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A novel eight amino acid insertion contributes to the hemagglutinin cleavability and the virulence of a highly pathogenic avian influenza A (H7N3) virus in mice

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ABSTRACT

In 2012, an avian influenza A H7N3 (A/Mexico/InDRE7218/2012; Mx/7218) virus was responsible for two confirmed cases of human infection and led to the death or culling of more than 22 million chickens in Jalisco, Mexico. Interestingly, this virus acquired an 8-amino acid (aa)-insertion (..PENPK-**DRKSRHRR**-TR/GLF) near the hemagglutinin (HA) cleavage site by nonhomologous recombination with host rRNA. It remains unclear which specific residues at the cleavage site contribute to the virulence of H7N3 viruses in mammals. Using loss-of-function approaches, we generated a series of cleavage site mutant viruses by reverse genetics and characterized the viruses in vitro and in vivo. We found that the 8-aa insertion and the arginine at position P4 of the Mx/7218 HA cleavage site are essential for intracellular HA cleavage in 293T cells, but have no effect on the pH of membrane fusion. However, we identified a role for the histidine residue at P5 position in viral fusion pH. In mice, the 8-aa insertion is required for Mx/7218 virus virulence; however, the basic residues upstream of the P4 position are dispensable for virulence. Overall, our study provides the first line of evidence that the insertion in the Mx/7218 virus HA cleavage site confers its intracellular cleavability, and consequently contributes to enhanced virulence in mice.

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Introduction

Influenza A viruses including 16 of the 18 hemagglutinin (HA) subtypes and 9 of the 11 neuraminidase (NA) subtypes primarily have their natural reservoir in wild aquatic birds (Webster et al., 2006). Although infection in their natural hosts is typically asymptomatic, influenza viruses, especially the H5 and H7 subtypes, can spread to domestic poultry and cause mild to severe disease, resulting in significant economic losses (Kalthoff et al., 2010). In addition, H5 and H7 subtype viruses have repeatedly jumped the species barrier from poultry to humans. The first well-documented case of human infection by an avian influenza virus through direct contact with infected birds occurred in 1996 (Kurtz et al., 1996). Since then, multiple subtypes of avian influenza viruses including H5, H6, H7, H9 and H10 have caused infection in humans, with severe and fatal disease possible with selected strains (Ozawa and Kawaoka, 2013; Subbarao and Joseph, 2007; Yuan et al., 2013). The increasing number of human influenza cases caused by avian influenza viruses underscores the need to better evaluate the risk of virulence, transmissibility and adaptation of these subtype viruses in mammalian hosts.

During June–August 2012, outbreaks of a highly pathogenic avian influenza A (HPAI) H7N3 subtype virus were reported in poultry farms throughout Jalisco State, Mexico, resulting in the deaths of approximately 22 million birds through either disease or culling (FAO, 2012). During the H7N3 outbreaks in Mexico, two poultry farm workers with direct contact to infected poultry developed conjunctivitis without fever or respiratory symptoms. Influenza H7N3 virus, A/Mexico/InDRE7218/2012 (Mx/7218) was isolated from one of the human cases (Lopez-Martinez et al., 2013; CDC, 2012) and found to be closely related to the circulating avian HPAI virus A/Chicken/Jalisco/CPA/2012 (H7N3) (Lopez-Martinez et al., 2013). Compared to other H7 viruses isolated from the outbreaks in poultry farms in the Americas in recent years, the 2012 H7N3 viruses isolated in Mexico are of special interest because the virus has demonstrated an ability to transmit to humans through direct contact or close proximity with infected animals (Lopez-Martinez et al., 2013). Experimentally, the closely related avian A/Chicken/Jalisco/CPA/2012 (H7N3) virus caused severe clinical disease and high mortality in chickens, similar to results reported for

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other HPAI viruses (Kapczynski et al., 2013). Pathogenesis studies in mammalian models revealed that the Mx/7218 virus replicated efficiently in the ferret respiratory tract and caused lethal infection in mice (Belser et al., 2013), which is in contrast to pathogenesis results observed for the lesser virulent 2004 H7N3 virus isolated from a human conjunctivitis case in British Columbia, Canada (Belser et al., 2007).

The influenza HA displayed on the viral envelope is responsible for both receptor binding and fusion between the host endosome and the viral membrane (Wiley and Skehel, 1987). The HA is synthesized as the polypeptide precursor HA0, which requires post-translational cleavage at the conserved arginine (R329, H3 numbering) located at the HA1/HA2 boundary. Activation of the fusion protein results in release of the fusion peptide and subsequent insertion into the target host cell membrane (Wiley and Skehel, 1987). This is a process that is dependent on the HA cleavage efficiency of host proteases. Structurally, the HA is organized in the viral membrane as a homotrimer composed of a globular head domain, a stem domain, and the HA cleavage site – the latter of which is projected away from the stem domain and exposed into solution, where it is accessible to proteases (Steinhauer, 1999). Depending on the primary sequences around the HA1/HA2 boundary, the proteases responsible for cleavage and the locations at which the cleavage occurs may vary (Steinhauer, 1999). Most human and low pathogenic avian influenza viruses, which possess a single arginine (R) residue at the HA1/HA2 boundary, are cleaved by extracellular or membrane-bound airway trypsin-like proteases. Such proteases include tryptase Clara found in the respiratory tract (Chen et al., 2000; Kido et al., 1992) or the recently identified serine proteases, TMPRSS2 and HAT, from the human airway epithelium (Bottcher et al., 2006). In contrast, HPAI H5 or H7 subtype viruses typically possess a stretch of R or lysine (K) residues at the HA cleavage site and can be recognized by ubiquitous subtilisin-like intracellular proteases. Such proteases include furin and PC6, both of which are specific for a minimal consensus sequence R-X-K/R-R for cleavage and can lead to systemic virus spread and increased virulence (Hosaka et al., 1991; Horimoto and Kawaoka, 1995; Horimoto et al., 1995). Furthermore, the cleavability of the HA protein can also be affected by how accessible the cleavage site is to the proteases. For example, the removal of a glycan located in close proximity to the HA cleavage site is necessary for HA intracellular cleavage of an avian H5N1 virus containing the R-X-K/R-R motif (Kawaoka et al., 1984; Kawaoka and Webster, 1989). In addition to removing steric hindrance posed by glycans, the structural accessibility of the HA cleavage site can also be improved by projecting it further away from the trimer stem surface as seen in some H7 viruses, in which an insertion acquired by nonhomologous recombination can facilitate cleavage by further exposing the cleavage site to the solvent (Hirst et al., 2004; Orlich et al., 1994).

Unlike most HPAI viruses, the Mexico H7N3 virus acquired a unique 8-amino acid (aa) insertion through nonhomologous recombination with host 28S rRNA (Kapczynski et al., 2013; Maurer-Stroh et al., 2013). In general, HA insertions acquired by nonhomologous recombination are rare, but have been previously observed among influenza viruses from the North American H7 lineage, including the 2002 H7N3 viruses isolated in Chile (Suarez et al., 2004), the 2007 H7N3 viruses isolated in Canada (Hirst et al., 2004; Pasick et al., 2005; Berhane et al., 2009), and the H7 variants selected through *in vitro* adaptation (Orlich et al., 1994; Li et al., 1990; Khatchikian et al., 1989). The exact role of HA insertions generated following nonhomologous recombination in viral pathogenicity, especially in viral adaptation to mammalian hosts, has not been fully evaluated. Here, by using the Mx/7218 virus, we evaluated the role of specific basic residues in the HA insertion towards viral pathogenesis. Our study provides valuable insight into the molecular basis of the H7N3 cleavage site and its impact on the efficiency of HA cleavage, the pH of fusion, and virulence in mammals.

Results

Intracellular cleavage of Mx/7128 HA

Compared to other HA cleavage site insertions acquired by nonhomologous recombination, the insertion at the HA cleavage site of Mx/7218 virus is relatively short at 8 aa long. The aa insertion of..PENPK-**DRKSRHRR**-TR/GLF (located at positions P10-P3; shown in bold and underlined), is rich in basic residues (6 out of 8 aa are basic residues), including one ionizable histidine (H) residue at position P5 (Table 1). However, unlike the continuous stretch of basic residues (R, K) regularly seen in the HAs of HPAI (H5 or H7) viruses, this unique insertion in the Mx/7128 virus HA contains basic residues interspersed with non-basic residues. To determine whether the Mx/7218 HA can be cleaved intracellularly in the absence of exogenous trypsin, we infected 293T cells with Mx/7218 or reference H7 viruses and assessed HA cleavage. Similar to the HPAI H7N7 viruses NL/219 and NL/230, Western blot analysis showed that cell surface-expressed Mx/7218 HA was able

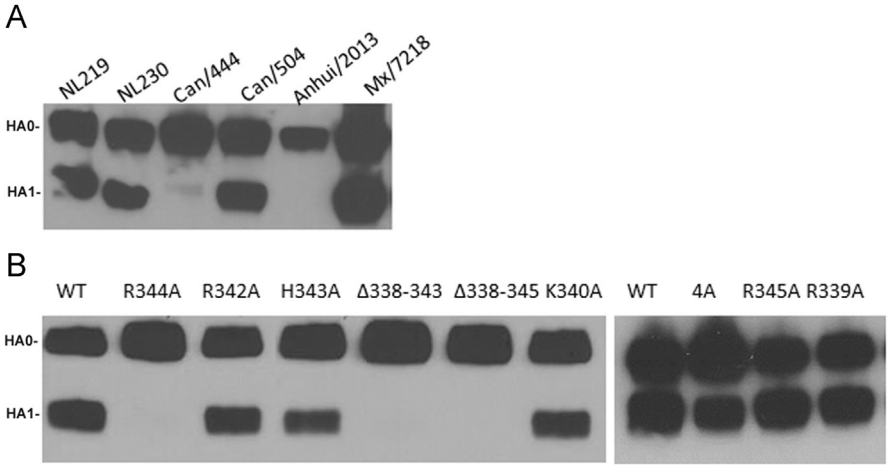

Fig. 1. The cleavability of H7 HAs in 293 T cells. The surface-expressed HAs in 293T cells from either viral infection (A) or transfection with the plasmids encoding wt or mutant forms of the Mx/7218 HA (B) were pulled down by streptavidin beads following biotin labeling and analyzed for the expression of precursor HA0 and the cleaved HA1 by Western blot with rabbit anti-HA monoclonal antibody.