

Influenza Virus M2 Protein Slows Traffic along the Secretory Pathway

pH PERTURBATION OF ACIDIFIED COMPARTMENTS AFFECTS EARLY GOLGI TRANSPORT STEPS*

(Received for publication, October 20, 1997, and in revised form, December 23, 1997)

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M2, an acid-activated ion channel, is an influenza A virus membrane protein required for efficient nucleocapsid release after viral fusion with the endosomal membrane. Recombinant M2 slows protein traffic through the Golgi complex (Sakaguchi, T., Leser, G. P.), and Lamb, R. A. (1996) *J. Cell Biol.* 133, 733–47). Despite its critical role in viral infection, little is known about the subcellular distribution of M2 or its fate following delivery to the plasma membrane (PM). We measured the kinetics of M2 transport in HeLa cells, and found that active M2 reached the PM considerably more slowly than inactive M2. In addition, M2 delayed intra-Golgi transport and cell surface delivery of influenza hemagglutinin (HA). We hypothesized that the effects of M2 on transport through non-acidified compartments are due to inefficient retrieval from the *trans*-Golgi of machinery required for intra-Golgi transport. In support of this, acutely activated M2 had no effect on intra-Golgi transport of HA, but still slowed HA delivery to the PM. Thus, M2 has an indirect effect on early transport steps, but a direct effect on late steps in PM delivery. These findings may help explain the conflicting and unexplained effects on protein traffic observed with other perturbants of intraorganelle pH such as weak bases and inhibitors of V-type ATPases.

The M2 protein is a 97-amino acid nonglycosylated integral membrane protein encoded on RNA segment 7 of influenza virus, which also encodes the M1 matrix protein (1). During synthesis, M2 is cotranslationally inserted into the endoplasmic reticulum and is transported to the plasma membrane of infected cells. The protein consists of a 24-amino acid luminal amino terminus, a single membrane spanning domain, and a 54-amino acid cytoplasmic tail. Some of the newly synthesized M2 is post-translationally palmitoylated and phosphorylated, but these modifications appear to have no effect on ion channel activity (2). Although large amounts of M2 are synthesized in influenza-infected cells, the molecule is poorly incorporated into budding virions; only about 20–60 molecules/virion are detected (3).

Recent data have shown that M2 forms a disulfide-bonded tetramer that can conduct protons across artificial lipid bilay-

ers and cell membranes when activated by low pH (4–6). This finding supported the hypothesis that the function of M2 is to modulate the pH of intracellular compartments in infected cells. The first clue came when M2 was shown to be the target of the anti-influenza drug amantadine (AMT),¹ which is known to block an early step in the viral replication cycle (7, 8). After virions are endocytosed, M2 is thought to channel protons from the low pH endosome to the virion interior. In the absence of M2-induced virion acidification, the influenza matrix protein M1 does not dissociate from ribonucleoproteins, and viral replication is inhibited (9, 10).

In addition to its role during viral infection, M2 activity is also required for the proper maturation of some strains of influenza virus hemagglutinin (HA), such as fowl plague virus HA (Rostock strain). Newly synthesized Rostock HA is normally proteolyzed in the *trans*-Golgi or *trans*-Golgi network into two fragments (HA1 and HA2) which remain associated during transit through the secretory pathway. By contrast, other HA isotypes are cleaved at the plasma membrane or not at all, depending on cell type. HA cleavage is required for the protein to become fusogenic, but also makes the protein susceptible to inactivation and aggregation at low pH. AMT causes Rostock HA to form aggregates in the *trans*-Golgi and to reach the cell surface in its inactive, low pH conformation (10), suggesting that Rostock M2 may actually function to raise the pH of the *trans*-Golgi. The proton ionophore monensin, which alkalinizes intracellular compartments, blocks the action of AMT in infected cells, supporting the idea that proper maturation of Rostock HA requires an elevated Golgi pH (10). Rostock HA maturation in various cell lines is differentially affected by AMT, suggesting that *trans*-Golgi pH differs between cell types. Grambas and Hay (11) estimated the pH of the *trans*-Golgi of Madin-Darby canine kidney cells to be approximately 6.0 by determining the ratio of native *versus* inactive HA for several influenza isotypes of known pH sensitivities in the presence and absence of the AMT analog rimantadine (11). These investigators estimated that Rostock M2 could increase the pH of this compartment by as much as 0.8 pH units, while M2 from other strains generated smaller increases (11).

Recent studies have shown that Rostock M2 slows several steps in intra-Golgi transport, as assayed by measuring the processing kinetics of Rostock HA. Both the acquisition of endoglycosidase H-resistant oligosaccharides and the cleavage of Rostock HA into HA1 and HA2 were significantly delayed in M2-expressing cells. Normal processing kinetics could be restored by including AMT in the medium to block M2 activity.

* This work was supported by grants from the Cystic Fibrosis Foundation and the Competitive Medical Research Fund of the University of Pittsburgh Medical Center (to O. A. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: AMT, amantadine; HA, hemagglutinin; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; COP, coat protein; ARF, ADP ribosylation factor.

These data suggested that M2 activity could affect early steps in Golgi transport. This result was surprising because these compartments are not thought to be acidified and therefore any M2 present in these compartments should not be activated.

We have monitored the effect of Rostock M2 activity on its own intracellular distribution. Interestingly, we find that newly synthesized active M2 reaches the cell surface far less efficiently than inactive M2 (expressed in the presence of AMT). M2 also caused a delay in the surface appearance of other newly synthesized proteins. To investigate which steps in transport were affected, we monitored the transport of glycosylated marker proteins at various steps along the secretory pathway. Our results show that M2 activity affects both early and late steps in protein delivery to the cell surface. However, whereas late steps in transport appear to be directly due to M2-mediated pH changes, early effects on transport may be an indirect result of effects on downstream (acidified) compartments. We hypothesize that M2-mediated accumulation of itinerant proteins in the *trans*-Golgi network results in inefficient retrieval to the early Golgi of protein or membrane components required for anterograde intra-Golgi transport. Our findings may also explain the conflicting data on the role of acidification in protein transport obtained by others using different perturbants of organelle pH.

EXPERIMENTAL PROCEDURES

DNA and Antibodies—The plasmids pTM3-FM2 and pSV73E/KM2-10, which encode Rostock and Udorn M2, respectively, and monoclonal antibody 5C4, which recognizes the luminal domain of both forms of M2, were generous gifts of Dr. R. Lamb (Northwestern University, Evanston, IL). The pTM3 vector contains both the EMC leader sequence, which allows cap-independent translation of mRNA and the T7 terminator, and generates very high levels of protein expression. For experiments that required M2 coexpression with other proteins, Rostock M2 was subcloned into the Bluescript vector (pBS (SK), Stratagene, Inc., La Jolla, CA) behind the T7 promoter (pBS-M2R). The polymerase chain reaction was used to isolate a fragment containing the entire sequence of M2 flanked by *Bam*HI from pTM3-FM2; this fragment was purified and subcloned into *Bam*HI-digested pBS. Orientation of the inserted sequences in pBS was determined by sequencing using the T7 primer (Stratagene, Inc.). Bluescript containing M2 inserted in the reverse orientation (pBS-M2rev) was also isolated for use as a control. A *Bam*HI fragment containing Udorn M2 was isolated from pSV73E/KM2-10 and subcloned into pBS as above to generate plasmid pBS-M2U. A *Hind*III-*Bam*HI fragment encoding HA from the Japan strain of influenza virus was purified after digestion of plasmid pCB6-HA (from Dr. M. Roth, Southwestern Medical Center, via Dr. S. Sisodia) and subcloned behind the T7 promoter of pBS. Monoclonal anti-HA antibody Fc125 was a generous gift of Dr. T. Braciale (University of Virginia, Charlottesville, VA).

Cell Culture and Transfection—HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells (40–80% confluent in 35-mm dishes) were infected in serum-free Dulbecco's modified Eagle's medium with recombinant vaccinia virus vTF7.3 encoding the T7 RNA polymerase gene at a multiplicity of infection of 10–20 (12). After 30 min at 37 °C, the inoculum was replaced with serum-free medium containing 3–6 μ g of DNA encoding the appropriate genes behind the T7 promoter, and 5–10 μ l of LipofectAMINE (Life Technologies, Inc.). Where indicated, 5 μ M amantadine (Sigma; prepared as a 5 mM stock in 95% ethanol and stored at 4 °C) was added to some dishes immediately following transfection. In coexpression experiments, 2 μ g of pBS-HA and 4 μ g of either pBS-M2 or pBS-M2rev were added per dish with 10 μ l of LipofectAMINE. The amount of HA synthesized upon coexpression with either pBS-M2 or pBS-M2rev was comparable as assessed by immunoprecipitation.

Indirect Immunofluorescence—Indirect immunofluorescence of vaccinia-infected HeLa cells was performed as described previously (13). Briefly, vaccinia virus-infected HeLa cells transfected with pTM3-FM2 were rinsed once with PBS, fixed for 20 min at ambient temperature in 3% paraformaldehyde, then incubated briefly with PBS containing 10 mM glycine and 0.02% sodium azide (PBS-G). Where indicated, cells were permeabilized for 3 min in 0.5% Triton X-100 in PBS-G. Nonspecific binding was blocked with 0.25% ovalbumin in PBS-G prior to incubation with antibodies. M2 was detected using the monoclonal

antibody 5C4 at a dilution of 1:250 followed by Cy3-conjugated affinity-purified goat anti-mouse IgG (2 mg/ml, 1:1000 dilution; Jackson ImmunoResearch Laboratories, Inc., Avondale, PA). Cells were viewed using a Nikon FXL microscope with an integrating 3 chip color CCD and images were captured using Optimus (Optimus, Seattle, WA).

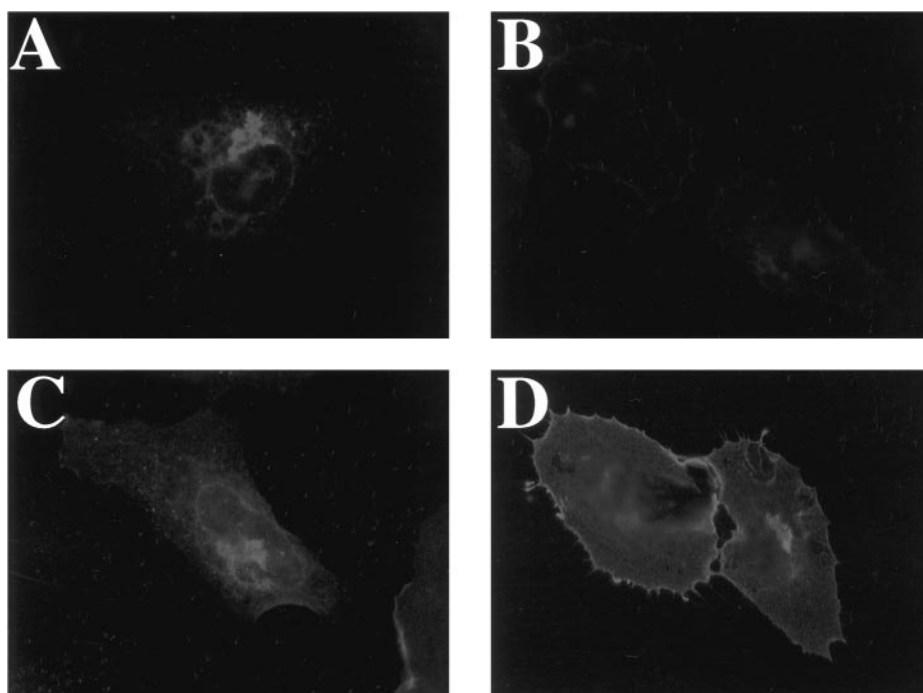
Cell Surface Immunoprecipitation of M2—At 4–5 h postinfection, cells were rinsed once with phosphate-buffered saline (PBS), then starved for 30 min in medium A (cysteine-free, methionine-free minimal essential medium containing 0.35 g/liter NaHCO₃, 10 mM HEPES, and 10 mM MES, pH 7.0 (14)). Cells were metabolically labeled with 50–100 μ Ci/ml [³⁵S]EXPRESS (NEN Life Science Products) in the same medium, then chased in the same medium supplemented with four times the normal amount of cysteine and methionine (medium B). At the indicated times, individual dishes were rapidly chilled, rinsed with PBS supplemented with 1% bovine serum albumin and 0.02% sodium azide (PBA), and incubated with 10 μ g of 5C4 antibody in 0.3 ml of PBA for 2 h at 0 °C on a rotating platform. Cells were then washed five times with PBA, rinsed with PBS, and lysed in 0.5 ml of detergent solution (50 mM Tris-HCl, 2% Nonidet P-40, 0.4% deoxycholate, 62.5 mM EDTA, pH 8.0) containing 1 μ g/ml aprotinin. Lysates were centrifuged briefly to remove nuclei, and SDS was added to the supernatant to a final concentration of 0.2%. Antibody-antigen complexes (surface M2) were collected immediately from four-fifths of the sample by incubation for 20 min at 4 °C with fixed *Staphylococcus aureus* (Calbiochem, La Jolla, CA). Total M2 was immunoprecipitated from the remainder of the sample using 3 μ g of 5C4 and collected as described above. After washing three times with RIPA buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS), bound material was eluted by boiling in Laemmli sample buffer containing 5% β -mercaptoethanol and electrophoresed on 12% SDS-polyacrylamide gels. Gels were scanned and the amount of M2 precipitated at the cell surface at each time point was quantitated using a PhosphorImager (GS-363 Molecular Imager System, Bio-Rad).

Endo H Kinetics—Vaccinia virus-infected HeLa cells co-transfected with pBS-HA and pBS-M2R or pBS-M2rev were starved for 30 min in medium A starting at 4 h postinfection. Dishes were radiolabeled for 10 min with 50–200 μ Ci/ml [³⁵S]EXPRESS, then chased in medium B for the indicated times. Dishes were rinsed with PBS, solubilized in detergent solution containing aprotinin, and HA immunoprecipitated using monoclonal antibody Fc125 (gift of Dr. Thomas Braciale). After collection of antibody antigen complexed using *S. aureus*, bound material was eluted by boiling for 3 min in 25 μ l of 1% SDS in 50 mM Tris buffer, pH 6.8, and the eluate was divided into two aliquots. An equal volume of 0.15 M citrate buffer, pH 5.5, containing approximately 50 units of endoglycosidase H (endo H, New England Biolabs, Inc., Beverly, MA) was added to one tube, while its control received buffer only. After overnight incubation at 37 °C, Laemmli sample buffer was added and the samples were boiled and resolved by electrophoresis on 10% SDS-polyacrylamide gels.

Kinetics of Sialylation—Cells were lysed in detergent solution and HA immunoprecipitated as described above. Antibody-antigen complexes were washed once with 50 mM sodium acetate, pH 5.5, 9 mM CaCl₂, then resuspended in 100 μ l of the same buffer and divided into two aliquots. Neuraminidase (1 milliunits of *Vibrio cholerae*, Calbiochem) was added to one aliquot, and the samples were incubated for 2 h at 37 °C. After washing once with RIPA, bound material was eluted by boiling in Laemmli sample buffer, and resolved by electrophoresis on 10% SDS-polyacrylamide gels. Immediately after synthesis, HA migrated as a single band; however, with increasing chase times, a second, more slowly migrating band began to accumulate. Neuraminidase treatment converted all of the slowly migrating form of HA to the more rapidly migrating form. Thus the kinetics of sialylation could be determined by quantitating the percentage of total HA migrating as the mature (slowly migrating) form in the untreated samples.

Cell Surface Trypsinization—Cell surface delivery of HA was measured using a well characterized trypsinization assay (15). Vaccinia virus-infected HeLa cells transfected with pBS-M2R or pBS-M2rev and pBS-HA were starved in medium A for 30 min at 37 °C starting at 4 h postinfection. Cells were metabolically radiolabeled with 100–200 μ Ci/ml [³⁵S]EXPRESS for 15 min, then chased in medium B. At various times, dishes were removed, rapidly chilled to 0 °C by rinsing with ice-cold PBS, and incubated on ice for 30 min in 1 ml of medium B containing 100 μ g/ml TPCK-trypsin (Sigma) for 30 min. Cells were then rinsed 5 times over a 30-min period with medium B containing 10% fetal bovine serum, rinsed with PBS, solubilized in detergent solution supplemented with 200 μ g/ml soybean trypsin inhibitor (Sigma), and HA immunoprecipitated using monoclonal antibody Fc125. This antibody quantitatively immunoprecipitates HA as well as both of its cleav-

FIG. 1. Localization of M2 expressed in HeLa cells in the presence and absence of amantadine. HeLa cells were infected with ν TF7.3 and transfected with 3 μ g of pTM3-FM2. Amantadine (5 μ M) was added to some dishes immediately after transfection (panels C and D). At 4 h postinfection, dishes were rinsed once with PBS, then fixed. Cells in panels A and C were permeabilized prior to processing for indirect immunofluorescence using a monoclonal antibody directed against the extracellular domain of M2. Cells in B and D were not permeabilized but otherwise processed identically.



age products (HA1 and HA2, which remain associated via disulfide bonds). After electrophoresis on 12% SDS-polyacrylamide gels, the percentage of cleaved HA was quantitated.

Reversible Inhibition of M2 with BL-1743—BL-1743 was kindly provided by Dr. Mark Krystal (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT) and prepared as a 10 mg/ml stock in ethanol. HeLa cells were infected with ν TF7.3 and transfected as described above. BL-1743 (10 μ M) was added to some dishes immediately following transfection and in all subsequent steps unless otherwise indicated. In some cases, BL-1743 was washed out by replacing the medium with fresh drug-free medium. At 4 h postinfection, cells were rapidly chilled, starved in medium A for 30 min, and pulse labeled for 10 min. In some experiments, BL-1743 was washed out immediately after the pulse label by incubating the cells for up to 2 h in ice-cold drug-free chase medium. The medium was then replaced with prewarmed chase medium (with or without BL-1743), the cells were returned to 37 °C, and endo H kinetics determined as described above.

RESULTS

M2 Activity Delays Its Appearance at the Cell Surface—Our initial observation that led to the studies described here was that M2 activity potentially blocked its own delivery to the cell surface. We transfected vaccinia-infected HeLa cells with plasmid pTM3-FM2 and examined total and cell surface expression of M2 at 5 h postinfection by indirect immunofluorescence. When M2 was expressed in the absence of AMT (Fig. 1, A and B), there was considerable staining of M2 in intracellular compartments; however, little M2 staining could be detected in nonpermeabilized cells, suggesting that this protein was inefficiently delivered to the cell surface (Fig. 1B). Surprisingly, when 5 μ M AMT was added to the cells immediately after transfection, considerably more M2 was detected at the plasma membrane (compare Fig. 1, B and D). These results suggest that M2 activity influences its own transport to the cell surface. Immunoprecipitation of M2 from similarly transfected cells confirmed that there was no difference in M2 expression in the presence or absence of AMT (not shown). Because M2 is not readily internalized from the plasma membrane,² the altered distribution of M2 between untreated and AMT-treated cells likely reflects a difference in cell surface delivery rate of M2.

To quantify this observation, we used cell surface immuno-

precipitation to measure the rate of M2 delivery to the cell surface in the presence and absence of AMT (the 24-amino acid luminal domain of M2 contains no lysine residues and cannot be biotinylated using NHS-biotin derivatives). HeLa cells expressing M2 in the TM3 vector were metabolically radiolabeled, then chased for various times. At each time point, dishes were rapidly chilled, then incubated with anti-M2 antibody. After washing, cells were lysed and antibody-antigen complexes (surface M2) were collected and the remainder of the sample (intracellular M2) was immunoprecipitated (Fig. 2). In control experiments, addition of excess unlabeled cell lysate from M2-expressing cells did not reduce the amount of radiolabeled M2 recovered at the cell surface, suggesting that antibody-antigen complexes remained stable during the lysis and recovery procedures. In addition, incubation with 3-fold more antibody did not increase the amount of M2 recovered at the cell surface.

Quantitation of M2 delivery to the cell surface confirmed the qualitative results from indirect immunofluorescence. Whereas approximately 16% of the total M2 expressed using the TM3 vector reached the cell surface by 2.5 h in the presence of AMT, less than 4% was detected at the surface in untreated cells. Inclusion of AMT only during the antibody-binding step did not affect the amount of M2 recovered at the cell surface, suggesting that the affinity of antibody binding to M2 was unaffected by the presence of this inhibitor. These kinetics of delivery represent a minimum estimate of the amount of M2 present at the plasma membrane, as cell surface immunoprecipitation is unlikely to be 100% efficient. Nonetheless, this approach allows us to compare cell surface delivery of M2 in the presence and absence of AMT, and confirms our qualitative observations by immunofluorescence.

M2 Expression Delays Transport of Membrane and Secreted Proteins—Because M2 activity clearly slows its own delivery to the plasma membrane, we asked whether transport of other proteins would be similarly affected. To this end, we coexpressed a glycosylated marker protein, influenza hemagglutinin (HA, Japan strain), with M2 and examined the kinetics of its transport through the Golgi complex and to the cell surface in the presence and absence of AMT. Because the high effi-

² J. R. Henkel and O. A. Weisz, unpublished observations.

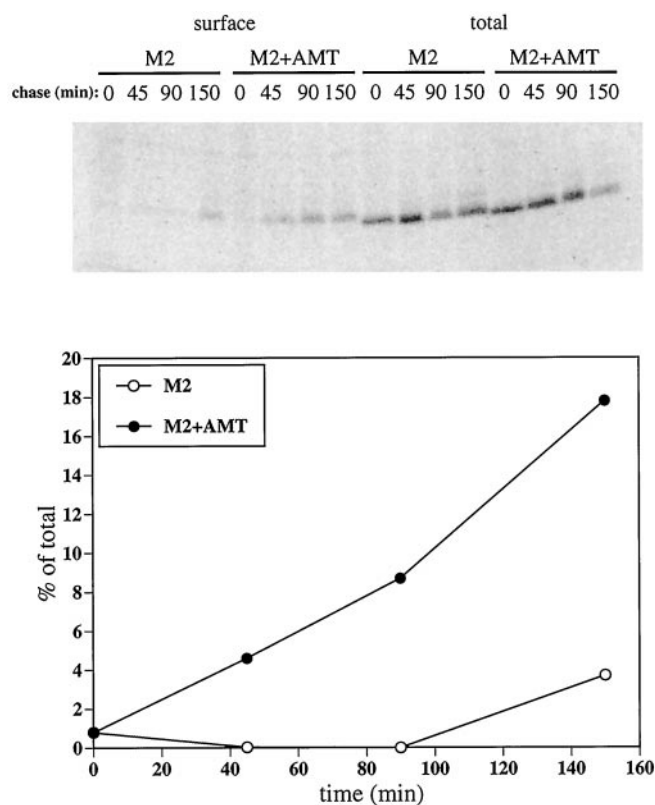


FIG. 2. Quantitation of M2 delivery to the plasma membrane in the presence and absence of amantadine. HeLa cells expressing Rostock M2 in the presence or absence of 5 μ M AMT were metabolically labeled with [35 S]EXPRESS and chased as described under "Experimental Procedures." At various times, dishes were rapidly chilled and cells were incubated with 10 μ g of mAb 5C4 for 2 h at 0 $^{\circ}$ C. After extensive washing, cells were lysed and surface and total M2 collected as described under "Experimental Procedures." After SDS-polyacrylamide gel electrophoresis, the dried gel was imaged (top panel) and quantitated using a PhosphorImager (bottom panel). This experiment was performed three times with similar results.

ciency vector pTM3-FM2 overwhelms expression from pBS-HA, we subcloned Rostock M2 into Bluescript so that expression of both proteins could be easily regulated. Expression of M2 from pBS was approximately 20-fold lower than from pTM3 (not shown). Vaccinia virus-infected HeLa cells were cotransfected with pBS-HA and either pBS-M2R, or as a control, with pBS-M2rev, which contains the Rostock M2 sequence inserted into pBS in the reverse orientation. The amount of HA synthesized when coexpressed with either Rostock M2 or M2rev was very similar.

As a first step in determining whether the relatively low level of M2 expressed using pBS-M2R affected protein transport through the secretory pathway, we compared HA transport to the *cis*/*medial* compartment of the Golgi complex in the presence or absence of active M2. HA transport through the early Golgi was followed by quantitating the acquisition of resistance to oligosaccharide cleavage by endoglycosidase H, an event that occurs in the *cis* and/or *medial* cisternae of the Golgi. The rate of conversion of HA to its endo H-resistant form was modestly but reproducibly inhibited when the protein was coexpressed with M2 compared with M2rev (Fig. 3A). Inclusion of 5 μ M AMT immediately post-transfection and in subsequent steps partially restored the processing kinetics, suggesting that the effect of M2 expression on HA endo H kinetics was due to its ion channel activity. These results are similar to those reported recently by Sakaguchi *et al.* (14) and therefore suggest that the effect of M2 activity on this step of protein traffic is maximal

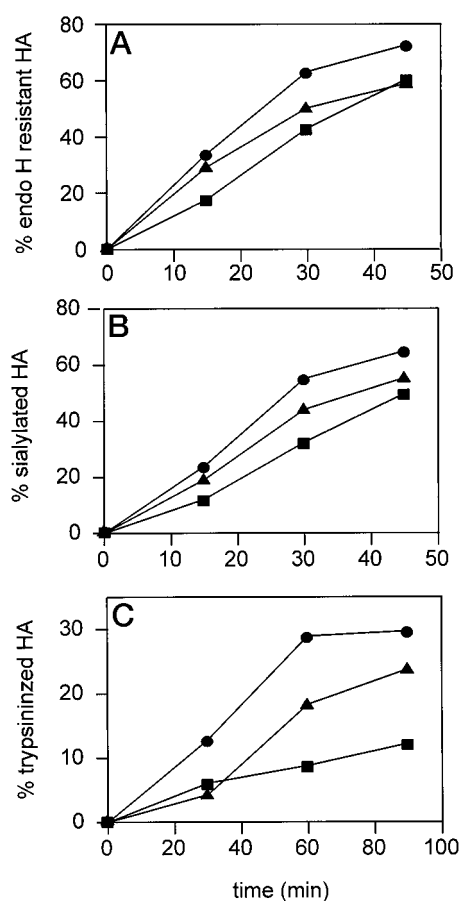


FIG. 3. Kinetics of protein transport in M2-expressing cells. Vaccinia-infected HeLa cells were cotransfected with 2 μ g of pBS-HA and 4 μ g of either pBS-M2R (squares, triangles) or pBS-M2rev (circles). Amantadine (5 μ M) was added to the indicated M2-expressing samples immediately after transfection (triangles). At 4 h postinfection, cells were starved for 30 min, radiolabeled for 15 min, then chased for the indicated times. A, endo H kinetics. Cells were lysed in detergent solution and HA was immunoprecipitated from the samples and treated with endo H as described under "Experimental Procedures." B, kinetics of sialylation. Cells were lysed in detergent solution and HA immunoprecipitated as described above. After electrophoresis, the percentage of total HA migrating as the mature (sialylated) form at each time point was determined as described under "Experimental Procedures." C, kinetics of cell surface delivery. At the indicated chase times, individual dishes of cells were rapidly chilled, then treated with 100 μ g/ml TPCK-trypsin for 30 min at 0 $^{\circ}$ C to cleave surface HA. Cells were washed extensively, lysed, and HA and its proteolytic fragments were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis. The percent of total HA cleaved at each time point is plotted. Although the kinetics of protein glycosylation and cell surface delivery varied somewhat between experiments, the rank order of the kinetic profiles for each treatment was highly reproducible. All three experiments were performed at least four times with similar results.

even at the presumably lower expression level used here. AMT at the concentration used here is not completely effective at blocking Rostock M2 activity (16); thus it is not surprising that only a partial restoration of transport kinetics was observed. Higher levels of AMT were not tested as this compound by itself can raise intracellular pH at increased concentrations (17).

The next step along the secretory pathway that we monitored is transport of HA to the *trans*-Golgi, where sialylation of *N*-linked oligosaccharides occurs. We compared the kinetics of sialylation of HA coexpressed with either M2 or M2rev. As shown in Fig. 3B, the rate of HA sialylation was slowed in the presence of active M2 and could be partly restored to normal by inclusion of AMT in the medium.

We then examined whether M2 activity altered the rate of

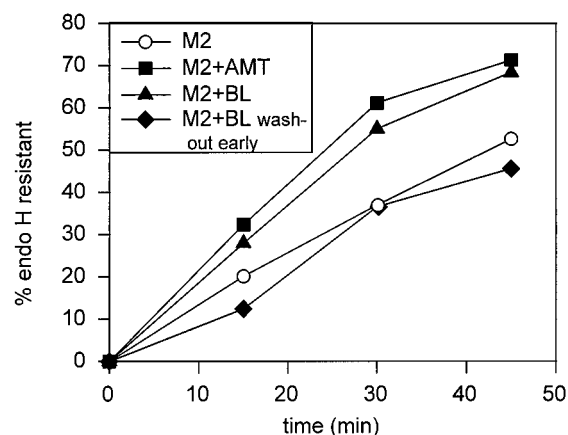


FIG. 4. The effects of BL-1743 on M2 activity are reversible. Vaccinia-infected HeLa cells were cotransfected with pBS-M2U (4 μ g/dish) and pBS-HA (2 μ g/dish). Either AMT (5 μ M) or BL-1743 (10 μ M) was added to some dishes immediately following transfection as indicated. At 3 h postinfection, the medium in some dishes containing BL-1743 was replaced with drug-free medium (diamonds). At 4 h postinfection, cells were rapidly chilled and starved in medium A for 30 min, pulse labeled for 10 min, chased for the indicated times, and the kinetics of endo H resistance determined. This experiment was performed three times with similar results.

appearance of newly synthesized HA at the cell surface. HA was coexpressed with M2 or M2rev as above, and cell surface delivery monitored using a surface trypsinization assay (Fig. 3C). The rate of cell surface delivery of HA was dramatically reduced in the presence of active M2, and could be partially restored by including AMT in the medium. We estimate that M2 activity caused a 70% decrease in the initial rate of HA cell surface delivery based on analysis of several individual experiments.

The M2-mediated Delay of Intra-Golgi Transport Is Due to Downstream Effects—The observation that M2 affects early steps in protein transport was surprising, as these compartments are not thought to be acidified. We hypothesized that an M2-mediated accumulation of coat proteins or other common components of the protein transport machinery in acidified regions of the Golgi complex could ultimately lead to decreased efficiency in transport through earlier compartments. If this were the case, then the first round of protein transport through the Golgi complex after activation of preaccumulated but inactive M2 should be unaffected by these downstream effects. To test this idea, we took advantage of the newly described compound BL-1743, which is a potent but rapidly reversible inhibitor of M2 (18). The affinity of this inhibitor for M2 is comparable to that of AMT, but unlike AMT, BL-1743 rapidly dissociates from M2 (the $t_{1/2}$ for reversal of M2 ion channel inhibition upon washout of BL-1743 is about 12 min (18)). Because inhibition by BL-1743 has been most extensively characterized for Udorn M2, we used this isoform of M2 for these studies. Rostock and Udorn M2 had similar effects on all of the steps in protein transport measured, including intra-Golgi transport and cell surface delivery of HA, and BL-1743 had no effect on the transport rate of HA expressed in the absence of M2 (not shown). To confirm that BL-1743 was reversible, HeLa cells cotransfected with pBS-M2U and pBS-HA were incubated in the presence or absence of BL-1743. At 3 h postinfection, the medium in some dishes that had been treated with BL-1743 was replaced with fresh medium without drug, and the incubation continued for an additional 60 min. Subsequently, the cells were starved, pulse labeled, chased for the indicated times, and the kinetics of endo H resistance determined (Fig. 4). When HA was coexpressed with M2, the rate of acquisition

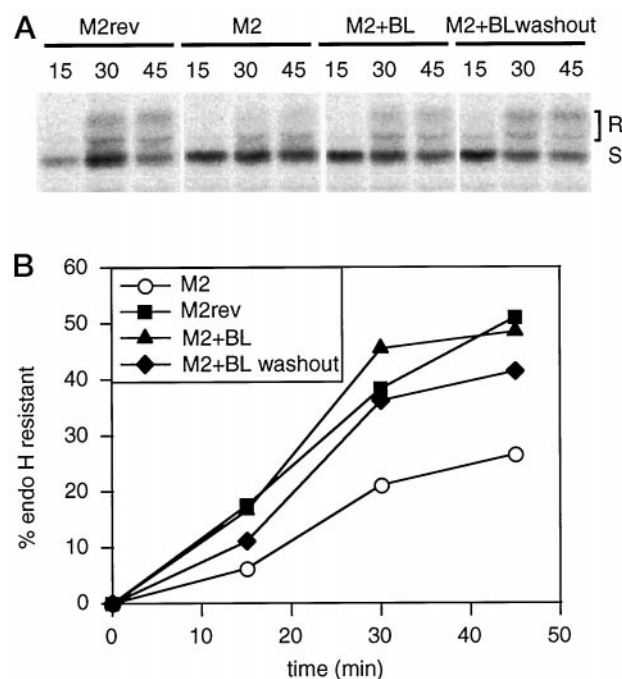


FIG. 5. Early steps in protein transport are insensitive to newly activated M2. Vaccinia-infected HeLa cells were cotransfected with pBS-M2U or pBS-M2rev and pBS-HA as described in the legend to Fig. 4. Some dishes were treated with 10 μ M BL-1743 immediately following transfection and during subsequent steps. At 4 h postinfection, cells were rapidly chilled, starved for 30 min, then pulse labeled for 10 min as described under "Experimental Procedures." The cells were then rapidly chilled to 0 $^{\circ}$ C by the addition of ice-cold chase medium with (triangles) or without BL-1743 (circles, squares, and diamonds in panel B). After a 30-min incubation, the medium was replaced with prewarmed chase medium (with or without BL-1743) and the cells were returned to 37 $^{\circ}$ C. At various times, cells were solubilized, HA immunoprecipitated, and endo H kinetics determined. Panel A depicts the appropriate region of a typical SDS-PAGE gel; endo H sensitive (S) and resistant (R) forms of HA are marked at the right. Quantitation of the labeled bands on this gel is shown in panel B. This experiment was performed four times with similar results.

of endo H-resistance of newly synthesized HA was markedly slowed compared with control samples in which HA was coexpressed with M2 in the presence of amantadine or BL-1743. However, when BL-1743 was washed out prior to the pulse label, the rate of acquisition of endo H resistance of HA was similar to that observed in samples expressing untreated (active) M2. Therefore, the effects of BL-1743 were completely reversible in this system. To determine whether protein transport through the early Golgi was selectively affected immediately upon M2 activation, we performed the following experiments. HeLa cells cotransfected with either pBS-M2U or pBS-M2rev and pBS-HA in HeLa cells were incubated in the presence or absence of BL-1743. At 4 h postinfection, cells were starved for 30 min prior to radiolabeling. Subsequently, the cells were rapidly chilled to 0 $^{\circ}$ C, and BL-1743 washed out of the indicated samples for 30 min. The cells were then rapidly warmed to 37 $^{\circ}$ C and the kinetics of endo H resistance (Fig. 5) and cell surface delivery (Fig. 6) of HA were determined in the continued presence or absence of BL-1743. As in Fig. 4, HA coexpressed with M2 which was active throughout the experiment had a slower rate of acquisition of endo H-resistance of newly synthesized HA compared with control samples in which HA was coexpressed with M2rev or M2 in the continued presence of BL-1743 (Fig. 5). However, when BL-1743 was washed out immediately prior to the chase period, the rate of acquisition of endo H resistance of HA was similar to that of controls, suggesting that the effects on early Golgi transport we and

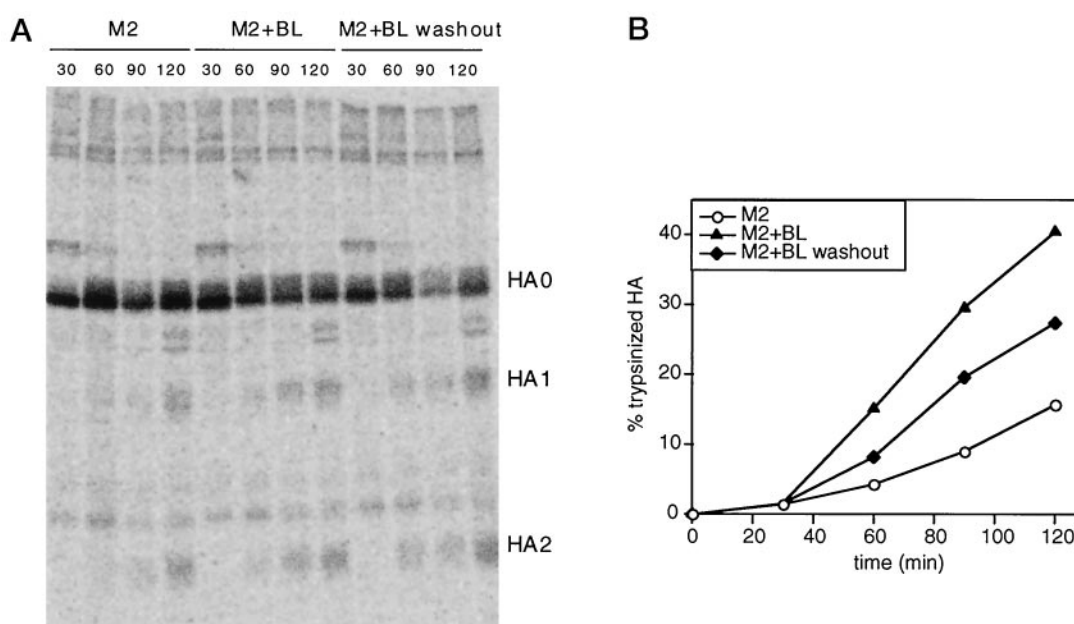


FIG. 6. Cell surface delivery of HA is slowed in the presence of newly activated M2. Vaccinia-infected HeLa cells were cotransfected with pBS-M2U and pBS-HA as described above. Some dishes were treated with 10 μ M BL-1743 immediately following transfection and during subsequent steps. At 4 h postinfection, cells were starved for 30 min, pulse labeled for 15 min, then rapidly chilled to 0 °C by the addition of ice-cold chase medium with (triangles) or without BL-1743 (circles and diamonds in panel B). After a 30-min incubation, the medium was replaced with prewarmed chase medium (with or without BL-1743) and the cells were returned to 37 °C. Cell surface delivery kinetics of HA were measured using the trypsinization assay described under "Experimental Procedures." A typical gel is shown in panel A; uncleaved HA (HA0) and the HA trypsin cleavage products (HA1 and HA2) are marked at the right. Quantitation of the labeled bands on this gel is shown in panel B. This experiment was performed four times with similar results.

others observed require prolonged activation of M2. Similar results were obtained when BL-1743 was washed out for up to 2 h instead of 30 min prior to initiating the chase (not shown). Furthermore, the kinetics of sialylation were also normal in cells expressing newly activated M2, suggesting that transport as far as the *trans*-Golgi was insensitive to newly activated M2 (not shown). By contrast, cells expressing newly activated M2 still showed decreased kinetics of HA cell surface delivery (Fig. 6). As expected, the kinetics of surface delivery in cells expressing newly activated M2 were faster than those observed for cells expressing active M2 throughout the experiment, presumably because indirect effects on early transport steps were avoided. These kinetics likely represent the true effect of M2 on *trans*-Golgi network to plasma membrane transport. Together, these data suggest that M2 slows steps in early Golgi transport only after protein traffic is allowed to proceed for extended periods in the presence of active M2. These findings are consistent with the idea that the direct target(s) of M2 activity is downstream of the early Golgi.

DISCUSSION

Our results demonstrate that M2 expression significantly delays cell surface delivery of membrane proteins. Both intra-Golgi transport and cell surface delivery were affected by M2 activity. The effects on early Golgi transport were observed at relatively low expression levels of M2, and were reversible (14). The delay in transport increased at each successive step, suggesting that multiple steps along the secretory pathway were affected as opposed to a single early step. Overall, delivery of HA to the cell surface was reduced by approximately 70% in the presence of active M2 compared with control. Low concentrations of the ion channel inhibitor AMT blocked most of the effect of M2, suggesting that the delay in transport was due to M2 ion channel activity. Of particular interest was our observation that active M2 itself was delivered to the cell surface far less efficiently than M2 expressed in the presence of AMT. This

finding has important implications for our understanding of influenza virus assembly, and may explain why M2 is incorporated very poorly into virions.

Our data confirm the recent report by Sakaguchi *et al.* (14) that M2 activity can delay acquisition of endo H-resistant oligosaccharides of newly synthesized proteins. Sakaguchi *et al.* (14) conclude that M2 ion channel activity must therefore be functional in the *cis*/*medial*-Golgi. By contrast, our findings suggest that the delay in traffic through the early Golgi is an indirect result of M2 activity in the late Golgi. We suggest that M2 ion channel activity directly blocks traffic in the *trans*-Golgi, where it has previously been demonstrated to function as an ion channel (11). A selective delay in transport to or from this compartment could result in accumulation of components of the transport machinery in this compartment. Recent studies have shown that anterograde and retrograde transport through the Golgi are inextricably linked, as the coat proteins and fusion machinery required for forward traffic through the secretory pathway must be continually recycled (19, 20). Therefore, an M2-mediated delay in late Golgi transport is likely to eventually affect the efficiency of earlier steps. This hypothesis may also explain the surprising observations that specific inhibitors of vacuolar H⁺-ATPases also delay early steps in protein traffic (21–23).

Interestingly, Sakaguchi *et al.* (14) report that whereas monensin causes osmotic swelling of all Golgi cisternae, M2 expression caused only the *trans*-Golgi cisternae to swell. The swelling of the *trans* region of the Golgi could be due to M2-mediated osmotic swelling as hypothesized by Sakaguchi *et al.* (14). Alternatively, if traffic from the *trans*-Golgi to the cell surface is the primary step affected by M2 activity, one would expect newly synthesized plasma membrane and secretory proteins to accumulate in this compartment. This could explain the increase observed in the surface area and volume of the *trans*-Golgi. Furthermore, whereas monensin treatment alters the

pattern of protein glycosylation of itinerant proteins (14), we did not detect any obvious effect of M2 on protein glycosylation apart from the decrease in the kinetics of oligosaccharide maturation. In addition, indirect immunofluorescence studies did not detect any gross changes in Golgi morphology or glycosyltransferase distribution (not shown). These observations reinforce the idea that unlike monensin, which alters the ionic composition of all Golgi cisternae, M2 does not affect the microenvironment of early Golgi compartments.

Regardless of its site of action, M2 could affect secretory traffic via several mechanisms. One possibility is that M2 activity somehow disrupts efficient protein concentration into budding vesicles. Alternatively, the formation, targeting, or fusion of coated vesicles could be affected. Recent studies support a role for vesicular acidification in these processes. Neutralization of endosomal pH either with specific inhibitors of the vacuolar ATPase or with the ionophore nigericin prevented binding of β and ϵ COPs to isolated endosomal membranes and inhibited the formation of endosomal carrier vesicles in an *in vitro* system (24, 25). This suggests that coat protein binding to the cytosolic face of a vesicle can be disrupted by inhibiting acidification (24). Notably, COP binding to membranes containing the intermediate compartment marker ERGIC-53 was insensitive to these inhibitors, suggesting that luminal pH *per se* does not regulate COP binding (24). This idea is supported by the finding that acidification of the cytosol also delays *trans*-Golgi network to cell surface transport (26). In other studies, ARF binding to microsomal membranes *in vitro* was shown to be dependent on vesicular acidification (27). ARF binding to membranes causes activation of phospholipase D activity, which has been implicated in the recruitment of coatomer to membranes (28). Thus it is possible that M2 activity somehow interferes with the generation or maintenance of ionic conditions required for coat protein binding, perhaps by equilibrating a gradient of pH or other ions between the inside of a compartment and the cytosol. We are currently testing this hypothesis in our laboratory.

Acknowledgments—O. A. W. thanks Dr. Carolyn Machamer for continued encouragement and mentoring over the past several years. We thank Dr. Bernard Moss for permission to use vTF7.3, Dr. Robert Lamb

for gifts of plasmids encoding Rostock and Udorn M2 as well as a very generous supply of 5C4 antibody, Dr. Michael Roth for pCB6-HA, Dr. Thomas Braciale for Fc125 antibody, Paul Poland for assistance with polymerase chain reactions, Dr. Mark Krystal for his kind gift of BL-1743, Drs. Gerard Apodaca and Simon Watkins for assistance with fluorescence imaging, and Drs. Gerard Apodaca and Rebecca Hughey for critical review of the manuscript.

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