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Rescue of Vector-Expressed Fowl Plague Virus Hemagglutinin in Biologically Active Form by Acidotropic Agents and Coexpressed M_2 Protein

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The hemagglutinin of the Rostock strain of fowl plague virus was expressed in CV-1 cells by a simian virus 40 vector, and its stability in the exocytotic transport process was examined by a fusion assay. A 50-fold increase in the fusion activity of the hemagglutinin was observed when expression occurred in the presence of ammonium chloride, Tris-HCl, or high doses of amantadine. When chloroquine, another acidotropic agent, was used, the hemagglutinin exposed at the cell surface had to be activated by trypsin, because intracellular cleavage was inhibited by this compound. Hemagglutinin mutants resistant to intracellular cleavage did not require acidotropic agents for full expression of fusion activity, when treated with trypsin after arrival at the cell surface. These results indicate that fowl plague virus hemagglutinin expressed by a simian virus 40 vector is denatured in the acidic milieu of the exocytotic pathway and that cleavage is a major factor responsible for the pH instability. Coexpression with the M_2 protein also markedly enhanced the fusion activity of the hemagglutinin, and this effect was inhibited by low doses of amantadine. These results support the concept that M_2, known to have ion channel function, protects the hemagglutinin from denaturation by raising the pH in the exocytotic transport system. The data also stress the importance of acidotropic agents or coexpressed M_2 for the structural and functional integrity of vector-expressed hemagglutinin.

Influenza virus enters cells by a mechanism of membrane fusion that is mediated by the hemagglutinin (HA). The fusogenic activity depends on posttranslational proteolytic cleavage (14, 17) and on a conformational change (27) of HA. The observation that this change requires low pH (9, 18) has led to the concept that fusion takes place in endocytotic vesicles (19, 34). Once the acid-induced conformational change has occurred, it renders HA in an irreversibly denatured form (5, 27). These observations were difficult to reconcile with the fact that virions released from host cells contain native HA, although on its passage through the Golgi apparatus and the *trans*-Golgi network (TGN) the newly synthesized glycoprotein is exposed to an acidic environment, too. The problem was particularly pertinent with fowl plague virus (FPV) and other strains in which cleavage already occurs during exocytosis, since it had been reported that cleaved HA was more susceptible to the low-pH change than uncleaved HA (4).

The available evidence indicates that the factor that prevents HA from being irreversibly denatured during exocytosis is the M_2 protein. M_2 is an integral membrane protein of influenza A virus (16, 35) that has the function of an ion channel (23, 30). Our knowledge on the role of M_2 in virus replication stems mainly from studies involving amantadine, an inhibitor of the channel function, and amantadine-resistant virus mutants. From the pioneering study by Kato and Eggers on the neutral red sensitivity of input virus, it has been known for a long time that amantadine interferes with virus uncoating (11). As has been shown with several influenza A strains this is accomplished by two different mechanisms. At high concentrations ($>$0.1 mM), amantadine neutralizes the acidic environment in endocytotic vesicles, thereby preventing the conformational change of HA required for fusion. At low concentrations ($<$10 μM), amantadine is believed to block the M_2 channels of virions present in endocytotic vesicles and thus to prevent the low-pH-dependent disassembly of nucleocapsid and M_1 protein (20, 36). Studies of FPV have demonstrated that amantadine interferes also with the late stages of virus replication and that HA produced under these conditions is present in the acid-denatured form. These observations led to the concept that M_2 channels present in Golgi and TGN membranes of infected cells raise the pH in these organelles and thereby stabilize HA on the exocytotic transport route (7, 29).

Since the concept of HA stabilization by M_2 has been derived from studies in which replicating virus was analyzed, it appeared desirable to evaluate it under conditions in which the other viral gene products are not expressed. It was also of interest to see whether expression of M_2 and its presumptive consequences on intracellular pH affect the production of recombinant HA. To this end, we examined in the present study the structural integrity of FPV HA expressed from a simian virus 40 (SV40) vector by assaying its fusion activity. It was found that the fusion activity of the FPV HA that is cleaved in this system was dramatically stimulated by acidotropic agents or by coexpression with M_2. The effects were less distinct when uncleaved HA mutants were analyzed. The data support the concept that M_2 stabilizes the HA by modulating the intracisternal pH during exocytosis. They also illustrate that vector-mediated expression of only the HA may be inappropriate for functional studies on this glycoprotein. Similar conclusions concerning the stabilizing effect of coexpressed M_2 have been drawn in an accompanying paper in which the pH-dependent conformation of the FPV HA has been examined by analyzing its susceptibility to proteolytic degradation (31).

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MATERIALS AND METHODS

Expression of HA and M_2 genes in CV-1 cells by SV40 recombinants. Preparation of cDNA of the HA gene of influenza virus A/FPV/Rostock/34 (H7N1) (15), preparation of HA mutants with altered cleavage sites by site-directed mutagenesis by using the M13 system, and cloning of wild-type HA and HA mutants into the SV40 vector pA11SVL3 for expression in CV-1 cells (33) have been described previously. The M_2 gene, which is part of segment 7 of FPV, was obtained by PCR. First cDNA was synthesized with spliced M_2 mRNA isolated from FPV-infected MDCK I cells and a primer specific for the 3′ end of the mRNA (sequence 5′-GATCG GATTCAAGATGAGTCTTCTA-3′). After a second primer (sequence 5′-GATCAGATCTTTACTCCAGCTCTAT-3′) was added, the cDNA was amplified via PCR. Both primers comprised restriction sites for *Bam*HI and *Bgl*2, respectively, that allowed cloning into the baculovirus vector pVL 1393. The M_2 gene was then recloned into the vertebrate expression vector pA11SVL3.

CV-1 cells infected with the SV40 recombinants were incubated in Dulbecco's minimal essential medium (DMEM) at 37°C in a 5% CO_2 atmosphere. Two days after infection, CV-1 cells were labeled for 3 h with [^{35}S]methionine or with [^{35}S]methionine and [^{35}S]cysteine (each at a concentration of 10 μCi/ml) with DMEM lacking the respective cold amino acids. After cells were solubilized in radioimmunoprecipitation buffer, HA was precipitated with anti-FPV hyperimmune rabbit serum and protein A-conjugated Sepharose 4B (Pharmacia Co. Ltd.). M_2 was precipitated with rabbit antisera prepared against synthetic N- and C-terminal peptides that were kindly provided by A. Hay, London, United Kingdom. The precipitates were washed extensively with radioimmunoprecipitation buffer and analyzed on 10 or 12% polyacrylamide gels as described previously (22).

When ammonium chloride, amantadine (1-aminoadamantane hydrochloride), Tris-HCl (pH 7.5), and chloroquine [7-chloro-4-(4-diethyl-amino-1-methylbutylamino)quinoline] were used, they were added to the media at the concentrations indicated at 34 h postinfection (p.i.) and left on the cell cultures for the rest of the incubation period.

Fusion assay. The fusion assay was done at 50 to 55 h p.i. with the HA-SV40 recombinant. Monolayers of HA-expressing CV-1 cells in 5-cm petri dishes were treated with acidic fusion medium {RPMI 1640 medium (Flow Laboratories), 0.1% bovine serum albumin (BSA), 5 mM MES [2-(*N*-morpholino) ethanesulfonic acid; pH 5.0] (Sigma)} at 37°C for 3 min and then neutralized by incubation in fresh medium containing 5% fetal calf serum at 37°C for 5 to 14 h. The CV-1 cell cultures were fixed with ethanol and stained with 1:10-diluted Giemsa solution. Cells expressing HA were monitored by immunofluorescent staining. Giant cells which contained more than three nuclei were regarded as fused cells. The extent of fusion was calculated by determining the fusion index, which is the total number of nuclei in syncytia divided by the total number of HA-positive cells.

Immunofluorescent staining. CV-1 cells infected with the HA-SV40 recombinant were fixed with 2% paraformaldehyde at 4°C for 1 h and permeabilized by treatment with 0.1% Triton-X 100 at room temperature for 30 min. For cell surface staining, treatment with Triton-X was omitted. The cells were washed with phosphate-buffered saline containing 0.1% BSA and incubated with 1:200 diluted antivirus hyperimmune rabbit serum at 37°C for 1 h. As the second serum, biotinylated anti-rabbit donkey antibody (Amersham, United Kingdom) was used at a dilution of 1:200. After 1 h of incubation with the second serum, the cells were stained with 1:400 diluted streptavidin-fluorescein (Amersham) for 30 min at 37°C. The cell cultures for immunofluorescent staining were made on glass discs deposited in a 24-well culture plate. The diameter of each well was 1.5 cm. The well, accordingly, had 1/11th of the area of the 5.0-cm petri dish in which the fusion test was done. To assay the number of antigen-positive cells, the wells were therefore inoculated with 1/11th of the volume of the virus suspension used for the infection of cultures in the petri dishes. Antigen-positive cells were counted under a microscope after permeabilization of the cells.

RESULTS

Acidotropic agents enhance cell fusion activity of FPV HA expressed from an SV40 vector. Evidence that FPV HA expressed from an SV40 vector exerts fusion activity has already been obtained in previous studies. However, the syncytia observed in cell monolayers were few in number and small in size (24, 32). This is also demonstrated here in Fig. 1a, which shows a monolayer of CV-1 cells that has been exposed to brief pH 5 treatment 55 h after infection with the HA-SV40 recombinant virus. Only occasionally, three or four cells that underwent fusion could be seen. In contrast, when HA was expressed in the presence of 10 mM ammonium chloride, a large number of giant cells containing at least 20 nuclei could be detected (Fig. 1b). The extensive increase in fusion occurring under these conditions becomes even more apparent when the fusion index is calculated as the total number of nuclei present in syncytia relative to the total number of nuclei present in HA-expressing cells. As shown in Fig. 2, the specific fusion activity of the FPV HA increased by incubation not only with ammonium chloride, but also with 10 mM Tris-HCl (pH 7.5) or relatively high doses (67 μM) of amantadine. These results support the view that the fusion activity of FPV HA expressed from an SV40 vector is greatly diminished by denaturation in the acidic milieu of the exocytotic transport system and that acidotropic agents, such as ammonium chloride, Tris-HCl, and amantadine, abolish this detrimental effect, presumably by raising the pH in the Golgi apparatus and the TGN.

It was therefore surprising to see that chloroquine, another acidotropic agent, did not increase the fusion activity of the FPV HA, as also shown in Fig. 2. This observation was explained when HA expression was analyzed by polyacrylamide gel electrophoresis (Fig. 3). Whereas the FPV HA was cleaved in CV-1 cells in the presence of ammonium chloride, as was the case in the presence of Tris-HCl and amantadine (data not shown), cleavage was not observed when expression occurred in the presence of chloroquine. However, CV-1 cells expressing FPV HA in the presence of chloroquine showed extensive fusion when monolayers were exposed 50 h p.i. to controlled trypsin treatment (Fig. 2). These observations indicate, firstly, that chloroquine protects the HA from acid denaturation as do the other acidotropic agents; secondly, that it does not interfere with the surface transport of HA; and, thirdly, that it prevents proteolytic activation by a mechanism not yet understood.

Acidotropic agents enhance accumulation of vector-expressed FPV HA at the cell surface. The observation that the acidotropic agents stimulate cell fusion raised the question of whether these compounds have an effect on the amount of HA present at the cell surface. HA expressed by the SV40 vector was therefore examined by immunofluorescence in permeabilized and nonpermeabilized cells. As demonstrated in Fig. 4, ammonium chloride did not have a significant effect either on

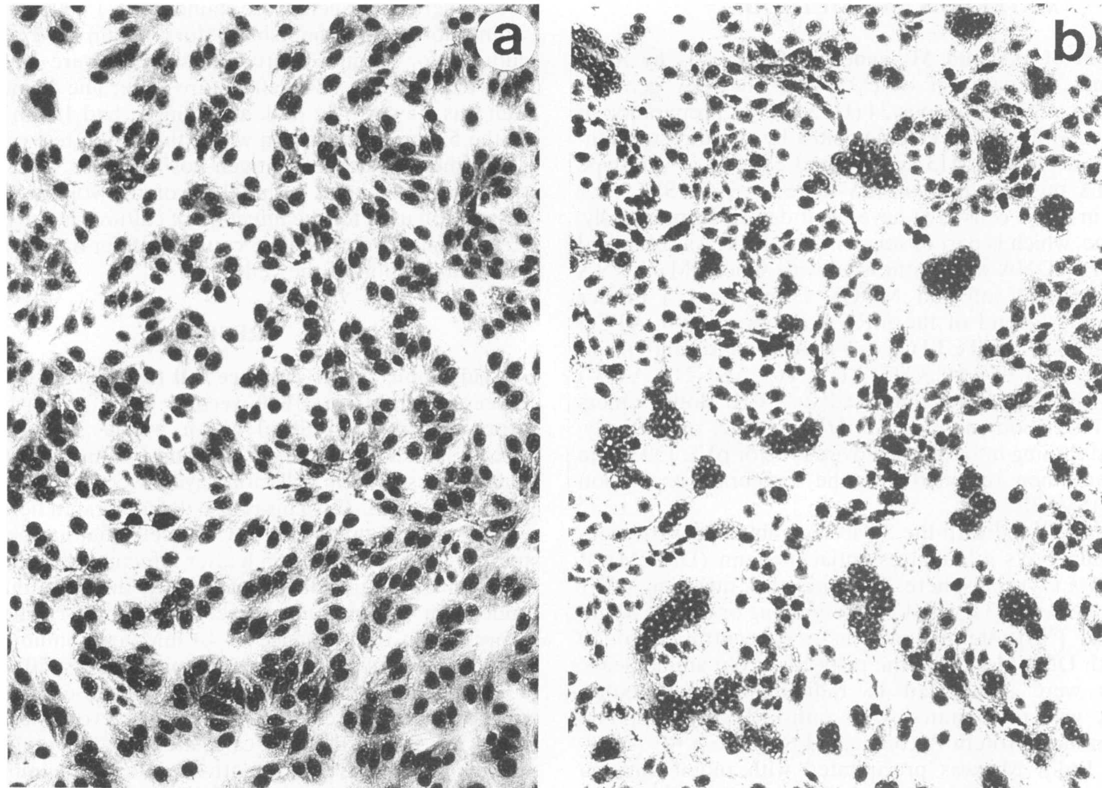

FIG. 1. Effect of ammonium chloride on cell fusion observed in CV-1 cells expressing A/FPV/Rostock/34 HA. Wild-type HA was expressed in CV-1 cells in the absence (a) and presence (b) of 10 mM ammonium chloride, and fusion was induced by acid treatment at 55 h after infection with recombinant virus. Cells were stained with Giemsa solution and inspected with a microscope.

the number of permeabilized cells expressing HA or on the amount of HA present in these cells. However, when nonpermeabilized cells were analyzed, it became clear that the addition of ammonium chloride to the medium resulted in an increase in the number of cells expressing HA at their surface. Similar observations have been made when HA was expressed in the presence of the other weak bases, including chloroquine (data not shown). Since the antiserum used for immunofluorescent staining did not discriminate between the low-pH and the neutral-pH forms of FPV HA, it can be concluded from these data that there is an increased accumulation of HA at the cell surface in the presence of the acidotropic agents.

It has been reported previously that the transport of influenza virus HA to the apical surface of polarized cells is retarded in the presence of ammonium chloride (21). This observation is not necessarily in conflict with the data presented here, since the H3 HA used in that study is relatively insensitive to acid denaturation and may therefore not depend on stabilization by acidotropic agents during exocytosis. In the case of FPV HA, however, a possible retarding effect of ammonium chloride on transport appears to be abrogated by the protective effect that allows the accumulation of structurally and functionally intact HA at the cell surface.

Effect of ammonium chloride on fusion depends on intracellular cleavage of FPV HA. As has been pointed out above, influenza virus HAs vary considerably in acid sensitivity, and the available evidence indicates that these differences depend on the subtype as well as on proteolytic cleavage of HA. It was therefore of interest to see whether the relatively high lability of the FPV HA and, hence, the distinct stabilizing effects of the acidotropic agents result from the obligatory cleavage by the ubiquitous intracellular protease furin, which is typical for this HA, or from another specific structural trait. To throw light on this problem, we have selected from a panel of HA mutants, in which the cleavage site was altered by site-directed mutagenesis (33), two variants that are no longer susceptible to furin but still can be activated by trypsin. The wild-type cleavage site REKR was changed in mutant Mu7 to RNRK and in mutant 23 to GKKR. Both mutants were expressed in CV-1 cells in uncleaved form, but the fractions present at the cell surface were cleaved after exposure to trypsin (10 μg/ml for 15 min) (Fig. 5). We then compared the fusion activity of the mutants with that of wild-type HA (Fig. 6). In the absence of stabilizing acidotropic agents, the fusion indices of both mutants were 20 times higher than that of the wild type. When expressed in the presence of ammonium chloride, fusion of mutant HA could still be increased by a factor of about 2, now reaching the fusion index of wild-type HA expressed under the same conditions (cf. Fig. 2). These results indicate that intracellular proteolytic cleavage is the major but is not the only factor responsible for the high sensitivity of the FPV HA to acid denaturation during exocytosis.

Coexpression with M_2 protein enhances fusion activity of FPV HA. Since the acidotropic agents used in this study appear to mimic the effect of M_2 on HA biosynthesis in FPV-infected cells, cell fusion was analyzed in cells in which HA was coexpressed with M_2. For this purpose, an SV40 recombinant that contained the M_2 gene of the Rostock strain of FPV was constructed. CV-1 cells were infected first with the HA recombinant and then 5 h later with the M_2 recombinant. At 50 h p.i.

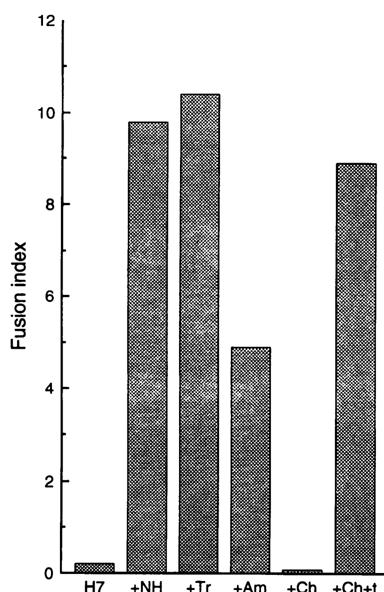

FIG. 2. Quantitative analysis of effects of ammonium chloride and other acidotropic agents on fusion activity of vector-expressed A/FPV/Rostock/34 HA. Wild-type HA was expressed in CV-1 cells in the absence of an acidotropic agent (H7) or in the presence of 10 mM ammonium chloride (+NH), 10 mM Tris-HCl (+Tr), 67 μM amantadine (+Am), or 80 μM chloroquine (+Ch). HA expressed at the cell surface in the presence of chloroquine was cleaved by incubation of the cultures with trypsin (10 μg/ml) for 15 min (+Ch+t). Fusion was induced as described in the legend to Fig. 1, and fusion indices were calculated as described in Materials and Methods.

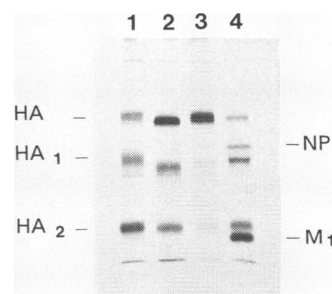

FIG. 3. Polypeptide profiles of A/FPV/Rostock/34 HA expressed in CV-1 cells in the absence and presence of acidotropic agents. Wild-type HA was labeled at 50 h after infection with recombinant virus for 3 h with [^{35}S]methionine and analyzed on a 10% polyacrylamide gel after immunoprecipitation. HA was expressed in the absence of an acidotropic agent (lane 1), in the presence of 10 mM ammonium chloride (lane 2), and in the presence of 80 μM chloroquine (lane 3). Lane 4, [^{35}S]methionine-labeled virus proteins were immunoprecipitated from infected CV-1 cells.

with the HA recombinant, cells were radiolabeled, and proteins were analyzed by immunoprecipitation followed by polyacrylamide gel electrophoresis. Figure 7 demonstrates that both proteins are expressed and that HA is cleaved under these conditions. The sequence of HA-SV40 infection followed by M_2-SV40 infection proved to be essential for optimal expression of both proteins. After simultaneous infection, the level of HA expression was significantly reduced, an observation supporting the view that overexpression of M_2 is toxic for cells and thus diminishes their biosynthetic potential (1).

To examine the effect of M_2 on fusion, cells were again sequentially infected with both SV40 recombinants. The fusion activity of FPV HA coexpressed with M_2 was 25 times higher than that of hemagglutinin expressed alone (Fig. 8). The observation that coexpression with M_2 did not enhance fusion activity quite as much as ammonium chloride is probably due to the fact that each protein was expressed by only about 70% of the cells present in a culture and that the proportion of coexpressing cells was therefore still lower. Figure 8 shows also that enhancement of fusion activity by coexpression with M_2 was abolished by amantadine. This indicates that stimulation of fusion is a specific effect of the ion channel activity of M_2. It has thus been clearly demonstrated that M_2 has an important helper function in the biosynthesis of functional FPV HA.

DISCUSSION

We have demonstrated in this study that FPV HA expressed from cDNA in vertebrate cells requires coexpression of M_2 for a dramatic increase in fusion activity. We saw similar effects when HA was expressed in the presence of acidotropic agents. These observations support the concept that the M_2 protein has the function of an ion channel (23), which, by raising the pH in the TGN, protects the acid-labile FPV HA from denaturation during exocytosis (7, 8, 29, 30). The examination of cell fusion revealed that the acidotropic agents and M_2 caused a 25- to 50-fold increase in the biological activity of HA. Because of its high sensitivity, this assay may therefore be a useful instrument for analyzing the ion channel function of M_2 as well as the pH sensitivity of HA.

When expressed without protection by M_2 or acidotropic agents from exposure to low pH, wild-type HA undergoing proteolytic cleavage in CV-1 cells had lost most of its fusion potential after arriving at the cell surface, whereas this was not the case with uncleaved HA mutants. This observation indicates that cleavage is an important factor in the pH sensitivity of FPV HA. The role of cleavage in HA stability has also been a matter of interest in several other studies. Thus, the antigenicity of the B epitope has been found to be modified by acid treatment in cleaved, but not in uncleaved, H3 HA (4). Ruigrok and coworkers (25) reported that, after low-pH treatment, cleaved H3 HA became susceptible to tryptic degradation, whereas uncleaved HA remained resistant. On the other hand, by using a panel of monoclonal antibodies recognizing different regions of HA, it could be shown that uncleaved H3 HA also underwent conformational changes at low pH, although in a manner somewhat different from that of the cleaved form (10). Finally, Boulay and coworkers (2) examined the pH stability of cleaved and uncleaved HA intensively and concluded on the basis of antigenic modifications and the acquisition of trypsin susceptibility that uncleaved H3 HA was also sensitive to low pH. These observations taken together indicate that cleavage is a major, but not the only, factor responsible for HA instability at low pH, and our observations of FPV HA support this view.

Acidotropic agents proved to be useful tools to protect vector-expressed FPV HA from low-pH-induced destabilization. These compounds include ammonium chloride, which has also been shown to interfere with amantadine-induced HA denaturation in FPV-infected cells (29), Tris-HCl at pH 7.5, amantadine at high concentrations, and chloroquine. Since FPV HA acquires low-pH sensitivity by furin-mediated cleavage in the trans-Golgi region (28) and since this is also the cell compartment in which conversion into the low-pH form has indeed been localized (3), it is obvious that the acidotropic

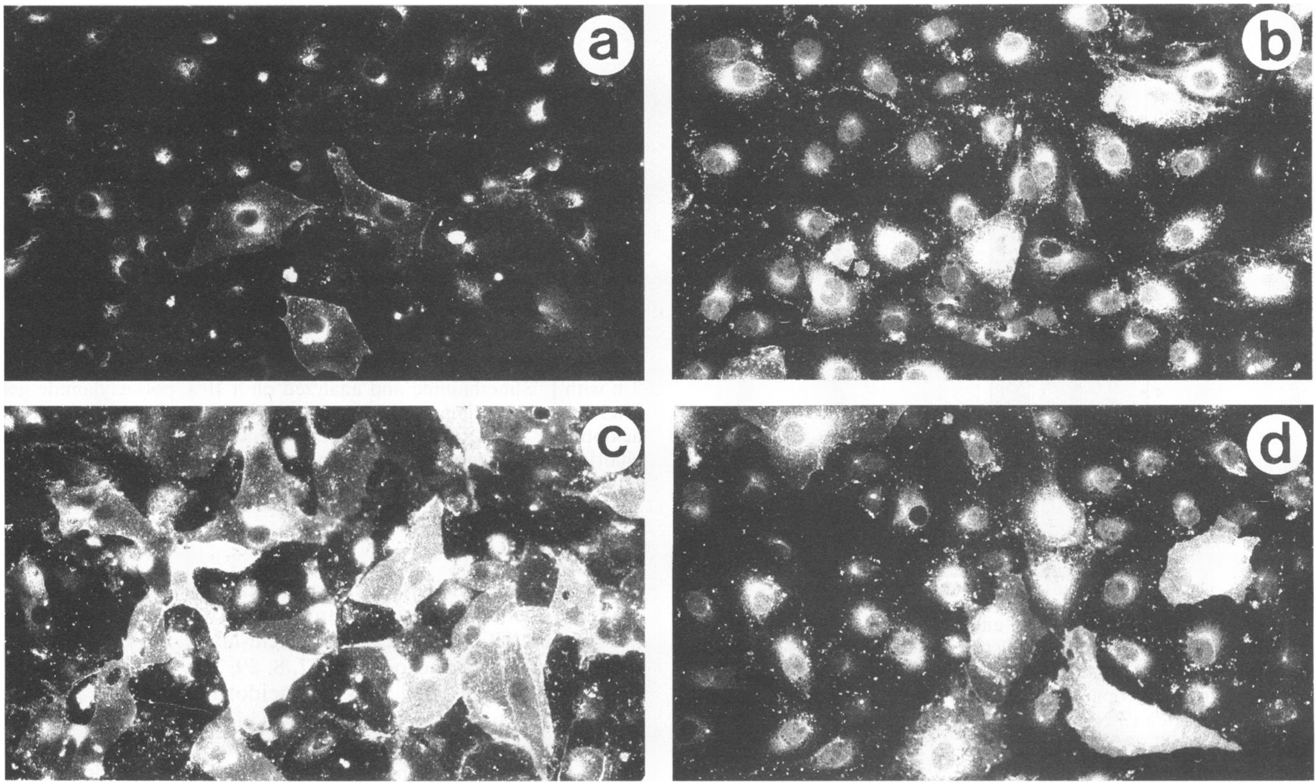

FIG. 4. Effect of ammonium chloride on cell surface accumulation of vector-expressed A/FPV/Rostock/34 HA. Wild-type HA was expressed in CV-1 cells in the absence (a and b) and presence (c and d) of 10 mM ammonium chloride. At 50 h p.i., immunofluorescence staining was done with nonpermeabilized (a and c) and permeabilized cells (b and d) as described in Materials and Methods.

agents protect HA by raising the pH in the acidic compartment. However, there are also other effects of these compounds on the processing machinery of the cell which are reflected by the structural variations of HA. When expressed in the presence of ammonium chloride, HA had a higher electrophoretic mobility than HA synthesized in the absence of this base (Fig. 3). Although this effect has not been further analyzed, it appears that ammonium chloride has some effect on HA glycosylation. Chloroquine, on the other hand, interfered with proteolytic cleavage. Since HA is transported under these conditions to the cell surface, its passage through the compartment in which cleavage actually should occur is most likely not prevented. It remains therefore to be seen whether chloroquine is an inhibitor of the activating protease furin or whether it interferes with HA cleavage by some other mechanism.

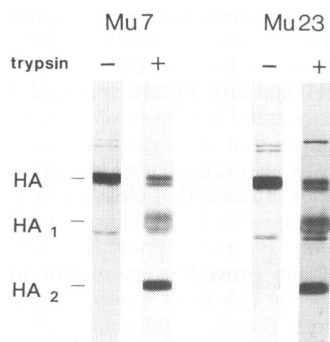

FIG. 5. Polypeptide profiles of cleavage mutants Mu7 and Mu23. HA mutants Mu7 and Mu23 with modified cleavage sites were expressed in CV-1 cells by using SV40 recombinants. At 50 h p.i., cells were labeled for 3 h with [^{35}S]methionine. They were then incubated for 15 min with trypsin (10 μg/ml) or mock treated. HA proteins were immunoprecipitated from cell lysates and analyzed by polyacrylamide gel electrophoresis.

As has been pointed out in the introduction, amantadine has been known for some time to have three different effects on replication of influenza A viruses: at high concentrations, in an M_2-independent fashion, it prevents the conversion of HA into the fusogenic form during endocytosis, thus interfering with virus entry at the stage of uncoating. At low concentrations, it prevents disassembly of infecting virus by blocking M_2 channels, thus again interfering with virus uncoating. On the other hand, amantadine at low concentrations also interferes with HA maturation by preventing M_2 channels from pH adjustment to neutrality in the exocytotic pathway, an effect that was also observed in the present study in the experiment for which the results are shown in Fig. 8. We describe here another mechanism of amantadine action that has not been reported previously: HA protection by raising the pH during exocytosis. Not mediated by M_2, this effect requires relatively high doses of amantadine. It has to be emphasized that the amantadine effects that interfere with virus entry as well as the effect on the M_2 function during exocytosis suppress virus replication. In contrast, by stabilizing newly synthesized HA the amantadine effect described for the first time here is expected to rather upregulate virus replication. Thus, there appears to be an antagonism between the M_2-mediated and the M_2-independent modes of amantadine action on the late steps in FPV

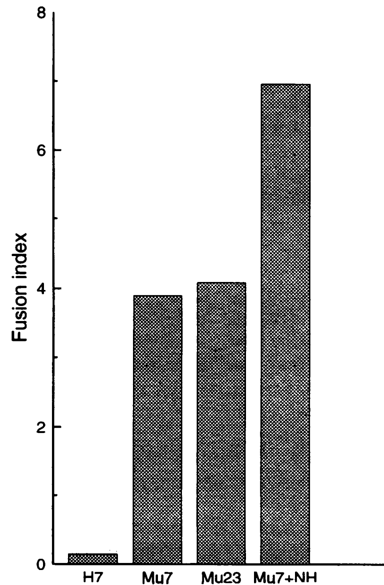

FIG. 6. Cell fusion activities of mutants Mu7 and Mu23 after trypsin treatment. HA mutants Mu7 and Mu23 and wild-type HA were expressed in CV-1 cells. At 55 h after infection with the recombinants, cells were exposed first to trypsin treatment (10 μg/ml) for 15 min and then to acidic medium to induce cell fusion. Fusion indices were determined as described in Materials and Methods. Wild-type HA (H7), mutant Mu7 (Mu7), mutant Mu23 (Mu23), and mutant Mu7 in the presence of 10 mM ammonium chloride (Mu7+NH) were expressed.

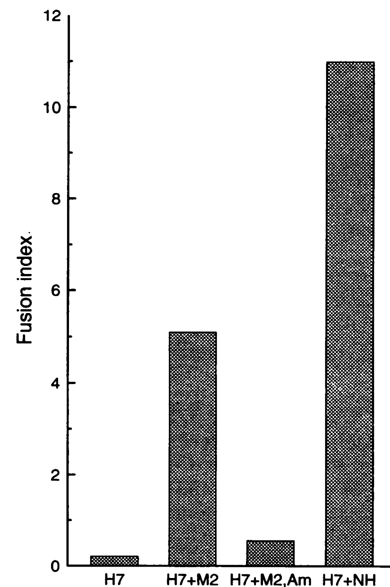

FIG. 8. Cell fusion activity of A/FPV/Rostock/34 HA after coexpression with M_2. Wild-type HA was expressed in CV-1 cells alone or with M_2 as described in the legend to Fig. 7. Incubation in the presence of ammonium chloride (10 mM) or amantadine (5 μM) was done as described in Materials and Methods. Fusion was induced as described in the legend to Fig. 1, and fusion indices were calculated for the fusion activity of HA expressed alone (H7), after coexpression with M_2 (H7+M_2), after coexpression with M_2 in the presence of amantadine (H7+M2, Am), and alone in the presence of ammonium chloride (H7+NH).

replication. This may explain why the inhibitory effect of amantadine on the late phase of FPV replication has escaped detection in some previous studies (11, 12, 26).

Evidence has been presented that the hemagglutinin of the Rostock strain of FPV that undergoes its conformational change at pH 5.9 requires a highly efficient M_2 to keep the intracisternal pH in the TGN above the denaturation threshold, whereas the Weybridge strain of FPV has an M_2 with lower levels of activity and HA undergoing conformational change at a lower pH (6). Thus, it appears that HA and M_2 of a given virus match each other. Examination of the effect of coexpressed M_2 on HA stability by the highly sensitive fusion assay presented here should provide a suitable system to analyze this problem in more detail. It will be of particular interest to find out how stringent the link is between the FPV HA and the homologous M_2. If the link would be stringent, it should preclude easy spread of the FPV HA into the other influenza A strains. This problem is of considerable biological interest because of the importance of the FPV HA as a determinant for high pathogenicity (13).

The experiments presented here underline the important role of the M_2 protein in the biosynthesis of FPV HA. Whether M_2 has a similar role in the biosynthesis of HA of other influenza A virus strains remains to be seen. It appears that cooperative effects between viral membrane proteins have not yet attracted the attention they deserve. This is particularly relevant when individual proteins are expressed from cDNA. Our data show that the information obtained from such studies may be incomplete if the cooperative partner is neglected.

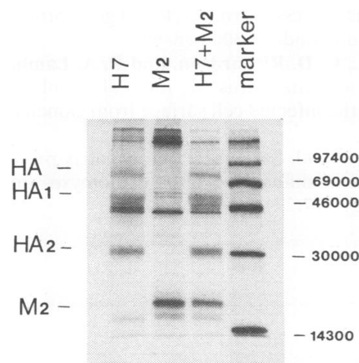

FIG. 7. Coexpression of HA and M_2 of influenza virus A/FPV/Rostock/34. CV-1 cells were infected with recombinants HA-SV40 (H7) and M_2-SV40 (M_2) individually or with both recombinants (H7+M_2). For coexpression, cells were infected first with the recombinant HA-SV40 and 5 h later with the recombinant M_2-SV40. At 50 h after the onset of the experiment, cells were labeled for 3 h with [^{35}S]methionine and [^{35}S]cysteine. HA and M_2 were immunoprecipitated with specific antisera and analyzed on a 12% polyacrylamide gel.

ACKNOWLEDGMENTS

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REFERENCES

1. **Black, R. A., P. A. Rota, N. Gorodkova, A. Cramer, H.-D. Klenk, and A. P. Kendal.** 1993. Production of the M_2 protein of influenza A virus in insect cells is enhanced in the presence of amantadine. J. Gen. Virol. **74:**1673–1677.
2. **Boulay, F., R. W. Doms, I. Wilson, and A. Helenius.** 1987. The influenza hemagglutinin precursor as an acid-sensitive probe of the biosynthetic pathway. EMBO J. **6:**2643–2650.
3. **Ciampor, F., P. M. Bayley, M. V. Nermut, E. M. A. Hirst, R. J. Sugrue, and A. J. Hay.** 1992. Evidence that the amantadine induced, M_2-mediated conversion of influenza A virus hemagglutinin to the low pH conformation occurs in an acidic trans Golgi compartment. Virology **188:**14–24.
4. **Daniels, R. S., A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1983. Analyses of the antigenicity of influenza haemagglutinin at the pH optimum for virus-mediated membrane fusion. J. Gen. Virol. **64:**1657–1662.
5. **Doms, R. W., A. Helenius, and J. White.** 1985. Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change. J. Biol. Chem. **260:**2973–2981.
6. **Grambas, S., M. S. Bennett, and A. J. Hay.** 1992. Influence of amantadine resistance mutations on the pH regulatory function of the M_2 protein of influenza A viruses. Virology **191:**541–549.
7. **Hay, A. J.** 1989. The mechanisms of action of amantadine and rimantadine against influenza viruses, p. 561–567. *In* A. L. Notkins and M. B. A. Oldstone (ed.), Concepts in viral pathogenesis, vol. III. Springer, N.Y.
8. **Hay, A. J., A. J. Wolstenholme, J. J. Skehel, and M. H. Smith.** 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. **4:**3021–3024.
9. **Huang, R. T. C., R. Rott, K. Wahn, H.-D. Klenk, and T. Kohama.** 1980. The function of the neuraminidase in membrane fusion induced by myxoviruses. Virology **107:**313–319.
10. **Jackson, D. C., and A. Nestorowicz.** 1985. Antigenic determinants of influenza virus hemagglutinin. XI. Conformational changes detected by monoclonal antibodies. Virology **145:**72–83.
11. **Kato, N., and H. J. Eggers.** 1969. Inhibition of uncoating of fowl plague virus by 1-adamantanamine hydrochloride. Virology **37:** 632–641.
12. **Kendal, A. P., and H.-D. Klenk.** 1991. Amantadine inhibits on early, M_2 protein-dependent event in the replication cycle of avian influenza (H7) viruses. Arch. Virol. **119:**265–273.
13. **Klenk, H.-D., and R. Rott.** 1988. The molecular biology of influenza virus pathogenicity. Adv. Virus Res. **34:**247–281.
14. **Klenk, H.-D., R. Rott, M. Orlich, and J. Blödorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426–439.
15. **Kuroda, K., C. Hauser, R. Rott, H.-D. Klenk, and W. Doerfler.** 1986. Expression of the influenza virus haemagglutinin in insect cells by a baculovirus vector. EMBO J. **5:**1359–1365.
16. **Lamb, R. A., S. L. Zebedee, and C. D. Richardson.** 1985. Influenza virus M_2 protein is an integral membrane protein expressed on the infected cell surface. Cell **40:**627–633.
17. **Lazarowitz, S. G., and P. W. Choppin.** 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology **68:**440–454.
18. **Maeda, T., and S. Ohnishi.** 1980. Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. FEBS Lett. **122:**283–287.
19. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107–151.
20. **Martin, K., and A. Helenius.** 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell **67:**117–130.
21. **Matlin, K. S.** 1986. Ammonium chloride slows transport of the influenza virus hemagglutinin but does cause missorting in a polarized epithelial cell line. J. Biol. Chem. **261:**15172–15178.
22. **Ohuchi, R., M. Ohuchi, W. Garten, and H.-D. Klenk.** 1991. Human influenza virus hemagglutinin with high sensitivity of proteolytic activation. J. Virol. **65:**3530–3537.
23. **Pinto, L. H., L. J. Holsinger, and R. A. Lamb.** 1992. Influenza virus M_2 protein has ion channel activity. Cell **69:**517–528.
24. **Roberts, P. C., W. Garten, and H.-D. Klenk.** 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. J. Virol. **67:**3048–3060.
25. **Ruigrok, R. W. H., A. F. M. Cremers, W. E. P. Beyer, and F. M. de Ronde-Verloop.** 1984. Changes in the morphology of influenza particles induced at low pH. Arch. Virol. **82:**181–194.
26. **Scholtissek, C., and G. P. Faulkner.** 1979. Amantadine-resistant and -sensitive influenza A strains and recombinants. J. Gen. Virol. **44:**807–815.
27. **Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, L. A. Wilson, and D. C. Wiley.** 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. USA **79:**968–972.
28. **Stienecke-Gröber, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H.-D. Klenk, and W. Garten.** 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J. **11:**2407–2414.
29. **Sugrue, R. J., G. Bahdur, M. C. Zambon, M. H. Smith, A. R. Douglas, and A. J. Hay.** 1990. Specific structural alteration of the influenza hemagglutinin by amantadine. EMBO J. **9:**3469–3476.
30. **Sugrue, R. J., and A. J. Hay.** 1991. Structural characteristics of the M_2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology **180:**617–624.
31. **Takeuchi, K., and R. A. Lamb.** 1994. Influenza virus M_2 protein ion channel activity stabilizes the native form of fowl plague virus hemagglutinin during intracellular transport. J. Virol. **68:**911–919.
32. **Veit, M., E. Kretzschmar, K. Kuroda, W. Garten, M. F. G. Schmidt, H.-D. Klenk, and R. Rott.** 1991. Site-specific mutagenesis identifies three cysteine residues in the cytoplasmic tail as acylation site of influenza virus hemagglutinin. J. Virol. **65:**2491–2500.
33. **Vey, M., M. Orlich, S. Adler, H.-D. Klenk, R. Rott, and W. Garten.** 1992. Hemagglutinin activation of pathogenic avian influenza virus serotype H7 requires the protease recognition motif R-X-K/R-R. Virology **188:**408–413.
34. **White, J., A. Helenius, and M. J. Gething.** 1982. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature (London) **300:**658–659.
35. **Zebedee, S. J., C. D. Richardson, and R. A. Lamb.** 1985. Characterization of influenza virus M_2 integral membrane protein and expression at the infected cell surface from cloned cDNA. J. Virol. **56:**502–511.
36. **Zhirnov, O. P.** 1990. Solubilization of matrix protein M_1/M from virions occurs at different pH for orthomyxo- and paramyxoviruses. Virology **177:**274–279.