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Reverse Genetics System for Generation of an Influenza A Virus Mutant Containing a Deletion of the Carboxyl-Terminal Residue of M2 Protein

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We established a reverse genetics system for the M gene of influenza A virus, using amantadine resistance as a selection criterion. Transfection of an artificial M ribonucleoprotein complex of A/Puerto Rico/8/34 (H1N1), a naturally occurring amantadine-resistant virus, and superinfection with amantadine-sensitive A/equine/Miami/1/63 (H3N8), followed by cultivation in the presence of the drug, led to the generation of a transfectant virus with the A/Puerto Rico/8/34 (H1N1) M gene. With this system, we attempted to generate a virus containing a deletion in an M-gene product (M2 protein). Viruses lacking the carboxyl-terminal Glu of M2, but not those lacking 5 or 10 carboxyl-terminal residues, were rescued in the presence of amantadine. These findings indicate that carboxyl-terminal residues of the M2 protein play an important role in influenza virus replication. The M-gene-based reverse genetics system will allow the study of different M-gene mutations to achieve a balance between attenuation and virus replication, thus facilitating the production of live vaccine strains.

The matrix (M) gene of influenza A viruses encodes two proteins, M1 and M2 (15). M2, an integral membrane protein (31), is a homotetramer (11, 22, 29) that is abundantly expressed at the surfaces of virus-infected cells but is a relatively minor component of virions (31). Sharing eight amino-terminal residues with M1, the M2 protein comprises 97 amino acids—24 as the ectodomain, 19 as the transmembrane domain, and 54 as the cytoplasmic domain. M2 proteins have been proposed to function as an ion channel that permits protons to enter the virion during uncoating and modulates the pH of intracellular compartments, the latter being an essential function for prevention of the acid-induced conformational change of the intracellularly cleaved hemagglutinin (HA) in the *trans*-Golgi network (9, 20, 30). The activity of the M2 ion channel is blocked by the anti-influenza drug amantadine hydrochloride (8).

The functional role of the M2 cytoplasmic region is poorly understood. This 54-amino-acid structure is the longest among the influenza virus membrane proteins (12 amino acids for the HA and six for the neuraminidase). M2 coprecipitates with the ribonucleoprotein core prepared from purified virus (1), indicating high affinity for ribonucleoprotein, presumably involving the M2 cytoplasmic tail. These lines of evidence suggest that the M2 tail could play an important role in virus replication and influenza pathogenesis.

Palese and colleagues were able to generate influenza A viruses that contained the influenza virus genes (5, 6) derived from cDNA clones (reverse genetics). With this technique, influenza A viruses with mutations in the coding and noncoding regions of the PB2 (28), HA (16, 17, 32), neuraminidase (2, 18, 19), and NS (7) genes have been made, allowing one to analyze the roles of structural features of viral proteins in the replication. Here we describe the establishment of a reverse genetics system for the M gene of influenza A virus and its use to generate a virus containing a deletion of the carboxyl-terminal residue of the M2 protein.

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MATERIALS AND METHODS

Viruses and cells. Influenza A/equine/Miami/63 (H3N8) (Eq/MIA), A/Puerto Rico/8/34 (H1N1) (PR8), and A/duck/Oklahoma/4/77 (H1N4) viruses were obtained from a repository at St. Jude Children's Research Hospital. The Madin-Darby bovine kidney (MDBK) cell line was cultured in Eagle's minimal essential medium containing 10% fetal calf serum. Madin-Darby canine kidney (MDCK) cells were cultured under the same conditions as MDBK cells, except that 5% calf serum was used.

Reverse genetics. A plasmid (pPR8M-10) containing the PR8 M gene was constructed as described by Huddleston and Brownlee (12). A second plasmid (pUCT3PRM), containing the PR8 M gene flanked by the *Ksp*632I site and T3 RNA polymerase promoter sequence, was made by cloning the PCR (24) product made with pPR8M-10 as a template and with primers 5′-ATCGATGAAT TCTCTTCGAGCGAAAGCAGGTAGATATTG-3′ and 5′-GAGGACAAGC TTATTAACCCTCACTAAAAGTAGAAACAAGGAGTTTTTTACT-3′. pUCT3PRM contains a T3 RNA polymerase promoter upstream and a *Ksp*632I site downstream of the M gene, so that viral sense RNA transcripts are generated when digested with *Ksp*632I, filled in with Klenow fragment, and transcribed with T3 RNA polymerase (5). pUCT3COOH-1, pUCT3COOH-5, and pUCT3COOH-10 were constructed by replacing M-gene nucleotides, which convert the M2 carboxyl-terminal Glu, amino acid residue 93, and amino acid residue 88 codons, respectively, to stop codons by oligonucleotide-directed mutagenesis (14) (Fig. 1).

Nucleoprotein (NP) and polymerase (P) proteins were purified from A/duck/Alberta/35/76 by glycerol and glycerol-cesium chloride (CsCl) gradients as previously described (23). An artificial M ribonucleoprotein complex was prepared by transcribing pUCT3PRM, pUCT3COOH-1, pUCT3COOH-5, or pUCT3COOH-10 with T3 RNA polymerase in the presence of the NP and P after these plasmids were digested with *Ksp*632I and filled in with Klenow fragment as described previously (5, 6). The M ribonucleoprotein complex was then transfected into 70 to 90% confluent MDBK cells infected 1 h before transfection with Eq/MIA at a multiplicity of infection of 1. Eighteen hours after transfection, MDCK cells were infected with transfectants in supernatant fluid in the presence of amantadine (1 μg/ml). Three days later, viruses in the supernatant were plaque purified in MDCK cells three times in the presence of amantadine (1 μg/ml) and then inoculated into embryonated eggs. The M gene of the viruses

		Rescue
	83 90 97	
wt PR8 M2	... Ala Val Asp Ala Asp Asp Gly His Phe Val Ser Ile Glu Leu Glu .	+
	... gct gtg gat gct gac gat ggt cat ttt gtc agc ata gag ctg gag taa	
COOH-1	... Ala Val Asp Ala Asp Asp Gly His Phe Val Ser Ile Glu Leu .	+
	... gct gtg gat gct gac gat ggt cat ttt gtc agc ata gag ctg tag taa	
COOH-5	... Ala Val Asp Ala Asp Asp Gly His Phe Val .	-
	... gct gtg gat gct gac gat ggt cat ttt gtc tga ata gag ctg gag taa	
COOH-10	... Ala Val Asp Ala Asp .	-
	... gct gtg gat gct gac taa ggt cat ttt gtc agc ata gag ctg gag taa	

FIG. 1. Amino acid and nucleotide sequences of wild-type (wt) M2 and M2 mutants. The carboxyl-terminal portion of each M2 protein is shown. Nucleotides mutated to introduce a stop codon are underlined. +, virus was generated; −, virus was not generated.

was sequenced as described previously (13) to confirm the origins of the gene and the intended mutations and to ensure that no unwanted mutations had occurred.

Immunologic methods. HA titration and HA inhibition tests were performed with receptor-destroying enzyme-treated antisera in microtiter plates (21).

Studies with ferrets. To measure viral replication in vivo, we anesthetized 5-month-old ferrets (four per group; Marshall Farms, North Rose, N.Y.) intramuscularly with ketamine-HCl (50 mg) and infected them intranasally with 1 ml of virus (approximately 10^7 PFU). The animals' nostrils were washed out with 1 ml of phosphate-buffered saline, pH 7.2, and the virus in nasal wash samples was titrated in MDCK cells.

For protection assays, ferrets were infected with the wild-type virus (10^7 PFU) 3 weeks after initial infection, and virus titers in nasal wash samples were determined as described above.

RESULTS

Generation of an influenza virus containing the M gene derived from cloned cDNA. To generate a transfectant virus with the M gene derived from cloned cDNA, we followed the basic reverse genetics procedure of Palese and colleagues (5, 6), which requires a strong selection system for the virus with the rescued gene because the majority of virus in the transfection supernatant is helper virus. We therefore chose selection for amantadine resistance conferred by the M2 protein of the PR8 strain, a naturally amantadine-resistant virus, as a method to rescue its M gene, with amantadine-sensitive Eq/MIA as a helper virus. PCR amplification and partial sequencing of the M gene of viruses derived from individual plaques grown in the presence of amantadine showed that approximately 50% of the plaques represented viruses with the PR8 M gene (designated Eq/MIA-PR8M). All remaining plaques were considered amantadine-resistant mutants of the helper Eq/MIA virus and were not examined further. Thus, our selection system permits the generation of influenza A viruses containing the M gene derived from cloned cDNA, allowing the generation of a virus with mutations introduced in this gene.

The carboxyl-terminal Glu in the M2 protein is not essential for viral replication. Using the selection system described above, we examined the importance of the carboxyl-terminal residues of the M2 protein for virus replication. The total number of M2 amino acid residues is identical among all influenza A viruses examined (9), suggesting that the entire M2 protein is important for normal functioning. We therefore attempted to generate influenza A viruses with carboxyl-terminal deletions in their M2 proteins. Attempts to generate viruses with either 5 or 10 residues deleted (COOH-5 or COOH-10) were unsuccessful; however, a mutant with a carboxyl-terminal Glu deletion (COOH-1) was rescued with an efficiency comparable to that of viruses with the parental (PR8 M) gene.

The COOH-1 mutant produced slightly smaller plaques on MDCK cells than did the transfectant virus with the PR8 M gene (2-mm compared with 3-mm diameter on day 3). When examined in ferrets, the mutant had a 10-fold-lower titer (2.3 ± 0.5 $\log_{10}$ PFU/ml) than did the Eq/MIA-PR8M parent (3.5 ± 0.8 $\log_{10}$ PFU/ml) 3 days after infection. It also replicated for a shorter period than did the wild-type virus (3.0 ± 0.0 compared with 4.5 ± 0.5 days). Taken together, these findings suggest that the carboxyl-terminal Glu deletion may have attenuated the virus.

To determine if the COOH-1 mutant could confer protective immunity against the wild-type virus, we inoculated ferrets with Eq/MIA-PR8M (10^7 PFU) 3 weeks after infection with COOH-1. No virus was recovered from nasal wash samples from animals previously infected with the mutant 3 days after challenge, whereas uninfected control ferrets ($n = 2$) shed more than 10^3 PFU of virus. Thus, the COOH-1 virus retained sufficient immunogenicity to protect animals against subsequent challenge with the wild-type virus.

We next examined the stability of the mutation during virus replication in ferrets. Sequencing of the M gene of the COOH-1 mutant recovered from two ferrets on the last day of shedding (3 days after infection) failed to reveal additional mutations (i.e., the stop codon was retained) in five plaque-purified clones (two from ferret 1 and three from ferret 2).

DISCUSSION

In this study, we established a rescue system for the M gene of influenza A viruses whose efficiency is adequate for generating a transfectant that contains a mutation in the M2 protein. Failure to generate a virus with either a 5- or a 10-amino-acid deletion of the M2 carboxyl-terminal residues indicates that these deletions either are lethal or affect virus replication to such a degree that the rescue of viruses with such deletions is inhibited in the reverse genetics system used here. These findings suggest that the M2 tail plays an important role in virus replication and influenza virus pathogenesis. The reason for the failure to generate the COOH-5 and COOH-10 mutants is

unknown. One possibility is that specific signals required for interaction of the M2 carboxyl terminus with other viral proteins were obliterated by the mutations. Another is that such deletions may have resulted in misfolding of the proteins, inhibiting their transport to the cell surface.

Cold-adapted, live influenza virus vaccines have been extensively investigated and hold promise for use in the general population (25–27). The major concern with these vaccines is that the limited number of attenuating mutations (4, 10) could permit the generation of a revertant virus in the field, although in clinical trials the viruses have maintained the attenuating phenotype. One way to avoid this hazard would be to introduce multiple attenuating mutations into the viral genes. It would be desirable to generate viruses that contain mutations in multiple genes encoding the internal proteins. This strategy would yield more stably attenuated mutants that could serve as donor strains for vaccine viruses that contain the HA and neuraminidase genes of currently circulating viruses. The level of attenuation of COOH-1 virus in ferrets was limited. However, the establishment of an M-gene rescue system should facilitate efforts to further identify attenuating mutations in the M-gene products. With the system described here, together with previously reported systems with PB2 (28) and NS (7), it will be possible to screen different combinations of mutations for an optimal balance between attenuation and viral replication.

Amantadine resistance, the selection pressure used to rescue the M gene from cloned cDNA, is not a desirable feature for vaccine strains, because amantadine and its derivative, rimantadine, are the only licensed drugs against influenza A virus. Thus, an alternative system that would generate viruses with attenuating mutations in the M gene and not introduce amantadine resistance is needed. This objective could be met if viruses with a specific M-gene-determined host range were available. Several influenza viruses with mutations in the neuraminidase gene have been generated by reverse genetics, and their host ranges differ from that of the parent virus (3, 18). Thus, one should be able to produce similar viruses with mutations in the M gene, which could be used as helper viruses in the generation of amantadine-sensitive transfectant viruses.

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