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Influenza Virus Hemagglutinin and Neuraminidase Glycoproteins Stimulate the Membrane Association of the Matrix Protein

MASAYOSHI ENAMI* AND KAZUE ENAMI

Department of Biochemistry, Kanazawa University School of Medicine, Takaramachi, Kanazawa, Ishikawa 920, Japan

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We have analyzed the mechanism by which the matrix (M1) protein associates with cellular membranes during influenza A virus assembly. Interaction of the M1 protein with the viral hemagglutinin (HA) or neuraminidase (NA) glycoprotein was extensively analyzed by using wild-type and transfectant influenza viruses as well as recombinant vaccinia viruses expressing the M1 protein, HA, or NA. Membrane binding of the M1 protein was significantly stimulated at the late stage of virus infection. Using recombinant vaccinia viruses, we found that a relatively small fraction (20 to 40%) of the cytoplasmic M1 protein associated with cellular membranes in the absence of other viral proteins, while coexpression of the HA and the NA stimulated membrane binding of the M1 protein. The stimulatory effect of the NA (>90%) was significant and higher than that of the HA (>60%). Introduction of mutations into the cytoplasmic tail of the NA interfered with its stimulatory effect. Meanwhile, the HA may complement the defective NA and facilitate virus assembly in cells infected with the NA/TAIL(−) transfectant. In conclusion, the highly conserved cytoplasmic tails of the HA and NA play an important role in virus assembly.

The assembly and budding of enveloped viruses at the plasma membrane is a complex process which is not well understood. The structure of the cytoplasmic tails of viral glycoproteins became the target of extensive study, since the interaction of the cytoplasmic tails with the matrix (M) protein or the viral nucleocapsid was thought to trigger the budding of infectious virus particles (11). The importance of the cytoplasmic tails of viral glycoproteins has been described for the influenza virus hemagglutinin (HA) (7, 32, 44, 52) and neuraminidase (NA) (3, 31), the Newcastle disease virus F (38), the Sendai virus F and HN (40, 41, 50), the vesicular stomatitis virus (VSV) G (30, 35, 49), the Semliki Forest virus E2 (29, 51), and the Sindbis virus E2 (18) proteins.

Influenza A virus is an enveloped virus in which eight species of viral nucleocapsids are coated with the plasma membrane of infected cells. The envelope contains two major glycoproteins, the HA and the NA (reviewed in reference 25). The majority of host proteins are excluded in the budding process.

The influenza virus NA is a type II membrane glycoprotein with an uncleaved amino-terminal signal/anchor domain and a cytoplasmic tail (1, 8). The three-dimensional structure of the NA indicates a tetrameric conformation consisting of a stalk and a globular head (9, 48). The globular head contains the enzyme-active site which is involved in the release of sialic acids from cellular and viral substrates (36). The transmembrane region contains the signal anchor domain required for the membrane association of the protein (4, 24, 33, 45). Although the amino-terminal six residues consisting of the cytoplasmic tail are highly conserved among different influenza A virus NAs, deletion of these amino acids did not alter the signal anchor function (10, 23). Several reports concluded that the NA activity is not required for virus assembly (26, 27, 36); however, fragments of the NA containing the transmembrane and cytoplasmic domain could facilitate the budding process. A similar mechanism was reported for the budding of spikeless VSV particles (30). Recently, mutations were introduced into the cytoplasmic tail of the NA, and the rescued viruses were shown to be attenuated (3, 19). However, the moderate degree of attenuation of these viruses (10^{-1} to 10^{-2}) did not help in defining the importance of this region for assembly.

The cytoplasmic tail of the HA contains 10 to 11 amino acids, of which 5, including two palmitylated cysteines, are highly conserved (34). Previous reports concluded that the HA may not be essential for virus assembly and that the cytoplasmic tail of the HA is dispensable for virus assembly (21, 22, 37). However, influenza virus formation was inhibited by a peptide which corresponded to sequences in the cytoplasmic tail of the HA (7). In addition, amino acid changes of the conserved cysteines in the cytoplasmic tail of the HA (H1 subtype) interfered with the formation of infectious virus (52).

In VSV and Sendai virus assembly, membrane binding of the matrix protein has been analyzed via sucrose flotation centrifugation (5, 6, 40, 41). In the case of Sendai virus, the matrix protein interacts with the F and HN glycoproteins at the cellular membranes (41). In the present study, we analyze the mechanism of M1-membrane association via sucrose flotation centrifugation.

MATERIALS AND METHODS

Viruses and cells. Influenza reassortant WSN-HK virus (15, 42) was grown in embryonated 10-day-old chicken eggs at 37°C for 2 days. The WSN virus was amplified in Madin-Darby bovine kidney (MDBK) cells in reinforced minimal essential medium (REM) containing 0.2% bovine serum albumin (BSA) at 37°C (47). Recombinant vaccinia viruses M1-VAC (46) and vTF7-3 (16), expressing the PR8 virus M1 and T7 RNA polymerase, respectively, were kindly provided by B. Moss. Recombinant vaccinia viruses which express influenza A/chicken/Germany/34 (FPV Rostock) virus HA (VV-HA) and NA (VV-NA) were generously provided by H.-G. Klenk. Vaccinia viruses were amplified in HeLa cells in Eagle's minimal essential medium (MEM) containing 2.5% fetal calf serum (FCS) and plaque titrated on CV-1 cells in Dulbecco's modified Eagle's medium containing 2.5% FCS. MDBK cells were maintained in REM containing 10% FCS. HeLa and 293 cells were maintained in MEM containing 10% FCS.

Preparation of plasmids. pT3/WSN-NA was previously described (14). pT3/

* Corresponding author. Mailing address: Department of Biochemistry, Kanazawa University School of Medicine, 13-1 Takaramachi, Kanazawa, Ishikawa 920, Japan. Phone: (81) 762-62-8151, ext. 2250. Fax: (81) 762-34-4225. Electronic mail address: menami@kenroku.ipc.kanazawa-u.ac.jp.

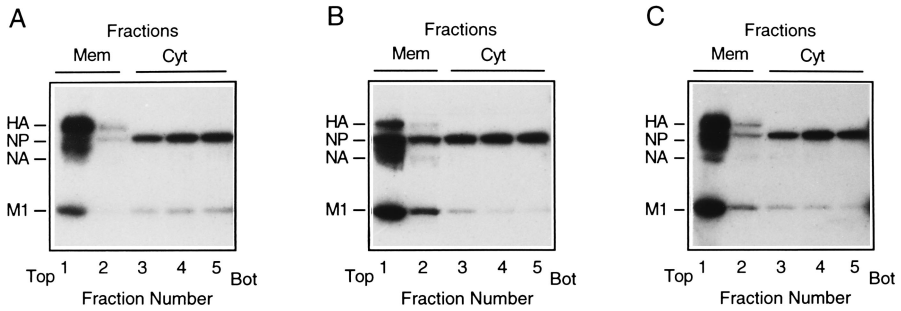

FIG. 1. Membrane association of viral proteins in WSN virus-infected MDBK cells. MDBK cells were infected with WSN virus. After incubation at 39.5°C for 4 h (A and B) or 7 h (C), cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min. Labeled cells were then chased for 3 h (B). Cells were lysed, and postnuclear fractions were subjected to sucrose flotation centrifugation as described in Materials and Methods. Viral proteins were immunoprecipitated with anti-WSN serum supplemented with anti-M1 and anti-NP antibodies. Proteins were analyzed by SDS-PAGE (10 to 20% gel). Membrane (Mem) and cytosolic (Cyt) fractions are indicated.

WSN/BTAIL-NA was constructed by PCR (39) using pT3/WSN-NA DNA as a template and the primer pair universal M13 primer M4 (5′-GTTTTCCCAGTC ACGAC-3′) and 5′-CGCGCGAATTCTCTTCGAGCGAAAGCAGGAGTTT AAATGCTTCCAAGCACGGTAATAATAACCATTGGGTCAATC-3′. Mutant WSN/BTAIL NA contains the amino-terminal six residues of the influenza B/Lee/40 virus NA (MLPSTV) (43) instead of the cytoplasmic tail of the WSN virus NA (MNPNQK) (20). The PCR product was digested with *Eco*RI and *Hin*dIII, and was inserted into plasmid pUC119.

pGEM-derived plasmids pGEM/WSN-M1, pGEM/WSN-NA, pGEM/WSN-NA/Tail(−) and pGEM/WSN-HA/Tail(−) were constructed as follows. A cDNA for the WSN virus M1 gene was obtained by reverse transcription-PCR using primers 5′-GCGCGAATTCAGCGAAAGCAGGTAGATATTG-3′ and 5′-CA ATGTCTAGAGGATCACTTGAATC-3′ as previously described (12). The PCR product was digested with *Eco*RI and *Xba*I and was inserted into plasmid pGEM-3Z (Promega) containing a T7 RNA polymerase promoter (pGEM/WSN-M1). Plasmid pT3/WSN-NA and pT3/WSN-NA/Tail(−) DNAs were digested with *Eco*RI and *Hin*dIII, and the cDNAs obtained were inserted into plasmid pGEM-3Z, resulting in pGEM/WSN-NA and pGEM/WSN-NA/Tail(−), respectively. Two cDNA fragments were obtained by PCR using the primer pair 5′-CAAAGCTTATTAAGAACACATCCAGAAACTGAT-3′ and universal M13 primer RV (5′-CAGGAAACAGCTATGAC-3′) or the primer pair 5′-TA ATGAAGCTTTGCAGTGCAGAATATGC-3′ and universal M13 primer M4. The PCR products were digested with *Hin*dIII and *Xba*I and were inserted into plasmid pGEM-4Z (Promega), resulting in pGEM/WSN-HA/Tail(−).

NA transfectant viruses. The WSN/TAIL(−) transfectant was described previously (19). WSN/BTAIL virus was obtained by ribonucleoprotein (RNP) transfection as described previously (14, 15, 28). Briefly, RNPs were reconstituted in vitro, using purified influenza virus core proteins and plasmid pT3/WSN/BTAIL-NA. RNPs were then transfected into MDBK cells infected with WSN-HK virus. Transfectant viruses were plaque purified and amplified in MDBK cells. The WSN/BTAIL virus is essentially identical to the previously described FLUBCYT virus (3). Expression of these mutant NAs at the cell surface was previously characterized and was not significantly reduced (3, 19). We confirmed these results (data not shown).

Labeling of influenza virus-infected cells. MDBK cells (2×10^6 in a 60-mm-diameter dish) were infected with influenza WSN, WSN/BTAIL, or WSN/TAIL(−) virus (multiplicity of infection [MOI] of 10) for 30 min at room temperature. Supernatants were removed, and the cells were incubated in REM containing 0.2% BSA for 4 or 7 h at 39.5°C. Cells were washed three times with prewarmed PBS(−) and were labeled for 30 min in 1 ml of MEM containing 100 μCi of [^{35}S]methionine and 25 μCi of [^{35}S]cysteine (NEN EXPRE^{35}S^{35}S Protein Labeling Mix). In pulse-chase experiments, cells were then incubated in REM containing unlabeled methionine and cysteine for 3 h at 39.5°C. Cells were washed with ice-cold PBS(−) and suspended in 0.4 ml of TMK buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM KCl, and 0.2 trypsin inhibitor unit (TIU) of aprotinin (Sigma) per ml. The cells were disrupted by repeated passage (50 times) through a 26-gauge needle. After centrifugation at 5,000 rpm for 2 min, supernatants were subjected to sucrose gradient flotation centrifugation to separate the membranes (endoplasmic reticulum, Golgi, and plasma membranes) and the cytosolic fractions.

Expression of M1, HA, and NA proteins by using vaccinia virus recombinants. HeLa cells (2×10^6 in a 60-mm-diameter dish) were infected with recombinant vaccinia viruses expressing influenza virus M1 (MOI of 20), HA (MOI of 10), or NA (MOI of 10) for 1 h at 37°C. After incubation with medium for 7 h at 37°C, proteins were labeled for 30 min in 1 ml of MEM containing 200 μCi of [^{35}S]methionine and 50 μCi of [^{35}S]cysteine. The cells were washed with PBS(−), suspended in TMK buffer, and disrupted as described above.

Expression of M1, NA, and HA proteins from plasmids by using vTF7-3 virus. 293 cells (2×10^6 in a 60-mm-diameter dish) were infected with vTF7-3 (MOI of 20) for 1 h at 37°C. Cells were washed twice with Opti-MEM I medium (Gibco BRL) and were transfected with 2 μg of pGEM/WSN-M1, 4 μg of pGEM/WSN-NA, 4 μg of pGEM/WSN-NA/Tail(−), and/or 4 μg of pGEM/WSN-HA/Tail(−) DNAs, using 20 μl of Lipofectamine (Gibco BRL) in 2 ml of Opti-MEM I for 5 h at 37°C. After 7 h of incubation with Opti-MEM I containing 2.5% FCS at 37°C, proteins were labeled for 30 min or for 2 h in 1 ml of MEM containing 200 μCi of [^{35}S]methionine and 50 μCi of [^{35}S]cysteine. Cells were washed with PBS(−), suspended in TMK buffer, and disrupted as described above.

Sucrose flotation centrifugation and immunoprecipitation. Sucrose flotation centrifugation was performed as described previously (40, 41). Postnuclear supernatants prepared as described above (approximately 0.4 ml) were dispersed in 2 ml of 70% (wt/wt) sucrose in LSB containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 mM KCl, and 0.2 TIU of aprotinin per ml. Samples were overlaid with 2 ml of 55% (wt/wt) sucrose in LSB and 0.6 ml of 10% (wt/wt) sucrose in LSB. Gradients were then centrifuged in a Beckman SW55 Ti rotor for 7 h at 45,000 rpm at 4°C. Fractions of 1 ml were collected from the top of the gradient. Aliquots were diluted with an equal amount of 2× radioimmunoprecipitation assay buffer (1× radioimmunoprecipitation assay buffer contains 10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 0.2 TIU of aprotinin per ml, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS] [2]). Proteins were immunoprecipitated with antibodies (2 μl of rabbit anti-WSN serum, 1 μl of rabbit anti-FPV serum, 2 μl of rabbit anti-M1 serum, and/or 0.2 μl of monoclonal anti-NP antibody) together with 10 μl of protein G-Sepharose 4 Fast Flow (Pharmacia LKB). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (2), using a 10 to 20% gradient gel or an 11% gel.

RESULTS

Membrane association of the M1 protein in influenza A/WSN virus-infected cells. Membrane association of the viral proteins in WSN virus-infected cells was investigated by separating the membrane fraction from cytosolic proteins by sucrose flotation centrifugation. Virus-infected cells were labeled at 4 h postinfection (hpi), and the fate of the labeled M1 protein was analyzed after a 3-h chase period (Fig. 1A and B). Thirty to 50% of the cytoplasmic M1 protein was associated with the membranes (Fig. 1A). After the 3-h chase period (i.e., at 7 hpi), the majority of the labeled M1 protein became associated with the membranes (Fig. 1B). The membrane association of the NP protein suggests virus assembly at the cellular membrane (Fig. 1B). It should be mentioned that some M1 proteins are incorporated into the nucleus and then exported to the cellular membranes during the chase period; viral proteins at the membranes are then incorporated into virions and released from the cells.

To investigate the requirement of the 3-h chase period for the increased M1-membrane association, the virus-infected cells were then labeled at 7 hpi (Fig. 1C). In contrast, >90% of the newly synthesized cytoplasmic M1 was immediately associated with the membrane. It is thus likely that membrane association of the M1 protein involves the accumulation of late

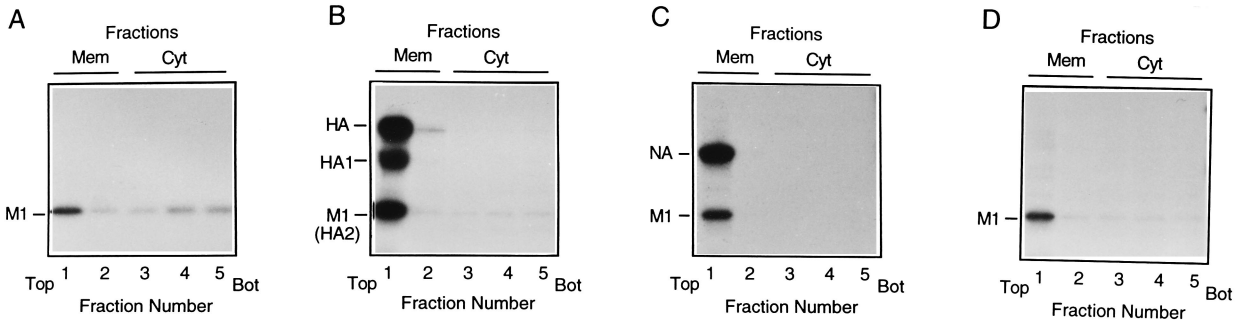

FIG. 2. Coexpression of HA and NA stimulates the membrane association of the M1 protein. HeLa cells were infected with the recombinant vaccinia viruses M1-VAC (A to D), VV-HA (B, D), and VV-NA (C). Infected cells were incubated at 37°C for 7 h before being labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min. Labeled cells were lysed, and postnuclear fractions were subjected to sucrose flotation centrifugation as described in Materials and Methods. Proteins were immunoprecipitated with anti-M1 serum (A and D) or anti-FPV serum supplemented with anti-M1 serum (B and C). Proteins were analyzed by SDS-PAGE (10 to 20% gel). Membrane (Mem) and cytosolic (Cyt) fractions are indicated.

viral proteins. An additional, minor possibility is that the membrane-bound M1 protein is due to budding viruses appearing during influenza virus replication. To investigate these possibilities, influenza virus proteins were selectively expressed in cells without influenza virus infection.

HA and NA proteins stimulate the membrane association of the M1 protein. To facilitate the selective expression of influenza virus proteins, three recombinant vaccinia viruses which encode either influenza virus M1, HA, or NA protein were used to infect HeLa cells. Cells were labeled, and the labeled proteins were analyzed by sucrose gradient flotation centrifugation (Fig. 2). When the M1 protein was expressed without other viral proteins, 20 to 40% of the cytoplasmic M1 protein was associated with the membranes (Fig. 2A). On the other hand, coexpression of either the HA (Fig. 2B and D) or the NA (Fig. 2C) stimulated the membrane association of the M1 protein. In repeated experiments, >60% of the M1 protein was found to be membrane associated in cells coexpressing the HA, and >90% of the M1 protein was membrane associated in cells coexpressing the NA. Therefore, significant involvement of the NA protein was suggested. In addition, other viral proteins, including RNPs, were not required for the increased binding of the M1 protein.

Introduction of mutations into the cytoplasmic tail of the NA protein altered the membrane association of the M1 protein. The NA protein was shown to significantly stimulate the membrane association of the M1 protein. The cytoplasmic tail of the NA protein is highly conserved among different influenza A viruses. This conserved domain has been speculated to play a role during virus assembly. In previous studies, site-directed mutations were introduced into the cytoplasmic tail of the NA protein, and rescued viruses were characterized (3, 19). Although the rescued viruses were attenuated, the mechanism of attenuation had not been clearly identified. Thus, we used the WSN/BTAIL and WSN/TAIL(−) transfectants to characterize the involvement of the cytoplasmic tail of the NA in the M1-membrane association (Fig. 3). In cells infected with the mutant viruses at 7 hpi, membrane binding of the M1 protein was significantly altered. The dissociation of the M1 protein can be ascribed to the mutation, but it was moderate in repeated experiments.

Since the HA protein has been shown to play a role in M1-membrane association (Fig. 2D), we analyzed the effect of the mutation in the absence of the HA or in the presence of mutant HA/TAIL(−). 293 cells were infected with vTF7-3 and were transfected with plasmids expressing M1, NA, NA/TAIL(−), and/or HA/TAIL(−). Proteins were analyzed by sucrose flotation centrifugation (Fig. 4). When the WSN NA was coexpressed, the majority of the cytoplasmic M1 protein was bound to the membrane [Fig. 4B and E(b)]. On the other hand, coexpression of NA/TAIL(−) or HA/TAIL(−) did not stimulate M1-membrane binding [Fig. 4C, D, E(c), and E(d)].

Stability of the membrane-bound M1 protein. The VSV matrix protein was previously shown to tightly associate with the membrane, and the binding was not altered by high-salt treatment (5). To investigate the stability of the membrane binding of influenza virus M1 protein, the membrane fraction of the WSN virus-infected cells at 7 hpi (Fig. 1B, fractions 1 and 2) was treated with 2 M KCl, and the fraction was then analyzed via a second sucrose flotation centrifugation (Fig. 5). The majority of the membrane-bound M1 protein was not dissociated by the high-salt treatment.

DISCUSSION

We have shown that the HA and the NA independently stimulate M1-membrane association. Once the HA or the NA has accumulated at the membranes, the M1 protein immediately associates with the membranes. Significance of the cytoplasmic tail was also indicated. Consequently, we conclude that the HA and NA cytoplasmic tails facilitate virus assembly. Recently, several groups reported functional characterization

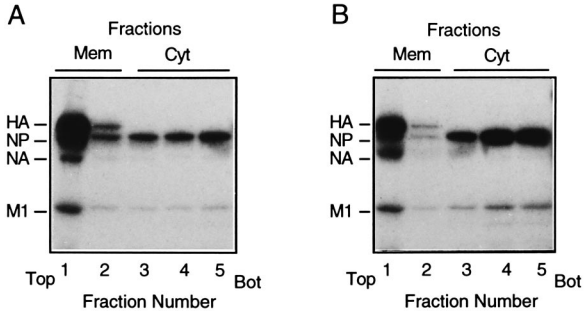

FIG. 3. Characterization of transfectant viruses containing mutations in the cytoplasmic tail of the NA. MDBK cells were infected with WSN/BTAIL virus (A) or WSN/TAIL(−) virus (B). Cells were incubated at 39.5°C for 7 h before being labeled with [^{35}S]methionine and [^{35}S]cysteine at 37°C for 30 min. Cells were lysed, and postnuclear fractions were subjected to sucrose gradient centrifugation as described in Materials and Methods. Proteins were immunoprecipitated with anti-WSN serum supplemented with anti-M1 and anti-NP antibodies. Proteins were analyzed by SDS-PAGE (10 to 20% gel). Membrane (Mem) and cytosolic (Cyt) fractions are indicated.

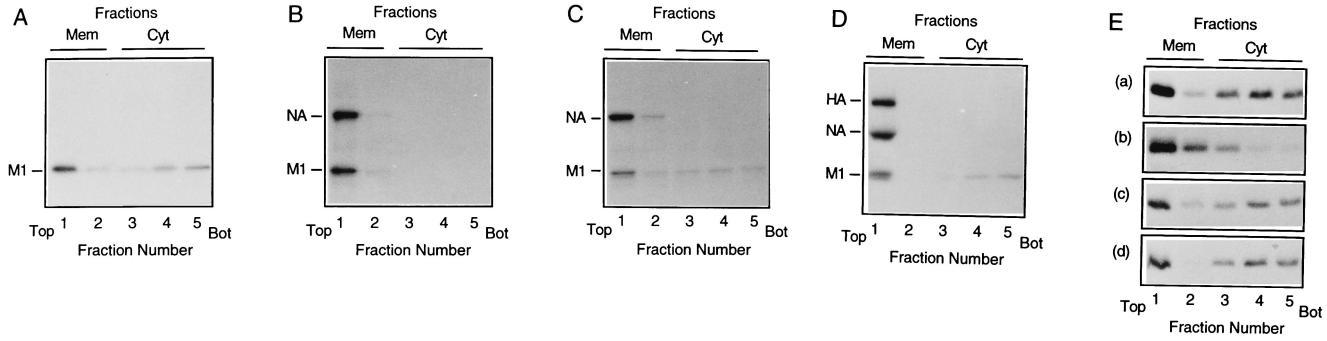

FIG. 4. Characterization of the mutant NA protein. 293 cells were infected with vTF7-3 and then transfected with pGEM/WSN-M1 (A to E), pGEM/WSN-NA [B and E(b)], pGEM/WSN-NA/TAIL(−) [C, D, E(c), and E(d)], and pGEM/WSN-HA/TAIL(−) [D and E(d)]. After a 12-h incubation at 37°C, proteins were labeled with [^{35}S]methionine and [^{35}S]cysteine for 30 min (A to D) or for 2 h (E). Cells were lysed, and postnuclear fractions were subjected to sucrose flotation centrifugation as described in Materials and Methods. Proteins were immunoprecipitated with anti-M1 serum (A and E) or anti-WSN serum supplemented with anti-M1 serum (B to D). Proteins were analyzed by SDS-PAGE on a 10 to 20% (A to D) or 11% (E) gel. Membrane (Mem) and cytosolic (Cyt) fractions are indicated.

of these viral glycoproteins in influenza A virus assembly (3, 19, 21, 22, 31, 52). Some groups argued significance of the cytoplasmic tails of the HA (52) or the NA (3, 31); however, others concluded that the cytoplasmic tails of the HA (21, 22) or the NA (19) are dispensable. Our data are consistent with the latter argument, since either the HA or the NA is sufficient for membrane binding of the M1 protein and minimum assembly.

Expression of the M1 protein and the NA is stimulated in late in virus infection via translational regulation (13). This regulation is reasonable, considering the function of these proteins in influenza virus replication.

Sucrose flotation analysis of Sendai virus assembly demonstrated that the F and HN glycoproteins independently stimulate the membrane binding of the matrix protein (41). In the case of Sendai virus, a 2- to 3-h chase period was required for the M-membrane binding. However, influenza virus M1-membrane interaction was accomplished within a 30-min labeling time. The difference could be attributed to the difference of the system or the difference of the virus.

The amino acid sequence of the NA cytoplasmic tail is highly conserved among different influenza A viruses. It is reasonable to think that this conserved region interacts with the M1 protein during virus assembly. The stimulatory effect of the NA protein was significantly reduced by a mutation in this region, suggesting the involvement of the direct interaction of the M1 protein with the NA cytoplasmic tail.

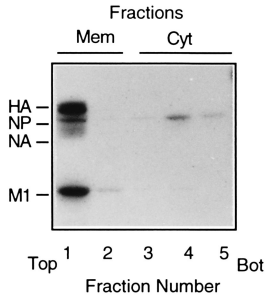

FIG. 5. Stability of the membrane-bound M1 protein. Membrane fractions 1 and 2 were obtained as described for Fig. 1B and treated with 2 M KCl–10 mM Tris-HCl (pH 7.4) for 1 h at 0°C. The sample was then subjected to second sucrose flotation centrifugation. Viral proteins were immunoprecipitated with anti-WSN serum supplemented with anti-M1 and anti-NP antibodies. Proteins were analyzed by SDS-PAGE (10 to 20% gel). Membrane (Mem) and cytosolic (Cyt) fractions are indicated.

The influenza virus M1 protein was tightly associated with the membranes, and the binding was resistant to salt treatment which dissociates peripherally associated proteins, suggesting that the M1 protein may partially integrate into the membrane. A similar result was obtained in studies of the membrane binding of the VSV matrix protein (5). One possibility is that the interaction of the M1 protein with the NA facilitates the membrane integration of the peripherally associated M1 protein. In support of this view, the six residues of the transmembrane region of the NA following the amino-terminal cytoplasmic tail are relatively conserved among different influenza A viruses. Interaction of the M1 protein with the amino-terminal 12 residues of the NA is therefore possible. In a recent study (31), the cytoplasmic and transmembrane region of the NA was shown to be involved in virus assembly and to be related to viral morphology, since the mutation in this domain led to the increase of long filamentous particles which was previously reported for the M1 mutant. In addition, recent electron cryomicroscopic analysis demonstrated that the infectious influenza virus envelope consists of a thin outer single phospholipid layer and an inner thick layer, suggesting alteration of the inner layer by the M1 protein (17). Consequently, the interaction of the M1 protein with the amino-terminal 12 residues of the NA was speculated (31). Their data support our conclusion.

Our data did not suggest the requirement of posttranslational modification of the M1 protein; however, our data do not rule out its involvement. For example, interaction of the M1 protein with the NA may lead to the conformational change of the protein and facilitate posttranslational modification.

Finally, in this study, the HA protein was also shown to have a stimulatory effect, but the effect was lower than that of the NA protein. This finding is consistent with a previous study (21) in which deletion of the HA cytoplasmic tail did not significantly altered virus infectivity. The HA protein probably facilitates virus assembly in place of the defective NA in NA mutant-infected cells. In addition, without HA or NA, 20 to 40% of the M1 protein was shown to associate with the membrane. This amount of the M1 protein might be enough to facilitate minimum viral assembly. It might be possible to obtain a double mutant containing mutations in the cytoplasmic tails of HA and NA. Such a mutant obtained should be highly attenuated and might be a good candidate for live attenuated vaccines.

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