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Sialic acid recognition is a key determinant of influenza A virus tropism in murine trachea epithelial cell cultures

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ABSTRACT

Influenza A virus interacts with specific types of sialic acid during attachment and entry into susceptible cells. The precise amino acids in the hemagglutinin protein that control sialic acid binding specificity and affinity vary among antigenic subtypes. For H3 subtypes, amino acids 226 and 228 are critical for differentiating between α2,3- and α2,6-linked forms of sialic acid (SA). We demonstrate that position 190 of the HA from A/Udorn/307/72 (H3N2) plays an important role in the recognition of α2,3-SA, as changing the residue from a glutamic acid to an aspartic acid led to alteration of red blood cell hemagglutination and a complete loss of replication in differentiated, murine trachea epithelial cell cultures which express only α2,3-SA. This amino acid change had a minimal effect on virus replication in MDCK cells, suggesting subtle changes in receptor recognition by the H3 hemagglutinin can lead to significant alterations in cell and species tropism.

Introduction

Influenza A virus is a significant human pathogen that causes annual epidemics in the human population (Thompson et al., 2004; Thompson et al., 2003). While only two antigenic subtypes of influenza A virus circulate in humans (H3N2 and H1N1), a number of different antigenic subtypes exist and circulate widely in avian populations (Dugan et al., 2008). Through reassortment of virus gene segments, influenza A virus strains containing hemagglutinin (H or HA) and neuraminidase (N or NA) antigenic subtypes that are novel to humans can emerge, leading to influenza A virus pandemics that are associated with significantly higher morbidity and mortality (Rajagopal and Treanor, 2007).

In order for an avian influenza A virus strain to replicate and transmit efficiently in humans, a number of barriers must be overcome (Parrish and Kawaoka, 2005). One of the critical barriers appears to be the recognition of appropriate forms of sialic acid (SA), the influenza A virus receptor, by the HA protein. Human influenza A virus strains preferentially recognize sialic acid linked via an α-2,6 glycosidic linkage (2,6 SA) to the penultimate carbohydrate while avian influenza A viruses preferentially recognize α-2,3 SA (2,3 SA) (Rogers et al., 1983) (Nicholls et al., 2008). The recognition of SA by HA is in fact, much more complex than simply differentiating between a 2–3 and 2–6 glycosidic linkage. There are very important interactions of HA with saccharides outside the terminal SA and modifications of the SA and other saccharides can also affect the ability of HA to recognize SA containing carbohydrates (Russell et al., 2006; Stevens et al., 2006b).

While the receptor binding pocket of the influenza A virus HA protein is structurally conserved among a number of HA subtypes the precise amino acids which dictate receptor binding specificity and affinity vary to some degree (Russell et al., 2006; Stevens et al., 2006a). Amino acids 226 and 228 of H3 subtypes of influenza HA have been implicated as critical residues important for differentiating between 2,3 SA and 2,6 SA receptors (Vines et al., 1998). However, a number of other H3 amino acids can modulate receptor recognition or affinity (Martin et al., 1998; Matrosovich et al., 2000; Meisner et al., 2008; Nakajima et al., 2003; Suzuki et al., 2000) (Busch et al., 2008; Lu et al., 2006; Lu et al., 2005; Medeiros et al., 2001; Medeiros et al., 2004; Widjaja et al., 2006). In particular, amino acid 190 has been associated with changes in 2,3 versus 2,6 SA recognition in H3 subtype viruses (Martin et al., 1998; Nobusawa et al., 2000; Yassine et al., 2007). A glutamic acid (E) is present in many avian influenza virus strains while an aspartic acid (D) is often found in human H3 viruses at this position (Matrosovich et al., 2000; Stevens et al., 2006a). The A/Udorn/307/72 influenza virus strain has affinity for both 2,3 SA and 2,6 SA (Matrosovich et al., 2000; Suzuki et al., 2000) and has amino acids associated with 2,6 SA receptor binding at positions 226 and 228 but not at position 190.

Since receptor recognition is believed to be one, if not the, key barrier for cross-species transmission of influenza A viruses, we

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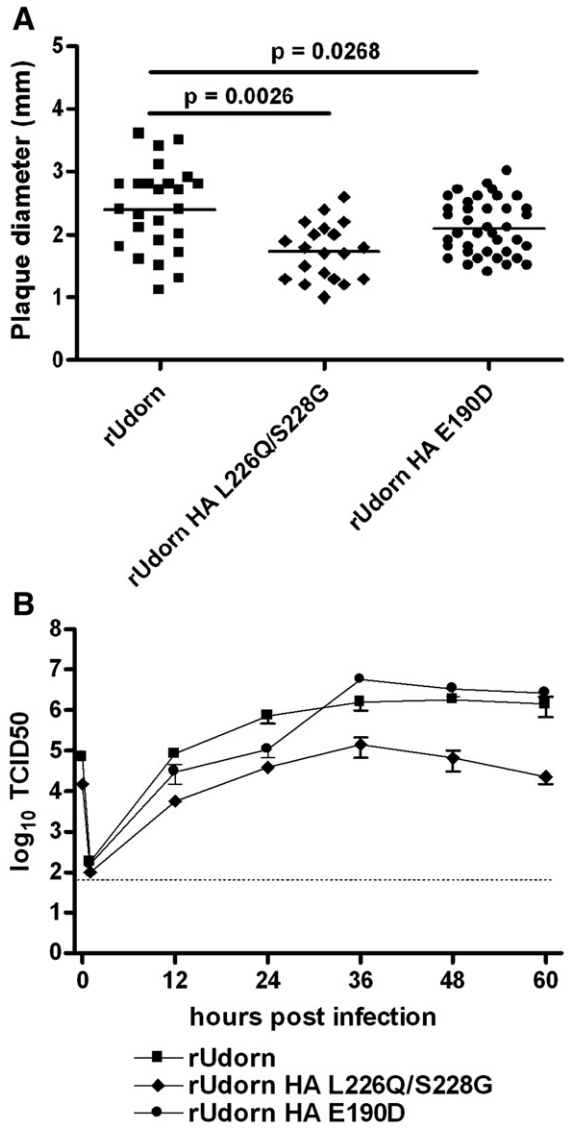

Fig. 1. Replication of rUdorn viruses encoding HA proteins with mutations in the receptor binding site. A) The indicated recombinant viruses were analyzed by plaque assay on MDCK cells. The plaque diameter was measured with a micrometer, average plaque diameter (n=25 for rUdorn; n=20 for rUdorn HA L226Q/S228G; n=39 for rUdorn HA E190D) calculated and statistical significance determined by student's t test. B) Virus replication in MDCK cells after infection at an MOI=0.01. Infected cell supernatants were harvested at the indicated times and $TCID_{50}$ titers determined on MDCK cells. The dotted line indicates the limit of detection. The mean and standard error of the mean are graphed.

initiated a study to determine if recognition of 2,3 SA could account for the ability of influenza A/Udorn/307/72 to productively infect murine tracheal epithelial cell (mTEC) cultures. Our results suggest that amino acid changes outside of positions 226 and 228 of H3 HA proteins, in particular position 190, can profoundly impact the ability of influenza A virus to recognize and utilize 2,3 SA as a virus receptor.

Results

The influenza A/Udorn/307/72 (rUdorn) virus HA protein can recognize 2,6 and 2,3 SA and is able to replicate in mTEC cultures (Ibricevic et al., 2006; Newby et al., 2007). The mTEC cultures express only 2,3 SA and minimally tissue culture passaged influenza virus strains that recognize only 2,6 SA are not able to replicate in mTEC cultures (Ibricevic et al., 2006; Newby et al., 2007), suggesting the recognition of 2,3 SA is key for influenza virus replication in these cultures. The rUdorn HA protein contains consensus residues at positions 226 and 228 which confer binding to 2,6 SA, however, it encodes an E at position 190 which has been shown to increase HA affinity for 2,3 SA (Martin et al., 1998; Matrosovich et al., 2000; Nobusawa et al., 2000; Yassine et al., 2007).

Site-directed mutagenesis was used to introduce amino acid changes that would alter the SA recognition of the rUdorn HA to solely 2,3 SA (HA L226Q/S228G). A mutation at position 190 (E190D) was introduced in order to confer greater selectivity for 2,6 SA. Recombinant influenza viruses encoding these mutations were rescued and characterized for their replication in MDCK cells since this cell type is routinely used to propagate a number of different influenza A virus strains. The recombinant virus encoding HA L226Q/S228G had significantly smaller plaques (p=0.0026) than the rUdorn wt virus while the virus encoding HA E190D formed plaques that were not significantly different in size (p=0.0268) from those of rUdorn wt (Fig. 1A). These data indicated the rUdorn HA L226Q/S228G virus was not as efficient at infecting MDCK cells as its parental virus.

The viruses were then characterized for their replication in MDCK cells (Fig. 1B) after a low multiplicity of infection (MOI). The rUdorn HA E190D virus replicated to similar titers as the rUdorn wt virus while the rUdorn HA L226Q/S228G virus replicated to titers that were consistently 10 to 100 fold lower. MDCK cells express higher levels of 2,3 SA when compared to 2,6 SA (Matrosovich et al., 2003; Oh et al., 2008), so the reduced replication of the rUdorn HA L226Q/S228G virus in both plaque assays and after low MOI infection implies that these mutations have a detrimental effect for virus replication in the rUdorn genetic background.

To determine if the rUdorn HA L226Q/S228G virus had altered levels of viral protein expression, MDCK cells were infected at an MOI of 5, harvested 6 hpi and the levels of HA and M2 protein expression

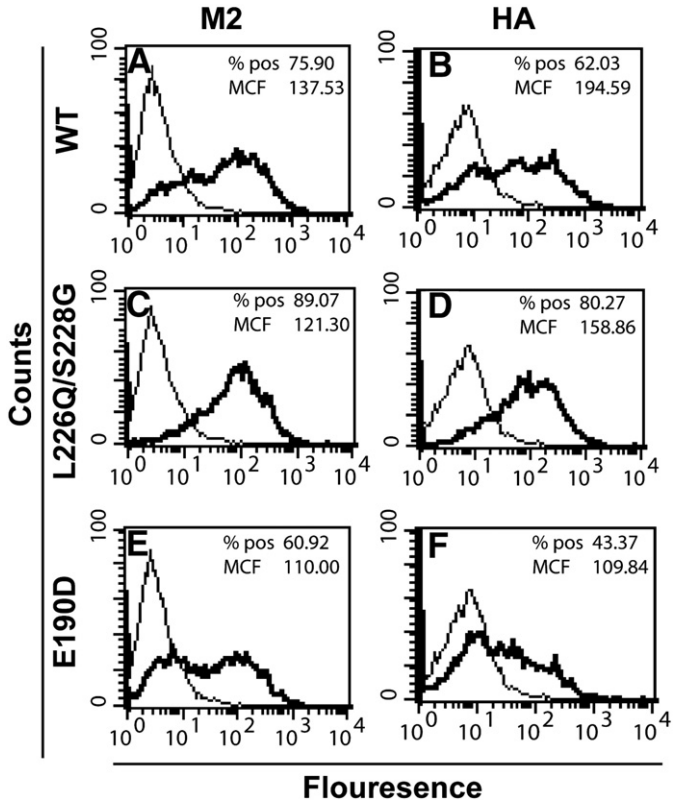

Fig. 2. Viral protein expression. MDCK cells were infected at an MOI of approximately 5 and harvested for flow cytometry at 6 hpi. The cells were immunostained for the M2 (A, C and E) or HA (B, D, F) protein and analyzed by flow cytometry. The percent positive (% pos) and mean channel fluorescence (MCF) were determined using CellQuest software. The data shown are representative of results from 4 independent experiments. The dark traces represent virus-infected cells while the light traces represent mock-infected cells.