A Mutation on Influenza C Virus M1 Protein Affects Virion Morphology by Altering the Membrane Affinity of the Protein[▽]

Yasushi Muraki,^{1*} Toshio Murata,² Emi Takashita,¹ Yoko Matsuzaki,¹
Kanetsu Sugawara,¹ and Seiji Hongo¹

Department of Infectious Diseases, Yamagata University School of Medicine, Iida-Nishi, 990-9585, Yamagata, Japan,¹ and
Department of Microbiology, Yamagata Prefectural Institute of Public Health, Toka-machi, 990-0031, Yamagata, Japan²

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Reverse genetics has been documented for influenza A, B, and Thogoto viruses belonging to the family *Orthomyxoviridae*. We report here the reverse genetics of influenza C virus, another member of this family. The seven viral RNA (vRNA) segments of C/Ann Arbor/1/50 were expressed in 293T cells from cloned cDNAs, together with nine influenza C virus proteins. At 48 h posttransfection, the infectious titer of the culture supernatant was determined to be 2.51×10^3 50% egg infectious doses/ml, which is lower than the number of influenza C virus-like particles (VLPs) (10^6 /ml) generated using the same system. By generating influenza C VLPs containing a given vRNA segment, we showed that each of the vRNA segments was similarly synthesized in the plasmid-transfected cells but that some segments were less efficiently incorporated into the VLPs. This finding leads us to speculate that the differences in incorporation efficiency into VLPs between segments might be a reason for the inefficient production of infectious viruses. Second, we generated a mutant recombinant virus, rMG96A, which possesses an Ala→Thr mutation at residue 24 of the M1 protein, a substitution demonstrated to be involved in the morphology (filamentous or spherical) of the influenza C VLPs. As expected, rMG96A exhibited a spherical morphology, whereas recombinant wild-type of C/Ann Arbor/1/50, rWT, exhibited a mainly filamentous morphology. Membrane flotation analysis of the cells infected with rWT or rMG96A revealed a difference in the ratio of membrane-associated M1 proteins, suggesting that the affinity of M1 protein to the cell membrane is a determinant for virion morphology.

Reverse genetics, a method for the generation of infectious virus particles from cloned cDNA, has been widely reported. For the viruses belonging to the family *Orthomyxoviridae*, successful reverse genetics was initially documented for influenza A virus (6, 9, 20), influenza B virus (8, 10, 13), and Thogoto virus (31). However, reverse genetics of influenza C virus have not yet been reported, although we previously reported the generation of virus-like particles (VLPs) of the virus (19).

In a number of studies involving influenza virus reverse genetics, in order to obtain a higher number of recombinant viruses, transfected cells were cocultured with susceptible cells such as MDCK cells, or the culture media containing recombinant viruses generated from the transfected cells were directly inoculated onto susceptible cells. Some original reports, however, documented the infectious titer of the culture medium of the transfected 293T cells. Neumann et al. (20) reported that 10^6 to 10^7 PFU/ml of infectious influenza A viruses were generated from the transfected 293T cells at 48 h posttransfection (p.t.), whereas Hatta et al. (8) reported $10^{3.5}$ 50% tissue culture infectious doses of influenza B viruses in the culture supernatant at 48 h p.t. Thus, there seems to be differences in the initial titers of the generated recombinant viruses between influenza A and B viruses, although similar procedures were adopted in both sets of experiments.

Influenza A virions exhibit a remarkable degree of structural

variation. The virus proteins, such as hemagglutinin (HA), neuraminidase (NA), M1, and M2, have been demonstrated to be involved in virion morphology. Truncation of either of HA or NA leads to a change in morphology (14, 18). A mutant containing a truncation in both the HA and NA cytoplasmic domains was shown to display a particularly exaggerated phenotype (14). In addition, there have been several lines of evidence suggesting that the M1 protein is a determinant of virion morphology. Studies on the Udorn strain showed that the M1 mutation correlates with morphology (26). Using reverse genetics, Bourmakina and García-Sastre (2) showed that amino acid residues 95 and 204 of the M1 protein of A/WSN/33 are critical in determining filamentous virus particle formation. Combination of the amino acids at position 41, 95, and 218 on the M1 protein were reported to control morphology of A/Victoria/3/75 (4). Another study of several mutants in the helix six domain of the M1 protein of A/WSN/33 indicated that changes at position 102 can affect virus morphology (11).

The association of influenza A virus M1 protein with the plasma membrane of transfected cells has been studied extensively. M1 has the inherent ability to associate with the membrane, and studies have shown that there is a great deal of variation in the membrane-binding ability of M1. Enami and Enami (5), Kretzschmar et al. (16), and Zhang and Lamb (33) reported that 20 to 30%, 15%, and 45 to 60%, respectively, of M1 protein was associated with the membrane. Furthermore, one report has demonstrated that the coexpression of the viral glycoproteins was found to stimulate the membrane association of M1 (5), whereas this phenomenon was not noted in other studies (16, 33).

* Corresponding author. Mailing address: Department of Infectious Diseases, Yamagata University School of Medicine, Iida-Nishi, 990-9585, Yamagata, Japan. Phone: 81-23-628-5249. Fax: 81-23-628-5250. E-mail: ymuraki@med.id.yamagata-u.ac.jp.

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The association of influenza A virus proteins with lipid rafts has also been recently documented. Lipid rafts, a major budding site of influenza A virus (30), are cholesterol- and glycosphingolipid-rich microdomains originally characterized by their insolubility at 4°C in nonionic detergents such as Triton X-100 (3). M1 was found to be associated with lipid rafts, and this association was decreased in cells infected with viruses lacking the cytoplasmic tails of HA or NA (34). The interaction of M1 with mature glycoproteins that associated with the lipid rafts was found to be responsible for the detergent resistance of membrane-bound M1 (1).

We have demonstrated that residue 24 of the influenza C virus M1 protein is involved in the formation of cord-like structures (CLS), bundles of filamentous VLPs, from the cells, as well as in the morphology of influenza C VLPs (19). The expression of M1 protein having an Ala at residue 24, together with the other eight virus proteins (PB2, PB1, P3, HE, NP, CM2, NS1, and NS2) and green fluorescent protein (GFP)-viral RNA (vRNA) in 293T cells, resulted in the formation of CLS from the cells and the generation of filamentous VLPs, whereas expression of M1 having a Thr instead of an Ala at residue 24 did not lead to CLS formation, and the morphology of the VLP was spherical. However, the effect of the M1 mutation on virus replication and virion morphology remains to be clarified.

To address the above question, we first attempted to establish reverse genetics of influenza C virus. Furthermore, we report here the generation of a recombinant influenza C virus possessing a mutation at residue 24 of the M1 protein and provide evidence that the affinity of the M1 protein to the plasma membrane affects the morphology of the virions and CLS formation from the infected cells.

MATERIALS AND METHODS

Cells, viruses, and antibodies. 293T cells, the HMV-II line of human malignant melanoma cells and MDCK cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum (19), RPMI 1640 medium with 10% calf serum (22) and minimal essential medium with 10% fetal bovine serum (23), respectively. The Ann Arbor/1/50 (AA/50) strain of influenza C virus was grown in the amniotic cavity of 8-day-old embryonated chicken eggs (32). Monoclonal antibodies to the HE (J14), NP (H27, H31), and M1 (L2) proteins of AA/50 and antiserum against AA/50 virions were prepared previously (28, 29, 32).

Construction of plasmid DNAs. The vRNA was extracted from egg-grown AA/50 virions as described previously (17). The 5' and 3' ends of the nucleotide sequences of the RNA segment 1 (PB2 gene), 2 (PB1), 3 (P3), 4 (HE), 6 (M), and 7 (NS) were determined by 5'RACE (rapid amplification of cDNA ends), as reported previously (19). The cDNAs of each segment were then synthesized as reported (19), and the whole region of each segment was PCR amplified by using a pair of primers containing the determined sequences flanked by BsmBI sites at their 5' ends. The obtained PCR products were digested with BsmBI, followed by insertion in antisense orientation between the PolI promoter and the terminator of the vector pHH21 (20). For each segment, the nucleotide sequences of three independent clones in the library were determined, and the plasmid with the consensus sequence, designated pPolI/PB2, pPolI/PB1, pPolI/P3, pPolI/HE, pPolI/M, and pPolI/NS, was selected for the subsequent experiments. Plasmids pPolI/NP and pPolI/NP-AA.GFP(−) and the nine plasmids for expression of the virus proteins (pCDNA/PB2-AA, pCDNA/PB1-AA, pCDNA/P3-AA, pME18S/HE-AA, pCAGGS.MCS/NP-AA, pCAGGS.MCS/M1-AA, pME18S/Met-CM2-YA, pME18S/NS1-YA, and pME18S/NS2-YA) were described previously (19). Plasmid pPolI/M-G96A, which has a G-to-A-mutation at residue 96 of the M gene, was constructed based on pPolI/M. Details of the primers and PCR protocols will be provided on request.

Generation of recombinant influenza C viruses. To rescue recombinant AA/50, 0.5-μg portions of pPolI/PB2, pPolI/PB1, pPolI/P3, pPolI/HE, pPolI/NP, pPolI/M, and pPolI/NS were transfected into 10⁶ 293T cells, together with four

(PB2, PB1, P3, and NP) or nine (PB2, PB1, P3, HE, NP, M1, CM2, NS1, and NS2) virus protein-expressing plasmids according to a procedure reported previously (19). The total amount of plasmids was 4.4 μg to 10.9 μg, depending on the constructs. At 48 to 60 h p.t., the culture medium was treated with 20 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin/ml at 37°C for 10 min, and then the infectious titers were determined.

Determination of infectious titers of viruses. The supernatant of the plasmid-transfected 293T cells was serially 10-fold diluted and inoculated into the amniotic cavity of 8- or 9-day-old chicken eggs. After incubation at 34°C for 72 h, the amniotic fluids were recovered from the eggs and tested for hemagglutination with 0.5% chicken red blood cells. The 50% egg infectious dose (EID₅₀)/ml was calculated by the method of Reed and Muench.

The stock of the egg-grown recombinant viruses or the supernatant of the virus-infected HMV-II cells were serially 10-fold diluted with minimal essential medium, followed by inoculation onto MDCK monolayer cultures on a 24-well plate (Nunc). At 48 h postinfection (p.i.), the cells were fixed with 4% paraformaldehyde and stained with a primary antibody, anti-HE monoclonal antibody (J14), and a secondary antibody, Alexa Fluor 488 goat anti-mouse immunoglobulin G (Molecular Probes). The fluorescence-positive cells in each well were measured by a fluorescence microscope (Leica), and the 50% tissue culture infectious dose(s) (TCID₅₀)/ml was calculated by the method of Reed and Muench.

Reverse transcription-PCR of vRNAs extracted from transfected 293T cells and VLPs. To obtain influenza C VLPs containing a given segment of vRNA, 293T cells were transfected with one of the PolI plasmids [pPolI/PB2, pPolI/PB1, pPolI/P3, pPolI/HE, pPolI/NP, pPolI/M, pPolI/NS, or pPolI/NP-AA.GFP(−)], together with the nine virus protein-expressing plasmids. At 48 h p.t., the total RNA in the cytoplasm of the transfected cells was extracted by using an RNeasy minikit (QIAGEN) according to the manufacturer's instructions. Simultaneously, the VLPs generated in the medium were collected as reported previously (19), and vRNAs were extracted from the VLPs. Each of the obtained RNA preparations was treated with DNase I (Fermentas) to degrade the residual plasmid DNAs. The cDNA was synthesized using a primer complementary to the 12 nucleotides of the 3' end of vRNA (15), and PCR was performed with a pair of primers specific for the gene of interest. The products were then electrophoresed in 2.0% agarose gel.

Electron microscopy. Recombinant viruses propagated in eggs or HMV-II cells were pelleted by ultracentrifugation at 35,000 rpm for 60 min. The obtained pellet was resuspended in TSE buffer, layered onto a 30/60% sucrose cushion, and centrifuged at 25,000 rpm for 90 min. The virus band was collected and pelleted by ultracentrifugation, and the obtained virions were negatively stained (19), followed by observation in an electron microscope (JEOL, Ltd.) at 80 kV.

Membrane flotation analysis of infected cells. Flotation analysis was performed according to the procedures described previously (12, 25). The HMV-II cells infected with recombinant viruses were washed twice with ice-cold phosphate-buffered saline at 20 h p.i. The cells were then resuspended in 0.3 ml of lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 5 mM MgCl₂, and a protease inhibitor cocktail [Calbiochem]). After incubation on ice for 30 min, the cells were disrupted by repeated passage (35 times) through a 26-gauge needle. Unbroken cells and nuclei were removed by centrifugation at 1,000 × g for 5 min at 4°C. The postnuclear supernatant (0.25 ml) was dispersed into 1.75 ml of 80% sucrose (wt/vol) in TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA), placed at the bottom of the tube, and then overlaid with 6.5 ml of 65% sucrose (wt/vol) in TE and 3.25 ml of 10% (wt/vol) of sucrose in TE. The gradient was centrifuged to equilibrium at 180,000 × g for 18 h at 4°C. Fractions (1.17 ml) were collected from the top, and the density of each fraction was determined on a refractometer (ATAGO Co., Ltd., Tokyo, Japan). Aliquots of the fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting (19). For immunoprecipitation, at 20 h p.i. the recombinant virus-infected cells were pulse-labeled with [³⁵S]methionine for 15 min, chased for 2.5 h, and treated as described above. After fractionation, each fraction was added to an equal volume (1.17 ml) of 2× radioimmunoprecipitation assay buffer, and immunoprecipitated with anti-AA/50 serum. The immunoprecipitates were analyzed by SDS-PAGE and processed for fluorography (32). For analysis of the association of the virus proteins with lipid rafts, the infected cells were labeled with [³⁵S]methionine for 15 min at 24 h p.i., chased for 4 h, and lysed in NTE buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl₂) containing 0.45% Triton X-100 and a protease inhibitor cocktail. The lysate was then fractionated and immunoprecipitated as described above, except that the sucrose solutions were made with NTE.

Nucleotide sequence accession number. The nucleotide sequence newly determined in the present study has been submitted to the DDBJ/EMBL/GenBank databases and assigned the accession number AB283001 (Table 1).

TABLE 1. The number of nucleotides of C/Ann Arbor/1/50 consensus sequence^a

RNA segment (gene)	No. of nucleotides				Accession no.
	Total	3'NCR	Coding region	5'NCR	
1 (PB2)	2,365	21	2,325	19	AB126191
2 (PB1)	2,363	17	2,265	81	AB126192
3 (P3)	2,183	21	2,130	32	AB126193
4 (HE)	2,073	21	1,968	84	AB126194
5 (NP)	1,807	29	1,698	80	AB126195
6 (M)	1,180	25	1,125	30	AB126196
7 (NS)	935	26	862	47	AB283001 ^b

^a The consensus sequence of this strain was determined as described in Materials and Methods. The total number of the nucleotides was 12,906. NCR; noncoding region.

^b The consensus sequence of the RNA segment 7 was determined in this study.

RESULTS

Generation of recombinant C/Ann Arbor/1/50. Prior to generation of the recombinant C/Ann Arbor/1/50 (AA/50), we determined the consensus sequence of the strain. As reported for RNA segment 5 (NP gene) (19), we determined the nucleotide sequences of the 5' and 3' ends of the remaining six segments by 5'RACE and then determined the consensus sequence of the whole region of each segment as described in Materials and Methods. As a result, the seven RNA segments of AA/50 consisted of 12,906 bases (Table 1). To the best of our knowledge, this is the first report in which the total nucleotide sequences of an influenza C virus strain have been documented.

To rescue the recombinant AA/50 (recombinant wild type [rWT]), the seven PolI plasmids were transfected into 293T cells together with four (PB2, PB1, P3, and NP) virus-protein expressing plasmids, as reported for influenza A and B viruses. At 48 to 60 h p.t., the supernatant of the 293T cells was serially diluted and inoculated onto MDCK or LLC-MK₂ monolayers for plaque purification (27), which resulted in no apparent plaque formation. Therefore, in order to detect infectious recombinant viruses using a more sensitive method, the serially 10-fold-diluted supernatants were inoculated into the amniotic cavity of 8- or 9-day-old chicken eggs. After incubation for 3 days, the amniotic fluids collected from each egg were tested for hemagglutination. The fluids positive for hemagglutination were reproducibly detected, enabling us to calculate the infectious titer of the culture medium. Three independent experiments revealed that the EID₅₀/ml of the medium was 1.62×10^1 to 1.10×10^2 . We prepared virus stock of the rWT by using chicken eggs and confirmed that the whole nucleotide sequences of the stock rWT were identical to those of the cDNAs in the PolI plasmids used for transfection (data not shown).

Attempts were made to generate rWT more efficiently, since the generation efficiency of rWT (10^1 to 10^2) was much lower than that (10^6 to 10^7) of recombinant influenza A virus (20; our unpublished results). The seven PolI plasmids and nine (PB2, PB1, P3, HE, NP, M1, CM2, NS1, and NS2) virus-protein expressing plasmids were transfected into 293T cells. In each of three independent experiments, rWT was actually rescued, and the titers of the media were 1.58×10^3 , 2.51×10^2 , and 2.51×10^3 EID₅₀/ml. A number of independent transfections with higher amounts of plasmids (up to 15 µg per dish) resulted in

the reproducible recovery of rWT, but the titer did not exceed 10^3 EID₅₀/ml. Longer incubation of transfected 293T cells for up to 7 days in the presence or absence of trypsin did not lead to the recovery of rWT with higher infectious titers (data not shown). Thus, our system of inoculating a transfected 293T cells culture medium into the amniotic cavity of chicken eggs at 48 to 60 h p.t. provides the most time-saving and efficient recovery of recombinant influenza C virus.

Analysis of 293T cells and VLPs containing a given segment of vRNA. The number of influenza A and C VLPs produced from the transfected 293T cells is more than 10^4 per dish (21) and approximately 10^6 /ml (19), respectively. In contrast, the infectious titer of the plasmid-transfected supernatant is 10^6 to 10^7 PFU/ml for recombinant influenza A virus (20) and 10^1 to 10^3 EID₅₀/ml for influenza C virus (see above). Thus, in contrast to influenza A virus, recombinant infectious influenza C viruses seem to be generated much less efficiently than influenza C VLPs.

To verify that each PolI plasmid in our system functions, we examined the individual vRNAs synthesized in the transfected cells, since the presence of limited amounts of a vRNA segment(s) in the cells may account for the low efficiency of infectious virus generation. Each of the PolI plasmids of PB2, PB1, P3, HE, NP, M, NS, or GFP was transfected into 293T cells, together with the nine virus-protein expressing plasmids. At 48 h p.t., vRNA in the 293T cytoplasm was extracted and examined by reverse transcription-PCR. As shown in Fig. 1A, the amount of PCR products was similar in all constructs, indicating that all vRNA segments existed in the cytoplasm in similar amounts. Furthermore, Western blotting analysis of the transfected 293T cells revealed no significant differences in the amount of major virus proteins—such as HE, NP, and M1—between the constructs (data not shown).

At the same time, we analyzed the VLPs generated from transfected 293T cells, because the presence of vRNA segment(s) with limited incorporation efficiency into VLPs should result in a lower infectious virus generation efficiency. The VLPs were collected as described previously (19) and analyzed by Western blotting and RT-PCR. A representative result from three independent experiments is shown in Fig. 1B. There were no specific vRNA segments incorporated into VLPs with significantly reduced efficiency. However, the amount of the segment 1, 2, and/or 3 vRNA tends to be less than that of the other constructs in each experiment, suggesting that these segments were incorporated less efficiently than the others (Fig. 1B). Taken together, these data suggest that the presence of less efficiently incorporated vRNA(s) may be one of the reasons for inefficient infectious virus production.

Growth kinetics and morphology of a recombinant virus possessing an Ala→Thr substitution at residue 24 of M1 protein. In our previous study on the generation of influenza C VLPs, we demonstrated that the amino acid (Ala/Thr) at residue 24 of the M1 protein is involved in the morphology (filamentous or spherical) of the VLPs (19). In the present study, to investigate the role of this residue in virus replication and morphology, we attempted to rescue a recombinant virus, rMG96A, in which the amino acid Ala at residue 24 of the M1 protein was substituted to Thr. The plasmid, pPolI/M-G96A, was constructed and transfected into 293T cells with the other six PolI plasmids (pPolI/PB2, pPolI/PB1, pPolI/P3, pPolI/NP,

FIG. 1. Generation of influenza C VLP containing a given vRNA segment. 293T cells were transfected with one of the indicated PolI plasmids [pPolI/PB2, pPolI/PB1, pPolI/P3, pPolI/HE, pPolI/NP, pPolI/M, pPolI/NS, or pPolI/NP-AA.GFP(-)], together with the nine virus protein-expressing plasmids. A representative result from three independent experiments is shown. (A) At 48 h p.t., RNA was extracted from the cytoplasm of the transfected cells, treated with DNase, reverse transcribed, and then PCR amplified with a set of primer specific for each gene segment. The PCR products were electrophoresed in an agarose gel (upper panel). To rule out the possibility that the RNA preparation was contaminated with residual plasmids, preparations without reverse transcription were used for PCR (middle panel). As an internal control, aliquots of extracted RNA were each applied to reverse transcribed-PCR for chicken β -actin mRNA (lower panel). The number of PCR cycles for chicken β -actin mRNA was adjusted to 18, at which number the amount of the PCR product increased in a log phase. To detect vRNAs, the amount of template from each construct was adjusted according to the ratio of β -actin PCR product, and then PCR (18 cycles) was performed. The sizes of the obtained PCR products were 505 bp for the PB2 gene, 482 bp for the PB1 gene, 528 bp for the P3 gene, 711 bp for the HE gene, 509 bp for the NP gene, 508 bp for the M gene, 502 bp for the NS gene, and 504 bp for the GFP gene. (B) At 48 h p.t., influenza C VLPs were collected from the culture medium and analyzed by Western blotting with antiserum against AA/50 virions (upper three panels). The RNA was extracted from the aliquots of the VLP preparation, treated with DNase, reverse transcribed, and then PCR amplified with a set of primer specific for each gene segment (lowest panel).

pPolI/HE, and pPolI/NS) and four (PB2, PB1, P3 and NP) virus protein-expressing plasmids. Two independent experiments showed that at 48 h p.t. the culture media contained 3.9×10^1 and 1.4×10^2 EID₅₀ of infectious viruses/ml. We extracted vRNA from the rMG96A stock, determined the nucleotide sequences of the M gene, and confirmed that no unwanted mutations had been introduced (data not shown).

FIG. 2. Infection experiments with recombinant viruses. (A) Growth kinetics of recombinant viruses. HMV-II cells were infected with rWT or rMG96A at a multiplicity of infection of 0.005 TCID₅₀ and then incubated in the presence of trypsin (20 μ g/ml). At 4, 6, and 7 days p.i., the culture media were used for the determination of infectious titers (TCID₅₀/ml, see the text). A representative result of three independent experiments is shown. (B) CLS on the infected cells. HMV-II cells were infected with rWT (left panel) or rMG96A (right panel) as described above, and the cells were observed under a light microscope (magnification, $\times 350$) at 4 days p.i. The CLS protruding from the cells are indicated as triangles. (C to F) Electron micrographs of recombinant viruses. The rWT grown in HMV-II cells (C) or the amniotic cavity of chicken eggs (F) and the rMG96A grown in eggs (D) or HMV-II cells (E) were concentrated, negatively stained, and examined under an electron microscope. Bar, 100 nm.

We then investigated the growth kinetics and morphology of the recombinant viruses. The rWT showed growth kinetics similar to that of C/Ann Arbor/1/50 strain (data not shown), and the rWT grew a little more efficiently (2.1×10^5 TCID₅₀/ml at 7 days p.i.) than did rMG96A (4.6×10^4 TCID₅₀/ml) (Fig. 2A). As expected, the cord-like structures (CLS), demonstrated to be a bundle of filamentous virions (19, 23, 24), were observed on rWT-infected, but not rMG96A-infected HMV-II cells (Fig. 2B). The electron microscopic analyses of rWT and rMG96A showed that rWT grown in HMV-II cells exhibited a mainly filamentous morphology and a rod-like shape (Fig. 2C), whereas all rMG96A virions

observed were spherical (Fig. 2E). In addition, when the growth and morphology of the recombinant viruses were investigated using chicken eggs, the rWT grew a little more efficiently (~4-fold) than did rMG96A (data not shown), and the rWT exhibited a mainly filamentous morphology (Fig. 2F), whereas rMG96A appeared spherical (Fig. 2D).

Membrane flotation analyses of infected and transfected cells. As shown above, residue 24 of the M1 protein is involved in the morphology of influenza C viruses. To detect any differences between the two recombinant virus-infected cells, we analyzed the infected cells by immunofluorescence and Western blotting at 6, 12, 24, 48, and 72 h p.i. with the monoclonal antibodies to HE, NP, and M1. No significant differences were observed in terms of the accumulation and localization of the virus proteins within the cells (data not shown). Therefore, we conducted an experiment on membrane association of the virus proteins because the morphology of the virions is likely to be affected during the assembly process at the cell membrane.

The recombinant virus-infected HMV-II cells were subjected to equilibrium centrifugation. Prior to detection of the virus proteins, we examined the optic ratio of the 10 fractions obtained. The sucrose concentration rose sharply at fractions 3 and 4 as described previously (25) (data not shown), indicating that the plasma membranes floated up to the 10 to 65% interface (fractions 3 and 4) and were absent from the bottom of the gradient.

Western blotting analysis of the fractionated samples revealed a difference between rWT- and rMG96A-infected cells. The distributions of HE and NP proteins throughout the fractions were similar to each other (Fig. 3A). The amount of membrane-associated M1 proteins from rWT in fractions 3 and 4 accounted for 90% of the total M1 proteins present, whereas that from rMG96A-infected cells accounted for nearly 70% of total M1 protein, as measured by NIH Image v.1.62 (Fig. 3A). To confirm this result, the infected cells were metabolically pulse-labeled, chased, and analyzed by immunoprecipitation. No significant difference in the kinetics of the HE and NP proteins was observed between rWT- and rMG96A-infected cells (Fig. 3B and C). The majority of HE glycoproteins were recovered in the membrane fractions, and the pulse-labeled NP was recovered in the bottom fractions, though some floated up to the membrane fractions during the chase period. On the other hand, unlike the HE and NP proteins, the pulse-labeled M1 proteins were recovered both in membrane and in bottom fractions. In the chase experiment, the amount of membrane-associated M1 proteins in the fractions 3 and 4 was 90% of the total M1 in the rWT-infected cells, which is higher than that (63%) in rMG96A-infected cells (Fig. 3C).

To investigate the intrinsic nature of influenza C virus M1 protein to associate with the plasma membrane, the M1 proteins of AA/50 or C/Taylor/1233/47 (TAY/47), the latter of which has Thr at residue 24 of the M1 protein (19), were expressed in 293T cells from cloned cDNAs and analyzed for membrane flotation. Approximately 68 and 48%, respectively, of the AA/50 and TAY/47 M1 was recovered in the membrane fractions (Fig. 3D). When the M1 proteins were expressed together with the HE glycoprotein of AA/50, the proportion of membrane-associated M1 proteins did not increase in either constructs (data not shown).

The virus proteins in the recombinant virus-infected cells

were analyzed for association to the lipid rafts of the plasma membrane, known to be a budding site for the influenza A virus (30). In our system, the main fractions containing lipid rafts were demonstrated to be fractions 3 and 4, since 19% of A/Aichi/2/68 HA floated up to these fractions in the chase experiments (Fig. 4C). On the other hand, in the rWT- and rMG96A-infected HMV-II cells, only a trace amount of virus proteins were recovered in fractions 3 and 4 (Fig. 4A and B).

DISCUSSION

In the present study, we first described the successful generation of infectious recombinant influenza C virus from cloned cDNAs. However, the culture supernatant of the transfected 293T cells contained less infectious viruses than did those of influenza A virus. The fact that at 48 h p.t. we can reproducibly rescue 10^6 to 10^7 PFU of A/WSN per ml from 293T cells transfected with 12 plasmids (data not shown) indicates (i) that the transfection procedure adopted in our laboratory does not pose any problems in virus generation and (ii) that some intrinsic mechanism(s) unique to influenza C virus generation may exist. The latter notion was supported by the finding that much less influenza C virus (10^1 to 10^2 EID₅₀/ml) was obtained upon transfection of 11 (7 PolII and 4 virus-protein expressing) plasmids (see above). Considering the number of plasmids transfected (11 plasmids for influenza C and 12 for influenza A), it is likely that transfection efficiency itself is not lower in the case of influenza C virus generation than in that of influenza A virus generation.

In the present study, by generating VLPs containing a given vRNA segment, we demonstrated that there were no significant differences in the synthesis of individual vRNAs in the transfected cells but that there were slight differences in incorporation efficiency between vRNA segments. Therefore, the inefficient production of infectious recombinant viruses may be attributable to the presence of inefficiently incorporated vRNA segment(s).

Alternatively, another mechanism underlying the inefficient production of infectious influenza C viruses should also be taken into account. In the case of influenza A virus, 10^4 VLPs and 10^6 to 10^7 PFU of infectious viruses/ml were rescued, respectively, with viruses containing seven or six segments being rescued with lower efficiency (7), suggesting that influenza A viruses containing a full set (eight) of vRNA segments were generated most efficiently. In the present study, the amount of NP protein and vRNA in the VLPs containing GFP-vRNA was reproducibly lower than those of VLPs containing a given vRNA segment (PB2, PB1, P3, HE, NP, M, or NS) (Fig. 1B), indicating that the number of each VLP containing a given vRNA segment was at least 10^6 /ml. Despite this fact, infectious influenza C viruses were rescued less efficiently (10^3 EID₅₀/ml at most), suggesting that influenza C viruses containing a full set (seven) of vRNA segments were generated much less efficiently than were VLPs. Based on these findings, we postulate the possibility that, unlike influenza A virus, another reason for the lower efficiency of infectious influenza C virus generation is the less efficient incorporation of more than two vRNAs into one particle, and we are currently addressing this problem.

In our previous report, we provided evidence that the amino acid at residue 24 of the M1 protein (Ala/Thr) is involved in

FIG. 3. Membrane flotation analyses of infected or transfected cells. (A) HMV-II cells were infected with rWT or rMG96A at a multiplicity of infection of 5 TCID₅₀, and the postnuclear supernatants were subjected to flotation analysis at 20 h p.i. as described in Materials and Methods. Virus proteins in each fraction were analyzed by Western blotting with antiserum against the AA/50 virion. Membrane-bound material floats up to the interface between 10 and 65% sucrose (fractions 3 and 4). A representative result of three independent experiments is shown. (B and C) HMV-II cells infected with rWT or rMG96A were pulse-labeled with [³⁵S]methionine for 15 min at 20 h p.i. (B) and chased for 2.5 h (C). The cells were subjected to flotation analysis. Virus proteins in each fraction were immunoprecipitated with antiserum against the AA/50 virion and analyzed by SDS-PAGE. A representative result of two independent experiments is shown. (D) 293T cells transfected with pCAGGS.MCS/M1-AA or pCAGGS.MCS/M1-TAY were subjected to flotation analysis and analyzed by Western blotting. The latter plasmid expresses the M1 of C/Taylor/1233/47, which has a Thr at residue 24.

the morphology (filamentous or spherical) of the VLPs, as well as in the formation of the cords, which are composed of bundles of filamentous virions on the transfected cells (19). In the present study, to investigate the role of the substitution, a mutant recombinant influenza C virus, rMG96A, was generated. As expected, the cords were observed only on rWT-infected cells (Fig. 2B), and the rWT exhibited a mainly filamentous morphology, whereas all rMG96A virions observed were spherical (Fig. 2C to F), indicating that residue 24 of the

M1 protein is involved in virion morphology. Furthermore, rWT grew a little more efficiently than did rMG96A (Fig. 2A), suggesting that filamentous virions may have some advantage over spherical virions in terms of replication.

The association of M1 proteins with the plasma membrane of virus-infected cells was investigated, and differences in the affinity of the M1 protein to the membrane were demonstrated (Fig. 3A to C). These data suggest that the amino acid Ala or Thr at residue 24 of the M1 protein affects the morphology of

FIG. 4. Raft association of virus proteins. HMV-II cells infected with rWT (A) or rMG96A (B) were pulse-labeled and chased for 4 h. The cells were treated with an NTE buffer containing 0.45% Triton X-100 on ice, and the lysates were subjected to flotation analysis. Virus proteins in each fraction were immunoprecipitated with antiserum against the AA/50 virion and analyzed by SDS-PAGE. (C) HMV-II cells infected with A/Aichi/2/68 were pulse-labeled for 15 min at 10 h p.i. and chased for 1.5 h. After fractionation, the HA protein in each fraction was immunoprecipitated with antiserum against the A/Aichi/2/68 virion and analyzed.

the virion by altering the affinity of the protein to the cell membrane, although the possibility cannot completely ruled out that the mutated M1 protein tends to be more readily aggregated, resulting in the increased amount found in the bottom fractions 9 and 10. Since influenza C virus M1 protein is located beneath the membrane and may play a central role in virus budding, as has been reported for influenza A virus, it is likely that the affinity to the membrane influences the morphology of the influenza C virions. The M1 protein of rWT, which associated firmly with the membrane, may have stronger propensity to virus budding, leading to filamentous virion formation. The M1 of rMG96A, which associated weakly with the membrane, may readily allow pinching off, resulting in spherical virion formation. If this is the case, the amino acid sequences around residue 24 of the M1 protein may provide a novel motif for the late domain (L domain). The possibility of the involvement of the other virus protein(s) and/or cellular

factor(s) in CLS formation, as well as the determination of virion morphology, remains to be investigated.

We previously hypothesized that residue 24 is involved in membrane association since the residue is located in a stretch of hydrophobic region (23-TAIISAITGGK-33, where 24 is underlined) of the M1 protein (19, 24). Computer-assisted analysis (Genetyx Mac) of the M1 protein, however, revealed that a mutation of Ala to Thr at residue 24 did not alter the regional hydrophilicity/hydrophobicity profile (data not shown), indicating that a substitution that does not affect the hydrophilicity/hydrophobicity is involved in membrane association. Using a series of mutated influenza A virus M1 proteins, Kretzschmar et al. (16) showed that specific hydrophobic domains are apparently not required for membrane binding. Taken together, affinity to the membrane appears to be not solely determined by the hydrophilicity/hydrophobicity of a protein.

As shown in Fig. 3D, the AA/50 M1 protein had a slightly higher affinity to the plasma membrane than did TAY/47 M1, suggesting that the difference in M1 membrane association observed in virus-infected cells may be due in part to an intrinsic nature of influenza C virus M1 proteins. Unlike the observation on influenza A virus M1 protein (5), coexpression of HE glycoprotein with the M1 proteins did not lead to an increase in the proportion of membrane-associated M1 proteins (data not shown). This discrepancy in the data between infected and transfected cells suggests that another virus protein, such as NP (RNP), may be involved in M1 membrane association.

Raft association of virus proteins in the infected cells was also investigated, but only a small amounts of the proteins were recovered in fractions 3 and 4 (Fig. 4A and B). Therefore, we are not able to discuss the differences based on such a small amounts of proteins. This finding was consistent with the previous result that only a little HE glycoprotein of C/Ann Arbor/1/50 was recovered in the insoluble fraction of virus-infected MDCK cells (34). Taken together, these data suggest that the budding of influenza C virus may occur at the regions other than the lipid rafts and/or, alternatively, only a very limited amount of the virus proteins associated with the lipid rafts is also used for virus budding. The budding site of influenza C virus remains to be elucidated.

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