Cleavability of Hemagglutinin from an Extremely Virulent Strain of Avian Influenza Virus Containing a Unique Cleavage Site Sequence

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ABSTRACT. An avian influenza virus, A/turkey/England/50–92/91 (H5N1), showed extremely high virulence in chickens, although its hemagglutinin (HA) cleavage site sequence (R-K-R-K-T-R), having a nonbasic (Thr) residue at the second position (P-2) from the carboxyl terminus of HA1, does not conform to the previously established concensus sequence motif, X-X-R/K-X-R/K-R (X=nonbasic residue), for highly virulent phenotype of the H5 virus. When we evaluated the HA cleavability of this strain in chicken embryo fibroblast culture, we observed that, unlike other HAs with a Thr residue at P-2, this HA was efficiently cleaved. These findings suggest that a nonbasic residue at the P-2 does not affect its recognition and catalyzation by cleavage enzymes that are otherwise influenced by steric structure around the cleavage site.—KEY WORDS: avian influenza virus, cleavability, hemagglutinin.

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Some avian influenza A viruses are lethal to poultry, whereas others are not. The latter group includes the majority of field isolates, which replicate only in the respiratory and intestinal organs and result in mild or asymptomatic disease with low mortality. The degree of symptoms depends on the species and age of the birds, and whether or not there is an accompanying bacterial infection. By contrast, highly virulent viruses of H5 or H7 subtypes, which are able to replicate in most organs, produce systemic "fowl plague" disease with high mortality, although epidemics of this type are rare [reviewed in Ref. 25]. One such outbreak occurred in 1983, in Pennsylvania and the surrounding states in the United States, killing over 17 million birds at a cost of over 60 million dollars [2]. Avian influenza epidemics have also affected poultry in Australia in 1985 [17] and 1992, Ireland in 1983 [8], and Great Britain in 1991 [1]. In Japan, no confirmed outbreaks by highly virulent avian influenza virus have been reported since 1930 [22].

Although polygenic in origin, the virulence of influenza virus is influenced greatly by the hemagglutinin (HA) molecule and particularly its cleavability [reviewed in Refs. 13, 26]. Posttranslational proteolytic cleavage of the precursor HA molecule (HA0) into HA1 and HA2 subunits generating a fusogenic domain at the HA2 amino terminus is essential for entry of the virus into cells, with fusion occurring between the virus envelope and the endosomal membrane [27]. This HA cleavage event is mediated by host cell proteases and the distribution of which determines the tissue tropism of avian influenza virus. HAs of virulent viruses are cleaved by ubiquitous intracellular proteases, whereas HAs of avirulent viruses are cleaved only by the trypsin-like proteases that are secreted into airways or the intestinal tract, that is, HAs cleavable by intracellular proteases are essential for the virulent phenotype of the virus [reviewed in Ref. 12].

The HA cleavability of influenza viruses has been extensively studied in a number of ways: 1) by selecting variants on the basis of their cleavage properties in cell culture [11, 14, 18, 20]; 2) by site-specific mutagenesis of the HAs in *in vitro* expression systems [9, 10, 23]; and 3) by generating site-specific mutant viruses by reverse genetics [3]. The results have indicated that two structural features – a series of basic amino acids at the cleavage site, and the presence of a carbohydrate side chain in the near vicinity– are crucial for determining HA cleavability by proteases. A minimal sequence requirement for H5 HA cleavage by intracellular proteases is R/K-X-R/K-R (X=nonbasic residues) in the absence of a carbohydrate side chain. In its presence, at least two amino acid insertions (X-X-R/K-X-R/K-R) or additional basic residues [B (X)-X (B)-R/K-X-R/K-R (B=basic residues)] at upstream positions are required.

In these motifs, a basic residue at the second position (P-2) from the HA1 carboxyl terminus is important for optimal recognition of HA by the intracellular proteases, because mutant HAs with nonbasic residue at P-2 show reduced cleavage in cell culture (Table 1); HA cleavability of a virulent virus, A/turkey/Ireland/1378/83 (H5N8) [TI(WT): R-K-R-K-K-R sequence at the cleavage site] was reduced by a Lys to Thr substitution at P-2 [TI(MT-1)] when examined in an *in vitro* expression system [9]. Furthermore, a mutant virus with the same (Lys to Thr) P-2 mutation [A/turkey/Ontario/7732/66 (H5N9) R(MO-1)] was less virulent compared to wild-type virus [R (36–2)], leading to a conclusion that the degree of HA cleavability in cell culture

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Table 1. Comparison of cleavage site sequence and biological properties of H5 viruses

Virus [Abbreviation]	Potential glycosylation at Asn 11	HA cleavage site sequence[a] HA1 / HA2	Plaque formation in CEF[b]	HA cleavage[c] (%)	Reference[d]
A /turkey / England / 50–92 / 91 (H5N1)					
[Ty / Eng / 91]	+	Q R K R K T R / G	+	92	[this study]
A / turkey / Ontario/ 7732 / 66 (H5N9)					
wild-type [R (36–2)]	+	Q R R R K K R / G	+ (Large)	95	[3]
mutant [R (MO-1)]	+	Q R R R K T R / G	+ (Small)	30	
A / turkey / Ireland / 1378 / 83 (H5N8)					
wild-type [TI (WT)]	+	Q R K R K K R / G	N. A.	86	[9]
mutant [TI (MT-1)]	+	Q R K R K T R / G	N. A.	51	
A / environment / Pennsylvania / 937302 / 93 (H5N2)					
wild-type [EP93]	+	Q – – R K T R / G	–	<10	[4]
mutant [EP93-M1]	–	Q – – R K T R / G	+	65	

a) Slashes indicate the cleavage site. Dashes are included for alignment purposes.
b) Assessed in trypsin-free CEF culture. N. A., not available.
c) Assessed in trypsin-free CEF culture and determined by RIP gel shown in Fig. 1. Percentage of total HA, calculated from gels scanned with a Phosphorimager (Molecular Dinamycs).
d) All data, except those with Ty/Eng/91, are from the references shown.

predicts avian influenza virus virulence [3]. Similarly, a mutant [EP93-M1] of A/environment/Pennsylvania/937302/93 (H5N2) containing an R-K-T-R sequence at the cleavage site and no nearby carbohydrate side chain, showed incomplete HA cleavage (only 65%; Table 1) [4].

A virulent virus, A/turkey/England/50–92/91 (H5N1) [Ty/Eng/91], isolated from an outbreak in turkeys in Norfolk, England [1], was reported to contain an R-K-R-K-T-R sequence at the HA cleavage site [28, 29]. Thus, this virus is expected to show reduced HA cleavability, like TI(MT-1) which has the same cleavage site sequence (Table 1), even though the virus showed extremely high virulence. The aims of this study are to investigate the HA cleavability of Ty/Eng/91 in cell culture and its virulence in chickens to further understand the relationship between HA cleavage site sequence, cleavability, and virulence of avian influenza virus.

The original isolate of Ty/Eng/91 was reported to be a mixed population of avirulent and virulent viruses [28]. We therefore inoculated the original isolate intravenously (i.v.)/tracheally to an adult (5-month-old) white leghorn chicken to obtain a pure virulent virus population by an *in vivo* passage. The inoculated chicken died within 36 hr. The virus was then reisolated by injection of brain homogenate from the dead chicken to chicken embryonated eggs (10-day-old). Harvested virus was serially diluted and inoculated to chicken embryo fibroblasts (CEF) cultures for plaque purification of the virus. Virus recovered from the brain produced plaques in CEF culture in the absence of trypsin. After three plaque-to-plaque passages, the virus was then inoculated to eggs to obtain a biologically cloned virus population for use in subsequent experiments.

An earlier report described that some Ty/Eng/91 clones were extremely virulent; they killed 6-week-old chickens within 1 day of exposure through i.v. inoculation [28]. To confirm this observation, we examined the virulence of our Ty/Eng/91 clone using 3-month-old or 1-day-old specific-pathogen-free (Spatas) chickens. Two chickens (#1 and #3) were inoculated i.v. or intramuscularly (i.m.) respectively, and died within 23 hr. All 1-day-old chicks also died within 23 hr regardless of inoculation routes (Table 2). We did not observe typical "fowl plague" clinical signs except depression in the chickens that died within 2 days. Only #2 chicken, which survived for 3 days after inoculation, manifested severe clinical signs comprising depression, necrosis of the comb and wattle, and hemorrhagic change in the legs. These results confirmed the extremely high virulence of Ty/Eng/91, compared to that of previously isolated highly virulent strains, which require at least 2–3 days to kill chickens [25].

To ascertain the reported cleavage site sequence to Ty/Eng/91, the HA gene of our virus clone was amplified by reverse transcriptase — polymerase chain reaction (RT-PCR), followed by direct sequencing of the RT-PCR product

Table 2. Virulence of Ty/Eng/91 in chickens[a]

Chicken No.	Age	Inoculation route	Time to death (hour)
1	3-month	i.v.	< 23
2	3-month	i.v.	72
3	3-month	i.m.	< 23
4	3-month	i.m.	26
5	3-month	i.n. / o.	30
6	3-month	i.n. / o.	48
7–12	1-day	i.m.	< 23
13–18	1-day	i.n. / o.	< 23

a) Three-month-old or one-day-old specific-pathogen-free white leghorn chickens were inoculated intavenously (i.v.), intramuscularly (i.m.), or intranasally/orally (i.n. / o.) with $10^{7.0}$ or $10^{6.5}$ median egg infectious dose of the virus, respectively.

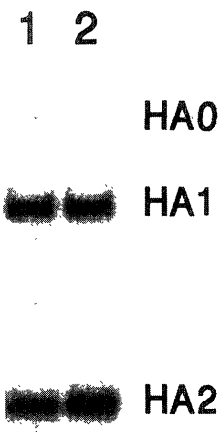

Fig. 1. HA cleavability of Ty/Eng/91 in CEF culture. Infected cells were incubated in the presence of Tran[^{35}S] label (ICN) for 20 hr. Progeny viruses in the culture supernatant were purified through 30% sucrose, lysed, and either untreated (1) or treated (2) with trypsin (2.5 μg/m*l* 37°C 10 min), prior to being immunoprecipitated with anti-H5 antibodies and protein A beads, and analyzed on a 10% gel by SDS-PAGE. The resulting gel was treated with 1 M sodium salicylate prior to fluorography and exposed to Kodak X-Omat film.

with Taq polymerase, as described previously [5, 7]. In agreement with the previous report [28, 29], our Ty/Eng/91 clone contained R-K-R-K-T-R sequence at the HA cleavage site (data not shown).

This cleavage site sequence was expected to show reduced HA cleavability due to the presence of a nonbasic (Thr) residue at P-2. Accordingly, we performed radioimmunoprecipitation (RIP) analysis to determine HA cleavability. The radiolabeled viruses, propagated in trypsin-free CEF culture, were purified, lysed, and immunoprecipitated with anti-H5 monoclonal antibodies and protein A beads [3]. The immunoprecipitated HA molecules were then analyzed by SDS-PAGE and visualized by fluorography (Fig. 1). Almost all of the HA molecules (92%) were cleaved, indicating that intracellular HA cleavage enzymes in CEF recognize this sequence optimally, that is, unlike other HAs (Table 1), Thr at P-2 of the Ty/Eng/91 HA cleavage site did not affect the protease catalyzation.

The HA cleavage site sequence of avian influenza viruses is as an important criterion for determining virus virulence potential [25]. Based on the findings with naturally isolated virulent viruses, the R/K-X-R/K-R motif is considered as a virulent-type sequence by agricultural officials who can then take action to prevent further spread of such viruses. However, here we have demonstrated that Ty/Eng/91 HA is efficiently cleaved and the virus is extremely virulent, despite the fact that its HA cleavage site sequence (R-K-R-K-T-R) does not conform to the established motif. We should, therefore, be aware of such virulent viruses when diagnosing virulence potential of avian influenza virus isolates. The recent observation that HAs of H7 laboratory mutants with B-X-R-A-T-R or R-R-T-R cleavage site sequences are cleavable by intracellular proteases [16, 19] supports this proposal.

Two subtilisin-like serine proteases, furin and PC6, are prime candidates for the enzymes responsible for HA cleavage of virulent influenza viruses *in vivo*, because the sequence requirements of both proteases are similar to those of endogenous HA cleavage proteases in cell culture [6, 21, 24]. Both proteases recognize a mutant substrate [TI (MT-1): R-K-R-K-T-R] less efficiently than they recognize wild-type HA (R-K-R-K-K-R), suggesting an important role for the basic residue at P-2 optimal recognition and catalyzation by these proteases [6, 24]. However, Molloy *et al.* reported that furin processed a mutant form of anthrax toxin protective antigen that contained a nonbasic residue at P-2 (R-A-A-R) as efficiently as it processed the native substrate (R-K-K-R), and proposed a minimal motif sequence requirement of R-X-X-R for the protease [15]. Our finding that Ty/Eng/91 HA was cleaved almost completely (92%) by endogenous proteases in cell culture, together with that of Molloy *et al.*, suggests that a basic residue at P-2 is not necessary for efficient processing of some substrates by intracellular proteases, but is needed for optimal recognition of other substrates by these proteases. Thus, the contribution of the P-2 residue to proprotein processing depends upon substrate structural features other than the cleavage site sequence.

What factors govern the high virulence of Ty/Eng/91? To address this question, we performed preliminary experiments on Ty/Eng/91 replication in cell culture. The virus appears to grow rapidly in CEF culture; an infectivity titer of 5×10^8/m*l* was produced even at 8 hr postinoculation at a multiplicity of infection of 10, whereas at least 16 hr were required for other strains to reach the equivalent infectivity titer. The rapid growth of this virus was also demonstrated by monitoring its plaque size, reaching 2.5 mm in diameter at 2 days postinoculation in trypsin-free CEF culture, which is larger than other virulent viruses such as A/chicken/Pennsylvania/1370/83 (H5N2) (1.5 mm) or R (36–2) (1.0 mm). These observations suggest that the extremely high virulence of Ty/Eng/91 may cause peracute damage of target tissues, as a result of rapid growth of the virus in these organs. Pathologic evaluation of Ty/Eng/91-infected birds may provide evidence to support this theory.

Is the HA molecule a determinant for high virulence of Ty/Eng/91? Wood *et al.* isolated both avirulent and virulent virus clones from an outbreak in Norfolk [28]. HA sequencing revealed that the cleavage site sequence (R-K-R-K-T-R) was identical in both virus types and that there appeared to be no significant sequence changes in other HA regions, which could affect HA cleavability, between these two viruses [28]. Indeed, both the avirulent and the virulent viruses produced plaques in trypsin-free CEF culture, suggesting that HA cleavability does not play a role in virulence difference between highly virulent and avirulent Ty/Eng/91 strains [28]. Together, these findings suggest that the high virulence of Ty/Eng/91 is due to its marked rapid growth *in vivo*, which is regulated by gene product (s) other than HA. To confirm this hypothesis, further studies

such as reassortment studies between avirulent and virulent viruses are needed.

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