Reverse Genetics Provides Direct Evidence for a Correlation of Hemagglutinin Cleavability and Virulence of an Avian Influenza A Virus

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To obtain direct evidence for a relationship between hemagglutinin (HA) cleavability and the virulence of avian influenza A viruses, we generated a series of HA cleavage mutants from a virulent virus, A/turkey/Ontario/7732/66 (H5N9), by reverse genetics. A transfectant virus containing the wild-type HA with R-R-R-K-K-R at the cleavage site, which was readily cleaved by endogenous proteases in chicken embryo fibroblasts (CEF), was highly virulent in intramuscularly or intranasally/orally inoculated chickens. By contrast, a mutant containing the HA with an avirulent-like sequence (R-E-T-R) at the cleavage site, which was not cleaved by the proteases in CEF, was avirulent in chickens, indicating that a genetic alteration confined to the HA cleavage site can affect cleavability and virulence. Mutant viruses with HA cleavage site sequences of T-R-R-K-K-R or T-T-R-K-K-R were as virulent as viruses with the wild-type HA, whereas a mutant with a two-amino-acid deletion but retention of four consecutive basic residues (R-K-K-R) was as avirulent as a virus with the avirulent-type HA. Interestingly, although a mutant containing an HA with R-R-R-K-T-R, which has reduced cleavability in CEF, was as virulent as viruses with high HA cleavability when given intramuscularly, it was less virulent when given intranasally/orally. We conclude that the degree of HA cleavability in CEF predicts the virulence of avian influenza viruses.

Although polygenic in origin, the virulence of influenza viruses is influenced greatly by the hemagglutinin (HA) molecule (23, 46, 57), its cleavability in particular (4, 5). Posttranslational proteolytic cleavage of the precursor HA molecule (HA0) into HA1 and HA2 subunits, which generates a fusogenic domain at the amino terminus of HA2, is essential for entry of the virus into cells, with fusion occurring between the virus envelope and the cell endosomal membrane (58). Virulent avian influenza viruses, restricted to the H5 and H7 subtypes, cause systemic, lethal infection in poultry, whereas mammalian and avirulent avian viruses cause local infection in the respiratory or intestinal tract or both. In tissue culture, the HAs of virulent viruses are cleaved in the absence of exogenous proteases, such as trypsin, whereas those of avirulent viruses are not, indicating different sensitivities to endogenous cellular proteases. These findings implicate HA cleavability as one of the determinants of tissue tropism of influenza viruses and suggest that tissue variability among the proteases is responsible for the cleavage of different HAs.

The HA cleavability of influenza viruses has been studied extensively either by selecting variants on the basis of their cleavage properties in cell culture (19, 27, 39, 41, 45, 51) or by site-specific mutagenesis of the HAs in *in vitro* expression systems (17, 18, 40, 51, 52). The results have indicated that two structural features—a series of basic amino acids at the cleavage site and the presence or absence of a carbohydrate side chain in the near vicinity—are crucial for determining HA cleavability by proteases. In order for the HA to be cleaved completely by the endogenous proteases in cell culture, at least

six basic amino acids have to be present at the cleavage site if a carbohydrate side chain is nearby. Otherwise, only four basic amino acids are needed. It has also been shown that a minimal sequence requirement for the HA cleavage by proteases in CV-1 cells, derived from African green monkey kidney, is the motif R/K-X-R/K-R.

The reverse genetics system, by which cloned genes can be incorporated into influenza viruses (10, 11, 32, 35), allows one to generate mutant viruses by strategic engineering of the influenza virus genes. With this system, several mutant viruses (3, 8, 30, 33, 36) or chimeric recombinant viruses carrying foreign epitopes (7, 28, 29, 31) have been generated. Reverse genetics allows one to examine the biologic effects of specific mutations by using viruses with otherwise identical genetic backgrounds. We have used this system to directly assess the relationship between HA cleavability and the virulence of avian influenza viruses. Specifically, we wished to know whether genetic alterations limited to the HA cleavage site can affect virulence. Also, are viruses with reduced HA cleavability virulent, and can one identify the sequence requirement at the HA cleavage site for high virulence?

MATERIALS AND METHODS

Viruses and cells. The A/turkey/Ontario/7732/66 (H5N9) influenza virus (Ty/Ont) (25) was obtained from the repository at St. Jude Children's Research Hospital. A reassortant virus, WSN-Ty/Ont (H1N9), which contains the HA gene from A/WSN/33 (H1N1) and all remaining genes from Ty/Ont (2), was used as a helper virus for rescue of the Ty/Ont HA gene by reverse genetics. Madin-Darby bovine kidney (MDBK) cells were cultured in Eagle's minimum essential medium (MEM; GIBCO) supplemented with 10% newborn calf serum. Madin-Darby canine kidney (MDCK) cells were cultured in MEM with 5% calf serum. Chicken embryo fibroblasts (CEF), pre-

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FIG. 1. Schematic diagram of construction of plasmids for Ty/Ont HA rescue. pUC19-derived plasmid pT3TOH36-2 contains the complete sequence (nucleotides 1 to 1773) of the wild-type HA gene of Ty/Ont, the bacteriophage T3 RNA polymerase promoter, and a *Ksp632I* site. The striped boxes indicate the regions (nucleotides 1043 to 1061) that encode the amino acid sequence immediately upstream of the HA cleavage site. pT3TOH(MO-C) contains two silent mutations (nucleotides 1028 and 1063) resulting in *KspI* and *StuI* sites, respectively. pT3TOH(MO-1), -(MO-2), -(MO-4), -(MO-12), and -(MO-41) contain altered sequences replaced between *KspI* and *StuI* sites of pT3TOH(MO-C).

pared from 10-day-old chicken embryos, were cultured in MEM with 10% calf serum. Experiments with infectious Ty/Ont and its HA cleavage mutant viruses were performed in a P3 containment laboratory at St. Jude Children's Research Hospital, approved for such use by the U.S. Department of Agriculture.

Construction of plasmids. A full-length cDNA copy of the Ty/Ont HA gene was cloned by a previously described method (13). Sequencing of two full-length clones revealed an amino acid sequence of P-Q-R-R-K-K-R at the HA cleavage site and another of P-Q-R-R-R-K-K-R. Other regions of the HA contained identical sequences. Our HA clone differed from the Ty/Ont HA gene in GenBank (43) (accession number M30122) as follows: A-18→G (noncoding region), G-142→A (silent), A-1077→T (K→I), and C-1608→A (N→T). Ty/Ont HA is most likely to have a carbohydrate side chain in the near vicinity of cleavage site, which influences accessibility to proteases, as judged from the presence of a potential glycosylation site in the sequence (N-10-N-S-T). Other HAs containing this sequence in the same region were shown to be glycosylated by direct amino acid sequencing (9, 18). We selected the HA clone containing the P-Q-R-R-R-K-K-R sequence at the cleavage site (pTOH36-2) as the parent plasmid for rescue experiments because it allowed us to exploit the results of an earlier in vitro expression study with A/turkey/Ireland/1378/83 (H5N8) (Ty/Ire) HA (P-Q-R-K-R-K-K-R at the cleavage site with a carbohydrate side chain in the near vicinity) (16, 17, 52) in the design and evaluation of mutant viruses in the present analysis. A plasmid (pT3TOH36-2) used for the generation of viral sense RNA transcripts for in vitro reconstitution of HA-ribonucleoprotein (RNP) complex was prepared by cloning the PCR (48) product, using pTOH36-2 as a template with

primers 5'-ATATATGGATCCCTCTTCGAGCAAAAGCA GGGGTCTGAT-3' and 5'-GCGCGCGGATCCTTATTAAC CCTCACTAAAAGTAGAAACAAGGGTGT'TTTTA-3', into the pUC19 *BamHI* site (Fig. 1).

We then introduced two unique restriction enzyme sites, *KspI* and *StuI*, into the HA gene of pT3TOH36-2 at both sides of the region encoding the cleavage site (without altering the amino acid sequence) by site-directed mutagenesis (24) with the oligonucleotide 5'-TGGTCCTTGCAACAGGACCGCG GAACGTGCCTCAAAGAAGAAGAAAAAAGAGGC CTGTTTGGAGCAATAGCAGG-3'. The resulting plasmid, pT3TOH(MO-C), was used for the preparation of the plasmids containing mutations at the HA cleavage site by using successive cassette mutagenesis of double-strand oligonucleotides (Fig. 1). Mutant plasmids pT3TOH(MO-1), -(MO-2), -(MO-4), -(MO-12), and -(MO-41) include the sequences encoding amino acids R-R-R-K-T-R, R-K-K-R, R-E-T-R, T-T-R-K-K-R, and T-R-R-K-K-R, respectively, at the cleavage site (Table 1).

Reverse genetics system. Nucleoprotein (NP) and polymerase (P) proteins were purified from egg-grown WSN-Ty/Ont with the use of glycerol and glycerol-cesium chloride gradients as described previously (42). An artificial HA-RNP complex was prepared in vitro by transcription of the plasmids with T3 RNA polymerase in the presence of the purified NP and P, after the plasmids were digested with *Ksp632I* and filled in with Klenow fragment as described previously (11). The in vitro-reconstituted HA-RNP complex was then transfected into 80% confluent MDBK cells infected 1 h before transfection with WSN-Ty/Ont at a multiplicity of infection of 1. Eighteen hours after transfection, the culture supernatants were harvested and clarified by centrifugation (10,000 × g, 20

TABLE 1. Ty/Ont HA cleavage mutants generated by reverse genetics

Virus	HA cleavage site sequence ^a										HA cleavability in CEF ^b	Plaque formation ^c			
	HA1									HA2 P1		CEF		MDBK, Tr–	MDCK, Tr–
	P-8	P-7	P-6	P-5	P-4	P-3	P-2	P-1	Tr+			Tr–			
R(36-2)	P	Q	R	R	R	K	K	R	/	G	+	++	++	++	++
R(MO-C)	P	Q	R	R	R	K	K	R	/	G	+	++	++	++	++
R(MO-1)	P	Q	R	R	R	K	T	R	/	G	±	++	+	+	+
R(MO-2)	P	Q	—	—	R	K	K	R	/	G	–	++	–	±	±
R(MO-4)	P	Q	—	—	R	E	T	R	/	G	–	++	–	–	±
R(MO-12)	P	Q	T	T	R	K	K	R	/	G	+	++	++	++	++
R(MO-41)	P	Q	T	R	R	K	K	R	/	G	+	++	++	++	++

^a Slashes and dashes indicate the cleavage site and an amino acid deletion, respectively.

^b Summarized from results shown in Fig. 3. +, high cleavability; ±, reduced cleavability; —, not cleavable.

^c Assessed in the presence or absence of trypsin (Tr+ or Tr–) as large (++) , small (+) , very small, turbid (±) , or no (–) plaques.

s), and viruses with the rescued HA genes were selected as described below.

Antibody-mediated virus-trapping system. The selection system is outlined in Fig. 2. The transfection supernatant,

harvested 18 h after transfection, was treated with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (5 µg/ml) at 37°C for 15 min to cleave the HA, added to 24-well tissue culture plates which had been precoated overnight with an-

FIG. 2. Schematic diagram of antibody-mediated virus-trapping system for selection of mutant viruses with rescued H5 HA. Transfection supernatants consisting of helper viruses and phenotypically mixed viruses were treated with trypsin to cleave the HAs and then added to tissue culture plates coated with anti-H5 antibodies (Ab) to trap the viruses with the H5 HA. After washing out of helper viruses, MDCK cell suspension with trypsin was added to the plate. Further propagation of the virus with the H1 HA was prevented by the addition of anti-H1 antibody in the medium. Propagated viruses with the rescued H5 were harvested 2 to 3 days (d) later and cloned biologically in egg-limiting dilutions.

ti-H5 monoclonal antibodies (1:1,000 dilution), subsequently blocked with 3% bovine serum albumin (BSA), and incubated at 37°C for 90 min. Wells were washed five times with MEM containing 0.3% BSA to remove unadsorbed viruses. Then, the MDCK cell suspension (5×10^5 per well) in Opti-MEM (GIBCO)–0.3% BSA with trypsin (500 ng/ml) was added to the wells, and the plate was incubated at 37°C. Two hours later, rabbit anti-WSN (H1) serum (ranging from 1:250 to 1:1,000) was added to the culture medium to inhibit further replication of the helper virus, and the plate was incubated at 37°C. When cytopathic effects were advanced, the supernatant was harvested. The viruses with the rescued HA in the supernatants were biologically cloned by three successive limiting dilutions in eggs. The HA gene of the viruses after the third passages was sequenced to confirm the intended mutations and used for subsequent experiments.

HA cleavability of mutant viruses in cell cultures. Radioimmunoprecipitation (RIP) analysis was performed to determine the HA cleavability of the mutant viruses. The viruses were inoculated to confluent CEF and adsorbed for 60 min. Cells were washed to remove unadsorbed viruses and incubated with methionine-free MEM for 30 min. Then, 250 μ Ci of Tran[³⁵S] label (ICN Radiochemicals) per ml was added to the medium. The culture supernatant was harvested at 24 h postinfection and clarified by centrifugation ($10,000 \times g$, 1 min). Radiolabeled viruses were purified by equilibrium sedimentation through 25 to 60% (wt/vol) sucrose gradient by ultracentrifugation ($100,000 \times g$, 2 h). The purified viruses were mixed with lysis buffer (50 mM Tris-Cl [pH 7.2], 600 mM KCl, 0.5% Triton X-100 [54]) and then used as antigens for the RIP assay. Antigens were incubated with anti-H5 monoclonal antibodies overnight at 4°C and immunoprecipitated with protein A beads. Immunoprecipitated proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the resulting gel was treated with 1 M sodium salicylate for fluorography and exposed to Kodak X-Omat film (Kodak).

Experimental infection with mutant viruses in chickens. Four-day-old specific-pathogen-free chicks (Spafas) were inoculated intramuscularly (i.m.) or intranasally/orally (i.n./o.) with 10^6 PFU of parent or HA cleavage mutant viruses per chick to evaluate the virulence of each virus. Mortality and clinical signs, including lethargy, necrosis of comb, inability to stand, and nervous disorder, were examined during an observation period of 10 days. The median lethal dose (LD_{50}) of each virus in chickens was determined after i.m. or i.n./o. inoculation.

To determine the tissue tropism of the viruses, we collected organs from three infected chickens at 3 days postinoculation, pooled the tissue, and determined virus titers of each sample in eggs as described previously (55).

RESULTS

Generation of HA cleavage mutants by reverse genetics. To rescue the HA gene of the virulent virus Ty/Ont, we used a reassortant, WSN-Ty/Ont, as a helper virus. WSN-Ty/Ont has the HA gene from WSN (H1) and all other genes from Ty/Ont. Plasmid pT3TOH36-2 was transcribed in vitro in the presence of purified NP and P prepared from the helper virus, and the reconstituted HA-RNP complex was transfected to the helper virus-infected MDBK cells. Hence, the genotype of viruses with the rescued HA would be the same as that of the parent virus Ty/Ont. Although Palese and colleagues successfully generated viruses with the rescued HA by specific antibodies against a helper virus (11, 28, 29, 31), we were unable to use this method with the Ty/Ont HA gene. We therefore estab-

lished a selection procedure for the Ty/Ont HA, using antibodies against both the helper virus (H1) HA and the H5 HA (antibody-mediated virus-trapping system; Fig. 2). We first coated the tissue culture plates with anti-H5 antibodies to trap the viruses with the H5 HA in the transfection supernatant, although such viruses would most likely contain H1 HA as well (phenotypic mixing). MDCK cell suspension containing trypsin was added to the wells after unbound viruses were washed out. In this step, the viruses trapped by anti-H5 antibodies attach to cell receptors. Viruses then penetrate into cells. Further propagation of the helper virus was prevented by antibody to the H1 molecule, so that only the viruses with the rescued HA would undergo multiple cycles of replication. In most cases, pure populations of viruses with the rescued HAs were obtained after only one cycle of this procedure.

Using this selection system, we generated virus with the wild-type HA [R(36-2)] and then R(MO-C), which contains the same amino acid sequence at the HA cleavage site as does R(36-2) but includes two silent mutations in the HA gene, resulting in the generation of two unique restriction sites for the subsequent cassette mutagenesis. The remaining five mutants are shown in Table 1. Considering the data previously obtained with Ty/Ire HA cleavage mutants in an in vitro expression system (17, 18, 52), five HA cleavage mutant viruses were made. R(MO-4), containing the HA cleavage site sequence (R-E-T-R), was generated to examine whether an amino acid change only at the HA cleavage site from virulent to avirulent forms alters the HA cleavability and virulence. Two mutants, R(MO-41) and R(MO-12), which differ in the number of basic amino acids but retain the total number of residues at the cleavage site, were created to assess the importance of the number of basic amino acids in this region. R(MO-2) containing a deletion of two basic amino acids was constructed to test the role of these residues for abrogating inhibition of HA cleavage by the nearby carbohydrate. Finally, R(MO-1), which contains a Lys-to-Thr mutation at the second residue (P-2) from the carboxyl (C) terminus of HA1, was produced to evaluate virulence of the virus with reduced HA cleavability. In in vitro expression studies with the Ty/Ire HA, the HA with this mutation was partially cleaved in cell cultures in the absence of trypsin (17). For substitution of basic residues, we selected threonine because of its small and noncharged properties, which may prevent drastic structural alteration around the cleavage site. We generated multiple clones for each mutant virus, compared their biological properties in vitro (see below), and found that there were no significant differences among clones of each mutant. We therefore present the data for only one virus for each mutant in the following sections. The HA cleavage site sequences of these mutant viruses generated proved to be stable after five successive passages in eggs.

HA cleavability of mutant viruses in cell cultures. RIP analysis was performed to determine directly the cleavability of HA molecules on virions of mutant viruses grown in CEF culture (Fig. 3). Purified virus was used in all experiments because of previous studies showing that the HA of wild-type Ty/Ont in cell lysate was not completely cleaved (16). The HAs of R(36-2) and R(MO-C), which possess the wild-type sequence R-R-R-K-K-R, were completely cleaved, whereas alteration of the cleavage site sequence to the avirulent form [R-E-T-R; R(MO-4)] abolished the cleavability altogether (Fig. 3A), indicating that the sequence in this region is critical for recognition by endogenous proteases in CEF. By contrast, alteration of P-5 and P-6 from basic to nonbasic residues [R(MO-12) and R(MO-41)] did not affect HA cleavability. Thus, four successive basic residues from the C terminus of

FIG. 3. HA cleavability of Ty/Ont HA cleavage mutants in CEF culture. CEF culture, inoculated with each virus, was incubated in the presence of Tran^[35S] label (ICN) for 24 h. Radiolabeled purified viruses were lysed, immunoprecipitated without (A) or with (B) trypsin treatment using anti-H5 monoclonal antibodies and protein A beads, and analyzed on an SDS-10% polyacrylamide gel.

HA1 are sufficient for optimal cleavage by endogenous proteases, so long as a total of six amino acids are present in this region. The deletion of P-5 and P-6 residues [R(MO-2)] abolished cleavability, suggesting that the R-K-K-R sequence was no longer recognized by proteases. Hence, the presence of P-5 and P-6 residues is an important feature of sequence recognition by proteases in the presence of a nearby carbohydrate side chain. The reduced HA cleavability of R(MO-1) corresponded to previous observations with Ty/Ire HA mutant (17), confirming that a Lys-to-Thr substitution at P-2 reduces recognition by proteases. Taken together, these results establish that alteration of the cleavage site sequence is sufficient to alter HA cleavability and that the number and position of basic residues and the length of the sequence in this region are important features of cleavability, in good agreement with previous studies in *in vitro* expression systems (17, 18, 40, 51, 52).

Because the plaque-forming ability of influenza viruses in the absence of trypsin correlates with HA cleavability (5), we investigated the substrate specificity of endogenous proteases for HA cleavage in cells of diverse origins by examining plaque formation of HA cleavage mutants. In CEF, the plaque-forming ability of each virus clearly correlated with the HA cleavability determined by RIP assay (Table 1); viruses with HAs that were not cleaved by the endogenous proteases [R(MO-4) and R(MO-2)] did not produce plaques, whereas those with highly cleavable HAs [R(36-2), R(MO-C), R(MO-12), and R(MO-41)] produced large plaques, and the R(MO-1) with reduced HA cleavability produced small plaques. In MDBK and MDCK cells, the plaque-forming ability of viruses with either high or reduced HA cleavability was similar to that in CEF. By contrast, slight differences in the substrate specificity of proteases were observed among cell cultures with the HAs of R(MO-2) and R(MO-4), as indicated

TABLE 2. Virulence of Ty/Ont HA cleavage mutants in chickens

Virus	% Sick/% dead/total no. ^a		LD ₅₀ by inoculation route ^b	
	i.m.	i.n./o.	i.m.	i.n./o.
R(36-2)	100/90/10	70/50/10	10 ^{4.7}	10 ^{5.8}
R(MO-C)	100/100/10	55/36/11	10 ^{4.5}	ND
R(MO-1)	100/80/10	30/20/10	10 ^{3.9}	10 ^{7.1}
R(MO-2)	0/0/8	0/0/7	>10 ^{8.3}	>10 ^{8.3}
R(MO-4)	0/0/8	0/0/8	>10 ^{8.3}	>10 ^{8.3}
R(MO-12)	100/80/10	90/40/10	10 ^{4.8}	10 ^{5.4}
R(MO-41)	100/100/11	100/73/11	10 ^{3.0}	10 ^{4.6}

^a Four-day-old specific-pathogen-free chicks were inoculated i.m. or i.n./o. with each virus (10⁶ PFU) and observed for 10 days. *P* = 0.849, 0.349, 0.081, 0.058, 1.000, or 0.535 for the differences in the lethality by i.n./o. inoculation between R(36-2) and R(MO-C), R(MO-1), R(MO-2), R(MO-4), R(MO-12), or R(MO-41), respectively, with the two-tailed Fisher exact test; *P* = 0.170, 0.588, 2.000, 0.137, or 0.004 between R(MO-4) and R(MO-C), R(MO-1), R(MO-2), R(MO-12), or R(MO-41), respectively.

^b Expressed as median egg infectious dose of each virus. ND, not determined.

by the production of very small, turbid plaques by R(MO-2) in MDCK and MDBK cells and by R(MO-4) in MDCK cells.

Virulence of mutant viruses. The virulence of R(36-2) and R(MO-4) was compared in chickens to assess the effect of altering the cleavage site from wild-type (R-R-R-K-K-R) to avirulent (R-E-T-R) forms. Viruses were inoculated i.m., because intravenous inoculation, the routine method for virulence tests, would have been impractical in the 4-day-old chicks used in these experiments. R(36-2) was highly virulent, whereas R(MO-4) was avirulent (Table 2), indicating that only a change at the cleavage site was sufficient to alter virulence. Both R(MO-12) and R(MO-41), which showed high HA cleavability in CEF, and R(MO-1), which showed reduced HA cleavability in the same system, were highly virulent. R(MO-2) with uncleavable HA was avirulent. These results suggest that with i.m. inoculation, the viruses with CEF-cleavable HAs produce high morbidity and mortality regardless of the degree of HA cleavability.

When inoculated i.n./o., which likely simulates the natural infection route, R(MO-4) and R(MO-2) did not produce any signs of disease in chickens (Table 2) and therefore were avirulent, regardless of the inoculation route. By contrast, the mutants that were highly virulent after i.m. inoculation produced less morbidity and mortality when injected i.n./o. The reduction in pathology associated with R(MO-1) was remarkable. The chicken LD₅₀ after i.m. or i.n./o. inoculation (Table 2) confirmed these observations. Although the LD₅₀ of R(36-2), R(MO-12), and R(MO-41) was lower (up to 40-fold) after i.m. than after i.n./o. inoculation, the difference was 1,585-fold for R(MO-1). Hence, this mutant was less virulent than other viruses with highly cleavable HAs.

Considering that the nonlethal phenotypes of R(MO-2) and R(MO-4) might reflect limited replication at a chicken's body temperature, we compared the replication of all of the viruses with rescued HAs at 37 and 42°C in CEF culture. All of the viruses, including R(MO-2) and R(MO-4), replicated well at both temperatures (data not shown), indicating that temperature sensitivity does not account for the avirulence of R(MO-2) and R(MO-4).

TABLE 3. Tissue tropism of Ty/Ont HA cleavage mutants in chickens

Virus	Inoculation route	Virus titer (log ₁₀ EID ₅₀ /g) by organ ^a														
		Brain	Trachea	Lung	Heart	Liver	Kidney	Pancreas	Jejunum	Ileum	Cecum	Colon	Spleen	Thymus	Bursa	Blood
R(36-2)	i.m.	3.5	4.0	8.0	3.0	5.0	5.0	4.5	2.5	3.5	3.5	4.0	5.5	6.0	4.5	3.0
	i.n./o.	3.0	4.5	6.0	3.0	3.5	3.0	4.5	2.0	3.5	2.5	—	3.5	4.5	5.0	2.5
R(MO-C)	i.m.	6.0	6.5	7.5	6.0	7.5	7.5	6.5	6.0	5.0	5.0	4.0	7.5	7.0	7.0	6.0
	i.n./o.	5.0	6.0	6.0	4.5	3.5	5.0	5.0	4.5	4.0	5.0	4.0	4.0	6.0	5.0	4.0
R(MO-1)	i.m.	3.0	3.5	6.5	3.5	4.0	5.0	4.5	5.0	5.5	5.0	5.5	3.5	6.5	5.5	4.0
	i.n./o.	—	2.0	3.0	—	—	—	2.5	—	2.5	2.0	—	2.5	4.5	3.5	—
R(MO-2)	i.m.	—	—	—	—	—	—	—	—	2.0	2.0	—	—	—	—	—
	i.n./o.	—	—	2.5	—	—	—	—	—	—	—	—	—	—	—	—
R(MO-4)	i.m.	—	—	3.0	—	—	—	—	2.0	—	—	2.5	—	3.5	—	—
	i.n./o.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R(MO-12)	i.m.	3.0	6.0	7.5	3.0	6.0	6.5	6.0	5.0	4.0	4.5	4.0	5.0	5.5	4.5	2.0
	i.n./o.	2.5	4.0	5.5	3.5	4.0	4.5	4.0	2.0	4.5	4.5	2.5	2.5	3.5	4.5	2.0
R(MO-41)	i.m.	3.5	5.0	8.0	4.0	7.0	6.0	5.0	5.0	4.5	4.0	3.5	5.5	5.0	5.5	4.0
	i.n./o.	4.0	6.5	6.5	3.0	5.0	7.5	7.5	2.0	4.5	4.5	5.0	4.5	6.5	6.5	5.0

^a For each mutant, organs were collected from three infected chickens at 3 days postinoculation, pooled, and homogenated for virus titration. —, <10^{2.0} median egg infectious doses (EID₅₀)/g.

Tissue tropism of mutant viruses in chickens. Whether the tissue tropism of influenza virus is altered by sequence changes at the HA cleavage site is unclear. Thus, we collected organs from infected chickens at 3 days postinfection and determined the virus titer in each organ (Table 3). R(36-2), containing wild-type HA, was recovered from all organs tested regardless of the inoculation route, in agreement with a previous report on wild-type Ty/Ont (6). By contrast, the R(MO-4) mutant, defined by an avirulent-type sequence, was recovered only from lung, intestine, and thymus from chickens inoculated by the i.m. route. It was not found in any organ from birds inoculated i.n./o., although specific antibodies to Ty/Ont HA were detected 1 month after inoculation (data not shown). These findings indicate that sequence changes at the HA cleavage site influence the tissue tropism of influenza viruses and that all of the organs tested contain proteases for cleavage of the wild-type HA, but only some contain proteases for the HAs with avirulent-type sequences.

Similarly, the mutant viruses R(MO-12), R(MO-41), and R(MO-1), characterized by either high or reduced HA cleavability in CEF, were also recovered from all organs collected from chickens inoculated i.m. This finding suggests that each organ contained proteases for HA cleavage and that sequence differences among the mutants did not affect their tissue tropism. The result with R(MO-1) also suggests that reduced HA cleavability of the virus in CEF did not affect its spread in chickens inoculated i.m. However, with i.n./o. inoculation, R(MO-1) was recovered from only a limited number of organs, indicating that its spread from initial replication sites to internal organs was impeded, probably because of its reduced HA cleavability. Thus, the ability of the virus to convert the initial local infection into systemic infection is one of the determinants of virulence. On the other hand, R(MO-2) with four basic residues (R-K-K-R) at the HA cleavage site was isolated only from lung and intestine, suggesting that the same types of proteases *in vivo* were responsible for HA cleavability of R(MO-2) and R(MO-4).

DISCUSSION

We have directly determined the relationship of HA cleavability to the virulence of avian influenza A viruses. By using the reverse genetics method, it was possible to generate mutant viruses that were genetically identical except for sequence alterations at the HA cleavage site. The results obtained with this system support the hypothesis that HA cleavability determines the virulence of influenza viruses. All mutant viruses with HAs highly cleavable by endogenous proteases were virulent, whereas those with uncleavable HAs were avirulent. Even viruses whose HA cleavage site sequences differed from those of naturally virulent isolates were virulent, so long as their HAs were highly cleavable [e.g., R(MO-12) with a sequence of T-T-R-K-K-R]. Laboratory isolation of the A/turkey/Oregon/71 (H7N3) mutant, which became highly virulent after insertion derived from 28S rRNA at the HA cleavage site with only a single arginine (19), provides additional support for this concept.

Do viruses with reduced HA cleavability retain virulence? R(MO-1), characterized by a Lys-to-Thr mutation at P-2, resulting in reduced cleavability, was as virulent as the mutants with highly cleavable HAs in testing by i.m. inoculation but was less virulent than the others when inoculated i.n./o. This property may have a pathophysiological explanation. After i.n./o. inoculation, both virulent and avirulent viruses replicate in the epithelium of respiratory and intestinal organs. Subsequently, the virulent viruses replicate in the inner layers of these organs, such as lamina propria mucosa, which leads to penetration into blood vessels and systemic infection. Thus, the reduced virulence of R(MO-1) after i.n./o. inoculation may relate to its limited replication in the inner layers of respiratory and intestinal organs because of reduced HA cleavability, resulting in a delay of penetration into blood vessels. This idea is supported by the finding that upon chorionic inoculation of the embryonated eggs, virulent viruses spread from chorionic to allantoic epithelium through the mesenchyme, whereas avirulent viruses did not (47). Thus, when R(MO-1) is passed

by an artificial (i.m.) penetration step, it spreads in organs as fast as viruses with highly cleavable HAs inoculated by the i.m. route, even though it shows reduced cleavability in CEF. Alternatively, R(MO-1) undergoes mutations in vivo to a more virulent form, and thus the originally avirulent virus produces an intermediate virulent phenotype. Naturally occurring viruses with reduced HA cleavability have not been reported; however, other viruses with reduced HA cleavability can be generated by mutations at the cleavage site. Whether or not viruses containing such HAs would be virulent probably depends on the degree of HA cleavability in vivo.

Is there a sequence requirement for high virulence? Findings in this present study, combined with earlier observations (15–18, 27, 39, 51, 52, 59), indicated that an X-X-R/K-X-R/K-R motif (X = nonbasic residues) at the HA cleavage site is required for high cleavability when a carbohydrate side chain is nearby. Whether P-3, P-5, and P-6 nonbasic residues are equally acceptable at the X site remains uncertain. In a previous in vitro expression study, the Ty/Ire HA mutant MT-41 with T-E-R-K-K-R showed reduced cleavability in CV-1 cells (52), in contrast to the high cleavability of R(MO-12)-HA with T-T-R-K-K-R, suggesting that the acidic property of glutamic acid (P-5) may render the electrostatic environment of this region suboptimal for recognition by proteases. Alternatively, there may be selective incorporation of the cleaved form of the HA into virions. It should be possible to distinguish between these possibilities by examining the degree of HA cleavability on MT-41 virions, using the Ty/Ire HA reverse genetics system.

Determination of the amino acid sequence at the HA cleavage site is essential for assessing the potential virulence of avian influenza virus isolates (56). A recently isolated H5 virulent virus A/turkey/England/90-62/91 (H5N1) possesses an R-K-R-K-T-R sequence at the HA cleavage site (59), similar to that of R(MO-1) in our study. Surprisingly, the HA of the former virus was highly cleavable in CEF, and the virus showed high virulence, killing some chickens within 24 h after inoculation (12a, 59). This finding suggests structural differences between the HAs of A/turkey/England/91 and R(MO-1) in the vicinity of the cleavage site. Although R(MO-1) produced less morbidity and mortality in i.n./o.-inoculated than in i.m.-inoculated chickens, its virulence could increase substantially after a limited number of amino acid changes. Thus, we propose that viruses with the R-R/K-R-K-T-R motif should be considered in the same category as virulent viruses with the X-X-R/K-X-R/K-R motif.

HA cleavability is defined by the sensitivity of the HA to proteases, suggesting that the tissue distribution of specific proteases could be a codeterminant of virulence. Differences in the plaque-forming ability of R(MO-2) and R(MO-4) in CEF, MDBK, and MDCK cell cultures may reflect variations in the intracellular proteases. A previous study of the host range variants of an H7 virus suggested that mammalian cells, such as MDCK cells, may contain proteases with a broader substrate specificity than those found in avian cells, such as CEF (27). Similarly, cell cultures may contain multiple proteases that recognize a series of basic amino acid motifs (14, 20). Differences in plaque-forming ability might also be explained by differences in the specificity of a protease from cell type to cell type. Bovine furin, a subtilisin-like eukaryotic protease (1), was isolated from MDBK cells as an intracellular protease that cleaves the HA of an H7 virulent virus (49). It is likely that specificity of furin (or furin-related proteases) is slightly different among CEF, MDBK, and MDCK cells, resulting in the different plaque-forming ability of the viruses in these cells.

Two groups of proteases appear responsible for the HA

cleavage in vivo. One includes enzymes able to cleave avirulent-type as well as virulent-type HAs, such as plasmin (26), blood-clotting factor X-like protease (12, 38), trypsin (21), and bacterial proteases (50). The tissue tropism of R(MO-2) and R(MO-4) in the present study suggests that proteases of this group reside in lung, intestinal organs, and thymus. The second group comprises proteases that cleave only virulent-type HAs with multiple basic residues at the cleavage site. The ability of R(36-2), R(MO-C), R(MO-12), R(MO-41), and R(MO-1) to replicate in all chicken organs tested indicates a wide distribution for such proteases, which remain unidentified. Furin is a prime candidate for a ubiquitous protease (49, 53), although many similar enzymes could be involved in HA cleavage in vivo (20, 22, 34, 37). Better replication of R(MO-1) in lymphoid organs, such as thymus and bursa, in chickens inoculated by the i.n./o. route supports the existence of organ-specific proteases with different specificities. Wider identification of the proteases responsible for HA cleavage would lend impetus to attempts to understand the pathogenesis of influenza and other viral diseases that may share similar sequence motifs at the cleavage sites of viral glycoproteins (reviewed in reference 44).

Mutational studies with reverse genetics can provide fresh insight into the biologic functions of influenza virus proteins. Here we establish the antibody-mediated virus-trapping system as a reliable procedure for selecting mutants of Ty/Ont HA. Despite being trapped by antibodies, the virus can penetrate into cells and replicate further only if the binding between virus and antibody or between antibody and plate is dissociated after binding to the cell receptor. However, the precise mechanism of this process is unclear. The blocking of plate wells with BSA to prevent nonspecific attachment of helper viruses is an effective measure with WSN-Ty/Ont but not all viruses (data not shown). The reason for this difference in the nonspecific binding of viruses to tissue culture plates among virus strains is unknown. Nonetheless, this selection system can be used effectively to rescue the HA, neuraminidase, and possibly matrix (through the binding of M2 protein) genes, all of whose products are located in viral envelope.

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