ABSTRACT

Title of Document: ROLE OF FUSION PROTEIN IN
                                NEWCASTLE DISEASE VIRUS
                                PATHOGENESIS

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The fusion (F) protein of Newcastle disease virus (NDV) is a type I membrane glycoprotein that mediates the merger of the viral envelope to the host cell membrane. The F protein activation initiates a series of conformational changes in the F protein leading to membrane merge which occurs at the cell surface at neutral pH thus modulating NDV entry and spread. The present studies have given an insight to understand the role of F protein in NDV pathogenesis by using established reverse genetic techniques. The F gene of NDV has six glycosylation sites, two of which are present in heptad repeats that facilitate conformational changes during fusion process. To understand the importance of the glycosylation sites in NDV replication and virulence, each site was eliminated individually and in combination on a cDNA clone of NDV strain BC. Our results suggest that glycosylation of F protein plays a major role in virulence and some of the N-glycosylation sites are critical for fusogenicity of the F protein thus modulating NDV infectivity.

The F protein is synthesized as an inactive precursor, F_0, which is only

fusogenic after cleavage into disulfide-linked F_1 and F_2 polypeptides by host cell proteases. The amino acid sequence surrounding the F protein cleavage site determines the virulence of NDV, since different host proteases that cleave the F protein of virulent strains are present in more tissues than those that cleave the F protein of non-virulent strains. The role of conserved glutamine residue in NDV F protein cleavage site in viral pathogenesis has been examined. This study has helped us to understand the requirement of F protein cleavage site conserved amino acids in proteolytic processing and viral infectivity.

Further in this study, the role of F protein cytoplasmic domain and conserved cysteine residues in viral pathogenesis have been explored using reverse genetics. These regions have been suggested to play important roles in F protein conformation, stability and thus affecting the fusion process and viral infectivity.

In summary, the purpose of this work is to determine the important domains and residues of the NDV F protein that facilitates fusion process and regulates viral pathogenesis and immunogenicity. An understanding of how NDV F protein fusion process are regulated may lead to the creation of more effective therapies and better vaccine against NDV and other paramyxoviruses in general.

ROLE OF FUSION PROTEIN IN NEWCASTLE DISEASE VIRUS

PATHOGENESIS

By

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Dedication

I dedicate this work to my daughter, Ms. Saanvi Verma for her unconditional love and my Ph.D advisor, Dr. Siba K Samal to believe on me and for his guidance and support.

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LIST OF ABBREVIATIONS

aa	amino acid
APMV	avian paramyxovirus
APMV-1	avian paramyxovirus serotype 1
bp	base pair
BC	Beaudette C
BSL	Bio Safety Level
cDNA	complementary DNA
CEF	chicken embryo fibroblast
CPE	cytopathic effect
CT	cytoplasmic tail
DF1	Douglas Foster 1
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
ELISA	enzyme linked immunosorbent assay
EMEM	essential modified Eagle's medium
F	Fusion protein
FBS	fetal bovine serum
FP	fusion peptide
GE	gene-end
GS	gene-start
HA	hemagglutination
HDV	hepatitis delta virus
HI	hemagglutinin inhibition
HMPV	human metapneumovirus
HN	hemagglutinin-neuraminidase
HPIV-2	human parainfluenza type 2
HPIV-3	human parainfluenza type 3
HIV	human immunodeficiency virus
HR	heptad repeats
ICPI	intracerebral pathogenicity index
IGS	intergenic sequence
IRES	internal ribosome entry site
IVPI	intravenous pathogenicity index
kDa	kilodaltons

L	large polymerase
M	matrix protein
mRNA	message RNA
MDT	mean death time
MOI	multiplicity of infection
MV	measles virus
MVA-T7	recombinant modified vaccinia strain Ankara expressing T7 polymerase
NA	neuraminidase
NDV	Newcastle disease virus
nm	nanometer
N	nucleocapsid protein
nt	nucleotide
NV	Nipah virus
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming unit
PI	post infection
RBC	red blood cell
RNA	ribonucleic acid
RNP	ribonucleoprotein
RSV	Respiratory syncytial virus
RT-PCR	reverse transcription PCR
SeV	Sendai virus
SDS-PAGE	sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SPF	specific pathogen free
SV5	simian virus 5
SD	standard deviation
$TCID_{50}$ = 50%	Tissue culture infectivity dose
TM	transmembrane domain
UTR	untranslated region
VSV	vesicular stomatitis virus

Chapter 1

1.1 Title

General Introduction

1.2 Introduction

Newcastle disease is a highly contagious respiratory and neurologic disease in chickens that affects many domestic and wild avian species, leading to severe economic losses in the poultry industry worldwide (Alexander, 2000; Alexander et al., 1997; Samal, 2011a). Outbreaks of virulent Newcastle disease have a tremendous impact on chickens in developing countries, where these birds are a significant source of protein and income. This disease is endemic in Asia, Africa, Middle East, South and Central America. In the United States the virulent form of the disease is absent and the disease caused by virulent NDV strains is called Exotic Newcastle Disease (END). In developed countries, the more virulent forms of the virus cause significant economic losses during outbreaks. In the United States, the last END epidemic in 2002-2003, resulted in the death of more than three million birds and caused industry losses estimated at $35 million (Kapczynski and King, 2005).

Newcastle disease is caused by Newcastle disease virus (NDV). NDV strains cause a continuous spectrum of clinical signs and are categorized into three major pathotypes depending on the severity of the disease in chickens; the lentogenic (avirulent), mesogenic (moderately virulent) and velogenic (highly virulent) strains (Alexander, 2000). The

velogenic strains cause acute fatal infection of chickens of all age groups with clinical findings of nervous signs or extensive hemorrhagic lesions in the gastrointestinal tract. The mesogenic strains are of intermediate virulence and cause moderate respiratory signs with occasional nervous signs while the lentogenic strains cause mild to inapparent infections (Alexander, 2000; Lamb, 2001). The velogenic strains of NDV have been identified as potential select agents for bioterrorism and are a threat to nation's agro economy (http://www.cidrap.umn.edu/cidrap/content/biosecurity/agbiosec/biofacts/ agbiooviewhtml#Potential_Animal_Pathogens).NDV is an intrinsically tumor-specific virus, which is currently under investigation as a clinical oncolytic agent (Altomonte et al., 2010; Biswas et al., 2012). Thus, it is necessary to understand the pathobiology of NDV to design better vaccine vector for poultry, animal and human viruses and making it an ideal candidate for clinical application in cancer treatment. NDV is also a promising vaccine vector for animal and human pathogens (Samal, 2011a).

NDV is a member of the genus *Avulavirus* in the family *Paramyxoviridae* in the order *Mononegavirales* (Lamb, 2001). The genome of NDV is a nonsegmented, single-stranded, negative-sense RNA (Lamb, 2001). The genome contains at least six genes, which encode the nucleocaspid protein (NP),phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large RNA-dependent RNA polymerase protein (L). Two additional proteins, V and W, may be produced by RNA editing during P gene transcription (Hausmann et al., 1996). The NDV genes are arranged on the genomic RNA in the order 3′-NP-P-M-F-HN-L-5′. Flanking the genes are 3′ and 5′ extracistronic sequences, known as the leader and trailer, respectively. These leader and trailer regions are *cis*-acting regulatory elements involved in replication, transcription, and packaging of the

genomic and antigenomic RNAs.

NDV initiates infection by attaching to cell surface receptors and fusing viral and host cell membranes(Lamb et al., 2006). Viral attachment protein, HN binds to sialic receptors while F protein directs membrane fusion (Lamb, 1993b). The fusion process is pH independent and thus virus entry occurs at host cell plasma membranes. NDV requires co-expression of both the attachment protein and the F protein for fusion activity (Lamb et al., 2006; Russell et al., 2003). NDV has assumed increased importance as a prototype paramyxovirus because crystal structures of both the NDV F and HN proteins have been determined (Chen et al., 2001; Crennell et al., 2000).

NDV F protein is type 1 glycoproteins with an amino-terminal signal sequence, a hydrophobic transmembrane domain (TM) located near the carboxyl terminus and a 31 amino-acid cytoplasmic domain (CT) (Chen et al., 2001; Morrison, 2003) . The NDV F protein is a 553-amino-acid protein and is synthesized as a precursor, F_0 which must be proteolytically cleaved to F_1 and F_2 for fusion activity (reviewed in Reference (Morrison, 2003). The F glycoprotein of NDV contains six potential N-linked glycosylation sites (McGinnes et al., 2001). N-glycans of viral envelope glycoproteins are involved in many functions, such as promoting efficient expression, transport, folding, and binding to cell surface receptors and facilitating fusion and infectivity (Aguilar et al., 2006; Bagai and Lamb, 1995; Collins and Mottet, 1991; Eichler et al., 2006; Panda et al., 2004a; Sjolander et al., 1996; Zimmer et al., 2001). Though, the role of N-linked glycosylation of NDV F protein in biological activity and protein stability has been studied earlier, the contribution of NDV F protein N-linked glycosylation to virus pathogenesis in the natural host is unknown. It would

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have great significance to study the role of the functional glycosylation sites of F protein on the pathobiology of NDV.

The F protein cleavage site sequence has been shown to be a major determinant of NDV virulence (de Leeuw et al., 2003; Nagai, 1995). The F protein cleavage site of virulent strains contains polybasic amino acids that are recognized by intracellular proteases present in most cell types and in contrast, avirulent NDV strains have one or two basic residues and depends on extra cellular secretory proteases for cleavage (Panda et al., 2004b). It would be interesting to find out the role of conserved amino acids in the cleavage sites for the requirement of virulence.

The cysteine residues in viral proteins are involved in disulfide bond formation and thus play important role in structure and function of the protein. The F protein of NDV contains 13 cysteine residues of which 11 are conserved among the F proteins of other paramyxoviruses. However, the role of these conserved cysteine residues on NDV infectivity and pathogenesis is not known. Therefore, it would be important to know the role of each conserved cysteine residue on virus infectivity and pathogenesis.

The F protein along with the HN protein is a main target of immune response for NDV. The F protein possesses conserved domains like heptad repeats, transmembrane domain, and cytoplasmic tail and there are presence of conserved key amino acids which make the proper conformational stability thus facilitating fusion process. Thus, studies on F protein may prove to be useful for development of better NDV vaccines and NDV as an ideal viral vector.

1.3 Research Objectives:

The specific objectives of the present study were:

1. To study the importance of N-glycosylation sites in NDV F protein on virus replication and pathogenesis.

2. To study the role of conserved glutamine residue in the F protein cleavage site.

3. To investigate the role of F protein cytoplasmic tail on fusion activity and NDV virulence.

4. To study the role of conserved cysteine residues in the F protein on NDV infectivity and pathogenesis.

Chapter 2

2.1 Title

Review of Literature

2.2 Classification

NDV is a member of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Avulavirus* (Mayo, 2002**)**. NDV is the only member of the genus *Avulavirus*. The genus *Avulavirus* contains nine serotypes of avian paramyxoviruses (APMV-1-9) (Samal, 2011a). NDV represents type 1(APMV-1).Other important members of the family *Paramyxoviridae* are the mumps virus, SV5 and parainfluenza virus type 2, respiratory syncytial virus and recently emerging hendra and nipah virus.

2.3 Virion

The NDV particles are enveloped, pleomorphic in nature and range from 150-400 nm in size. The envelope is covered with viral glycoproteins which are 8-12 nm in diameter. The genome of NDV is a single strand of RNA of negative sense, and has a molecular weight of 5.2 to 5.7×10^{6} daltons (Lamb, 2001). The envelope contains two surface glycoproteins; HN protein and F protein. The HN protein, a receptor binding protein that is responsible for the attachment of the virion to the host cell receptor and F protein is required for the fusion of the virion to target host cell membranes (Lamb et al., 2006). The Matrix (M) protein is layered under the envelope,

which is thought to play major role in assembly and budding of the mature virion particles (Peeples, 1991). The ribo-nucleocapsid forms the core structure which acts as a template for the virus RNA synthesis. The core structure is formed by nucleocapsid protein (NP) tightly bound to RNA to which phosphoprotein (P) and large polymerase (L) proteins are attached (Fig.1).

2.4 Genome organization

The genome of NDV is a nonsegmented, negative-sense RNA genome consists of six transcriptional units arranged in (3' NP-P-M-F-HN-L 5') encoding at least eight proteins (Lamb, 2001)(Fig.2). The genomic RNA contains a 3' extracistronic region of 55 nucleotides, known as the leader, and a 5' extracistronic region of 114 nucleotides, known as the trailer (Krishnamurthy and Samal, 1998). These regions are essential for replication of the genome, and they flank the six genes. Each transcriptional unit contains a major open reading frame flanked by short 5' and 3' untranslated regions (UTRs), which are followed by conserved transcriptional initiation and termination control sequences, known as gene start (GS) and gene end (GE), respectively. Between the gene boundaries are non-coding intergenic sequences (IGS), which vary in length 1 to 47 nt (Chambers et al., 1986a; Krishnamurthy and Samal, 1998). The mRNAs are capped and have poly (A) tails. The genome consists of six genes: nucleocapsid protein (NP) gene, phosphoprotein (P) gene, matrix protein (M) gene, fusion protein (F) gene, hemagglutunin neuraminidase (HN) gene, and large RNA-dependent RNA polymerase protein (L) gene.

2.5 Viral proteins

The genome of NDV codes for at least six major proteins: NP, P, M, F, HN, L, and two minor proteins V and W.
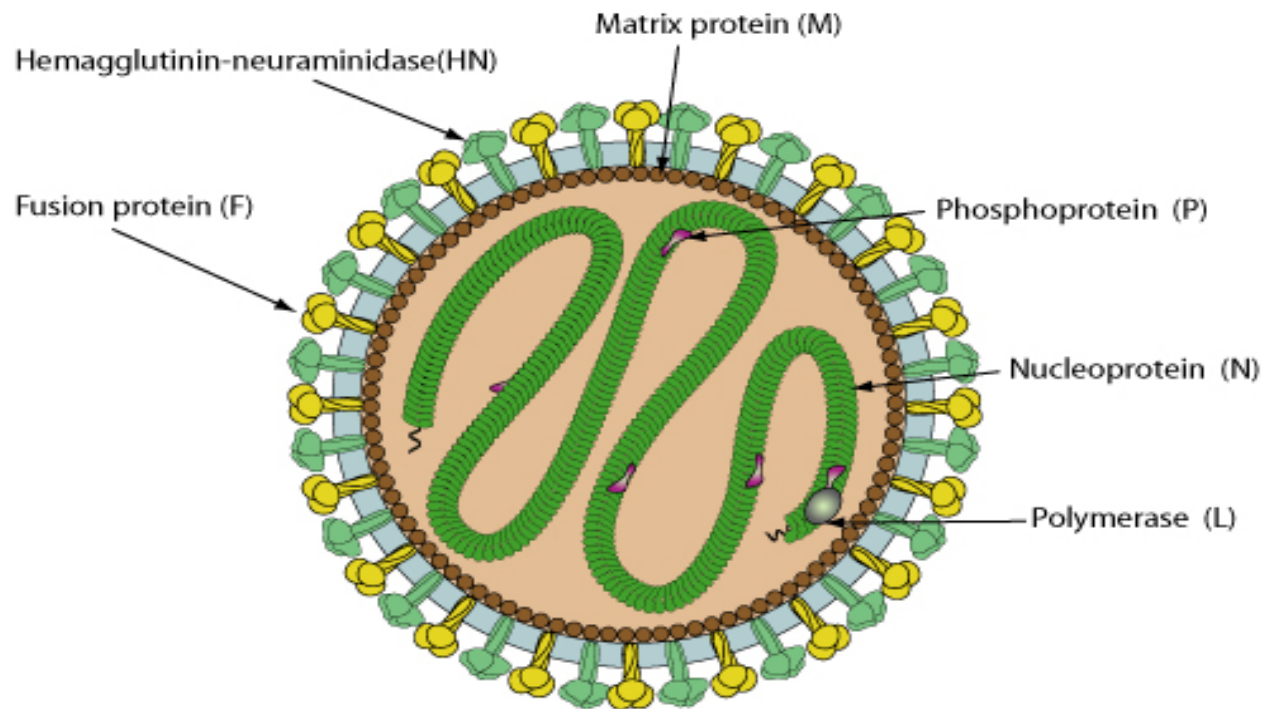
Figure 1. Schematic diagram of Newcastle disease virus particle (not drawn to scale).

(From ©Viralzone 2010)
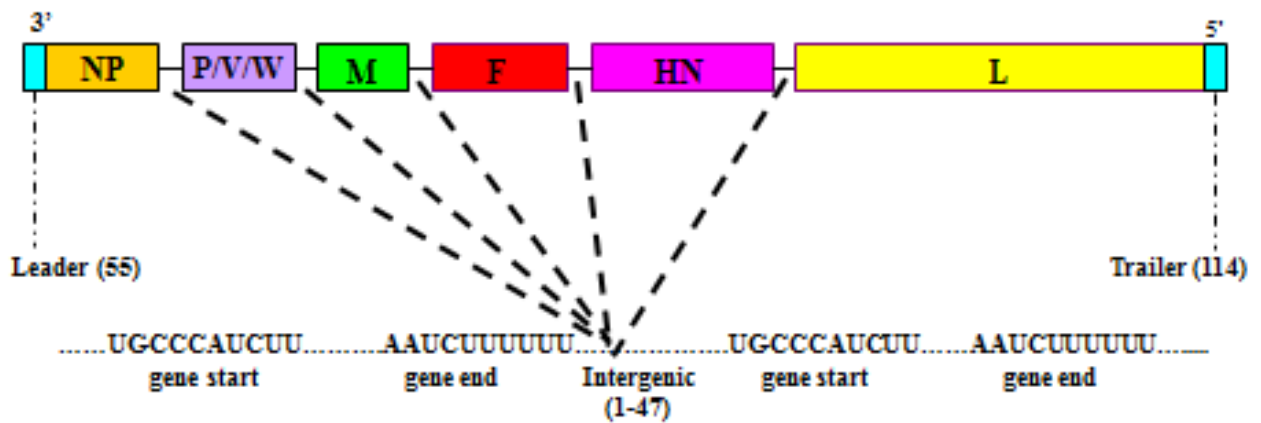
Figure 2. Genetic map of genomic RNA of NDV.

The single stranded, negative sense RNA genome consists of six major genes and two minor genes. The length of leader and trailer is shown in parenthesis. Each gene is flanked by conserved gene start and gene end. Between each gene boundary, nontranscribed intergenic sequences present which vary 1-47 nt in length.

The V and W proteins are the two additional proteins formed by non- template nucleotide addition through the RNA editing process during P gene mRNA transcription (Steward et al., 1993).

2.5.1 Ribonucleoprotein complex

The genomic RNA is associated with the NP, P and L proteins to form the ribonucleoprotein complex (RNP), which serves as a template for RNA synthesis (Lamb, 2001).

NP protein: The NDV genome is the template for two separate function in the life-cycle of the virus; RNA replication and transcription. The switch between RNA replication and transcription is considered to be controlled by the presence of *de novo* NP protein. The NP protein interacts with the P-L polymerase during transcription and replication and most likely, interacts with the M protein during virus assembly. The intracellular concentration of unassembled NP is also considered to be a major factor controlling the relative rates of transcription and replication from genome templates (Blumberg and Kolakofsky, 1981; Blumberg et al., 1981) The NP gene of NDV consists of 1747 nucleotides coding for 489 amino acids. The molecular weight of the protein is predicted to be 54 kilodaltons (kDa) (Krishnamurthy and Samal, 1998).

P protein: Essential component of the RNA polymerase transcription and replication complex. P protein binds to the viral ribonucleocapsid and positions the L polymerase on the template and acts as a chaperone for newly synthesized free N protein, so-called N_0. This property of P protein has been suggested to prevent NP from assembling RNA non-specifically. The P gene of

NDV is 1451 nt long and have a molecular weight of 53-56kDa (McGinnes et al., 1988). The P gene produces two additional proteins V and W, by RNA editing. The process of RNA editing is addition of one G nucleotide at the editing site (near the center of the ORF) which produces an mRNA that encodes the V protein, whereas addition of two G nucleotides produces an mRNA that encodes the W protein (Steward et al., 1993). The P protein is highly phosphorylated and acidic in nature(McGinnes et al., 1988).

L protein: The L protein is the least abundant but the largest structural protein (about 50 copies per virion) in the virion core (Banerjee, 1987). The P and L proteins form a complex, and both proteins are required for polymerase activity with NP:RNA templates. The L gene is 6704 nt long and predicted molecular mass is 242 kDa (Yusoff et al., 1987). The L protein is also responsible for capping and polyadenylation of the nascent viral mRNAs.

2.5.2 Matrix protein

The matrix protein is associated with the inner surface of the membrane and is the most abundant protein in the virion. The M gene of NDV is 1241 nt long. Its predicted molecular mass is 40 kDa. It is also believed that it interacts with the NP protein, but the exact binding domains are yet to be determined. The dimers of paramyxovirus M protein can form a grid-like array on the inner surface of the viral membrane, and probably interact with both the cytoplasmic tails of the HN and F glycoproteins as well as the nucleocapsid to initiate virus assembly and budding (Chambers et al., 1986b).

2.5.3 Envelope surface glycoproteins

The envelope of NDV virions have two transmembrane glycoproteins; the attachment protein termed HN and the F protein and they form spikes protruding from the lipid bilayer. HN is a dual-function hemagglutinin/ neuraminidase, capable of binding to cell surface sialic acids. F mediates pH-independent fusion with the host cell plasma membrane.

HN protein: The HN protein of NDV is a multifunctional protein. It possesses both the receptor recognition and neuraminidase (NA) activities associated with the virus. It recognizes sialic acid-containing receptors on cell surface; it promotes the fusion activity of the F protein, thereby allowing the virus to penetrate the cell surface; and it acts as an NA by removing the sialic acid from progeny virus particles to prevent self-agglutination of progeny virus. Thus, the HN protein plays an important role in viral infection (Lamb et al., 2006). The HN glycoprotein of NDV is a major antigenic determinant of the virus (Meulemans et al., 1986; Morgan et al., 1992). The HN gene is 1998 nt long with a coding region of 577 amino acid residues and a molecular weight of 74kDa. The HN of some strains of NDV is synthesized as a biologically inactive precursor (HN_0), and 90 residues from the C-terminal are removed to activate the molecule The HN proteins are type II integral membrane proteins that span the membrane once. The N-terminus of the HN protein consists of the cytoplasmic domain, followed by the transmembrane region and the stalk region. The HN attachment proteins are thought to form tetramers in their active form. Mutational studies of the NDV HN stalk have examined effects on membrane fusion, NA activity, hemadsorption, F-protein complex formation, and oligomerization. Although mutations in the NDV HN stalk can affect both NA

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and membrane fusion activities, it has not been clear how these two functions are coupled. The crystal structure of the intact NDV HN reveals a four-helix bundle (4HB) stalk packed between two NDV NA domain dimers, which provide insight into the structural basis for stalk-dependent HN NA and membrane fusion-promoting activities (Crennell et al., 2000).

F protein: The F protein directs membrane fusion between the viral and the cellular membranes (Morrison, 2003). NDV F proteins do not require the acid pH of endosomes for the activation of fusion activity; thus, other mechanisms for F protein activation must be invoked. Because of this acid pH independence, infected cells expressing both the HN and the F proteins can fuse with adjacent cells to form multinuclear cells or syncytia, a process that is assumed to be similar to virus-cell fusion (Baker et al., 1999). Syncytia formation is a hallmark of NDV infection in host cells(Horvath and Lamb, 1992). It is a typical cytopathic effect caused by the virus and can lead to tissue necrosis and might also be a mechanism of virus spread(Horvath et al., 1992).

The NDV F protein is synthesized as a precursor, F_0 which is 1792 nt long encoding 553 amino acids that must be proteolytically cleaved to activate F protein fusion activity. Cleavage at amino acid 117 produces disulfide-linked F_2 and F_1 polypeptides derived from the amino-terminal and carboxyl-terminal domains of F_0, respectively(de Leeuw et al., 2005; Nagai, 1995). The F_1 polypeptide has one fusion peptide. Upon initiation of fusion, fusion peptides are thought to insert into target membranes, docking the protein to

these membranes. Paramyxovirus F_1 polypeptides have two heptad repeat (HR) regions, one (HR1) located adjacent to carboxyl terminal to the more amino-terminal fusion peptide and the other adjacent to the transmembrane domain (HR2)(Chen et al., 2001). Studies of peptides with sequences of these HR domains and characterization of mutations within these domains have led to the hypothesis that F proteins are synthesized and transported to cell surfaces in a metastable conformation in which the HR domains are not associated and the fusion peptides are masked. Upon fusion activation, F proteins are thought to undergo a series of conformational changes that result in the insertion of fusion peptides into target membranes and the interaction of the HR1 and HR2 domains to form a very stable complex(Lamb et al., 1999; Morrison et al., 1987) The formation of this complex is thought to pull target and attack membranes in close proximity, allowing subsequent fusion events (Fig 3).The amino acid sequence at the F protein cleavage site is different among most lentogenic, mesogenic and velogenic NDV strains (de Leeuw et al., 2003; Panda et al., 2004b). The F protein of all lentogenic strains has a monobasic cleavage site, which is cleaved by extracellular proteases restricted to specific tissues; whereas, the F protein of all mesogenic and velogenic NDV strains has a multibasic cleavage site, which is cleaved by ubiquitous intracellular proteases. The fully glycosylated F protein of NDV contains a 470-amino-acid extracellular domain, a transmembrane domain near C-terminal, and a 29-amino-acid cytoplasmic tail. The molecular weights of F_0, F_1 and F_2 are
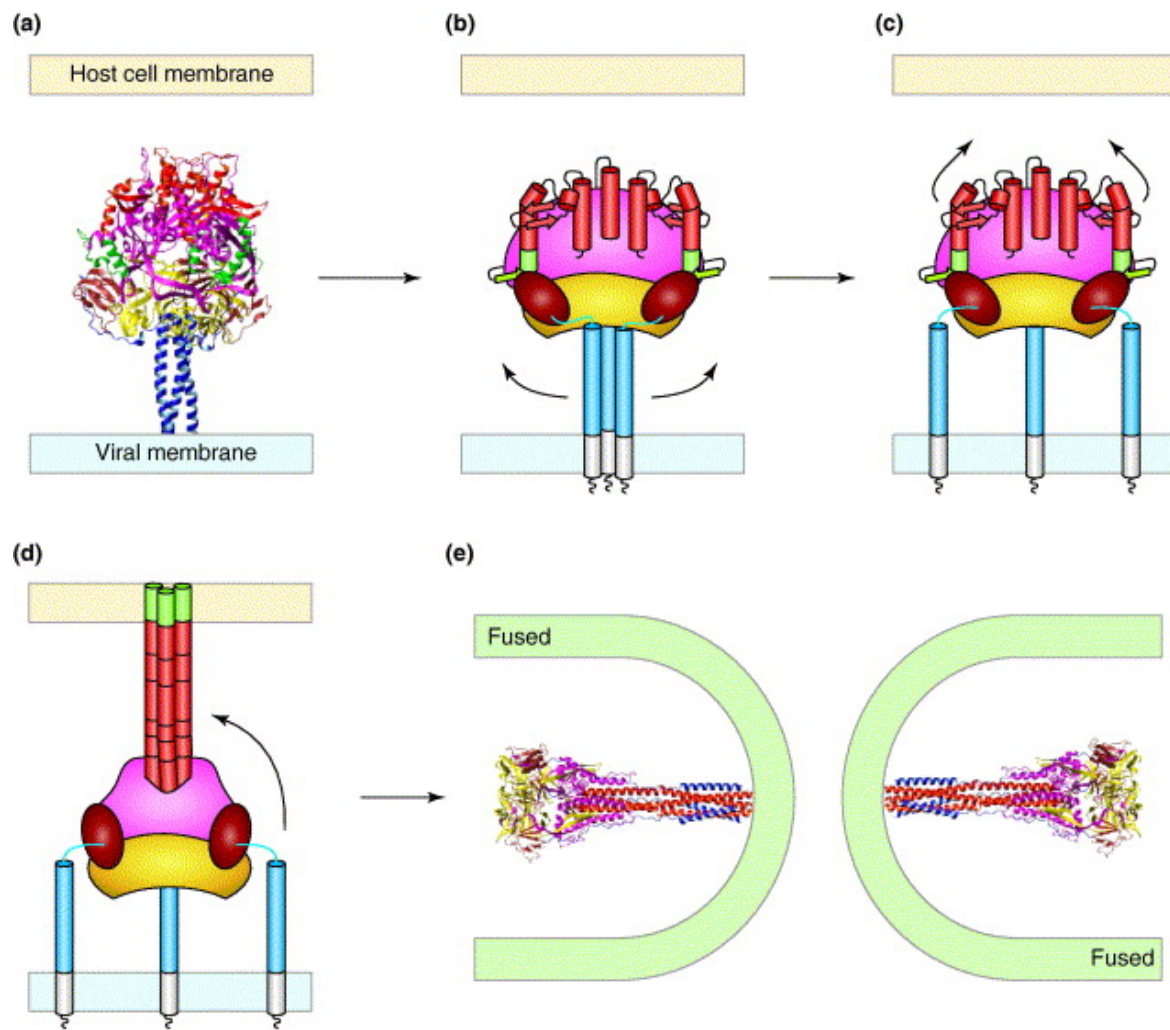
Figure 3. Model of Paramyxoviruses mediated fusion. (Modified from Trends of Mircobiology) Native state b) Prefusion activation c) Fusion activation d) Pre hairpin intermediate e) Fusion

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66kDa, 55kDa, and 12.5kDa respectively. The F protein is the major immunogenic protein of NDV.

2.5.4 V and W proteins

NDV produces two additional proteins, V and W, from the P gene by alternative mRNAs that are generated by RNA editing(Steward et al., 1993). In NDV, insertion of one nontemplate G residue gives rise to a V-encoding mRNA, while insertion of two nontemplate G residues generates a W-encoding mRNA. Analysis of mRNAs produced from the P gene showed that 68% were P-encoding mRNA, 29% were V-encoding mRNA, and 2% were W-encoding mRNA(Huang et al., 2003). All three P gene-derived proteins are amino coterminal but vary at their carboxyl terminus in length and amino acid composition. The V protein of NDV, in common with its counterparts in other paramyxoviruses, is cysteine rich within its unique carboxyl-terminal region and binds to zinc (Mebatsion et al., 2001). The V protein of NDV is found to be incorporated in virions, as are simian virus 5 (SV5) and mumps virus (Kubota et al., 2005; Sun et al., 2004). V and W proteins are present in infected cells but absent in viral particles. V protein is considered as a virulence factor acting as an interferon antagonist. The function of the W protein of NDV has not established, however work with other virus suggests it may be an inhibitor of replication.

2.6 Stages of replication of NDV

The replication of NDV occurs in the cytoplasm and is very similar to that of other non-segmented negative-strand RNA viruses of *paramyxoviridae* (Fig.4). Replication of NDV is initiated by the binding of the HN on the virion envelope to sialic acid on the cell surface glycolipids followed by fusion of the viral-host cell membrane (Huang et al., 2004; Morrison, 2003). The F protein promotes fusion of the envelope with the plasma membrane. The RNA polymerase is carried into the cell as part of the nucleocapsid. Transcription, protein synthesis, and replication of the genome all occur in the host cell cytoplasm. The genome is transcribed into individual messenger RNAs (mRNAs) and a full-length positive-sense RNA template. New genomes associate with the L, N, and NP proteins to form nucleocapsids, which associate with the M proteins on viral glycoprotein–modified plasma membranes. Finally, the progeny viruses matured by budding through plasma membrane.

2.6.1 Virus attachment, fusion and entry

Primary adsorption of the virus to the target cell is generally promoted by the attachment protein, with sialic acid residues or cell surface proteins serving as receptors. Upon adsorption of the virus to the cellular receptors, the viral membrane fuses with the host cellular plasma membrane at neutral pH. The F protein is then responsible for fusion of the viral membrane with a host cell membrane.NDV require their homotypic attachment protein for membrane fusion activity, suggesting a role for F-attachment protein interactions in control of fusion. This results the release of the viral nucleocapsids into the cytoplasm of the host cell. The M protein is considered to make several contacts

with the nucleocapsid. After the release of the nucleocapsid into the cytoplasm, disruption of the M-nucleocapsid complex occurs and the viral nucleocapsid is released into the host cell cytoplasm.

2.6.2 Transcription

The key feature of transcriptional control in the NDV RNA viruse is entry of the virus-encoded RNA-dependent RNA polymerase at a single 3' proximal site followed by obligatory sequential transcription of the linear array of genes. Levels of gene expression are primarily regulated by position of each gene relative to the single promoter and also by cis-acting sequences located at the beginning and end of each gene and at the intergenic junctions. Obligatory sequential transcription dictates that termination of each upstream gene is required for initiation of downstream genes. Therefore, termination is a means to regulate expression of individual genes within the framework of a single transcriptional promoter.The viral RNA polymerase has to first transcribe the leader RNA before beginning mRNA synthesis at the NP gene start signal. RNA replication of Sendai virus requires the genome length to be a multiple of six ("Rule of Six" theory), for efficient replication. NDV also follows the rule of six principle for efficient replication. This hexamer rule is most likely related to the finding that each NP subunit of the nucleocapsid is associated with exactly six nucleotides. Once the nucleocapsid is released in to host cell cytoplasm the leader mRNA is synthesized first, on entry of the NP and P/L polymerase at the 3′ end of the genome followed by re-initiation of NP gene mRNA synthesis from NP gene start sequence. The transcription undergoes a sequential start and stop

mechanism producing gradient mRNA production in which 3' proximal gene concentration is higher than those of downstream genes (Cattaneo et al., 1987).

2.6.3 Genome Replication

Viral RNA replication involves full length plus strand synthesis. This is used as a template for full length minus strand. Both full length strands are coated with nucleocapsid protein as they are made. New full length minus strands may serve as templates for replication, or templates for transcription, or they may be packaged into new virions. Both the genome and antigenome are assembled into encapsidated nucleocapsid. The leader and trailer regions of the genome contain specific sequences for initiating encapsidation. The leader and trailer regions of the genome contain specific sequences for initiating encapsidation. The processes of transcription and replication are tightly regulated. When unassembled NP is limiting, the viral RNA polymerase is preferentially engaged in mRNA synthesis, raising the intracellular levels of unassembled NP and all other viral proteins(Nagai, 1999). When unassembled NP levels are sufficient, some viral RNA polymerase activity switches from transcription to replication, thereby lowering the levels of unassembled NP, as each initiation of encapsidation utilizes many NP monomers to finish the assembled genome chain (Blumberg et al., 1981). The RNA synthesis of NDV is shown in Fig. 5.

2.6.4 Virus assembly and release

Once formed, RNPs must be incorporated into budding virus particles. The intracellular site of nucleocapsid assembly is in the cytoplasm. The first step in viral assembly is the encapsidation of genomic RNA into nucleocapsid. The nucleocapsids are thought to be assembled in two steps: first, there is an association of free NP subunits with the genome or template RNA to form the helical ribonucleoprotein (RNP) structure, followed by the association of the P-L protein complex. Selective genome incorporation based on the polarity of the viral RNA also occurs, with (−)-sense genomes incorporated more efficiently into budding particles than (+)-sense antigenomes. In contrast to the antigenomes, Paramyxovirus mRNAs are not encapsidated. Incorporation of genomes into budding virions is likely driven by interactions between viral matrix proteins and nucleocapsids at virus assembly sites. The assembly of the viral envelope takes place at the cell surface. The viral integral membrane glycoproteins (F and HN) are synthesized in the endoplasmic reticulum and undergo step-wise conformational maturation before transport through the secretory pathway. Only correctly folded and assembled proteins are transported out of the endoplasmic reticulum (Braakman and van Anken, 2000). In the Golgi apparatus, the carbohydrate chains on the HN and F glycoproteins are modified extensively. Cleavage of the F proteins with multiple basic cleavage sites, occurs in the trans Golgi apparatus (de Leeuw et al., 2005). Finally, the glycoproteins are transported to the plasma membrane. In NDV, the assembly of the envelope occurs at the cell surface and release of the virus takes place by budding.
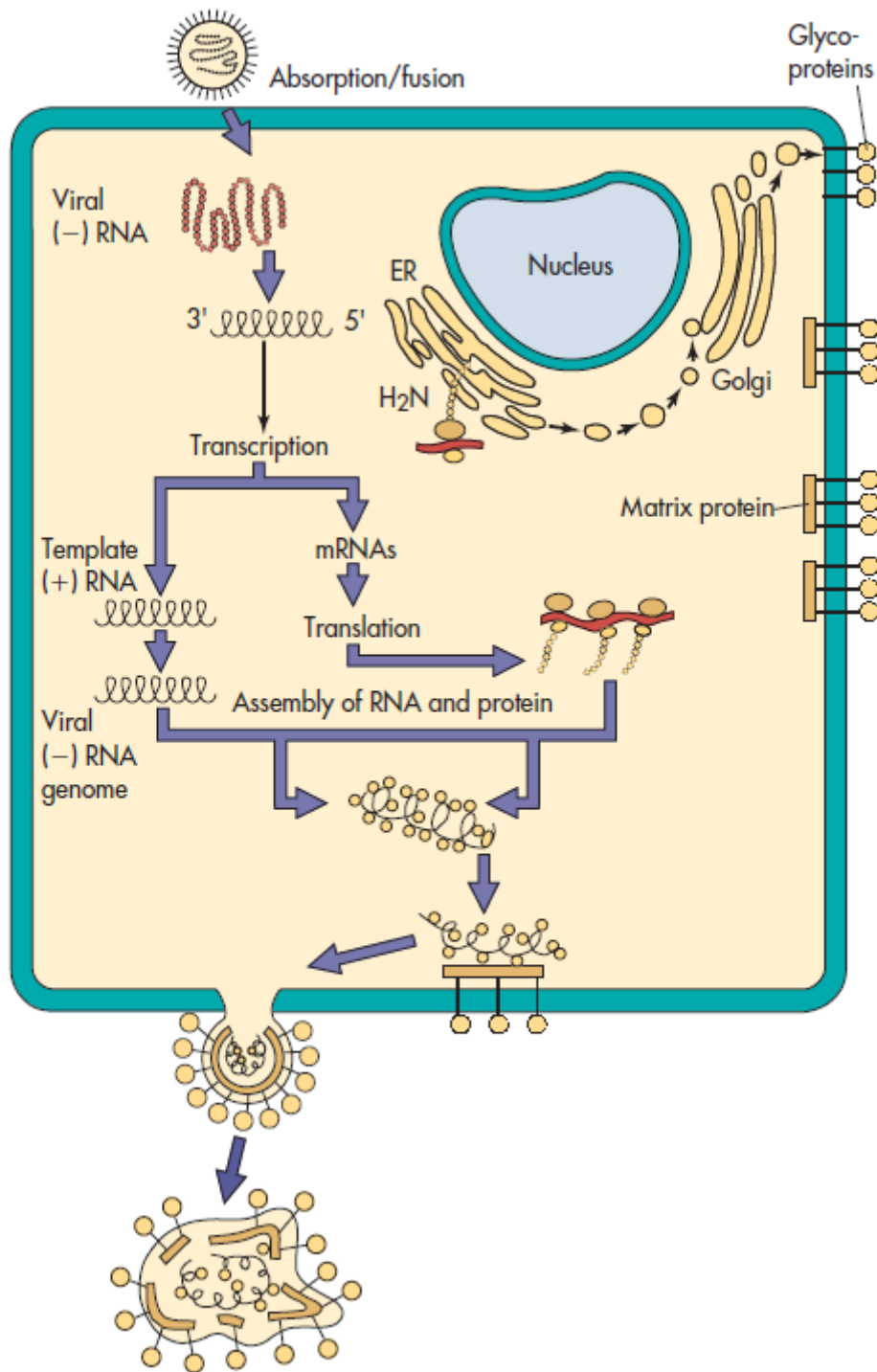
Figure 4. The life cycle of NDV

(Figure modified from www.uib.es/depart/dba/microbiologia/.../paramixoviruses.pdf)
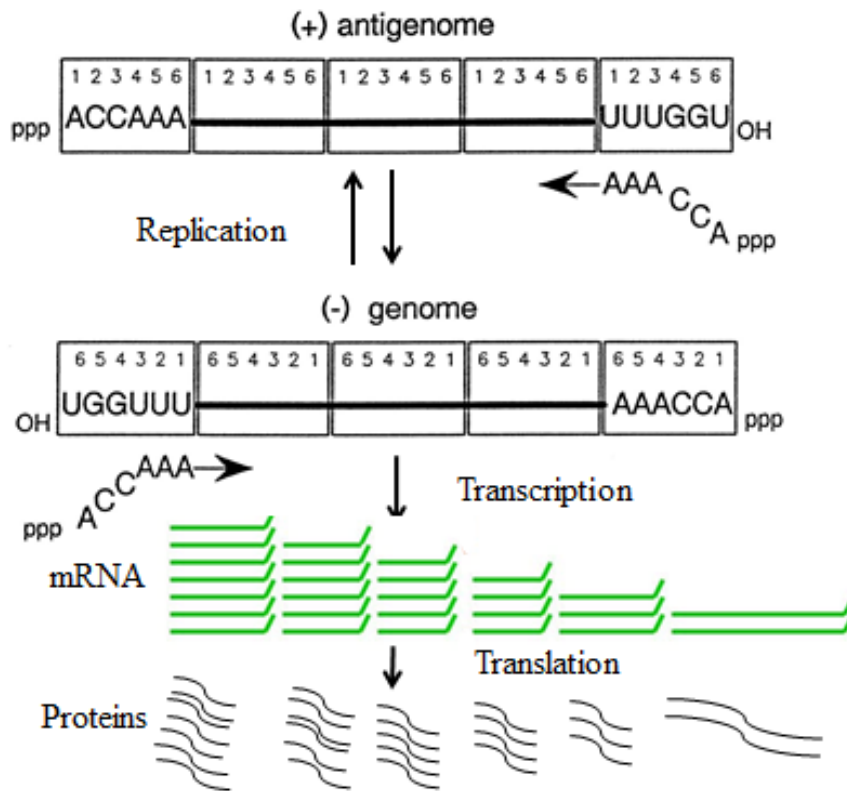
Figure 5. Schematic diagram of RNA replication and transcription in paramyxovirus.

Schematic diagram showing transcription and replication of a paramyxovirus. Genome and antigenome are shown encapsidated by nucleocapsid protein subunits. The vertical lines indicate the gene junctions. The polymerase complex (P-L complex) transcribes the genome to yield capped and poly A-tailed mRNAs. When sufficient amount of viral protein levels are achieved, the viral polymerase switches from its transcription mode to replicative mode to produce antigenome, which serves as the template for the synthesis of the progeny viral genome.

2.7 Reverse genetics

Reverse genetics is a method which is used for genetic manipulation of viruses by using the cloned cDNA of the viral genome that allows the generation of infectious virus *in vitro*. In nonsegmented negative-strand RNA viruses, the minimal unit that is able to initiate infection inside the host cell is genomic or anti-genomic RNP complexes with the viral RNA polymerase. Therefore, introduction of a reverse genetics system by transfecting plasmids expressing viral accessory proteins such as NP, P and L along with full length antigenome plasmid have made it possible to recover genetically engineered virus (Fig 6). Genetic manipulation of a negative strand-RNA virus was first made possible in 1990 for the segmented influenza-A virus using biological active viral RNP complexes that were reconstitute *in vitro* (made of synthetic RNA and purified nucleoprotein and polymerase protein) and then transfecting the complex into cells previous infected with a fully functional, helper, virus (Enami et al., 1990).Subsequently, recovery of several other viruses such as the vesicular stomatitis virus(Lawson et al., 1995; Whelan et al., 1995) simian virus 5(He et al., 1997), human respiratory syncytial virus (Collins et al., 1995) sendai virus(Garcin et al., 1995), rinderpest virus(Baron and Barrett, 1997), parainfluenza virus(Durbin et al., 1997; Hoffman and Banerjee, 1997) and measles virus(Radecke et al., 1995) have been achieved. The recoveries of infectious NDVs from cDNA using reverse genetics system were first reported in 1999 (Peeters et al., 1999; Romer-Oberdorfer et al., 1999). Currently reverse genetics systems are available for lentogenic strain LaSota (Peeters et al., 1999; Romer-Oberdorfer et al., 1999) B1 (Nakaya et al., 2001), mesogenic strain Beaudette C (Krishnamurthy et al., 2000)(Krishnamurthy *et al*., 2000) and velogenic strain Hert/33 (de Leeuw et al., 2005). The availability of a reverse genetics system for NDV as well as other viruses has provided essential information and tools to study the viral molecular mechanism in greater detail.
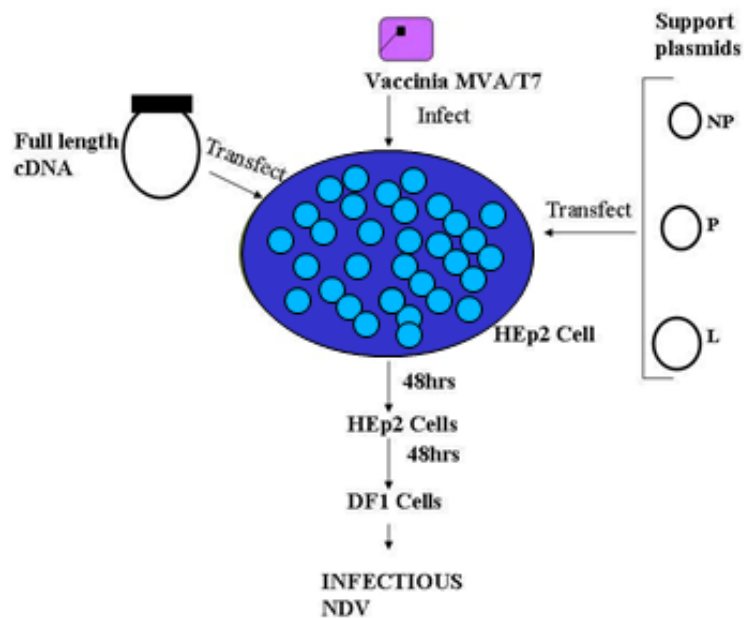
Figure 6. Schematic Diagram for the recovery of infectious NDV from cDNA.

Plasmids encoding for antigenome full length cDNA, NP, P and L mRNA were co-transfected into HEp-2 cells. All the plasmids are under control of the T7 RNA polymerase promoter. The T7 RNA polymerase is provided by the recombinant vaccinia MVA/T7 strain. Infectious NDV was generated entirely from cloned cDNA with procedures explained by Krishnamurthy *et al*., 2000.

Chapter 3

3.1 Title

Coordinated deletion of N-glycans from the heptad repeats of the F protein of

Newcastle Disease Virus affects virulence (Samal et al., 2012)

3.2 Abstract

The role of N-linked glycosylation of the NDV fusion protein in viral replication and

pathogenesis was examined by eliminating potential acceptor sites using a reverse genetics

system for the moderately pathogenic strain Beaudette C (BC). The NDV-BC F protein

contains six potential acceptor sites for N-linked glycosylation at residues 85, 191, 366, 447,

471, and 541 (sites Ng1-6, respectively). The sites at Ng2 and Ng5 are present in heptad

repeat (HR) domains HR1 and HR2, respectively, and thus might affect fusion. Each N-

glycosylation site was eliminated individually by substituting asparagine (N) with glutamine

(Q), and a double mutant (Ng2+5) involving the two HR domains also was made. Each

mutant was successfully recovered by reverse genetics except for the one involving Ng6,

which is present in the cytoplasmic domain. All of the F proteins expressed by the recovered

mutant viruses were efficiently cleaved and transported to the infected-cell surface. None of

the individual mutations affected viral fusogenicity, but the double mutation at Ng2 and Ng5

in HR1 and HR2 increased fusogenicity >12-fold. The single mutations at sites Ng1, Ng2,

and Ng5 resulted in modestly reduced multi-cycle growth *in vitro*. These three single

mutations also were the most attenuating in eggs and 1-day-old chicks, and were associated

with decreased replication and spread in 2-week-old chickens. In contrast, the combination of

the mutations at Ng2 and Ng5 yielded a virus that, compared to the BC parent, replicated >100-fold more efficiently *in vitro*, was more virulent in eggs and chicks, replicated more efficiently in chickens with enhanced tropism for the brain and gut, and elicited stronger humoral cell responses. These results illustrate the effects of N-glycosylation of the F protein on NDV pathobiology, and suggest that the N-glycans in HR1 and HR2 coordinately down regulates viral fusion and virulence.

3.3 Introduction

Newcastle disease virus (NDV) is a major avian pathogen affecting many species of birds and it causes severe economic losses to poultry industry worldwide (Alexander, 1997). NDV isolates cause a broad spectrum of disease ranging from fatal to asymptomatic infection. NDV strains are grouped as highly virulent (velogenic), moderately virulent (mesogenic) and low virulent (lentogenic) based on pathogenicity in chickens (Alexander, 1997). NDV is a member of the genus *Avulavirus* in the family *Paramyxoviridae* (R. Lamb, 2005). The genome of NDV is a single-stranded, non-segmented, negative-sense RNA of 15,186 nucleotides (de Leeuw and Peeters, 1999; Krishnamurthy and Samal, 1998; Nagai et al., 1989; Phillips et al., 1998). The genomic RNA contains six genes that encode at least seven proteins (Chambers et al., 1986a; Wilde, 1986). NDV initiates infection after attachment to susceptible cells and subsequent membrane fusion process directed by two virion glycoproteins associated with the envelope, the hemaglutinin-neuraminidase (HN) protein and the fusion (F) protein (Lamb, 1993a). The HN protein mediates attachment by binding to sialic acid receptor and has neuraminidase activity and plays a role in fusion promotion, whereas the F protein is responsible for membrane fusion and penetration through the host cell membrane (Lamb, 2007). The NDV F protein does not require the acidic pH of

endosomes for the activation of fusion process and because of this acidic pH independence, infected cells fuse with adjacent cells to form syncytia, a process very similar to virus-cell fusion (Baker et al., 1999). Although the trigger mechanism for F mediated membrane fusion is still unknown, it is postulated that interaction between the HN and F proteins stimulate conformational changes in the F protein that drives merger of viral and host cell membranes (Lamb et al., 2006).

The NDV F protein is a trimeric type I integral membrane protein that is synthesized as an inactive precursor F_0 (66 kDa) which is post translationally cleaved by host cell proteases into two disulfide-linked subunits, N-terminal F_2 (12.5kDa) and C-terminal F_1(55kDa)(Nagai et al., 1989). A stretch of hydrophobic amino acids at the N terminus of the F_1 subunit form a fusion peptide (FP) that interacts with the host cell membrane, thereby initiating the fusion process. The NDV F protein has two HR motifs in the F_1 subunit; HR1 is adjacent to the fusion peptide, and HR2 is adjacent to the transmembrane (TM) domain. Crystal structure of fusion proteins of different paramyxoviruses revealed that these heptad repeats assemble to form conserved six helix bundle and this assembly is tightly coupled to membrane fusion (Baker et al., 1999; Chen, 2001; Luque and Russell, 2007; Swanson et al.; Yin et al., 2006; Yu et al., 2002; Zhu et al., 2003a; Zhu et al., 2003b). Subsequent structural, biochemical, and functional studies of fusion protein of paramyxoviruses have led to the hypothesis that, prior to interaction of F protein with the host cell, the F protein is believed to fold in a pre-fusion, metastable conformation, which is then activated to undergo a large conformational rearrangement needed to accomplish membrane fusion (Lamb, 1993a; Lamb and Jardetzky, 2007; Lamb et al., 2006). However, the mechanistic details of the extensive conformational rearrangements of fusion protein are still not clear.

The F glycoprotein of NDV undergoes N-linked glycosylation in rough endoplasmic reticulum of host cells, in which N glycan chains are attached covalently to asparagines residues at the consensus sequence motif Asn-X-Ser/Thr (Baker et al., 1999; Braakman, 2000; Collins and Mottet, 1991; Doms et al., 1993) (where "X" can be any amino acid except proline). N-glycans of viral envelope glycoproteins are involved in many functions, such as promoting efficient expression, transport, folding, binding to cell surface receptors, facilitating fusion and infectivity (Aguilar et al., 2006; Bagai and Lamb, 1995; Braakman, 2000; Collins and Mottet, 1991; Eichler et al., 2006; Goffard and Dubuisson, 2003; Panda et al., 2004a). On the other hand, N-linked glycans also act in shielding the virus against antibody neutralization as reported in HIV, Hepatitis B and influenza viruses (Kniskern et al., 1994; Vigerust et al., 2007; Wei, 2003).

The F glycoprotein of NDV contains six potential N-linked glycosylation acceptor sites at residues 85, 191, 366, 447, 471 and 541 which are conserved in all strains (de Leeuw and Peeters, 1999; Paldurai et al.)(Fig.7). A previous study has predicted that four of these sites present at residues 85,191,366 and 471 are functionally active (McGinnes et al., 2001). Two of these residues at positions 191 and 471 are present within the heptad repeats HR1 and HR2, suggesting that N-glycosylation at these sites might play an important role in the fusion promotion. The previous study demonstrated the role of N-linked glycosylation of NDV F protein in the biological activity and protein stability using a plasmid transfection system (McGinnes et al., 2001). However, the contribution of NDV F protein N-linked glycosylation on virus replication, pathogenesis and virulence in the natural host is unknown.

In the present study, a reverse genetics system was used to generate a panel of recombinant viruses with mutations in the N-glycosylation sites of the NDV F protein. These

mutations eliminated each of the six N-glycosylation sites individually (Ng1, Ng2, Ng3, Ng4, Ng5 and Ng6) and in combination (Ng2 and Ng5). These mutant viruses allowed us to determine the usage of each N-glycosylation site and study the role of each N-glycan on functional activity of F protein and its effect in viral replication, pathogenesis, virulence and immunogenicity in chickens. We demonstrate that NDV F protein with deglycosylation of each N-glycan site remains completely functional. However, deletion of N-glycosylation sites in viruses modulates the viral pathogenesis to various extents.

3.4 Materials and Methods

3.4.1. Cells and viruses.

Chicken embryo fibroblast cell line (DF1) and human epidermoid carcinoma cell line (HEp-2) 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 cells were 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 (NIH, Bethesda, MD) and propagated in primary chicken embryo fibroblast cells in DMEM with 2% FBS. The moderately pathogenic (mesogenic) NDV strain Beaudette C (BC) and its recombinant derivatives were grown in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs.After 2 days, the allantoic fluid was harvested and the virus was plaque purified using our standard procedure (Krishnamurthy et al., 2000).

3.4.2 Construction of plasmids and recovery of mutant viruses

The construction of plasmid pNDVfl carrying the full length antigenome cDNA of the NDV strain BC has been described previously (Krishnamurthy et al., 2000). In the present study, a unique PacI site was created in the downstream untranslated region (UTR) of the M gene. To introduce mutations into the F gene of pNDVfl, a PacI-MluI fragment containing the F gene was amplified using each N-glycosylation mutant forward and reverse primers with desired mutations (Table 1). The PCR product was cloned into pCR 2.1-TOPO vector (Invitrogen, USA). The inserts bearing N-glycan mutation were released by digestion with PacI and MluI and then cloned into the full length cDNA of BC. A panel of N-glycosylation F mutants were generated. A double F mutant, Ng2+5, was also created by eliminating N-glycosylation sites 2 and 5. This was done by subjecting the Ng2 mutant to a second round of mutagenesis to remove Ng5. All mutant F cDNAs were sequenced in their entirety to confirm the presence of the desired mutations. Transfection and recovery of recombinant NDV mutants were performed by using reverse genetics technique described previously (Krishnamurthy et al., 2000).

3.4.3. RT-PCR and sequence analysis.

The recovered F mutant viruses were passaged in 9-day-old SPF chicken embryos for five times. From each passage total RNAs were isolated from mutant NDV-infected allantoic fluid of 9-day-old SPF chicken embryos, using TRIzol reagent (Invitrogen, USA). Reverse transcription-PCR

(RT-PCR) was performed using the Thermoscript RT-PCR kit (Invitrogen). The amplified cDNA fragments were then sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc, USA) in ABI 3130*xl* 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.

3.4.4. Production of anti NDV-F antisera

Three synthetic peptides were custom synthesized (Invitrogen): C-terminal tail (30 residues), corresponding to amino acids 524 to 553 of cytoplasmic tail of the F protein; peptide A (10 residues), corresponding to amino acids 27 to 36; and peptide B (10 residues), corresponding to amino acids 67 to 76. One rabbit was injected subcutaneously with 1 mg of KLH conjugated C-terminal tail peptide and another rabbit was injected with 0.5 mg of each of KLH conjugated peptide A and peptide B in Freund's complete adjuvant to raise anti-F_{cyt} and anti-F_{Nterm} antiserum, respectively. After 2 weeks, a booster immunization was given with 0.5 mg peptide in Freund's incomplete adjuvant and 2 weeks later the hyperimmune sera were collected. Western blot analysis was performed using NDV infected cell-lysates to confirm the specificity of the two antisera to NDV F protein.

Table 1. Primers used in this study*

Primer	Nucleotide sequence
Ng1 Forward…………5'ATGCATAC**CA**G**G**AGGACATTGACCACTTTGCTCACCCC3'	
Ng1 Reverse…………5'AATGTCCT**CT**G**G**TATGCATCCAAGGGGGCTTTCGCAC3'	
Ng2 Forward…… 5'ACCAATTT**CA**G**G**AAAACAGCTCAGGAATTAGGCTGCATCAG3'	
Ng2 Reverse…………5'AGCTGTTTT**CT**G**G**AAATTGGTCATTAACAAACTGCTG3'	
Ng3 Forward……….. 5'GGC**CA**G**G**ACATCGGCCTGTATGTACTCAAAGACCG3'	
Ng3 Reverse…………5'CAGGCCGATGT**CT**G**G**CCGCTCAAGCAGGAATAAATACC3'	
Ng4 Forward…………5'**CA**G**G**AAG**CA**G**G**ATCTCAATACAAGATTCTC3'	
Ng4 Reverse…………5'TATTGAGAT**CT**G**G**CTT**CT**G**G**ATAAGTTGCATCG3'	
Ng5 Forward……5'GAATGTC**CA**G**G**AACTCGATCAGTAATGCTTTGAATAAGTTAG3'	
Ng5 Reverse…………5'GAGTT**CT**G**G**GACATTCCCAAGCTCAGTTG 3'	
Ng6 Forward…………5'ATTATGGCTTGGG**CA**G**G**AATACCCTAGATC3'	
Ng6 Reverse ………...5'CTAGGGTATT**CT**G**G**CCCAAGCCATAATAAGGTC3'	
Ng7 Forward…………5' ATTATGGCTTGGG**A**A**T**AATACCCTAGATC3'	
Ng7 Reverse………… 5'CTAGGGTATT**A**TT**CCCAAGCCATAATAAGGTC3'	

*Bold and underline indicates mutations.

3.4.5 Western blot analysis and PNGase F digestion

Vero cells were infected with each mutant virus at an MOI of 1.0 and incubated for 1 h at 37^0C. Cells were washed with phosphate-buffered saline (PBS) and overlaid with 2% DMEM. After 36h post infection (PI), the cells were washed with PBS and were divided into two aliquots. From one aliquot proteins were extracted using cell lysis buffer (BD Biosciences, USA) and the second aliquot was treated with denaturing buffer (New England Biolabs, USA). 40ul of cell lysates from first aliquot were diluted in Laemmli sample buffer (Bio-Rad Lab, USA) in the presence of reducing agent and loaded onto 10% polyacrylamide gels. 40ul of cell lysates from second aliquot were digested with PNGaseF (New England Biolabs). Briefly, infected cell extracts were denatured at 100^0C for 10 min. The reaction mixture was put on ice for 5 min, and PNGaseF (2U) and 10x reaction buffer and 10% NP40 supplied by manufacturer were added to equal amounts of cell lysates from each recombinant virus in 40 µl reaction buffer and incubated overnight at 37^0C. The digestion was stopped by boiling and samples were diluted in Laemmli sample buffer in the presence of reducing agent and loaded onto 10% polyacrylamide gels along with equal amount of undigested cell lysates. After electrophoresis, the gels were equilibrated in transfer buffer and transferred onto nitro cellulose membrane. The membrane was blocked with blocking solution (5% skimmed milk in PBS) for 2 h at room temperature and incubated with primary antibody (1:100 dilution) anti-F_{cyt} overnight at 4°C. Membranes were washed three times in washing solution (0.05% Tween-20 in

PBS) and then incubated in secondary antibody, anti-rabbit IgG antibodies diluted (1:5000) in dilution buffer for 1 h at room temperature. Membranes were washed extensively and bound antibody was detected using the ECL Western blotting detection reagent system (Amersham,USA).

3.4.6 Cell surface expression of the F proteins of N-glycosylation mutant viruses

Cell surface expression of the F proteins of N-glycosylation mutants viruses were quantitatively determined by flow cytometry. Briefly, DF1 cells were infected with each recombinant virus at an MOI of 0.1. After 24 h the cells were detached with PBS containing 5 mM EDTA and centrifuged at $500 \times g$ for 5 min at 4 °C. Cells were then incubated with the anti-F_{Nterm} antiserum (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 immunoglobulin G antibodies. Cells were analyzed by using a FACSRIA II apparatus and Flowjo software (Becton Dickinson Biosciences).

3.4.7 Fusion assay and syncytia formation of N-glycosylation mutant viruses

The ability of each N-glycosylation mutant virus to form syncytia was determined according to a procedure described by Kohn (Kohn, 1965). 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% CO_2. Thirty six h
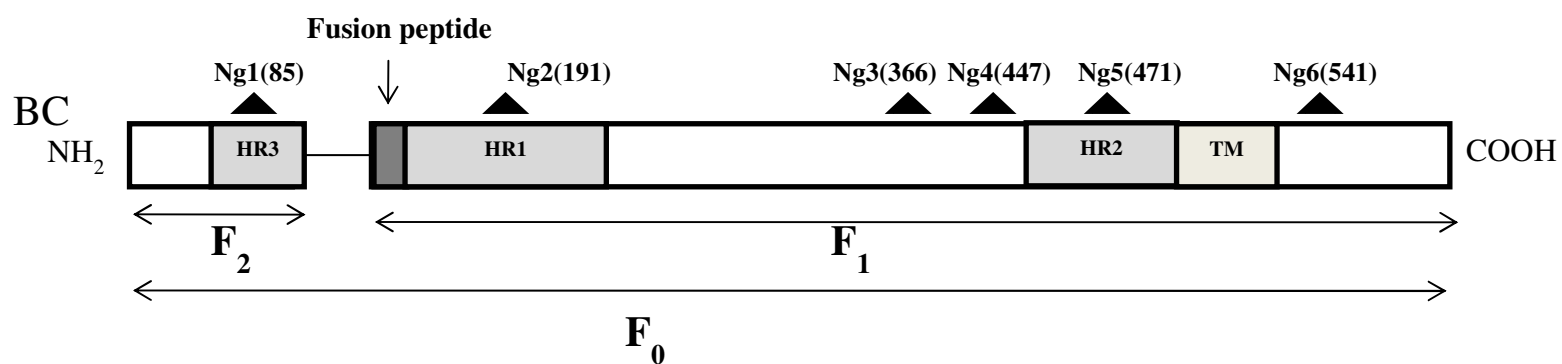
Figure 7. Linear diagram of the NDV F protein. ▲ indicates potential N-glycosylation sites Ng1 to Ng6, with the amino acid position of the Asn residue in parentheses. Grey boxes, heptad repeats; light box, transmembrane (TM) domain.

PI, 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).

3.4.8 Growth characteristics of N-glycosylation mutant viruses

The growth kinetics of N-glycosylation mutant viruses were evaluated by multiple-step growth assays. DF1 cells in duplicate wells of six-well plates were infected with each virus at an MOI of 0.01. After 1 hour of adsorption, the cells were washed with PBS and overlaid with DMEM containing 2% FBS at 37°C. Supernatant was collected and replaced with an equal volume of fresh medium every 8-h intervals until 64-h PI. The titer of virus in the sample was quantified by plaque assay on DF1 cells. All plaque assays were performed in six-well plates. Briefly, monolayers of DF1 cells were infected with 0.2 ml of 10-fold-diluted fresh virus infected allantoic fluid. After 1 h of adsorption, cells were covered with DMEM containing 2% FBS and 0.8% methylcellulose and then incubated at 37°C. Six days later, the cells were fixed with methanol and stained with crystal violet. The syncytia formation in DF1 cells was determined in duplicate wells of six-well plates infected with each virus at an MOI of 0.01. Cells were maintained in 5% DMEM at 37°C under 5% CO_2. Twenty four h PI 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.

3.4.9 Pathogenicity studies

The pathogenicity of the N-glycosylation mutant viruses was determined by mean death time (MDT) test in 9-day-old embryonated chicken eggs and the intracerebral pathogenicity index (ICPI) test in 1-day-old chicks (Alexander, 1997) and in 2-week-old chickens. The MDT is the mean time in hours for the minimum lethal dose to kill all inoculated embryos. For ICPI test, 0.05 ml (1:10 dilution) of fresh infective allantoic fluid of each virus was inoculated into groups of 10 1-day-old SPF chicks via the intracerebral route. The ICPI is the mean score per bird per observation over the 8-day period.

The pathogenicity of the F mutant viruses was further evaluated in 2-week-old chickens by a natural route of infection. Briefly, 2-week-old SPF chickens in groups of 10 were inoculated with 10^6 PFU (50µl in each nare and eye) of each virus per chicken via the occulonasal route. The birds were observed daily for clinical signs of disease until 14 days PI. In order to determine the replication efficiency of the mutant viruses, another 2-week-old chickens in groups of 5 were inoculated with 10^6 PFU of parental and each mutant virus per chicken via the occulonasal route. At 3 day PI, 3 birds from each group were sacrificed and organs (brain, nasal turbinate, lungs, and gut) were collected. The virus titers in these organs were determined by plaque assay in DF_1 cells. The extra 2 birds in each group were present to accommodate possible losses due to infection, which occurred in the rNg2+5

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group.

3.4.10 Measurement of humoral response of N-glycosylation mutant viruses by enzyme-linked immunosorbent assay (ELISA)

Four-week-old chickens in groups of 5 were inoculated with 10^6 PFU of wild type and N-glycosylation mutant viruses per bird via occulonasal route. Serum samples were collected on the 3rd, 7th and 14th days PI. Commercial NDV ELISA kits (Synbiotics Corporation, San Diego, CA) were used to detect antibodies against the NDV antigens. The assay was designed to measure NDV antibody bound to NDV whole antigen coated plates. Serum samples were diluted 1:100 in dilution buffer (Synbiotics Corporation) added to the plates, and incubated for 1 h at room temperature. The plates were washed three times with plate-washing solution (Synbiotics Corporation) and incubated for 1 h with an isotype-specific secondary antibody, namely, horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG. The plates were washed three times and developed with ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) peroxidase substrate solution (Synbiotics Corporation), development was stopped by the addition of peroxidase stop solution, and analysis was performed at 405 nm using an ELx800 ELISA plate reader (BioTek, Winooski, VT).

3.5 Results

3.5.1. Generation of recombinant NDVs containing mutations that eliminate potential N-glycosylation acceptor sites in the F protein

We investigated the role of N-linked glycosylation of the NDV F protein in viral biological activities and viral pathogenesis using a previously-described reverse genetics system for the mesogenic strain Beaudette C (BC) (Krishnamurthy et al., 2000). The NDV F protein has six potential acceptor N-glycosylation sites as indicated in Fig.7. Each of the six potential N-linked glycosylation sites at amino acid sequence positions 85, 191, 366, 447, 471, and 541 (Ng1-6, respectively) in the F protein was mutated by overlapping PCR to change asparagine, the first amino acid residue of the conserved sequence NXS/T, to glutamine. Glutamine was chosen because it is structurally similar to asparagine, differing by only a single methylene group. To make each mutation, the first and third positions (underlined) of the respective asparagine codon (<u>A</u>A<u>T</u> OR <u>A</u>A<u>C</u>, depending on the particular site) were substituted to create a codon for glutamine (<u>C</u>A<u>G</u>). Thus, each mutant would require two nucleotide changes in order to revert to any codon specifying asparagine, thereby reducing the likelihood of direct reversion during virus replication. A double N-glycosylation mutant also was created at positions Ng2 (191) and Ng5 (471) to examine the effect of combined loss of the two N-linked glycosylation sites in HR1 and HR2. The single-site mutants were designated Ng1, Ng2, Ng3, Ng4, Ng5, and Ng6, and the one double mutant was Ng2+5. The sequence of each mutant F gene was confirmed in the final cDNA clones. Recombinant viruses were recovered as described previously (Krishnamurthy et al., 2000). We were unable to recover a viable virus from the Ng6 cDNA in several attempts. To investigate this further, we

changed the glutamine to asparagine in Ng6 clone by using primer Ng7 forward and Ng7 reverse and found that infectious virus (Ng7 clone) could readily be recovered. This indicated that the inability to recover rNg6 virus was specific to that mutation, implying that it is severely debilitating or lethal. The presence of the introduced mutations in the mutant viruses was confirmed by RT-PCR and subsequent DNA sequence analysis of the F gene of each virus .

3.5.2 Determination of the N-glycosylation site usage in the NDV F protein

We examined the F proteins encoded by the N-glycosylation mutants to determine which of the potential N-linked glycosylation sites in the NDV-BC F protein were utilized. Vero cells were infected with wild type rBC and the N-glycosylation mutant viruses. The infected cell lysates were divided into two aliquots: one aliquot was left untreated and other was treated with PNGaseF, which cleaves high mannose and complex oligosaccharides from N-linked glycoproteins (Fig.8).The relative electrophoretic mobilities of the F proteins were examined by Western blot analysis in the presence of reducing agent by using rabbit antiserum raised against a synthetic peptide representing the NDV F cytoplasmic tail (anti-F_{cyt} antiserum). The sizes of the F_0 and F_1 proteins of wild-type rBC are 66 and 55KDAa, respectively. Our results showed that the F_0 protein was efficiently cleaved in wild type and all mutant viruses. In undigested cell lysates, mutation of single N-glycosylation sites resulted in faster electrophoretic migration of mutants F_1 protein compared to

the wild-type F_1 protein except rNg1, suggesting that each mutation resulted in the loss of an N-linked glycan (Fig. 8A and 8B). In case of rNg1 the N-glycan site at residue 85 is present in F_2 subunit, hence we observed similar migration pattern of rNg1 F_1 protein as that of rBC. In the double mutant, rNg2+5, the mobility shift of the F_0 and F_1 proteins was greater compared to that of F proteins of the single-site mutants, suggesting that N-glycans have been removed from both sites 191 and 471 (Fig.8B). The F proteins of mutants after treatment with PNGaseF co migrated with wild-type virus, supporting the interpretation that sites Ng1-5 are used in the NDV F protein for N-glycosylation, and that substitution of asparagine to glutamine at the respective sites prevented N-glycosylation.

3.5.3 Cell surface expression of F proteins encoded by the N-glycosylation mutant viruses

Cell surface expression of the F proteins of the N-glycosylation mutant viruses was quantified by flow cytometry. DF1 cells were infected with each of the mutant viruses and, 24 h PI; the cells were detached, treated with rabbit antiserum raised against a mixture of two synthetic peptides designed from the N-terminal region of the F protein (anti-F_{Nterm} antiserum), treated with Alexa Fluor-conjugated goat anti-rabbit antibodies, and analyzed by flow cytometry (Table 2).
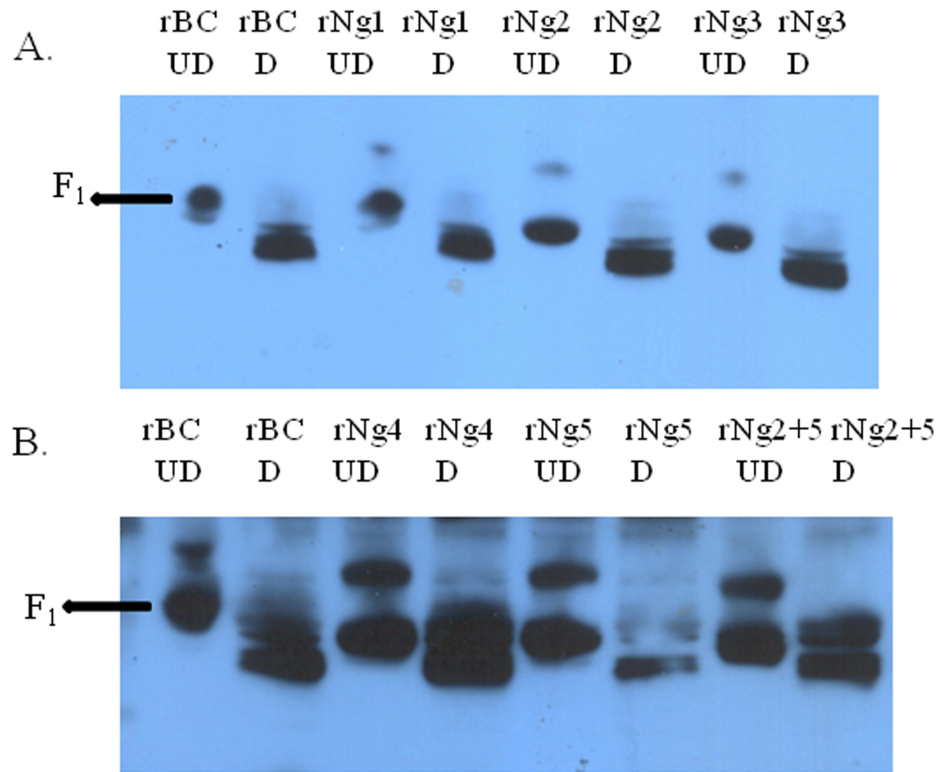
Figure 8. Analysis of the F proteins of the N-glycosylation mutants by Western blotting and PNGaseF digestion. Vero cells were infected with wild-type rBC and N-glycosylation mutant viruses at an MOI 1 and total proteins were collected after 36 h PI. Samples were resolved on 10% polyacrylamide gels in the presence of reducing agent (A) Samples from rBC, rNg1, rNg2, rNg3 (B) rBC, rNg4, rNg5, rNg2+5 were digested overnight with PNGaseF or kept as untreated controls, separated by 10% polyacrylamide gels in presence of reducing agent, and blotted onto nitrocellulose membrane. Western blot analysis was performed using a rabbit antiserum raised against a synthetic peptide designed from the F protein cytoplasmic tail. D: Digested;UD:Undigested.

The results showed that the percentages of cells expressing the different mutant F proteins were similar to that of the wild type rBC virus. The mean fluorescence intensities of rNg3, rNg4 and rNg2+5 ranged from 15% to 25% above the wild type rBC while cells infected with rNg1 and rNg2 had decreased mean fluorescence intensity of 5 and 7% lower than wild-type rBC. These results suggested that all the N-glycosylation mutant F proteins retained their ability to be transported efficiently to the cell surface.

3.5.4 Fusion activity of N-glycosylation mutant viruses

To determine the role of each N-glycan in the fusion activity of F protein, Vero cells were infected with the mutant viruses and at 36 h PI, the cells were fixed and stained with hematoxylin-eosin and examined microscopically to quantify the percentage of nuclei involved in syncytia as the fusion index. The fusion indices of the rNg1, rNg2, rNg3, rNg4, and rNg5 viruses were similar to that of the wild-type rBC virus (Fig. 9). Interestingly, the rNg2+5 double mutant virus exhibited a dramatically increased (>12-fold higher) fusion index compared to the parental rBC virus (Fig. 9). Thus, the individual elimination of N-linked glycosylation sites (including Ng2 and Ng5 in HR1 and HR2) did not significantly change the fusion activity of NDV F protein, but the dual loss of the sites in HR1 and HR2 in rNg2+5 resulted in a dramatic increase in fusion activity.

3.5.5 Growth characteristics of the N-glycosylation mutant viruses in cell culture

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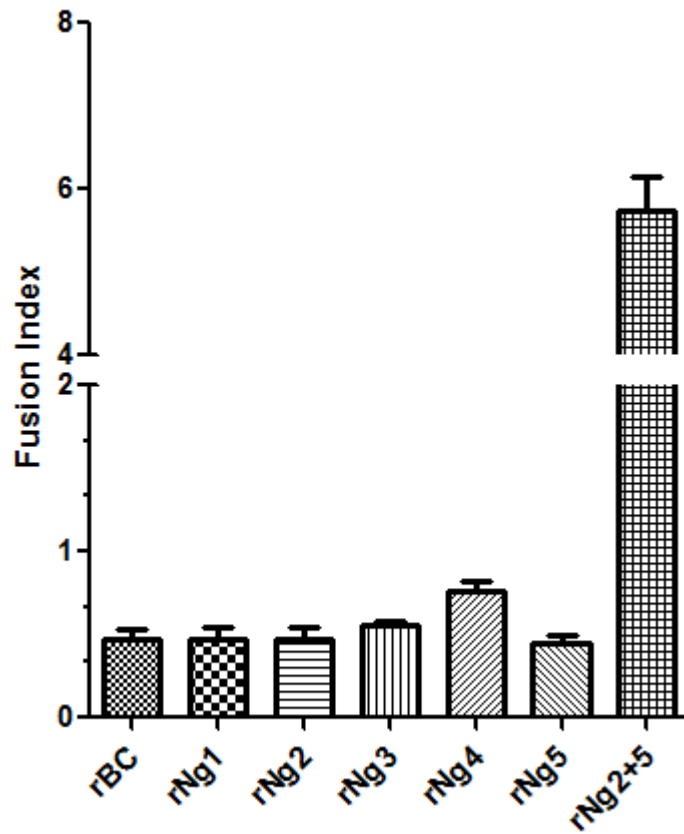
Figure 9. Comparison of the fusogenicity of wild-type rBC and the N-glycosylation mutant viruses. Vero cells were infected with the indicated viruses at an MOI of 0.1, fixed at 36 h.p.i, and stained with hematoxylin-eosin. The fusion index was calculated as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. Data were means from three independent experiments.
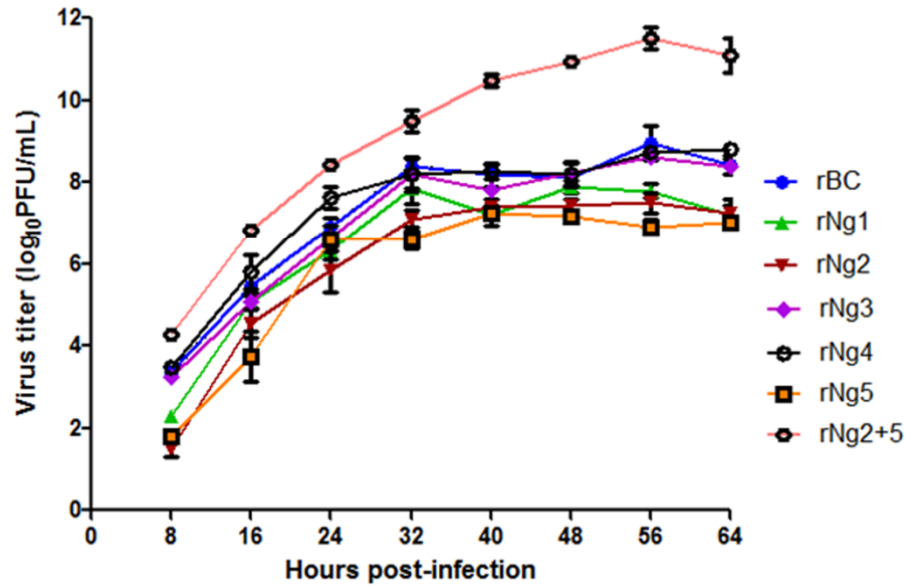
Figure 10A. Growth kinetics of viruses in tissue culture. (A) Comparison of multicycle growth kinetics of wild-type rBC and the N-glycosylation mutants viruses in DF1 chicken embryo fibroblast cells. Cells were infected with the indicated viruses at an MOI of 0.01 and cell culture media supernatant aliquots were harvested and replaced at 8 h intervals until 64 h PI. The virus titers in the aliquots were determined by plaque assay in DF1 cells. Values are averages from three independent experiments.

The *in vitro* replication of the wild-type and mutant viruses were compared in a multistep growth experiment in DF1 cells (Fig. 10A). Mutant viruses rNg1, rNg2, and rNg5 exhibited modestly delayed and reduced growth compared to the parental rBC virus: the yield of rNg1 was 0.5 $\log_{10}$ lower, whereas those of rNg2 and rNg5 were 1 $\log_{10}$ lower. In addition, syncytia formation by rNg1, rNg2, and rNg5 was evident by 36 h PI compared to 24 h PI for wild-type rBC in DF1 cells. For mutant viruses rNg3 and rNg4, there was no significant difference in growth kinetics or syncytia formation compared to wild-type rBC. Interestingly, the double mutant virus rNg2+5 replicated faster and attained a much higher titer than the wild-type virus. Specifically, at 64 h PI, the titer of mutant virus rNg2+5 was 2.5 $\log_{10}$ higher than that of the wild-type virus. Furthermore, the rNg2+5 virus initiated syncytia formation at 18 h PI compared to 24 h PI for the wild-type virus (Fig. 10 B). These results demonstrated that the individual elimination of Ng1, Ng2, and Ng5 of the NDV F protein decreased the replication of the virus to various extents, whereas the combination of two of these sites, Ng2 and Ng5, in the double mutant rNg2+5 strongly increased virus replication.

3.5.6 Pathogenicity of the N-glycosylation mutant viruses in chicken eggs and 1-day old chicks

The pathogenicity of the N-glycosylation mutant viruses and their wild-type rBC parent were evaluated by 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
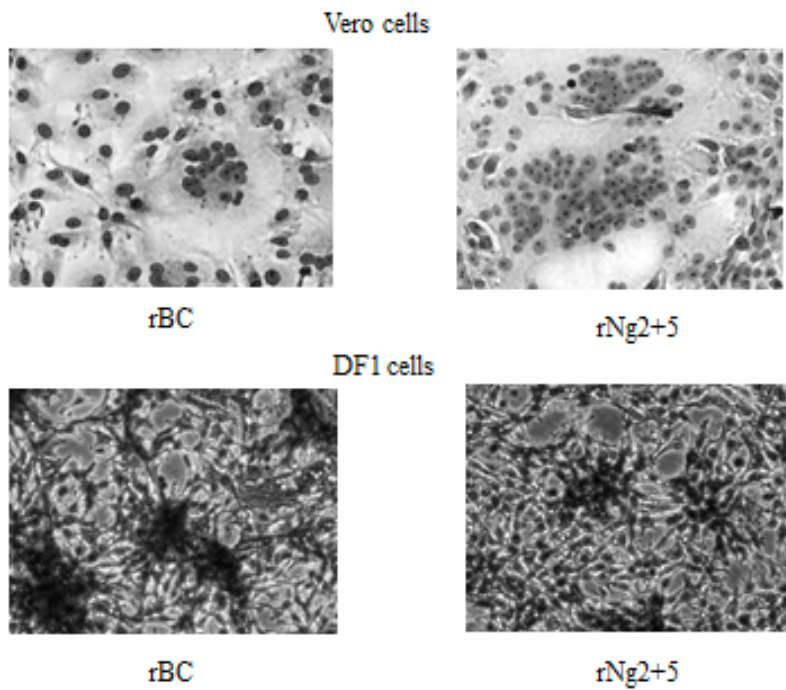
Figure 10B. Syncytia formation of rBC and rNg2+5 in Vero and DF1 cells. Vero and DF1 cells were infected in duplicate wells of six-well plates with each virus at an MOI of 0.01. Cells were maintained in 5% DMEM at 37°C under 5% CO_2. Twenty four h PI the medium was removed and the cells were washed with PBS, fixed with methanol for 20 min at room temperature, and stained with hematoxylin-eosin.

Table 2. Cell surface expression of the F proteins of the N-glycosylation mutant viruses

Viruses	% of positive cells ± SD	Relative mean fluorescence intensity
rBC	99±2.4	1.00
rNg1	98±3.8	0.95
rNg2	99±4.2	0.93
rNg3	96±3.1	1.15
rNg4	97±2.2	1.20
rNg5	99±1.8	1.00
rNg2+5	99±3.0	1.25
None (Mock infected cells)		0.01

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 24 hr PI with a cocktail of anti-F_{Nterm} antibody 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 3. Pathogenicity of the N-glycosylation mutant viruses in embryonated eggs and chicks

Viruses	MDT[a]	ICPI score[b]
rBC	59	1.52
rNg1	76	1.16
rNg2	78	1.12
rNg3	60	1.42
rNg4	56	1.48
rNg5	68	1.30
rNg2+5	51	1.88

[a] 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.

[b] Intracerebral pathogenicity index (ICPI). ICPI score= [(total number of sick chicks x1) + (total number of dead chicks 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. $P<0.05$

embryonated chicken eggs (Table 3). 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 values of rNg1 (76 h) and rNg2 (78 h) were increased compared to that of the wild type rBC parent (59 h), indicating a modest reduction in virulence. The rNg3, rNg4, and rNg5 viruses had MDT values of 60 h, 56 h, and 68 h, respectively, which were marginally increased (i.e., attenuated) compared to wild-type rBC. In contrast, the MDT value of the rNg2+5 double mutant was 51 h, indicating an increase in virulence compared to wild-type rBC. We also evaluated the pathogenicity of the recombinant viruses in 1-day-old chicks by the ICPI test. Velogenic strains give values approaching 2.0, whereas lentogenic strains give values close to 0. The ICPI values of the parental rBC, rNg1, rNg2, rNg3, rNg4, rNg5, and rNg2+5 viruses were 1.52, 1.16, 1.12, 1.42, 1.48, 1.30, and 1.88 respectively (Table 3) Thus, the results of the ICPI test were consistent with the results of MDT test: specifically, the rNg1 and rNg2 mutants were the most attenuated, followed by the rNg5 mutant, and the rNg3 and rNG4 mutants were the least attenuated, compared to the wild-type rBC parent. In contrast, the rNg2+5 mutant was more virulent than rBC.

3.5.7 Replication and virulence of the N-glycosylation mutant viruses in 2-week-old chickens

We examined the effect of mutations of the N-glycosylation sites of the F protein on replication and virulence in 2-week-old chickens. Chickens in groups of 5 were inoculated by the oculonasal route (mimicking natural

infection) at a dose of 10^6 PFU per bird. Three chickens from each group were euthanized on the 3rd day PI and tissue samples of the brain, lung, nasal turbinates, and gut were collected. All of the birds appeared to be healthy on the 3rd day PI except in case of birds infected with the rNg2+5 virus, where two birds were found dead and one paralyzed on the 3rd day PI (tissues were taken only from the 3 living birds). Virus titers in tissue samples were measured by plaque assay using DF1 cells (Fig. 11A). Differences in the presence of virus and in the virus titers in the different organs were observed between wild-type rBC and the N-glycosylation mutant viruses. The rNg1 mutant virus was not detected in the brain and gut, and its titers in the lung and nasal turbinate tissue was reduced by ~50% compared to those of wild type rBC. The rNg2 mutant virus was not detected in gut and there was 43%, 64% and 67% reduction in titers in the lung, nasal turbinates and brain compared to those of wild type rBC virus. The rNg3 virus replicated at similar titers in the brain, lungs and nasal turbinates compared to the wild type virus, whereas the rNg5 virus was somewhat reduced and was not detected in gut. The rNg4 virus replicated to 7% (nasal turbinates), 14% (lungs), 33 % (brain) to 62% (gut) higher titers as compared to wild-type rBC. The double mutant rNg2+5 virus replicated to significant higher titers in all of the sampled organs compared to the rBC parent: specifically, ~3 logs higher titer in the gut and brain , 65% and 33% greater in the lungs and nasal turbinates, respectively. These results are consistent with the MDT and ICPI tests, showing that, compared to wild-type rBC, rNg1 and rNg2 were the most attenuated,

followed by rNg5, whereas rNg2+5 exhibited increased replication. We also examined the effects of the N-glycosylation mutations on morbidity and mortality. Two-week-old chickens in groups of 10 were inoculated with each mutant virus via the oculonasal route with 10^6 PFU of virus per bird and were observed for 14 days for clinical signs (Fig. 11B). The birds inoculated with wild-type rBC showed clinical signs of depression, watery greenish diarrhea, drooping wings by the 9th day PI. The groups of birds infected with the single-site N-glycosylation mutant viruses remained normal throughout the observation period. In contrast, birds inoculated with the double mutant rNg2+5 virus first exhibited signs of sickness and paralysis at 2 day PI, and deaths were observed beginning at day 3 PI. By the 6th day PI, all 10 birds inoculated with the double mutant rNg2+5 virus had died (Fig. 11B).

3.5.8 Host immune responses following N-glycosylation mutant virus infection

N-glycosylation of viral proteins can influence immunogenicity. In order to examine the effect of loss of N-linked glycans from the F protein on the immune response to NDV, 4-week-old chickens in groups of 5 were inoculated via the occulonasal route with 10^6 PFU per bird of wild type and mutant viruses. Sera were collected on days 3, 7, and 14 PI and antibody levels were measured by an NDV-specific ELISA. As shown in Fig. 12, there were no significant differences in the total NDV-specific serum antibody responses elicited by wild type rBC or the mutant viruses on the 3rd PI. On the 7th day PI, chickens inoculated with the rNg2+5 virus
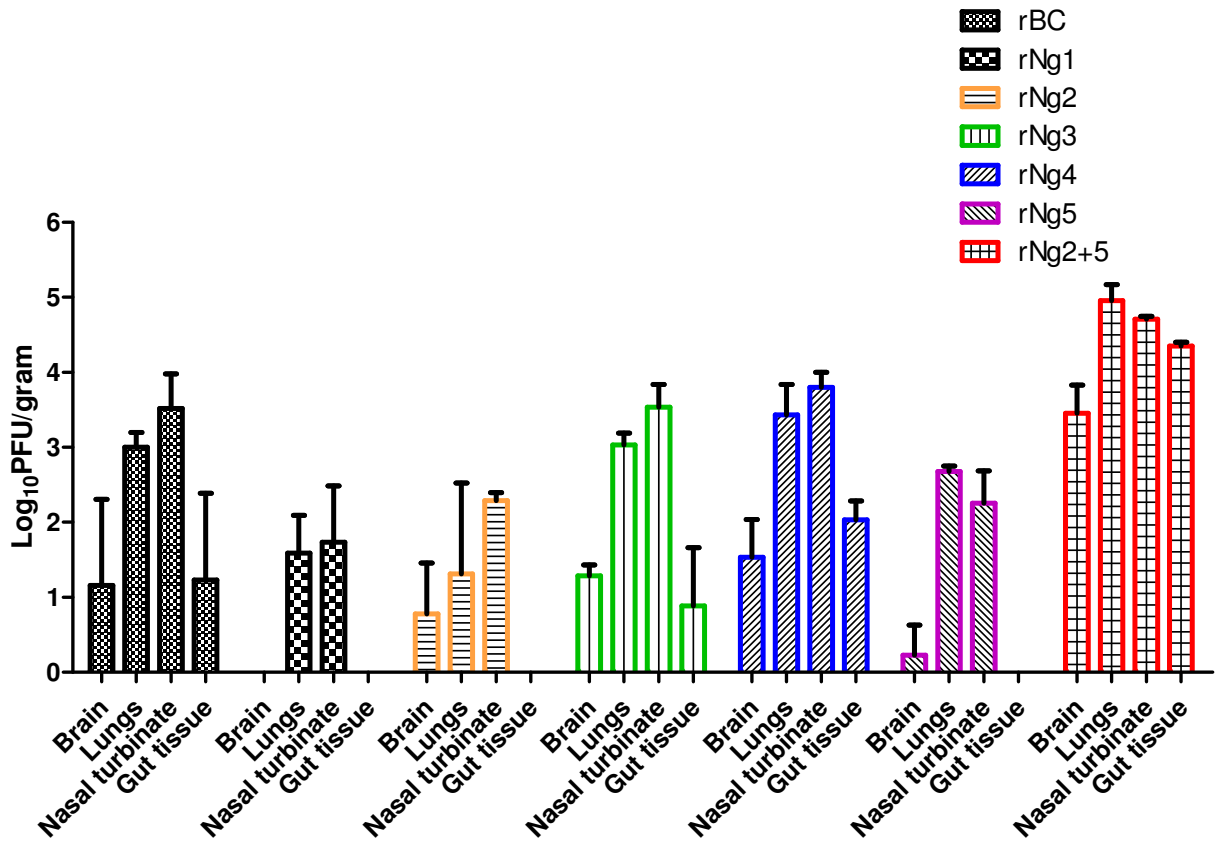
Figure 11A. Virus replication in the indicated organs. Two-week-old chickens in groups of 5 were inoculated with 10^6 PFU of virus per bird by the oculonasal route, mimicking natural infection. Three chickens per group were sacrificed 3 d.p.i and samples of the brain, lungs, trachea and gut were collected (more birds were inoculated than were sacrificed to allow for attrition). Virus titers were determined by plaque assay in DF1 cells. Values were averages from the results of three independent plaque assay experiments. * ($p < 0.05$).
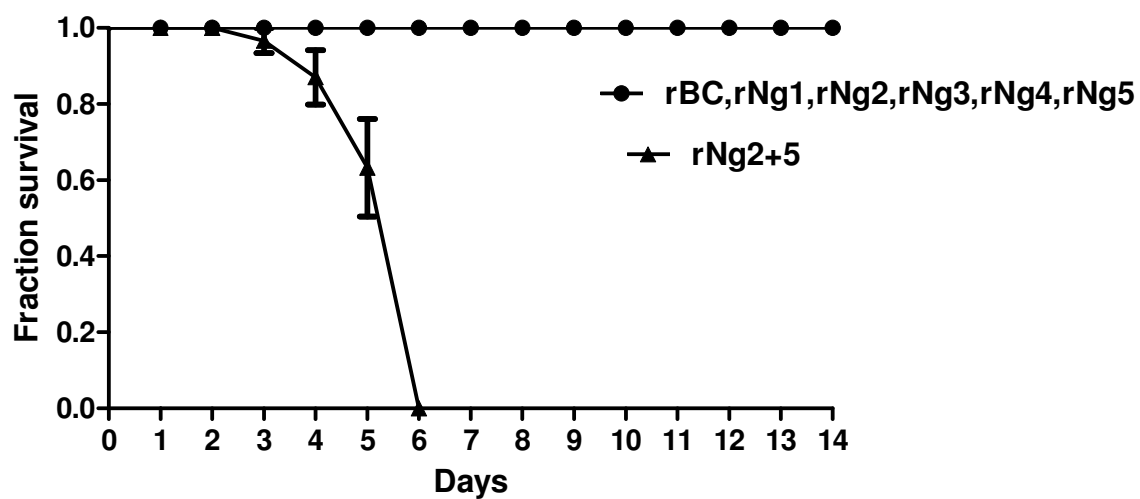
Figure 11B. Two-week-old chickens in groups of 10 were inoculated with 10^6 PFU of virus per bird and observed for 10 days for signs of disease and for mortality. Note that all of the birds survived in the rBC, rNg1, rNg2, rNg3, rNg4, rNg5 groups.

exhibited slightly higher antibody titers than the other groups, and on the 14th day PI there was a significantly higher mean antibody titer in the rNg2+5 group as compared to the other groups including the wild-type rBC group. This correlates with our earlier findings that increased replication of mutant virus rNg2+5 resulted in enhanced antibody production.

3.6. Discussion

N-glycosylation has been shown to have a key role in viral glycoprotein folding, proteolytic processing, and function, and also has been shown to influence viral infectivity, tropism and the immune response (Aguilar et al., 2006; Eichler et al., 2006; Goffard and Dubuisson, 2003; Lin et al., 2003; McGinnes et al., 2001; Oostra et al., 2006; Panda et al., 2004a). In the present study, we examined the role of N-glycosylation of the NDV F glycoprotein in the context of infectious virus using reverse genetics to construct mutants that were then analyzed in cell culture and, importantly, in the natural chicken host using standard pathogenicity tests in eggs and 1-day-old chicks, as well as inoculation of 2-week-old and 4-week-old chickens by the oculonasal route to mimic natural infection. Analysis of N-glycosylation site usage in NDV-BC F protein showed that five of the six potential N-glycosylation acceptor sites are utilized, one in the F_2 subunit and four in the F_1 subunit. Previously, it was reported that the sixth N-glycosylation site of NDV strain AV, which corresponds to site 541 (Ng6) in NDV-BC and is located in the cytoplasmic domain in both strains, was not utilized as an acceptor site for N-linked glycosylation (McGinnes et al., 2001). In the present study, we were unable to recover virus bearing the N541Q mutation, and the deleterious effect of this mutation was confirmed by the ability to rescue virus in
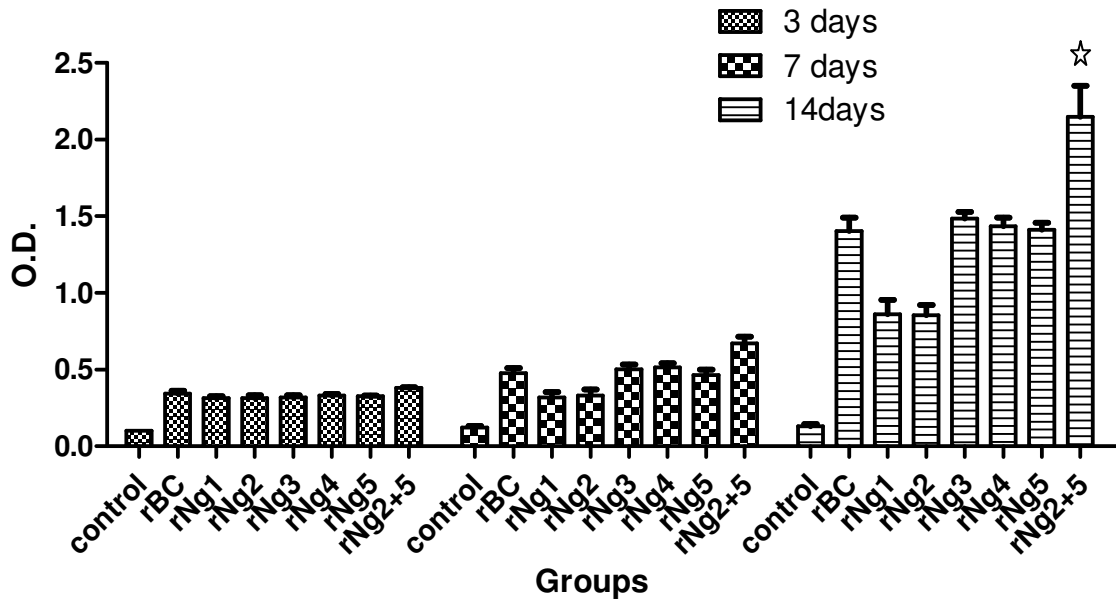
Figure 12. NDV-specific serum antibody responses in chickens infected with wild-type rBC and the N-glycosylation mutants. Four-week-old chickens in groups of 5 were inoculated with 10^6 PFU of virus per bird via the occulonasal route. Sera were collected on days 3, 7, and 14 PI and analyzed by an NDV-specific ELISA assay. Data represent mean absorbance values ± standard deviation. .☆The mean value for the rNg2+5 group was taken for 3 animals because 2 birds died by day 14 and were not included in the analysis. *P* values were <0.0001

which the assignment had been restored to that of wild-type virus. Taken together, these data suggests that this site probably does not normally contain a sugar side chain, which would be consistent with its location in the cytoplasmic domain, but that the assignment of N at position 541 is important for some function(s) of the cytoplasmic tail that is essential for virus replication. It was of particular interest that both previous and present studies indicated that sites Ng2 and Ng5 are utilized for N-glycosylation, since these sites are located in HR1 and HR2, respectively, and thus have the potential to affect fusion.

One of the important functions of N-glycans in glycoproteins is to facilitate protein processing and folding (Ellgaard, 1999). Our results showed that deletion of single or multiple N-glycans in the F glycoprotein in the context of whole virus had little effect on cell surface expression of the F protein. The results from the present study are similar to the findings reported previously for the respiratory syncytial virus F protein, where deletion of multiple N-glycans did not affect F protein transportation to the cell surface (Collins and Mottet, 1991; Zimmer et al., 2001). These results suggest that no single N-glycan (nor Ng2 and Ng5 together) was essential for NDV-BC F protein transport to the cell surface. In addition, none of the mutations significantly affected the efficiency of F protein cleavage. Biophysical and crystallographic studies of several paramyxovirus F proteins have revealed that the F protein is present in the virus particle in a metastable state that, upon viral contact with the target cell, undergoes a conformational shift to insert the fusion peptide present at the N-terminus of the F1 subunit into the target membrane. The F protein then make the transition to a post-fusion structure driven by association of HR1 with HR2, which brings the transmembrane domain and fusion peptide into close proximity resulting in the merger of viral and host cell membranes. Thus, one of the important phases of the fusion process is the

formation of coiled coil triple strand by the HRs, which is common structural features of the paramyxovirus, orthomyxovirus and retrovirus fusion proteins (Joshi et al., 1998; Lamb, 1993a; Matthews et al., 2000). The presence of N-glycan side chains in HR1 and HR2 thus might influence the conformational changes involved in the fusion process. Our fusion index assay of single N-glycan mutants showed that there were no major differences in fusion activity as compared to wild-type rBC. Interestingly, in the present study, while the individual removal of the N-linked site from HR1 or HR2 had little effect on fusion, the removal of both sites in rNg2+5 mutant virus resulted in hyperfusogenic phenotype. This result has some similarity with a previous study with Nipah virus in which the removal of multiple glycans from the Nipah virus F protein resulted in a hyperfusogenic phenotype (Aguilar et al., 2006). It was suggested that presence of N-glycans on the Nipah virus F protein decreases the rate of six-helix bundle formation, resulting in slower fusion kinetics. Similarly, for NDV, it may be that the presence of the N-linked glycans in HR1 and HR2 delays or otherwise reduces association between HR1 and HR2, resulting in reduced fusion. The more interesting finding was that, while the single mutations at sites Ng2 and Ng5 were somewhat inhibitory to growth individually *in vitro*, in combination they resulted in more rapid growth and a dramatic 2.5 $\log_{10}$ increase in viral titer. This presumably was primarily due to the increase in fusion, which may increase the efficiency and rate of infection as well as cell-to-cell spread by fusion. Evaluation of the pathogenicity of the N-glycan mutants *in vivo* provided results consistent with the *in vitro* growth study. The rNg1 and rNg2 mutants (and, to a lesser extent, the rNg5 mutant) were the most attenuated based on the standard MDT and ICPI tests. Similarly, in 2-week old chickens, the rNg1, rNg2, and rNg5 mutants were the most attenuated based on tissue tropism and the magnitude of virus replication.

Interestingly, while the Ng2 and Ng5 mutations were modestly attenuating on their own, when added together they resulted in a virus that replicated to substantially higher titers in every sampled tissue, was more virulent in the MDT and ICPI assays, and converted NDV-BC from a non-lethal virus into one that killed all of the inoculated 2-week-old chickens within 6 days. A similar observation was reported earlier in neurovirulent influenza virus strain A/WSN/33 in mice (Li S, 1993 Nov; Ward AC, 1995) and H5N2 influenza virus in chickens (Kawaoka et al., 1984): in both cases, loss of carbohydrate from HA gene increased the virulence of the virus. In the case of influenza virus, a suggested mechanism for this increased virulence was that the loss of carbohydrate resulted in improved accessibility of the receptor-binding site to cellular receptors. In the case of NDV, in which the attachment function is on the HN protein, the situation is probably different. Instead, this effect probably reflects the increased fusogenic nature of the F protein, which likely increases the efficiency and rate of infection in the various tissues.

The oligosaccharide chains on the glycoproteins of many viruses play important role in altering immune responses. They may form a barrier that shields viruses from immune recognition (Lee et al., 2003; Li et al., 2008; Wei, 2003). Conversely, deletion of some N-glycans in the glycoprotein of human immunodeficiency virus abrogated the *in vivo* priming of T cell recognition for a nearby epitope, indicating that carbohydrate side chains also can increase immunogenicity (Sjolander et al., 1996). Our study showed that the double mutant rNg2+5 virus elicited the highest NDV-specific serum antibody response whereas the attenuated rNg1 and rNg2 viruses had the lowest antibody responses, as measured by ELISA. We did not extend the study beyond 14 days PI because of the morbidity and mortality of the chickens in the rNg2+5 group. It seems likely that the increased immune response was

primarily due to the increased level of viral replication, which provided more antigenic stimulation. It is also possible that other factors are involved, such as greater exposure of epitopes due the loss of shielding glycans, or improved antigen processing. This might be investigated in further work by comparing the immunogenicity of the rNg2+5 mutant versus wild-type BC using UV-inactivated virus, where differences in viral replication would not be a factor.

In summary, the present study demonstrates the impact of N-glycosylation of the F protein on NDV pathogenesis and virulence in chickens. The most striking finding was that, whereas the individual removal of sites Ng2 and Ng5 in HR1 and HR2 was modestly attenuating, removal of both in combination resulted in a hyperfusogenic phenotype that was associated with increased replication *in vitro* and *in vivo* and converted a mesogenic strain into a velogenic strain. The simplest explanation is that the presence of N-glycans on both HR1 and HR2 normally impedes the conformational shifts in the F protein during the fusion process, and thus in effect reduces the efficiency of fusion. The crystal structure of NDV F published so far does not give a clear view of how the N-glycans have been decorated in the F protein. It is difficult to interpret whether N-glycans orientation is affecting at all the fusion process or N-glycans are responsible for any structural instability from pre-fusion to post-fusion state thus maintaining different energy state or they are interacting with some other host molecules. Previously, a hyperfusogenic phenotype also was observed with mutants of the F protein of parainfluenza virus 5 (previously called simian virus 5) in which the fusion peptide was modified by glycine-to-alanine substitutions, suggesting that the native glycine residues serve to reduce the efficiency of fusion (Horvath and Lamb, 1992). Wild-type Sendai virus also down-regulates its fusion activity, in this case due to a difference

in a transcription gene start signal that results in reduced F gene transcription and protein expression; and correction of this difference results in a virus that replicates more efficiently and is more lethal (Kato et al., 1999). A number of other paramyxoviruses also down-regulate F expression (Bousse et al., 2002; Rassa and Parks, 1998; Spriggs and Collins, 1986).The idea that paramyxoviruses contain structural elements that reduce the efficiency of fusion suggests that this is advantageous to the virus. The present study, as well as the previous study with Sendai virus, indicates that a hyperfusogenic phenotype can be associated with increased virulence and rapid death. It may be that reducing the severity of disease and prolonging the survival of the infected host may increase the opportunity for viral spread.

Chapter 4

4.1 Title

Role of conserved glutamine residue in the NDV F protein fusion cleavage site. (Samal et al., 2011)

4.2 Abstract

A key determinant of Newcastle disease virus (NDV) virulence is the amino acid sequence at the fusion protein (F) cleavage site. The NDV F protein is synthesized as an inactive precursor F_0 and is activated by proteolytic cleavage between amino acid positions 116 and 117 into two disulfide-linked subunits, F_1 and F_2. The consensus sequence of F protein cleavage site of virulent [^{112}(R/K)-R-Q-(R/K)-R↓F-I^{118}] and avirulent [^{112}(G/E)-(K/R)-Q-(G/E)-R↓L-I^{118}] strains contains a conserved glutamine residue (Q) 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-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 replication and pathogenicity of NDV using moderately pathogenic Beaudette C (BC) strain, by replacing Q114R, K115R, and I118V. Our results showed that change of glutamine to basic residue arginine (R) reduced the viral replication and attenuated the virus

pathogenicity in chickens. The pathogenicity was further reduced when isoleucine (I) at position 118 was substituted by valine.

4.3. Introduction

Newcastle disease virus (NDV) causes a highly contagious disease in chickens resulting in severe economic losses to the poultry industry worldwide (Alexander, 1989.). NDV is a prototype member of family *Paramyxoviridae* which belongs to genus *Avulavirus* (Lamb, 2007). NDV isolates can be differentiated into three clinicopathologic groups based on their pathogenicity in chickens; low virulent (lentogenic), moderately virulent (mesogenic) and highly virulent (velogenic) (Alexander, 1997). The envelope of NDV contains two transmembrane glycoproteins, the hemagglutunin-neuraminidase (HN) protein and the fusion (F) protein. The HN protein is involved in attachment to host cell sialic acid receptor and release of the virus and the F protein mediates fusion of the virion envelope with the host cell plasma membrane (Lamb, 2007). The NDV F protein is synthesized as an inactive precursor F_0 and is activated by proteolytic cleavage into two disulfide-linked subunits, F_1 and F_2. The amino acid sequence at the F protein cleavage site determines the substrate specificity for different types of cellular proteases (Kawahara et al., 1992). The F protein cleavage site sequence has been shown to be a major determinant of NDV virulence (Klenk, 1994.; Lamb and Jardetzky, 2007; Nagai et al., 1976; Panda et al., 2004b; Wakamatsu et al., 2006). The consensus sequence of the F protein cleavage site of virulent strains is ^{112}(R/K)-R-Q-(R/K)-R↓F-I^{118}, whereas the consensus sequence of the F protein cleavage site of avirulent strains is ^{112}(G/E)-(K/R)-Q-(G/E)-R↓L-I^{118}. The F protein cleavage site of virulent strains contains polybasic amino acids that are the preferred recognition site for furin R-X-(R/K)-R↓F, which is an intracellular protease that is present in most cell types.

This provides for efficient cleavage of F protein in a wide range of tissues, making it possible for virulent strains to spread systemically, resulting in fatal infection (Murakami, 2001.; Nagai et al., 1976; Ogasawara, 1992.). In contrast, avirulent NDV strains have one or two basic residues at the -1 and -4 positions relative to the cleavage site. These cleavage sequences are insensitive to intracellular proteases and depend on extra-cellular secretory proteases for cleavage. It limits the replication of avirulent strains to the respiratory and enteric tracts (de Leeuw et al., 2003; Klenk, 1994.; Panda et al., 2004b). The individual amino acids at the F protein cleavage site have been examined for their requirement to virulence. It was found that phenylalanine (F) at position 117, arginine (R) at 116, lysine (K) or R at 115 and R at 113 are required for virulence of NDV (de Leeuw et al., 2003). Interestingly, glutamine (Q) is present at position 114 of F protein in both avirulent and virulent strains but its role in NDV pathogenesis has not been evaluated (de Leeuw and Peeters, 1999; Paldurai et al.; Peeters et al., 1999). Recently some NDV strains from Africa and Madagascar were 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 (V) at position 118 instead of isoleucine (I) at fusion cleavage site, which is present in most virulent and avirulent NDV strains. This finding does not agree with our current understanding 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 Q 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.

4.4. Materials and Methods

4.4.1. Cells and viruses.

Chicken embryo fibroblast cell line (DF1) and human epidermoid carcinoma cell line (HEp-2) 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 cells were 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 (NIH, Bethesda, MD) and propagated in primary chicken embryo fibroblast cells in DMEM with 2% FBS. The moderately pathogenic (mesogenic) NDV strain Beaudette C (BC) and its recombinant 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 (Krishnamurthy et al., 2000).

4.4.2 Construction of plasmids and recovery of mutant viruses

The construction of plasmid pNDVfl carrying the full length antigenome cDNA of the NDV strain BC has been described previously (Krishnamurthy et al., 2000). In the present study, four NDV F cleavage site mutation clones were constructed as described in Table 4. 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 of full length cDNA clone on BC antigenome was then replaced with each mutagenized 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 as rNDV-Q114R, I118V (^{112}R-R-R-K-R↓F-V^{118}), rNDV-Q114R, K115R, I118V (^{112}R-R-R-R-R↓F-V^{118}). rNDV-Q114R (^{112}R-R-R-K-R↓F-I^{118}),rNDV-Q114R, K115R (^{112}R-R-R-R-R↓F-I^{118}) and The F genes from recovered viruses were sequenced which confirmed the presence of each introduced mutation and the lack of adventitious mutations in the F gene. To determine the stability of each F mutation, 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. All infectious virus research was performed in our USDA approved enhanced biosafety level 3 (BSL-3+) containment facility.

4.4.3 Surface expression of mutant F proteins

The surface expression of F protein of each mutant virus was determined by Flow cytometry in infected chicken embryo fibroblast (DF1) cells and was found similar to that of wild type BC virus (rNDV) (Table. 4). Briefly, DF$_1$ cells were infected with each recombinant virus at an MOI of 0.1. After 24 h the cells were detached with PBS containing 5 mM EDTA and centrifuged at $500 \times g$ for 5 min at 4 °C. Cells were then incubated with the

NDV anti-F$_{Nterm}$ specific antibodies (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 immunoglobulin G antibodies. Cells were analyzed by using a FACSRIA II apparatus and Flowjo software (Becton Dickinson Biosciences).

4.4.4 Pulse-chase experiment

DF1 cells were infected at a MOI of 10 for 24 h at 37^0C. Cells were washed and incubated in medium lacking methionine and cysteine for 1 h. Infected cells were pulse labeled with 100µCi of EXPRESS^{35}S (Perkin Elmer) for 30 min and then chased in nonradioactive medium containing excess methionine and cysteine for 0, 30, 60 and 90 min. Equal amounts of cell lysates were immunoprecipitated with polyclonal antiserum against the cytoplasmic tail of NDV F protein followed by incubation with *Staphylococcus aureus* protein A. The precipitated proteins were analyzed by 10% SDS-PAGE in the presence of reducing agent and labeled proteins were visualized by autoradiography.

4.4.5. Growth kinetics of mutant viruses

The growth kinetics of mutant viruses were evaluated by multiple-step growth assays. DF1 cells in duplicate wells of six-well plates were infected with each virus at an MOI of 0.01. After 1 hour of adsorption, the cells were washed with PBS and overlaid with DMEM containing 2% FBS at 37°C. Supernatant was collected and replaced with an equal volume of fresh medium every 8-h intervals until 64-h PI. The titer of virus in the sample was

quantified by plaque assay on DF1 cells. All plaque assays were performed in six-well plates. Briefly, monolayers of DF1 cells were infected with 0.2 ml of 10-fold-diluted fresh virus infected allantoic fluid. After 1 h of adsorption, cells were covered with DMEM containing 2% FBS and 0.8% methylcellulose and then incubated at 37°C. Six days later, the cells were fixed with methanol and stained with crystal violet.

4.4.6 Pathogenicity studies

The pathogenicity of the N-glycosylation mutant viruses was determined by the intracerebral pathogenicity index (ICPI) test in 1-day-old chicks (Alexander, 1997), intracerebral growth kinetics in 1 day-old-chickens and by natural route of infection in 1-day-old chicks. For growth kinetics in brain of groups of 10 one-day-old chicks were inoculated intracerebrally with 10^3 PFU of virus/chick. Two birds were sacrificed each day after infection; brains were collected and homogenized, and virus titrated by plaque assay in DF1 cells. For pathogenicity test of wild type and F protein cleavage site mutants of NDV in one-day-old chicks inoculated via intra-nasal and intra-ocular route. Groups of five one-day old chicks were inoculated with 10^6 PFU of virus per bird and observed daily for signs of disease and mortality for 8 days.

4.5 Results

4.5.1 Generation of recombinant NDV mutants containing cleavage site mutations.

Previously, recovery of recombinant NDV from an infectious cDNA clone (pNDVfl) derived from a mesogenic strain of NDV, BC, was reported from our laboratory (Krishnamurthy et al., 2000). In this study, the established reverse genetics system was used to determine the role of conserved glutamine on the biological activities of NDV. To achieve this goal, the PacI-MluI subclone containing the F gene derived from the full-length clone of BC (pNDVfl) was mutated by site-directed mutagenesis as described in Table.4. To ensure the presence of the introduced mutations, the entire F cDNA clone was sequenced. Recombinant viruses expressing wild-type and mutant viruses were recovered by transfection of HEp-2 cells with full-length mutant F cDNA clones and support plasmids and amplification of viruses in DF1 cells. Recovered viruses were subjected to RT-PCR, and the F genes were sequenced in their entirety to confirm the presence of the introduced mutations. To determine the stability of each F mutation, the recovered viruses were passaged five times in 9- to 11-day-old embryonated chicken eggs and the sequence of the F gene was determined in viruses recovered at each passage level. These sequence analyses showed that the introduced HN mutations were unaltered, even after five egg passages.

4.5.2 Cell surface expression and cleavage processivity of the F proteins of mutant viruses

The surface expression of F protein of each mutant virus was determined by Flow cytometry in infected chicken embryo fibroblast (DF1) cells and was found similar to that of wild type BC virus (rNDV) (Table. 4). The cell surface expressions of the mutant viruses were found to be similar to that of the wild type viruses.

To determine the F protein processivity by intracellular host cell proteases, we infected the DF1 cells with rNDV and cleavage site mutants for 24 h at a MOI of 10. The infected cells were labeled with a mixture of $[^{35}S]$ methionine and $[^{35}S]$ 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 10% SDS-PAGE and subjected to autoradiography (Fig.13A & 13B). The pulse-chase analysis revealed that after 30 min pulse both uncleaved precursor F_0 and cleaved F_1 were detected in both wild type and mutant viruses (0 min chase). In case of wild type rNDV, after 60 min of chase period all the F_0 proteins were processed completely (100%) into F_1-F_2 subunit. In contrast, F_0 proteins of cleavage site mutants remained incompletely cleaved even after 90 min chase, suggesting slow processivity of the cleavage site mutants F_0 protein by host cell proteases.

4.5.3 Cleavage site mutants showed slower growth rate in DF1 cells

To determine the *in vitro* growth characteristics of recombinant F mutant viruses, we performed mutlicycle growth kinetics in DF1 cells. The

Table 4. NDV F protein cleavage site mutants, surface expression of F protein cleavage site mutants of NDV, pathogenicity of F protein cleavage site mutants of NDV.

Viruses	Amino acid sequences at the cleavage site of F protein▲ 112 113 114 115 116↓117 118							Cell surface expression£	ICPI score▼
rNDV	R	R	Q	K	R	F	I	100.00	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 ± 1.2	1.36

▲Location of F protein cleavage site mutations. Shown at the top is the wild type mesogenic strain BC (rNDV) virulent cleavage site amino acids position. The amino acid changes in the mutants are in boldface and underlined. The mutant viruses generated from rNDV are (rNDV-Q114R,I118V), (rNDV-Q114R,K115R,I118V), (rNDV-Q114R), (rNDV-Q114R,K115R). Cleavage site is indicated as arrow.

£ Shown are the cell surface expression levels of F protein of cleavage site mutants relative to the level of the rNDV . Expression of the F protein was quantitated by flow cytometry using NDV-F_{Nterm} specific antibodies. All values are averages ± standard deviations of three independent experiments P<0.05.

▼The virulence of the mutant and wild type viruses was evaluated by ICPI in 10 1-day-old chicks. ICPI score= [(total number of sick chickens x1) + (total number of dead chickens x 2)]/80 observations. P<0.05
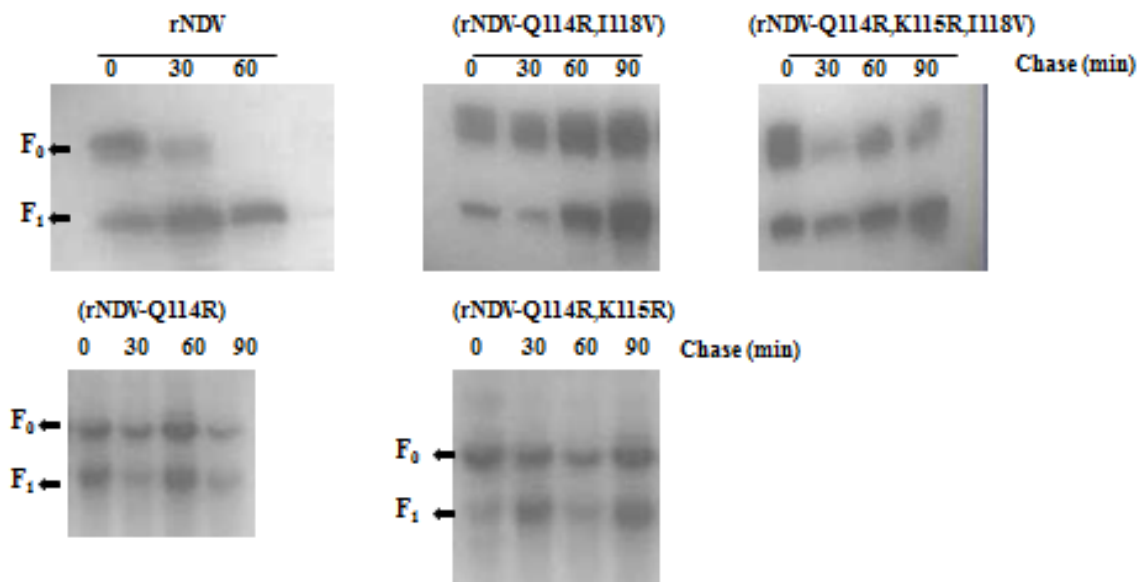
Figure 13A. Proteolytic processivity of F_0 proteins of wild type BC and cleavage site

mutants. Proteolytic processivity of F_0 protein was determined by pulse-chase radio labeling

and immunoprecipitation. The precipitated proteins were analyzed by 10% SDS-PAGE in the

presence of reducing agent and labeled proteins were visualized by autoradiography.
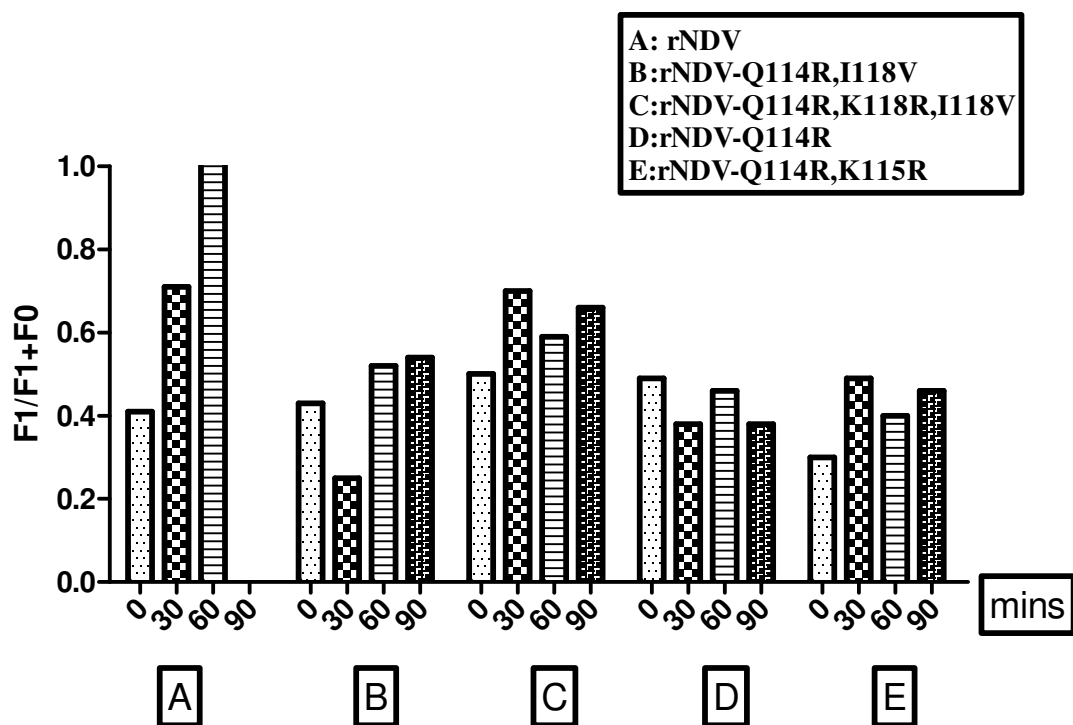
Figure 13B. Panel.13A was scanned and amount of F_0 and F_1 proteins were quantified by densitometry (using Adobe photoshop program). The amount of F_1 protein as a percentage of total F protein (F_1 plus F_0) was calculated to yield the percentage of cleavage.

results showed that each of the F cleavage site mutant viruses had delayed growth compared to rNDV (Fig.14). The mutants (rNDV-Q114R, I118V) and (rNDV-Q114R, K115R, I118V) had 1.5-2.0 log lower virus yield and the mutants (rNDV-Q114R) and (rNDV-Q114R, K115R) had 1.0 log lower virus yield as compared to rNDV at 32 h post infection (PI).Even after 64 h PI, the yield of cleavage site mutant viruses showed delayed growth than that of the rNDV. The virus titers of cleavage site mutant viruses from 8 h to 32 h PI were significantly different than that of the wild type virus (P<0.05). These results showed that not only the change of Q to R at position 114 decreased the replication of NDV but also the change of V to I at position 118 further decreased the replication of NDV. Further, K115R mutation did not appear to have an effect on the replication rate (rNDV-Q114R, K115R) vs (rNDV-Q114R) or (rNDV-Q114R, K115R, I118V) vs (rNDV-Q114R, I118V).

4.5.4 Pathogenecity studies of cleavage site mutants

We evaluated the effect of these F cleavage site mutations *in vivo* by performing intracerebral pathogenicity index (ICPI) test in 1-day-old chicks (Alexander, 1989.). Each virus was inoculated intracerebrally into groups of 10 one-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 of all the cleavage site mutants were significantly lower than that of rNDV (Table. 4). The day-old-chicks infected with rNDV showed signs of depression and paralysis at 24 h PI, whereas the day-old-chicks infected with cleavage site mutants showed the same signs at 48 h PI. To further compare

74
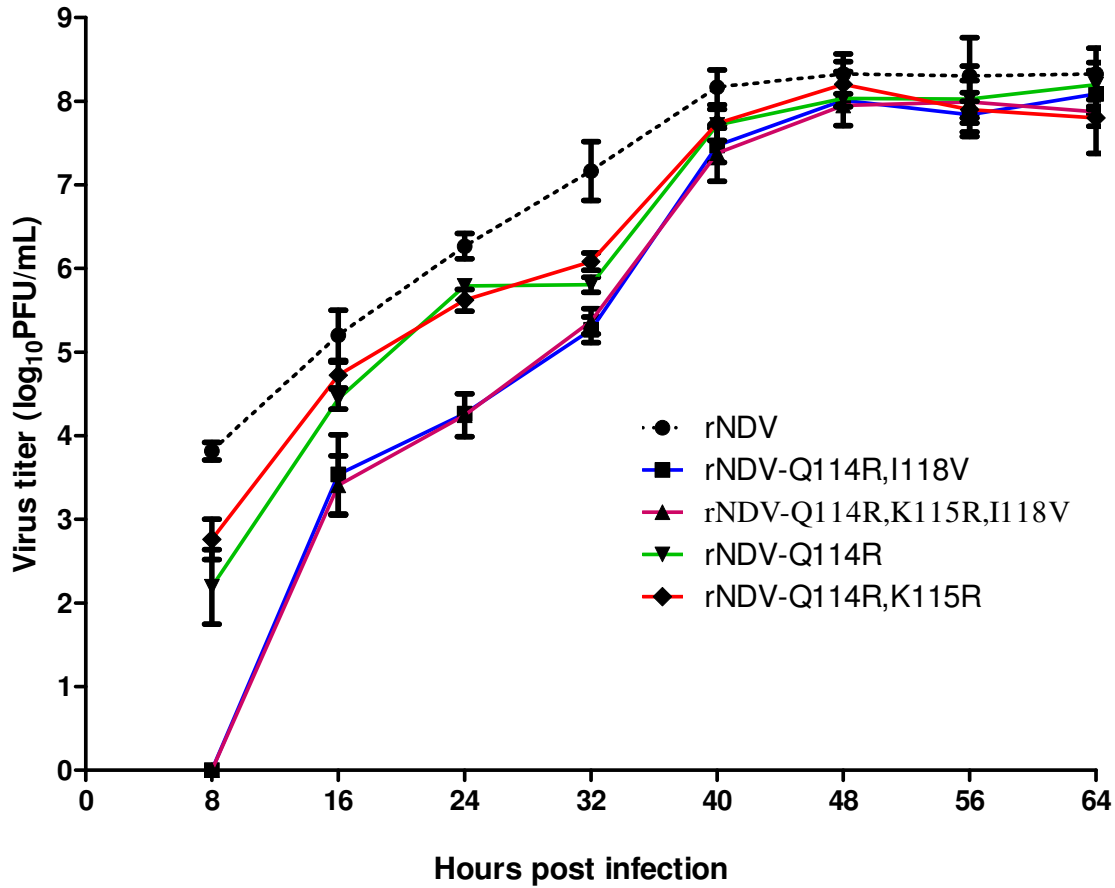
Figure 14. Multicycle growth kinetics of wild type mesogenic strain BC (rNDV) and F protein cleavage site mutants of NDV in chicken embryo fibroblast (DF1) cells. DF1 cells in six well plates were infected in duplicates with parental and mutant viruses at a multiplicity of infection (MOI) of 0.01. Supernatants were collected at 8 h intervals until 64 h post infection and virus titers were determined at each time points by plaque assay. Values are averages from three independent experiments. Error bar shows the standard deviation.

the replication of the mutant viruses in neuronal tissue, groups of 10 one-day-old chicks were inoculated with 50 µl of phosphate buffer saline (PBS) containing 10^3 PFU of each mutant virus/chick via the intracerebral route.

Two birds from each group were sacrificed every 12 h PI, and brain tissue samples were collected and snap-frozen on dry ice.The brain tissue samples were homogenized, and the virus titers in the tissue samples were determined by plaque assay in DF1 cells (Fig.15A). The cleavage site mutants exhibited marked decrease in their replication rate compared to rNDV. The mutant (rNDV-Q114R, K115R) had 1.0 log lower and (rNDV-Q114R, I118V), (rNDV-Q114R, K115R, I118V), (rNDV-Q114R) had 1.5-2.0 log lower virus yield compared to rNDV at 48 h PI. This result corroborated with our earlier *in vitro* results of slower growth replication rate of mutant viruses compared to rNDV in DF1 cells. To further evaluate the pathogenicity of cleavage site mutants through natural route of infection, groups of 5 one-day-old chicks were infected by natural (oculonasal) route with 100 μL of PBS containing 10^6 PFU of each mutant virus per bird. The chicks in each group were observed for clinical signs of disease until 8 days PI and the survival percentage were calculated (Fig. 15B). All the birds inoculated with rNDV showed clinical signs of depression, watery greenish diarrhea, drooping wings on 2nd day PI and died by 4th day PI, whereas the cleavage site mutants exhibited the same clinical signs on 4th day PI. In case of mutants (rNDV-Q114R, K115R, I118V), (rNDV-Q114R), (rNDV-Q114R, K115R) all the birds died by 7th day PI and in case of (rNDV-Q114R, I118V) all the birds
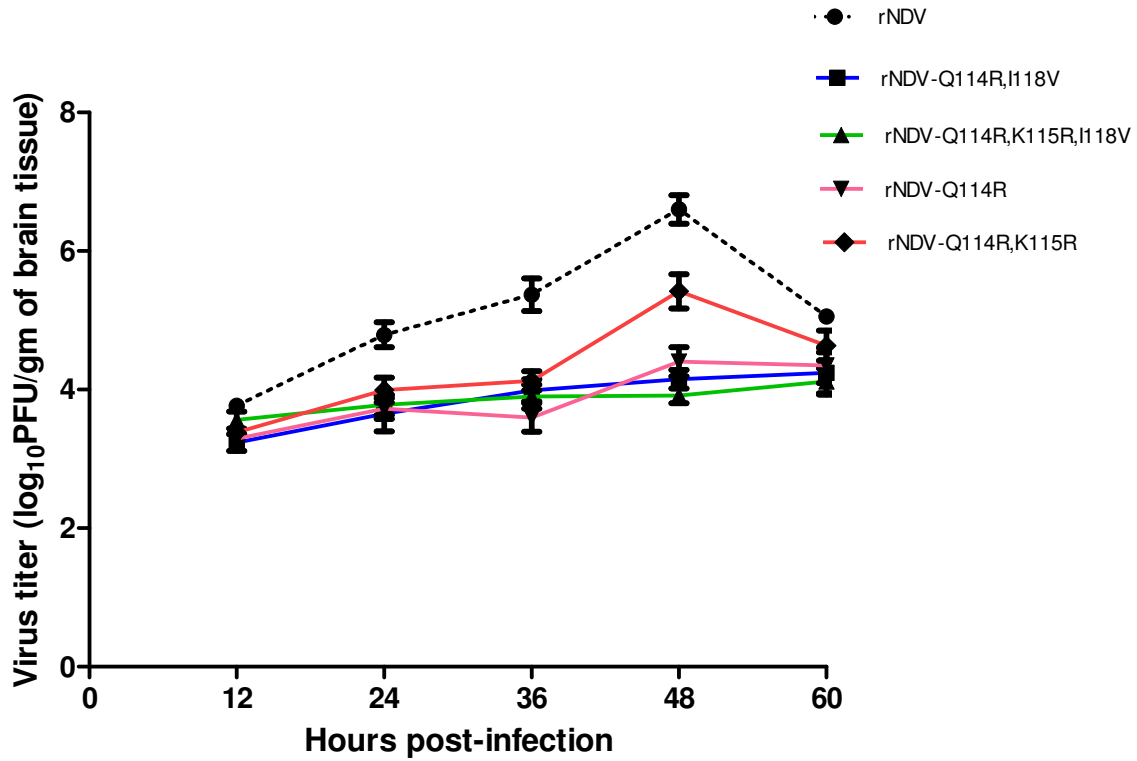
Figure. 15A. Growth kinetics of rNDV and and F protein cleavage site mutants of NDV in the brain of one-day old chicks. Groups of 10 one-day-old chicks were inoculated intracerebrally with 10^3 PFU of virus/chick. Two birds were sacrificed each day after infection; brains were collected and homogenized, and virus titrated by plaque assay in DF1 cells. Virus titers are shown as $\log_{10}$PFU/gm of brain tissue. Values are averages from three independent experiments. Error bar shows the standard deviation.
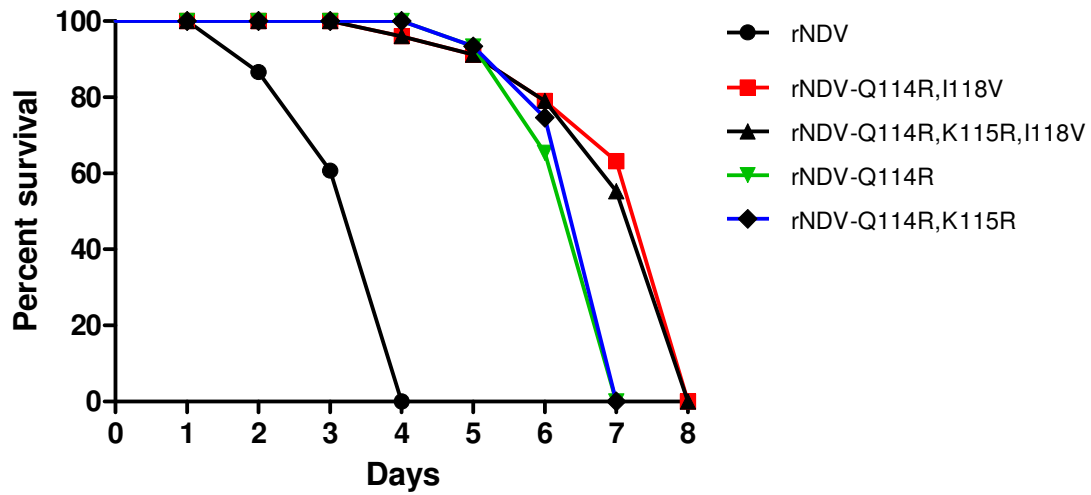
Figure 15B. Pathogenicity of wild type and F protein cleavage site mutants of NDV in one-day-old chicks inoculated via intra-nasal and intra-ocular route. Groups of five one-day old chicks were inoculated with 10^6 PFU of virus per bird and observed daily for signs of disease and mortality for 8 days. Percentage survival was calculated.

died by 8th day PI.

4.6 Discussion

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 F_0 of virulent strains of NDV are processed within the trans-Golgi network in mammalian cells to produce the disulphide linked F_1-F_2 active protein. The cleavage recognition sequence Basic-X-Basic-Basic represents a preferred substrate for the cellular enzyme furin (Durell et al., 1997; Klenk, 1994.; Nagai et al., 1976; Ortmann et al., 1994). Structural modeling 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 of positively charged basic amino acid present in substrate for furin proteolytic activity (Henrich et al., 2003; Schechter and Berger, 1967). The profusely distributed negatively charged amino acids in the furin substrate binding pocket are supposedly ideal for proper electrostatic bond formation with positively charged amino acid residues present in substrate. However, in our study, Q114R mutation reduced the viral pathogenicity. Furthermore, the attenuation is more pronounced in (rNDV-Q114R, I118V) and (rNDV-Q114R, K115R, I118V) which in addition to Q114R mutations also have I118V mutation. It should be noted that, in the hypothetical two dimensional model of furin substrate binding site domains, enzymatic sub-domain of furin which 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 enzyme sub-domains that interact with basic residues of viral substrates are very much 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 R at position 114 in the NDV F cleavage site might be disturbing the conformational stability of furin binding site thus influencing the host cell enzyme activity. It could be one of the reasons that many viral glycoproteins like human immunodeficiency virus gp160 (QR**E**KR↓AV), avian influenza virus A hemagglutinin (KR**E**KR↓GL), sindbis virus gpE2 (GR**S**KR↓SV), human parainfluenza virus type 3 F_0 (PR**T**KR↓FF) , ebola virus Zaire strain envelope glycoprotein (RR**T**RR↓EA) which are processed by furin protease maintain a neutral or acidic amino acid in the X position of consensus R-X-(R/K)-R furin cleavage site motif (Hallenberger et al., 1992; Klenk, 1994.; Nagai et al., 1976; Nakayama, 1997; Wool-Lewis and Bates, 1999). In addition, I118 might also be playing a role in conformational dependability and stability of furin protease for its processivity. It will be interesting to explore further the effect of conserved acidic or neutral amino acids flanking mono or dibasic residues in cleavage site motif in 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 F protein of paramyxoviruses; it was evident from our study that NDV also needs to maintain a neutral amino acid (Q) at conserved 114 residual sites 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 present study gives a new insight in understanding how NDV maintains a conserved residue for effective proteolytic processing of F glycoprotein thus modulating pathogenesis. In future the role of Q114 and V118 can be further exploited to produce a safe live attenuated vaccine.

Chapter 5

5.1 Title

Mutations in the cytoplasmic domain of the Newcastle Disease Virus Fusion Protein confer hyperfusogenic phenotypes modulating viral replication and pathogenicity

5.2 Abstract

The Newcastle disease virus (NDV) fusion protein (F) mediates fusion of viral and hosts cell membranes and is a major determinant of NDV pathogenicity. The cytoplasmic tail (CT) of the NDV F protein is 31 residues long (amino acid 523 to 553), and studies with transfected plasmids have indicated the role of F CT in fusion. In the present study, we used reverse genetics to investigate the effects of mutations in the F protein CT in the context of complete infectious virus, using the moderately pathogenic NDV strain Beaudette C (BC). Out of a series of progressively longer C-terminal deletions in the CT, we were able to rescue recombinant viruses lacking two or four residues (rΔ2 and rΔ4). We further generated and rescued 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 tyrosines (Y524 and Y527) residues and a di-leucine motif at position 536-537 (LL536-537). In other paramyxoviruses, these residues were shown to affect fusion activity and are central element in basolateral targeting signals thus modulating viral pathogenesis. We successfully rescued recombinant viruses with substitution of tyrosine residue (rY524A

and rY527A), but could not recover virus with mutations in the di-leucine motif. With the exception of one mutant (rT550A) that closely resembled wild-type virus (rWT), rest of these mutant viruses exhibited increased cell surface expression of the F protein, were hyperfusogenic, and had increased replication and increased pathogenicity in 9-day-old embryonated chicken eggs, 1-day-old chicks, and 2-week-old chickens. We conclude that these residues in the F CT have the effect of down-regulating fusion and virulence. These mutations may assist in the development of NDV as a vaccine vector and as an oncolytic agent.

5.3 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 (Alexander, 2000; Samal, 2011b). 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. NDV belongs to the genus *Avulavirus* within the family *Paramyxovirdae*, a family of enveloped, non-segmented, negative sense RNA viruses (Lamb, 2001). The entry and spread of Paramyxoviruses was regulated by two viral surface glycoproteins HN and F (Chen et al., 2001; Iorio et al., 2001). HN mediates viral attachment by binding to sialic acid cellular receptors, an activity that also promotes membrane fusion mediated by F (Crennell et al., 2000; Yuan et al., 2011). The F protein mediates pH-independent fusion of the viral membrane with the host cell plasma membrane, resulting in viral penetration, and also mediates fusion of the membranes of adjacent cells to form syncytia (Baker et al., 1999).

Fusion involves a series of major coordinated conformational changes in the F protein that bring together and merge the opposing membranes (Chen et al., 2001; Lamb, 1993b).

The NDV F protein is synthesized as an inactive precursor F_0 (66 kDa) that is cleaved post-translationally by host cell proteases into two disulfide-linked subunits, N-terminal F_2 and C-terminal F_1 (Morrison, 2003; Nagai et al., 1989). 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),measles virus, respiratory syncytial virus (RSV), and 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 (Baker et al., 1999; Carr and Kim, 1993; Collins and Mottet, 1991; Dutch, 2010; Joshi et al., 1998; Weissenhorn et al., 1998; Zhu et al., 2002).

Several reports on virus type I fusion glycoproteins (retrovirus, lentivirus, herpes virus, and other paramyxoviruses) have indicated the role of the cytoplasmic tail (CT) in regulating viral entry and facilitating conformational changes that can affect F protein cleavage and fusogenicity (Aguilar et al., 2007; Bagai and Lamb, 1996; Emerson et al., 2010; Fan et al., 2002; Neyt et al., 1989; Oomens et al., 2006; Saha et al., 2005; Tong et al., 2002; Vzorov et al., 2007; Yao and Compans, 1995). In recent years, tyrosine-containing signals, especially Y-X-X-aliphatic/aromatic consensus motifs, in the CT of viral membrane proteins have been also found to be associated with targeted protein delivery (Ball et al., 1997; Brewer and Roth, 1991; Weise et al., 2010). A second type of signal, a di-leucine (LL) motif, has similarly been shown to mediate processes including internalization and targeting to intracellular compartments and to the basolateral surface of polarized epithelial cells (Bello et al., 2001; Hunziker and Fumey, 1994). Mutagenesis of tyrosine and di-leucine motifs in the

CT of several viral envelope glycoproteins provided evidence that they can affect fusion and infectivity (Dylla et al., 2008; Javier, 2008; Runkler et al., 2009).

The NDV fusion protein CT is 31 amino acids long (amino acid positions 523 to 553, Fig. 15A) that is highly conserved among different strains of NDV (de Leeuw and Peeters, 1999; Dolganiuc et al., 2003; Krishnamurthy et al., 2000; Paldurai et al., 2010). It has been previously reported that deletions in the NDV F CT greatly reduced syncytia formation (Sergel and Morrison, 1995). In the present study we have demonstrated the possible effects on CT of F protein in NDV infectivity, spread, and pathogenicity. 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. We have further investigated the potential role(s) of the tyrosine and di-leucine motifs in CT in viral replication and pathogenicity. Using reverse genetics, we rescued eight NDV mutant viruses with truncation or point mutations in the CT involving conserved signals or possible motifs. The mutant viruses were characterized for intracellular processing, surface expression of F, membrane fusion and replication *in vitro* and *in vivo* in 1-day-old chicks and 2-week old chickens. Our results showed that truncation of C-terminal amino acids and substitution of CT tyrosine residues in F protein resulted in hyperfusogenic phenotypes with increased spread and pathogenicity in chickens.

5.4 Material and Methods

5.4.1 Cells and viruses.

The chicken embryo fibroblast DF1 cell line and human epidermoid carcinoma HEp-2 cell line 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 recombinant 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 (Krishnamurthy et al., 2000). Virus stocks were grown in 9-day –old embryonated eggs, as above, and titers were determined by plaque assay.

5.4.2 Construction of plasmids

The construction of plasmid pNDVfl carrying the full-length antigenome cDNA of NDV strain BC has been described previously (Krishnamurthy et al., 2000). The mutations that were introduced into the F protein CT are summarized in Fig.16. 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. The overlapping PCR was 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.

5.4.3 Recovery of mutant viruses

Plasmid transfection and recovery of recombinant NDV mutants were performed as described previously (Krishnamurthy et al., 2000). 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 (Invitrogen, USA). 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 3130*xl* 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.

5.4.4 Metabolic labeling and immunoprecipitation.

Vero cells in 6-well plates infected with rWT and the CT mutant viruses at an MOI of 1.0 and incubated at 37^0C. At 24 h post infection (PI), the cells were starved for 1 h in Met- and Cys-free DMEM and then were labeled with 100μCi per ml per well of a mixture of [^{35}S] methionine and cysteine for 30 min (pulse), washed, and incubated for 45 min in complete medium (chase). The cells were lysed using RIPA buffer (Tris 50mM, Nacl 150mM, SDS 0.1%, Na.Deoxycholate 0.5%, Triton X100 1%, 1mM PMSF)

and the F proteins were immunoprecipitated using rabbit anti-Fcyt antiserum (raised against a 30-amino acid synthetic peptide representing amino acids 524-553 of the F protein, representing nearly the complete CT) as described previously (Samal et al., 2011). The precipitated proteins were denatured and reduced, separated by 10% SDS-PAGE, and subjected to autoradiography.

5.4.5 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 24 h the cells were detached with PBS containing 5 mM EDTA and centrifuged at $500 \times g$ for 5 min at 4°C. Cells were then incubated with rabbit anti-F_{Nterm} antiserum (1:10 dilution) for 30 min at 4°C. This antiserum was raised against two synthetic peptides representing F amino acids 27-36 and 67-76, as previously described (Samal et al., 2011). Subsequently, cells were washed 3 times with phosphate-buffered saline (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).

5.4.6 Fusion assay of the CT mutant viruses

Syncytia formation was quantified as described by Kohn (Kohn, 1965). Briefly, Vero cells in 6-well plates were infected with each virus at an

Figure 16. 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.

MOI of 0.1. Cells were maintained in 5% MEM at 37°C under 5% CO_2. Twenty-four h PI, 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).

5.4.7 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 PI. Virus titers were quantified by plaque assay on DF1 cells.

5.4.8 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. The MDT is the mean time in h for the minimum lethal dose to kill all inoculated embryos. The definitions for the NDV pathotypes based on MDT are: <60 h, velogenic strains; 60 to 90 h, mesogenic strains; and >90 h, lentogenic strains. For the ICPI test, 0.05 ml of a 1:10 dilution of

fresh infective allantoic fluid of each virus was inoculated into groups of 10 1-day-old SPF chicks via the intracerebral route. The birds were observed for clinical symptoms and mortality once every 8 h for a period of 8 days. The ICPI is the mean score per bird per observation over the 8-day period. Highly virulent velogenic viruses give values approaching 2, and lentogenic strains give values close to 0.

5.4.9 Replication and pathogenicity in 2-week-old chickens

To determine the ability of rWT and the CT mutant viruses to replicate in 2-weeks-old chicken, SPF chicks in groups of 10 were inoculated with 10^6 PFU in 200 µl per bird of each virus via the occulonasal route (50µl in each nare and eye). The birds were observed daily for clinical signs of disease until 14 days PI. To evaluate tropism and viral spread, 3 birds per group were sacrificed on day 3 PI and selected organs (brain, trachea, lungs, gut and spleen) were collected. The virus titers in these organs were determined by $TCID_{50}$ measurement.

5.5 Results

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

Syncytium formation is the hallmark of paramyxovirus cytopathology (Hernandez et al., 1996; Horvath et al., 1992; Lamb, 1993b). It was shown earlier that truncations in the CT of the NDV F protein were inhibitory to

membrane fusion (Sergel and Morrison, 1995). In that study, progressive truncations in the CT indicated that a 10-amino-acid stretch between amino acids 540 to 550 was important for syncytium formation. In the present study, 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, with the rule of six maintained (Krishnamurthy et al., 2000). All mutant F cDNAs were sequenced in their entirety to confirm the presence of the desired mutations. Transfection and recovery of recombinant NDV mutants was performed as described previously (Krishnamurthy et al., 2000). Six mutants were constructed involving progressive deletion of 2, 4, 6, 12, 18, or 30 amino acids from the C-terminus (Fig. 16). However, of these six mutants, viable virus could be recovered two, namely the 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.We were able to recover all four of these mutants, designated rM553A, rK552A, rT551A, rT550A.

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. To investigate possible roles of these Y- and LL- motifs in regulating NDV fusion, we mutated each of the two tyrosine residues singly to alanine, and

mutated the di-leucine to di-alanine (Fig.16). We were able to rescue only the two viruses with substitution of tyrosine to alanine (rY524A and rY527A). The failure to recover the LL-motif mutant suggests that this motif is essential for viral viability. For each of the 8 recovered mutants, the presence of the introduced mutations in the recovered virus was confirmed by RT-PCR and sequence analysis of each F gene (data not shown). The HN gene of each mutant virus also was sequenced, and no adventitious mutations were detected in either F or HN gene. To determine the genetic stability of each F gene mutation, the recovered viruses were plaque purified and passaged five times in 9-day old SPF embryonated chicken eggs.

5.5.2 Intracellular processing and cell surface 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 DF1 chicken embryo cells. Twenty-four h PI, the cells were incubated for 30 min with [^{35}S] methionine and cysteine in medium deficient in both amino acids, followed by washing and an additional 45-min incubation in complete unlabeled medium. Following lysis of the cells, the glycoproteins in the cell lysates were immunoprecipitated with anti-Fcyt rabbit antiserum and resolved by SDS-PAGE.
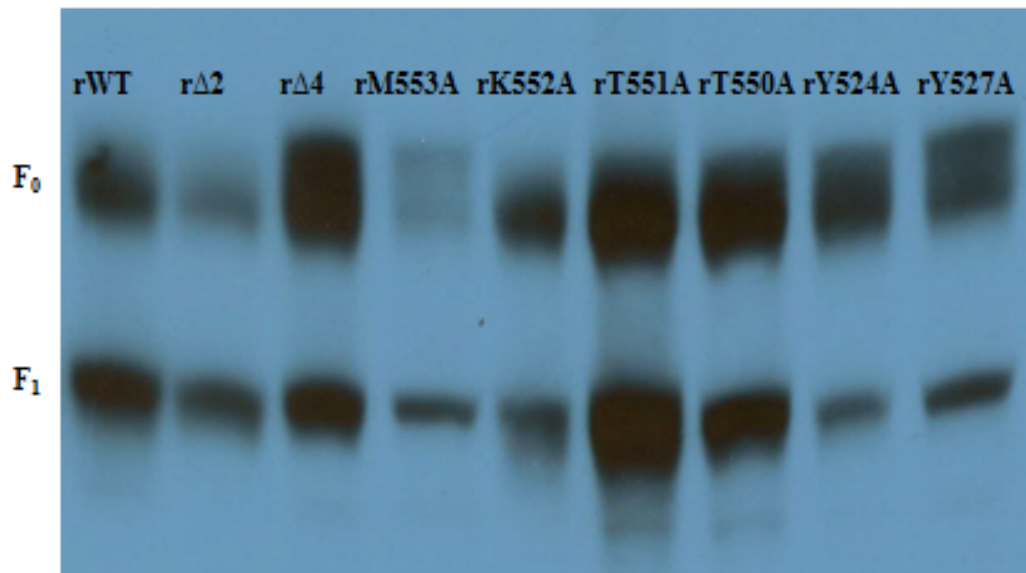
Figure 17. Analysis of the F proteins of the CT mutants by immunoprecipitation and SDS-PAGE. Vero cells were infected with rWT and the CT mutant viruses at an MOI of 1.0. Twenty-four hours PI the cells were starved for methionine and cysteine for 1 h, metabolically labeled with [^{35}S] methionine and cysteine for 30 min, and incubated in complete medium for 45 min. Cell lysates were prepared and subjected to immunoprecipitation using rabbit anti-F_{cyt} antiserum. The samples were denatured and reduced and subjected to SDS-PAGE on a 10% polyacrylamide gel followed by autoradiography.

under denaturing and reducing conditions, and visualized by autoradiography (Fig. 17). The F protein of each of the mutant viruses was expressed and cleaved efficiently, indicating normal intracellular transport. Note that, because the antiserum used in this experiment was specific to the CT, it was very possible that some of the mutations in the CT affected the efficiency of immunoprecipitation with this antiserum: thus, while all of the mutant F proteins reacted efficiently with this antiserum, this experiment could not be used to compare levels of expression.

The relative levels of expression of the F CT mutant viruses were measured by flow cytometric analysis of DF1-infected cells using anti-FNterm rabbit antiserum. The results showed that the efficiency of infection under these conditions, measured by 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 5). The rT550A virus was indistinguishable from rWT, but the other CT mutants exhibited levels of F protein expression that exceeded that of rWT by 16% to 62%, depending on the mutant. The highest levels of expression were observed with the two tyrosine mutants, rY527A and rY524A (62% and 51% increases, respectively), followed by the two deletion mutants, rΔ2 and rΔ4 (48% and 27%), followed by the four viruses with point mutations in the last 4 amino acids of the CT, rT551A (26%), rM553A (24%), rK552A (16%), and rT550

(no increase compared to rWT). These results confirmed that all the CT mutant F proteins retained the ability to be synthesized and transported efficiently to the cell surface, and indeed showed that most of the mutants did so more efficiently than rWT.

5.5.3. 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, Vero cells were infected with the mutant viruses at an MOI of 0.1 and, at 24 h PI, the cells were fixed and stained with hematoxylin-eosin and examined microscopically to quantify the percentage of nuclei involved in syncytia formation as the fusion index (Fig. 18A). All 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), which is consistent with these mutants having the highest levels of surface expression of the F protein (Table 5). Substantial increases also were observed with the rΔ2 and rΔ4 mutants (27% and 13%), consistent with the observed substantial increases in F protein surface expression for these mutants. The rM553A and rK552A mutants also exhibited substantial increases in fusion

Table 5. Cell surface expression of the F proteins of CT mutant viruses

Viruses	% of positive cells ± SD	Relative mean fluorescence intensity
rWT	99±2.4	1.00
rΔ2	99±2.0	1.48
rΔ4	99±2.2	1.27
rM553A	98±3.5	1.24
rK552A	95±1.4	1.16
rT551A	99±1.2	1.26
rT550A	99±3.0	1.00
rY524A	100±3.4	1.51
rY527A	99±1.7	1.62
None (Mock infected cells)		0.01

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 24 hr PI with a cocktail of anti-F_{Nterm} antibody 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 ($P<0.05$).

Figure 18A. 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 PI, 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 ± standard errors from three independent experiments.
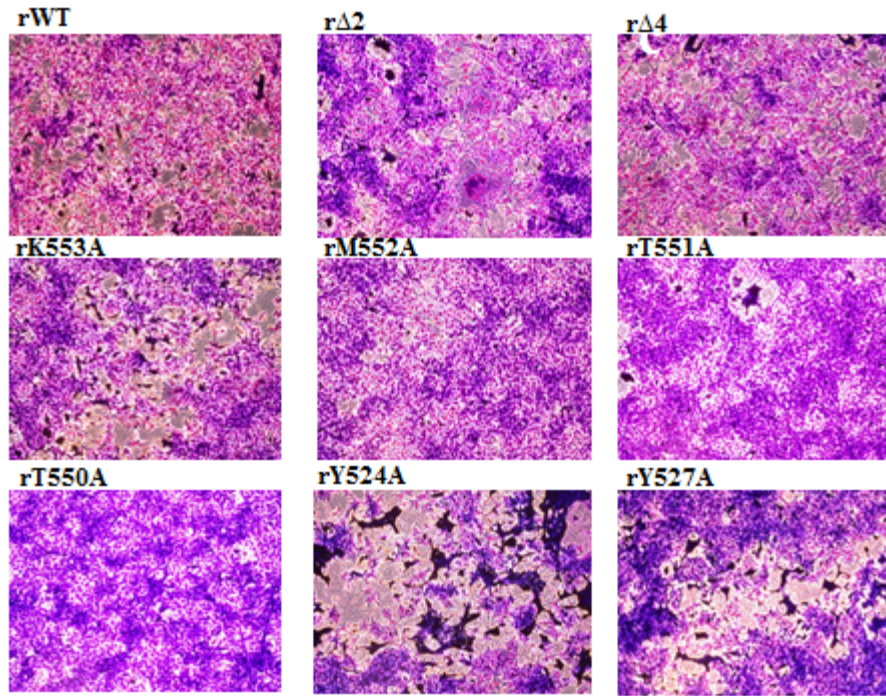
Figure 18B. Photomicrographs of cells infected and treated as in (Fig.18A), except that the cells were fixed 36 h post-infection.

activity (32% and 27%) that were proportionately somewhat greater than the observed increases in F protein surface expression (Table 5).

The CPE caused by rWT and the CT mutant viruses in Vero cells was evaluated following infection at an MOI of 0.1. The rY524A and rY527A viruses produced the most extensive CPE, with detachment of cells at 36 h PI. The rΔ2, rΔ4, r553A, and r552A viruses also produced more CPE than the rWT virus, whereas the rT550A and rT551A were similar to rWT. Fig. 18B shows photomicrographs of cells that were fixed 36 h post-infection and stained with hematoxylin-eosin.

5.5.4. Multicycle growth and CPE of the CT mutant viruses in DF1 cells

The multi-step growth kinetics and magnitude of replication of the CT mutant viruses were determined in DF1 cells (Fig.19). All of the viruses replicated exponentially until ~40 h PI, after which replication was at a plateau. The magnitude of replication was similar for rWT and the rT550A and rT551A viruses, 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 $\log_{10}$ higher compared to rWT at 16 h PI and 2.0 $\log_{10}$ higher compared to rWT at 40 h PI. The CPE associated with the CT mutant viruses in DF1 cells was evaluated following infection at an MOI of 0.1 PFU/cell. The mutant viruses (rΔ2, rΔ4, r553A, r552A, rY524A, rY527A) initiated syncytia at 18h PI and consistently

100
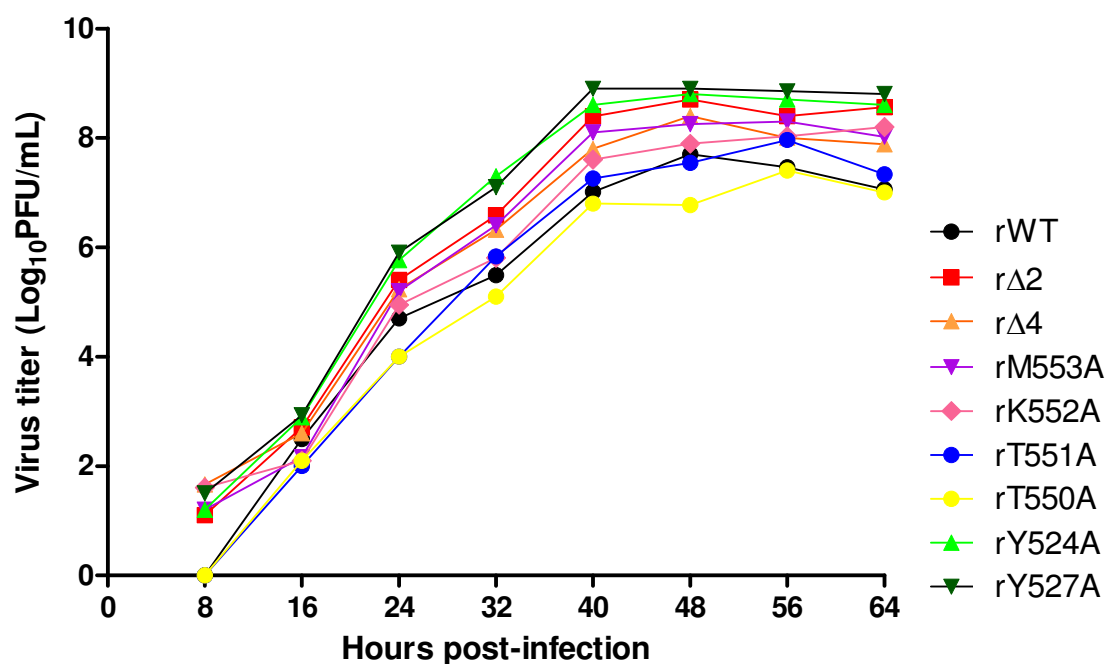
Figure 19A. Comparison of the multicycle growth kinetics and CPE of rWT and the CT mutant viruses in DF1 chicken embryo fibroblast cells. (A) Comparison of multicycle growth kinetics. Cells were infected with each virus at an MOI of 0.01, and cell culture media supernatant aliquots were harvested at 8-h intervals until 64 h PI. The virus titers in the aliquots were determined by plaque assay in DF1 cells. Data shown are averages of three independent experiments.
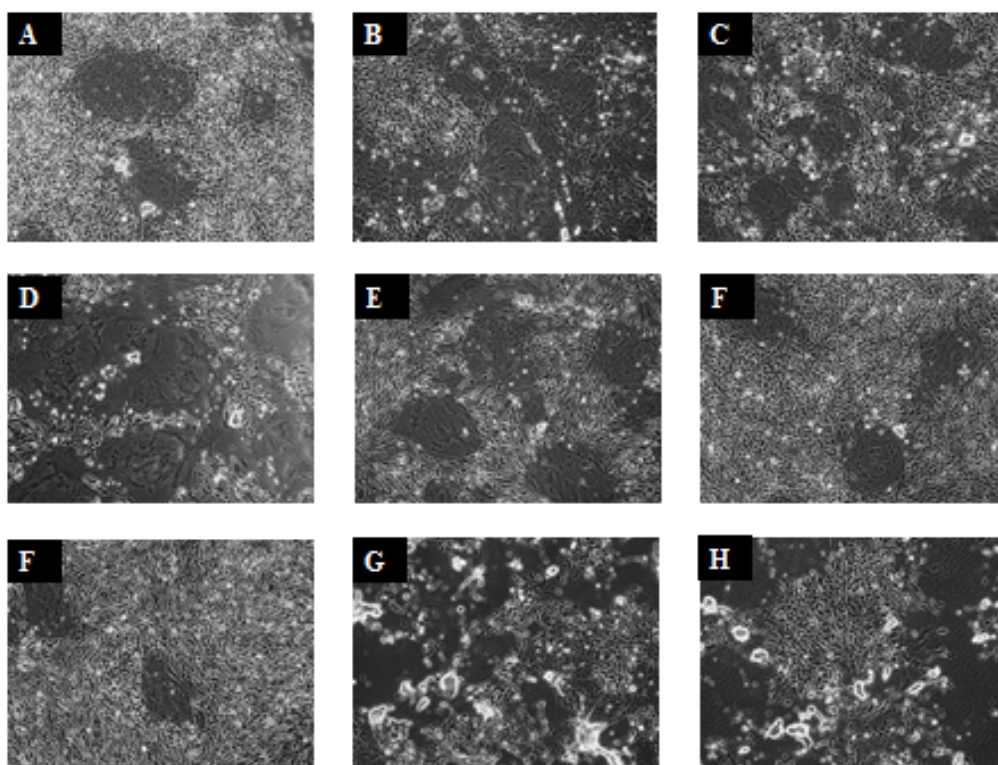
Figure.19B.CPE of rWT and CT mutants in DF1 cells. DF1 cells were infected with each

virus at an MOI of 0.1. Twenty-four h PI the cells were fixed with methanol and visualized

by microscopy. rWT (A), rΔ2 (B), rΔ4(C), rM553A(D), rK552A(E), rT551A(F), rT550A(G),

rY524A(H), rY527A (I).

induced more rapid and extensive syncytia as compared to rWT virus, which produced syncytia by 24 h PI in DF1 cells. The rT550A and rT551A viruses were similar to the rWT virus. Fig. 19B shows photomicrographs of cells that were fixed 24 h post-infection.

5.5.5 Analysis of fusion activity at different permissive temperatures

The paramyxovirus F proteins, like other class I viral fusion-mediating glycoproteins, are present on the surface of infected cells or virions as trimers that are trapped in a metastable (high energy) conformation (Colman and Lawrence, 2003; Lamb et al., 1999; Weissenhorn et al., 1999). To evaluate whether the hyperfusogenic mutants observed in this study had differences in the energy threshold needed to trigger fusion, we compared the rΔ2, rΔ4, rY524A and rY527A mutants with rWT virus in a fusion assay that was performed at different permissive temperatures, namely 25^0C, 30^0C, 33^0C and 37^0C (Fig.20). None of the viruses had detectable fusion at 25^0C. The fusion activity of rWT at 30^0C and 33^0C was 24% and 39%, respectively, as compared to 37^0C as 100%. In comparison, at 30^0C, the rΔ2, rY524A, and rY527A viruses were 30%, 30%, and 28% as compared to 37^0C; and at 33^0C the rΔ2, rY524A, and rY527A viruses were 45%, 54%, and 52% as compared to 37^0C. Thus, the hyperfusogenic mutants were marginally more fusogenic than rWT at reduced temperatures versus 37^0C, but the differences seemed marginal. This suggested that the hyperfusogenic CT mutant viruses did not exhibit a substantial change in the energy threshold to trigger fusion, as compared to rWT virus.

5.5.6. Pathogenicity of the CT mutant viruses in embryonated chicken 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 6). 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 (51.5 h), Y524A (52 h) and rΔ2 (54 h) mutants, which had values that were up to 15% less than that of rWT virus. 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 6). Velogenic strains give values approaching 2.0, whereas lentogenic strains give values close to 0. The ICPI values of the CT mutants were increased compared to rWT virus, which is indicative of increased pathogenicity, although the increases were modest. 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%
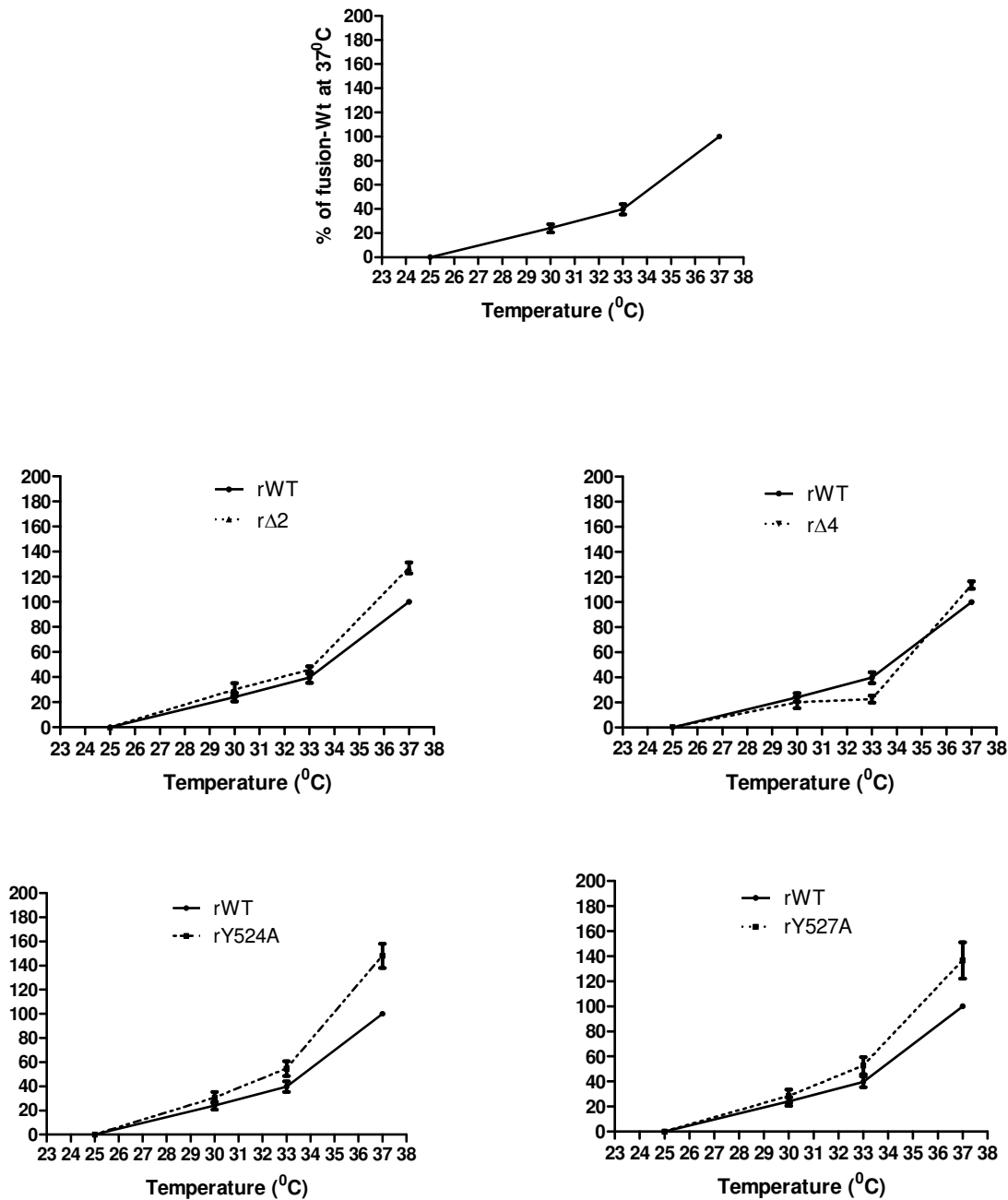
Figure 20. Temperature dependence of the hyperfusogenic rY524A and rY527A CT mutant viruses. The fusion assay was performed in Vero cells as described previously except that replicate cultures were incubated at the following temperatures: 25^0C, 30^0C, 33^0C, and 37^0C. The fusion indices were determined and normalized to that of rWT at 37^0C as 100%. Results are means of three independent experiments.

greater than that of rWT virus. The ICPI values of the other viruses were intermediate. Thus, 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.

5.5.7. Replication, tissue tropism, and pathogenicity of CT mutants in 2-week old chickens

We further evaluated the effects of the CT mutations on viral replication and virulence in 2-week-old chickens. Chickens in groups of 10 were inoculated by the oculonasal route (mimicking natural infection) at a dose of 10^6 PFU per bird. Three chickens from each group were euthanized on day 3 PI and tissue samples of the brain, lung, trachea, gut and spleen were collected and processed for virus titration by limiting dilution. The birds were observed daily for 10 days PI. In the groups infected with the rY524A and rY527A viruses, the chickens showed the clinical signs of paralysis of limbs and increased morbidity, and there was one mortality in the rY527A group. There were no apparent clinical signs in any of the other groups of chickens. Virus replication was detected in each of the sampled tissues (brain, lung, trachea, gut and spleen) for each of the viruses, including rWT (Fig. 21). The titers for the rT550A mutant were very similar to those of rWT, while the titers for the other viruses generally were increased. The highest virus titers were with the rY524A and rY527A viruses in the brain, lungs, trachea and gut. The increase in titer for these two viruses compared to rWT was,

respectively: brain ~ 10 and 50 fold; lungs ~ 100 and 120 fold; trachea ~ 100 and 150 fold; and gut ~0.5 and 10 fold. The induction of paralysis in chickens infected with rY524A and rY524A virus is most likely due to increased replication of these mutants in brain tissues, although the overall titer in the brain for the rY524A virus was not obviously much higher than for several other mutants. The mutants rΔ2, rM553A, rK552A, rT551A also grew to relatively high titers in the brain, trachea, lung, and gut (Fig. 21). None of the viruses recovered from the brain had reversion or other unintended mutations.

5.6 Discussion

The CTs of the several paramxyovirus 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 (Bagai and Lamb, 1996; Branigan et al., 2006; Cathomen et al., 1998). Various studies on the CTs of several viral enveloped proteins have been shown to harbor critical residues required for intracellular trafficking, virus assembly and budding (Ball et al., 1997; Popa et al., 2011; Seth et al., 2003; Waning et al., 2004; Weise et al., 2010). 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. Previous work has shown that deletion of entire CT of the NDV F protein resulted in no syncytia formation whereas deletions of different lengths of CT reduced syncytium formation to various extents (Sergel and Morrison, 1995). In our study, using infectious clones we were able to assess effects of mutations on CT on viral replication, fusion infectivity, tropism, and pathogenesis *in vivo* in a natural host. We attempted to recover viruses with C-terminal deletions of up to 30 amino

acids from the 31-amino acid CT of the NDV F protein. 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 higher levels of F protein expression. Apart from the higher levels of surface expression, these mutations did not appear to affect moderatley in F protein processing and stability. These results suggest that a slightly smaller CT has the effect of enhancing F protein expression and membrane fusion. These deletion mutants also exhibited increased viral growth *in vitro* and *in vivo*, and increased virulence in 1-day-old chicks and 2-week-old chickens. 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 (Fan et al., 2002; Vzorov et al., 2007; Wyss et al., 2005; Zingler and Littman, 1993).

We further investigated the role of these C-terminal four amino acids in the fusion phenotype and in viral replication and pathogenesis by individually substituting them with alanine. We found that the M553A (e.g., the C-terminal residue) and K552A (the penultimate residue) substitutions resulted in phenotypes similar to those of the rΔ2 and rΔ4 deletion viruses: increased surface expression of the F protein (without any other apparent effects on processing, stability and cleavability), hyperfusogenicity, increased viral replication in vitro and in vivo and increased virulence in 1-day-old chicks and 2-week-old chickens. Of the other two mutants, rT550A was generally very similar to rWT virus, whereas rT551A was intermediate between rWT and the rK552A/rM553A viruses.

Table 6. Pathogenicity of the N-glycosylation mutant viruses in embryonated chicken eggs and 1-day-old chicks

Viruses	MDT[a]	ICPI score[b]
rWT	60	1.51
rΔ2	54	1.68
rΔ4	56	1.65
rM553A	55	1.60
rK552A	54.8	1.53
rT551A	56	1.53
rT550A	59	1.53
rY524A	52	1.70
rY527A	51.2	1.78

[a] Mean embryo death time (MDT).

[b] Intracerebral pathogenicity index (ICPI).

The mechanism by which truncations or point mutations of the C-terminal four amino acids of CT mediated these effects is unclear. Increased surface expression of the F protein appeared to an important factor. This could contribute to increased fusion, which in turn could contribute to increased viral replication *in vitro* and *in vivo* and increased pathogenesis. For example, among these two deletion mutants and four substitution mutants, the mutants with higher levels of F protein surface expression relative to rWT tended to have the highest relative fusion indices. Thus, the small deletions or substitutions involving the last four residues of the CT may have conferred increased efficiency of synthesis or transport to the cell surface, resulting in higher levels of F protein surface expression that in turn mediated increased fusion. However, the increases in F protein surface expression and fusion index were not always proportional. For example, the relative levels of surface expression for the rΔ4, rM533A, and rT551A viruses were nearly identical (1.27, 1.24, and 1.26, respectively), but these viruses had substantial differences in their relative fusion indices (1.13, 1.32, and 1.06, respectively). Thus, other factors may have contributed to the increases in fusion. Possible effects might include increased incorporation into virus particles or effects on the fusion process. For example, in case of PIV5/SV5, the F CT is implicated in regulating fusion pore formation (Dutch and Lamb, 2001).

While the full mechanistic details are not known, it is clear that C-terminal residues in the NDV F protein CT, and in particular the two terminal residues, have the effect of down-regulating F protein surface expression, fusion, replication *in vitro* and *in vivo*, tropism, and pathogenesis. The virus presumably could readily mutate to lose expression of these terminal residues by the introduction of missense or nonsense mutations, but this apparently does not confer a selective advantage because the overall WT virus population retains the WT
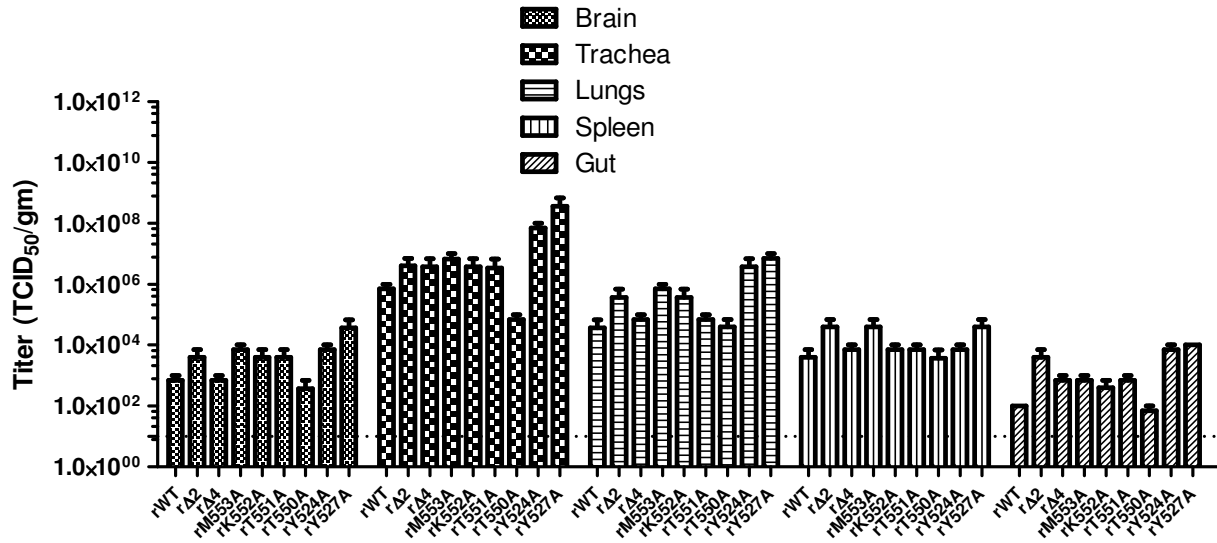
Figure 21. Virus titration from the indicated tissues harvested on day 3 following infection

of 2-week-old chickens with rWT and the CT mutants. Chickens were inoculated by the

oculonasal route. Three chickens for each virus were sacrificed on day 3 PI, and the indicated

organs were harvested and the titers of NDV were determined by limiting dilution assay on

DF1 cells. Titers are expressed in $\log_{10}TCID_{50}$ per gram of tissue with SEM indicated.

sequence. It may be that the increase in virus replication conferred by these mutations is offset by some other factor such as the increased virulence observed in the present study, or some other factor that was not examined here such as the physical stability of the virus particle.

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. In the present report, we also investigated the possible roles of these residues in viral replication and pathogenesis. We were able to recover virus in which either of the tyrosine residues was substituted with alanine. Our inability to recover the LL-motif mutants even after several attempts suggests the importance of LL- motif in F protein function and viability of virus.

The two recovered tyrosine mutants Y524A and Y527A exhibited phenotypes similar to those of the truncation and substitution mutants described above, including increased surface expression of the F protein (without any other apparent effects on processing, stability and cleavability), hyperfusogenicity, increased replication *in vitro* and *in vivo*, and increased pathogenesis. Indeed, of the mutants characterized in the present study, these phenotypes were the most pronounced for the tyrosine mutants, In particular, 2-week-old chickens infected with the tyrosine mutants exhibited paralysis and, in one case, death, that were not observed with rWT virus or with the other mutants. The most widely used tyrosine-based motif is YXX□ (where Y is tyrosine, X is any amino acid and □ 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. Whether these two tyrosines are involved in a signal, and whether these mutations have any effect on the trafficking of the F protein is presently being investigated.

In conclusion, our results provide evidence that deletions or substitutions involving the terminal 2 amino acids of the NDV F protein, or substitutions involving the two tyrosine residue found in the CT, resulted in increased F protein expression on the cell surface, increased fusion, increased replication and pathogenesis. Effects on the surface expression of the F protein appeared to play an important role. It is reasonable to suggest that increased surface expression of F was an important determinant of the hyperfusogenic phenotype observed for these mutants, although, as noted, the level of expression of F was not always proportional to the level of fusion. Thus, other factors may also contribute. It also is reasonable to suggest that increased fusion played an important role in the observed increased replication *in vitro* and *in vivo* and increased pathogenesis. 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 resides 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 vaccine vector and as an oncolytic agent.

Chapter 6

6.1 Title

The Conserved Cysteine Residues in Newcastle Disease Virus Fusion protein are essential for virus infectivity

6.2 Abstract

Newcastle disease virus (NDV) fusion protein (F) contains 13 cysteine residues, 11 of which are conserved across the member of the family *Paramyxoviridae*. The cysteine residues are critical for proper folding and structural stability of the fusion protein. In this study we have determined the individual contribution of conserved cysteine residues present in the F protein at positions 76,199,338,347, 362,370,394,399,401,424 and 523 on NDV infectivity and pathogenicity using reverse genetics techniques. Site-directed mutagenesis was employed to replace each cysteine residue by an alanine and the mutations were introduced into a full length clone of a moderately pathogenic NDV strain Beaudette C (BC). We were able to rescue only three mutants at positions C362A, C370A and C523A. Mutation of cysteine residues at positions C362A and C370A resulted in highly debilitated viruses with significantly impaired biological functions. The mutants rC362A and rC370A replicated in embryonated chicken eggs but failed to replicate further in cell culture. The mutant virus rC523A showed reduced fusogenicity and delayed growth kinetics compared to the wild type virus. The pathogenicity of the rC523A mutant virus was moderately decreased *in vivo*. To assess the effect of cysteine residues on protein expression, 293T cells were transfected with

plasmids encoding the parental and cysteine mutated F proteins. Mutations of C76A, C199A C347A, C362A, C370A, and C401A did not react with an antibody prepared against the cytoplasmic tail of F protein, suggesting profound effects on protein conformation. Mutations at C338, C394A, C399A, C424A and C523A showed different levels of protein expression. These results suggest that the conserved cysteine residues in NDV F protein play a critical role in structure and function of F protein thus regulating NDV infectivity. Further analysis of the polypeptides of rC362A and rC370A showed altered migration patterns of F protein, nucleoprotein (NP) and matrix (M) protein in 10% SDS-PAGE. Sequence analysis of NP and M genes of rC362A and rC370A showed compensatory mutations thus changing the open reading frames (ORF) of NP and M genes.

6.3 Introduction

Newcastle disease is a contagious and highly fatal viral disease, affecting most species of birds and leads to severe economic losses in the poultry industry worldwide (Alexander, 2000). The disease has a wide spectrum of clinical signs ranging from a mild, inapparent infection to 100% mortality. The aetiological agent Newcastle disease virus (NDV) belongs to the genus *Avulavirus* in the family *Paramyxoviridae* and has a nonsegmented, negative-sense RNA genome consisting of 15,186 nucleotides (Lamb, 2001; Samal, 2011a). Strains of NDV are classified into three major pathotypes, depending on the severity of disease produced in chickens. Avirulent strains are termed lentogenic, intermediately virulent strains are termed mesogenic, and highly virulent strains are termed velogenic (Lamb, 2001). The envelope of NDV contains glycoproteins hemagglutinin-neuraminidase (HN), a receptor binding protein that is responsible for attachment to

sialoglycoconjugates at the cell surface and fusion protein (F) which upon activation initiates the fusion of the viral and target membranes (Lamb, 2001; Lamb et al., 2006).

The F protein is well conserved among NDV strains (de Leeuw and Peeters, 1999; Krishnamurthy et al., 2000; Paldurai et al., 2010) and has common structural features with other paramyxovirus F proteins (Baker et al., 1999; Chen et al., 2001; Lamb et al., 1999). The NDV F protein is a type I homotrimeric integral membrane glycoprotein with a monomer length of 553 amino acids for most strains (de Leeuw and Peeters, 1999; Krishnamurthy et al., 2000; Paldurai et al., 2010). The F protein is synthesized as a biologically inactive precursor (F_0) that must be cleaved by host cell proteases (extracellular or intracellular proteases) to form an active fusion protein (Morrison, 2003). The cleavage which takes place at a fusion peptide sequence, generating two subunits F_1 (55kDa) and F_2 (14kDa) that remain covalently linked by at least one disulfide bond. Upon fusion activation, the F protein is thought to undergo major conformational changes that mediate fusion of the viral and cellular membranes (Dutch, 2010; Lamb, 1993b). Similar to other paramyxoviruses the mature F protein of NDV contains 13 cysteine residues, 11 of which are conserved across the F proteins of family *Paramyxoviridae* (Chen et al., 2001; Day et al., 2006; McGinnes and Morrison, 1986; Morrison, 2003; Paldurai et al., 2010). Two of these cysteine residues at positions 76 and 199 have been assigned to form disulfide bond thus linking F_2-F_1 polypeptide chains and hence form the fusion competent active protein (Morrison, 2003; Scheid and Choppin, 1977). The cysteine residues at positions 338,347,362,370, 394, 399,401 and 424 form four disulfide bonds on the F protein head region (Chen et al., 2001). A single cysteine residue is present in the cytoplasmic tail (CT) of NDV F protein at position 523. The cysteine residues present in the CT of diverse viral envelope glycoproteins have

been shown to be the targets for palmitoylation, which is necessary for the association with membrane lipid rafts (Bhattacharya et al., 2004; Caballero et al., 1998; Veit et al., 1989). However, it has been suggested that palmitoylation of NDV F cytoplasmic tail is not required for lipid raft association (Dolganiuc et al., 2003). The strict conservation of cysteine residues of NDV F protein is suggestive of their critical role played on structure and functions of F protein. Further, the cysteine residues are a prerequisite for the formation of conformational dependent antigenic epitopes necessary for NDV infectivity and virulence.

The individual contribution of conserved cysteine residues in NDV F protein to virus infectivity and pathogenesis has never been evaluated. In this study, we have employed reverse genetic techniques to evaluate the role of each conserved cysteine residue on virus recovery, fusion, replication and pathogenesis. Our results demonstrate that eight cysteine residues at positions 76,199,338,347,394,399,401 and 424 are indispensable for virus recovery. However, three cysteine residues at positions 362,370 and 523 were found to be tolerated for the recovery of NDV. The biological characteristics and replication of the mutant viruses with cysteine substitution at positions C362A, C370A and C523A were evaluated *in vitro* and *in vivo*. The mutant virus rC523A showed reduced fusion activity, delayed replication in cell culture and moderate decrease in pathogenicity compared to the wild type (WT) virus; whereas, the mutant viruses rC362A and rC370A grew only in embryonated eggs but showed complete abolishment of fusion activity and replication in cell culture, whereas Our results demonstrated that the conserved cysteine residues of NDV F protein are indispensable for virus infectivity except the cysteine residue in the cytoplasmic domain which is not essential for virus infectivity. The role of each conserved cysteine

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residue on structure and functions of F protein was further evaluated using a plasmid transfection system.

6.4 Materials and Methods

6.4.1 Cells and viruses

The chicken embryo fibroblast DF1 cell line and human epidermoid carcinoma HEp-2 cell line were grown in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) and maintained in DMEM with 2% FBS. The African green monkey kidney (Vero) cell line was grown in DMEM containing 10% FBS and maintained in DMEM with 2% FBS. 293T cells were grown at 37^0C in a humidified atmosphere of 5% CO_2 and maintained in 2% DMEM. 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 wild type (WT) mesogenic NDV starin Beaudette C (BC) and its recombinant 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. Two days after infection the allantoic fluid was harvested and the virus was plaque purified using our standard procedure (Krishnamurthy et al., 2000).

6.4.2 Construction of plasmids

The construction of plasmid pNDVfl carrying the full-length antigenome cDNA of NDV strain BC has been described previously

(Krishnamurthy et al., 2000). The mutations that were introduced into the F protein are summarized in Fig 22. Their introduction was facilitated by the presence of the unique restriction enzyme sites PacI and MluI located in the untranslated regions (UTRs) flanking the F and HN ORFs in the NDV cDNA. The PacI-MluI fragment containing the F-HN gene was mutagenized with primers containing the desired mutations and cloned into TOPO®-TA vector (Invitrogen, USA). The inserts bearing the desired mutation were then cloned into the full-length antigenomic cDNA of strain BC. The total number of nucleotides was adjusted to maintain the 'rule of six' in all full length cDNAs (Krishnamurthy et al., 2000; Peeters et al., 2000). All mutant F genes on full length clones were sequenced in their entirety to confirm the presence of the desired mutations.

For construction of F expression in plasmids, the WT and cysteine mutant F genes were amplified from TOPO-TA clones and were introduced into plasmid pCAGGS expression vector provided by J. Miazaki (Osaka University, *Japan*).

6.4.3 Recovery of mutant viruses

Recovery of recombinant NDV mutants were performed as described previously (Krishnamurthy et al., 2000). 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,USA) 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 (HA) 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 the virus was not recovered, at least three independent transfections were performed in parallel with the parental (WT) cDNA as a positive control before considering the construct negative for virus recovery. To assay the genetic stability, the recovered mutant viruses were passaged three times in 9-day-old SPF chicken embryos. From each passage total RNAs were isolated from NDV-infected allantoic fluid using TRIzol reagent (Invitrogen, USA). 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 3130*xl* genetic analyzer to confirm the presence of the introduced mutations in the recovered viruses. The HN gene from each recovered virus was also sequenced using available primers from our laboratory.

6.4.4 Expression of fusion proteins

To evaluate the expression of WT and mutant F proteins, Vero cells in 6-well plates infected with rWT and the mutant viruses at an MOI of 1.0 and

incubated at 37^0C. Twenty-four hours after infection the cells were lysed using cell lysis buffer (Clontech,USA) and the F proteins were immunoprecipitated using rabbit anti-Fcyt antiserum as described previously (Samal et al., 2011) or HN monoclonal antibody (kindly provided By Ron Iorio, University of Massachusetts Medical School), NP or M polyclonal anti-peptide antibody. The proteins were separated by 10% SDS-PAGE, and subjected to autoradiography. To confirm F protein expression using plasmids, 293T cells in six-well plates were transfected with 2 µg of plasmids expressing either WT F proteins, the panel of cysteine mutants or a vector only control. Twenty-four hours after transfection, cells were lysed and immunoprecipiated as described above.

The cell surface expression of WT and cysteine mutant F proteins was evaluated by flow cytometry. Briefly, DF1 cells were infected with each mutant virus at an MOI of 0.1.After 24 h the cells were detached with PBS containing 5 mM EDTA and centrifuged at $500 \times g$ for 5 min at 4°C. Cells were then incubated with rabbit anti-F_{Nterm} antiserum (1:10 dilution) for 30 min at 4°C as previously described (Samal et al., 2011). 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 then fixed in 2% paraformaldehyde and analyzed by using a FACSRIA II apparatus and Flowjo software (Becton Dickinson Biosciences). The expression of F protein in surface of 293T cells transfected with mutant and wild type F protein plasmids were quantitated by flow cytometry as described

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above.

6.4.5 Cell fusion assays

Syncytia formation was quantified as described by Kohn (Kohn, 1965). 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% CO_2. Twenty-four hours after infection, 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). To evaluate cell fusion activity of WT and mutant F proteins, 293T cells were co-transfected with pCAGGS vector-F (wild type or mutants) and pCAGGS vector-HN. The fusion assays were conducted as described above.

6.4.6 Growth characteristics of wild type and mutant viruses

The growth characteristics of rWT and mutant viruses were evaluated by multicycle growth kinetics. Briefly, 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 PI. Virus titers were quantified by plaque assay on DF1 cells.

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

The pathogenicity of the F cysteine mutant viruses was determined by the MDT test in 9-day-old embryonated chicken eggs and by the ICPI test in 1-day-old SPF chicks (Alexander, 2000; Samal, 2011a). All studies were conducted under enhanced biosafety level (EBSL-3) conditions at the University of Maryland. The MDT is the mean time in hours for the minimum lethal dose to kill all inoculated embryos. The criteria for classifying the virulence of NDV isolates are: <60 h, virulent strains; 60 to 90 h, intermediate virulent strains; and >90 h, avirulent strains.

For ICPI test, 0.05 ml (1:10 dilution) of fresh infective allantoic fluid of each virus was inoculated into groups of 10 1-day-old SPF chicks via the intracerebral route. The ICPI is the mean score per bird per observation over the 8-day period. Highly virulent velogenic 1 viruses give values approaching 2 and avirulent or the lentogenic strains give values close to 0.

6.4.8 DNA sequencing and analysis

Viral RNA was isolated from infected allantoic fluid using RNeasy kit according to the manufacturer's instructions (QIAGEN, USA). Reverse transcription (RT) was performed using Superscript II RT (Invitrogen) and an oligo dT primer. The resulting first strand cDNA was PCR-amplified. PCR products were gel purified and were sequenced directly or were cloned into the TOPO TA cloning vector (Invitrogen) and positive clones were sequenced using M13 forward and M13 reverse primers. DNA sequencing was

performed in a 3130*xl* genetic analyzer (Applied Biosystems Inc, USA) according to the manufacturer's instruction. The entire genome sequence was determined at least three times. Sequence analysis and prediction of ORFs were carried out using the SeqMan and EditSeq programs, and PCR primers were designed using the PrimerSelect program in DNASTAR Lasergene 8 (software suite for sequence analysis, version 8.0.2(13) 412).

6.5 Results

6.5.1 Each conserved cysteine residue in the NDV F protein is indispensable for virus infectivity

There are eleven conserved cysteine residues in the F protein of NDV (Day et al., 2006; Krishnamurthy et al., 2000).To examine the effects of these conserved cysteine residues on virus infectivity and pathogenesis, we performed site-directed mutagenesis by substituting individual cysteine (C) residue by alanine (A) to construct a series of mutant F genes (Fig.1). Each mutagenized F gene was then inserted into a full-length cDNA clone of the BC antigenome. These clones were transfected into HEp2 cells and mutant viruses were recovered using reverse genetics as previously described (Krishnamurthy et al., 2000). We were unable to recover infectious virus with cysteine substitutions at positions76, 199, 338, 347, 394, 394,399,401 and 424, suggesting that these cysteine residues are essential for virus recovery. However, the substitutions of three cysteine residues at positions C362A, C370A and C523A did not affect the virus recovery in 9-day-old embryonated chicken eggs, indicating that these residues are not crucial for virus viability.
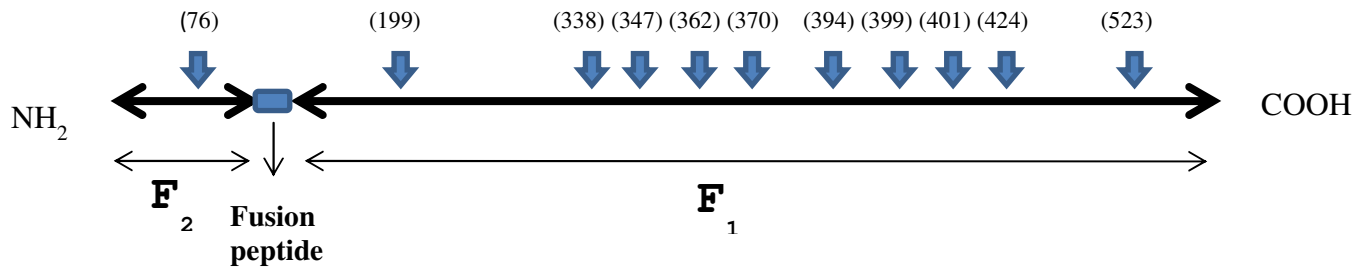
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C76A : Not recovered
C199A: Not recovered
C338A: Not recovered
C347A: Not recovered
C362A: Recovered
C370A: Recovered
C394A: Not recovered
C399A: Not recovered
C401A: Not recovered
C424A: Not recovered
C523A: Recovered
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Figure 22. Schematic representation of NDV F protein and locations of conserved cysteine

residues were marked with an arrow. Mutations correspond to the cysteine residues to alanine

in F protein and clones recovered or not recovered to viable virus listed below.

The sequence analysis of the F genes from recovered viruses confirmed the presence of each introduced mutation.

6.5.2 Effect of cysteine mutation on expression of F proteins

To evaluate the expression of F protein in cysteine mutant viruses, DF1 cells were infected with recombinant wild type (rWT) and mutant viruses. Twenty-four hours after infection cell extracts were collected and F protein expression was determined by Western blot (Fig.23A panel A). The expression level of F protein of rC523A mutant virus was similar to that of the rWT virus and in presence of reducing agent, both F_0 and F_1 proteins were detected. Whereas, in case of the mutant viruses rC362A and rC370A the synthesis of F protein was not detected by Western blot. To confirm viral protein synthesis in those two mutant viruses, the western blot experiment was repeated using a HN monoclonal, a NP polyclonal and M polyclonal antibody. Our results did not show synthesis of any of these viral proteins, confirming absence of rC362A and rC370A replication in DF1 cells (Fig.23A panel B, C and D). The cell surface expression of rC523A mutant F protein was determined by flow cytometry. We observed that the mutant F protein was expressed at 72% of wild type level (Table 7).

To further characterize the mutant F proteins, 293T cells were transfected with expression plasmids encoding WT and mutant F proteins.Twenty-four hour after transfection cell extracts were analyzed by western blot using an anti F-cyt polyclonal antibody (Samal et al., 2012).

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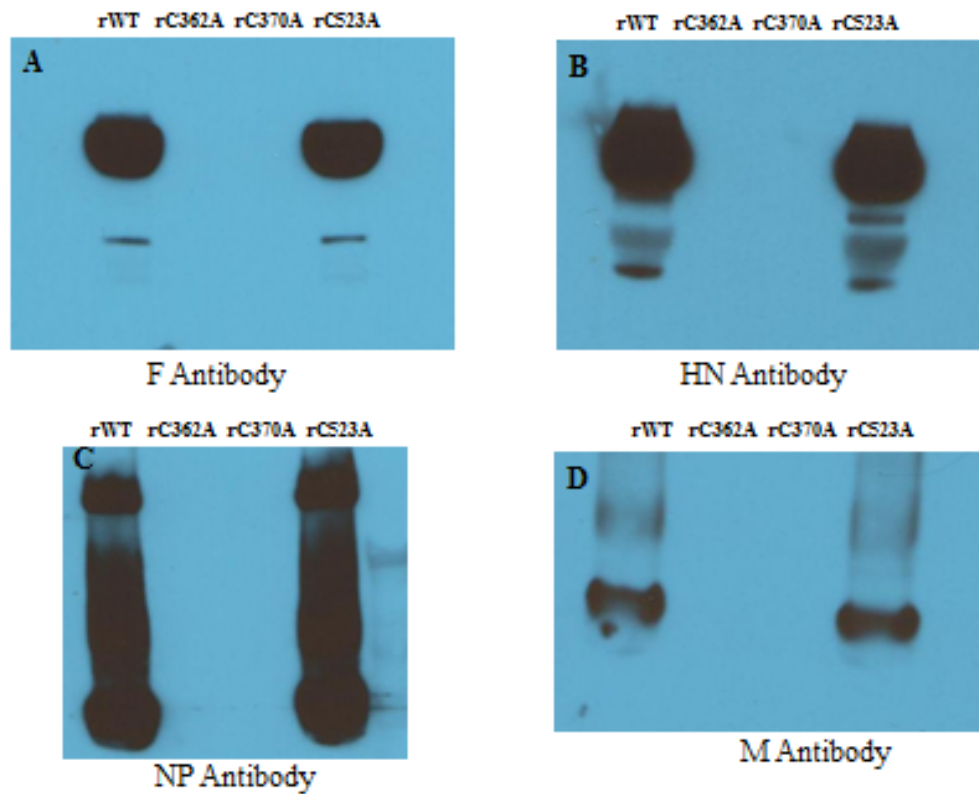
Figure 23A. Western blot analysis of WT and cysteine mutant F proteins (A) Vero cells in 6-well plates infected with rWT and the cysteine mutant viruses at an MOI of 1.0 and incubated at 37^0C. At 24 h post infection (PI), the cell lysates were precipitated with anti-Fcyt antibody or HN monoclonal antibody or NP polyclonal or M polyclonal antibody and analyzed by 10% SDS-PAGE. Panel A: F antibody; Panel B: HN antibody; Panel C: NP antibody; Panel D: M antibody.

Mutation of cysteine residues at C338A, C394A, C399A, and C424A showed reduced expression levels of F protein compared to that of WT F protein (Fig.23B). The F protein of mutant plasmid C523A was expressed at wild-type level. In contrast, we were not able to detect any expression of F protein in C76A, C199A, C347A, C362A, C370A, and C401A mutants. These results suggest that either these mutations are affecting the translation and/or stability of F protein or these mutations are changing the conformation of F protein thus leading to failure of binding to anti-Fcyt antibodies. To determine cell surface expression of the cysteine mutant F proteins, 293T cells were transfected with plasmids encoding WT or mutant F proteins. Twenty-four hours after transfection, cells were analyzed by flow cytometry using anti-F_{Nterm} antiserum as described previously (Samal et al., 2012). The cell surface expression of mutations at C424A, C338A, C394A, and C399A were 6%, 14%, 28% and 32%, respectively, of the WT F protein level (Table.7). The cell surface expression of C523A F protein was 78% of the WT F protein.

6.5.3 Effect of cysteine mutations on fusion activity of F protein

To assess the fusion activity of the mutant viruses, fusion assay was performed in Vero cells. The fusion activity of rC523A was reduced by 37% of the rWT virus. The mutant viruses rC362A and rC370A showed no fusion activity or growth in Vero cells (Fig.24A and 24B). We then determined the fusion activity of the mutant F proteins expressed from transfected plasmids, by performing syncytium assay in 293T cells as described in Methods. Mutant
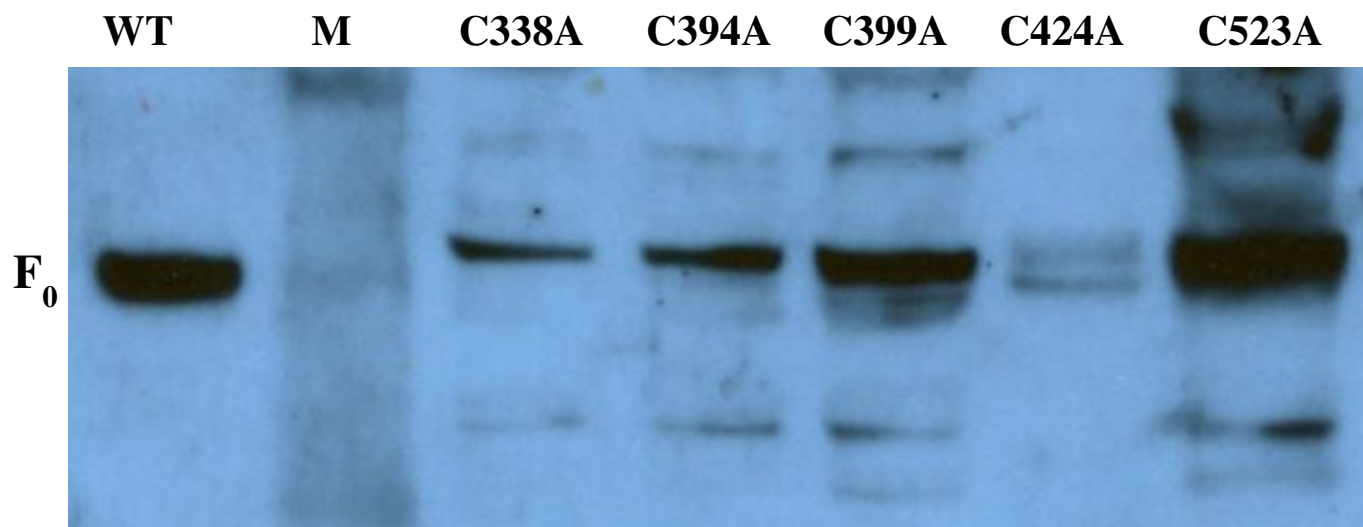
Figure 23B. Western blot analysis of 293T cells were transfected with the plasmids and after 24 h PI cell lysates were collected and precipitated with anti-Fcyt antibody and analyzed by 10% SDS-PAGE.
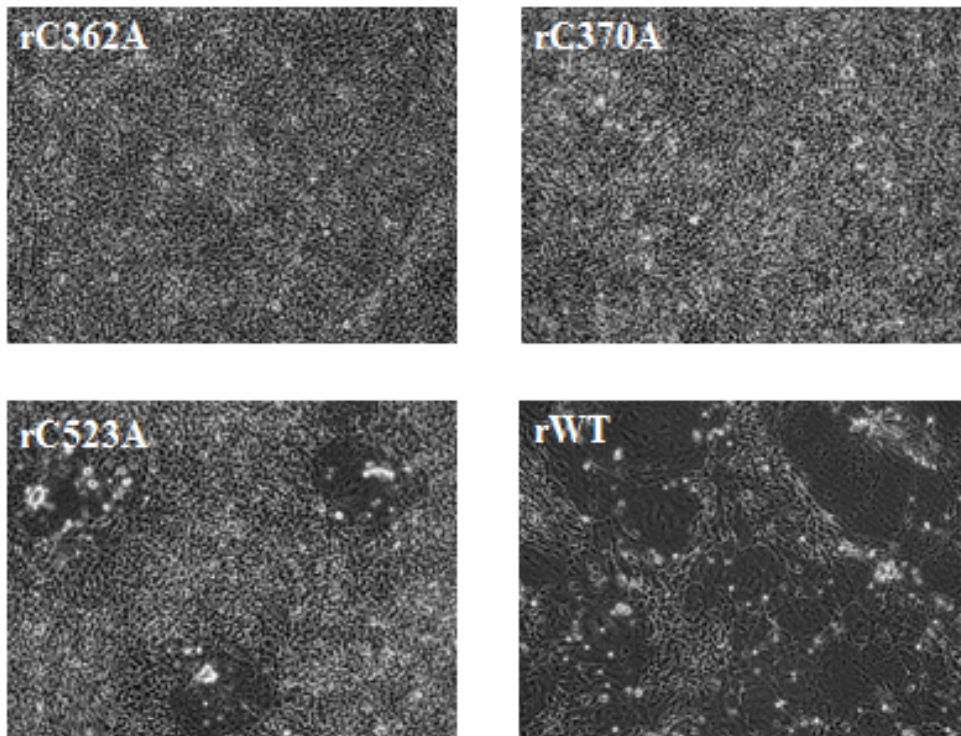
Figure 24A. Cell fusion assay (a) Photomicrographs of Vero cells infected with the indicated viruses at an MOI of 0.1, fixed at 24 h PI, and stained with hematoxylin-eosin.

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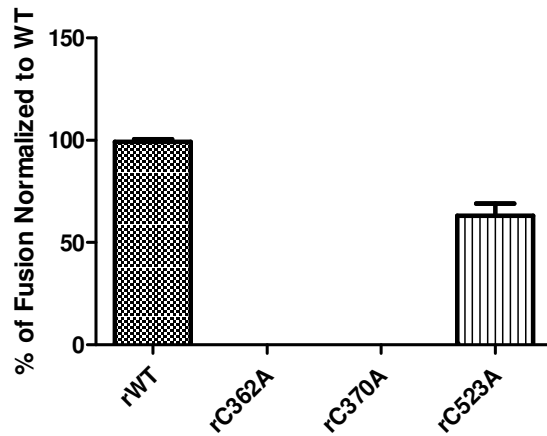
Figure 24B. Relative levels of fusion obtained for the mutant viruses compared to rWT. The fusion index was calculated as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. 10 fields were counted per condition. Fusion levels were normalized to WT at 100%. Data shown are averages ± standard errors from three independent experiments.
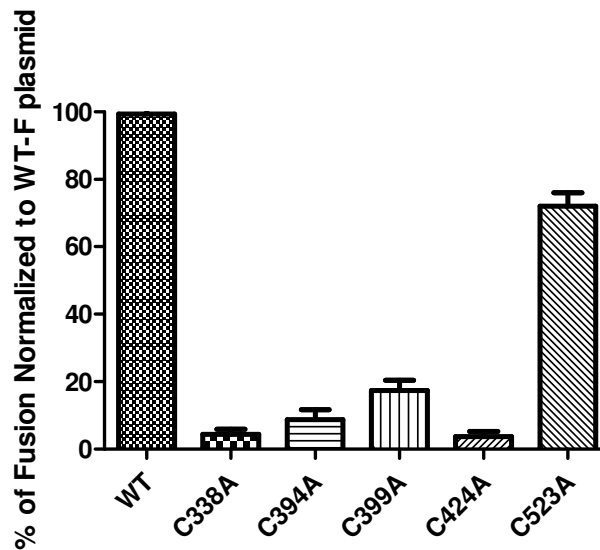
Figure 24C. Relative levels of fusion obtained for the mutant proteins compared to WT-F proteins. 293T cells were co-transfected with pCAGGS vector encoding mutant proteins and pCAGGS-HN protein and fusion index were calculated as described above.

proteins that failed to express matured conformation stable proteins (C76A, C199A, C362A, C370A, C399A, C401A) did not show syncytium formation. However the F protein expressed from plasmid C523A showed 28% reduction in syncytia formation compared to that of the WT F protein. The mutants F proteins of C338A, C394A, C399A and C394A showed no or minimal syncytium formation activity (Fig.24C).

6.5.4 Role of cysteine residues on virus infectivity

We have shown that three of the eleven cysteine mutant viruses were rescued in embryonated chicken eggs and all the three mutant viruses produced high levels of HA titer as the rWT virus (rC362A and rC370A; HA:2^7 and rWT and rC523A; HA: 2^9). We then sought to determine the ability of these mutant viruses to grow in cell culture. *In vitro* growth characteristics of cysteine mutant viruses were evaluated in DF1 cells (Fig.25A). The mutant rC362A and rC370A viruses failed to grow in DF1 cells. The rC523A virus showed a delayed growth kinetics and had lower virus yield (1.5 to 2.0 log U lower) than that of rWT virus. The size of the plaques produced by the mutant virus rC523A was also slightly smaller than that of rWT virus (Fig.25B). There were no plaque formation by mutant viruses rC362A and rC370A. These results suggest that the cysteine residues at postions 362 and 370 are essential for virus infectivity in cell culture.

We further determined the effect of cysteine mutations on the pathogenicity of NDV in embryonated chicken eggs and 1-day-old chicks (Table.8). The effect of the cysteine mutation on the pathogenicity of NDV in
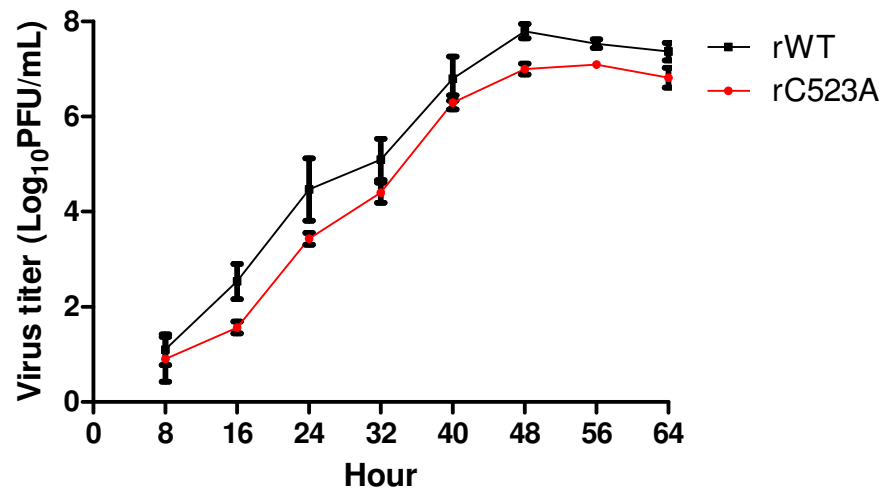
Figure 25A. Comparison of the multicycle growth kinetics of rWT and the mutant viruses in DF1 cells.
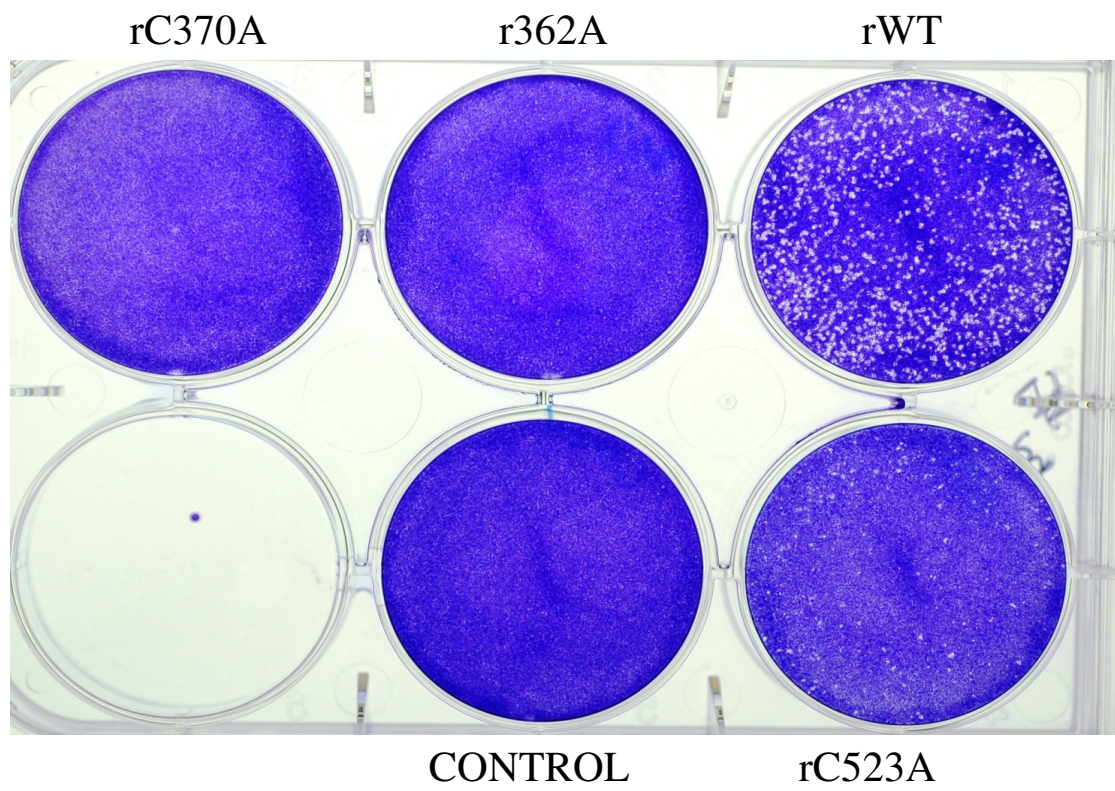
Figure 25B. Plaque morphology in DF1 cells by parental and cysteine mutant viruses 4 days PI.

Table 7. Cell surface expression were measured by flow cytometry in 293T cells

Plasmids	Cell surface expression (%)
WT	100
C338A	14
C394A	28
C399A	32
C424A	6
C523A	78

Table 8. Cell surface expression, MDT and ICPI values

Virus	Cell surface expression (%)	MDT(h)	ICPI
rWT	100	58	1.56
rC362A	0	More than 7 days; No Death	No death
rC370A	0	More than 7 days; No Death	No death
rC523A	72	71	1.40

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 24 hr PI 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. Values are expressed as % relative to parental F protein.

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 x1) + (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.

1-day-old SPF chicks was also determined by intracerebral pathogenicity index (ICPI) test (Alexander, 2000). The MDTs value of rC362A and rC370A were greater than 120h and ICPI values were 0.00, indicating that they are completely avirulent. In case of rC523A mutant virus the MDT value was 71h compared to 58h for rWT virus and ICPI value was 1.40 compared to 1.56 for rWT virus. These results suggested that the delayed growth kinetics of rC523A virus probably resulted in a moderately attenuated phenotype.

6.5.5 Effect of cysteine mutations on incorporation of viral proteins into envelope of virions

To analyze the incorporation of the mutant F proteins into the envelope of virions, the rWT and mutant viruses were harvested from allantoic fluid and were partially purified through a 30% sucrose cushion. The viral proteins separated on a 10% SDS-PAGE,were detected by Coomassie blue staining (Fig.5). The rC523A mutant virus proteins migrated at same rate of the rWT virus proteins. Densitometry analysis of the rC523A mutant virus proteins showed there was no difference in ratios of mutant virus proteins compared to ratios of rWT virus proteins. Surprisingly, the migration pattern of NP and M proteins of mutants rC362A and rC370A were different compared to the migration pattern of the respective proteins of rWT virus in 10% SDS-PAGE (Fig.5). The NP protein of mutant viruses migrated faster than the NP protein of rWT virus. The M protein of mutant viruses migrated slower than the M protein of rWT virus.

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6.5.6 Sequence analysis of cysteine mutant viruses

The stability of cysteine mutations was confirmed after three passages in 9-day-old embryonated chicken eggs. Sequence analysis of the F gene of the mutant viruses at each passage showed that the introduced mutations were unaltered (data not shown). To determine whether there were any compensatory mutations in other viral proteins, the complete genome of the recovered mutant viruses were sequenced and confirmed from uncloned and cloned RT-PCR products of allantoic fluid. The sequences were compared with rWT virus genome sequence. No change in genome sequence was found in the rC523A mutant virus (data not shown). However, in mutant viruses rC362A and rC370A, compensatory mutations in NP and M genes were found. In rC362A virus there were 15nt changes in NP gene and 24 nt changes in M gene and in rC370A virus there were 12 nt changes in NP and 21 nt changes in M gene. There were no compensatory mutations in other viral genes. Sequence analysis showed that these mutations have changed the ORF of the NP and M proteins of rC362A and rC370A viruses, which resulted in not only change in the length of the proteins but also amino acid composition of the two proteins. In both the rC362A and rC370A viruses there were introduction of new stop codon at position 313 which might be resulted in decrease in length of ORF of NP. There was 4.8% divergent (rC362A) and 5.6% divergent (rC370A) in M ORF as compared to M ORF of rWT. These results suggest that probably mutations of NP and M protein of rC362A and
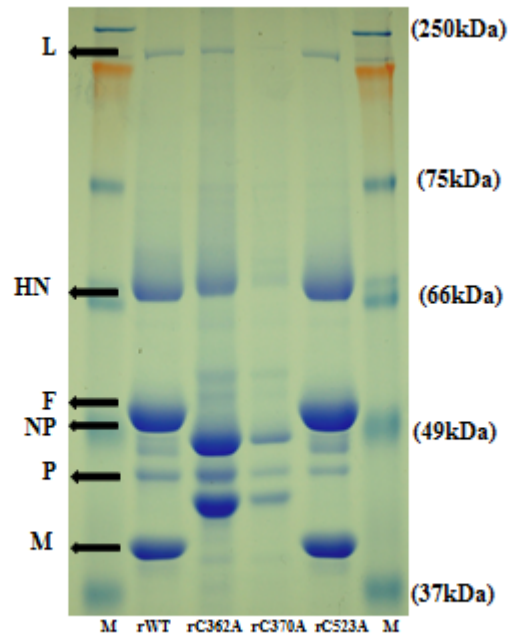
Figure 26. Expression of viral proteins of wild type and cysteine mutants. Equal amounts of proteins from wild type and mutant purified viruses were analyzed by SDS-PAGE. The migration and protein stability of the viral proteins from each virus were examined after Coomassie blue staining. A protein molecular mass marker was also run along with the purified viral samples to assess the viral proteins based on their molecular masses.

rC370A viruses have occurred to suppress the effects of cysteine mutations at positions 362 and 370 of F protein.

6.6 Discussion

The entry of paramyxovirus into the host cell is mediated by F protein, which upon activation undergoes major structural rearrangements to facilitate membrane fusion (Baker et al., 1999; Chen et al., 2001; Dutch, 2010; Lamb et al., 2006; Morrison, 2003). The cysteine residues present in the F protein have been shown to be essential for its biological functions in diverse paramyxoviruses (Day et al., 2006; Iwata et al., 1994; Segawa et al., 1998; Wild et al., 1994). The F protein of NDV has 13 cysteine residues, out of which 11 are conserved across the F proteins of paramyxovirus family and were suggested to form disulfide linkage thus is essential for trafficking, membrane binding, and conformational stability (Chen et al., 2001; McGinnes and Morrison, 1986; Morrison et al., 1987).

To analyze the effects of the cysteine residues in the NDV F protein on virus infectivity and pathogenesis, we generated a series of cysteine mutants by substituting each cysteine residue with alanine. Eight of the cysteine residues were found to be indispensable for the viability of NDV. The failure to recover these eight cysteine mutant viruses indicated that these residues play an important role in F protein folding and conformation, hence critical for survival of the virus. We were able to recover three cysteine mutants C362A, C370A, and C523A. Two of the cysteines, C362A and C370A, form a disulfide linkage in the head region (Chen et al., 2001). These cysteine mutant viruses replicated in 9-day-old embryonated chicken eggs as shown by HA test and RT-PCR, however, they failed to infect cell culture and 1-day-old chicks indicating complete inhibition of virus replication. The disulfide linker between C362A and C370A also had a potential N-linked glycosylation site

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at residue 366. Previous studies have reported that removal of this N-glycosylation site has a moderate effect on the biological functions of F proteins and slightly attenuated the wild type virus (McGinnes et al., 2001; Samal et al., 2012). Therefore, removal of the disulfide linker might be disrupting the F protein conformation and restricting virus fusion and replication. The mutant viruses rC362A and rC370A further showed different migration patterns of NP and M proteins in SDS-PAGE. The complete genome sequences of the mutant viruses revealed compensatory mutations only in NP and M genes leading to change in the ORFs of these genes. It has been earlier reported that membrane glycoproteins (HN and F) of NDV interact with M protein and M protein interacts with NP protein in virus assembly (Kim et al., 2009; Pantua et al., 2006). Therefore, it is possible that the mutation only in NP and M proteins were induced by the virus to suppress the effect of cysteine 362 and 370 mutations in F proteins. It was interesting to observe how NDV can quickly adapt to survive by inducing extragenic mutations in NP and M genes to suppress the deleterious effect of a cysteine mutation in F gene. Our results suggest a possible role of these cysteine residues in F protein in virus assembly by regulating F-M-NP interactions.

The mutant rC523A had the ability to produce syncytia in cell culture; however, the fusion activity was slightly decreased as compared to the wild type virus (Fig.24A). Although the mutation C523A had little effect on expression and cleavability of the F protein (Fig.2 and Table.1a), it delayed the replication of the virus in cell culture thus producing a slightly attenuated phenotype (Fig.25 and Table.8). The cysteine residue C523 is present in the F protein cytoplasmic tail. In previous studies with other paramyxoviruses, this residue is suggested to undergo fatty acid acylation and hence modulating fusion activity (Arumugham et al., 1989; Caballero et al., 1998; Veit et al., 1989). However, in NDV F protein, removal

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of the palmitate site had no effect on virus association with lipid rafts (Dolganiuc et al., 2003). In our study, we have observed a moderate decrease in fusion activity and delayed viral replication. It is possible that the substitution of cysteine with an alanine might be disrupting the overall cytoplasmic tail conformation of NDV F protein and thus affecting its interaction with the M protein in virus assembly.

To further investigate the role of cysteine residues in F protein expression and trafficking we generated F expression plasmids and studied the expression of mutant cysteine proteins after transfection in 293T cells. Our anti-Fcyt antibody recognized only five mutants that were expressed to various levels in cell culture (Fig.23B). These results suggested that mutations at residues 76,199,347,362,370,401 have modified the folding of F protein affecting the conformational epitopes. Furthermore, the mutations at residues 338, 394,399, 401 have significantly reduced cell surface expression and fusion activity when co-expressed along with NDV HN protein, indicating defect in F protein trafficking.

In summary, mutational analysis of the conserved cysteine residues of NDV F protein suggests that these residues play a key role in folding, trafficking and maturation of the F protein and are indispensable for virus infectivity. These residues might be contributing to the viral protein-protein interactions during assembly thus regulating virus infectivity and pathogenesis. It will be interesting to explore whether the rC523A mutation can be used to generate attenuated NDV strains for vaccine purpose.

Chapter 7

7.1 Title.

Conclusion and Future prospects

7.2 Conclusion and future prospects

Newcastle disease (ND) is an avian viral disease caused by Newcastle disease virus (NDV) that causes infection in over 8000 species of birds, including domestic and wild-type, thus resulting in substantial losses to the poultry industry worldwide. Newcastle disease is sufficiently serious disease to be included in List A of the Office Internationale dese-epizooties (OIE). NDV is an enveloped, negative-sense, single stranded RNA virus, belonging to the genus *Avulavirus* and family *Paramyxoviridae* family and (Lamb, 2001; Samal, 2011a). The family *Paramyxoviridae* also includes some important human pathogens, such as Mumps virus, Measles virus, and Respiratory syncytial virus and some important animal pathogens, such as Rinderpest virus, canine distemper virus, and recently emerging Nipah and Hendra viruses

The highly virulent form of Newcastle disease is one of the most important poultry diseases worldwide, which can cause morbidity and mortality rates up to 100%. Outbreaks of virulent Newcastle disease have a tremendous impact on backyard chickens in developing countries, where these birds are a significant source of protein and income. This disease is

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endemic in Asia, Africa, Central and South America. Vaccination can protect birds from clinical signs but does not necessarily prevent virus replication and shedding. Understanding of the biology of NDV can serve as an important guide for future research on the molecular principles that determine its virulence and it will help further to develop highly effective NDV vaccines.

Reverse genetic techniques allow the introduction of site-specific mutations into the genomes of viruses. This revolutionary technique is crucial for the study of the structure/function relationships of viral genes, for investigation of viral pathogenicity, and for development and manufacture of novel vaccines. The technique has significant implications in understanding and preparing for infectious disease pandemic. With the help of the reverse genetics, we have investigated the role of F protein in NDV pathogenesis.

The studies summarized in this project have indicated the importance of the F protein in NDV in virus pathobiology. Our results on N-glycosylation of NDV F protein demonstrated that N-linked carbohydrates are crucial for the fusion function, and this may provide important clues for the development of live attenuated vaccines. By deletion of N-glycans together from HR1 and HR2, we generated a hyperfusogenic phenotype which showed increased spread of the virus and infectivity. The F protein is the main target for immune response and possesses significant immunogenic property. The result of this study can be useful to design effective vaccine strains or avian paramyxovirus vaccine vectors (Kumar et al., 2011; Subbiah et al., 2011; Xiao et al., 2012) by increasing the fusion activity of the vaccine virus which can increase replication and immunogenicity. Similarly it can also

increase the replication of the NDV vector. NDV strains have wide spectrum of virulence in chickens from completely avirulent to highly virulent and also there is variation in tissue tropisms (viscerotropic, neurotropic, and some strains are restricted to only respiratory tract). In our study we have used the BC strain which is moderately pathogenic and the infection is restricted mostly to the brain and respiratory tract. However, in our study on N-glycans the (rNg2+5) hyperfusogenic phenotype had shown an significant spread in gut tissues; this gives an new insight to understand how N-glycans of NDV F protein possibly modify the structure and function thus influencing virus replication and tropism. Additional studies of N-glycans of other velogenic and lentogenic strains will provide better understanding towards the critical role of F protein in NDV pathogenesis.

The wide variation in NDV pathogenicity is primarily due to differences in the cleavage site sequence within the F protein. This protein is synthesized as a precursor (F0) in non-functional state, which then is cleaved by host proteases into two functionally active polypeptides (F1 and F2). However, recently the vast scale of sequence analysis and studies on NDV virulence factors have demonstrated that virulence of NDV is a multigenic trait. Our studies on cleavage site of NDV F protein have pointed out the importance of the conserved glutamine residue on the F protein cleavage site and have given a plausible explanation why many field isolates can harbor virulent strains of NDV without showing clinical signs, and that it consequently may act as silent carriers. This study can be extended in future to other strains of NDV and other avian paramyxoviruses to understand the role of cleavage site and to generate more genetically stable live attenuated NDV vaccines.

The paramyxovirus F protein is a class I viral membrane fusion protein which undergoes a significant refolding transition during virus entry. The availability of the crystal structures of two surface glycoproteins, F and HN, of NDV has allowed us to understand the complex mechanism by which these viruses initiate infection (Chen, 2001; Crennell et al., 2000; Swanson et al.). The exact molecular mechanism by which the F protein mediates the critical steps of fusion process is still unknown. Recombinant NDVs serve as an excellent model natural for understanding paramyxovirus pathogenesis, because pathobiology of NDV can be measured on a quantitative basis in its natural host chickens. Our studies on cytoplasmic domain and conserved cysteine residues of NDV F protein have broadened our understanding on the requirement of conserved amino acids in F protein functions. In the future, more detailed studies on this can have higher implications to understand the mechanism of paramyxovirus fusion and for strategies to prevent viral entry.

NDV has inherent oncolytic potential for the treatment of human cancers. NDV replicates selectively in human cancerous cells sparing normal cells and activates programmed cell death program in cancer cells. It is demonstrated that NDV selectively replicates in tumor cells and induces death while sparing normal cells. Due to this property, NDV has been exploited as a potential anti-cancer agent in humans. A previous study demonstrated that recombinant NDV expressing highly fusogenic F protein has enhanced oncolytic property (Vigil et al., 2007). For a successful virotherapy, virion production rates in the infected tumor cells must outstrip the growth rate of healthy tissue. Our studies on NDV F protein has demonstrated how various domains and conserved residues play a critical role in generating hyper or hypo fusogenic phenotypes and the possible interactions of F protein

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with HN and other internal proteins. These studies can help in improvement of therapeutic index of recombinat NDV strains as oncolytic agents. In the future, more detailed studies of NDV F protein will provide us with more knowledge to increase the oncolytic property of NDV.

An important application of reverse-genetic techniques is the generation of recombinant viruses for use as vaccine vectors. Recombinant NDV is an excellent vaccine vector expressing foreign proteins for both human and animal diseases (DiNapoli et al., 2007; Khattar et al., 2010; Khattar et al., 2011; Xiao et al., 2011) . Our study can be further exploited to increase the efficacy of the NDV as a vaccine vector. The HN and F glyocproteins of the paramyxoviruses are known to be responsible for initiation and progress of the infection process and thus are the potent immunogenic candidates. Our studies can be explored further to enhance the immunogenicity of F protein.

In summary, our studies on NDV F protein provide the basis for further investigation to understand overall F protein in NDV virulence. It will be interesting to explore further whether F protein determines NDV tropism and virus host interactions.

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