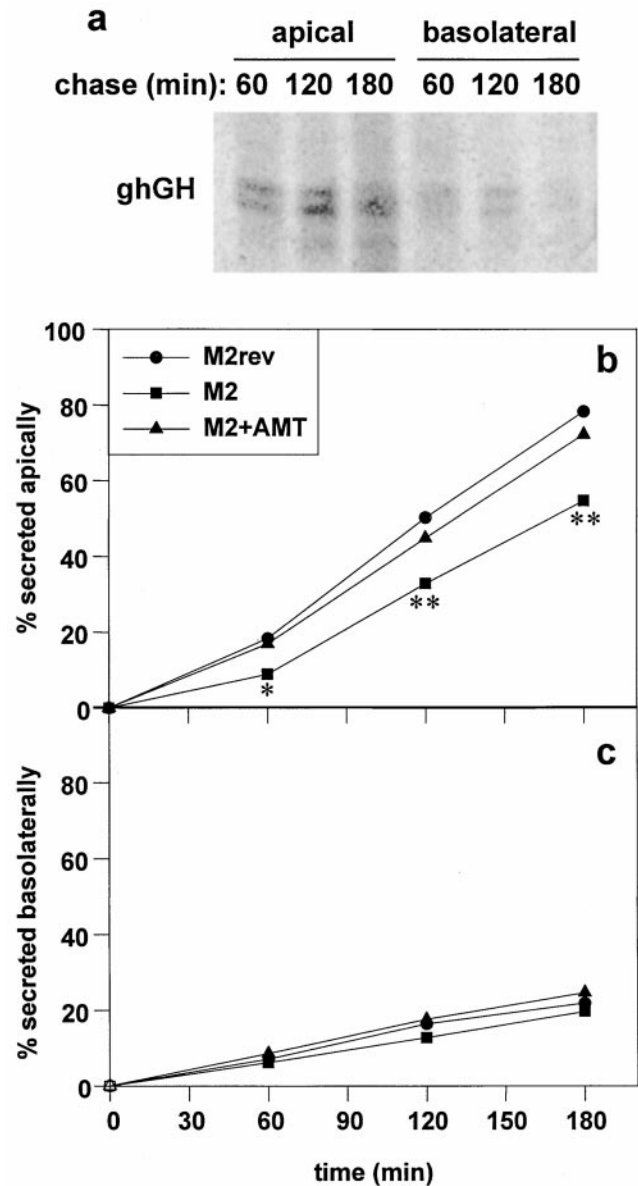


Figure 7. M2 slows apical secretion of ghGH. (a) ghGH is secreted predominantly apically. Stable MDCK cells expressing ghGH were infected with AV-TA and AV-M2rev and induced with 10 mM butyrate. The following day, cells were starved, radiolabeled for 30 min, then chased for the indicated periods. At each time point, the apical and basolateral medium were replaced and immunoprecipitated with anti-myc antibody. Approximately 85% of the secreted ghGH was released apically. (b and c) Secretion kinetics of ghGH. MDCK cells stably expressing ghGH were infected with AV-M2rev or AV-M2 and induced with 10 mM butyrate. Cells were starved, pulse labeled for 30 min, then chased for the indicated times at 37°C. AMT was included in all steps where indicated. Samples were collected and analyzed as described in the legend to Fig. 6. The mean of seven experiments is shown. On average, $68.4 \pm 5.9\%$ of the total ghGH was secreted by the end of the chase. Kinetics of apical (b) and basolateral (c) secretion are shown. Statistical analysis of the raw data by paired *t* test revealed that M2 significantly affected apical secretion of ghGH relative to M2rev-expressing cells. Asterisk indicates $P = 0.04$; two asterisks indicate $P = 0.02$.

nonglycosylated hGH is unaffected by M2 expression suggests that M2 does not affect the overall volume of apically destined vesicles, and by inference, the number of apically destined vesicles formed.

We attempted to reconstitute budding of hGH-containing vesicles in mechanically perforated cells; however, in our hands, release of hGH was ATP-independent. We suspect that this is due to inefficient concentration of hGH in the TGN during the 19°C chase, as ATP-independent release of immature HA (compared with the sialylated form) has been observed previously (Bennett et al., 1988). Thus, we cannot be certain that HA and hGH traffic to the apical surface in distinct vesicles. Therefore, we considered an alternative explanation for our results: that apically sorted cargo is delivered to the apical surface in distinct vesicles that do not contain hGH; and that budding of the former class of vesicles is selectively inhibited by M2. We do not favor this hypothesis, as it implies both that nonsorted cargo is actively excluded from apical vesicles (i.e., sorted, in a sense), and that a separate class of vesicles exists that is enriched in this nonsorted cargo. Because MDCK cells do not secrete endogenous proteins in a non-polarized fashion, this seems an unlikely possibility.

Because the rate of delivery but not the overall polarity of delivery of proteins that are preferentially targeted to the apical surface was selectively inhibited, M2 may interfere with recruitment of presorted cargo into apically destined vesicles. Lin et al. (1998) have recently postulated a two-step mechanism for HA sorting in which the ability to enter glycolipid rafts is a prerequisite for recognition by the apical sorting machinery. Our data are consistent with a model in which M2 does not interfere with either of these steps, but rather with the subsequent incorporation of these rafts into forming vesicles. Efficient sorting into glycolipid rafts but inefficient recruitment of these lipid rafts into apical vesicles could result in the observed delay in apical transport with little to no effect on the steady state distribution of HA. However, the mechanism by which cargo loading could be disrupted by M2 activity is unknown. One possibility is that TGN acidification could be important in limiting the size of glycolipid rafts. Formation of large oligomeric complexes containing Golgi-resident proteins has been proposed to block their entry into intra-Golgi transport vesicles (Machamer, 1993; Nilsson et al., 1993; Weisz et al., 1993). Alternatively, acidic pH might be important for the recognition of signals that direct rafts to forming apical vesicles. Finally, acidification could play a role in the active recruitment of rafts into forming vesicles.

In summary, we have demonstrated that expression of active M2 interferes with release of newly synthesized apical but not basolateral proteins from the TGN. The effect of M2 is consistent with a role for TGN acidification in the recruitment of presorted apical proteins into forming vesicles. Future experiments will be directed towards further understanding the role of TGN pH in this step of protein sorting.

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