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Influenza Virus M_2 Protein Ion Channel Activity Stabilizes the Native Form of Fowl Plague Virus Hemagglutinin during Intracellular Transport

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The influenza A/fowl plague virus/Rostock/34 hemagglutinin (HA), which is cleaved intracellularly and has a high pH threshold (pH 5.9) for undergoing its conformational change to the low-pH form, was expressed from cDNA in CV-1 and HeLa T4 cells in the absence of other influenza virus proteins. It was found, by biochemical assays, that the majority of the HA molecules were in a form indistinguishable from the low-pH form of HA. The acidotropic agent, ammonium chloride, stabilized the accumulation of HA in its native form. Coexpression of HA and the homotypic influenza virus M_2 protein, which has ion channel activity, stabilized the accumulation of HA in its pH neutral (native) form, and the M_2 protein ion channel blocker, amantadine, prevented the rescue of HA in its native form. These data provide direct evidence that the influenza virus M_2 protein ion channel activity can affect the status of the conformational form of cleaved HA during intracellular transport.

The influenza A virus M_2 integral membrane protein (31, 33) is minimally a homotetramer (21, 57) that is abundantly expressed at the surface of virus-infected cells but is a relatively minor component of virions (63). The M_2 protein has been proposed 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 (18, 57). Direct evidence that the M_2 protein has ion channel activity was provided by expressing the M_2 protein in oocytes of *Xenopus laevis* and measuring membrane currents. It was found that the M_2 ion channel activity is blocked by the anti-influenza virus drug amantadine hydrochloride and that the channel activity is regulated by changes in pH, with the channel being activated at the lowered pH found intralumenally in endosomes and the *trans*-Golgi network (TGN) (46, 60).

Influenza virus particles are internalized into cells by receptor-mediated endocytosis. After exposure of virions to low pH in endosomal compartments, the hemagglutinin (HA) undergoes a low pH-induced conformational change which releases the hydrophobic fusion peptide, mediating the fusion of the viral membrane with the endosomal membrane (reviewed in references 29 and 61). The consequence of this membrane fusion event is that the viral ribonucleoproteins are released into the cytoplasm. Amantadine blocks an early step in virus replication between the steps of virus penetration and uncoating (5, 23, 53), and recent findings suggest that in the presence of the drug, the influenza membrane (matrix) (M_1) protein fails to dissociate from the ribonucleoproteins (38). It is generally believed that once a virion particle has been endocytosed, the ion channel activity of the virion-associated M_2 protein permits the flow of ions from the endosome to the virion interior to disrupt protein-protein interactions and free the ribonucleoproteins from the M_1 protein (reviewed in references 18, 20, 37, and 52).

The HA polypeptide chain is synthesized on membrane-bound ribosomes and is translocated into the endoplasmic reticulum. Core carbohydrate chains are added, and HA oligomerizes to form a trimer ($t_{1/2}$, ~ 7 to 10 min) (14). As HA is transported through the exocytotic pathway to the plasma membrane, its carbohydrate chains are trimmed, and by the time of HA transport to the medial Golgi apparatus, some carbohydrate chains are terminally glycosylated (reviewed in reference 28). HA is synthesized as a precursor, HA_0, that is proteolytically cleaved to form the disulfide-linked subunits HA_1 and HA_2. This cleavage event is the first step in the pathway that permits HA to undergo its conformational change at low pH converting native HA to its fusogenic form. Thus, cleavage of HA is a key determinant for infectivity and pathogenicity (25, 35). For those influenza virus subtypes that contain the appropriate multi-basic amino acids at the cleavage site (largely H5 and H7 avian and equine subtypes) (24, 43), the HA_0 precursor is cleaved in the TGN by furin, a subtilisin-like endoprotease (55). On the other hand, human strains of influenza virus contain a single basic residue in the cleavage site, and when grown in tissue culture or in ovo, cleavage of HA occurs after HA is expressed at the cell surface.

In addition to the early effect of amantadine on influenza virus replication described above, for some subtypes of avian influenza virus, which have an HA that is cleaved intracellularly and have a high pH optimum of fusion (e.g., fowl plague virus [FPV] Rostock), there is a second effect of the drug late in replication. A large body of data indicates that addition of amantadine to cells late in infection brings about a premature conformational change in HA that occurs in the TGN during the transport of HA to the cell surface (6, 7, 15, 16, 56). By immunological and biochemical criteria, this form of HA is indistinguishable from the low pH-induced form of HA (53; reviewed in reference 62) that results following exposure of

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native HA to low pH. Thus, the M_2 ion channel activity (which is blocked by amantadine) is thought to function in the TGN and associated transport vesicles to regulate intracompartmental pH and keep the pH above the threshold at which the HA conformational change occurs (6, 56). The consequence of this irreversible conformational change, which brings about the extrusion of the fusion peptide at the wrong time in the infectious cycle and in the wrong subcellular compartment, is that the HA oligomers aggregate and viral budding is greatly restricted (48; reviewed in reference 62).

All the studies reported to date on the effect of amantadine at late times in infection and how it relates to the need to have functional M_2 protein ion channel activity to obtain FPV Rostock HA expressed at the cell surface in a native form have been performed with influenza virus-infected cells. We were interested in showing directly the role of expression of the M_2 protein on HA biogenesis. Thus, we examined the status of the FPV Rostock HA molecule when it was expressed from a cDNA in the absence of expression of other influenza virus proteins and measured the effect of coexpression of M_2 cDNA on the conformational form of the HA molecule in the presence and absence of amantadine. In the accompanying article, Ohuchi and coworkers (42) quantitate the effect of M_2 protein coexpression on the ability of HA to cause cell-cell fusion.

MATERIALS AND METHODS

Cells and viruses. Monolayer culture of CV-1 cells and a stable line of HeLa cells that express the human CD4 molecule (HeLa T4) (36) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and incubated at 37°C in 5% CO_2. Vaccinia recombinant virus vTF7.3 that expresses bacteriophage T7 RNA polymerase (12) was obtained from Bernard Moss (National Institutes of Health, Bethesda, Md.).

Construction of cDNA encoding M_2 protein. All procedures with the use of recombinant DNA technology, including subcloning of DNA, plasmid purification, PCR, and purification of DNA fragments, were performed by standard methods (3). The cDNA encoding the influenza A/chicken/Germany/34 (H7N1) (FPV Rostock) M_2 protein was constructed from eight oligonucleotides (seven 60-mers and one 48-mer) on the basis of the nucleotide sequence of FPV Rostock RNA segment 7 (40) but adjusted so that the M_2 protein sequence matched that reported by Hay and colleagues (19). The oligonucleotides were designed such that the M_2 cDNA could be cloned via a plasmid *Bam*HI site. A *Nsi*I site was introduced into the M_2 cDNA to facilitate subcloning of the synthetic oligonucleotides in two segments. Four pairs of partially overlapping oligonucleotides were mixed, and completely double-stranded DNA was synthesized by 20 cycles of a PCR (94°C for 1 min, 37°C for 2 min, and 72°C for 3 min). The resulting four double-stranded DNA fragments were purified by separation on and elution from a polyacrylamide gel, and a second round of PCR was performed by mixing pairs of DNA to synthesize the 5′-segment and 3′-segment of the M_2 cDNA. After digestion with *Bam*HI and *Nsi*I, both 5′ and 3′-DNA segments were cloned individually at the *Bam*HI and *Nsi*I sites of pGEM7Zf(+) (Promega Corp., Madison, Wis.). The 5′- and 3′-segments of the M_2 cDNA were excised from the plasmids by *Bam*HI and *Nsi*I digestion, and the M_2 cDNA segments were ligated into the *Bam*HI site of pGEM3 (Promega) such that M_2 cDNA is under control of the bacteriophage T7 RNA polymerase promoter. The nucleotide sequence of the FPV Rostock M_2 cDNA was confirmed by dideoxynucleotide chain-terminating sequencing (49). Construction of cDNAs encoding the influenza A/Udorn/72 M_2 protein and the deletion mutant (M_2 del_{28-31}) have been described previously (46, 64).

Expression of HA and M_2 proteins in eukaryotic cells. The cDNA encoding the influenza virus A/chicken/Germany/34 (FPV Rostock) HA (13, 27, 59) was kindly provided by Hans-Dieter Klenk and was expressed in mammalian cells with either simian virus 40 (SV40) recombinant viruses or the vaccinia virus T7 RNA polymerase hybrid expression system (vac-T7) (12). For SV40 expression, the HA cDNA was excised from the starting plasmid pUC18 vector by *Bgl*II digestion and was subcloned into the SV40 shuttle vector pSV103 at the *Xho*I site (44). Recombinant SV40-HA virus stocks were prepared, by using as helper virus SV40 dl1055, as described previously (51). For vac-T7 RNA expression, the FPV HA cDNA and the M_2 cDNA were each subcloned into pTM3, a plasmid vector which contains a T7 RNA polymerase promoter, the 5′ noncoding region from a picornavirus to facilitate enhanced translation of downstream sequences, *Nco*I and *Bam*HI cloning sites, and a bacteriophage T7 transcriptional terminator (41). An *Nco*I site could not be introduced into the HA or M_2 cDNA sequences without changing the N-terminal proximal residue. However, introduction of a *Bsp*HI site into the HA and M_2 cDNAs (by a PCR procedure) at the point of initiation of protein synthesis did not result in a change of amino acid sequence and permitted fusion of the cDNA into the pTM3 *Nco*I site. In the final constructions, the HA and the M_2 cDNA are under control of the T7 promoter.

To express the HA and the M_2 proteins by using the vac-T7 system, 3.5-cm dishes of subconfluent monolayers of HeLa T4 cells were infected with vaccinia virus vTF7.3 for 30 min and transfected with pTM3 recombinant plasmids with calcium phosphate as described previously (12). Cultures were incubate for 5 h at 37°C in a 5% CO_2 atmosphere.

Endo H digestion. CV-1 cells were infected with SV40-HA recombinant virus. At 48 h postinfection (p.i.) cultures were incubated in DMEM deficient in cysteine and methionine (DMEM Met^-/Cys^-) for 30 min, labeled for 10 min with Tran[^{35}S] label (100 μCi/ml) in DMEM Met^-/Cys^-, and incubated in chase medium (DMEM, 2 mM methionine, 2 mM cysteine) for various times. HA protein was immunoprecipitated with a rabbit antibody prepared against disrupted FPV (kindly provided by Hans-Dieter Klenk) and protein A-Sepharose beads, and the recovered HA protein was digested with 1 mU of endo H (endo-β-*N*-acetylglucosaminidase H) for 18 h as described previously (45).

Proteinase digestion of immature and low-pH form of HA. To radiolabel proteins, cells infected with SV40 recombinant virus or infected and transfected by using the vac-T7 expression system at 48 or 5.5 h p.i., respectively, were washed twice with DMEM Met^-/Cys^-. Then, the medium was replaced with pH-labeling medium (methionine- and cysteine-deficient minimal essential media [MEM] containing a low concentration of $NaHCO_3$ [0.35 g/liter] and 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]) with the pH adjusted to a stated pH, ranging from pH 6.5 to 8.5, and the cultures were incubated for 30 min with or without CO_2, depending on the pH. The cells were labeled with Tran[^{35}S] label (100 μCi/ml) in pH-labeling medium for 10 min. Then, the medium was replaced with pH-chase medium (DMEM containing a low concentration of $NaHCO_3$ [0.35 g/liter] and 25 mM HEPES) and adjusted to a stated pH, ranging from pH 6.5 to 8.5, and the cells were incubated at 37°C for 60 min with or without CO_2, depending on the pH. To minimize pH fluctuations during the starvation, labeling, and chase periods, cells were incubated in a 5% CO_2 atmosphere for pHs below

7.0 and without CO_2 for pHs above 7.5. After chase periods, cells were washed with phosphate-buffered saline (PBS) and lysed in 500 μl of 50 mM Tris-HCl (pH 7.4)–0.1% sodium dodecyl sulfate (SDS)–1% Nonidet P-40 (34), and lysates were clarified by centrifugation (14,000 × *g*, 10 min) and incubated with (+) or without (−) 50 μg of proteinase K or TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin per ml for 60 min at 30°C. Proteinases were inactivated by the addition of an equal volume of 2× radioimmunoprecipitation buffer containing 200 μg of soybean trypsin inhibitor per ml, 1% aprotinin, and 1 mM PMSF (phenylmethylsulfonyl fluoride). Lysates were immunoprecipitated with an anti-FPV polyclonal antiserum, and the polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Dithiothreitol dissociation of HA_1 from the low-pH form of HA. HeLa T4 cells were infected with the vaccinia recombinant virus vTF7.3 and transfected with pTM3 recombinant plasmids, and at 5 h posttransfection the cultures were incubated with pH-labeling medium (pH 6.9) for 30 min. Cells were metabolically labeled with Tran[^{35}S] label (100 μCi/ml) in pH-labeling medium (pH 6.9) for 2 h, and then the cells were washed twice with PBS and incubated in PBS containing 20 mM dithiothreitol for 10 min at room temperature. Supernatants were collected and clarified by centrifugation (14,000 × *g*, 4°C, 10 min), mixed with an equal volume of 2× radioimmunoprecipitation buffer, and further clarified by centrifugation (100,000 × *g*, 4°C, 10 min). HA was immunoprecipitated from lysates with an anti-FPV polyclonal antiserum, and polypeptides were analyzed by SDS-PAGE.

Immunoprecipitation and SDS-PAGE. Samples were analyzed by SDS-PAGE on 17.5% polyacrylamide gels plus 4 M urea or 10% polyacrylamide gels and processed for fluorography and autoradiography as described previously (30). Radioactivity was quantitated by using a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, Calif.).

Immunofluorescence. For indirect surface immunofluorescence, HeLa T4 cells were transfected with Lipofectin (Life Technologies, Gaithersburg, Md.) to avoid calcium phosphate precipitate interference. Indirect immunofluorescence was done as described previously (33).

RESULTS

Kinetics of intracellular transport and protease sensitivity of FPV Rostock HA expressed from cDNA. The HA cDNA clone of influenza virus A/chicken/Germany/34 (H7N1) (FPV Rostock) (26, 27, 47, 59) was expressed in CV-1 cells by using an SV40-HA recombinant virus. To confirm that HA was expressed and transported through the exocytotic pathway, the rate of transport of HA to the medial Golgi apparatus was monitored by analyzing the rate at which carbohydrate chains on HA acquired resistance to digestion by endo H, and concomitantly the time course of cleavage of HA was measured. SV40-HA-infected cells were metabolically pulse-labeled with Tran[^{35}S] label for 10 min at 48 h p.i. and incubated in chase medium for various periods, and the HA polypeptide species were analyzed by SDS-PAGE. As shown in Fig. 1A, the uncleaved HA_0 acquired resistance to endo H digestion with a $t_{1/2}$ of ~15 min, and cleavage of HA_0 to HA_1 and HA_2 occurred slightly more slowly ($t_{1/2}$, ~ 25 min), the latter kinetics being consistent with cleavage occurring in the TGN. In the CV-1 cells used, both HA_1 and HA_2 on endo H digestion migrated on SDS-PAGE as two species, suggesting that individual carbohydrate chains differ in the extent of their carbohydrate trimming and conversion to the complex form. This observation has also been made previously for FPV HA expressed in insect cells (26). Importantly, for the experiments to be described below, in many pulse-chase experiments it was observed that the amount of HA_1 recovered decreased with time, whereas the amount of HA_2 recovered was relatively constant (cf. the 40-min time point with the 60-, 90-, and 120-min time points in Fig. 1A). The FPV HA expressed at the cell surface was biologically active and caused fusion when cultures of SV40-HA-infected CV-1 cells were treated with low-pH media, and it was found that the pH of transition to cause syncytium formation was pH 5.85 (data not shown). However, the extent of cell-cell fusion caused by HA has not been quantitated, but in the accompanying paper by Ohuchi and coworkers (42) it can be seen that the fusion index determined from expression of HA alone is quite low. Thus, these data confirm that the FPV HA cDNA expresses a biologically active protein (59) and confirm the pH of transition (pH 5.9) of the FPV HA to a fusion competent form (16).

Addition of amantadine hydrochloride to FPV-infected cells, which blocks the M_2 protein-associated ion channel activity (46), causes HA to undergo its transition to the low-pH

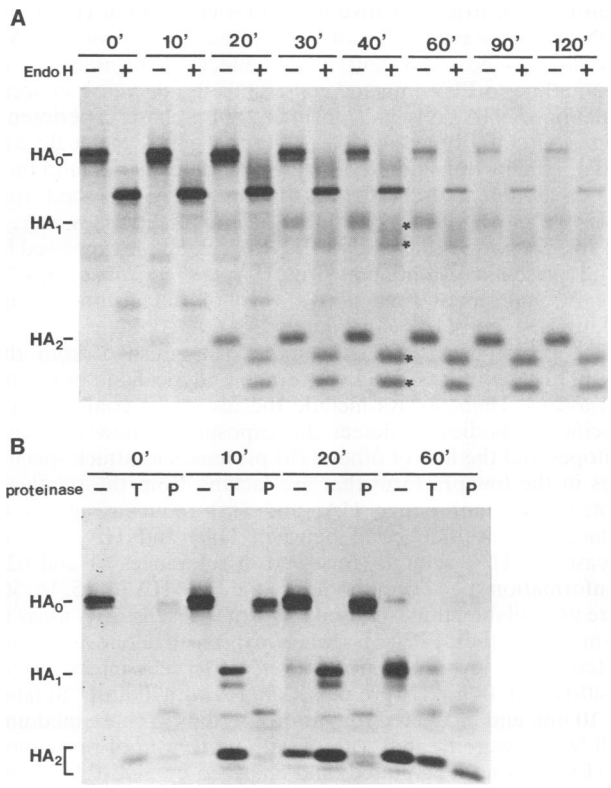

FIG. 1. Kinetics of intracellular transport of FPV Rostock HA expressed from cDNA. (A) Kinetics of resistance of FPV HA carbohydrate chains to endo H digestion. CV-1 cells were infected with SV40-HA recombinant virus and were labeled at 48 h p.i. with Tran[^{35}S] label for 10 min and incubated for various periods of time (in minutes as indicated above each pair of lanes) in chase medium. HA was immunoprecipitated with a rabbit antiserum against FPV virions, precipitates were digested with (+) or without (−) endo H, and polypeptides were analyzed by SDS-PAGE. *, endo H-resistant HA species. (B) Kinetics of resistance of HA to digestion by proteases. HA was metabolically labeled as described in panel A, and cell lysates were incubated with TPCK-trypsin (T) or proteinase K (P) and without either (−), as described in Materials and Methods, for various periods of time (in minutes as indicated above the lanes).

conformation from its native form after cleavage of HA in the TGN (56). The expression of HA without coexpression of M_2 protein may be equivalent to the effect of the addition of amantadine to FPV-infected cells. Thus the decrease in accumulation of HA_1 observed in many pulse-chase experiments (Fig. 1A) could be explained if either the HA molecule was undergoing its transition to the low-pH conformation and then was susceptible to protease degradation or HA_1 was shed from cells. We have not investigated either possibility. However, it has been reported that when FPV Rostock HA is expressed by using a vaccinia recombinant virus, HA_1 is shed from cells (47). The mechanism of reduction in vivo of the disulfide bond linking HA_1 and HA_2 is not known.

The native form of HA can be distinguished from the low-pH HA conformation by several biophysical and biochemical assays. These assays include the use of (i) conformation specific antibodies to detect the exposure of new antigenic epitopes and the loss of others, (ii) proteases to attack specific sites in the low-pH form that are lacking from the relatively protease-resistant native HA, and (iii) reducing agents to reduce the disulfide bond between HA_1 and HA_2 and to release the HA_1 subunit (reviewed in references 61 and 62). Conformation specific antibodies to the FPV HA (6, 15, 16, 56) were unavailable; thus, we used the protease sensitivity assay to examine whether FPV HA when expressed alone was converted to the low-pH conformation. CV-1 cells infected with SV40-HA at 48 h p.i. were pulse-labeled with Trans[^{35}S] label for 10 min and incubated for various periods in chase medium. Cell lysates were treated with or without trypsin or proteinase K, HA immunoprecipitated, and analyzed by SDS-PAGE. As shown in Fig. 1B, immediately after the pulse-label, HA_0 is susceptible to trypsin and proteinase K digestion, as expected for HA molecules that have yet to form a trimer (10). However, by 10 and 20 min after the pulse-label, HA_0 is cleaved by trypsin only at a single exquisitely sensitive site to form HA_1 and HA_2, and the majority of HA_0 is relatively unaffected by proteinase K digestion. However, after 60 min when HA has been transported beyond the TGN and cleavage of HA_0 to HA_1 and HA_2 has occurred, HA_1 is susceptible to trypsin and proteinase K digestion. The SDS-PAGE mobility of HA_2 of both native HA and low-pH HA becomes slightly faster on proteinase K digestion, suggesting that a terminal region of HA_2 is sensitive to protease digestion under the conditions used.

Reversal by ammonium chloride of protease sensitivity of FPV Rostock HA expressed from cDNA. It has been shown previously that NH_4Cl, an agent that can cause an elevation in the pH of intracellular vesicular compartments, can reverse the deleterious effect of amantadine on FPV Rostock HA in that production of low-pH HA is abolished (56). To address further the notion that the reduced accumulation of HA_1 observed in many pulse-chase experiments when HA is expressed alone in the absence of a functional M_2 protein is related to a low pH environment to which HA is exposed after cleavage in the TGN, CV-1 cells expressing HA were incubated with 2 mM NH_4Cl before, during, and after the pulse-label. As shown in Fig. 2, incubation with 2 mM NH_4Cl dramatically increased the amount of HA_1 that accumulated at 60 min and rendered the species resistant to proteinase K digestion (cf. control versus 2 mM NH_4Cl at 60 min, with and without proteinase K treatment). Thus, taken together the data shown in Fig. 1B and 2 suggest that when HA is expressed in CV-1 cells in the absence of a functional M_2 protein, ~85% of HA after cleavage is converted to the low-pH conformation.

Effect of extracellular pH on formation of low-pH HA conformation. In initial experiments in which the FPV HA was

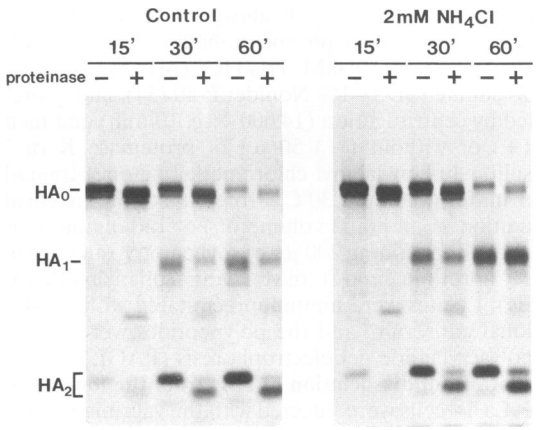

FIG. 2. Effect of NH_4Cl on sensitivity of FPV HA to convert to low-pH form. CV-1 cells were infected with SV40-HA recombinant virus, labeled at 48 h p.i. with Tran[^{35}S]-label for 10 min, and incubated for various periods (in minutes as indicated above the lanes) in chase medium. Where indicated, 2 mM NH_4Cl was added to the medium prior to metabolic labeling and maintained in subsequent medium changes. At the times indicated after metabolic labeling, cell lysates were digested with (+) or without (−) proteinase K, and HA was immunoprecipitated and analyzed by SDS-PAGE.

expressed, we observed that at 60 min after the pulse-label, HA_1 was not as proteinase K sensitive (data not shown) as that shown in Fig. 1B and Fig. 2 (control). In considering technical aspects of the protocol, it seemed quite likely that variation in the pH of the bicarbonate-buffered chase medium could affect the outcome of the experiment. To investigate the effect of extracellular pH on the proteinase K sensitivity of HA, CV-1 cells were infected with SV40-HA, at 48 h p.i. were incubated in a specially buffered pH-labeling medium (see Materials and Methods) at a stated pH (ranging from pH 6.5 to 8.5) for 30 min, labeled with Tran[^{35}S]-label for 10 min, and incubated in pH-chase medium (see Materials and Methods) for 60 min. Then, the HA polypeptides were immunoprecipitated and analyzed by SDS-PAGE. As shown in Fig. 3, changing the extracellular pH had a marked effect on the conversion of HA to the low-pH conformation as measured by susceptibility of HA_1 to digestion by proteinase K. As the medium became more alkaline (pH >7.5), the amount of HA found in the low-pH conformation was greatly reduced. Thus, the simplest explanation of these observations is that in tissue culture cells, the extracellular pH can influence intracellular pH.

Coexpression of FPV Rostock HA and M_2 protein to rescue the HA native form. The SV40-HA recombinant virus stock infected ~70% of the cells, and an SV40 recombinant virus stock that expresses the FPV Rostock M_2 cDNA also infected ~70% of the cells (data not shown). However, it seemed likely that the opportunity to observe changes in the amount of HA in the low-pH form would be enhanced if there was a high probability of coexpression of HA and M_2. Thus, the FPV Rostock HA cDNA and the FPV Rostock M_2 cDNA were subcloned into the pTM3 vector (41). Transient expression of HA and M_2 was obtained by using the vac-T7 system that involved infecting HeLa T4 cells with a recombinant vaccinia virus (vTF7.3) that expresses bacteriophage T7 RNA polymerase (12) and then cotransfecting these infected cells with pTM3-HA and pTM3-M_2 DNAs. At 5 h posttransfection, cells were labeled with Tran[^{35}S] label for 30 min and lysed, and HA and M_2 were immunoprecipitated and analyzed by SDS-PAGE (Fig. 4a). Indirect immunofluorescent double labeling of HA

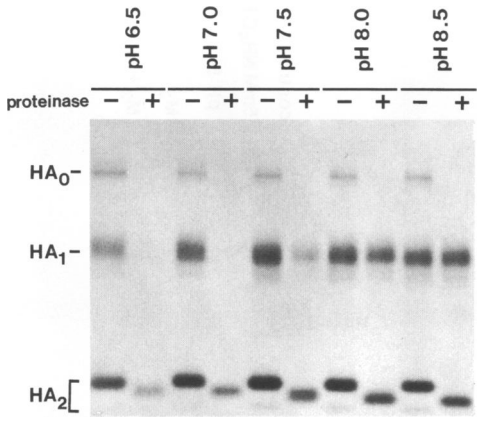

FIG. 3. Effect of varying extracellular pH on sensitivity of FPV HA to convert to low-pH form. CV-1 cells were infected with SV40-HA recombinant virus. At 48 h p.i. The cells were incubated for 30 min in pH-labeling medium with the pH adjusted to the stated pH, ranging from pH 6.5 to 8.5, and labeled with Tran[^{35}S] label for 10 min in pH-labeling medium. Then, cultures were incubated for 60 min in pH chase medium and adjusted to the stated pH, ranging from pH 6.5 to 8.5. Cell lysates were digested with (+) or without (−) proteinase K, and HA was immunoprecipitated and analyzed by SDS-PAGE.

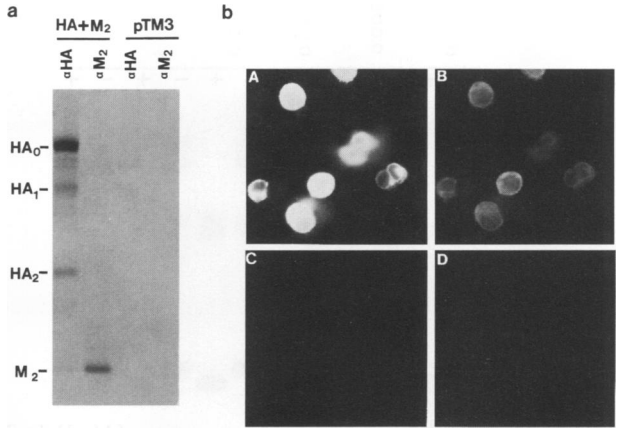

FIG. 4. Coexpression of HA and M_2 in HeLa T4 cells by using vac-T7 expression system. HA and M_2 cDNAs were cloned into the pTM3 expression vector such that HA and M_2 cDNAs are under the control of the bacteriophage T7 RNA polymerase promoter. HeLa T4 cells were infected with vaccinia recombinant virus vTF7.3, which expresses T7 RNA polymerase, and after 30 min were transfected with pTM3-HA and pTM3-M_2 DNAs or pTM3 vector alone. (a) At 5 h posttransfection, cells were labeled for 30 min with Tran[^{35}S] label and lysed in radioimmunoprecipitation buffer, and HA and M_2 were immunoprecipitated with rabbit antisera against FPV virions (αHA) or mouse monoclonal antibody (1F1) (21) against M_2 (αM_2), respectively. (b) Cell surface expression of HA and M_2. Cells were infected with vaccinia recombinant virus vTF7.3 and transfected with pTM3-HA and pTM3-M_2 (A and B) or with pTM3 (C and D). At 5 h posttransfection, cells were fixed with formaldehyde, and M_2 surface staining was done as described previously (33) by using an αM_2 primary antibody (1F1) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin as a secondary antibody (A and C). HA surface staining was done with rabbit αHA sera and Texas red-conjugated goat anti-rabbit immunoglobulin as a secondary antibody (B and D). All four panels show the same field of cells photographed with a fluorescence microscope equipped with optics to detect FITC and Texas red. The exposure time for all four panels was identical.

and M_2 at the surface of cells, when expressed by the vac-T7 system, indicated that >95% of the cells expressing one DNA also expressed the second DNA (Fig. 4b).

To examine the ability of M_2 to restore HA to its native form, vTF7.3-infected HeLa T4 cells were transfected with 5 μg of pTM3-HA and various amounts of pTM3-M_2 DNA (0 to 5 μg): the total amount of DNA transfected was adjusted to 10 μg by the addition of pTM3 DNA. At 5 h posttransfection, cells were labeled with Tran[^{35}S] label for 10 min and incubated in pH-chase medium (pH 6.9) for 60 min to allow for intracellular transport of HA, a treatment which caused the conversion of the majority of HA to the low-pH form (Fig. 3). Cells were lysed and treated with and without proteinase K, and HA and M_2 were immunoprecipitated and analyzed by SDS-PAGE. An increasing gradient of M_2 polypeptide expression (accumulation) that correlated in an approximately linear manner with the amount of pTM3-M_2 DNA transfected was observed (data not shown). Expression of HA by using the vac-T7 system and a 10-min labeling and 60-min chase protocol caused HA_0 to migrate as two species (Fig. 5 and 6) which differ in the extent of carbohydrate modification as shown by peptide *N*-glycanase digestion (data not shown). Both of these HA_0 species were cleaved to HA_1 and HA_2. Cotransfection of 0.04 to 0.2 μg of pTM3-M_2 DNA greatly increased the amount of HA_1 which was resistant to proteinase K digestion compared with HA expression in the absence of M_2 expression (Fig. 5, lane 0). When greater amounts (1 to 5 μg) of pTM3-M_2 DNA were transfected, large amounts of HA_0 accumulated. The conformational form of this HA_0 will be the subject of another study; but the sensitivity of HA_0 to proteinase K digestion (Fig. 5, lanes 1+ and 5+) and its continued sensitivity to endo H digestion (data not shown) suggest that high levels of M_2 expression alter normal folding or oligomerization and, in turn, affect intracellular transport from the endoplasmic reticulum. At the present time, it is not known whether this effect is due to the M_2 protein ion channel activity or is a consequence of overexpression of integral membrane proteins. Even in the absence of M_2 expression, after 60 to 90 min of chase ~5 to 10% of HA does not become cleaved and is sensitive to proteinase K digestion (Fig. 3 and 5), and as described previously, these HA molecules probably represent the ~5 to 10% of HA molecules that are malfolded and fail to be transported out of the endoplasmic reticulum (14; reviewed in reference 11). Because of the deleterious effect of transfecting large quantities of pTM3-M_2 DNA, in all experiments described below the amount of pTM3-M_2 DNA transfected was kept at or below 0.2 μg per 3.5-cm-diameter culture dish.

Amantadine prevents rescue of the native form of HA on coexpression of HA and M_2. To confirm that the rescue of the native form of HA by coexpression of M_2 was due to the ion channel activity of the M_2 protein, the M_2 channel blocker amantadine (46) was used. HeLa T4 cells expressing HA or HA and M_2 were treated with or without 10 μM amantadine for 2 h prior to labeling, and the drug was maintained in all subsequent steps. Cells were labeled with Tran[^{35}S] label for 10 min and incubated for 60 min in pH-chase medium (pH 6.9), and HA was immunoprecipitated and analyzed by SDS-PAGE. As shown in Fig. 6, when HA was expressed alone, amantadine had no effect on the level of HA expression or on its sensitivity to proteinase K digestion. However, when HA and M_2 were coexpressed, amantadine treatment reversed the effect of M_2 expression on stabilizing HA in its native form and caused most HA to adopt its low-pH conformation. Thus, these data indicate that the ion channel activity of the M_2 protein per se

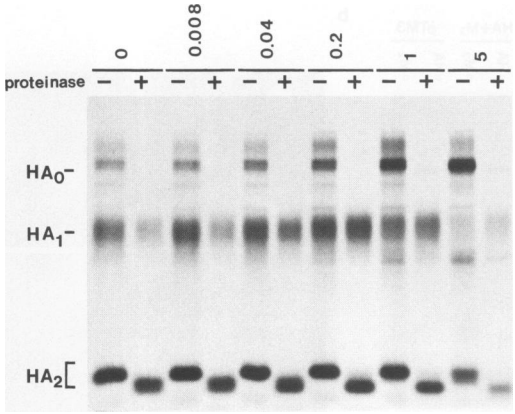

FIG. 5. Rescue of HA native form by coexpression of M_2. HeLa T4 cells (10^6 cells) were infected with vaccinia recombinant virus vTF7.3. After 30 min the cells were transfected with 5 μg of pTM3-HA and different amounts (0 to 5 μg as indicated above the lanes) of pTM3-M_2, and the total DNA was adjusted to 10 μg per dish with pTM3 DNA. After 5 h of incubation, the cells were incubated in pH-labeling medium (pH 6.9) for 30 min and then metabolically labeled with Tran[^{35}S] label for 10 min. Cultures were incubated in pH-chase medium (pH 6.9) for 60 min. Cell lysates were digested with (+) or without (–) proteinase K, and HA was immunoprecipitated and analyzed by SDS-PAGE.

prevents HA from undergoing its conformational change to the low-pH form.

Reductive dissociation of HA_1 as an assay for the low-pH conformation of HA. To confirm the data obtained by using proteinase K sensitivity as an assay for the low-pH conformational form of HA and the effect of M_2 coexpression in stabilizing the native form of HA, the reductive dissociation of HA_1 (17) was also used. Cells expressing HA or HA and M_2 were labeled with Tran[^{35}S] label for 2 h and incubated in PBS containing 20 mM dithiothreitol. Supernatants were collected, and HA was immunoprecipitated and analyzed by SDS-PAGE.

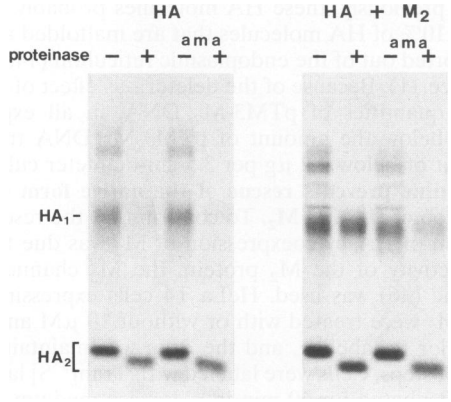

FIG. 6. Effect of amantadine on HA native form-stabilizing activity of M_2 protein. HeLa T4 cells were infected with vaccinia recombinant virus vTF7.3. At 30 min p.i. the cells were transfected with 5 μg of pTM3-HA or pTM3-HA and 0.2 μg pTM3-M_2, and the total DNA amount was adjusted to 10 μg per dish with pTM3 vector DNA. Cells were treated with (+) or without (–) 10 μM amantadine (ama) for 2 h prior to metabolic labeling and maintained in all subsequent medium changes. Cells were radioactively labeled and treated with proteinase K, and HA was analyzed as described in the legend to Fig. 5.

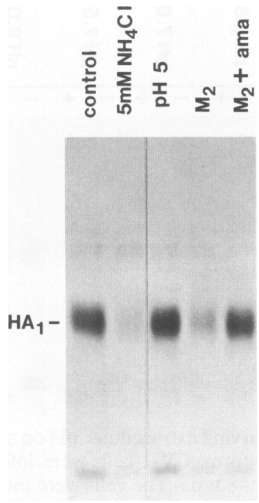

FIG. 7. Reductive dissociation of HA_1 on the surface of infected cells and stabilizing activity of M_2 protein. HeLa T4 cells were infected with 5 μg of pTM3-HA or both 5 μg of pTM3-HA and 0.2 μg of pTM3-M_2, and the total amount of DNA was adjusted to 10 μg per dish with pTM3 vector DNA. Cells were incubated for 4 h, and where indicated, the medium was adjusted to contain 5 mM NH_4Cl or 10 μM amantadine (ama). At 5 h posttransfection, cells were labeled for 2 h with Tran[^{35}S] label as described in the legend to Fig. 5. Either 5 mM NH_4Cl or 10 μM amantadine was maintained as appropriate during the labeling period. Cells were then incubated for 3 min at 37°C with either PBS containing either 10 mM MES (morpholineethanesulfonic 10 mM HEPES (pH 5.0 acid)–to induce the low-pH HA conformation or with 10 mM MES–10 mM HEPES (pH 7.0) for all other cultures. Cells were then incubated for 10 min at room temperature with PBS containing 20 mM dithiothreitol. Supernatants were collected and clarified by centrifugation (14,000 × *g*, 10 min), and HA was immunoprecipitated and analyzed by SDS-PAGE, as described in the legend to Fig. 5. Control, cells expressing HA and untreated; 5 mM NH_4Cl, cells expressing HA and incubated with 5 mM NH_4Cl before and during the labeling period; pH 5, cells expressing HA that were treated with pH 5-medium after the labeling period; M_2, cells cotransfected with HA and M_2 cDNAs; M_2 + ama, cells cotransfected with HA and M_2 cDNAs and incubated with 10 μM amantadine before, during, and after the labeling period.

As shown in Fig. 7, when HA was expressed alone (control) almost as much HA_1 could be released from the cell surface as when cell surfaces were treated with pH 5 medium to convert HA to the low-pH conformation (lane labeled pH 5). Incubation of cells expressing HA with 5 mM NH_4Cl greatly reduced the amount of HA_1 that could be released by reductive dissociation (lane labeled 5 mM NH_4Cl). Coexpression of M_2 with HA also greatly reduced the amount of HA_1 that could be released by reductive dissociation (lane labeled M_2), confirming that M_2 expression stabilized the native conformation of HA. Addition of the inhibitor of M_2 ion channel activity, amantadine (lane labeled M_2 + ama), to cells coexpressing HA and M_2 caused a considerable increase in amount of HA_1 released on reductive dissociation. Thus, the reductive dissociation assay confirmed all the data described above by using proteinase K sensitivity as an assay for the low-pH form of HA.

Comparison of expression of different M_2 ion channels on stabilizing the HA native form. From analysis of the amount of low-pH HA expressed in influenza FPV-infected cells, in cells infected with FPV gene reassortants, and in coinfection experiments, it has been suggested that the FPV Rostock M_2 ion channel has a level of activity (in terms of the level to which it

can raise the intracellular pH of the TGN) higher than that of the M_2 ion channel of the closely related influenza FPV Weybridge (A/chicken/Germany/27) (H7N7) (16). The experimental system described here for stabilizing HA in its native form on coexpression of FPV M_2 made it feasible to test other M_2 ion channels by using this system. Firstly, the cDNA to the M_2 ion channel of influenza A/Udorn/72 virus (46, 64) was tested for its ability to stabilize HA in its native form. Secondly, a cDNA encoding the Udorn M_2 protein that contains a four-residue deletion in the transmembrane pore region of the ion channel was tested. This deletion occurs naturally in an amantadine-resistant mutant of FPV Rostock (19). When this deletion was incorporated into the Udorn M_2 protein (M_2 del_{28-31}), it yielded channel activity upon expression in oocytes of *X. laevis* that was voltage activated as opposed to the activity of the wild-type M_2 ion channel, which is pH activated. To test for possible differences in the ability in these M_2 ion channel activities to stabilize HA in its native form, a constant amount of pTM3-HA DNA was transfected together with various amounts of the pTM3 FPV M_2, pTM3 Udorn M_2, and pTM3 del_{28-31} DNAs. Radioactively labeled cell lysates were treated with and without proteinase K, and HA species and M_2 were immunoprecipitated and analyzed by SDS-PAGE. Radioactivity was quantitated by using a phosphorimager, and the percentage of native (proteinase K-resistant) HA compared with the total amount of untreated HA species was calculated for each concentration of M_2 DNA transfected. In addition, the amount of M_2 protein that accumulated as a consequence of transfection with each pTM3M_2 DNA concentration was determined. As shown in Fig. 8, approximately five-fold more Udorn M_2 protein expression and approximately 25-fold more M_2 del_{28-31} protein expression were needed to obtain 50% of HA in its native form than were required for the FPV Rostock M_2 protein. Thus, this indirect assay supports the notion that different M_2 proteins have various levels of ion channel activity.

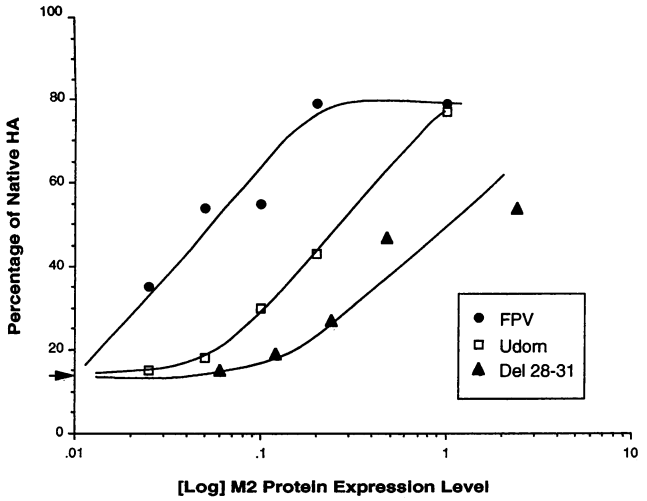

FIG. 8. Comparison of the effect of different M_2 ion channels on their ability to stabilize HA in its native form. HeLa T4 cells were infected with vaccinia recombinant virus vTF7.3 and then transfected with 5 μg pTM3-HA and different amounts (0 to 5 μg) of pTM3-M_2 FPV Rostock, pTM3-M_2 Udorn, or pTM3-M_2 del_{28-31}. The total amount of DNA transfected was adjusted to be the same (10 μg) by the addition of pTM3 DNA. At 5 h posttransfection, cells were labeled with Tran[^{35}S] label for 10 min and incubated for 60 min in pH-chase medium (pH 6.9) to allow for intracellular transport, lysates were made, and proteinase K digestions were performed as described in the legend to Fig. 5. Polypeptides were analyzed by SDS-PAGE, and the radioactive bands of proteinase K-resistant HA species, untreated HA species, and M_2 were quantitated by using a phosphorimager (Molecular Dynamics, Inc.). The percentage of native (proteinase K-resistant) HA_1 species compared with total amount of untreated HA species was calculated for each concentration of M_2 DNA used. The relative amounts (arbitrary units) of M_2 polypeptide that accumulated from the different DNA concentrations transfected are indicated. The amount of M_2 protein accumulated from transfection of 1 μg of pTM3-M_2 FPV is designated as 1. The arrowhead indicates the percentage of native HA in the absence of added M_2 DNA.

DISCUSSION

The role of the influenza virus M_2 protein in regulating intracompartmental pH during transport of HA through the TGN had been studied previously in influenza virus-infected cells (6, 16, 56). In the absence of functional M_2 protein ion channel activity (due to amantadine block), it has been estimated that HA is exposed to pH 5.2 in chick embryo fibroblasts and pH 5.6 in MDCK cells. These pH values are below those estimated for Golgi compartments of uninfected cells (1, 2, 7, 50). Thus, influenza virus infection of cells may cause a lowering of intracellular pH.

In setting up a system to reconstruct the effect of M_2 protein expression on HA maturation, it was necessary to use an HA that is cleaved intracellularly, as cleavage is prerequisite to the low-pH conformational change which leads to fusion activity (reviewed in reference 62), even though uncleaved HA can undergo limited acid-induced conformation changes when cells are treated at pH 5 (4). Normally, in an influenza virus infection, structural changes in uncleaved HA do occur on exit from the Golgi complex (8), but these changes do not lead to production of low-pH HA. A second criterion for establishing the experimental test system was that the HA would have a high pH of transition to the low-pH form. In the absence of an influenza virus infection to lower pH, the ability of HA to undergo its conformational change would dependent on the pH of the TGN, if the assumption is made that the vector system to be used has no effect on the TGN lumenal pH. Thus, the FPV Rostock HA was chosen for study because it is cleaved intracellularly and has a high pH of transition (pH 5.9) and its conformational forms have been studied extensively in influenza virus-infected cells treated with amantadine (16, 56). As it has been suggested that FPV Rostock M_2 protein may have a higher level of activity than those of other M_2 proteins (16), a cDNA encoding the FPV Rostock M_2 protein was constructed so that expression of the homotypic HA and M_2 could be analyzed.

The FPV Rostock HA expressed from cDNA by using either the SV40-HA recombinant virus or the vac-T7 expression system (data not shown) was transported through the exocytotic pathway with kinetics similar to that shown previously (47, 59). Sufficient HA was expressed at the cell surface in a form that on pH 5 treatment of cell surfaces, could cause syncytium formation. However, on cleavage of HA to HA_1 and HA_2, much of HA_1 could not be recovered by immunoprecipitation. We have not investigated whether the loss of HA_1 is due to shedding, as has been reported previously for FPV Rostock HA (47), or is due to proteolytic degradation. Nonetheless, both the assays used for monitoring the low-pH conformation of HA, proteinase K digestion and reductive dissociation of HA_1, indicated that the majority of HA had undergone its transition to the low-pH form. Treatment of cells expressing HA with NH_4Cl, which raises the pH of intracellular compo-

nents, caused an increased amount of HA_1 to accumulate, suggesting that the loss of HA_1 is a pH-related occurrence.

To establish a reproducible system to test directly the effect of M_2 protein coexpression on HA, the extracellular pH was rigorously controlled. For all experiments in which HA and M_2 were coexpressed, the extracellular pH was held at 6.9, as this treatment caused the majority of HA to be converted to the low-pH form. It has been established previously that extracellular pH changes can alter the pH in the TGN (6). Thus, the effect of manipulating the external pH of the cell is probably analogous to lowering the pH of the TGN by influenza virus infection. Coexpression of limited amounts of M_2 protein with HA stabilized the native form of HA, and this effect was abolished by the addition of the M_2 ion channel activity blocker amantadine. Thus, these data support the conclusion that the M_2 ion channel activity regulates the pH of intracellular compartments during influenza virus infections. Expression of larger quantities of M_2 protein in cells caused an HA_0 species that was sensitive to proteinase K digestion to accumulate, suggesting that the molecule either had not folded correctly or had failed to oligomerize. Although we cannot distinguish at the present time whether this observation is due to the M_2 ion channel activity or is a consequence of integral membrane protein overexpression, it has been shown previously that reduced extracellular pH (pH 5.8) inhibits HA trimerization (39). HA oligomerization occurs in a pre-Golgi compartment (9), and thus it seems possible that when M_2 protein is expressed at high levels there is a sufficient population of molecules in transport through the exocytotic pathway to alter the intracellular pH of pre-Golgi compartments: this remains to be rigorously tested. However, the deleterious effect of overexpression of the M_2 protein shown here may, in part, explain the tight control of splicing of the precursor M_1 mRNA to form the M_2 mRNA which is observed in influenza virus-infected cells (22, 32, 58).

Considerable evidence that suggests the Rostock M_2 ion channel can raise the intralumenal pH of the TGN to a pH higher than that achieved by the Weybridge M_2 ion channel (15) has been obtained and implies that the Rostock M_2 ion channel has a higher level of activity than the Weybridge M_2 ion channel. It would be ideal in discussing specific activity measurements of the M_2 ion channel to calculate the flux of an individual ion channel, and to measure the fraction of time each individual functional M_2 ion channel complex spends in the open state, by electrophysiological techniques; these studies are in progress. In the interim, the assay system established here for M_2 function in stabilizing HA permitted testing of other M_2 ion channels, and by using various M_2 expression levels it can be deduced that approximately 5-fold-more Udorn M_2 and 25-fold-more M_2 del_{28-31} than Rostock M_2 protein are required to obtain the same amount of HA in its native form. Thus, this assay may be amenable for testing other viral integral membrane proteins for putative pH-altering ion channel activity.

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