The Ion Channel Activity of the Influenza Virus M_2 Protein Affects Transport through the Golgi Apparatus

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Abstract. High level expression of the M_2 ion channel protein of influenza virus inhibits the rate of intracellular transport of the influenza virus hemagglutinin (HA) and that of other integral membrane glycoproteins. HA coexpressed with M_2 is properly folded, is not associated with GRP78-BiP, and trimerizes with the same kinetics as when HA is expressed alone. Analysis of the rate of transport of HA from the ER to the *cis* and medial Golgi compartments and the TGN indicated that transport through the Golgi apparatus is delayed. Uncleaved HA_0 was not expressed at the cell surface, and accumulation of HA at the plasma membrane was reduced to 75–80% of control cells. The delay in intracellular transport of HA on coexpression of M_2 was not observed in the presence of the M_2-specific ion channel blocker, amantadine, indicating that the Golgi transport delay is due to the M_2 protein ion channel activity equilibrating pH between the Golgi lumen and the cytoplasm, and not due to saturation of the intracellular transport machinery. The Na^+/H^+ ionophore, monensin, which also equilibrates pH between the Golgi lumen and the cytoplasm, caused a similar inhibition of intracellular transport as M_2 protein expression did for HA and other integral membrane glycoproteins. EM data showed a dilation of Golgi cisternae in cells expressing the M_2 ion channel protein. Taken together, the data suggest a similarity of effects of M_2 ion channel activity and monensin on intracellular transport through the Golgi apparatus.

THE influenza A virus M_2 integral membrane protein is abundantly expressed in virus-infected cells (Lamb and Choppin, 1981; Lamb et al., 1985; Zebedee et al., 1985), although it is only a minor, but nonetheless essential, component of virions (Zebedee and Lamb, 1988). The M_2 protein is a disulfide-linked homotetramer with each chain consisting of 97 amino acids, with 24 NH_2-terminal extracellular residues, 19 transmembrane $(TM)^1$ residues, and 54 COOH-terminal intracellular cytoplasmic residues (Holsinger and Lamb, 1991; Lamb et al., 1985; Sugrue and Hay, 1991).

The M_2 protein is thought to function as an ion channel that permits ions to enter the virion during uncoating and also to act as an ion channel that modulates the pH of intracellular compartments (Hay, 1992; Sugrue and Hay, 1991). Direct evidence that the M_2 protein has ion channel activity was provided by expressing the M_2 protein in either oocytes of *Xenopus laevis* (Holsinger et al., 1994; Pinto et al., 1992; Wang et al., 1993) or in mammalian cells (Wang et al., 1994) and by measuring cell surface currents. The M_2 protein ion channel activity was specifically blocked by the anti-influenza virus drug amantadine and was activated at the lowered pH found intraluminally in endosomes and the TGN (Pinto et al., 1992; Wang et al., 1995; Shimbo et al., 1996). In addition, introduction of purified M_2 protein or the TM domain peptide into planar lipid bilayers resulted in an amantadine-sensitive ion channel activity that was activated by low pH (Duff and Ashley, 1992; Schroeder et al., 1994; Tosteson et al., 1994).

The M_2 ion channel of influenza A virus is thought to play an essential role in the uncoating of the virus. Influenza virus enters cells by the viral hemagglutinin (HA) glycoprotein binding to sialic acid on cell surfaces, and virus particles are internalized via the endocytic pathway into endosomes. In the acidic environment of the secondary endosome, the M_2 ion channel contained in the virion envelope is activated by low pH. In addition, the pH-neutral (and metastable) form of HA undergoes a low pH–induced conformational change that releases the hydrophobic fusion peptide, mediating fusion of the viral mem-

1. *Abbreviations used in this paper.* BiP, binding protein; DSP, dithiobis(succinimidylpropionate); endo D or H, endoglycosidase D or H; FPV, fowl plaque virus; HA, hemagglutinin; PBSN, PBS containing 0.02% sodium azide; SV5, simian virus 5; TM, transmembrane.

brane with the endosomal membrane (Bullough et al., 1994; Skehel et al., 1982). The activated M_2 channel permits the passage of protons across the virion membrane into the virion core. It is generally thought that the consequence of the M_2 ion channel activity is that protein–protein interactions between the matrix (M_1) protein and the ribonucleoprotein core are weakened (for review see Helenius, 1992). When the M_2 ion channel is blocked by amantadine, uncoating of the virion is incomplete and viral replication is blocked (for review see Hay, 1992; Lamb et al., 1994).

The influenza virus HA glycoprotein is synthesized as a precursor, HA_0, that has to be proteolytically cleaved to form the disulfide-linked subunits HA_1 and HA_2. Cleavage of HA is a prerequisite step in the pathway that permits HA to undergo its conformational change to its low pH–induced form. Human strains of influenza virus HA contain a single basic residue in the HA cleavage site, and cleavage of HA occurs after the molecule is expressed at the cell surface. However, for some HA subtypes (largely H5 and H7 avian and equine subtypes), HA_0 is cleaved in the TGN by furin, a subtilisin-like protease (Stieneke-Gröber et al., 1992). Observations on the effect of amantadine late in viral infection on the HA of influenza fowl plaque virus (FPV) Rostock, an HA that is cleaved intracellularly and has a high pH (pH 5.9) of transition to the low pH–induced form, led to the notion that the M_2 protein functioned as an ion channel in the Golgi apparatus (Sugrue et al., 1990). A large body of immunological and biochemical data indicated that addition of the M_2 channel inhibitor amantadine to cells, late in infection, caused HA to undergo its conformational change to the low pH–induced form in the TGN (Ciampor et al., 1992a,b; Grambas et al., 1992; Grambas and Hay, 1992; Sugrue et al., 1990). Addition of the Na^+/H^+ ionophore monensin at low concentrations (nanomolar) to amantadine-treated infected cells restored HA to its pH-neutral form, without affecting HA transport, functionally overcoming the amantadine block of the M_2 ion channel, and suggesting a similarity in mechanism of monensin and the M_2 ion channel (Grambas et al., 1992; Sugrue et al., 1990). Thus, the M_2 ion channel activity is thought to function in the TGN and associated transport vesicles to keep the pH above the threshold at which the HA conformational change occurs (Ciampor et al., 1992a; Sugrue et al., 1990).

To obtain evidence that the M_2 protein could alter the pH of intracellular compartments in the absence of an influenza virus infection, the effect of expressing M_2 on the biogenesis of HA was examined (Ohuchi et al., 1994; Takeuchi and Lamb, 1994). When FPV HA was expressed from cDNA in cells, it was found that the majority of the HA molecules were in the low pH form of HA (Takeuchi and Lamb, 1994). In addition, the fusion capacity of the FPV HA expressed from cDNA was greatly diminished (Ohuchi et al., 1994). The lysosomaltropic agent, ammonium chloride, stabilized the accumulation of HA in its pH neutral form (Takeuchi and Lamb, 1994) and increased its fusion ability (Ohuchi et al., 1994). Coexpression of HA and the FPV M_2 protein stabilized the accumulation of HA in its pH-neutral form, indicating that expression of M_2 affects intracellular pH, and amantadine prevented the rescue of HA in its pH-neutral form (Ohuchi et al., 1994;

Takeuchi and Lamb, 1994). In our studies, we observed that transfection of increasing amounts of M_2 cDNA caused the inhibition of cleavage of the HA_0 to HA_1 and HA_2 (Takeuchi and Lamb, 1994). This observation raised the possibility that overexpression of M_2 has an effect on intracellular processing of FPV HA.

In the present study, we show that high level expression of the M_2 protein slows the rate of intracellular transport, and this is due to the M_2 protein ion channel activity. The deleterious effect of M_2 ion channel activity on the rate of intracellular protein transport is similar to the effect of the monensin, which at micromolar concentrations delays intracellular protein transport through the medial Golgi apparatus.

Materials and Methods

Cells and Viruses

CV-1 cells and HeLa T4 cells (a stable line of HeLa cells that express the human CD4 molecule) (Maddon et al., 1986) were grown in DME supplemented with 10% FCS. CHO 15B cells (a cell line deficient in N-acetylglucosamine transferase I) (Gottlieb et al., 1975) were grown in α-MEM supplemented with 10% FCS.

Recombinant vaccinia virus vTF7.3, which contains the bacteriophage T7 RNA polymerase gene (Fuerst et al., 1986), was provided by Bernard Moss (National Institutes of Health, Bethesda, MD). Influenza virus A/WSN/33 was grown in MDBK cells.

Plasmids, Site-specific Mutagenesis, and Construction of Plasmids

pTM3-HA and pTM3-M_2 encode the HA and M_2 proteins, respectively, of influenza A/chicken/Germany/34 (H7N1) (FPV Rostock) virus (Takeuchi and Lamb, 1994). The original plasmid encoding HA was kindly provided by Dr. Hans-Dieter Klenk (Institute for Virology, Marburg, Germany). pT7.5 HA-Udorn encodes the HA of influenza A/Udorn/72 virus and was derived from pSV103 HA-Udorn (Simpson and Lamb, 1992), such that HA coding sequences were under the control of the T7 polymerase promoter (Fuerst et al., 1986). For site-specific mutagenesis of M_2 cDNA, PCR (Saiki et al., 1988) was performed by using pTM3-M_2 as a template and using primers possessing the mutation and either a BspHI site (5' end primer) or BamHI site (3' end primer). The product was digested with BspHI and BamHI and ligated into the NcoI (compatible with BspHI) and BamHI sites of pTM3, a modified vector for T7 RNA polymerase–mediated expression (Moss et al., 1990). To subclone F cDNA (Paterson et al., 1984, 1985) of simian virus 5 (SV5) into pTM3, a novel NcoI site was introduced at the first translation start codon of the F cDNA using the unique site elimination (U.S.E.) system (Deng and Nickoloff, 1992) and performed as described in the U.S.E. mutagenesis protocols of Pharmacia-LKB, Inc. (Piscataway, NJ). The cDNA was then ligated into the NcoI site of pTM3. The nucleotide sequences of the altered cDNA were confirmed.

Flow Cytometry

Cells expressing HA or HA + M_2 were chilled on ice and washed with ice-cold PBS containing 0.02% sodium azide (PBSN). mAb HC2 (kindly provided by Alan Hay [National Institute of Medical Research, Mill Hill, UK]) was added to the monolayer at 1:500 dilution in PBSN containing 1% BSA and incubated on the plate for 30 min at 4°C. The cell monolayers were washed five times with PBSN, incubated with FITC-conjugated goat anti–mouse IgG for 30 min at 4°C, and washed five times with PBSN and twice with PBS deficient in calcium and magnesium (PBS–) to remove unbound secondary antibody. To remove cells from the dish, PBS containing 50 mM EDTA (0.5 ml) was added, and cells were incubated at 4°C until they dissociated easily from the plate with aspiration. Cells were transferred to flow cytometry tubes containing 0.5 ml of 1% methanol-free formaldehyde and dispersed by pipetting. Fluorescence intensity of 10,000 cells was measured by a FACS®can flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Protein Expression in Mammalian Cells, Metabolic Labeling, Immunoprecipitation, and SDS-PAGE

Proteins were expressed in HeLa T4 cells by using the vaccinia virus T7 RNA polymerase (vac/T7)–mediated transient expression system (Fuerst et al., 1986). Subconfluent monolayers of HeLa T4 cells in a 3.5-cm dish were infected with vaccinia virus vTF7.3 at an input multiplicity of infection of 10 PFU per cell for 30 min and transfected with plasmid DNA (5 μg) by using a cationic liposome reagent (Rose et al., 1991) synthesized in our laboratory. At 5-h posttransfection, the cells were incubated for 30 min in pH-labeling medium (methionine- and cysteine-free MEM containing low $NaHCO_3$ [0.35 g/l], 10 mM Hepes, and 10 mM MES, pH 7.0). The cells were then labeled with Tran[^{35}S] label (100 μCi/ml; ICN Radiochemicals, Inc., Irvine, CA) and incubated for various times in chase medium (DME) containing low $NaHCO_3$ (0.35 g/l), 10 mM Hepes, and 10 mM MES, pH 7.0. The Hepes/MES-buffered media were used in pulse-label and chase experiments because a change of extracellular pH alters the pH of the TGN, which results in fluctuations in the effect of the M_2 ion channel activity (Takeuchi and Lamb, 1994). The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM iodoacetamide and 1% (vol/vol) aprotinin (Sigma Chemical Co., St. Louis, MO) as described (Paterson and Lamb, 1993), and proteins were immunoprecipitated as described (Lamb and Choppin, 1979). For analysis of FPV HA, either a polyclonal antibody against disrupted FPV virions was used, or mAbs 5C1-1F8 (Garten, W., and H.-D. Klenk, unpublished reagent) and mAb HC2 (Sugrue et al., 1990) were used; for analysis of FPV M_2 protein, mAbs 1F1 or 1D6 raised against influenza A/Udorn/72 M_2 protein and which cross-react with FPV M_2 protein were used (Holsinger and Lamb, 1991); for analysis of A/Udorn/72 HA polyclonal sera, SP31 was used (Simpson and Lamb, 1992); and for SV5 F protein, an anti-F_2 peptide antisera was used (Horvath and Lamb, 1992). To analyze the complex of the cellular heavy-chain binding protein (BiP), a rat anti-BiP mAb (Bole et al., 1986) was used under ATP-depleting conditions as described previously (Ng et al., 1989). Polypeptides were analyzed by SDS-PAGE using 10, 15, or 17.5% polyacrylamide gels (17.5% gels containing 4 M urea). Radioactivity was analyzed and quantified either by using an image analyzer (Fujix BAS 1000; Fuji Medical Systems, Inc., Stamford, CT) and MacBAS software (Fuji Medical Systems, Inc.) or by fluorography and quantified by densitometry (Lamb et al., 1976; Paterson and Lamb, 1993).

Proteinase Digestion of Neutral pH and Low pH Forms of HA

For analysis of the HA conformational form, FPV HA was expressed by using the vac/T7 expression system, and cells were labeled with Tran[^{35}S] label for 10 min and incubated in pH-label-DME for 60 min. The cells were lysed with 50 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% NP-40 and incubated with or without 50 μg/ml proteinase K for 60 min at 4°C or 30°C as described (Takeuchi and Lamb, 1994). The proteinase K was inactivated by the addition of 2× RIPA buffer containing 1 mM PMSF. Polypeptides were immunoprecipitated and analyzed by SDS-PAGE.

Glycosidase Digestions

Proteins were immunoprecipitated, and the recovered proteins were treated with 0.1 U/ml endo-β-N-acetylglucosaminidase H (endo H) (ICN Biomedicals, Inc., Costa Mesa, CA), 0.4 U/ml endo-β-galactosidase (endo β) (Boehringer Mannheim Biochemicals, Indianapolis, IN), or 0.4 U/ml neuraminidase (Boehringer Mannheim Biochemicals) in the presence of 50 mM sodium citrate, pH 5.5, at 37°C for 18 h as described previously (Paterson and Lamb, 1987). Immunoprecipitated proteins were digested with 0.1 U/ml endoglycosidase D (endo D) (Boehringer Mannheim Biochemicals) in 20 mM Pipes, pH 6.5, 5 mM EDTA, and 0.1% Triton X-100 as described (Beckers et al., 1987).

Chemical Cross-linking

Cross-linking of FPV HA was performed as described (Gething et al., 1986; Russell et al., 1994). Metabolically labeled cells were solubilized with 1% Triton X-100 in 50 mM Tris-HCl, pH 8.0, and 100 mM NaCl and incubated with 1 mM dithiobis(succinimidylpropionate) (DSP) for 30 min on ice. The reaction was quenched with 50 mM glycine, proteins were immunoprecipitated, and polypeptides were analyzed on 3.5% borate-acetate gels as described previously (Gething et al., 1989; Russell et al., 1994).

Electron Microscopy

Colloidal gold was prepared essentially as described (Slot and Geuze, 1985), and BSA was coupled to 6 nm colloidal gold as described (Ghitescu et al., 1986). Cells were fixed, embedded, and prepared for EM as described previously (Jin et al., 1994) with the following exceptions. Before fixation cells were incubated for 1 h in the presence of 5 μg/ml peroxidase-labeled lectin from *Ricius communis* (agglutinin RCA_{120}; Sigma Chemical Co.) and BSA coupled to 6 nm gold (used at a final dilution of 1:7.5). Ricin was detected by HRP staining performed as described (Sandvig and van Deurs, 1990). After fixation, unless otherwise indicated, cells were treated for 1 h with 1% tannic acid at room temperature, and during dehydration cells were stained en bloc with 1% uranyl acetate in 70% ethanol for 4 h at 4°C.

Results

Coexpression of HA and M_2 Protein Rescues the pH-neutral Form of HA, but Increasing Levels of M_2 Protein Expression Block Cleavage of the HA_0 Precursor

The FPV HA has a high pH threshold (pH 5.9) for undergoing its low pH–induced conformational change and requires coexpression of the M_2 protein ion channel to rescue HA in its pH-neutral form. Our initial observation that led to the series of experiments described here was that coexpression of HA with high levels of M_2 protein blocked cleavage of the HA_0 precursor to its subunits HA_1 and HA_2 in the TGN. When FPV HA was expressed in HeLa T4 cells in the absence of other influenza virus proteins, upon cleavage of HA_0 in the TGN, the majority of the HA molecules were found to be in a form indistinguishable from the low pH–induced conformation of HA (Takeuchi and Lamb, 1994). The amount of the HA_1 subunit recovered was less than stoichiometric (presumably due to endogenous protease digestion), and the HA_1 subunit was sensitive to digestion by exogenous proteinase K (Takeuchi and Lamb, 1994; see Fig. 1), a diagnostic indicator of the HA low pH–induced conformation (Skehel et al., 1982). An increasing gradient of concentration of expressed M_2 ion channel protein with a constant amount of coexpressed HA (Fig. 1 *A*) showed that low levels of M_2 coexpression increased the amount of HA_1 that was resistant to proteinase K digestion (and in the HA pH-neutral form) (M_2 expression shown in Fig. 1 *B*). High level expression of M_2 protein (1–2.5 μg DNA transfected) (Fig. 1, *A* and *B*, lanes *1* and *2.5*) resulted in accumulation of the uncleaved HA precursor, HA_0.

Uncleaved HA_0 Is Not Expressed at the Cell Surface

It was considered possible that the effect of M_2 protein coexpression could be to permit expression of uncleaved HA at the cell surface. To examine this possiblity, fluorescent flow cytometry was performed on cells expressing HA + M_2 or HA alone. The mAb HC2 was used for flow cytometry, as this antibody recognizes a loop (antigenic site A) in mature HA that is found in the HA_1 domain of both uncleaved and cleaved HA, and it is also present in the low pH–induced form of HA (Sugrue et al., 1990). The data indicated that the overall accumulation of HA at the plasma membrane in cells coexpressing HA + M_2 was 75–80% of that of control cells expressing HA alone. To investigate if coexpression of HA + M_2 prevented intracellular cleavage
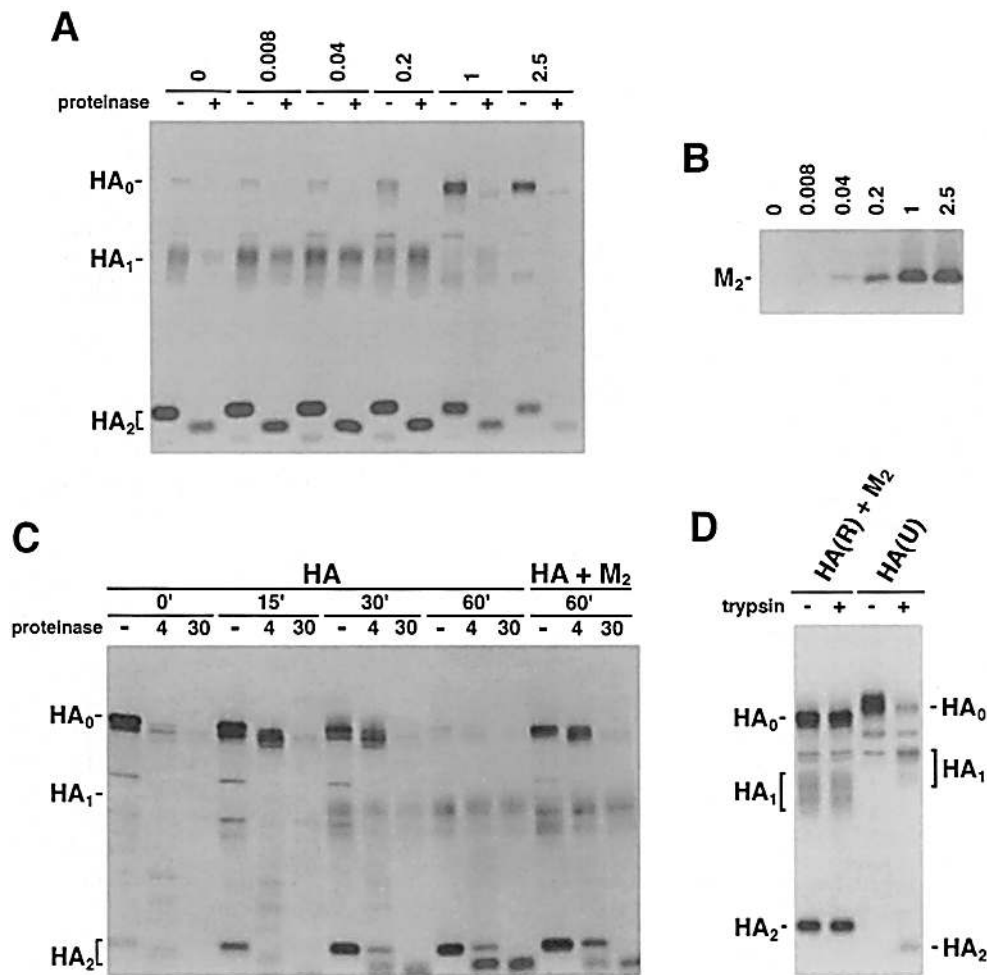
Figure 1. Effect of varying concentration of M_2 protein on both rescue of the pH-neutral form of HA and the effect on HA cleavage. (*A* and *B*) HA and M_2 proteins were coexpressed in HeLa T4 cells by using the Vac/T7 expression system. Cells were infected with vaccinia virus vTF7.3 and transfected with 2.5 μg of pTM3-HA DNA, together with various amounts (0–2.5 μg as indicated above the lanes) of pTM3-M_2 DNA, with the total amount of DNA adjusted to 5 μg per dish by adding pTM3 vector DNA. At 5.5-h posttransfection, the cells were metabolically labeled with Tran[^{35}S] label for 10 min in pH-labeling medium (pH 7.0) and incubated in pH-chase medium (pH 7.0) for 60 min. (*A*) Cell lysates were digested with (+) or without (−) proteinase K, and HA was immunoprecipitated and analyzed by 10% SDS-PAGE under reducing conditions. (*B*) A fraction of the cell lysate was immunoprecipitated using M_2-specific mAb 1F1, and M_2 protein was analyzed by 17.5% + 4 M urea SDS-PAGE under reducing conditions. (*C*) HA alone or HA and M_2 were expressed in HeLa T4 cells using 2.5 μg of each plasmid and labeled with Tran[^{35}S] label for 15 min and incubated in chase medium for various periods as indicated. Cell lysates were digested with proteinase K (50 μg/ml) for 30 min at 4°C (*4*) or 30°C (*30*) or mock treated at 30°C (−). (*D*) FPV HA and M_2 were coexpressed using pTM3-HA (2.5 μg) and pTM3-M_2 (2.5 μg) [*HA(R)+M_2*], and A/Udorn/72 HA was expressed using 2.5 μg of pT7.5 HA-Udorn plasmid [*HA(U)*]. Proteins were metabolically labeled for 10 min and incubated in chase medium for 120 min. The cells were then treated with (+) or without (−) 50 μg/ml TPCK-trypsin in Dulbecco's PBS for 10 min at 37°C. Digestions were stopped with 2 mM PMSF, cells were lysed and HA immunoprecipitated, and polypeptides were analyzed on 10% SDS-PAGE.

of HA_0, cell surfaces were treated with trypsin to cleave HA_0 to HA_1 and HA_2, a diagnostic test used for HA subtypes that are not cleaved intracellularly. Whereas the FPV Rostock HA coexpressed with M_2 could not be cleaved to HA_1 and HA_2 by cell surface trypsin treatment, a control HA (A/Udorn/72) that in influenza virus–infected cells is not cleaved intracellularly but can be cleaved extracellularly by exogenous trypsin treatment was cleaved using this assay (Fig. 1 *D*). Thus, coexpression of HA + M_2 did not cause accumulation of uncleaved HA_0 at the cell surface.

The M_2 Protein Ion Channel Activity Delays HA Acquisition of Endo H Resistance and Delays the Kinetics of HA Cleavage

As coexpression of M_2 with HA did not cause presentation of uncleaved HA at the cell surface (Fig. 1 *D*), it seemed likely that the reduced amount of HA cleavage (Fig. 1 *A*), rather than resulting from an inhibition of cleavage per se,

was due to delayed transport of HA to the TGN where cleavage occurs. Thus, the rate at which HA carbohydrate chains became resistant to digestion with endo H, indicative of transport to the medial Golgi apparatus, and the rate of HA cleavage was determined. Most importantly, to show that the effects observed were due to the M_2 ion channel activity and not due to a consequence of artifactual effects such as a possible competition for the intracellular transport machinery because of overexpression of integral membrane proteins, or a consequence of the vector system used, we examined the effect of the M_2 ion channel blocker amantadine on the kinetics of endo H resistance and cleavage. As shown in Fig. 2, coexpression of M_2 + HA delayed the kinetics of HA acquiring endo H resistance (Fig. 2 *A*; *HA* + M_2 [*1* μg], nonreducing gel; Fig. 2 *C*; *HA* + M_2 [*2.5* μg], reducing gel), and the effect of M_2 on HA transport was nearly completely reversed by addition of amantadine (Fig. 2 *A*, data quantified in Fig. 2 *F*). Furthermore, coexpression of M_2 + HA delayed cleavage of HA_0
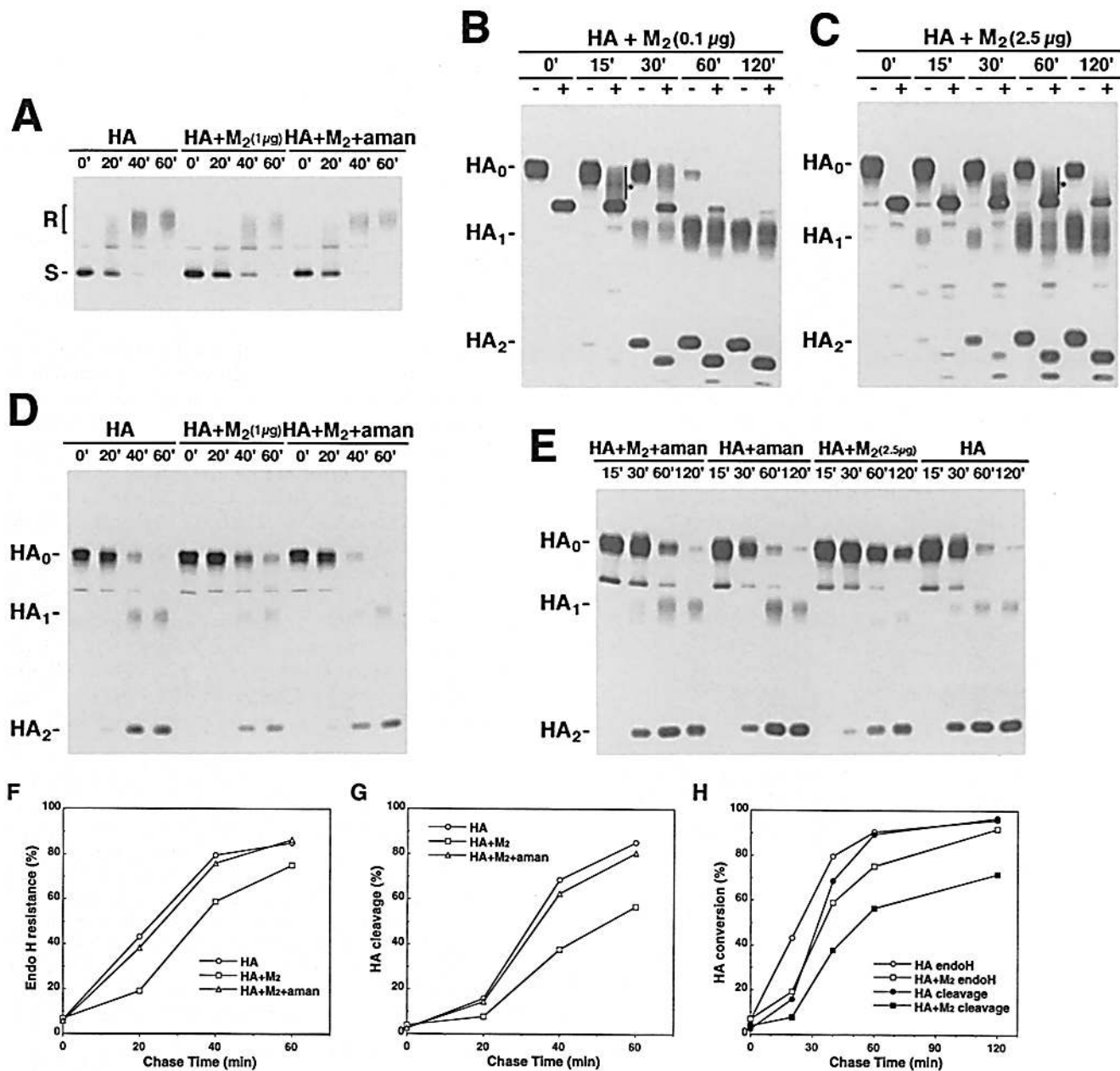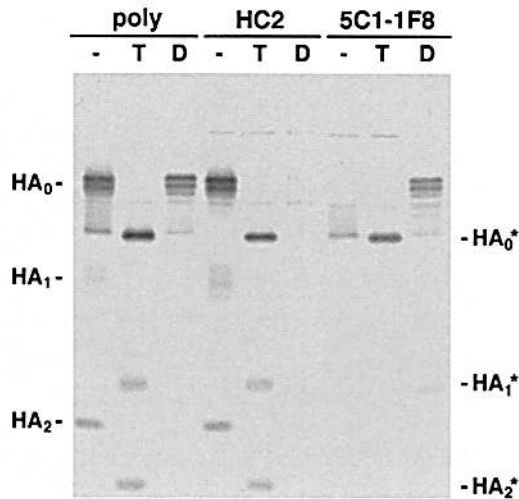
Figure 2. The M_2 protein ion channel activity slows the kinetics of HA acquiring endo H resistance and slows the kinetics of HA cleavage. HeLa T4 cells were infected with vaccinia virus vTF7.3 and transfected with 2.5 µg of pTM3-HA DNA in the presence or absence (as indicated above the lanes) of 1 µg or 2.5 µg of pTM3-M_2 DNA, and the total amount of DNA was adjusted to 5 µg per dish with pTM3 vector DNA. At 5.5-h posttransfection, the cells were metabolically labeled with Tran[^{35}S] label for 10 min and incubated in chase medium for various periods as indicated. Where indicated, amantadine (*aman*) was added to the medium (final concentration 5 µM) at 2.5 h before metabolic labeling, and the drug was maintained in the subsequent washing, starving, labeling, and chase media. (*A–C*). HA was immunoprecipitated and digested with endo H, and polypeptides were analyzed by 10% SDS-PAGE under nonreducing conditions (*A*) or reducing conditions (*B* and *C*). In *A*, endo H–resistant (*R*) and –sensitive (*S*) species are indicated. In *B* and *C*, the asterisk indicates uncleaved and endo H–resistant HA. In *B*, 0.1 µg M_2 plasmid was cotransfected to stabilize HA and to prevent it from undergoing the low pH–induced conformational change that results in loss of HA_1. (*D* and *E*) Cleavage kinetics of HA on coexpression of 1 µg (*D*) or 2.5 µg (*E*) of pTM3-M_2 DNA. HA was immunoprecipitated and analyzed by 10% SDS-PAGE under reducing conditions. (*F*) Quantification of HA endo H resistance kinetics shown in *A*; (*G*) quantification of HA cleavage kinetics shown in *D*. (*H*) Average of three independent experiments of endo H resistance kinetics and cleavage kinetics. Radioactivity determined on a Fuji Bio Imager.
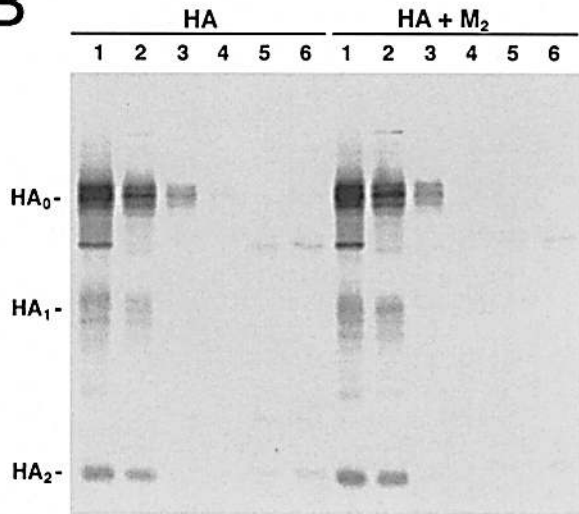
(Figs. 2, *D* and *E*; data quantified in Fig. 2, *G* and *H*), and the delayed rate of cleavage was nearly completely reversed by addition of amantadine, the specific blocker of the M_2 ion channel. A possible artifact of the experimental design is that high concentrations of amantadine can have an amine effect and raise intracellular pH; e.g., FPV HA in the absence of M_2 protein coexpression was rescued to the HA pH-neutral form by 67 µM amantadine (Sugrue et al.,

A

poly | HC2 | 5C1-1F8

- T D | - T D | - T D

HA₀-

-HA₀*

HA₁-

-HA₁*

HA₂-

-HA₂*

B

HA | HA + M₂

1 2 3 4 5 6 | 1 2 3 4 5 6

HA₀-

HA₁-

HA₂-

C

HA | HA+M₂

0' 10' 20' 60' | 0' 10' 20' 60'
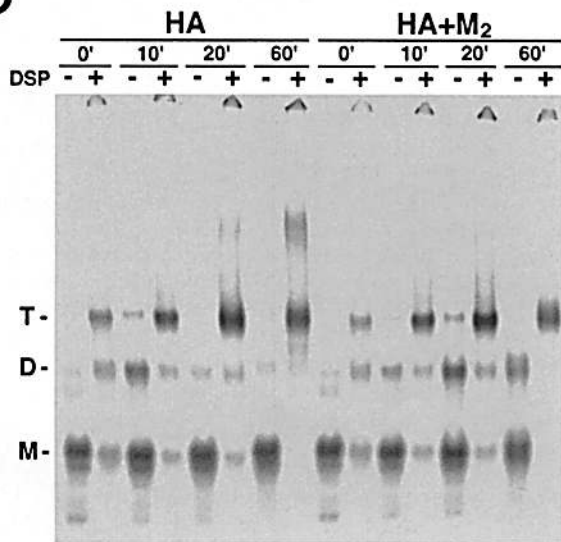
DSP - + - + - + - + | - + - + - + - +

T-

D-

M-

1990; Ohuchi et al., 1994). However, for our experiments, this possibility was eliminated by showing that when cells expressing FPV HA alone were treated with 10 μM amantadine, the amounts of HA₁ observed in the presence of amantadine at 60- and 120-min chase were not greatly different from HA expressed in the absence of the drug (Fig. 2 E), and HA₁ was sensitive to proteinase K digestion (unpublished observation).

Coexpression of M₂ Ion Channel Protein with HA Does Not Affect HA Folding or Oligomerization

The above data suggested that the M₂ ion channel activity caused a delay in intracellular transport of HA to the medial Golgi apparatus. We considered it likely that the ability of the pH-activated M₂ ion channel activity to equilibrate pH between the lumen and cytoplasm was responsible for the effects observed. However, it was essential to eliminate the possibility that overexpression of M₂ protein was having a pleiotropic effect on HA folding or oligomerization in the ER, which, in turn, would affect transport of HA to the Golgi apparatus. Thus, we examined in detail the ability of HA to fold and to oligomerize upon coexpression of M₂ protein.

Protease Sensitivity. Although proteinase K sensitivity of HA is a diagnostic indicator of the low pH–induced conformation of HA, unfolded or malfolded HA is also susceptible to proteinase K digestion. It has been shown that in the ER HA progresses from an unfolded and proteinase K–sensitive form to an oligomeric, proteinase K–resistant

Figure 3. HA coexpressed with M₂ is properly folded and oligomerized. (*A*) HA was expressed in HeLa T4 cells and metabolically labeled with Tran[³⁵S] label for 15 min and incubated in chase medium for 20 min. (Lanes −) Untreated cells. (Lanes *T*) To inhibit addition of N-linked carbohydrate chains, tunicamycin (1 μg/ml) was added to the media 2 h before labeling, and the drug was maintained in subsequent washing, labeling, and chase media. (Lanes *D*) To prevent disulfide bond formation, 5 mM DTT was included in the labeling and chase media (Tatu et al., 1993). The cell lysates were immunoprecipitated with either HA-specific polyclonal antisera, mAb HC2, or mAb 5C1-1F8, and polypeptides were analyzed by 10% SDS-PAGE under reducing conditions. The migration of unglycosylated HA₀, HA₁, and HA₂ is indicated as HA₀*, HA₁*, and HA₂*, respectively. (*B*) HA or HA + M₂ were expressed in HeLa T4 cells using 2.5 μg pTM3-HA and 2.5 μg pTM3-M₂. Cells were labeled with Tran[³⁵S] label for 10 min and incubated in chase medium for 15 min, HA was immunoprecipitated with conformation-specific mAb HC2 in three sequential cycles to deplete the precipitable HA, and the remaining supernatant was further precipitated with either mAb 5C1-1F8 or HA-specific polyclonal antisera. (Lanes *1*) HA precipitated with polyclonal antisera in the starting lysate. (Lanes *2, 3,* and *4*) HA precipitated with HC2 in the first, second, and third cycles, respectively. (Lanes *5* and *6*) HA precipitated in the fourth cycle with either 5C1-1F8 or HA polyclonal antisera. (*C*) HA alone or HA and M₂ were expressed in HeLa T4 cells using 2.5 μg of each plasmid, metabolically labeled, and chased for various periods as described above. Cells were lysed in 1% Triton, 50 mM Tris-HCl, pH 8.0, and 100 mM NaCl and chemically crosslinked with 1 mM DSP for 30 min on ice (+) or incubated without DSP (−). Proteins were immunoprecipitated and analyzed on a 3.5% borate-acetate gel. Positions of monomer, dimer, and trimer were shown as *M, D,* and *T*, respectively.

form (Copeland et al., 1988; Gething et al., 1986). To investigate the possibility that the effect of M_2 coexpression on delaying cleavage of HA_0 was because it induced malfolding of HA, the sensitivity of FPV HA to proteinase K was examined. After a short pulse-label (Fig. 1 C; $0'$), all HA species were susceptible to proteinase K digestion (at 4°C or 30°C), indicating HA had yet to fold or oligomerize. However, after 15 min of chase period, HA_0 was observed to be resistant to proteinase K digestion at 4°C. By 30 min HA_0 was cleaved to HA_1 and HA_2, and a proportion of HA_1/HA_2 was digested by proteinase K at 4° or 30°C, indicating that HA had undergone the transition to the low pH–induced form. However, HA_0, either in the absence (30-min chase) or presence of M_2 (60-min chase), was resistant to proteinase K digestion at 4°C, unlike at 0-min chase period where HA_0 was sensitive to proteinase K digestion. This finding suggests that M_2 coexpression in the large part does not prevent HA folding or cause HA malfolding. It is noted that FPV HA_0 remains sensitive to proteinase K digestion at 30°C, whereas cleaved HA_1/HA_2 (particularly with M_2 coexpression) is proteinase K resistant. Thus, the difference in proteinase K sensitivity between FPV HA_0 and HA_1/HA_2 suggests this assay may be a means of detecting the differences in conformation of these molecules (see Wilson et al., 1981). In Fig. 1, A and C, a minor HA species that is proteinase K–sensitive at 4° and 30°C and migrates between HA_0 and HA_1 was observed. As will be shown below, this species is probably unglycosylated HA_0.

Reactivity with Antibodies. To examine further the possibility that the effect of high level M_2 expression on HA cleavage was due to interference with folding of HA, conformation-specific HA antibodies were used. A rabbit polyclonal HA serum was used that recognized all forms of HA (i.e., uncleaved and cleaved HA, unglycosylated HA species synthesized in cells treated with tunicamycin, and HA_0 synthesized in cells treated with 5 mM DTT, the latter treatment preventing HA folding) (Tatu et al., 1993) (Fig. 3 A). Conformation-specific mAb (HC2), which recognizes antigenic loop A of the HA_1 domain in mature uncleaved HA and cleaved HA (Sugrue et al., 1990), was found to recognize glycosylated and unglycosylated, uncleaved and cleaved HA species, but mAb HC2 did not recognize HA synthesized in DTT-treated cells (Fig. 3 A). The third antibody used, mAb 5C1-1F8, did not recognize uncleaved and cleaved HA species, but it did recognize the minor HA species synthesized in untreated cells that comigrates with unglycosylated HA synthesized in cells treated with tunicamycin. Furthermore, mAb 5C1-1F8 recognizes HA_0 synthesized in the presence of DTT (Fig. 3 A). Thus, operationally, mAbs HC2 and HC1-1F8 can be used to discriminate between folded and unfolded HA.

The conformation-specific mAbs were then used to screen for unfolded/malfolded HA in cells expressing HA alone or in cells coexpressing HA and M_2. An aliquot of the cell lysate was immunoprecipitated with the polyclonal HA serum (Fig. 3 B, lanes *1*). Another aliquot of the cell lysate was immunoprecipitated using three sequential rounds of immunodepletion with mAb HC2 to deplete the lysate of mature HA species (Fig. 3 B, lanes *2–4*). Then the HA-specific species remaining in the supernatant of the immunoprecipitation reaction were finally immunoprecipitated

with either mAb 5C1-1F8 (Fig. 3 B lanes *5*) or with the polyclonal HA serum (Fig. 3 B, lanes *6*). When the immunoreactiovity profiles of HA expressed alone or HA + M_2 were compared, they were found to be virtually identical, and only a very small amount of unfolded/malfolded HA (unglycosylated) was observed. Thus, coexpression of M_2 with HA does not lead to malfolding of HA.

Coexpression of M_2 with HA Does Not Cause HA Association with GRP78-BiP. The resident ER chaperone protein GRP78-BiP forms a stable association with many malfolded proteins and prevents their transport from the ER, and for some proteins as they undergo folding in the ER, it is possible to show a specific and transient association with GRP78-BiP (de Silva et al., 1990; Gething et al., 1986, 1994; Machamer et al., 1990; Marquardt et al., 1993; Ng et al., 1989; for review see Doms et al., 1993). To determine if coexpression of M_2 with HA caused an association with GRP78-BiP, coimmunoprecipitation studies under ATP-depleting conditions were performed. When HA was expressed alone, or when HA was coexpressed with M_2, the polyclonal HA-specific serum did not coprecipitate GRP78-BiP, and the mAb specific for BiP (Bole et al., 1986) only precipitated GRP78-BiP (and not HA) (data not shown). Thus, coexpression of the M_2 + HA does not cause association of HA with BiP, which provides further evidence that M_2 protein does not cause an accumulation of unfolded HA molecules.

Coexpression of M_2 with HA Does Not Affect the Rate or Extent of HA Oligomerization. Mature HA is a homotrimer (Wiley et al., 1977; Wilson et al., 1981), and oligomerization is thought to occur in the ER (Copeland et al., 1988; Gething et al., 1986). Oligomerization of HA can be followed by using the bifunctional protein cross-linking reagent DSP (Gething et al., 1986). To determine if coexpression of M_2 causes an alteration in HA trimerization, cell lysates were cross-linked with DSP at various times after a 5-min metabolic pulse-label. As cross-linking to the HA trimeric species went almost to completion, it was possible to analyze the kinetics of oligomerization (Fig. 3 C). When HA was expressed alone, 70–80% of the HA molecules formed a trimer within 10 min of the pulse-label, kinetics that are comparable with those reported previously (Gething et al., 1986) (Fig. 3 C). It can be seen in Fig. 3 C that under nonreducing conditions and without addition of cross-linker, some HA molecules form SDS-resistant oligomers as had been observed previously, but the differences observed between expressing HA alone or HA + M_2 varied among experiments, as did the detection of the higher molecular weight species of HA observed in the 60' chase lane (Gething et al., 1986). On coexpression of HA + M_2, the kinetics of oligomerization of HA were very similar to those observed for expression of HA alone (data not shown).

Retention of M_2 Protein in the ER Has No Effect on HA Transport

Another possibility that had to be eliminated for the delayed transport of HA to the medial Golgi apparatus on high level expression of the M_2 protein was that gross competition for the intracellular transport machinery in the ER was occurring. To investigate this possibility, the M_2
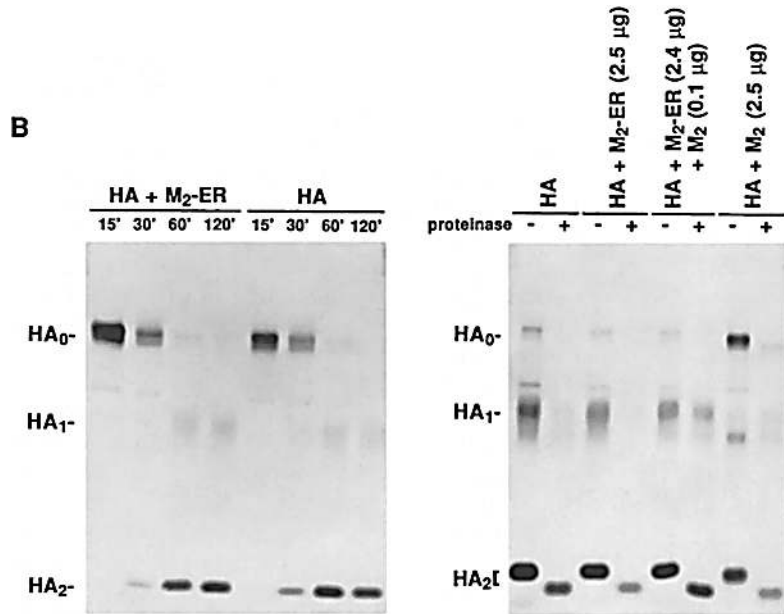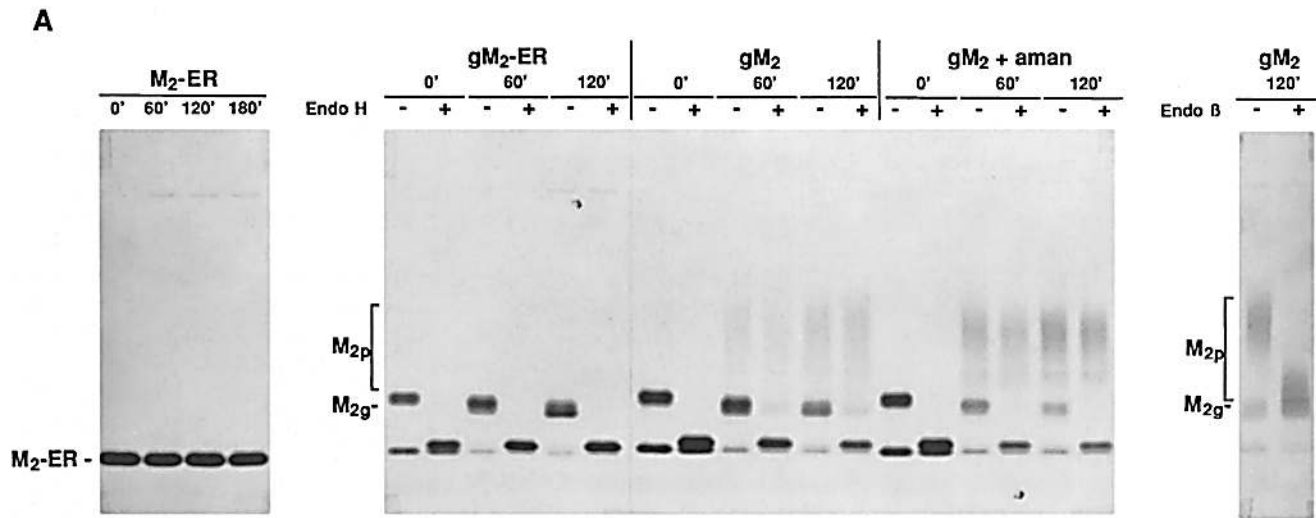
A

B

Figure 4. Retention of M_2 protein in the ER does not affect transport of coexpressed HA. (*A*) M_2 protein modified to contain a double lysine, ER retention signal at the COOH terminus (M_2-*ER*), M_2 protein modified to contain an NH_2-terminal site for N-linked glycosylation (*gM_2*), and M_2 protein modified to contain both mutations (*gM_2-ER*) were expressed in HeLa T4 cells by using the Vac/T7 expression system. 1 μg of mutant DNA and 4 μg of pTM3 vector DNA were transfected into vaccinia virus vTF7.3-infected HeLa T4 cells. The cells were metabolically labeled with Tran[^{35}S] label for 10 min and incubated in pH chase medium (pH 7.0) for various periods as indicated. Cell lysates were immunoprecipitated with M_2 cytoplasmic tail–specific mAb 1D6, and the recovered M_2 protein was incubated with (+) or without (−) 2 mU endo H at 37°C for 18 h and analyzed by SDS-PAGE in 17.5% + 4 M urea gels under reducing conditions. M_{2g}, M_2 with one high-mannose carbohydrate chain; M_{2p}, M_2 containing heterogeneous glycan additions. (*B, left*) Kinetics of HA cleavage on coexpression of M_2-ER. HA alone or HA + M_2-ER was expressed in HeLa T4 cells by the Vac/T7 system using 2.5 μg of pTM3-HA DNA and 2.5 μg of pTM3-M_2-ER DNA. HA was metabolically labeled, immunoprecipitated, and analyzed by SDS-PAGE under reducing conditions. (*B, right*) Analysis of the conformational form of HA on coexpression with M_2-ER. HeLa T4 cells were infected with vaccinia virus vTF7.3 and transfected with 2.5 μg of pTM3-HA DNA and 2.5 μg of various combinations of pTM3-M_2-ER and pTM3-M_2 DNA as indicated. The cells were metabolically labeled for 10 min and incubated in chase medium for 60 min. Cell lysates were treated with (+) or without (−) proteinase K, and HA was immunoprecipitated and analyzed by 10% SDS-PAGE under reducing conditions.

protein was modified to contain an ER retention signal at its COOH terminus (M_2-ER) (Jackson et al., 1990, 1993) (lysine substitutions at residue −3 and −4; M_2 residues 94 and 95). The M_2-ER protein when expressed in cells was stable in a pulse-chase protocol and was not degraded within 3 h after the pulse-label (Fig. 4 *A*). To facilitate further characterization of the M_2-ER protein, a site for addition of N-linked carbohydrate was introduced into the NH_2-terminal ectodomain of both M_2 and M_2-ER proteins as described previously (Holsinger et al., 1994; Hull et al., 1988), and these proteins were designated gM_2 and gM_2-ER, respectively. As shown in Fig. 4 *A* (lanes *gM_2-ER* and *gM_2*), significant populations of these molecules were glycosylated (*M_{2g}*), and digestion with endo H indicated M_{2g} was in the high mannose endo H–sensitive form. In a

pulse-chase protocol, even after prolonged chase periods the carbohydrate chain on gM_2-ER remained endo H sensitive, and, with time, the gM_2-ER glycosylated species had a faster electrophoretic mobility (Fig. 4 *A*) consistent with retention of gM_2-ER in the ER and the trimming of the glucose and mannose residues on the high mannose core oligosaccharide chain. Immunofluorescent staining indicated that M_2-ER and gM_2-ER, unlike M_2 and gM_2 proteins, were not expressed at the cell surface (unpublished observations). Thus, taken together the available data suggest M_2-ER and gM_2-ER are localized predominantly to the ER.

In contrast with the endo H sensitivity of gM_2-ER, in a pulse-chase protocol a population of gM_2 molecules had a heterogeneous gel mobility after a chase period, and this

population of molecules was resistant to endo H digestion (M_{2p}). The M_{2p} species could be digested with endo-β-galactosidase (Fig. 4 A, *right panel*), diagnostic of polylactosaminoglycan addition to the high mannose carbohydrate chain (Fukuda et al., 1984; Williams and Lamb, 1988), and this modification occurs in the Golgi apparatus (Fukuda, 1985). Treatment of cells expressing gM_2 with amantadine caused an increase in the amount of M_{2p} species with a concomitant decrease in the amount of the endo H–sensitive M_{2g} species (Fig. 4 A, *middle panel*; gM_2 + *aman*): quantification by densitometry of the loss of endo H–sensitive species indicated a 2.5-fold increase in rate at both 60- and 120-min chase upon addition of the M_2 channel blocker amantadine. These data suggest that the M_2 ion channel protein, in addition to delaying HA transport, affects its own intracellular transport.

When HA and M_2-ER were coexpressed, it was found that M_2-ER expression had no effect on the kinetics of cleavage of HA_0 and that the amount of HA_1 that accumulated was less than stoichiometric (Fig. 4 B, *left panel*). Examination of the conformational form of HA, using the proteinase K digestion sensitivity assay, indicated that M_2-ER (or gM_2-ER; unpublished observations) could not prevent HA from undergoing its low pH–induced conformational change, and an accumulation of HA_0 due to delayed cleavage was not observed. When M_2-ER (2.4 μg DNA transfected) was coexpressed with a small amount of wild-type M_2 protein (0.1 μg DNA transfected), HA was stabilized in its pH-neutral form. Parenthetically, this latter observation suggests that even though M_2-ER molecules were present at high concentration, they were not free to form mixed oligomers with the low concentration of wild-type M_2 molecules. Thus, these data support the view that the delayed intracellular transport of HA upon coexpression of high levels of M_2 protein is not due to gross saturation of the intracellular transport machinery in the ER. In addition, it seems reasonable to infer that the M_2 ion channel activity was not functioning in the ER, and there is no evidence to suggest that the ER intraluminal pH is sufficiently low to activate the M_2 ion channel. However, the caveat that it cannot be proven definitively that M_2-ER is still a functional and pH-activated ion channel has to be added, because electrophysiological measurements require cell surface expression. However, as discussed above, the M_2 TM domain peptide alone has functional ion channel activity (Duff and Ashley, 1992), and modifications to the M_2 protein cytoplasmic tail (addition of an HA-epitope tag and a His_6 tag) do not affect the ion channel activity (unpublished observations). Thus, it seems most unlikely that substitution of two lysine residues into the immediate COOH terminus of the M_2 protein will affect its ion channel activity.

Thus, taken together the data shown in Figs. 3 and 4 indicate coexpression of M_2 with HA does not have a major effect on HA folding, does not cause an altered association of HA with the cellular chaperone GRP78-BiP, does not cause a change in HA oligomerization, and the ER transport machinery is not grossly saturated due to overexpression of integral membrane proteins. Thus, the delay in cleavage of HA_0 observed on M_2 protein expression (Fig. 1) most likely reflects changes in events that occur in the exocytic pathway after those we have measured in the ER.

Effect of Coexpression of M_2 on Kinetics of Transport of HA to the cis Golgi Apparatus

The effect of M_2 ion channel activity on intracellular transport of HA could be due to either a gross delay in delivery of trimerized HA <u>to</u> the *cis* Golgi apparatus or it could be due to delayed transport of HA <u>through</u> the Golgi cisternae. To distinguish between these possibilities, the transport of HA between the ER and Golgi compartments in the CHO clone 15B cell line was measured by monitoring the trimming of HA N-linked carbohydrates from the high mannose ($Man_{8-9}GlcNAc_2$) ER form to the $Man_5GlcNAc_2$ species by the *cis* Golgi-associated α1,2-mannosidase I (Balch et al., 1986; Beckers et al., 1987). The assay for trimming was based on the specificity of the enzyme endo D, which will not hydrolyze the $Man_{8-9}GlcNAc_2$ oligosaccharide form but will hydrolyze the $Man_5GlcNAc_2$ form (Mizuochi et al., 1984), and results in an easily detectable shift in HA mobility on SDS-PAGE. While the endo D–sensitive form of the oligosaccharides is only a transient intermediate in the wild-type cells, it is stable in the CHO clone 15B cell line as the $Man_5GlcNAc_2$ species is not further processed in CHO 15B cells, as these cells lack the enzyme N-acetylglucosamine transferase I that is required for processing of carbohydrates to the complex form (Gottlieb et al., 1975). The kinetics of endo D sensitivity of HA carbohydrate chains and the kinetics of transport of HA to the *trans* Golgi (as measured by HA cleavage) were monitored when HA was expressed alone in CHO 15B cells or when HA + M_2 were coexpressed in CHO 15B cells. As shown in Fig. 5, coexpression of M_2 did not change appreciably the rate (as measured by the slope of the line) or extent of HA endo D sensitivity (HA analyzed under nonreducing conditions; Fig. 5 A), but there was a lag (5–7 min) in transport from the ER to the *cis* Golgi. However, coexpression of M_2 with HA had a dramatic effect on the rate of transport of HA to the *trans* Golgi, as observed by the delay in the rate (note difference in slopes of lines) and extent of HA cleavage. In Fig. 5 B, HA was analyzed under reducing conditions: the trimmed $Man_5GlcNAc_2$ HA_0 intermediate is denoted as species T. Quantification of the sum of all HA species at each time point shown in Fig. 5 B indicated that when HA was expressed alone, 40–45% of total HA species were lost between 60 and 180 min of chase period, and this was due to a loss of the HA_1 subunit. When HA + M_2 were coexpressed, the sum of the HA species was constant for 120 min, and a small ($\sim$10%) loss of HA_1 was observed at 180 min. The instability of HA_1 in the absence of M_2 expression probably reflects the conversion of HA to the low pH–induced form in CHO-15B cells. Thus, these data indicate that a major effect of coexpression of the M_2 ion channel protein on HA transport is to dramatically delay and partially prevent transport through the Golgi cisternae. We do not know the reason for the delay in arrival of HA to the *cis* Golgi, but it may be that a delay in intra-Golgi transport events impacts delivery from the ER.

Comparison of Effect of M_2 Coexpression and Monensin Treatment on Intracellular Transport of HA

The effect of high level M_2 protein expression on intracellular transport appeared similar to that reported for mon-
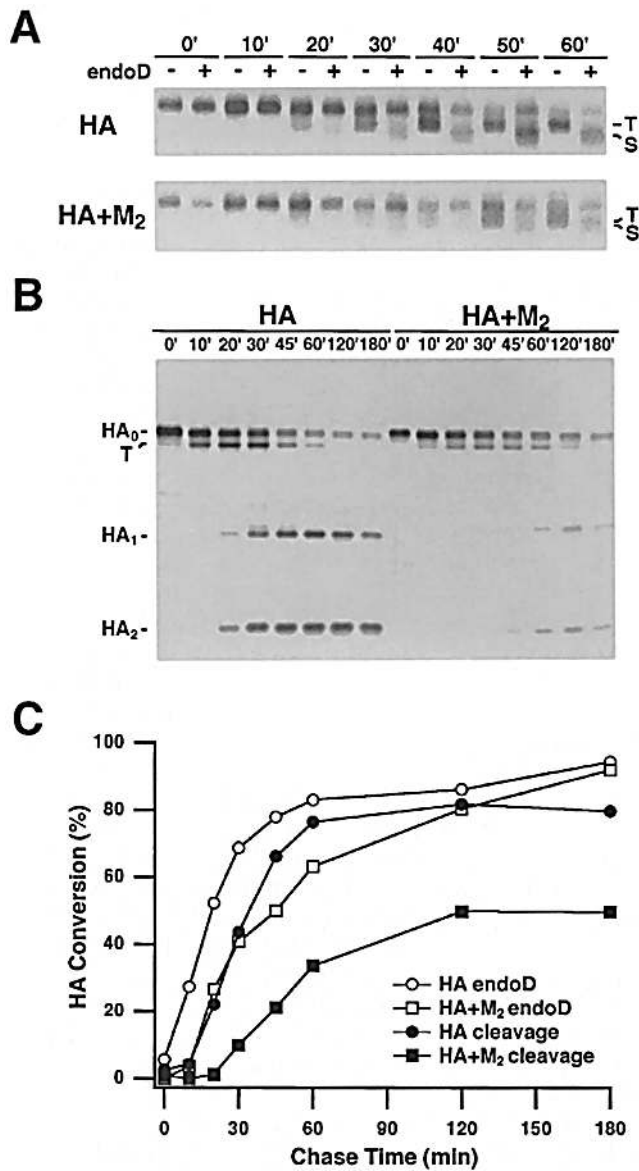
Figure 5. Coexpression of M_2 delays HA transport through the Golgi apparatus. HA alone or HA + M_2 (2.5 μg of each plasmid) were expressed in CHO clone 15B cells (a mutant line which lacks *N*-acetylglucosamine transferase I). The cells were metabolically labeled with Tran[^{35}S] label for 10 min and subjected to a chase protocol for various periods as indicated. HA was immunoprecipitated with HA-specific polyclonal antiserum and divided into two aliquots. (*A*) Immunoprecipitated HA was incubated with (+) or without (−) endo D at 37°C for 16 h and analyzed by SDS-PAGE under nonreducing conditions. *T*, HA species with carbohydrate chains trimmed by Golgi mannosidase I; *S*, endo D–sensitive HA. (*B*) HA was analyzed by SDS-PAGE under reducing conditions. *T*, HA species with carbohydrate chains trimmed by Golgi mannosidase I. (*C*) Quantification of data from two experiments for kinetics of HA endo D sensitivity and HA cleavage in CHO 15B cells. Radioactivity determined using a Fuji Bio Imager.

Figure 6. Monensin treatment slows the kinetics of HA transport. HA alone or HA + M_2 (2.5 μg of each plasmid) was expressed in HeLa T4 cells using the Vac/T7 system, and the cells were metabolically labeled with Tran[^{35}S] label for 10 min and incubated in chase medium for various periods as indicated. For monensin treatment, the drug (final concentration 10 μM) was added to the medium at 2.5 h before metabolic labeling, and the drug was maintained in all subsequent washing, starving, labeling, and chase media. (*Top*) Cell lysates were immunoprecipitated with an HA-specific polyclonal antiserum and analyzed by 10% SDS-PAGE under reducing conditions. (*Bottom*) Cell lysates were immunoprecipitated with HA-specific polyclonal antisera, and the recovered HA was treated with (+) or without (−) 2 mU endo H at 37°C for 18 h and analyzed by 10% SDS-PAGE under reducing conditions.

ensin treatment of cells; thus, the treatments were directly compared. The Na$^+$/H$^+$ ionophore monensin disrupts intracellular pH gradients and causes the Golgi cisternae to form greatly enlarged vacuoles (Tartakoff, 1983). Mon-
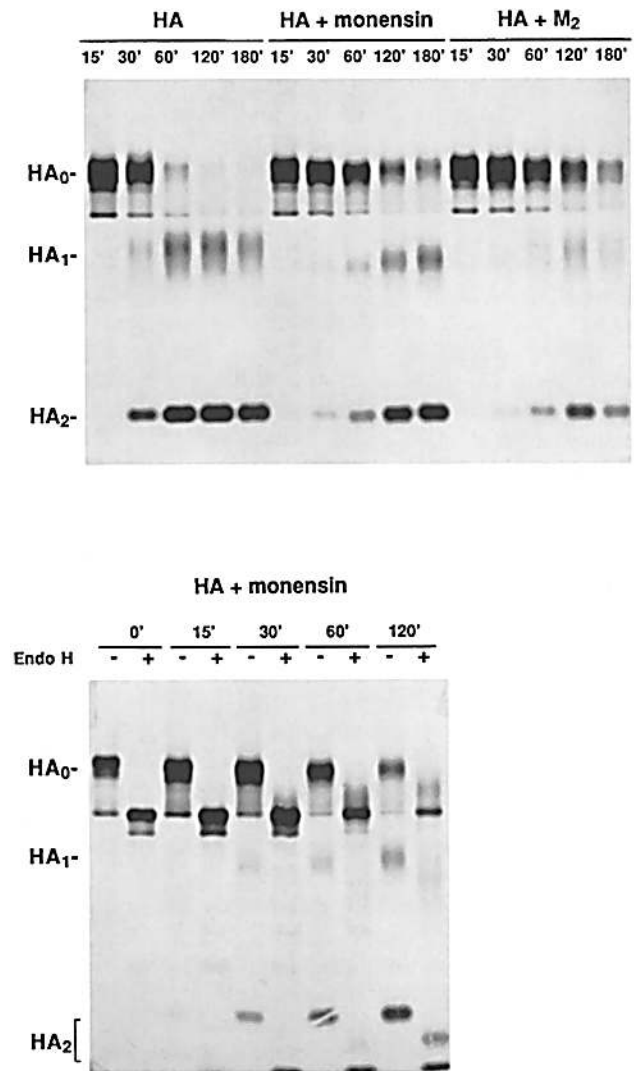
ensin blocks transport of many integral membrane proteins in the medial or *trans* Golgi, and, as a secondary effect, glycoproteins do not receive terminal carbohydrate additions (Griffiths et al., 1983; Strous and Lodish, 1980; Tartakoff, 1983). However, influenza virus HA is expressed at the cell surface in monensin-treated cells even though transport through the Golgi apparatus is delayed (Alonso and Compans, 1981; Rodriguez-Boulan et al., 1984; un-
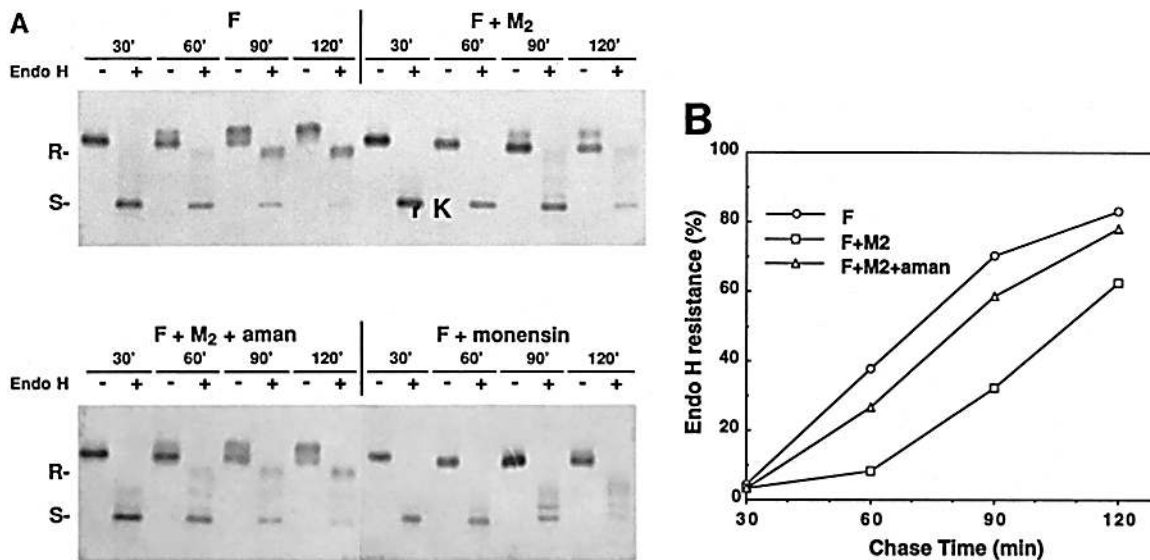
Figure 7. Effect of M_2 ion channel activity and monensin treatment on F glycoprotein intracellular transport. (*A*) SV5 F protein alone or F + M_2 proteins were expressed in HeLa T4 cells using 1 μg of pTM3-F DNA and 2.5 μg of pTM3-M_2 DNA. The cells were metabolically labeled with Tran[^{35}S] label for 10 min and incubated in chase medium for various periods as indicated. In amantadine- or monensin-treated cultures, the drugs (final concentration 10 μM) were added to the medium at 2.5 h before metabolic labeling and maintained in all of the subsequent steps. Cell lysates were immunoprecipitated with F-specific peptide sera (Horvath et al., 1992), and the recovered F was digested with (+) or without (−) endo H and analyzed by 10% SDS-PAGE under nonreducing conditions. *R* and *S* denote endo H–resistant and –sensitive bands, respectively. (*B*) Quantification of the data shown in *A* from densitometric scanning of the autoradiograph (LKB Ultroscan XL Densitometer; Pharmacia-LKB).

published results). As shown in Fig. 6, addition of 10 μM monensin to cells expressing HA delayed the rate at which HA acquired endo H resistance (Fig. 6, *bottom panel*) (c.f. Fig. 2 for the control of HA expressed alone and for the effect of M_2 protein coexpression on HA) and delayed the rate of cleavage of HA_0 (Fig. 6, *top panel*), in both cases with kinetics (quantified data not shown) similar to that observed on coexpression of HA + M_2 protein.

Effect of M_2 Coexpression and Monensin on Intracellular Transport of Other Glycoproteins

To investigate the effect of M_2 protein coexpression on the rate of intracellular transport of glycoproteins not related to influenza virus, the cDNA of the F glycoprotein of the paramyxovirus SV5 was expressed in HeLa T4 cells. In a pulse-chase protocol, F protein acquired endo H resistance with a $t_{1/2}$ ~55 min (Fig. 7, lanes *F*), a rate comparable to that observed previously when using the vac/T7 expression system (Bagai and Lamb, 1995). Coexpression of a high level of M_2 protein delayed the rate at which F protein acquired endo H resistance ($t_{1/2}$ ~100 min) (Fig. 7, lanes *F* + M_2), and addition of amantadine (10 μM) to the F + M_2–coexpressing cells largely overcame the delay in transport kinetics (Fig. 7, lanes *F* + M_2 + *aman*). Monensin (10 μM) treatment of cells expressing F also caused a slower rate of acquisition of endo H resistance.

In Figs. 6 and 7, it can be observed that monensin treatment of cells expressing HA and F causes the glycoproteins to have an altered gel mobility as compared to untreated cells. However, for HA, after a prolonged chase period (6 h), the gel mobility of HA_1 in monensin-treated cells became similar to HA_1 in untreated cells. After a 6-h chase period, HA_1 exhibited an altered mobility on diges-

tion with bacterial neuraminidase, indicative of addition of sialic acid to HA (unpublished observations). Thus, the mobility differences of the polypeptides after a 2-h chase period and observed in Figs. 6 and 7 probably reflect the delayed transport of the proteins through the TGN.

Morphology of the Golgi Apparatus in Cells Expressing M_2 Protein

As the M_2 ion channel protein and monensin both have a commonality of function in equilibrating pH gradients between the lumen of the Golgi and the cytoplasm, the morphology of the Golgi in cells expressing the M_2 ion channel protein was examined by EM. As shown in Fig. 8, the Golgi apparatus was found to be dilated, with the greatest amount of swelling occurring on one face of the Golgi cisternae (Fig. 8, *B* and *C*). Quantification of the effect of M_2 expression is described in the legend to Fig. 8: 88% of Golgi in cells expressing M_2 exhibited dilation. As a marker for the Golgi apparatus, peroxidase-labeled ricin staining was performed (specific for galactose, a carbohydrate moiety that is localized to the *trans* Golgi cisternae and also to endosomal compartments). To distinguish between endosomal and Golgi compartments, the endosomal system was loaded with BSA-gold. As shown in Fig. 8 *D*, the dilated membranous compartments were stained with peroxidase-ricin and did not show BSA–gold labeling and thus are considered Golgi compartments. Treatment of cells expressing the M_2 protein with amantadine largely prevented dilation of the *trans* Golgi cisternae (Fig. 8 *E*). For comparison, cells were treated with 10 nM monensin (Fig. 8 *F*) and 10 μM monensin (Fig. 8 *G*), and as found previously for monensin treatment (Orci et al., 1984), there was little selectivity as to which Golgi cisternae were
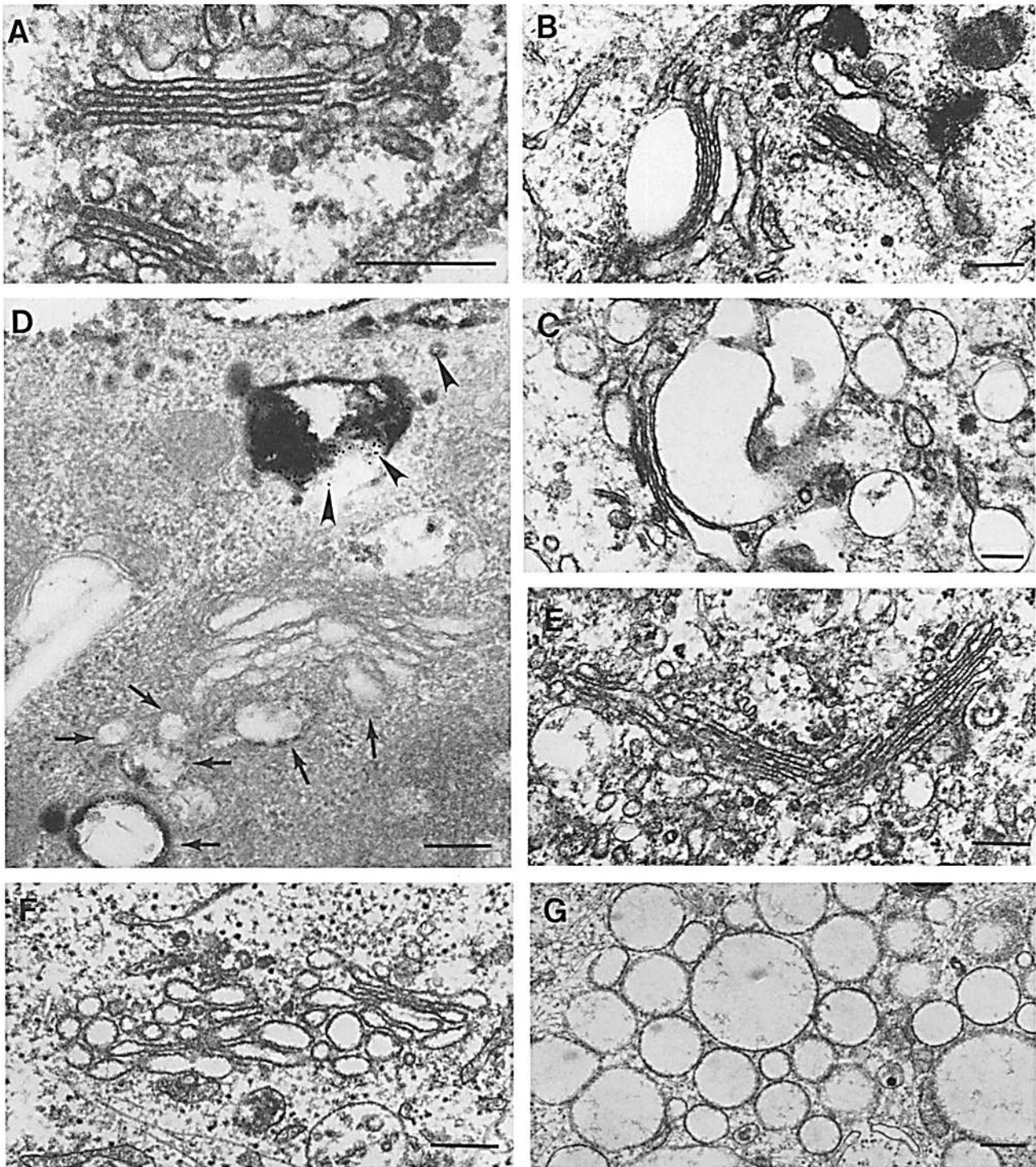
Figure 8. M$_2$ protein expression causes dilation of the Golgi apparatus. BHK cells were incubated with BSA–colloidal gold to provide a marker for endocytic compartments, and then infected with vTF7-3 and either mock transfected (*A*), transfected to express M$_2$ using 2.5 μg pTM3-M$_2$ DNA (*B–D*), transfected to express M$_2$ (2.5 μg pTM3-M$_2$ DNA) and incubated with 5 μM amantadine (*E*), or treated with 10 nM (*F*) or 10 μM (*G*) monensin. Cells were incubated with 5 μg/ml ricin-HRP for 1 h, fixed with glutaraldehyde, incubated in substrate solution, and postfixed with osmium tetroxide. Samples were treated with 1% tannic acid and stained en bloc with uranyl acetate (except *D*). Cells were dehydrated, embedded in epoxy resin, and sectioned (~50–60 nm) for EM. Sections were contrasted with uranyl acetate and lead citrate. To more clearly illustrate the ricin-HRP staining, the sample shown in *D* was more thickly sectioned (~120 nm) and contrasted only with uranyl acetate. Arrows denote dilated ricin-containing TGN, and arrowheads denote BSA–gold. Profiles of 114 cells were examined. The number of Golgi per cell varied from one to four, although where multiple Golgi apparati were observed, they could be continuous from connections out of the plane of the section. A total of 93 Golgi apparati were examined, and none contained BSA–gold, although BSA–gold was readily detected in endosomes: 71% of the Golgi had some elements that were clearly dilated, and the transfection efficiency was determined by fluorescence for M$_2$ protein expression to be ~80%. Thus, M$_2$ expression causes the vast majority of Golgi apparati to be dilated. Bar 0.25 μm.

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dilated, but the extent of dilation observed was dependent on the concentration of monensin used.

Discussion

When influenza virus–infected cells are treated with amantadine to block the M_2 ion channel activity, the HA (FPV subtype) undergoes a structural rearrangement in the TGN from its pH-neutral form to the low pH–induced form of HA (Ciampor et al., 1992a,b; Hay, 1992; Ohuchi et al., 1994; Sugrue et al., 1990; Sugrue and Hay, 1991; Takeuchi and Lamb, 1994). Treatment of virus-infected cells with the Na^+/H^+ ionophore monensin (at nanomolar concentrations) overcomes the effect of the amantadine block of the M_2 ion channel and keeps HA in its pH-neutral form (Sugrue et al., 1990). Thus, from these experiments it was proposed that the M_2 ion channel equilibrates the pH of the lumen of the TGN with the cytoplasm, and it was suggested that there is a similarity in the mechanism of monensin and the M_2 ion channel (Ciampor et al., 1992a,b; Grambas et al., 1992; Sugrue et al., 1990).

Cleavage of the precursor HA_0 to the disulfide-linked chains HA_1 and HA_2 by furin, a TGN resident endoprotease (Molloy et al., 1994), is a prerequisite for the irreversible conformational change to the HA low pH–induced form (Bullough et al., 1994; Skehel et al., 1982; for review see Wiley and Skehel, 1987). Thus, as an indicator for the M_2 ion channel activity, the HA conformational change assay is only useful for monitoring events after transport through the TGN. The M_2 ion channel activity is progressively activated by lowering pH until a plateau is reached at $\sim$pH 5.4 (Pinto et al., 1992; Wang et al., 1993, 1994, 1995; Shimbo et al., 1996) and the lumen of the Golgi apparatus has an acidic pH (pH 5.4–5.9) with a decreasing pH gradient from the cis to the trans cisternae (Anderson et al., 1984; Anderson and Orci, 1988). Thus, it seems possible that the M_2 ion channel may be activated across the Golgi cisternae.

We observed that the M_2 ion channel protein did not interfere with folding or oligomerization of HA. However, coexpression of HA + M_2 caused a marked delay in transport of HA_0 to the medial Golgi apparatus (as monitored by susceptibility of carbohydrate chains to endo H digestion) and delayed the rate and reduced the extent of cleavage of HA_0 in the TGN. Coexpression of HA + M_2 caused a lag in transport of HA from the ER to the cis Golgi, but the rate of transport once it began, and the extent of transport of HA, was very similar to when HA was expressed alone. It is possible that intra-Golgi events impact on delivery from the ER. The delay in intracellular transport we observed upon coexpression of HA + M_2 was not just due to the special relationship of the M_2 protein and HA, as the M_2 ion channel activity also significantly delayed transport of another protein, F glycoprotein. Furthermore, it can be inferred from the delayed modification of carbohydrate chains on a biologically active M_2 protein derivative (gM_2) (Holsinger et al., 1994) that the M_2 protein intrinsic ion channel activity affects its own intra-Golgi transport. That the delay in intra-Golgi transport was due to the M_2 ion channel activity and not due to the expression level of proteins causing gross competition for the intracellular transport machinery was shown by finding that the intra-

Golgi transport delay could be overcome by addition of the M_2 ion channel blocker amantadine; an increased surface of expression of M_2 in the presence of amantadine when using a recombinant baculovirus has also been observed (Black et al., 1993). In addition, M_2 protein engineered to be retained in the ER had no effect on intracellular transport of HA through the Golgi cisternae, which suggests that the machinery in the ER was not saturated. Taken together, the data indicate that the M_2 ion channel is sufficiently activated in the medial Golgi so that expression of the M_2 ion channel activity affects glycoprotein transport through the medial Golgi cisternae.

The expression level of M_2 used here was not greatly different from those found in influenza virus–infected cells. When the level of M_2 protein expression was quantified by using Western blotting (Holsinger et al., 1994), the amount of M_2 protein accumulated in transfected HeLa T4 cells was found to be 900 pg M_2 protein per mg cell protein, and for HeLa T4 cells infected with influenza A/WSN/33 virus (FPV is prohibited from use on the mainland USA by the U.S. Department of Agriculture), the amount was 180 pg M_2 protein per mg cell protein. Although this is a four- to fivefold difference in expression level, the activity of the M_2 ion channel among influenza virus subtypes varies (Grambas et al., 1992; Takeuchi and Lamb, 1994). Thus, the effects described here of M_2 expression on cells are likely to be relevant to effects of virus infection on cells. In this regard, it has been observed that influenza FPV only infects Vero cells abortively; it was also found that the level of M_2 protein mRNA and M_2 protein synthesis was greatly increased over normal, and the level of cell surface HA expression was greatly decreased over normal (Lau and Scholtissek, 1995). We believe that the observations on HA in abortive FPV infection of Vero cells are directly related to those we report here.

The effect of M_2 protein expression on transport of the HA and F glycoproteins was found to parallel the effect of monensin (micromolar concentrations) on intra-Golgi transport for each protein. It is well established that the degree to which monensin treatment impairs intracellular transport varies with the protein examined (Alonso-Caplen and Compans, 1983; Griffiths et al., 1983; Niemann et al., 1982; Srinivas et al., 1982; Strous and Lodish, 1980; Uchida et al., 1979). However, examination by EM of the morphology of the Golgi cisternae showed that the effects of M_2 protein expression and monensin treatment were different. As observed previously (Orci et al., 1984), monensin causes a swelling of all cisternae of the Golgi, whereas the M_2 ion channel activity caused a preferential swelling of the trans Golgi cisternae. This was not unexpected given that the trans cisternae of the Golgi apparatus is the most acidic and the M_2 ion channel activity is activated monotonically to pH 5.4 (Wang et al., 1995). Although the biochemical data concerning the delay in intra-Golgi transport indicate that the M_2 ion channel activity was active in the medial Golgi cisternae, presumably this was not at a sufficient level to cause an observable morphological effect. Monensin treatment is thought to cause a swelling of the Golgi cisternae as a consequence of the Na^+/H^+ ionophore dissipating the pH gradient between the Golgi lumen and the cytoplasm; in doing so, it allows the exit from the Golgi of protons (osmotically inactive if stripped from buffered

proteins) and, at the same time, the influx of osmotically active Na$^+$ (accompanied by water) from the cytoplasm. Although permeability to protons is the ion of most biological relevance for the M$_2$ ion channel (Pinto et al., 1992; for review see Hay, 1992), the swelling of the *trans* Golgi cisternae upon high level M$_2$ protein expression may occur by a mechanism similar to that for monensin.

The M$_2$ protein is synthesized from an alternatively spliced mRNA that is derived from a precursor RNA that is the mRNA for the influenza virus matrix (M$_1$) protein (Lamb and Choppin, 1981). The deleterious effect of high level M$_2$ protein expression on intracellular protein transport provides a rationale for the tightly regulated splicing that generates the M$_2$ mRNA in influenza virus–infected cells (<10% of M$_1$) and that is restricted to times late in the virus replicative cycle (Lamb and Lai, 1982; Shih et al., 1995; Valcarcel et al., 1991).

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