Mutations in the Cytoplasmic Domain of the Newcastle Disease Virus Fusion Protein Confer Hyperfusogenic Phenotypes Modulating Viral Replication and Pathogenicity

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ABSTRACT

The Newcastle disease virus (NDV) fusion protein (F) mediates fusion of viral and host cell membrane and is a major determinant of NDV pathogenicity. In the present study, we demonstrate the effects of functional properties of F cytoplasmic tail (CT) amino acids on virus replication and pathogenesis. Out of a series of C-terminal deletions in the CT, we were able to rescue mutant viruses lacking two or four residues (rΔ2 and rΔ4). We further rescued viral mutants with individual amino acid substitutions at each of these four terminal residues (rM553A, rK552A, rT551A, rT550A). In addition, the NDV F CT has two conserved tyrosine residues (Y524 and Y527) and a di-leucine motif (LL536-537). In other paramyxoviruses, these residues were shown to affect fusion activity and are central elements in basolateral targeting. The deletion of CT 2 and 4 amino acids and single tyrosine substitution resulted in hyperfusogenic phenotypes and increased viral replication and pathogenesis. We further found that in rY524A and rY527A viruses, disruption of the targeting signals did not reduce the expression of apical or basolateral surface in polarized Madin-Darby canine kidney cells; whereas in double tyrosine mutant, it was reduced on both the apical and basolateral surface. Interestingly, in rL536A and rL537A mutants the F protein expression was more on apical than basolateral surface and this effect was more pronounced in rL537A mutant. We conclude that these wild type residues in the NDV F CT have effect on regulating F protein biological functions and thus modulating viral replication and pathogenesis.
INTRODUCTION

Newcastle disease virus (NDV) is a highly prevalent avian pathogen that infects essentially all species of birds and is of major economic importance to the poultry industry (3, 47). The disease varies in degree of severity, ranging from an inapparent infection to outbreaks of severe respiratory and neurologic disease that can have 100% mortality. Based on severity of the disease, NDV strains are grouped into lentogenic (non-pathogenic or mildly pathogenic), mesogenic (moderately pathogenic), and velogenic (highly pathogenic) pathotypes (2). NDV belongs to the genus *Avulavirus* within the family *Paramyxoviridae*, a family of enveloped, non-segmented, negative sense RNA viruses (33).

The envelope of NDV contains with two surface glycoproteins HN and F (16, 26). HN mediates viral attachment by binding to sialic acid cellular receptors and F protein facilitates viral entry into the cells by fusing viral envelope with host cell membrane (34, 36). The virus fusion process involves a series of major coordinated conformational changes in the F protein that bring together and merges the opposing membranes (16, 32).

The NDV F protein (Fig. 1) is synthesized as an inactive precursor F₀ (66 kDa) that is cleaved post-transnationally by host cell proteases into two disulfide-linked subunits, N-terminal F₂ (12.5kDa) and C-terminal F₁ (55kDa) (36, 37). The F cleavage site is a major determinant of NDV tropism and virulence: virulent strains typically have cleavage sites with multiple basic residues that are readily cleaved in most cell types and provide for systemic spread, whereas avirulent stains depend on secreted protease for cleavage and usually are restricted to the mucosal surfaces of the respiratory and enteric tracts. The F₁ subunit contains two hydrophobic domains, the fusion peptide (FP) present at the N-terminus created by protease cleavage, and the transmembrane (TM) domain that is located near the C-terminus and anchors the protein in the
membrane of the virus or infected cell. The FP initiates the process of fusion by inserting into the
target host cell membrane (7). The F1 subunit has two heptad repeat (HR) motifs: HR1 is
immediately C-terminal to the FP, and HR2 is immediately N-terminal to the TM domain. Upon
triggering, HR1 and HR2 domains undergo coordinated conformational changes necessary for
fusion (36, 43). The NDV F protein is a class I fusion protein that has structural and functional
characteristics that are highly related to those of the F proteins of other paramyxoviruses
including parainfluenza type 5 (PIV5, previously known as SV5), measles virus, respiratory
syncytial virus (RSV), Nipah and Hendra viruses, and also has general similarity to gp41 of
human immunodeficiency type 1 virus (HIV), the hemagglutinin (HA) of influenza virus, and
GP2 of Ebola virus (7, 14, 17, 20, 28, 56, 59).

NDV F protein has structural features in addition to the cleavage site, FP, and HR that
affect the efficiency of the fusion process, and thus may influence viral infectivity, replication,
and pathogenicity (5, 19, 38, 45, 46, 49). While it is well known that structural features in the
ectodomain of the F protein can have a major impact on fusion (5, 34), several reports on other
type I fusion glycoproteins (retrovirus, lentivirus, herpes virus, and other paramyxoviruses) have
also indicated the role of cytoplasmic tail (CT) in regulating viral entry, F protein cleavage and
fusogenicity (1, 6, 23, 24, 38, 39, 44, 52, 53, 57, 58). In addition, the CTs of several other viral
envelope glycoproteins contain sequence motifs that target the transport of newly synthesized
glycoproteins from the endoplasmic reticulum (ER) to different intracellular compartments and
to the cell surface (4, 11). In recent years, tyrosine-containing signals, especially Y-X-X-
aliphatic/aromatic consensus motifs, in the CT of viral membrane proteins have been found to be
associated with targeted protein delivery (8, 13, 55). A second type of signal, a di-leucine (LL)
motif, has similarly been shown to mediate internalization and targeting of viral glycoproteins to
intracellular compartments and to the basolateral surface of polarized epithelial cells (10, 25).

Mutagenesis of tyrosine and di-leucine motifs in the CTs of several other viral envelope
glycoproteins provided evidence that they can affect fusion and infectivity (22, 27, 42).

The NDV fusion protein CT is 31 amino acids long (amino acid positions 523 to 553, Fig.1) and is highly conserved among different strains of NDV (18, 19, 31, 40). It has been previously reported that deletions in the NDV F CT greatly reduced syncytia formation (48). In addition, the NDV F protein CT has tyrosine residues at positions 524 and 527 and a di-leucine motif at 536-537, and their possible roles in fusion and viral pathogenicity were not known.

In the present study we investigated the potential role(s) of the NDV F protein CT in viral replication and pathogenicity. Using reverse genetics, we rescued eleven NDV mutant viruses with truncated CT or with point mutations in the CT involving conserved signals or possible motifs. The mutant viruses were characterized for intracellular processing and surface expression of F, and for membrane fusion and replication in vitro and in vivo. Our results showed that truncation of 2 and 4 C-terminal amino acids and substitution of CT tyrosine residues in F protein resulted in hyperfusogenic phenotypes with increased pathogenicity. We further analyzed the significance of tyrosine and di-leucine motifs in polarized cells for apical and basolateral transport. Substitution of both the tyrosine residues together resulted in impaired apical expression of F glycoprotein; however substitution of single leucine residue moderately reduced basolateral targeting of F glycoprotein.

**MATERIALS AND METHODS**

**Cells and viruses.**
The chicken embryo fibroblast DF1 and human epidermoid carcinoma HEp-2 cell lines were grown in Dulbecco’s minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and maintained in DMEM with 5% FBS. The African green monkey kidney Vero cell line was grown in Eagle’s minimal essential medium (EMEM) containing 10% FBS and maintained in EMEM with 5% FBS. The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr. Bernard Moss (NIAID, Bethesda, MD) and propagated in primary chicken embryo fibroblast cells in DMEM with 5% FBS. The moderately pathogenic (mesogenic) NDV strain Beaudette C (BC) and its mutant derivatives were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs in an enhanced BSL-3 containment facility certified by the USDA following the guidelines of IACUC, University of Maryland. After 2 days, the allantoic fluid was harvested and the virus was plaque purified using our standard procedure (31).

**Construction of plasmids and recovery of mutant viruses.**

The construction of plasmid pNDVfl carrying the full-length antigenome cDNA of NDV strain BC has been described previously (31). The mutations that were introduced into the F protein CT are summarized in Fig.1. Their introduction was facilitated by the presence of the unique restriction enzyme sites PacI and AgeI located in the untranslated regions (UTRs) flanking the F and HN ORFs in the NDV cDNA. The Pac I-Age I fragment containing the F-HN gene was mutagenized with primers containing the desired mutations in a two-step procedure. First, two PCR products were generated: one PCR product was generated using a non-mutant forward oligonucleotide that primed upstream of the PacI site combined with a mutation-bearing reverse primer, and the second PCR product was generated using a mutation-bearing forward primer and a non-mutant reverse oligonucleotide that primed downstream of the AgeI site.
Second, overlapping PCR was then used to generate the ~4kb PacI-AgeI fragment containing the desired mutation, which was cloned into TOPO®-XL vector (Invitrogen, USA). The inserts bearing the desired mutation were cloned into the full-length antigenomic cDNA of strain BC. The rule of six was maintained in all of the mutants. All mutant F cDNAs were sequenced in their entirety to confirm the presence of the desired mutations.

**Recovery of viruses and confirmation of genetic stability and lack of adventitious mutations.**

Plasmid transfection and recovery of mutant NDV mutants were performed as described previously (31). Briefly, HEp-2 cells were transfected with three plasmids individually encoding the N, P, and L proteins (3.0 µg, 2.0 µg, and 1.0 µg per single well of a six-well dish, respectively) and a fourth plasmid encoding the full-length antigenome (5.0 µg) using Lipofectamine (Invitrogen, Carlsbad, CA) and simultaneously infected with vaccinia MVA expressing T7 RNA polymerase at a multiplicity of infection (MOI) of 1 PFU/cell. Two days after transfection, the cell culture medium supernatant was harvested and inoculated into the allantoic cavities of 9-day-old SPF embryonated chicken eggs. Recovery of the virus was confirmed by hemagglutination assay using 1% chicken red blood cells (RBCs). The sequences of the F and HN genes in the recovered chimeric viruses were confirmed by RT-PCR and nucleotide sequencing. In cases where virus was not recovered, at least three independent transfections were performed in parallel with the wild type (WT) cDNA as a positive control before considering the construct negative for virus recovery.

To assay genetic stability, the recovered CT mutant viruses were passaged in 9-day-old SPF chicken embryos for five times. From each passage total RNAs were isolated from NDV-infected allantoic fluid of 9-day-old SPF chicken embryos, using TRIzol reagent.
Reverse transcription-PCR (RT-PCR) was performed using the Thermoscript RT-PCR kit (Invitrogen) with specific forward and reverse primers to amplify the F gene. The amplified cDNA fragments were then sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc, USA) in ABI 3130xl genetic analyzer to confirm the presence of the introduced mutations in the recovered viruses. The HN gene from each recovered virus was also sequenced with available primers from our laboratory.

**Western blot analysis and domain-selective surface biotinylation and immunoprecipitation**

Expression of F protein was examined by Western blot analysis. Briefly, Vero cells were infected at a multiplicity of infection (MOI) of 0.01 PFU. The cells were harvested at 24 hour postinfection (h.p.i), the lysed proteins were denatured and reduced, separated by 10% SDS-PAGE and analyzed by Western blotting using a 1:100 dilution of anti-Fcyt terminal specific antibodies (45). The blot was stripped using stripping buffer (Restore™ PLUS western blot stripping buffer, Thermo Scientific) and re-immunoblotted using anti-β tubulin antibodies (Invitrogen).

Madin-Darby canine kidney (MDCK) cells were seeded on 0·4 μm pore size Transwell polycarbonate filters (BD Falcon™) and polarization of the cell monolayer was tested every day by monitoring the electrical resistance between upper and lower chambers (World Precision Instruments, WPI, USA). Cells were infected with rWT and mutant viruses. At 48 h.p.i., cells were washed three times with phosphate-buffered saline (PBS) and either the apical or the basolateral side of the filter membranes was incubated twice for 20 min at 4 °C with PBS containing 2 mg S-NHS-Biotin ml⁻¹ (Thermo Scientific, USA). After biotinylation, cells were washed with cold PBS containing glycine (0·1 M) to the opposite membranes. After washing the cells once with 0·1 M glycine and three times with PBS, filter membranes were cut out and lysed.
in 0.5 ml radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM EDTA, 10 mM iodoacetamide, 1 mM PMSF, and 20 mM Tris/HCl, pH 8.5). Cell lysates were clarified by centrifugation for 20 min at 19,000 X g. Supernatants were immunoprecipitated using anti-FNterm antibodies and 50 µl protein A–Sepharose beads (Calbiochem). Following separation on a 10% SDS-PAGE and blotting on to nitrocellulose, biotinylated proteins were detected with streptavidin–biotinylated horseradish peroxidase complex and enhanced chemiluminescence (Amersham).

**Cell surface expression of the CT mutant viruses.**

Cell surface expression of the F proteins of the CT mutant viruses was quantified by flow cytometry. Briefly, DF1 cells were infected with each mutant virus at an MOI of 0.1. After overnight infection the cells were detached with PBS containing 5 mM EDTA and centrifuged at 500 × g for 5 min at 4°C. Cells were then incubated with rabbit anti-FNterm antiserum (1:10 dilution) for 30 min at 4°C (45). Subsequently, cells were washed 3 times with PBS, and incubated for 30 min on ice with 1:500 diluted Alexa Fluor 488 conjugated goat anti rabbit immunoglobulin G antibodies. Cells were analyzed by using a FACSRIA II apparatus and Flowjo software (Becton Dickinson Biosciences).

**Surface immunofluorescence analysis**

MDCK cells were grown on 0.4 µm-pore size filter supports and infected with rWT and mutant viruses. At 48 h p.i., infected cells were fixed with 2% paraformaldehyde (PFA) in DMEM for 2h and then incubated from both sides with rabbit antibodies directed against the NDV F for over night at 4°C. The primary antibodies were detected using AlexaFluor 555-conjugated secondary antibodies (Invitrogen) for 1.5 h at 4°C. To visualize cell junctions, cells were permeabilized for 10 min with 1:1 methanol and acetone and stained with a monoclonal antibody against E-
cadherin (BD Biosciences) and AlexaFluor 488-conjugated secondary antibodies (Invitrogen) and DAPI (BD Biosciences). Filters were cut out from their supports, mounted onto microscope slides in anti-fade (Invitrogen) and were analyzed using a Zeiss Axiovert200M microscope or with a confocal laser scanning microscope (Zeiss, LSM510).

**Fusion assay of the CT mutant viruses.**

Syncytia formation was quantified as described by Kohn (30). Briefly, Vero cells in 6-well plates were infected with each virus at an MOI of 0.1. Cells were maintained in 5% MEM at 37°C under 5% CO₂. Twenty-four h.p.i, the medium was removed and the cells were washed with PBS, fixed with methanol for 20 min at room temperature, and stained with hematoxylin-eosin. The fusion index of each mutant virus was calculated by observing 10 fields per well in duplicate. The fusion index is the ratio of the total number of nuclei to the number of cells in which these nuclei are present (i.e., the mean number of nuclei per cell).

**Multi-cycle growth in DF1 cells.**

DF1 cells in duplicate wells of six-well plates were infected with each virus at an MOI of 0.01. After 1 h of adsorption, the cells were washed with PBS and overlaid with DMEM containing 5% FBS at 37°C. The medium was collected and replaced with an equal volume of fresh medium at 8-h intervals until 64 h.p.i. Virus titers were quantified by plaque assay on DF1 cells.

**Virus purification**

The wild type and hyperfusogenic mutant viruses were harvested from allantoic fluid, were clarified by centrifugation at 5,000 rpm for 5 min, and then overlaid with 30% sucrose (wt/vol) and subjected to centrifugation at 100,000 × g for 3 h at 4°C, using a Beckman SW 25 rotor.
Viral proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was then stained with Coomassie brilliant blue. Purified viruses were further analyzed by Western blot using a 1:100 dilution of anti-Fcyt antibodies. The blots were stripped using stripping buffer and again blotted with 1:200 dilution anti-NP polyclonal antibodies. Amounts of F and NP proteins were quantified by densitometry.

Mean death time (MDT) and intracerebral pathogenicity index (ICPI)

The pathogenicity of the F cytoplasmic mutant viruses was determined by the MDT test in 9-day-old embryonated chicken eggs and the ICPI test in 1-day-old SPF chicks. All studies were conducted under enhanced biosafety level (EBSL-3) conditions at the University of Maryland (45).

Statistical analysis

Statistically significant differences in data from different recombinant virus groups were evaluated by one-way analysis of variance (ANOVA). Growth kinetics of various groups was analyzed using correlation of XY pairs (Pearson); P value (two tailed). P values below 0.05 were regarded as being significant for all analyses. Experiments were repeated a minimum of three times. Statistical analysis for mean, standard deviation of data, one-way ANOVA were analyzed by using computer software Prism 5.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

Construction and recovery of F cytoplasmic tail (CT) mutant viruses.

In the present study, we investigated the importance of the CT of the NDV F protein in viral replication and pathogenesis using reverse genetics. We created constructs with mutations in the NDV F protein CT using a cDNA encoding the full-length antigenome cDNA of the
moderately virulent (mesogenic) NDV strain BC (31). Six mutants were constructed involving progressive deletion of 2, 4, 6, 12, 18, or 30 amino acids from the C-terminus (Fig. 1). However, of these six mutants, only two viable viruses were recovered with 2 and 4-amino-acid deletions (rΔ2 and rΔ4, respectively). This implied that deletion of 6 amino acids or more from the CT was lethal for the production of infectious NDV. Since the C-terminal 4 amino acids of the CT were dispensable for virus replication, we constructed four more mutants in which these 4 residues were individually replaced by alanine (Fig.1). We were able to recover all four of these mutants, designated rM553A, rK552A, rT551A, rT550A.

Several viral membrane proteins have been shown to contain tyrosine (YXX) or di-leucine (LL) based targeting motifs involved in protein trafficking in both the secretory and endocytic pathways. The NDV F protein CT contains two tyrosine residues (Y) at positions 524 and 527 and one di-leucine (LL) motif at positions 536-537. These residues are highly conserved between NDV isolates. To investigate possible roles of these Y- and LL- motifs in regulating apical sorting and fusion, we mutated each of the two tyrosine residues singly to alanine and double substitution to alanine and mutated the di-leucine singly each to alanine and double substitution to di-alanine (Fig.1). We were able to rescue viruses with substitution of tyrosine to alanine (rY524A, rY527A and rYY524-527AA) and single substitution of leucine to alanine (rL536A, rL537A).

**Intracellular processing and expression of the F proteins of the CT mutant viruses.**

In order to investigate the effects of CT deletions and point mutations on F protein synthesis and processing, the rWT and mutant CT viruses were used to infect Vero cells and were analyzed by Western blot using Fcyt specific antibody which was raised against 30 amino acid long C-terminal synthetic peptides of the F protein (45).
mutant viruses was expressed and cleaved to F1-F2; although the extent of cleavage varied among these mutants (Fig. 2). In rΔ2, rΔ4, rM553A, rK552A, rT551A, rT550A mutants, both F0 and F1 were detectable however there were reduced processivity in rM553A, rK552A, rT551A and rT550A resulting in more percentage of F0 than F1. In contrast, in rY524A and rY527A mutants there were more percentage of F1 than F0, suggesting enhanced processivity of the F protein. In case of rYY524-527AA, rL536A and rL537A the intracellular expression of F0 and F1 were less as compared to rWT.

The relative levels of expression of the F CT mutant viruses were measured by flow cytometric analysis of DF1-infected cells using anti-F Nterm rabbit antiserum. The results showed the percentage of cells expressing F protein on the cell surface, was indistinguishable for rWT and the CT mutant viruses. However, the level of F protein expression per cell, measured by mean fluorescence intensity, varied considerably (Table 1). After 12 h.p.i the deleted CT mutants F protein expression exceeded that of rWT by 15% to 52%, depending on the mutant. The highest levels of expression were observed with the two tyrosine mutants, rY527A and rY524A (52% and 45% increases, respectively). In contrast, in case of tyrosine double mutant rYY524-527AA there was reduction of fluorescence intensity by 42%; followed by moderate reduction of intensity in single leucine mutants rL536A and rL537A (11% and 18% respectively). These data suggest that all the mutants are capable of spreading under these conditions.

Our results indicate that CT mutants retained the ability to be synthesized and transported to the cell surface, and indeed showed that most of the mutants did so more efficiently than rWT. The single tyrosine mutants cleaved more efficiently than wild type F protein which might be enhancing the transport of the F protein through Golgi and therefore increasing cell surface expression; whereas in double tyrosine and single leucine mutants the cleavage of F protein was...
reduced which resulted in decreased cell surface expression. However, in this study the cytoplasmic tail antiserum was used for the immunoblot and it is possible that the apparent variation in quantity of F protein produced in some mutants might be due to loss of major epitope.

**Fusion activity and CPE of the CT mutant viruses in Vero cells.**

To investigate possible effect of the CT mutations on the fusion activity of F protein, mutant viruses were examined in Vero cells (Fig.3). The CT deletion/substitution and single tyrosine mutant viruses exhibited increased (13% to 48% higher) fusion indices compared to the rWT virus with the exception of the rT550A mutant, which was essentially identical to rWT, and the rT551A mutant, which was only marginally increased. The most efficient fusion was observed with the rY527A and rY524A mutants (48% and 36% increase, respectively, compared to rWT). Substantial increases also were observed with the rΔ2 and rΔ4 mutants (27% and 13%) and rM553A, rK552A mutants (32% and 27% respectively). In contrast, in tyrosine double mutant rYY524-527AA there was reduction in fusion by 32% and in rL536A and rL537A there were reduction in fusion activity by 8% and 12% respectively. These date indicate that CT of F protein is capable of modulating cell-cell fusion mediated by rWT F glycoprotein.

**Replication kinetics of the CT mutant viruses**

The multi-step growth kinetics and magnitude of replication of the CT mutant viruses were determined in DF1 cells (Fig.4). The CT deleted/substituted mutant viruses and single tyrosine mutants replicated exponentially until ~40 h.p.i, after which replication was at a plateau. The magnitude of replication was similar for rWT and the rT550A and rT551A viruses (Fig.4A), but was substantially higher for the other CT mutant viruses. The highest viral titers were seen.
with the tyrosine mutant viruses rY524A and rY527A, followed closely by the rΔ2, rM553A, rK552, and rΔ4 viruses. For example, the titer of the rY527 virus was 1.0 log₁₀ higher compared to rWT at 16 h.p.i and 2.0 log₁₀ higher compared to rWT at 40 h.p.i (Fig.4B). Interestingly, the double substituted tyrosine had a reduced replication rate and had maximum titer reduced by 1 log₁₀; where as in case of rL536A and rL537A mutants replicate at a lower titer of 0.5 log₁₀ till 48 h.p.i (Fig.4B) after which the replicate rate were similar to wild-type virus.

Surface distribution of F glycoproteins in infected polarized MDCK cells

To study the role of tyrosine and dileucine motifs in the transport of NDV F glycoprotein in polarized epithelial cells, MDCK cells were grown on polycarbonate filters and infected with rWT or the tyrosine and leucine mutants, subjected to domain specific surface biotinylation assay as described in Materials and Methods. In rWT infected cells F glycoprotein is abundantly expressed both in apical and basolateral surfaces (Fig.5 panel A). Similar results were seen in single tyrosine mutants where F glycoproteins were found in both the apical and basolateral surfaces; suggesting substitution of single tyrosine residue did not have any significant effect in transporting the F glycoprotein to either of the cell surfaces. In contrast, substitution of both the tyrosine residues reduced apical surface expression of F glycoprotein and there was also reduction in F protein expression in basolateral surface as compared to rWT and single tyrosine residue mutant F protein expression (Fig.5). This indicates that in context of virus infection, in NDV F protein, abundant expression of F protein in apical and basolateral surface is prevented by mutation of both the tyrosine residues. However, in leucine mutants infected MDCK cells, more percentage of F glycoproteins were expressed apically (Fig.5 panel B). These data
indicated that the di-leucine motif in F glycoprotein might be influencing the basolateral targeting of F protein.

**NDV F protein in single tyrosine mutants are expressed predominantly on apical and basolateral surfaces of polarized epithelial cells**

To further evaluate the spread of single tyrosine mutants and distribution of these mutants F proteins on apical and basolateral surfaces, polarized MDCK cells were grown on filters and were infected with wild type and mutant viruses from the apical side. At 48 h.p.i the samples were fixed with 2% PFA and immunostained with anti-F_Nterm antiserum and Alexa fluor 555-conjugated secondary antibodies. Cell junctions were visualized by co-staining with E-cadherin specific monoclonal antibodies. As shown in confocal analysis of the distribution of F protein (Fig.6A), fluorescent signals were detected more at apical surface in single tyrosine mutant infected MDCK cells as compared to wild type F protein. In infected MDCK cells, there was disruption of the cell junctions which might be a result of viral replication and fusion of adjacent cells. In addition, horizontal sections of confocal through apical to basal in MDCK cells infected with rY527A mutant showed extensive expression of F protein in apical surface as compared to wild type (Fig.6B). In addition, the fluorescent signals for F protein in rY527A mutant infected MDCK cells did not reduce in central and basal part as compared to wild type rather there were more expression on basolateral surface. (Vertical sections Fig.6B).

**Incorporation of F protein in hyperfusogenic viruses**

To examine the amount of incorporation of the F proteins into hyperfusogenic viruses, the wild type and hyperfusogenic mutant viruses were partially purified from infected allantoic fluid by
sucrose gradient centrifugation and analyzed by Coomassie staining and then quantified by Western blotting. We examined the level of F protein incorporation in different mutant viruses by performing the ratios of the F protein to other viral protein NP (Fig.7). As compared to wild type virus F protein band, there were 8% and 6% increase in relative intensities of F protein bands in rΔ2 and rΔ4 mutant viruses. The highest intensities of F protein band was seen in rY527A mutant (25% increase than rWT ) followed by 20% in in rY524A virions.

**Pathogenicity of the CT mutant viruses in embryonated chicken eggs and 1-day old chicks**

We evaluated the effect of the CT mutations on viral pathogenicity using two standard pathogenicity assays, namely the mean embryo death time (MDT) assay and the intracerebral pathogenicity index (ICPI) test. MDT values were determined in 9-day-old embryonated chicken eggs (Table 2). NDV strains are categorized into three pathotypes on the basis of their MDT values: velogenic (less than 60 h), mesogenic (60 to 90 h), and lentogenic (greater than 90 h). The MDT value of the rT550A mutant (59 h) was essentially identical to that of rWT virus mutants, The MDT of the other mutants were reduced to varying extents compared to rWT, suggestive of modest increases in virulence. The greatest differences were observed with the MDT of the rY527A (51.2 h), rY524A (52 h) and rΔ2 (54 h) mutants, which had values that were up to 15% less than that of rWT virus. The tyrosine double mutant rYY524-527AA showed moderate decrease in MDT (68h) as compared to rWT. The other viruses had intermediate values.

The pathogenicity of the CT mutant viruses also was evaluated by the ICPI test in 1-day-old chicks (Table 2). Velogenic strains give values approaching 2.0, whereas lentogenic strains give values close to 0. The differences in ICPI values compared to rWT virus (1.51) were
greatest with the rY527A (1.78), rY524A (1.70), and rΔ2 (1.68) viruses, whose values were up to 18% greater than that of rWT virus. The ICPI values of the CT mutants were increased compared to rWT virus (except the rYY524-527AA (1.30) and leucine mutants) which is indicative of increased pathogenicity, although the increases were modest. The ICPI values of the other viruses were intermediate. The results of the ICPI test were consistent with the results of MDT test, and in particular the two tyrosine mutants, rY527A and rY524A, and the deletion mutant rΔ2, were the most virulent.

DISCUSSION

The CTs of the several paramyxovirus F proteins have been shown to play an important role in modulating membrane fusion and hence are a significant determinant in the replication of these viruses (6, 12, 15). Various studies on the CTs of paramyxovirus F proteins and other virus envelope proteins have revealed effects on syncytium, fusion pore formation, oligomerization, protein folding, fusion promotion and infectivity (1, 9, 12, 15, 29, 48, 60, 61). In addition, the CTs of several viral enveloped proteins have been shown to harbor critical residues required for intracellular trafficking, virus assembly and budding (8, 41, 50, 54, 55). It was shown earlier, that truncations in the CT of the NDV F protein were inhibitory to membrane fusion (48). In the present study, we have evaluated the effect of truncations and point mutations in the CT of F protein on NDV replication and pathogenesis. We attempted to recover viruses with C-terminal deletions of upto 30 amino acids from the 31-amino acid CT of the NDV F protein maintaining rule of six. However, we were only able to recover virus with the two smallest deletions, of 2 and 4 amino acids. Thus, while infectious NDV can readily tolerate deletions of up to 4 amino acids in the F protein CT tail, longer deletions apparently were lethal. Deletion of the first 2 and 4 amino acids from the CT resulted in mutant virus that had a hyperfusogenic phenotype, with
moderate levels of increase in F protein cleavability and expression as compared to wild type. These deletion mutants also exhibited increased viral growth in vitro and in vivo, and increased virulence in 1-day-old chicks. This was especially evident with the rΔ2 virus. In case of HIV-1, SIV and HSV-1, truncations of C-terminal CT of envelope proteins also resulted in increased cell fusion (24, 53, 57, 60). For, RSV virus deletion or substitution of F protein CT severely impaired virus growth in cell culture and it has been suggested that deleted CT might be effecting the F protein conformation change leading to premature fusion (39). However, in measles virus, deletion of CT resulted in enhanced cell to cell fusion without significant effect in virus replication(15); whereas in SV5, the F protein CT is absolute necessary for virus propagation(21). In this study, deletion of 2 and 4 amino acids from CT resulted in enhanced cell to cell fusion and which might have affected the increased replication rate of viruses in cell culture.

We further investigated the role of these C-terminal four amino acids in the fusion phenotype and found moderate increase in fusogenicity, viral replication and pathogenicity by individually substituting them with alanine. The mechanism by which truncations or point mutations of the C-terminal four amino acids of CT mediated these effects is unclear. In purified virions we found moderate increased in incorporation of mutant F proteins into virus particles (Fig.7). However, it is possible that other mechanisms might be present, such as interaction of F with other viral proteins M and HN which is modulating the fusion process. For example, in PIV5, the F CT was implicated in regulating fusion pore formation (21). We also note that NDV F protein processing is increased in these mutant viruses (Fig.2). In future studies it would be interesting to determine how the deletion of CT can affect the processing through Golgi apparatus.
The CT of NDV F harbors two tyrosine residues and one di-leucine motif that are highly conserved among strains and have the potential to be signals involved in processing and transport. Our experiment revealed that in wild type infected MDCK cells F proteins were abundantly present both in apical and basolateral surface. Substitution of single tyrosine residues did not reduce the expression of F protein in either of the surface rather we found they were over expressed in basolateral surface as compared to wild type virus F protein; in contrast substitution of both the tyrosine residues resulted in preferential transport of F protein to basolateral surface. Similar results were found in Measles virus where though viruses were released from apical membrane but the F protein is majorly transported to basolateral surface (35). These results suggest the functional redundancy of tyrosine residues in F glycoprotein signaling to apical surface; however did not lead to changes in expression of basolateral sorting. We further noted that in single leucine substitution the F protein is accumulated more in apical surface and is more pronounced in L537A substitution; suggesting dileucine motif might be playing the predominant role in basolateral sorting of NDV F protein. However, it is yet to be determined how this is affecting the budding of NDV virus from apical and/or basolateral surface.

The significance of polarized based signals on course of disease was supported by previous works in Sendai virus (51). Our inability to recover the LL- motif mutants even after several attempts suggests the importance of both the leucine residues in LL- motif in F protein function and viability of virus. The two recovered tyrosine mutants rY524A and rY527A exhibited hyperfusogenic phenotypes with increased replication in vitro and increased pathogenesis in vivo. Indeed, of the mutants characterized in the present study, these phenotypes were most pronounced for the tyrosine mutants. In contrast substitution of double tyrosine
residues and single leucine residues moderately decreased the fusion process and the virus replication in cell culture.

The most widely used tyrosine-based motif is YXX (where Y is tyrosine, X is any amino acid and (phi) is an amino acid with bulky hydrophobic group). In the NDV F CT, Y524 and Y527 are both present in the motif YLMY, and Y527 also is present in the motif YKQK. Thus, neither of these conforms to the YXX motif. Thus, other structural and functional domains of F protein such as transmembrane domain and N-linked glycosylation might influence apical/basolateral sorting. Besides the transport of NDV F protein in polarized epithelial cells may be affected by presence of HN and other viral proteins.

It also is reasonable to suggest that increased fusion played an important role in the observed increased replication and pathogenicity. These results show that the NDV F protein has features that restrain the fusogenic phenotype. Since mutations to the terminal residues and to the tyrosine residues were well-tolerated and presumably could readily occur and be selected for in nature, there apparently is a lack of selective advantage for these mutations in nature. This study has increased our understanding of NDV virulence mediated by the F protein, but also has raised new questions about the mechanism by which the CT restrains fusion. These hyperfusogenic viruses may be useful in developing NDV as a better vaccine vector and as an oncolytic agent.

Acknowledgements

We thank Daniel Rockemann and other laboratory members for their technical assistance and help. This research was supported by NIAID contract no. N01A060009 (85% support) and NIAID, NIH Intramural Research Program (15% support). The views expressed herein neither necessarily reflect the official policies of the Department of Health and Human Services; nor
REFERENCES


Figure legends

**Figure 1:** Schematic diagram of the NDV F protein, and mutations that were introduced into the CT. Linear diagram of the NDV F protein, and sequences of the intact WT CT and of progressive deletion mutations (Δ). Grey boxes: heavy shading, fusion peptide; intermediate shading, heptad repeats (HR); light shading, transmembrane (TM) domain. Sequences of amino acid point mutations; alanine substitutions are in bold and in red color.

**Figure 2.** Western blot analysis of F CT mutants expressed in Vero cells. Vero cells were infected with virus at an MOI of 0.01 PFU. After 24 h.p.i, the cells were collected and processed to prepare cell lysates. The samples were denatured and reduced and subjected to Western blot analysis using anti-Fcyt specific antibodies. The positions of F₀ and F₁ are indicated by arrows in the left margins. The immunoblot was stripped using stripping buffer and again immunostained using monoclonal antibody against β-tubulin as a cellular control (bottom panel).

**Figure 3.** Comparison of the fusogenicity and CPE of rWT and the CT mutant viruses in Vero cells. Relative levels of fusion obtained for the CT mutants compared to rWT. Vero cells were infected with the indicated viruses at an MOI of 0.1, fixed at 24 h.p.i, and stained with hematoxylin-eosin. The fusion index was calculated as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. 10 fields were counted per condition. Fusion levels were normalized to WT at 100%. Data shown are averages of three independent experiments. Error bars refer to SEM. P value < 0.0001, reflects comparison of all groups through ANOVA.
Figure 4. Comparison of the multicycle growth kinetics and CPE of rWT and the CT mutant viruses in DF1 (A) Comparison of multicycle growth kinetics of rWT, rΔ2, rΔ4, rM553A, rK552A, rT551A, rT550A. Cells were infected with each virus at an MOI of 0.01, and cell culture media supernatant aliquots were harvested at 8-h intervals until 64 h.p.i. The virus titers in the aliquots were determined by plaque assay in DF1 cells. P=0.0001 (B) Comparison of multicycle growth kinetics of rWT, rY524A, rY527A, rYY524-527AA, rL536A, rL537A. Data shown are averages of three independent experiments with standard error bars. P=0.002

Figure 5. Surface selective biotinylation of wildtype or mutant F CT glycoproteins in polarized MDCK cells. MDCK cells were grown on permeable filter supports and infected with viruses at MOI of 0.01 PFU. After 48 h.p.i, cells were surface biotinylated from either the apical (Ap) or the basolateral (Bl) side. After cell lysis, proteins were immunoprecipitated with F specific antibodies and were analyzed by SDS-PAGE using peroxidase-conjugated streptavidin. (A) Cell lysates of rY524A, rY527A and rYY524-527AA compared with rWT. (B) Cell lysates of rL536A and rL537A compared with rWT.

Figure 6. Surface distribution of wild type and single tyrosine mutants F proteins in polarized MDCK cells (A and B). MDCK cells were grown on filter supports until full polarization was reached and then infected with wild type or single tyrosine mutants. (A) At 48 h.p.i, cells were fixed with 2% PFA and subsequently stained with anti-F specific antiserum and Alexa Fluro 555 secondary conjugated antibodies. After permeabilization with methanol-acetone, cell junctions were visualized with anti-E cadherin monoclonal antibodies and with Alexa Fluor 488 conjugated secondary antibodies and co stained with DAPI. (B) Confocal horizontal sections
through the apical part of the cell monolayer to bottom. (i) wild type F protein and (ii) rY527A F protein surface distribution on apical and basolateral sides.

**Figure 7.** Incorporation of the F protein into viral particles. (A) Ultracentrifuge-purified viruses from infected allantoic fluid samples were separated by electrophoresis, and the gel was then stained with Coomassie brilliant blue. Lane 1: rWT, 2: rΔ2,3: rΔ4, 4: rY524A, 5: rY527A (B) Purified virus samples were analyzed by Western blot using anti-Fcyt antibodies (Top) and anti-NP polyclonal antibodies (bottom). (C) Relative intensities of NP and F protein in wild type and mutant viruses as measured by densitometry. *P value for rY524A and rY527A <0.05.
WT : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM
Δ2 : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM (RECOVERED)
Δ4 : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM (RECOVERED)
Δ6 : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM
Δ12 : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM
Δ18 : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM
Δ30 : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM
M553A : CYSMYKQGAKQQTLLWLGNNTLDQMRAATKA (RECOVERED)
K552A : CYSMYKQGAKQQTLLWLGNNTLDQMRAATAM (RECOVERED)
T551A : CYSMYKQGAKQQTLLWLGNNTLDQMRAATKM (RECOVERED)
T550A : CYSMYKQGAKQQTLLWLGNNTLDQMRAATKM (RECOVERED)
Y524A : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM (RECOVERED)
Y527A : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM (RECOVERED)
L536-537A : CYSMYKQGKQGTKAAWLGNNTLDQMRAATTKM
YY524-527AA : CYSMYKQGAKQQTLLWLGNNTLDQMRAATTKM (RECOVERED)
L536A : CYSMYKQGKQGTKAAWLGNNTLDQMRAATTKM (RECOVERED)
L537A : CYSMYKQGKQGTKAAWLGNNTLDQMRAATTKM (RECOVERED)
Figure 3: Bar graph showing % Fusion relative to WT for various mutants.
Figure 4B.

Virus titer (log$_{10}$PFU/mL) over time for different strains:
- rWT
- rY524A
- rY527A
- rYY524-527AA
- rL536A
- rL537A

The graph shows the progression of virus titer from 0 to 64 hours, with error bars indicating variability.
Figure 6A

<table>
<thead>
<tr>
<th>DAPI</th>
<th>E-cadherin</th>
<th>F</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rWT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rY524A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rY527A</td>
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Table 1. Cell surface expression of the F proteins of CT mutant viruses

<table>
<thead>
<tr>
<th>Viruses</th>
<th>% of positive cells ± SD</th>
<th>Relative mean fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>rWT</td>
<td>99±1.8</td>
<td>1.00</td>
</tr>
<tr>
<td>rΔ2</td>
<td>99±1.2</td>
<td>1.30</td>
</tr>
<tr>
<td>rΔ4</td>
<td>99±1.5</td>
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</tr>
<tr>
<td>rM553A</td>
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<td>1.19</td>
</tr>
<tr>
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<td>1.15</td>
</tr>
<tr>
<td>rT551A</td>
<td>99±2.0</td>
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</tr>
<tr>
<td>rT550A</td>
<td>99±2.0</td>
<td>1.08</td>
</tr>
<tr>
<td>rY524A</td>
<td>99±1.8</td>
<td>1.45*</td>
</tr>
<tr>
<td>rY527A</td>
<td>99±1.2</td>
<td>1.52*</td>
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<tr>
<td>rYY524-527AA</td>
<td>96±1.5</td>
<td>0.58</td>
</tr>
<tr>
<td>rL536A</td>
<td>95±1.2</td>
<td>0.89</td>
</tr>
<tr>
<td>rL537A</td>
<td>92±2.4</td>
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</tr>
<tr>
<td>None (Mock infected cells)</td>
<td>0.01</td>
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Cell surface expression of the F protein was determined by flow cytometry. DF1 cells were infected with each mutant virus at an MOI of 0.1. Surface expression of the F proteins was assessed by flow cytometry at 12 h.p.i with rabbit anti-F_{Nterm} antiserum followed by anti-rabbit Alexa Fluor 488 conjugated antibodies. Surface immunofluorescence was quantitated by FACS analysis. Uninfected DF1 cells were used as negative controls. Values shown are averages of results from three independent experiments. SD; standard deviation. * P< 0.05.
Table 2. Pathogenicity of the CT mutant viruses in embryonated chicken eggs and 1-day-old chicks

<table>
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<tr>
<th>Viruses</th>
<th>MDT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ICPI score&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>rWT</td>
<td>60</td>
<td>1.51</td>
</tr>
<tr>
<td>rΔ2</td>
<td>54</td>
<td>1.68*</td>
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<tr>
<td>rΔ4</td>
<td>56</td>
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<tr>
<td>rM553A</td>
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<td>1.53</td>
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<td>1.53</td>
</tr>
<tr>
<td>rY524A</td>
<td>52</td>
<td>1.70*</td>
</tr>
<tr>
<td>rY527A</td>
<td>51.2</td>
<td>1.78*</td>
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<tr>
<td>rYY524-527AA</td>
<td>68</td>
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<tr>
<td>rL536A</td>
<td>62</td>
<td>1.56*</td>
</tr>
<tr>
<td>rL537A</td>
<td>64</td>
<td>1.50</td>
</tr>
</tbody>
</table>
Mean embryo death time (MDT). The mean time (h) for the minimum lethal dose of virus to kill all of the inoculated embryos. Pathotype definition: virulent strains, <60 h; intermediate virulent strains, 60 to 90 h; avirulent strains, >90 h.

Intracerebral pathogenicity index (ICPI). ICPI score = [(total number of sick chicks at each observation x 1) + (total number of dead chicks at each observation x 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 mean of three independent experiments. SD; * P<0.05