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Molecular Basis for High Virulence of Hong Kong H5N1 Influenza A Viruses

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In 1997, an H5N1 influenza A virus was transmitted from birds to humans in Hong Kong, killing 6 of the 18 people infected. When mice were infected with the human isolates, two virulence groups became apparent. Using reverse genetics, we showed that a mutation at position 627 in the PB2 protein influenced the outcome of infection in mice. Moreover, high cleavability of the hemagglutinin glycoprotein was an essential requirement for lethal infection.

An outbreak of H5N1 influenza A virus in Hong Kong in 1997 resulted in 6 deaths among 18 people infected (*1–3*). The virulence seen in mice when infected with these human isolates showed some correspondence with the severity of disease in adult patients, although there are exceptions (*3–7*). To determine the molecular basis for the difference in virulence among Hong Kong H5N1 viruses, we generated reassortants between virulent and avirulent viruses (Fig. 1) using a plasmid-based reverse genetics system (*8*). First, we constructed plasmids in which a human RNA polymerase I promoter and a mouse polymerase I terminator flanked cDNAs for the full-length RNAs of A/Hong Kong/483/97 (HK483) (which caused lethal systemic infection in mice) or A/Hong Kong/486/97 (HK486) (which produced nonlethal respiratory infection) viruses (*9*). Two forms of the HA gene were identified in our HK486 virus stock: one possessing Ser at position 227 (H3 numbering) (486HA227S) and another with Ile at position 227 (486HA227I). Using these plasmids, we produced HK483 and HK486 transfectant viruses (*10*)—designated HK483RG, HK486RG(HA-227S), and HK-486RG(HA-227I)—and inoculated them into Madin-Derby canine kidney (MDCK) cells to produce virus stocks.

Figure 1 and Table 1 compare the pathogenicity of the transfectants, based on the virus dose lethal to 50% of infected mice (50% mouse lethal dose, MLD_{50}) and virus growth in the organs of mice infected intranasally with 100 plaque-forming units (PFU) of virus. HK483RG, like the original HK483 virus, produced lethal systemic infection in mice (MLD_{50}, 1.7) (Table 1). By contrast, the HK486 transfectants [HK486RG(HA-

227S) and HK486RG(HA-227I)] did not kill mice even at a dose of 10^3 PFU, and both were recovered only from respiratory organs (Table 1). However, the MLD_{50} for HK-486RG(HA-227S) was 4.6×10^4, whereas HK486RG(HA-227I) failed to kill mice at the highest dose tested, 1.3×10^6 PFU.

To elucidate the molecular basis of the virulence discrepancy between the HK483 and HK486 viruses, we generated a spectrum of single-gene reassortants (Fig. 1) and tested their pathogenicity in mice. Among nine single-gene reassortant viruses, each containing one gene segment from HK486 virus and the remaining segments from HK483 virus, only those possessing the PB2 (HK3/6PB2) or HA-227I (HK3/6HA227I) gene from the HK486 virus were appreciably attenuated (MLD_{50}, 1.0×10^4 or 1.1×10^2 PFU, respectively, compared with <5 PFU for all other transfectants) (Fig. 1). Moreover, although more than 10^6 PFU/g of virus was detected at 6 days after infection in the lungs of mice infected with either HK3/6PB2 or HK3/6HA227I virus (Table 1), virus was not recovered from other nonrespiratory organs (with the exception of the heart in mice infected with HK3/6PB2 virus). By contrast, the single-gene reassortants containing the HK486HA227S (HK3/6HA227S) gene or the HK486 NA (HK3/6NA) gene (both tested as representatives of virulent single-gene reassortants) were not attenuated and caused systemic infection (Table 1).

We next tested which gene from HK483 virus converts the HK486 virus to high virulence, using an approach similar to the one described above. The reassortant possessing the PB2 gene from HK483 virus, 486HA227S HA gene, and the remaining segments from the HK486 virus (HK6HA227S/3PB2) was the only construct with noteworthy virulence (MLD_{50}, 0.4 PFU), killing all mice by day 5 after infection and being recovered from all organs tested (Fig. 1 and Table 1); however, the reassortant possessing the PB2 gene from HK483, 486HA227I, and the remaining genes from HK486 virus (HK6HA227I/3PB2) was

attenuated with an MLD_{50} of 3.4×10^3, causing only respiratory infection. None of the other single-gene reassortants, possessing only one gene from HK483 virus and the remaining genes from HK486 virus, was as virulent as the HK483 virus (MLD_{50}, $>10^2$ PFU). The single-gene reassortants possessing 486HA227I were more attenuated than those possessing 486HA227S. The HK6HA227S/3NA and HK6HA227I/3NA reassortants, possessing the HK483 NA gene and all remaining genes from HK486 (tested as representatives of avirulent single-gene reassortant viruses), were recovered only from respiratory organs (Table 1). These results indicate that the PB2 viral protein is responsible for the difference in virulence between the two Hong Kong H5N1 viruses, and that a Ser-to-Ile substitution at position 227 of the HK486 HA can reduce the virulence potential of the virus.

There are eight amino acid differences between the PB2 proteins of HK483 and HK486 viruses (Fig. 2). To identify the specific changes that give rise to virulence, we generated mutant HK483 viruses possessing a chimeric PB2 protein (Fig. 2) and determined their MLD_{50} values. The mutant viruses possessing chimera 2, 4, or 5 were attenuated (MLD_{50}, $>10^3$ PFU), whereas those with chimera 1, 3,
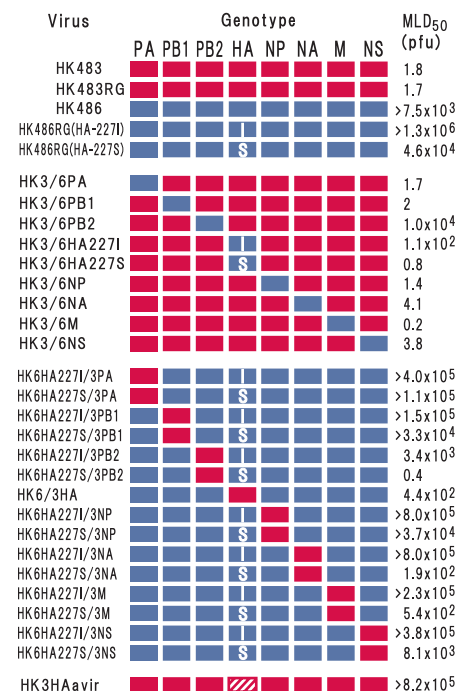

Fig. 1. Pathogenicity of transfectant viruses. Bars indicate the origin of the viral gene: A/Hong Kong/483/97 (HK483, red), A/Hong Kong/486/97 (HK486, blue). I and S in blue bars indicate the HK486 HA possessing either Ile or Ser at position 227, respectively. Red hatched bar, amino acid sequence at the HA cleavage site of HK483 changed from PQRERRRKKR/G to PQ----RETR/G, as described in the text. The virus dose lethal to 50% of mice (MLD_{50}) was determined as described (*5*).

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or 6 were virulent (MLD$_{50}$, <3.1 PFU). These results implicated amino acid residue 627 or 675 (or both) in contributing to the pathogenicity of HK483 virus. A mutant HK483 virus containing a single Leu-to-Ile substitution at position 675 of PB2 (HK3PB2-675I) and another mutant virus containing an Ile-to-Leu substitution at position 675 of HK486 PB2, with all remaining genes from the HK483 virus (HK6PB2-675L), were constructed. These mutations failed to alter the ability of PB2 to contribute to viral virulence (Fig. 2). However, a Lys-to-Glu substitution at position 627 in PB2 (HK3PB2-627E) resulted in marked attenuation of HK483 virus (MLD$_{50}$, 2.3×10^3). Finally, we tested a mutant of HK486RG(HA-227S) characterized by a single Glu-to-Lys substitution at position 627 in PB2 (HK6PB2-627K). It killed all mice by day 5 after infection (MLD$_{50}$, 5.8 PFU) and was recovered in high titers from all organs examined (Fig. 1 and Table 1).

For avian influenza A viruses, cleavability of the HA molecule plays a major role in virulence in birds, although other genes also contribute to this property (11–13). Thus, we generated a mutant HK483 virus in which the amino acid sequence at the HA cleavage site, PQRERRRKKR/G, was converted to the sequence of typical avirulent avian viruses, PQ----RETR/G (where a dash indicates a deletion). When tested in mice, this HA mutant (HK3HAavir) was highly attenuated (MLD$_{50}$, $>10^5$ PFU) (Fig. 1), and none of the infected mice showed signs of disease. On intranasal

inoculation into mice (100 PFU), this mutant was recovered from lung and nasal turbinates, but not from any other organs 3 days after infection. By 6 days after infection, the virus could not be recovered from any organ (Table 1). The virulence of HK483 virus in mice appears to involve HA cleavability, as well as an amino acid substitution in the PB2 viral protein. We suggest that this mutation enables HK483 virus to adapt to efficient growth in mice and possibly humans. Indeed, a mutation at position 627 of PB2 has been linked to the host range of influenza A viruses (14). The virulent HK483 and avirulent HK486 viruses also differed in their ability to invade systemic organs, suggest-

ing that the PB2 protein may be a determinant of cell tropism, in accordance with previous findings (14–17). Whether the mechanisms for these effects are discrete or overlapping and rely on known properties of PB2 [e.g., cap-binding (18, 19)] remains to be determined.

The HK486 virus consists of two types of HA molecules that differ by a single amino acid, resulting in different abilities to support virulence in mice. The Ile at position 227 of the HA, which reduced the ability to support virulence, was found only in the HK486 virus. This amino acid is located in the receptor-binding pocket on the distal tip of the HA (20) and therefore may affect the ability of

Fig. 2. Schematic diagram of chimeric and single–amino acid PB2 mutants, with their virulence in mice (MLD$_{50}$). Differences in PB2 amino acid residues between A/Hong Kong/483/97 and A/Hong Kong/486/97 are shown as single-letter amino acid codes with their positions indicated at the top of the diagram. The red and blue bars indicate whether the amino acid regions originated from HK483 or HK486, respectively. MLD$_{50}$ values are reported at the far right. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; I, Ile; K, Lys; L, Leu; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val.
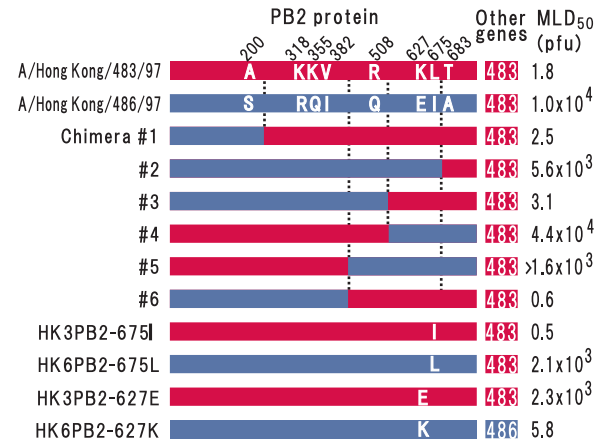
Table 1. Tissue tropism of H5N1 transfectant viruses. BALB/c mice, anesthetized with methoxyflurane, were infected intranasally with 50 μl of virus (100 PFU). Three mice from each infected group were killed on day 3 or day 6 after infection (or both) for virus titration. When virus was not recovered from all three mice, individual titers were recorded. (–) Virus not isolated.

Virus	Day	Virus titer [(mean log PFU $\pm$ SD)/g] in							
		Lungs	Nasal turbinates	Spleen	Heart	Kidneys	Brain	Pancreas	Colon
A/Hong Kong/483/97	3	6.7 ± 0.1	4.0, 4.3	3.3 ± 0.4	–	–	–	–	–
	6	6.3 ± 0.3	5.7 ± 0.2	1.7	4.0 ± 0.2	1.7	3.8 ± 1.0	2.9 ± 0.6	3.2 ± 0.2
A/Hong Kong/486/97	3	4.0 ± 0.1	–	–	–	–	–	–	–
	6	5.5 ± 0.5	–	–	–	–	–	–	–
HK483RG	3	7.1 ± 0.2	3.4 ± 0.6	4.1 ± 0.2	2.2 ± 0.3	1.7	1.4, 1.4	–	–
	6	6.1 ± 0.1	5.4 ± 1.3	–	4.8 ± 0.3	2.9 ± 0.7	3.9 ± 1.0	2.3 ± 0.5	3.7 ± 1.0
HK486RG(HA-227S)	3	5.3 ± 0.3	–	–	–	–	–	–	–
	6	6.5 ± 0.2	5.3	–	–	–	–	–	–
HK486RG(HA-227I)	3	3.8 ± 0.7	–	–	–	–	–	–	–
	6	5.3 ± 0.3	–	–	–	–	–	–	–
HK3/6PB2	6	6.7 ± 0.7	3.8 ± 0.7	–	4.5, 3.1	–	–	–	–
HK3/6HA227I	6	6.5 ± 0.2	–	–	–	–	–	–	–
HK3/6HA227S	6	7.6 ± 0.1	6.8 ± 1.0	2.3	5.6 ± 0.8	2.7 ± 0.5	5.6 ± 0.4	3.9	4.5 ± 0.4
HK483/6NA	6	6.4 ± 0.1	2.7 ± 0.7	–	3.4 ± 0.1	2.1 ± 0.2	2.4 ± 0.5	2.5	2.5 ± 0.2
HK6HA227S/3PB2	4*	8.3 ± 0.2	7.1 ± 0.4	3.6 ± 0.1	5.9 ± 0.2	6.1 ± 0.3	6.5 ± 0.2	4.7 ± 0.3	5.4 ± 0.2
HK6HA227I/3PB2	6	6.1, 6.7	3.2	–	–	–	–	–	–
HK6HA227S/3NA	6	7.3 ± 0.1	2.1, 3.8	–	–	–	–	–	–
HK6HA227I/3NA	6	5.1 ± 0.9	–	–	–	–	–	–	–
HK6PB2–627K	5*	7.7 ± 0.1	6.6 ± 0.8	–	5.4 ± 0.4	4.2 ± 0.6	6.6 ± 0.2	2.6, 5.1	5.4 ± 0.9
HK3HAavir	3	6.4 ± 0.1	1.5, 2.2	–	–	–	–	–	–
	6	–	–	–	–	–	–	–	–

*Mice were killed on days 4 and 5 because all mice died on days 5 and 6 in the preliminary experiments.

the virus to recognize and bind to target cells. It will be important in future experiments to compare the receptor binding of the two types of HK486 HAs.

Here we have demonstrated that single amino acid substitutions in PB2 and HA are principal determinants of the difference in virulence between the two viruses tested. However, genes other than PB2 and HA may also contribute to this difference to a lesser extent, as suggested by the limited, but appreciable, increase in virulence upon replacement of the HK486 NA with that of HK483 virus (HK6HA227S/3NA) (Fig. 1), consistent with the concept that influenza virus pathogenicity is multigenic (*11–13*).

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9. HK483 and HK486 viruses isolated from patients during the Hong Kong outbreak of 1997 were obtained from the Centers for Disease Control and Prevention (CDC). They were grown in MDCK cells maintained in minimum essential medium with 5% newborn calf serum. All experiments with live Hong Kong H5N1 viruses and with transfectants generated by reverse genetics were performed in a biosafety level 3 containment laboratory approved for such use by the CDC and U.S. Department of Agriculture. The cDNAs of HK483 and HK486 were synthesized by reverse transcription of viral RNA with an oligonucleotide (Uni 12) complementary to the conserved 3′ end of viral RNA, as described (*21*). The cDNA was amplified by polymerase chain reaction (PCR) with gene-specific oligonucleotide primers containing BsmB I sites, and PCR products were cloned into the pT7Blueblunt vector (Novagen, Madison, WI). After digestion with BsmB I, the fragment was cloned into the BsmB I sites of a plasmid vector, which contains the human RNA polymerase I promoter and the mouse RNA polymerase I terminator, separated by BsmB I sites (*8*). All of the constructs were sequenced to ensure that unwanted mutations were not present.
10. Transfectant viruses were generated in the 293T human embryonic kidney cell line, a derivative of the 293 line constitutively expressing the gene for the simian virus 40 T antigen (*22*), and maintained in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum as reported earlier (*8*). The mutant viruses were sequenced to confirm the presence of the intended mutations and to determine that no unwanted mutations were present.
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Recombination in the Hemagglutinin Gene of the 1918 "Spanish Flu"

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When gene sequences from the influenza virus that caused the 1918 pandemic were first compared with those of related viruses, they yielded few clues about its origins and virulence. Our reanalysis indicates that the hemagglutinin gene, a key virulence determinant, originated by recombination. The "globular domain" of the 1918 hemagglutinin protein was encoded by a part of a gene derived from a swine-lineage influenza, whereas the "stalk" was encoded by parts derived from a human-lineage influenza. Phylogenetic analyses showed that this recombination, which probably changed the virulence of the virus, occurred at the start of, or immediately before, the pandemic and thus may have triggered it.

The 1918 "Spanish flu" pandemic was the most severe recorded outbreak of acute human disease and was also infamous because it killed an unusually high number of young adults (*1, 2*). Fragments of the genomic RNA of the 1918 virus were recently recovered from preserved tissues of three of its victims, and complete sequences for three genes, including the hemagglutinin (HA) gene, were reported (*3–5*). These sequences confirmed that the 1918 Spanish flu was caused by an influenza A of the H1 N1 subtype, but they did not reveal why the virus was so virulent (*3–7*).

The virulence of influenza A viruses is largely determined by their HA. Mutations in the HA gene have produced highly pathogenic strains, and the major pandemics of 1957 and 1968 were largely caused by the introduction of antigenically novel HA genes from bird-infecting influenzas (*8–11*). It has been suggested that the 1918 pandemic was similarly caused by the introduction of genes from an avian strain (*6, 12*), but this theory was not supported when sequences from the virus were obtained (*3–5*). Phylogenetic analyses showed that the 1918 virus was most closely related to H1 influenzas from mammals and suggested that progenitors of the virus had infected mammals for several years before 1918, implying that some additional event must have triggered the pandemic (*3–7*). New virulent variants of some other viruses have been generated by homologous recombi-

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nation (*13–15*), but no evidence of this kind of genetic change has been found before in influenza virus populations (*16, 17*). Here, we report that the 1918 HA gene was a recombinant, and that the start of the 1918 pandemic and the recombination event were probably linked.

Complete HA gene sequences were analyzed from 30 H1-subtype isolates from the three main lineages (*3*): the lineages of isolates mostly from people, pigs, and birds. Sequences were aligned (*18*) and gaps (two codons) removed, producing an alignment 1695 nucleotides long. The mature HA protein consists of the NH_2-terminal HA1 and COOH-terminal HA2 polypeptides; the first 1026 nucleotides of our alignment encoded the HA1 and the remainder the HA2.

Every possible combination of three sequences from the aligned set was examined by the sister-scanning method (*19*) using, as outlier, a fourth sequence generated by local randomization. Four HA gene sequences were identified as likely recombinants—those of the 1918 influenza (A/South Carolina/1/18) and three Iowa-cluster sequences: A/swine/Iowa/15/30 (Iowa), A/Alma Ata/1417/84 (Alma Ata), and A/swine/St-Hyacinthe/148/90 (St-Hyacinthe). Different regions of these genes contained dominant signals that were conflicting (Fig. 1) but significant (Z scores >3.0) when compared with several combinations of HA sequences from isolates from pigs and humans. Two possible recombination sites were found in the 1918 sequence and three in the Iowa-cluster sequences; all of these, except one of the sites in the Iowa cluster, were also found using a maximum likelihood (ML) method for detecting re-