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Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: Evidence for virus-specific regulation of the pH of glycoprotein transport vesicles

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ABSTRACT **Mutants of influenza Rostock virus (H7N1 subtype) were selected for resistance to amantadine hydrochloride at concentrations of the antiviral drug known to affect the function of the virus M2 transmembrane protein. Sequence analysis revealed that three mutants had no changes in M2 but contained a lysine to isoleucine substitution in the hemagglutinin (HA) membrane glycoprotein at position 58 of HA_2. The mutant viruses were found to fuse membranes at a pH value 0.7 lower than wild type and to exhibit changes in the conformation of their HAs specifically at the lower pH. The homologous lysine to isoleucine substitution was introduced by site-specific mutagenesis into the HA of X-31 influenza virus (H3 subtype), which was expressed by using vaccinia virus recombinants. The expressed HA also mediated membrane fusion and changed in conformation at a pH value 0.7 lower than wild type. These results indicate that increased acid stability of the HA obviates the consequences of the inhibition of M2 function by amantadine and provide further evidence for the role of M2 in regulating the pH of vesicles involved in glycoprotein transport to the cell surface.**

The antiviral drug amantadine inhibits influenza replication at a number of different stages (1). At millimolar concentrations, like other weak bases, incubation of cells with amantadine results in an increase in endosomal pH, which prevents the acid-dependent activation of the membrane fusion potential of influenza hemagglutinin (HA). As a consequence, fusion between virus and endosomal membranes is blocked and the initiation of infection is prevented. Influenza virus mutants resistant to this action of amantadine contain HAs that fuse membranes at higher pH than wild type and a number of these have been characterized in detail (2–4).

At micromolar concentrations, similar to those achieved in antiviral therapy, amantadine inhibits two different steps in the replication of certain strains of influenza A viruses. The first of these steps appears to involve a requirement for acidification of the interior of infecting virus particles (5–9); the second occurs at a later stage of replication at which a virus-specified modification of the membranes of vesicles involved in glycoprotein transport to the cell surface is proposed to result in an increase of vesicular pH. Influenza A virus mutants selected at these lower concentrations of amantadine contained amino acid substitutions in the transmembrane region of the M2 protein (10–12), a spliced product of the M gene, which is expressed in virus-infected cells and is present in low numbers in virus particles (13, 14). Analyses of these mutant viruses and their replication have provided evidence that the M2 membrane protein can function as an ion channel (1, 15) to allow protons into virus particles and to elevate the pH of vesicles through which glycoproteins are transferred to the surface membranes of infected cells.

The experiments reported in this paper are based on considerations of the latter activity. We have presented evidence previously that in the presence of amantadine the proposed ion-channel function of M2 is blocked and as a consequence influenza-infected cells express HAs at the cell surface and in the trans Golgi, exclusively in the conformation characteristic of that induced at the pH of membrane fusion (16, 17). It is therefore possible that the surface expression of native HAs could be achieved in infections by mutant influenza viruses containing HAs stable to the low pH of transport vesicles in infected cells treated with amantadine. The isolation and characterization of such mutants is reported here.

MATERIALS AND METHODS

Virus and Cells. Influenza A (H7N1 Rostock strain) was grown and amantadine-resistant variants were selected by plaque titration in the presence of 5 μM amantadine (10). HA and M genes were sequenced by the dideoxynucleotide chain-termination method and with reverse transcriptase as described (2, 10). Vaccinia viruses (Copenhagen strain) were propagated on CV1 cells grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum.

Mutagenesis and Construction of Recombinant Viruses. Site-specific mutagenesis was carried out as described (18). Wild-type and mutant HA genes were cloned into vaccinia virus expression vectors that direct transcription from the 7.5K promoter (19) or the cowpox p160 late promoter (20). HA-expressing recombinants were generated as described (21) and screened by Western blotting, and positive samples were plaque purified once more prior to experimental use.

Conformational Change and Membrane Fusion Assays. ELISAs on influenza virus-infected cells were done as described by Sugrue *et al.* (16) and ELISAs on vaccinia recombinant-infected cells were carried out according to the procedures used previously (22). Soluble membrane anchorless HA prepared by bromelain digestion (BHA) was purified from expressing cells as follows. Expressing cells were harvested 48 hr after infection with vaccinia recombinants expressing HA from the cowpox p160 late promoter. Membranes were prepared by flotation through discontinuous sucrose gradients of 55% and 35% and smooth and plasma membranes were harvested. Membranes were washed three times with phosphate-buffered saline, trypsin treated, and bromelain digested as described (23). Digests were layered over continuous sucrose gradients of 5–25%. After centrifugation, HA-containing fractions were concentrated, and trypsin susceptibility assays were carried out as described (24). BHK-21 cells were used for heterokaryon formation

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Abbreviations: HA, hemagglutinin; BHA, soluble bromelain-digested HA.

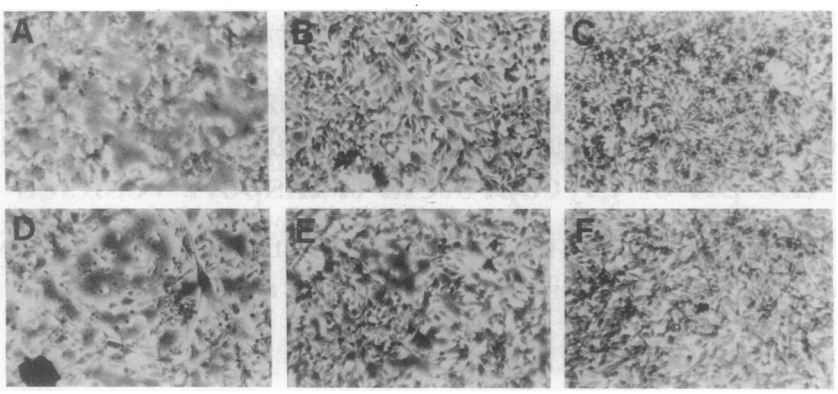

FIG. 1. Syncytia formation of BHK-21 cells infected with wild-type Rostock virus incubated at pH 5.8 (*A*), 5.9 (*B*), and 6.0 (*C*), or by mutant virus HA_2 K58I at pH 5.1 (*D*), 5.2 (*E*), and 5.3 (*F*).

both with influenza-infected and recombinant vaccinia-infected cells. HA-expressing vaccinia-infected cells had to be trypsin treated (5 μg/ml; 37°C; 5 min) to cleave HA_0 to HA_1 and HA_2. For both vaccinia- and influenza-infected cells, pH was dropped for 1 min and then neutralized. Medium with 5% fetal calf serum was added and after 10–15 min for influenza-infected cells or 1–2 hr for vaccinia-infected cells, monolayers were fixed with 0.25% glutaraldehyde and stained with 1% toluidine blue. Erythrocyte hemolysis assays were carried out as described by Daniels *et al.* (2).

RESULTS

Isolation of Amantadine-Resistant Mutants. Twenty-five amantadine-resistant isolates of Rostock influenza virus were selected by plaque titration of the amantadine-sensitive wild-type virus in the presence of 5 μM amantadine as described elsewhere (A.J.H. *et al.*, unpublished data). Three of the isolates were distinguished by nucleotide sequence analysis from the other 22 by the absence of any mutations in their genes for the M1 and M2 proteins and by the presence of the same single mutation in their genes for HA. This mutation resulted in the substitution of isoleucine for lysine at residue 58 of HA_2 (HA_2 K58I). These isolates grew more slowly than wild-type virus as reflected in smaller plaque size and were stable to six passages in chicken embryo fibroblast monolayers. The passaged viruses were indistinguishable from the initial isolates with respect to the mutation in HA, the pH at which they fused with membranes, and their amantadine resistance.

pH Dependence of Membrane Fusion by Wild-Type and Mutant Viruses. The pH dependence of virus-mediated membrane fusion was analyzed *in vitro* by an erythrocyte hemolysis assay (2) and by recording syncytia formation by virus-infected cells. The results of hemolysis assays indicated that wild-type Rostock virus mediated fusion at pH 6.1, while the mutant viruses fused with erythrocytes at pH 5.4. Similarly, BHK-21 cells infected with wild-type virus formed syncytia at pH 5.9 compared with mutant virus-infected cells that only formed syncytia at pH 5.2 (Fig. 1).

Previous studies (24–26) have indicated that at the pH of fusion, influenza HAs undergo irreversible changes in conformation that result in extrusion of the hydrophobic amino terminus of HA_2, the fusion peptide, from its buried location in the native structure (27). These changes can be specifically detected at the pH of fusion by analysis of increased susceptibility to proteolysis, of soluble HA aggregation as a result of fusion peptide-mediated intermolecular association, and of differences in reactivity with conformation-specific monoclonal antibodies. The transition of wild-type and mutant HAs from their native to low pH conformations was assayed in ELISAs by using one antibody, HC 58, that recognizes native HA and another, H9, that only recognizes HA in the low pH conformation. For wild-type HA the transition in structure occurred at pH 5.9; for the mutant HAs, it occurred at pH 5.3 (Fig. 2).

These observations indicate that the mutant viruses, presumably as a result of the single amino acid substitution HA_2 K58I in their HAs, are more acid stable than wild type and that their membrane fusion potential is activated only at a pH 0.7 below that required to activate the wild-type molecule.

Construction of a Mutant H3 Subtype HA Containing the HA_2 K58I Amino Acid Substitution. Lysine 58 of HA_2 (Fig. 3) is conserved in HAs of all 14 subtypes of influenza A except H11, which has arginine at this position (28, 29). To verify that the mutation HA_2 K58I was responsible for the acid-stable phenotype of the mutant viruses selected in the presence of amantadine, we introduced the homologous nucleotide change into the gene for HA of X-31 virus (H3 subtype) by site-directed mutagenesis. The membrane fusion properties of this HA are well characterized (2, 3, 24) and its crystal structure has been determined (27). The genes for mutant and wild-type X-31 HAs were cloned into two vaccinia virus

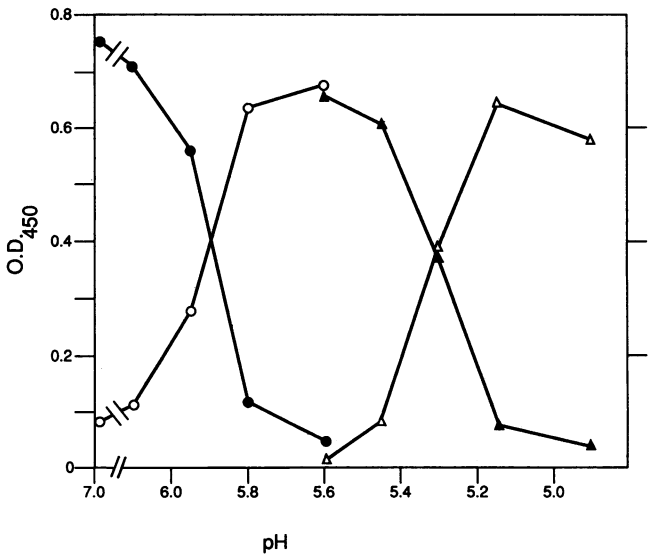

FIG. 2. pH of the conformational change of HAs of the mutant K58I and wild-type viruses. Five hours after infection, cells in duplicate microtiter plate wells were incubated at 37°C for 10 min with phosphate-buffered saline/citrate at different pH values, ranging from 4.9 to 7.0. Cells were fixed with 0.05% glutaraldehyde and HA was detected by ELISA using monoclonal antibodies specific for either native HA, HC58 (solid symbols), or the low pH form of HA, H9 (open symbols); circles, wild-type Rostock; triangles, mutant K58I.

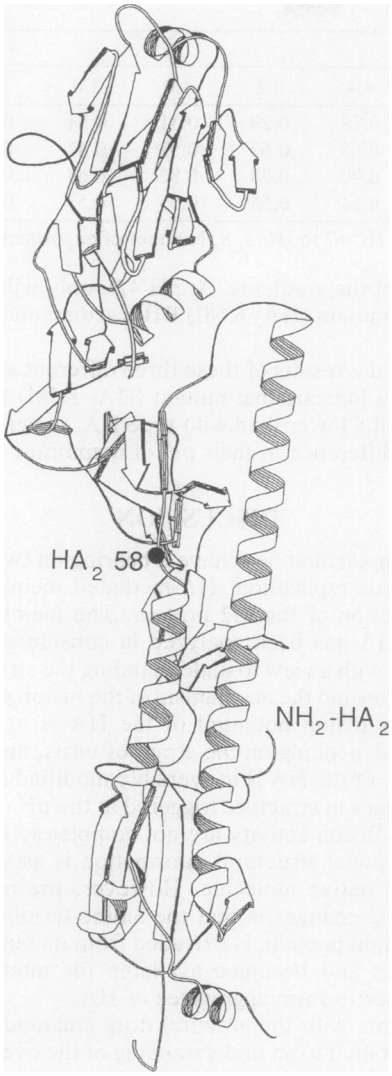

FIG. 3. Structure of a HA monomer showing the location of residue 58 of HA_2 and the amino terminus of HA_2.

shuttle vectors, one that directs transcription from the 7.5K early-late promoter (19) and one that directs transcription from the promoter and cis-acting elements of cowpox p160 late promoter (20). Vaccinia virus recombinants were constructed (21) and used to characterize HAs expressed in recombinant-infected cells.

Membrane Fusion Activity of the Site-Directed Mutant, H3 Subtype, HA. To determine the pH of membrane fusion by mutant and wild-type HAs of the H3 subtype, heterokaryon formation by vaccinia recombinant-infected BHK-21 cells was determined as a function of pH. Since the biosynthetic precursors (HA_0) of this subtype, unlike those of the H7 subtype, are not processed intracellularly, it is necessary to incubate cells expressing these HAs with trypsin to cleave HA_0 into HA_1 and HA_2 and so generate HAs with membrane fusion potential. At 15 hr postinfection, therefore, cells were treated with trypsin and adjusted to various pH values between 4.0 and 5.8. The pH was then returned to neutral and the monolayers were incubated in standard medium. Heterokaryon formation was clearly observed ≈1 hr later in monolayers that had been incubated at or below the pH of fusion (Fig. 4). Thus, wild-type HA mediated fusion at pH 5.2, while no heterokaryons formed in mutant HA_2 K58I infected cells above pH 4.5. No heterokaryons were seen in control cells infected with vaccinia virus or in cells infected with the HA recombinant viruses that had not been incubated with trypsin to cleave the HA_0 precursors.

ELISA, trypsin susceptibility, and aggregation assays were used to monitor the pH of conformational changes in HA that are required for membrane fusion activity.

(*i*) The conformations of expressed HAs were analyzed as a function of pH in ELISAs by using monoclonal antibodies that recognize only native HA (HC67) or that recognize HA in both native and low pH conformations (HC3). The ratios of HC67 to HC3 reactivities at different pH values are shown in Table 1. For wild-type, X-31, HA this ratio was observed to decrease significantly below pH 5.2. Changes in the mutant HA, however, were not detected until the pH was adjusted below pH 4.6. Two controls were included to ensure that the loss of HC67 reactivity at low pH resulted specifically from changes in HA conformation required for membrane fusion. As noted above, cells expressing HAs of the H3 subtype that had not been treated with trypsin to cleave the precursor HA_0

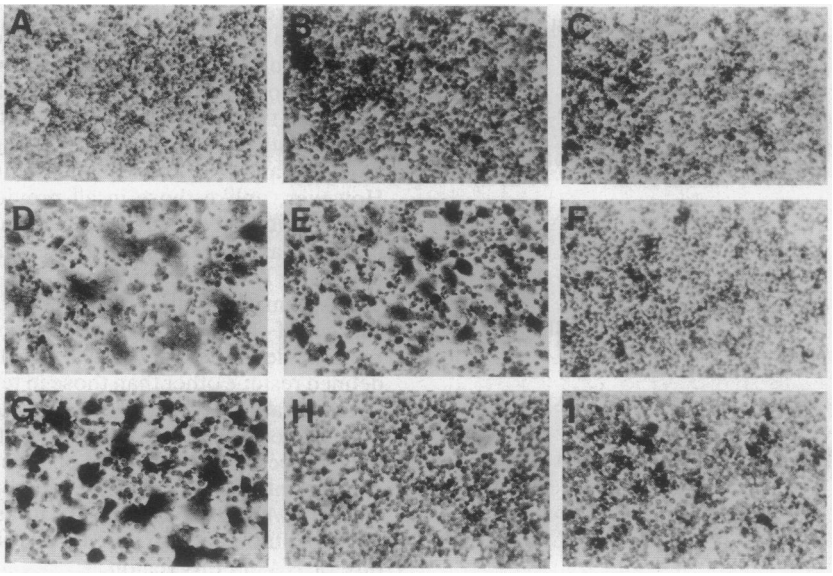

FIG. 4. Heterokaryon formation of BHK-21 cells infected by vaccinia virus (VV) or vaccinia virus–HA recombinants (VV-HA). wt, Wild type. (*A*) VV (pH 4.2). (*B*) VV-wtHA (pH 4.2), no trypsin. (*C*) VV-HA_2 K58I (pH 4.2), no trypsin. (*D*) VV-wtHA plus trypsin (pH 5.1). (*E*) VV-wtHA plus trypsin (pH 5.2). (*F*) VV-wtHA plus trypsin (pH 5.3). (*G*) VV-HA_2 K58I plus trypsin (pH 4.4). (*H*) VV-HA_2 K58I plus trypsin (pH 4.5). (*I*) VV-HA_2 K58I plus trypsin (pH 4.6).

Table 1. Analysis of conformational change by ELISA

HA (*n*)	pH									
	5.6	5.4	5.2	5.0	4.6	4.4	4.2	4.0	3.8	3.6
Wild type (9)	0.56	0.56	0.50	0.32	0.28	0.28	0.29	0.31	0.34	0.38
HA_2 K58I (13)	0.75	0.77	0.74	0.72	0.71	0.65	0.62	0.53	0.47	0.41
HA_2 K58I, no trypsin (1)	0.75	0.71	0.76	0.82	0.66	0.70	0.78	0.82	0.68	0.72
T212C/N216C (3)	0.49	0.50	0.54	0.61	0.49	0.54	0.55	0.55	0.57	0.52

The values given represent the ratio of reactivity with monoclonal antibody HC 67 to HC 3. *n*, Number of experiments.

were unable to form heterokaryons and no changes in the conformation of HAs expressed by these cells were detected by ELISA (Table 1). Similarly, a mutant HA (HA_1 T212C/N216C), which contained two cysteine residues at HA_1 212 and 216 resulting in covalent disulfide crosslinking of the membrane–distal domains of the HA, was unable to fuse membranes even after cleavage of HA_0 unless the disulfide bonds were reduced (L. Godley, J. Pfeifer, D.A.S., B. Ely, G. Shaw, R. Kaufmann, E. Suchanek, C. Pabo, J.J.S., D.C.W., and S.A.W., unpublished work). Without reduction, no changes in the conformation of this HA were detected at low pH by ELISA.

(*ii*) Changes in HA conformation at the pH of fusion were also determined in assays based on the differences in susceptibility to tryptic digestion of native and low pH HA (24). Soluble BHA (23) is resistant to proteolysis in its native state but is susceptible to trypsin and a variety of proteases (25) at the pH of fusion. Fig. 5 shows the pH dependence of trypsin susceptibility of wild-type X-31 and mutant (HA_2 K58I) BHAs. Wild-type BHA was degraded after incubation at pH 5.2; mutant BHA was degraded only after incubation at or below pH 4.4.

(*iii*) At the pH of fusion, BHA forms aggregates as a result of the extrusion of the hydrophobic fusion peptide at the amino terminus of HA_2 (24). The pH of aggregation of wild-type and mutant (HA_2 K58I) BHAs was determined by sucrose density gradient centrifugation of BHAs after incubation at pH 5.6, 4.9, or 4.4 (Fig. 6). At pH 5.6, both wild-type and mutant BHAs sedimented as native trimers (sedimentation coefficient, ≈9 *s*) and, at pH 4.4, both BHAs were detected as aggregates (sedimentation coefficient, ≈30 *s*) at the bottom of the gradients. At pH 4.9, only wild-type BHA aggregated; mutant (HA_2 K58I) BHA sedimented as a native trimer.

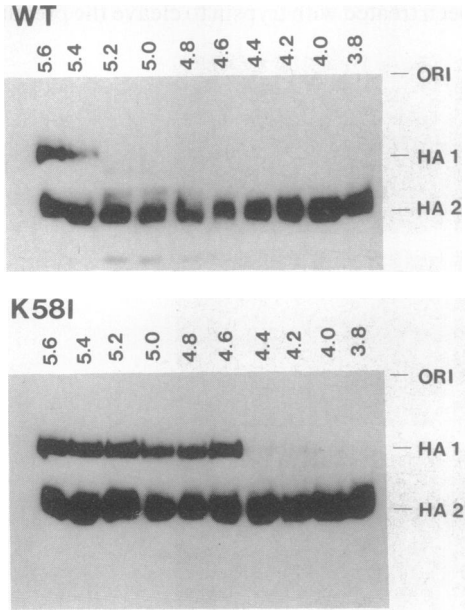

FIG. 5. Western blot analysis of 12% polyacrylamide gels run under reducing conditions showing trypsin susceptibility of the HA_1 subunit of wild-type and mutant BHA at the indicated pH. ORI, origin.

Together, the results of these three different assays of HA conformation indicate that mutant (HA_2 K58I) HA is stable at a pH 0.6–0.8 lower than wild-type HA, in agreement with the 0.7 pH difference in their pH of membrane fusion.

DISCUSSION

The results presented here have a bearing on two aspects of influenza virus replication: HA-mediated membrane fusion and the function of the M2 protein. The membrane fusion activity of HA has been analyzed in considerable detail in recent years with a view to understanding the structural basis of HA function and the mechanism of the fusion process itself (30–33). The fusion potential of the HA is activated at a specific pH depending on the strain of virus, and at this pH the structure of the HA is irreversibly modified (24–26). The precise changes in structure triggered at the pH of fusion and required for fusion activity are not completely known since three-dimensional structural information is as yet available only for the native molecule. However, the major consequence of the changes is that the amino-terminal region of HA_2, the fusion peptide, is extruded from its buried location in the trimer and becomes available for interaction with membranes or to form aggregates of HA.

Experiments with the antiviral drug amantadine have already contributed to an understanding of the overall changes in HA structure required for fusion peptide extrusion since viruses resistant to high concentrations of the drug contain mutant HAs that fuse membranes at higher pH than wild type (2). The amino acid substitutions that these mutant HAs contain are located throughout the length of the molecule, leading to the conclusion that the changes in structure required for fusion peptide extrusion are similarly extensive. The amino acid substitution HA_2 K58I in the mutant described in this paper extends the list of locations where amino acid substitutions appear to change the stability of the molecule and as a consequence influence the pH of fusion. However, unlike those in all previously reported mutants, this substitution stabilizes rather than destabilizes the native molecule and prevents the extrusion of the fusion peptide at pH > 4.5. The residue involved, HA_2 58, is ≈20 Å membrane distal from the amino terminus of HA_2, located near the surface of the molecule at the top of a short α-helix. It is remarkable in the native X-31 HA structure as the least well defined residue other than those in terminal sequences (atomic temperature factors > 65 $Å^2$) and for its proximity to an unidentified region of electron density between the short and long helices (34), which are prominent features of the HA_2 component of HA. The three-dimensional structure of the mutant HA will be determined to assess the significance of the substitution and to give further information on the structure of HA_2 in this region.

Perhaps more importantly for the present study is the manner in which the mutant was selected. The results of a variety of experiments have indicated that during the permissive replication of Rostock influenza virus in tissue cul-

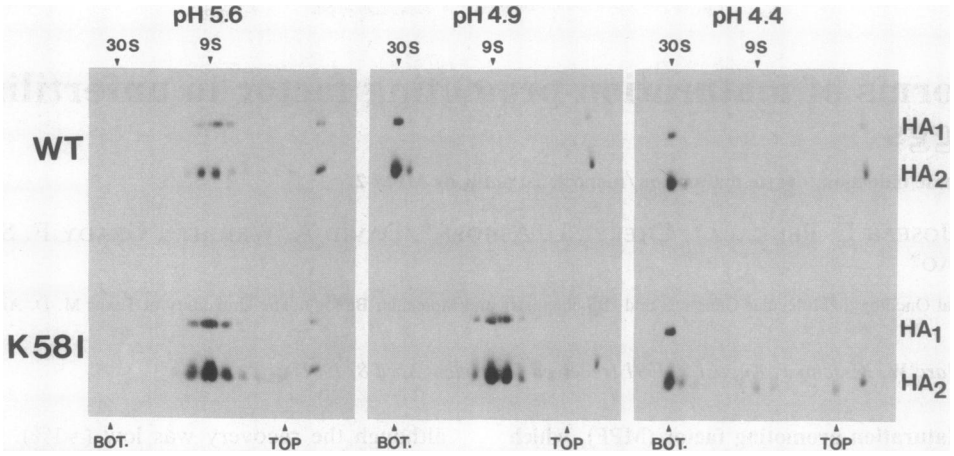

FIG. 6. Western blot analysis showing BHA aggregation experiments of wild-type and mutant BHA at indicated pH as determined by sedimentation on 5–25% sucrose density gradients. Bottom and top fractions are indicated. Virus BHA marker is to the right side of fractions from the top of each gradient.

ture cells incubated in the presence of amantadine, the HA detected in the trans Golgi and at the cell surface is in a conformation characteristic of that specifically induced at the pH of fusion (refs. 16 and 17; F. Ciampor and A.J.H., unpublished work). Previously, Rostock mutants selected for resistance to the antiviral effects of amantadine contained amino acid substitutions in the transmembrane region of the M2 protein and the hypothesis was proposed that M2 functions as a membrane channel (15) through which protons are released from a trans Golgi compartment. The reduced acidity would allow transfer of functional, native HA to the cell surface rather than inactivated, low pH HA produced during replication of a sensitive virus in the presence of amantadine. Our observations that amantadine-resistant mutants with no changes in M2 proteins but containing acid-stable HAs can also be selected under the same virus growth conditions add strong support to this hypothesis. They also support the propositions that the M2 protein functions as a proton channel and that amantadine blocks this function to inhibit influenza virus replication.

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