A single amino acid change, Q114R, in the cleavage-site sequence of Newcastle disease virus fusion protein attenuates viral replication and pathogenicity

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A key determinant of Newcastle disease virus (NDV) virulence is the amino acid sequence at the fusion (F) protein cleavage site. The NDV F protein is synthesized as an inactive precursor, F0, and is activated by proteolytic cleavage between amino acids 116 and 117 to produce two disulfide-linked subunits, F1 and F2. The consensus sequence of the F protein cleavage site of virulent [112(R/K)-R-Q-(R/K)-R ↓ F-I118] and avirulent [112(G/E)-(K/R)-Q-(G/E)-R ↓ L-I118] strains contains a conserved glutamine residue at position 114. Recently, some NDV strains from Africa and Madagascar were isolated from healthy birds and have been reported to contain five basic residues (R-R-K-R ↓ F-I/V or R-R-R-R-R ↓ F-I/V) at the F protein cleavage site. In this study, we have evaluated the role of this conserved glutamine residue in the replication and pathogenicity of NDV by using the moderately pathogenic Beaudette C strain and by making Q114R, K115R and I118V mutants of the F protein in this strain. Our results showed that changing the glutamine to a basic arginine residue reduced viral replication and attenuated the pathogenicity of the virus in chickens. The pathogenicity was further reduced when the isoleucine at position 118 was substituted for valine.
reported to contain five basic residues (R-R-R-K-R ↓ F-I/V or R-R-R-R-R ↓ F-I/V) at the F protein cleavage site but were isolated from apparently healthy, unvaccinated poultry birds (Servan de Almeida et al., 2009; Snoeck et al., 2009). Furthermore, some of these strains contain valine at position 118 of the F protein cleavage site instead of isoleucine, which is present in most virulent and avirulent NDV strains. This finding does not agree with our current understanding, which is that the number of basic amino acid residues at the F protein cleavage site determines the virulence of NDV. Therefore, this study was undertaken to examine the role of glutamine at the F protein cleavage site in NDV pathogenicity by using reverse genetics. We have also evaluated the role of V118 in conjunction with Q114 in NDV pathogenesis.

In the present study, four NDV F cleavage-site-mutant clones were constructed, as described in Table 1. Site-directed mutagenesis was used to introduce individual amino acid substitutions into a cDNA of the F gene of mesogenic NDV strain Beaudette C (BC). The F gene within a full-length cDNA clone of the BC antigenome was then replaced with each mutated F gene. These clones were transfected into HEp-2 cells, and mutant viruses were recovered as previously described (Krishnamurthy et al., 2000). These viruses were designated rNDV-Q114R, I118V (112R-R-R-K-R ↓ F-V118), rNDV-Q114R, K115R, I118V (112R-R-R-R-R ↓ F-V118), rNDV-Q114R (112R-R-R-K-R ↓ F-118) and rNDV-Q114R, K115R (112R-R-R-R-R ↓ F-118). The F genes from recovered viruses were sequenced, which confirmed the presence of each mutation introduced and the lack of adventitious mutations in the F gene. To determine the stability of each F gene mutant, the recovered viruses were plaque purified and passaged five times in 9-day-old embryonated chicken eggs. Sequence analysis of the F gene in the mutant viruses after five passages showed that the introduced mutations were unaltered (data not shown). All infectious virus research was performed in our USDA approved enhanced biosafety level 3 (BSL-3+) containment facility.

The cell-surface expression of the F protein of each mutant virus was determined by flow cytometry in infected chicken-embryo fibroblast (DF-1) cells and was found to be similar to that of wild-type BC virus (rNDV) (Table 1). Briefly, DF-1 cells were infected with each recombinant virus at an m.o.i. of 0.1. After 24 h, the cells were detached with PBS (pH 7.2) containing 5 mM EDTA and centrifuged at 500 g for 5 min at 4 °C. Cells were then incubated with NDV anti-FNterm-specific antibody (1:10 dilution) for 30 min at 4 °C. Subsequently, cells were washed with PBS and incubated for 30 min on ice with 1:500 diluted Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody. Cells were analysed by using a FACS Aria II apparatus and FlowJo software (Becton Dickinson Biosciences).

To analyse whether the attenuation of the F protein cleavage-site mutant viruses is correlated with the rate of processing of the F protein by intracellular host cell proteases, we infected DF-1 cells with rNDV and the cleavage-site mutants for 24 h at an m.o.i. of 10. The infected cells were labelled with a mixture of [35S]methionine and [35S]cysteine for 30 min (‘pulse’) and were incubated for different times (‘chase’) to allow the proteins to be processed. The cells were lysed and F proteins were immunoprecipitated, separated by SDS-PAGE (10% acrylamide gel) and subjected to autoradiography (Fig. 1a, b). Analysis of the pulse–chase experiment revealed that after the 30 min pulse both uncleaved precursor F0 and cleaved product F1 were detected in both wild-type and mutant viruses (0 min chase). In the case of wild-type rNDV, after 60 min of the chase period all the F0 proteins had been completely processed (100%) into F1–F2 subunits. In contrast, F0 proteins of cleavage-site mutants

| Table 1. NDV F protein cleavage-site mutants, surface expression of F protein cleavage-site mutants of NDV and pathogenicity of F protein cleavage-site mutants of NDV |
|-----------------|-----------------|-----------------|
| Virus           | Amino acid sequence at the cleavage site of F protein* | Cell-surface expression† | ICPI score‡ |
|                 | 112 113 114 115 116 ↓ 117 118 |                  |             |
| rNDV            | R R Q K R ↓ F I | 100.0           | 1.58        |
| rNDV-Q114R, I118V | R R R K R ↓ F V | 99.8 ± 1.5     | 1.33        |
| rNDV-Q114R, K115R, I118V | R R R R R ↓ F V | 99.7 ± 2.2     | 1.37        |
| rNDV-Q114R     | R R R K R ↓ F I | 99.6 ± 4.1     | 1.33        |
| rNDV-Q114R, K115R | R R R R R ↓ F I | 100.0 ± 1.2    | 1.36        |

*Location of F protein cleavage-site mutations. Cleavage site is indicated by an arrow. Wild-type mesogenic strain BC (rNDV) virulent cleavage-site amino acid positions are shown at the column heads. The amino acid changes in the mutants are shown in boldface type.

†Cell surface expression levels of F protein of cleavage-site mutants relative to the level of the rNDV are shown. Expression of the F protein was quantified by flow cytometry using NDV-FNterm-specific antibodies. All values are means ± SD of three independent experiments where P<0.05.

‡Virulence of mutant and wild-type viruses was evaluated by ICPI in ten 1-day-old chicks. ICPI score=[(total number of sick chickens × 1) + (total number of dead chickens × 2)]/80 observations. ICPI values for velogenic strains approach the maximum score of 2.00, whereas lentogenic strains give values close to 0. Values were means of three independent experiments where P<0.05.
remained incompletely cleaved even after a 90 min chase period, suggesting slower processing of the F₀ protein by host-cell proteases of the cleavage-site mutants.

To determine the in vitro growth characteristics of recombinant F protein mutant viruses, we performed multicycle growth kinetics in DF-1 cells. The results showed that each of the F cleavage-site mutant viruses had delayed growth compared with rNDV (Fig. 2a). The mutants rNDV-Q114R, I118V and rNDV-Q114R, K115R, I118V had 1.5–2.0 log₁₀ lower virus yield and the mutants rNDV-Q114R and rNDV-Q114R, K115R had 1.0 log₁₀ lower virus yield compared with rNDV at 32 h post-infection (p.i.). Even after 64 h p.i., the yield of cleavage-site mutant viruses showed more delayed growth than that of rNDV. The virus titres of cleavage-site mutant viruses from 8 to 32 h p.i. were significantly different from that of the wild-type virus (P<0.05). These results showed not only that the change from glutamine to arginine at position 114 decreased the replication rate of NDV but also that the change from valine to isoleucine at position 118 further decreased the replication rate of NDV. Furthermore,
Fig. 2. (a) Multicycle growth kinetics of wild-type mesogenic strain BC (rNDV) and F protein cleavage-site mutants of NDV in DF-1 cells. DF-1 cells in six-well plates were infected in duplicate with parental and mutant viruses at an m.o.i. of 0.01. Supernatants were collected at 8 h intervals until 64 h p.i. and virus titres were determined at each time point by plaque assay. Values are means from three independent experiments; error bars indicate SD. (b) Growth kinetics of rNDV and the F protein cleavage-site mutants of NDV in the brains of 1-day-old chicks. Groups of ten 1-day-old chicks were inoculated intracerebrally with $10^3$ p.f.u. of virus per chick. Two birds were sacrificed each day after infection; brains were collected and homogenized, and virus titrated by plaque assay in DF-1 cells. Virus titres are shown as log$_{10}$ p.f.u. per gram of brain tissue. Values are means from three independent experiments; error bars indicate SD. (c) Pathogenicity of wild-type and F protein cleavage-site mutants of NDV in 1-day-old chicks inoculated via the intra-nasal and intra-ocular routes. Groups of five 1-day-old chicks were inoculated with $10^6$ p.f.u. of virus per bird and observed daily for signs of disease and mortality for 8 days.
mutation K115R did not appear to have an effect on the replication rate, as shown by rNDV-Q114R, K115R versus rNDV-Q114R or rNDV-Q114R, K115R, I118V versus rNDV-Q114R, I118V.

We then evaluated the effect of these F protein cleavage-site mutations in vivo by performing intracerebral pathogenicity index (ICPI) tests in 1-day-old chicks (Alexander, 1989). Each virus was inoculated intracerebrally into groups of ten 1-day-old chicks. The birds were observed and scored for paralysis and death once every 12 h for 8 days, and ICPI values were calculated. The ICPI values for all the cleavage-site mutants were significantly lower than that for rNDV (Table 1). The 1-day-old chicks infected with rNDV showed signs of depression and paralysis at 24 h p.i., whereas the 1-day-old-chicks infected with cleavage-site mutant viruses showed the same signs at 48 h p.i. To further compare the replication of the mutant viruses in neuronal tissue, groups of ten 1-day-old chicks were inoculated with 50 μl of PBS (pH 7.2) containing 10^3 p.f.u. of each mutant virus per chick via the intracerebral route. Two birds from each group were sacrificed every 12 h p.i., and brain-tissue samples were collected and snap-frozen on dry ice. The brain-tissue samples were homogenized, and virus titres in the tissue samples were determined by plaque assay in DF-1 cells (Fig. 2b). The cleavage-site mutants exhibited a marked decrease in their replication rate compared with rNDV. The mutant rNDV-Q114R, K115R had 1.0 log10 lower and rNDV-Q114R, I118V, rNDV-Q114R, K115R, I118V and rNDV-Q114R had 1.5–2.0 log10 lower virus yield compared with rNDV at 48 h p.i. This result corroborated our earlier in vitro results of the slower replication rates of mutant viruses compared with rNDV in DF-1 cells. To evaluate the pathogenicity of cleavage-site mutants through the natural route of infection further, groups of five 1-day-old chicks were infected by the natural (oculonasal) route with 100 μl of PBS containing 10^6 p.f.u. of each mutant virus per bird. The chicks in each group were observed for clinical signs of disease until 8 days p.i. and the survival percentages were calculated (Fig. 2c). All the birds inoculated with rNDV showed clinical signs of depression, watery greenish diarrhoea and drooping wings on day 2 p.i. and died by day 4 p.i., whereas the cleavage-site mutants exhibited the same clinical signs on day 4 p.i. In the case of mutants rNDV-Q114R, K115R, I118V, rNDV-Q114R and rNDV-Q114R, K115R, all the birds died by day 7 p.i. and in the case of rNDV-Q114R, I118V all the birds died by day 8 p.i.

In summary, we have examined the role of Q114 and I118 in the F protein cleavage-site motif in NDV pathogenesis. The precursor envelope glycoprotein F0 of virulent strains of NDV is processed within the trans-Golgi network in mammalian cells to produce the disulphide-linked F1–F2 active protein. The cleavage recognition sequence, wherein the amino acid residues follow the pattern basic-X-basic-basic, represents a preferred substrate for the cellular enzyme furin (Durell et al., 1997; Klenk & Garten, 1994; Nagai et al., 1976; Ortmann et al., 1994). Structural modelling of human furin and the crystal structure of mouse furin suggested that the catalytic domain of furin is surrounded in and around with abundant negatively charged amino acids in the substrate-binding region. This explains the stringent requirement for positively charged basic amino acids to be present in the substrate for furin proteolytic activity (Henrich et al., 2003; Schechter & Berger, 1967). The profusely distributed negatively charged amino acids in the furin substrate-binding pocket are supposedly ideal for proper electrostatic bond formation with the positively charged amino acid residues present in the substrate. However, in our study, mutation Q114R reduced viral pathogenicity. Furthermore, the attenuation is more pronounced in mutants rNDV-Q114R, I118V and rNDV-Q114R, K115R, I118V, which in addition to Q114R mutations also have an I118V mutation. It should be noted that, in the hypothetical two-dimensional model of furin substrate binding-site domains, the enzymic subdomain of furin that interacts with glutamine and also with valine is not a distinct site and the substrate points away from the enzyme towards the solvent, whereas the enzymic subdomains that interact with the basic residues of viral substrates are very much more distinct and form a well-defined pocket (Roebroek et al., 1994; Siezen et al., 1994). We proposed that the presence of a strong positively charged amino acid, arginine, at position 114 in the NDV F protein cleavage site might be disturbing the conformational stability of the furin-binding site, thus influencing the host-cell enzyme activity. It could be one of the reasons that many viral glycoproteins, such as human immunodeficiency virus gp160 (QREKR↓AV), avian influenza virus A haemagglutinin (KR↓GL), Sindbis virus gpE2 (GRSKR↓SV), human parainfluenza virus type 3 F0 (PRTKR↓FF) and Ebola virus Zaire strain envelope glycoprotein (RRTRR↓EA), which are processed by furin protease, maintain a neutral or acidic amino acid in the X position of the consensus furin cleavage-site motif [R-X-(R/K)-R] (Hallenberger et al., 1992; Klenk & Garten, 1994; Nagai et al., 1976; Nakayama, 1997; Wool-Lewis & Bates, 1999). In addition, I118 might also be playing a role in the conformational dependability and stability of furin protease in terms of its activity. It will be interesting to explore further the effect of conserved acidic or neutral amino acids flanking mono or dibasic residues in the cleavage-site motif of other viral glycoproteins where furin is the major proteolytic processor. Although it has been known that the presence of paired basic amino acids residues is a prerequisite for proteolytic processing and infectivity of the F protein of paramyxoviruses, it was evident from our study that NDV also needs to maintain a neutral amino acid (glutamine) at conserved position 114 for efficient proteolytic processing by host cell proteases. The observation that substitution of V118 along with Q114 further attenuates the virus indicates the dependence of proteolytic activation on the overall catalytic domain structure. Our study gives a new insight into understanding how NDV maintains a conserved residue for effective proteolytic processing of the F glycoprotein, thus modulating...
pathogenesis. In future the role of Q114 and V118 might be further exploited to produce a safe live-attenuated vaccine.

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References


