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Two single mutations in the fusion protein of Newcastle disease virus confer hemagglutinin-neuraminidase independent fusion promotion and attenuate the pathogenicity in chickens

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ABSTRACT

The fusion (F) protein of Newcastle disease virus (NDV) affects viral infection and pathogenicity through mediating membrane fusion. Previously, we found NDV with increased fusogenic activity in which contained T458D or G459D mutation in the F protein. Here, we investigated the effects of these two mutations on viral infection, fusogenicity and pathogenicity. Syncytium formation assays indicated that T458D or G459D increased the F protein cleavage activity and enhanced cell fusion with or without the presence of HN protein. The T458D- or G459D-mutated NDV resulted in a decrease in virus replication or release from cells. The animal study showed that the pathogenicity of the mutated NDVs was attenuated in chickens. These results indicate that these two single mutations in F altered or diminished the requirement of HN for promoting membrane fusion. The increased fusogenic activity may disrupt the cellular machinery and consequently decrease the virus replication and pathogenicity in chickens.

1. Introduction

Newcastle disease (ND) is a highly contagious disease in chickens which causes huge economic losses to the poultry industry. ND is widely spread all over the world and consistently reported from different countries. The causative agent of ND is Newcastle disease virus (NDV) which belongs to the Avulavirus genus of the *Paramyxoviridae* family. Eighteen different genotypes (I –XVIII) (Dimitrov et al., 2016; Snoeck et al., 2013) have been identified to date according to the characteristic of nucleotide sequence of the fusion (F) protein gene although NDV only has one serotype. The NDV genome is about 15 kb and contains six genes which encode at least seven proteins. NDV infection is initiated by receptor recognition and binding to the host cell surface, which is followed by fusion mediated by the hemagglutinin-neuraminidase (HN) and F glycoproteins. These two glycoproteins on the NDV surface make major contributions to virulence, contagiousness, host range, and tissue tropism.

The F protein of NDV is a class I integral membrane protein present as a trimer in the virion (Lamb and Jardetzky, 2007; Swanson et al., 2010). Initially, an inactive precursor F0 is synthesized and subsequently cleaved by cellular proteases into disulfide-linked F1-F2 complex. The F1 subunit contains the fusion peptide (FP), the transmembrane (TM) domain and two hydrophobic heptad repeat (HR) domains (HRA and HRB) (Baker et al., 1999). Upon triggering, HRA forms a triple-stranded coiled coil that mediates insertion of the FP into the target membrane, thus forming the so-called transient intermediates. Subsequently, HRB binds into the grooves between adjacent HRA monomers in an antiparallel orientation, forming a six-helix bundle that pull the target and effector membranes together (Chen et al., 2001; Swanson et al., 2010). Generally, this process is dependent on the interaction of the F and homologous HN proteins (Iorio and Mahon, 2008; Iorio et al., 2009).

Previous studies reported that L289A mutation in the F protein of NDV could alter the requirement for HN protein in fusion (Li et al., 2005; Sergel et al., 2000), but the effects of the L289A mutation on the NDV biological property was not investigated. Recently, Manoharan et al. (2016) reported that a Y527A mutation in F resulted in a hyperfusogenic virus with increased replication and immunogenicity.

In the present study, we identified two single mutations in the HRB linker domain of genotype VII NDV F protein could independently

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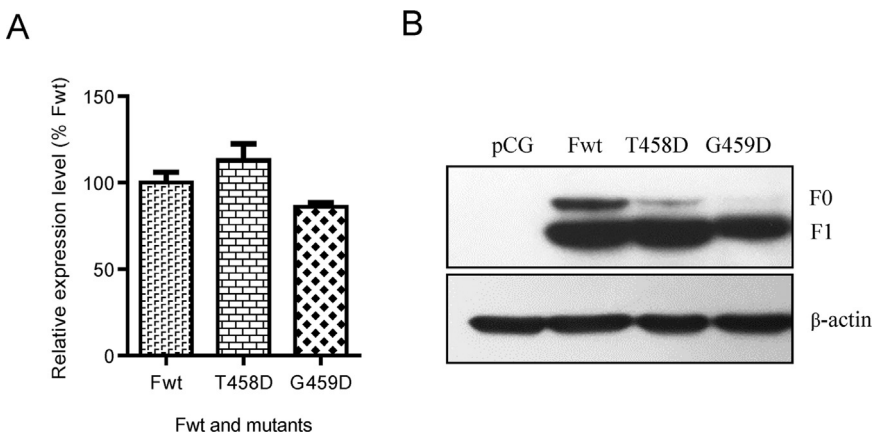

Fig. 1. Expression of Fwt and F mutant proteins. (**A**) Surface expression of T458D and G459D F mutants was determined by FACS analysis at 8 h post-transfection of BHK-21F cells by using anti-NDV polyclonal antibody. Data are representative of at least three independent experiments. (**B**) At 16 h post-transfection, the T458D and G459D F mutant-transfected BHK-21F cells were harvested and lysed. Expression and cleavage of NDV F proteins from the cell lysates were detected by Western blotting. The positions of precursor (F0) and cleaved subunit (F1) of the F protein are shown.

promote fusion in the absence of its homologous HN protein and decrease the pathogenicity of mutated NDVs in chickens.

2. Results

2.1. Newly-identified mutations in the fusion protein

In our previous study on the role of the HN of G7 strain in the pathogenicity in chickens (Liu et al., 2015), we rescued a G7 mutant with highly increased fusogenic activity. Sequence analysis of the mutant identified two amino acid mutations (T458D and G459D) in the HRB linker domain of the F protein. In this study, we aimed to further investigate the effects of these two mutations in the F protein on the biological property of the virus. First, we generated F protein expression clones containing one of these two mutations and quantitated the cell surface expression of the F protein from the mutants by FACS analysis and comparison with that of Fwt. The results showed that all mutated proteins were efficiently expressed on the cell surface with a similar level to Fwt, ranging from 86% to 113% (Fig. 1A). Subsequently, we detected the cleavage abilities of the NDV Fwt, T458D and G459D mutants expressed in BHK-21F cells. The result from Western blotting assay showed that T458D and G459D mutants were cleaved more efficiently than Fwt protein (Fig. 1B). Thus, the data suggest that two mutants were readily expressed and transported to the cell surface, and efficiently cleaved by the cellular proteases.

2.2. Two single mutations promote fusion independently

To detect which of the two mutations affects the fusion activity, we transfected T458D, G459D, or parental Fwt plasmid with or without its homologous HN into BHK-21F cells. The results showed that either of mutated F proteins could induce significantly higher fusion activities than the Fwt when co-expressed with the homologous HN. Surprisingly, these two mutants were also able to promote the fusion in the absence of HN protein (Fig. 2A and B) although the fusion activities were lower than that in the co-expression with HN. To detect if the increased fusion activities mediated by these two mutants are cell type-specific, the same experiments were performed in Vero and DF-1 cells. Interestingly, the similar results obtained from BHK-21F cells were observed in Vero and DF-1 cells as well (Fig. 2A and B). Therefore, our data indicate that T458D and G459D mutations could alter or diminish the requirement for the homologous HN protein in fusion promotion, and mediate the membrane fusion regardless of cell types.

2.3. Two single mutations decreased virus replication or release from infected cells

To explore if the increased fusogenicity resulted from the F mutations affects other biological properties of the virus, we generated two NDV recombinant viruses bearing T458D or G459D mutation (designed as rG7-T458D and rG7-G459D, respectively) based on the rG7 infectious clone (Liu et al., 2015). First, we examined the capability of the attachment and replication of the mutated NDVs in BHK-21 cells at 0 h and 12 h post-infection by measuring the NP mRNA. The qPCR data showed that rG7-T458D mutant had a similar capability of viral attachment to cells and replication in the cytoplasm as the parental rG7 virus during the early hours of infection (Fig. 3A). Virus titration showed that the titers of two mutant viruses in the supernatants were markedly lower than that of rG7 from 16 h to 48 h post-infection (Fig. 3B). These data suggest that the two mutations in the F protein may impair the viral replication or release from the cells after 16 h post-infection, but not viral attachment or replication in the early stage of infection.

2.4. The F mutations attenuate the pathogenicity in chickens

To assess the effects of the F mutations on viral virulence in chickens, we evaluated the pathogenicity of the NDV mutants by performing the standard intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) assays. The results showed that ICPI of the rG7-T458D and rG7-G459D strains was 1.59 and 1.51, respectively, which are lower than that of rG7 (ICPI = 1.7). The IVPI of two mutated viruses was 1.94 and 2.01, respectively, which are also lower than that of rG7 (IVPI = 2.4). In addition, the data obtained from the intranasal infection experiments showed that virus shedding from pharynx and cloaca, and replication in spleen and intestinum tenue from the mutant virus-infected chickens were significantly lower than those of rG7-infected chickens at day 4 post-infection (Fig. 4). Taken together, our data suggest that the two mutations in the F protein impaired the viral replication or release and attenuated the pathogenicity *in vitro* and *in vivo*.

3. Discussion

A previous study had reported that an L289A mutation in the NDV F protein confers the independent fusion promotion without the requirement for its homologous HN protein (Sergel et al., 2000). Subsequently, Li et al. (2005), demonstrated that this HN-independent fusion mode is cell specific in the absence of HN. In our present study,