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Acquisition of a Polybasic Hemagglutinin Cleavage Site by a Low-Pathogenic Avian Influenza Virus Is Not Sufficient for Immediate Transformation into a Highly Pathogenic Strain▽

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Highly pathogenic avian influenza viruses (HPAIV) differ from all other strains by a polybasic cleavage site in their hemagglutinin. All these HPAIV share the H5 or H7 subtype. In order to investigate whether the acquisition of a polybasic cleavage site by an avirulent avian influenza virus strain with a hemagglutinin other than H5 or H7 is sufficient for immediate transformation into an HPAIV, we adapted the hemagglutinin cleavage site of A/Duck/Ukraine/1/1963 (H3N8) to that of the HPAIV A/Chicken/Italy/8/98 (H5N2), A/Chicken/HongKong/220/97 (H5N1), or A/Chicken/Germany/R28/03 (H7N7) and generated the recombinant wild-type and cleavage site mutants. In contrast to the wild type, multicycle replication of these mutants in tissue culture was demonstrated by positive plaque assays and viral multiplication in the absence of exogenous trypsin. Therefore, in vitro all cleavage site mutants resemble an HPAIV. However, in chicken they did not exhibit high pathogenicity, although they could be reisolated from cloacal swabs to some extent, indicating enhanced replication in vivo. These results demonstrate that beyond the polybasic hemagglutinin cleavage site, the virulence of HPAIV in chicken is based on additional pathogenicity determinants within the hemagglutinin itself or in the other viral proteins. Taken together, these observations support the notion that acquisition of a polybasic hemagglutinin cleavage site by an avirulent strain with a non-H5/H7 subtype is only one among several alterations necessary for evolution into an HPAIV.

Highly pathogenic avian influenza viruses (HPAIV) cause fowl plague in poultry (2), resulting in devastating losses. Moreover, transmission of H5N1 or H7N7 HPAIV to humans (5, 6, 9, 19) raises the concern that these viruses might evolve into a new human pandemic strain. An essential step in the replication of influenza A viruses is proteolytic processing of the trimeric hemagglutinin (HA). Only cleaved HA is able to expose the N terminus of the HA2 fragment within the endosome at low pH for mediating fusion of the virion envelope with the endosomal membrane (14, 22, 34). Most influenza virus strains contain an HA cleavage site (HACS) with only a single basic amino acid residue which is cleaved by tissue-restricted proteases only (16), thereby limiting spread in the infected host. In contrast, HPAIV carry a polybasic HACS leading to proteolytic activation by the ubiquitous protease furin (16, 32). This different type of activation results in very broad, nonrestricted organ tropism and a highly pathogenic phenotype in poultry (31). Intriguingly, beside the polybasic HACS, the second common feature of all HPAIV is the H5 or H7 subtype. None of the other HA subtypes have so far been found to be associated with HPAIV. Beside the polybasic HACS (17), additional molecular correlates of virulence were found within the HA itself (18, 24), the neuraminidase (3, 4, 7, 13), the NS1 protein (8, 15, 20, 21, 26), and the polymerase complex together with the nucleoprotein (9–11, 24, 25, 27, 28, 33). However, for natural hosts such as the chicken, such studies are rather scarce. In this study, we investigated whether the acquisition of a polybasic HACS by an avirulent avian strain with a serotype different from H5 or H7 would be sufficient for transformation into a highly pathogenic phenotype or result in low pathogenicity and, thus, reveal the existence of additional pathogenicity determinants. Therefore, we introduced the polybasic cleavage sites from three different HPAIV into the HA of the avirulent avian strain A/Duck/Ukraine/1/1963 (H3N8) and analyzed the obtained HACS mutants and the wild type in regard to in vitro properties and virulence in chicken.

MATERIALS AND METHODS

Recombinant viruses. The cloning of all eight viral gene segments of strain A/Duck/Ukraine/1/1963 (H3N8) into the vector pHW2000 (12) has been described previously (29). Modification of the HACS regions was performed by site-directed QuikChange mutagenesis; the primer sequences are available on request. Recombinant viruses were rescued essentially as described previously (10) and propagated in 11-day-old embryonated chicken eggs. After virus rescue, the gene composition and presence of the expected cleavage sites of the mutants were verified by sequencing following reverse transcription-PCR from isolated viral RNA (data not shown).

Plaque assays and growth curves. Plaque assays were performed as described previously (30) either in the presence of 2 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma, Taufkirchen, Germany) or in the absence of any exogenous protease. The volume of inoculum was 333 μl. Growth curves on Madin-Darby canine kidney (MDCK) cells and quail fibrosarcoma (QT05 6) cells in the presence of 2 or 1 μg/ml TPCK-treated trypsin, respectively, and in the absence of any exogenous protease in the supernatant were determined by titration of cell culture supernatants at 0, 8, 48, and 96 h pastinoculation at a multiplicity of infection of 10^{-2} by plaque assay on MDCK cells without trypsin in case of the HACS mutants and with trypsin in case of the wild-type A/Duck/Ukraine/1/1963 (H3N8). The reported plaque titers are the averages from two independent experiments.

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TABLE 1. Generated recombinant viruses with their HACSs and adjacent amino acid residues

Virus	HACS[a]
DkUkr63-Wt	NVPEKQT----**R**
DkUkr63-It98$_{HACS}$	NVPQ--**RRRKKR**
DkUkr63-Hk220$_{HACS}$	NTPQ**RERRRKKR**
DkUkr63-R28$_{HACS}$	NVPEIP-**KRRRR**

[a] Underlining and boldface indicate amino acid residues differing from the cleavage site and close vicinity of HA of DkUkr63-Wt and basic amino acid residues, respectively.

Western blotting. MDCK cell cultures were infected with virus in the presence of either 2 μg/ml TPCK-treated trypsin or no protease for 16 h in minimal essential medium containing 0.2% bovine serum albumin. Proteins from supernatant were separated on sodium dodecyl sulfate–8% polyacrylamide gels and electrotransferred to nitrocellulose membranes. For detection of HA, a monoclonal mouse antibody to A/Shangdong/9/93 (H3N2) (3HG3, 1:100,000, incubated overnight at room temperature; HyTest Ltd., Turku, Finland) and as a secondary antibody a mouse-specific goat immunoglobulin G Fab fragment conjugated with horseradish peroxidase (1:20,000, 1 h at room temperature; Dianova, Hamburg, Germany) were used, followed by chemiluminescence (Supersignal West Pico chemiluminescent substrate kit from Pierce).

Animal experiments. Ten 4-week-old White Leghorn specific-pathogen-free chickens were infected oculonasally with each virus. Each bird was observed daily for clinical signs and classified as healthy (0), sick (1) (exhibiting one of the following: respiratory symptoms, depression, diarrhea, cyanosis, edema, or nervous symptoms), severely sick (2) (severe or more than one of the previously mentioned symptoms), or dead (3), as described previously (1). In addition, chickens exhibiting slight depression were given a classification of 0.5. For virus isolation, cloacal swabs were taken and diluted in 1 ml phosphate-buffered saline (PBS) prior to plaque titration.

RESULTS

Generation of recombinant viruses. In order to obtain different HACS mutants of an avirulent avian strain, we replaced the HACS and adjacent amino acid residues of influenza virus A/Duck/Ukraine/1/1963 (H3N8) with those of A/Chicken/Italy/8/98 (H5N2), A/Chicken/Hong Kong/220/97 (H5N1), or A/Chicken/Germany/R28/03 (H7N7) by site-directed mutagenesis. To address possible structural constraints within the HA of the parent virus, we chose three polybasic HACSs of various lengths and with different types of adjacent amino acids (Table 1). The wild type (DkUkr63-Wt) or the HACS mutants (DkUkr63-It89$_{HACS}$, DkUkr63-Hk220$_{HACS}$, or DkUkr63-R28$_{HACS}$) were rescued by cotransfection of plasmids encoding the wild-type or mutated HA genes together with plasmids encoding the PB2, PB1, PA, NP, NA, M, and NS genes of A/Duck/Ukraine/1/1963 (H3N8).

Introduction of a polybasic cleavage site leads to trypsin-independent replication in vitro similar to that of HPAIV. In order to investigate whether the cleavage site mutants are dependent on trypsin for multicycle replication, plaque assays were performed with DkUkr63-Wt and mutants DkUkr63-It89$_{HACS}$, DkUkr63-Hk220$_{HACS}$, and DkUkr63-R28$_{HACS}$ on MDCK cells in the presence and in the absence of trypsin. Whereas DkUkr63-Wt proved to be dependent on trypsin for plaque formation, as expected for a low-pathogenic avian influenza virus, all three HACS mutants formed plaques independent of exogenous trypsin (Fig. 1A). For demonstration of activation cleavage of the HA precursor HA0, Western blot analyses of supernatants from virus-infected MDCK cells were performed. In accordance with the plaque assays, the HA0 of DkUkr63-Wt remained uncleaved in the absence of trypsin, whereas the HA0 precursors of the HACS mutants were cleaved into the HA1 and HA2 fragments to various extents. DkUkr63-It89$_{HACS}$ displayed efficient HA0 cleavage, whereas in the case of DkUkr63-Hk220$_{HACS}$ and DkUkr63-R28$_{HACS}$, the cleavage was incomplete (Fig. 1B). Moreover, the latter two mutants displayed an additional peptide with a molecular weight slightly higher than that of the HA1 fragment, in particular in the presence of trypsin, suggesting aberrant HA maturation. Analysis of multicycle growth kinetics corresponded to the plaque formation and proteolytic activation pattern of the HA. In contrast to DkUkr63-Wt, which required trypsin for productive replication in both MDCK and QT6 cells, all cleavage site mutants replicated independently of the addition of trypsin (Fig. 2). However, mutant DkUkr63-R28$_{HACS}$ grew considerably less efficiently in QT6 cells irrespective of the presence or absence of trypsin, correlating with the formation of very small plaques and inefficient proteolytic HA activation (Fig. 1 and 2).

Nevertheless, in contrast to the wild type, all three HACS mutants undergo multicycle replication in vitro, and their HA0 is processed in the absence of an exogenous protease. The independence of viral replication from exogenous trypsin is indicative of proteolytic activation by furin (32). These are features attributed only to HPAIV.

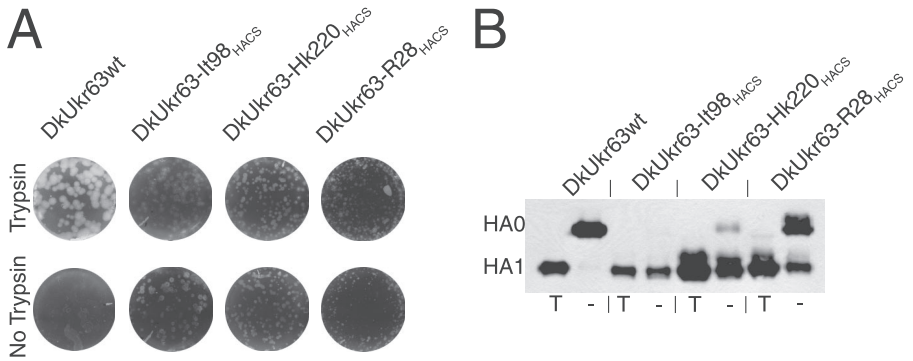

FIG. 1. In vitro replication of DkUkr63-Wt in comparison with the HACS mutants DkUkr63-It98$_{HACS}$, DkUkr63-Hk220$_{HACS}$, and DkUkr63-R28$_{HACS}$. (A) Plaque assays on MDCK cells in the presence and in the absence of trypsin. (B) Western blots of supernatants from infected MDCK cells incubated in the presence (T) and in the absence (−) of trypsin, using a monoclonal antibody specific to an HA of the H3 serotype.

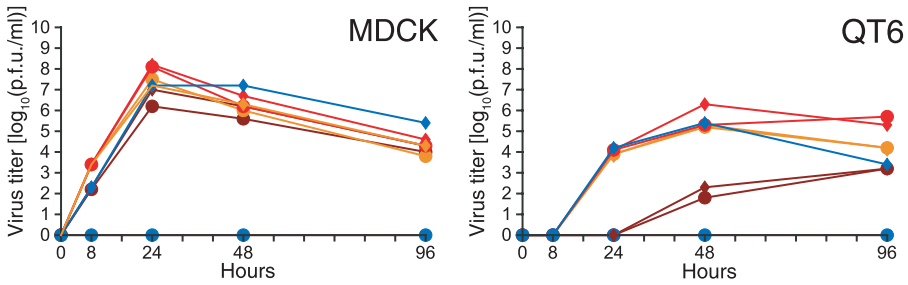

FIG. 2. Multicycle growth curves of DkUkr63-Wt (blue), DkUkr63-It98$_{HACS}$ (red), DkUkr63-Hk220$_{HACS}$ (orange), and DkUkr63-R28$_{HACS}$ (brown) on MDCK and QT6 cells in the presence (diamonds) and in the absence (circles) of trypsin.

Pathogenicity in chicken. To investigate the virulence of the cleavage site mutants in vivo, five groups of 10 chickens each were infected oculonasally with either DkUkr63-It89$_{HACS}$ (1.7×10^6 PFU/animal), DkUkr63-Hk220$_{HACS}$ (1.7×10^6 PFU/animal), DkUkr63-R28$_{HACS}$ (7.2×10^4 PFU/animal), or DkUkr63-Wt (1.7×10^6 PFU/animal) or mock infected by the administration of PBS and were observed for 10 days. (Due to its inefficient growth, DkUkr63-R28$_{HACS}$ had to be administered at a lower dosage.) All animals inoculated with PBS, DkUkr63-Wt, and DkUkr63-Hk220$_{HACS}$ exhibited no symptoms. Eight DkUkr63-It89$_{HACS}$-infected animals displayed slight transient depression; two animals (no. 3 and 9) further developed central nervous symptoms (Fig. 3). Chicken no. 3 exhibited a head inclination on day 9 after infection, whereas chicken no. 9 showed a slight head inclination on day 6, which decreased during the following days. We then determined the titers of shed virus in cloacal samples from day 5 by plaque assay beginning with undiluted inoculum from four animals of each group, including those with central nervous symptoms. Animals which had been inoculated with PBS, DkUkr63-Wt, or DkUkr63-Hk220$_{HACS}$ did not shed virus, whereas in the cloacal samples from four animals inoculated with DkUkr63-It89$_{HACS}$ and from one animal inoculated with DkUkr63-R28$_{HACS}$, virus could be detected at a titer of up to $10^{3.7}$ PFU per swab. Remarkably, virus could be reisolated only from animals which already had developed or proceeded to show signs of disease (Table 2). In contrast to trypsin-independent replication in vitro, the insertion of a polybasic cleavage site into the HA of the avirulent DkUkr63-Wt did not cause immediate transformation into an HPAIV; however, cloacal shedding of virus in accordance with clinical symptoms suggests enhanced replication in chicken.

DISCUSSION

In this study, we addressed the question of whether the acquisition of a polybasic HACS by an avirulent strain with a serotype different from H5 or H7 is sufficient for its transformation into an HPAIV or whether additional pathogenicity determinants within the HA itself or other viral proteins would

PBS

	1	2	3	4	5	6	7	8	9	10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
Sum	0	0	0	0	0	0	0	0	0	0
DCI	0	0	0	0	0	0	0	0	0	0

DkUkr63-Wt

	1	2	3	4	5	6	7	8	9	10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
Sum	0	0	0	0	0	0	0	0	0	0
DCI	0	0	0	0	0	0	0	0	0	0

DkUkr63-It98$_{HACS}$

	1	2	3	4	5	6	7	8	9	10
1	0	0	0	0	0	0	0.5	0	0.5	0
2	0	0	0	0.5	0.5	0	0	0.5	0	0
3	0	0	0	0	0	0	0	0.5	1	1
4	0	0	0	0.5	0	0	0.5	0	0	0
5	0	0	0	0	0	0	0	0.5	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0.5	0	0
9	0	0	0	0.5	0	1	1	1	1	1
10	0	0	0	0	0	0	0	0.5	0.5	0.5
Sum	0	0	0	1.5	0.5	1	2	3.5	3	2.5
DCI	0	0	0	0.15	0.05	0.1	0.2	0.35	0.3	0.25

DkUkr63-Hk220$_{HACS}$

	1	2	3	4	5	6	7	8	9	10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
Sum	0	0	0	0	0	0	0	0	0	0
DCI	0	0	0	0	0	0	0	0	0	0

DkUkr63-R28$_{HACS}$

	1	2	3	4	5	6	7	8	9	10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	1	1	1	1	1	1
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
Sum	0	0	0	0	1	1	1	1	1	1
DCI	0	0	0	0	0.1	0.1	0.1	0.1	0.1	0.1

FIG. 3. Virulence in chickens. Survival and disease after oculonasal inoculation with PBS (mock), DkUkr63-Wt, DkUkr63-It98$_{HACS}$, DkUkr63-Hk220$_{HACS}$, or DkUkr63-R28$_{HACS}$. The birds were observed for 10 days for clinical signs and classified as healthy (0), slightly ill (0.5), or ill (1); the daily clinical index (DCI) was calculated from the sum of individual clinical scores from all birds divided by the number of animals per group (10 chickens). Chickens 3 and 9 from the group infected with DkUkr63-It98$_{HACS}$ developed central nervous symptoms.

TABLE 2. Virus titers from cloacal swabs taken from chickens on day 5 after infection in relation to occurrence of clinical symptoms during the experiment

Virus	Chicken	Symptoms	Virus titer [$\log_{10}$ (PFU/swab)]
DkUkr63-Wt	1	No	—[a]
	2	No	—
	4	No	—
	6	No	—
DkUkr63-It98$_{HACS}$	1	Yes	2.6
	2	Yes	3.1
	3	Yes	3.7
	9	Yes	3.4
DkUkr63-Hk220$_{HACS}$	1	No	—
	2	No	—
	3	No	—
	4	No	—
DkUkr63-R28$_{HACS}$	1	No	—
	2	No	—
	3	No	—
	4	Yes	2.6

[a] —, no virus detected.

be required. To take into account possible structural constraints within the HA of the parental wild-type virus, we selected three polybasic HACSs of various lengths and with different adjacent amino acids. Accordingly, we replaced the HACS of the avirulent avian H3 strain A/Duck05 /Ukraine/1/1963 (H3N8) and neighboring amino acid residues with those of the HPAIV A/Chicken/Italy/8/98 (H5N2), A/Chicken/Hong Kong/220/97 (H5N1), or A/Chicken/Germany/R28/03 (H7N7). Previously, it has been shown in transient expression studies that the insertion of additional basic amino acids into the cleavage site of an H3 HA from a human strain results in intracellular proteolytic cleavage (23). However, at present, the consequence of this finding for in vivo replication and virulence could not be tested. In agreement with the previous observation, we demonstrated that a polybasic cleavage site introduced into the HA of an apathogenic avian influenza virus can result in equivalent cleavage activation and thus support multicycle replication in vitro independent of trypsin, a common feature of HPAIV strains. Cleavage of the HA0 precursor of DkUkr63-Hk220$_{HACS}$ and DkUkr63-R28$_{HACS}$ was incomplete in MDCK cells in the absence of trypsin. However, this did not affect viral growth in cell culture. This incomplete cleavage could be attributed to steric incompatibilities of the inserted polybasic HACS with the HA of DkUkr63-Wt. The extent of proteolytic activation of the HA may determine the efficiency of replication in vivo, i.e., cloacal shedding and pathogenicity as observed with DkUkr63-It89$_{HACS}$. Simulating the natural route of infection, oculonasal infection of chicken did not result in death, nor did the animals show severe symptoms indicative of fowl plague. Thus, despite HPAIV-like properties in vitro and cloacal shedding to some extent, in contrast to the wild-type parental virus, replication of the HACS mutants in the animal is still restricted compared with that of an HPAIV. Currently it is unclear whether after infection at a higher dose, more of the DkUkr63-R28$_{HACS}$-infected animals would have exhibited severe signs of disease. Virus reisolation from cloacal swabs failed after infection with the parental DkUkr63-Wt or DkUkr63-Hk220$_{HACS}$. The absence of detectable infectivity in cloacal swabs does not per se exclude viral genome replication but could also be due to impairment of later stages of viral replication, resulting in inefficient release of infectious virions. However, virus could be reisolated from one animal after infection with DkUkr63-R28$_{HACS}$ and from four animals after infection with DkUkr63-It98$_{HACS}$. Remarkably, virus isolation coincided with the occurrence of clinical symptoms in these animals. Taken together, the results indicate that although all three cleavage site mutants did not display a highly pathogenic phenotype in chicken, the virus reisolation from cloacal swabs indicates enhanced replication in vivo, suggesting incomplete adaption.

In conclusion, our data support the notion that the presence of a polybasic HACS is not the sole determinant for virulence in chicken. These results demonstrate that beyond the polybasic HACS, the virulence of HPAIV in chicken is based on additional pathogenicity determinants within the HA itself or other viral proteins. Therefore, the evolution of an HPAIV from a low-pathogenic avian influenza strain involves more alterations than the acquisition of a polybasic cleavage site.

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