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NEWCASTLE DISEASE VIRUS VIRULENCE: MECHANISM OF THE INTERFERON ANTAGONISTIC ACTIVITY OF THE V PROTEIN AND CHARACTERIZATION OF A PUTATIVE VIRULENCE-SPECIFIC ANTIBODY TO THE ATTACHMENT PROTEIN

A Dissertation Presented
By

Judith Gallego Alamares

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 5, 2008

Department of Molecular Genetics and Microbiology

NEWCASTLE DISEASE VIRUS VIRULENCE: MECHANISM OF THE
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ABSTRACT

Newcastle disease virus (NDV) is a member of the genus *Avulavirus* of the *Paramyxoviridae* family of enveloped negative-stranded RNA viruses. The virus causes respiratory, neurological, or enteric disease in many species of birds, resulting in significant losses to the poultry industry worldwide. Strains of the virus are classified into three pathotypes based on the severity of disease in chickens. Avirulent strains that produce mild or asymptomatic infections are termed lentogenic, whereas virulent strains are termed velogenic. Strains of intermediate virulence are termed mesogenic.

The envelope of NDV virions contains two types of glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins. HN mediates three functions: 1) virus attachment to sialic acid-containing receptors; 2) neuraminidase activity that cleaves sialic acid from progeny virions to prevent self-aggregation; and, 3) complementation of the F protein in the promotion of fusion.

Though it is widely accepted that cleavage of a fusion protein precursor is the primary determinant of NDV virulence, it is not the sole determinant. At least two other proteins, HN and the V protein, contribute to virulence. The V protein possesses interferon (IFN) antagonistic activity. The long-range goal of these studies is to understand the roles of HN and V in the differential virulence patterns exhibited by members of the NDV serotype.

The first aim is to compare the IFN antagonistic activity of the V protein from a lentogenic and a mesogenic strain of the virus. The results of this study demonstrate that the V protein of the mesogenic strain Beaudette C (BC) exhibits greater IFN antagonistic activity than that of the lentogenic strain La Sota. Hence, the IFN antagonistic activities of the two V proteins correlate with their known virulence properties.

Comparison of the C-terminal regions of La Sota and BC V proteins revealed four amino acid differences. The results demonstrate that the IFN antagonistic activity of La Sota V increases when any one of these residues is mutated to the corresponding residue in BC V. Conversely, the IFN antagonistic activity of BC V decreases when any one of these four residues is mutated to the corresponding residue in La Sota V. However, no single residue accounts for the difference in IFN antagonistic activity between the two V proteins. Also, analysis of La Sota V and BC V proteins with multiple mutations in these positions revealed that the four residues are collectively responsible for the difference in the IFN antagonistic activity of the two V proteins. Finally, characterization of chimeric La Sota/BC V proteins showed that the N-terminal region also contributes to the IFN antagonistic activity of V.

Contrary to an earlier report, results described here demonstrate that the NDV V protein does not target STAT1 for degradation. However, both La Sota and BC V proteins target interferon regulatory factor (IRF)-7 for degradation and promote the conversion of full-length IRF-7 to a lower molecular weight form

(IRF-7∗). This is the first demonstration that IRF-7 is targeted by a paramyxovirus V protein. The amount of IRF-7∗ decreases in a dose-dependent manner in the presence of a proteasome inhibitor, suggesting that IRF-7∗ is a degradation product of IRF-7. Furthermore, the BC V protein promotes complete conversion of IRF-7 to IRF7∗, whereas the La Sota V protein does so less efficiently. Again, this is consistent with the difference in IFN antagonistic activity of the two V proteins, and in turn, with their virulence.

The second aim is to characterize an HN-specific monoclonal antibody called AVS-I. A previous study suggested that AVS-I recognizes an epitope that is conserved in lentogenic strains and raises the possibility that this epitope may colocalize with a determinant of virulence in HN. To further characterize antibody AVS-I and the epitope it recognizes, we (i) determined its specificity for several additional strains of the virus, (ii) mapped its binding to HN in competition with our own antibodies, (iii) determined its functional inhibition profile, and (iv) isolated and sequenced an AVS-I escape mutant. The results demonstrate that AVS-I binds to a conformational epitope at the carboxy terminus of HN. This suggests that this region of HN may define a determinant of virulence. However, it was also shown that AVS-I, which was previously thought to be specific for avirulent strains of NDV, actually recognizes individual mesogenic and velogenic strains.

In conclusion, the data presented in this dissertation contributes to a greater understanding of the molecular basis for NDV virulence and may aid in

development of antiviral strategies and generation of recombinant NDVs suitable

for use in cancer and gene therapy.

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LIST OF ABBREVIATIONS

Aequorea coerulescens green fluorescent protein 1	AcGFP1
Baby hamster kidney	BHK
Beaudette C	BC
Beta propeller	BP
Canine distemper virus	CDV
Caspase recruitment domains	CARD
Chicken embryo fibroblast	CEF
Cullin 4A	Cul4A
Cyclic-AMP-responsive-element-binding protein (CREB)-binding protein	CBP
UV-damaged DNA binding protein 1	DDB1
Dendritic cells	DCs
Deoxyribonucleic acid	DNA
Dimethyl sulfoxide	DMSO
DNA-dependent activator of IFN regulatory factors	DAI
Double-stranded DNA	dsDNA
Double-stranded RNA	dsRNA
Dulbecco's Modified Eagle medium	DMEM
Enzyme-linked immunosorbent assay	ELISA
Fetal calf serum	FCS
Fusion protein	F
Green fluorescent protein	GFP

Hemadsorption	HAd
Hemagglutination	HA
Hemagglutination inhibition	HI
Hemagglutinin-neuraminidase	HN
Heptad repeat	HR
Human papilloma virus	HPV
Human parainfluenza virus 2	hPIV2
IκB kinase-ε	IKK-ε
Interferon	IFN
Interferon regulatory factor	IRF
Interferon regulatory factor-associated domain	IAD
Interferon -sensitive response element	ISRE
Interferon -stimulated gene	ISG
Interferon -stimulated gene factor 3	ISGF3
Internal ribosome entry site	IRES
Intracerebral pathogenicity index	ICPI
Intravenous pathogenicity index	IVPI
Kilodalton	kDa
Large RNA-dependent RNA polymerase	L
Matrix protein	M
Mean embryo death time	MDT
Melanoma differentiation-associated gene 5	MDA-5

Messenger ribonucleic acid	mRNA
Mitogen-activated protein	MAP
Monoclonal antibody	MAb
Mouse embryonic fibroblasts	MEFs
Multiplicity of infection	MOI
Neuraminidase	NA
Neurotropic velogenic	NV
Newcastle Disease Virus	NDV
Nucleocapsid protein	NP
2',5'-oligoadenylate synthetase	OAS
Open reading frame	ORF
Pattern recognition receptors	PRR
pBluescript SK(+)	pBSK
Phenylmethylsulfonyl fluoride	PMSF
Phosphate buffered saline	PBS
Phosphoprotein	P
RNA-dependent protein kinase	PKR
Rabbit anti-mouse immunoglobulin	RAM Ig
Respiratory syncytial virus	RSV
Retinoic acid-inducible gene I	RIG-I
Reverse transcription polymerase chain reaction	RT-PCR
Ribonucleic acid	RNA

Rinderpest virus	RPV
Sendai virus	SV
Severe acute respiratory syndrome-associated coronavirus	SARS-CoV
Signal transducer and activator of transcription	STAT
Simian virus 5	SV5
Single-stranded DNA	ssDNA
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Specific-pathogen-free	SPF
Src homology 2	SH2
TANK-binding kinase 1	TBK1
Toll-like receptor	TLR
Transcriptional activation domain	TAD
V-dependent degradation complex	VDC
Viscerotropic velogenic	VV
Wild type	Wt

CHAPTER I

Introduction

1.1 Newcastle Disease Virus

1.1.1 Classification and Overview

Newcastle disease is one of the most devastating avian diseases and causes substantial economic losses in the poultry industry worldwide (Alexander, 1988). The causative agent of the disease is Newcastle Disease Virus (NDV), a member of the genus *Avulavirus* in the subfamily *Paramyxovirinae* within the *Paramyxoviridae* family (Mayo, 2002). Paramyxoviruses are enveloped viruses with a non-segmented single-stranded RNA genome of negative polarity (Lamb and Kolakofsky, 2001). Other members of this family include animal pathogens such as Sendai virus (SV), simian virus 5 (SV5), canine distemper virus (CDV), rinderpest virus (RPV) and peste-des-petits-ruminants virus, and human pathogens such as measles virus, mumps virus, respiratory syncytial virus (RSV) and various parainfluenza viruses. The newly emerged Hendra and Nipah viruses also belong to this family (Table 1).

NDV can infect a wide variety of domestic and wild birds, but chickens seem to be the most susceptible species. Vaccines against NDV are based on avirulent strains of the virus and are administered to birds through drinking water, aerosols, or eye drops or by parenteral routes. However, these methods are labor intensive. The in ovo method, which allows automated   vaccine delivery in

Table 1. Members of the *Paramyxoviridae* family

Family *Paramyxoviridae*

    Subfamily *Paramyxovirinae*
        Genus Avulavirus
            Newcastle Disease virus

        Genus Henipavirus
            Hendra virus
            Nipah virus

        Genus Morbillivirus
            Measles virus
            Canine distemper virus
            Peste-des-petits-ruminants virus
            Rinderpest virus

        Genus Paramyxovirus
            Sendai virus
            Human parainfluenza virus type 1 and type 3
            Bovine parainfluenza virus type 3

        Genus Rubulavirus
            Simian virus 5 or Parainfluenza virus 5
            Mumps virus
            Human parainfluenza virus type 2, type 4a and 4b

    Subfamily *Pneumovirinae*
        Genus Pneumovirus
            Human respiratory syncytial virus
            Bovine respiratory syncytial virus

embryonated eggs, provides a labor saving alternative to posthatching vaccination. At present, there is no live NDV vaccine licensed for use in embryonated eggs due to high embryo mortality and low hatchability. Despite availability of live attenuated vaccines, the inherent ability of attenuated strains to revert in virulence makes control of this disease difficult. Virulent strains of NDV have been classified as select agents by the Centers for Disease Control (CDC) and the United States Department of Agriculture (USDA) because of their potential as agents of agricultural bioterrorism. These issues have led to a continued effort to develop alternative vaccination strategies and antiviral treatments.

NDV is not only an economically important pathogen, but is also being developed as a vaccine vector. Several characteristics of NDV make it suitable for the latter. NDV grows well in a wide variety of cells and elicits strong humoral and cellular immune responses in vivo. It also naturally infects the respiratory tract, making it especially useful for delivery of protective antigens from other respiratory disease pathogens. Unlike vaccine vectors that are based on human pathogens, such as measles virus and adenovirus, NDV is expected to be more immunogenic in adults due to the absence of existing host immunity, which reduces immunogenicity of the vector. NDV replicates in the cytoplasm without a DNA intermediate, which eliminates the problem of integration of viral DNA into the host genome. In addition, the availability of a reverse genetics system to create recombinant viruses that express foreign proteins has enhanced the

development of NDV as a vaccine vector (reviewed in Huang et al., 2003). Several studies have already shown some success in using NDV as a vector to express proteins from other viruses such as infectious bursal disease virus (Huang et al., 2004), influenza virus (Nakaya et al., 2001), human parainfluenza virus type 3 (Bukreyev et al., 2005), respiratory syncytial virus (Martinez-Sobrido et al., 2006) and severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (DiNapoli et al., 2007).

NDV can selectively kill tumor cells making it a good candidate for use in anticancer therapy. Some strains of NDV (natural or derived) caused regression of human tumors without affecting the surrounding normal tissue (Cassel and Garrett, 1965; Schirrmacher et al., 2001; Pecora et al., 2002). Several Phase I/II studies in patients with tumors also demonstrated the oncolytic ability of NDV (Cassel et al., 1983; Freeman et al., 2006; Pecora et al., 2002). It has also been shown that the oncolytic properties of NDV can be improved by using reverse genetics to generate recombinant NDV that expresses immunostimulatory molecules such as interleukin-2 (Vigil et al., 2007).

1.1.2   Virion Structure and Genome Organization

The RNA genome of NDV is 15,186 nucleotides in size (de Leeuw and Peeters, 1999) and contains six genes in the order 3'-NP-P-M-F-HN-L-5', which encode six major structural proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and the large (L) RNA-dependent RNA polymerase (Lamb and Kolakofsky,

2001). In addition to these gene products, NDV produces two additional proteins (designated V and W proteins) by an RNA editing event that occurs during transcription of the P gene (Steward et al., 1993).

NDV particles are pleomorphic as revealed by electron microscopy (Figure 1A). Generally, they are rounded and vary in diameter from 100-500 nm, although filamentous forms of about 100 nm across and of variable length are often seen (Alexander, 1988). The virion (Figure 1B) consists of a core of genomic RNA encapsidated by nucleocapsid protein to which is bound an RNA polymerase complex composed of L protein and phosphoprotein. This core is surrounded by a lipid envelope coated with matrix protein on its inner surface. The F and HN glycoproteins form spike-like protrusions on the outer surface of the virion. Both proteins play important roles in the initiation of infection (Nagai et al.,1989).

1.1.2.1   NP, L, P and M proteins

NP Protein

The nucleocapsid protein (NP) is the most abundant protein in NDV-infected cells and in virus particles. The NP gene is 1.747 kb in size and is located at the 3' end of the genome adjacent to a 53-nucleotide leader sequence in the genomic RNA of NDV (Ishida et al., 1986; Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999). The NP protein is composed of 489 amino acids with a predicted molecular weight of 53 kDa (Ishida et al., 1986; Errington and Emmerson, 1997). The N-terminal region of the NP protein is involved with

A.

B.

Larger glycoprotein (HN, H, G) (viral attachment protein)

Smaller glycoprotein (fusion [F])

Lipid bilayer

Nucleocapsid

Matrix (M) protein
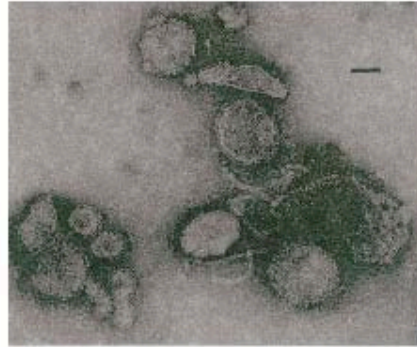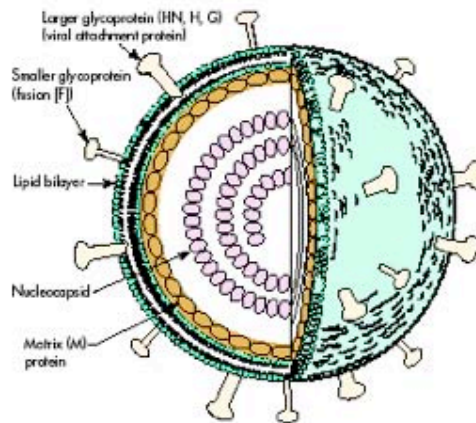
Figure 1. Virion structure of Newcastle Disease Virus
(A) Electron micrograph of pleomorphic particles of NDV (from Newcastle Disease, edited by D.J. Alexander). B) Diagram of a paramyxovirus (from Medical Microbiology, 5th edition).

encapsidation of RNA and forms the helical nucleocapsid, whereas the C-terminal region binds P (Buchholz et al., 1994). The NP protein is essential for viral replication and serves several functions, including encapsidation of the genomic RNA into an RNase-resistant nucleocapsid which acts as the template for RNA synthesis, interaction with P and L proteins during transcription and replication, and association with M protein during virus assembly (Lamb and Kolakofsky, 2001).

L protein

The L protein is the least abundant protein in virus particles. The L gene is the last to be transcribed in the viral genome and encodes a protein consisting of 2200 amino acids (Lamb and Kolakofsky, 2001). The L protein associates with P to form the active viral polymerase (Hamaguchi et al., 1983). The polymerase complex recognizes the helical nucleoprotein complex wherein the NP protein is tightly associated with the genomic RNA (Poch et al., 1990). L also has 5' capping and poly(A) polymerase activities on the nascent mRNA (Ishihama and Barbier, 1994).

Phosphoprotein

The phosphoprotein of NDV is composed of 395 amino acids and has a predicted molecular weight of 42 kDa (McGinnes et al., 1988; Daskalakis et al., 1992; Steward et al., 1993). However, it has a relative mobility of 53 kDa by SDS-PAGE analysis (Collins et al., 1980; Chambers and Samson, 1980; Morrison and Simpson, 1980). P is an essential subunit of the viral RNA-

dependent RNA polymerase (Lamb and Kolakofsky, 2001). In complex with the L protein, the P protein plays multiple roles during transcription and replication of the viral genome. The P protein acts as a chaperone to prevent uncontrolled encapsidation of non-viral RNA by NP (Errington and Emmerson, 1997).

Matrix Protein

The M protein is composed of 341 to 375 amino acids with a molecular weight of approximately 38.5 to 42.5 kDa. It is a basic protein (net charge at neutral pH of +14 to +17) and somewhat hydrophobic. M proteins are peripherally associated with membranes and are seen underlying the viral lipid bilayer (Lamb and Kolakofsky, 2001). The M protein is thought to orchestrate the assembly of virus particles at the plasma membrane of infected cells through interactions with the plasma membrane, the viral glycoproteins and the nucleocapsid (Peeples, 1991).

1.1.2.2 Viral Glycoproteins

A. Structure and Function of HN

The HN glycoprotein is a type II integral membrane protein with a molecular weight of 74 kDa (Schuy et al., 1984). The ectodomain consists of a stalk region that supports a terminal globular head. HN exists on the surface of virions and infected cells as a tetramer consisting of pairs of homodimers (Thompson et al., 1988). HN is a multifunctional protein. It is responsible for attachment of the virus to sialic acid-containing cell surface receptors. It also possesses neuraminidase (NA) activity that cleaves sialic acid from progeny

virus particles to prevent viral self-aggregation. HN also promotes the fusion activity of the F protein (Scheid and Choppin, 1974).

The attachment and neuraminidase activities of HN reside in the globular head (Thompson and Portner, 1987; Mirza et al.,1993). The crystal structure of the globular head region (residues 124-570) of HN from the Kansas strain of NDV was solved both ligand-free and in complex with either sialic acid or a NA inhibitor (Crennell et al., 2000). The ligand-free HN dimer was crystallized at pH 4.6, whereas the liganded dimer was co-crystallized at pH 6.5. (Figure 2). Comparison of these structures led the authors to conclude that both receptor binding and NA activities are mediated by a single site, which is able to switch between two different states through a conformational change. However, the ligand-free structure poses a problem because the distance between amino acid residues at position 124 of each monomer is too large to allow disulfide bond formation between cysteine residues at position 123 in several NDV isolates (Sheehan et al., 1987). This led the authors to believe that the ligand-free structure may be an artifact of the low pH at which the crystals were grown. Subsequent studies by the same group showed evidence for a second sialic acid binding site, composed of residues from each monomer at the membrane-distal end of the dimer interface (Figure 2) (Zaitsev et al., 2004).

Our lab previously characterized a panel of monoclonal antibodies (MAbs) raised against the HN glycoprotein of the Australia-Victoria (AV) strain of NDV. These MAbs have been used in competition antibody binding assays and
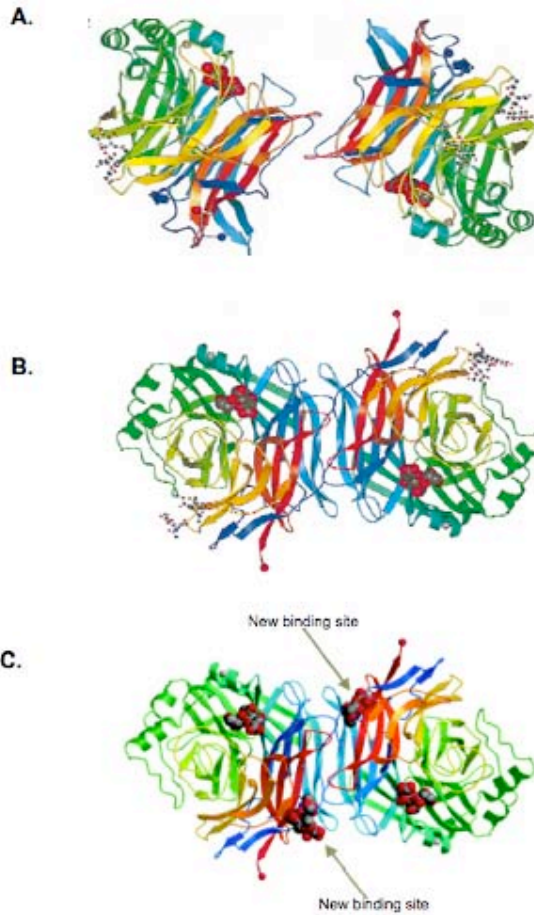
Figure 2. Schematic representations of the crystal structure of NDV HN
(A) The ligand-free HN dimer at pH 4.6. Taken from Crennell et al., 2000.
(B) The HN dimer complexed with 2-deoxy-2,3-dehydro-N-acetylneuraminic acid at pH 6.5. Taken from Crennell et al., 2000. (C) The HN dimer showing the location of the second sialic acid binding site (indicated as new binding site). Taken from Zaitsev et al., 2004.

additive neutralization assays to delineate several antigenic sites on HN. Initially, we identified four antigenic sites: 1, 2, 3 and 4. Subsequent MAbs were shown to map to sites, which overlap two of the original four sites. Hence, they were named to reflect this. Three additional sites (12, 14, 23) were identified resulting in a total of seven antigenic sites that form a continuum in the three dimensional conformation of the molecule (Figure 3)(Iorio et al., 1986; Iorio and Bratt, 1983; Iorio et al., 1989). Escape mutants were selected with MAbs to each site and sequenced to identify the following epitopes: site 1 (residue 345), site 2 (residues 513, 514, 521 and 569), site 3 (residues 263, 287 and 321), site 4 (residues 332, 333 and 356), site 12 (residues 494 and 516), site 14 (residues 347, 350 and 353) and site 23 (residues 193, 194 and 201) (Iorio et al., 1991). Only site 14 MAbs recognize a linear epitope, defined by residues 341 to 355; all other sites are conformational (Iorio et al., 1991). In addition, antibodies to sites 1, 4 and 14 recognize a broad range of strains, while those to the other sites exhibit various degrees of strain specificity (Iorio et al., 1986; Iorio et al., 1984).

B. Structure and Function of F

The F glycoprotein is a type I integral membrane protein composed of 553 amino acids. It is synthesized as an inactive precursor, F_0, which must be proteolytically cleaved to produce the active fusion protein, which consists of disulfide-linked F_1 and F_2 polypeptides (Scheid and Choppin, 1974). Cleavage of F_0 to F_1 and F_2 by host cell proteases is required for progeny virions to become infectious (Garten et al., 1980; Nagai et al., 1976).
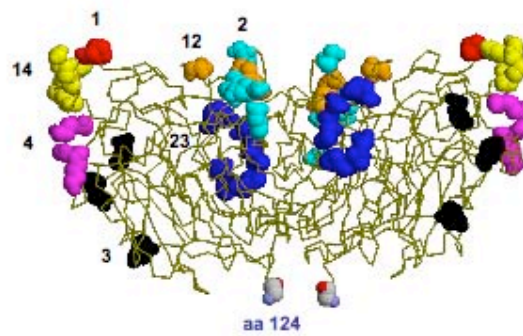
Figure 3. Schematic representation of HN dimer, showing seven overlapping antigenic sites.
Each antigenic site is shown in a different color. Residue 124 is at the top of the stalk of HN. This diagram was generated using RasMol.

The crystal structure of F from the Queensland strain (V4) of NDV has been solved (Chen et al., 2001). The structure revealed that F exists as a homotrimer organized into head, neck and stalk regions. The head region is composed of a highly twisted β domain and an immunoglobulin-like β domain. The neck consists of the C-terminal extension of the heptad repeat region HR-A, capped by a bundle of four α helices. The C-terminus of HR-A is enclosed by helix HR-C and a four-stranded β domain. The stalk is composed of the remaining portion of the HR-A coiled coil and by a polypeptide immediately N-terminal to HR-B (Figure 4).

1.1.2.3 Accessory Proteins V and W

Two accessory proteins, designated V and W, are produced from the P gene of NDV by an RNA editing event that occurs during transcription (Steward et al., 1993). RNA editing, which involves insertion of pseudotemplated G residue(s) occurs by a polymerase stuttering mechanism (Hausmann et al., 1999; Hausmann et al., 1999; Vidal et al., 1990). The P gene mRNA of NDV is edited by insertion of one or two G residues into a run of G residues within the conserved editing site (5′-AAAAAGGG) (Mebatsion et al., 2001; Steward et al., 1993). Insertion of one G residue generates the V-encoding mRNA, whereas insertion of two G residues gives rise to the W-encoding mRNA. The unedited mRNA codes for P. As a result, P, V and W proteins are amino coterminal, but vary at their carboxyl termini in both length and amino acid composition (Figure 5A). Analysis of mRNAs generated from the P gene showed that 68% were

Figure 4. Crystal Structure of NDV F trimer

(A) The left panel shows the NDV F trimer, with the head pointing up. The head, neck and stalk regions are indicated. The right panel shows the top view of the F trimer , with the head pointing out. The three monomers of the F trimer are colored red, yellow and purple. (B) Diagram of the head, neck and stalk regions of NDV F, showing the domains that make up each region. Taken from Chen et al., 2001.

Figure 5. Paramyxovirus V proteins
(A) Diagram shows that the P, V and W proteins of NDV are amino coterminal, but differ at their carboxy termini in length and amino acid composition. The frequency of mRNA encoding each protein is also indicated. (B) Sequence alignment of the conserved cysteine-rich C-terminal domains of paramyxovirus V proteins. Amino acids in black are conserved in at least seven V proteins. Amino acids in grey are conservative changes. Taken from Childs et al., 2007.

P-encoding mRNA, 29% were V-encoding mRNA, and 2% were W-encoding mRNA (Mebatsion et al., 2001).

The V protein of NDV is composed of 239 amino acids and has a molecular weight of 36 kDa (Samson et al., 1991). The NDV V protein, like other paramyxovirus V proteins, has a cysteine-rich C-terminal domain that binds two atoms of zinc (Steward et al., 1995). The sequence of the C-terminal domain of the V protein is highly conserved among paramyxoviruses (Figure 5B). This domain, which contains seven cysteine residues, is similar to a classic zinc finger domain (Klug and Rhodes, 1987). The V protein of NDV, SV5 and mumps virus is incorporated into virions, unlike that of Sendai virus and measles virus (Kubota et al., 2001; Mebatsion et al., 2001; Paterson et al., 1995; Horikami et al., 1996; Valsamakis et al., 1998). The V protein plays a role in viral replication and virulence of NDV (Mebatsion et al., 2001; Huang et al., 2003). The W protein of NDV consists of 181 amino acids (Steward et al., 1993). Its function is currently unknown.

1.1.2.4   Replication

Replication of NDV (Figure 6) is initiated by binding of HN to sialic acid-containing receptors on the host cell surface. Fusion of the viral and cell membranes is mediated by the F protein with the help of the HN protein. Transcription, protein synthesis, and replication of the genome take place in the host cell's cytoplasm. The genome is transcribed into messenger RNAs (mRNAs) and a full-length positive-sense RNA strand. The viral mRNAs are translated to
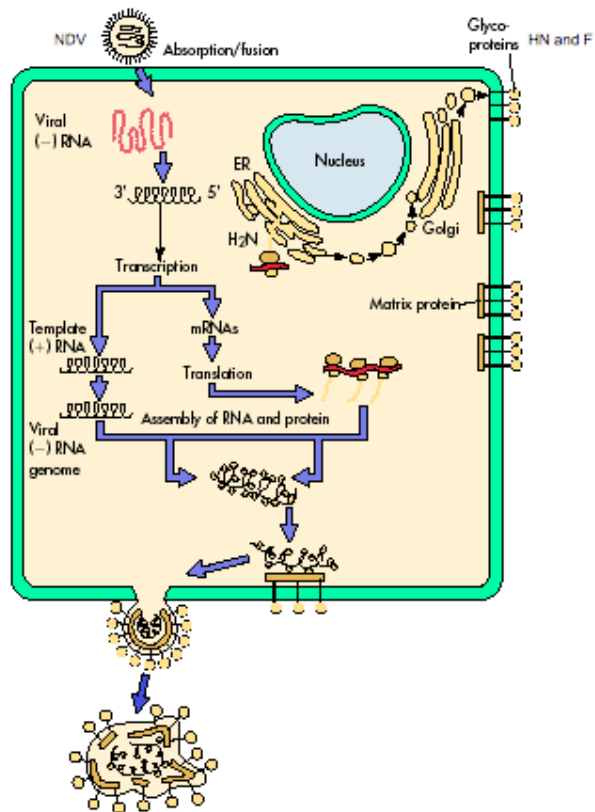
Figure 6. Diagram of NDV replication cycle
NDV binds to sialic acid-containing receptors and fuses with the host cell
membrane. Transcription, protein synthesis, and replication of the genome
occur in the cytoplasm. Mature virions exit the cell by budding. (adapted
from Medical Microbiology, 5th edition).

produce viral proteins. The full-length positive-sense strand is used as a template for full-length negative-sense strand synthesis. New full-length negative-sense strand may serve as templates for replication or transcription, or they may be packaged into new virions. The viral glycoproteins (HN and F) are transported to the cell membrane. New genomes associate with the L, P and NP proteins to form nucleocapsids. The M protein enables nucleocapsids to interact with regions of the cell membrane that had been modified with the viral glycoproteins. Mature virions then bud from the cell membrane and exit the cell (Lamb and Kolakofsky, 2001).

1.2 Pathotypes and Determinants of Virulence

1.2.1 NDV strains and pathogenicity tests

NDV causes respiratory, neurological, or enteric disease in many species of birds. Strains of the virus are classified into three pathotypes based on the severity of disease in chickens. Avirulent strains that produce mild or asymptomatic infections are termed lentogenic, whereas virulent strains that cause acute infections with high mortality are termed velogenic. Strains of intermediate virulence are termed mesogenic. Velogenic strains are further subdivided into two categories: viscerotropic velogenic (VV) strains that cause an acute lethal disease with frequent visceral hemorrhage, and neurotropic velogenic (NV) strains that cause an acute lethal disease preceded by neurological signs (Alexander, 1988).

The pathogenicity of a given NDV isolate can be determined using several assays. One way is to measure the mean embryo death time (MDT). In this assay, virus is injected into embryonated chicken eggs and the average time it takes for the virus to kill the embryo is used as a measure of virulence. The MDT for lentogens is >90 hours, for mesogens is 60-90 hours and for velogens is <60 hours. Another way is to assess the intracerebral pathogenicity index (ICPI). Virus is injected intracerebrally into 1-day-old, specific-pathogen-free (SPF) chickens. The birds are scored for disease symptoms and mortality (0 if normal, 1 if sick, and 2 if dead) over a period of 8 days. The ICPI value is the mean score per bird per observation. Isolates with an ICPI value of ≤0.5 are classified as lentogenic whereas those with ICPI values of 1.0-1.5 and 1.5-2.0 are classified as mesogenic and velogenic strains, respectively. Another method is to determine the intravenous pathogenicity index (IVPI). Virus is inoculated intravenously into 6-week old SPF chickens. The birds are scored for disease symptoms and mortality (0 if normal, 1 if sick, 2 if paralyzed, and 3 if dead) over a period of 10 days. The IVPI value is the mean score per bird per observation. Isolates with IVPI values of ≤0.5 are categorized as lentogens and those with IVPI values of 0.5-2.0 and 2.0-3.0 are classified as mesogens and velogens, respectively (Alexander, 1988; Saif, 2003). Examples of NDV strains and their pathotypes are shown in Table 2.

NDV strain	Pathotype	Mean embyo death time (MDT)	Intracerebral pathogenicity index (ICPI)	Intravenous Pathogenicity Index (IVPI)
Ulster 2C	Lentogenic	>150	0.0	0.0
Queensland V4	Lentogenic	>150	0.0	0.0
Hitchner B1	Lentogenic	120	0.2	0.0
F	Lentogenic	119	0.25	0.0
La Sota	Lentogenic	103	0.4	0.0
H	Mesogenic	48	1.2	0.0
Mukteswar	Mesogenic	46	1.4	0.0
Roakin	Mesogenic	68	1.45	0.0
Beaudette C	Mesogenic	62	1.6	1.45
Texas GB	Velogenic	55	1.75	2.7
NY parrot 70181	Velogenic	51	1.8	2.6
Italien	Velogenic	50	1.85	2.8
Milano	Velogenic	50	1.9	2.8
Herts 33/56	Velogenic	48	2.0	2.7

Table 2. Pathogenicity indices for some strains of Newcastle Disease Virus.
(from Diseases of Poultry, by Y.M. Saif)

1.2.2 Role of the F protein in virulence

It has been established that cleavage of the fusion protein precursor (F_0) is the primary determinant of NDV virulence. F_0 is cleaved at a basic amino acid-rich region, resulting in the formation of the active fusion protein consisting of disulfide-linked F_1 and F_2 polypeptides (Scheid and Choppin, 1974). Virulent strains have two pairs of basic amino acids (RRQRR or RRQKR) at the cleavage site (Glickman et al., 1988; Toyoda et al., 1989). This structural feature permits host proteases such as furin and PC6 (Gotoh et al., 1992; Sakaguchi et al., 1994), which are found in a wide range of tissues, to cleave F_0 and render the virus infectious and able to spread to various organs, leading to fatal systemic infection. In contrast, avirulent strains do not possess a pair of dibasic amino acids at the cleavage site. The F_0 of avirulent strains is cleaved only by trypsin-like proteases secreted by a limited number of tissues in the respiratory and intestinal tracts, so that these strains only produce localized infections, resulting in mild or asymptomatic infection (Nagai et al., 1976).

1.2.3 Role of HN and V proteins in virulence

Although cleavage of F is the major determinant of virulence, several studies have shown that it is not the sole determinant. Modification of a lentogenic F cleavage site to a velogenic one increased virulence, but not to the level of velogenic strains (Panda et al., 2004; Peeters et al., 1999). This indicates that other viral proteins in addition to F also contribute to virulence. Huang et al. (2004) showed that the HN protein plays a role in viral tropism and

virulence. The HN gene of the mesogenic recombinant Beaudette C (BC) strain was exchanged with that of the lentogenic recombinant La Sota strain, creating a BC virus having the HN of La Sota and a La Sota virus having the HN of BC. Pathogenicity studies showed that the BC virus having the HN of La Sota decreased in virulence and the La Sota virus having the HN of BC increased in virulence, indicating that HN plays a role in this process.

Srinivasappa et al. (1986) previously isolated a monoclonal antibody (AVS-I) raised against the avirulent La Sota strain of NDV. In a hemagglutination inhibition (HI) assay, they found that AVS-I reacted exclusively against lentogenic strains of NDV (B1-Hitchner, La Sota, Queensland V4, and Ulster), though it did not react with two other lentogenic strains (ENG F and NEB GOL). AVS-I also exhibited HI activity towards ten commonly employed commercial B1-Hitchner and La Sota vaccine strains. Most importantly, AVS-I did not react with three mesogenic (ENG P3R10, Roakin and Kimber) or six velogenic (GB Texas, Largo, Calif 1083, KM, P1307, and P5658) strains tested. These data suggested that this antibody recognizes an epitope that is more conserved in lentogenic strains and have led to its use in identifying strains of NDV belonging to this pathotype. These findings raise the possibility that antibody AVS-I may identify a domain on HN that contributes to virulence.

Several studies have shown that the V protein also plays a role in virus replication and virulence. Recombinant viruses lacking V protein expression have impaired growth in cell cultures and 6-day-old chicken embryos, and no growth in

9- to 11-day-old embryonated chicken eggs (Mebatsion et al., 2001; Huang et al., 2003). In addition, pathogenicity studies showed that V-deficient recombinant viruses were highly attenuated in 1-day-old and 6-week-old chickens, indicating that the V protein plays an important role in NDV virulence. These mutant viruses also showed increased sensitivity to the antiviral effects of exogenous interferon (IFN) (Huang et al., 2003). Using an IFN-sensitive NDV-GFP-based assay, it was demonstrated that the V protein of NDV exhibits IFN antagonistic activity and that this activity is located in the carboxy-terminal region of the protein (Park et al., 2003). Therefore, the mechanism by which V protein plays a role in NDV virulence is presumably due, at least in part, to its ability to antagonize interferon.

1.3 IFN signaling

1.3.1 Overview of type I IFN signaling

The interferon response is the first line of host defense against viral infection. When a virus infects a host cell, double-stranded RNA (dsRNA) is generated during transcription and /or replication. One of the signaling pathways activated by dsRNA results in induction of type I IFN (IFNα/β) (Goodbourn et al., 1985; Ryals et al., 1985). Double-stranded RNA is recognized by pattern recognition receptors (PRR), such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) and Toll-like receptor (TLR) 3 (Yoneyama et al., 2004; Yoneyama et al., 2005; Kato et al., 2006; Alexopoulou

et al., 2001). Recognition of dsRNA triggers a cascade that leads to phosphorylation of transcription factors called interferon regulatory factor (IRF)-3 and IRF-7 (Figure 7). Activated IRF-3 and IRF-7 homodimerize or heterodimerize and translocate to the nucleus and induce expression of small amounts of IFNα and IFNβ. Secreted IFNα/β bind to the type I IFN receptor and activate the receptor-bound kinases Jak1 and Tyk2, which phosphorylate signal transducer and activator of transcription (STAT)1 and STAT2. The activated STAT proteins associate with IRF-9 to form a transcription factor complex known as IFN-stimulated gene factor 3 (ISGF3). The ISGF3 complex then translocates to the nucleus and induces transcription of more IRF7. Following recognition of dsRNA, newly synthesized IRF-7 is activated and leads to induction of large amounts of type I IFN, creating a positive feedback loop. The ISGF3 complex also binds to IFN-sensitive response elements (ISRE) within the promoters of IFN-stimulated genes (ISGs), resulting in transcriptional upregulation of these genes and establishment of an antiviral state (reviewed in Honda and Taniguchi, 2006). Among the antiviral proteins induced in response to IFN are 2',5'-oligoadenylate synthetase (OAS), RNA-dependent protein kinase (PKR), and the Mx proteins (Clemens, 1997; Floyd-Smith et al., 1981; Haller et al., 1998).

The IFN response is also induced by DNA derived from pathogens or the host. Recent studies demonstrate the existence of a cytosolic DNA-sensing machinery which is independent of that mediated by TLR9, the membrane
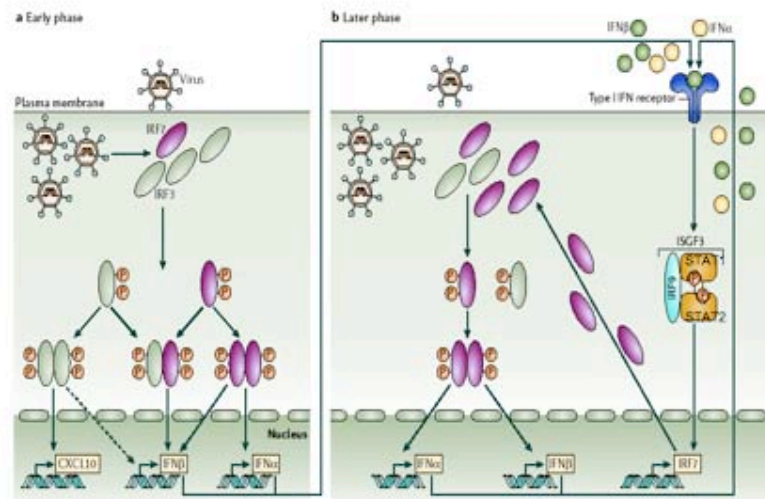
Figure 7. Positive feedback regulation of type I interferon genes
(adapted from Honda and Taniguchi, 2006, Nature Reviews Immunology)

receptor activated by hypomethylated DNA. Martin and Elkon (2006) showed that DNA transfection of dendritic cells (DCs) resulted in production of IFNα in a TLR9-independent manner. Ishii et al. (2006) demonstrated that TLR9-deficient mouse embryonic fibroblasts (MEFs) produced IFNβ upon transfection with genomic DNA isolated from viruses, bacteria or mammals. Synthetic double-stranded DNA (dsDNA), but not single-stranded DNA (ssDNA), stimulated stromal cells and DCs, leading to production of type I IFN. In addition, DNA-transfected cells were resistant to DNA (modified vaccinia virus Ankara) and RNA (vesicular stomatitis virus) viral infection. Shirota et al. (2006) also showed that dsDNA-transfected MEFs were protected against herpes simplex virus-2 infection. Takaoka et al. (2007) have identified DAI (DNA-dependent activator of IFN regulatory factors) as a cytosolic DNA sensor that can activate type I IFN and other immune responses. DAI binds to dsDNA from a variety of sources and associates, either directly or indirectly, with IRF-3 and TANK-binding kinase 1 (TBK1).

1.3.1.1   Some components of IFN signaling

A. STAT proteins

The STAT proteins are a family of cytoplasmic transcription factors consisting of seven mammalian members (STAT1, 2, 3, 4, 5a, 5b and 6). STATs range in size from 748 to 851 amino acids (90-115 kDa) and share a conserved structure consisting of six domains. The amino terminal domain stabilizes STAT dimer-to-dimer interactions. The coiled coil domain is important for interaction

with other proteins. The DNA binding domain contains several residues conserved in all members of the family. The linker domain separates the DNA-binding domain from the Src homology 2 (SH2) domain and plays a role in transcription. The SH2 domain is important for receptor binding and dimerization. It contains a conserved tyrosine residue. Phosphorylation of this residue activates the STAT protein and allows it to interact with the SH2 domain of another STAT. The transcriptional activation domain (TAD) mediates interactions with nuclear coactivators to facilitate chromatin modifications and transcriptional activation (reviewed in Brierley and Fish, 2005).

Although type I IFN induces activation of all members of the STAT family, STAT1 and STAT2 are the ones that are important in type I IFN signaling. Binding of type I IFN to its receptor activates a cascade that leads to phosphorylation of STAT2 on Y690 and STAT1 on Y701. The activated STAT1 and STAT2 associate with IRF-9 (also known as p48 or ISGF3γ) to form the ISGF3 complex that localizes to the nucleus. STAT1 and IRF-9 of the ISGF3 complex bind the ISRE AGTTTN$_3$TTTC to induce gene transcription. STAT2 of this complex does not contribute to DNA binding but provides a potent TAD (reviewed in Brierley and Fish, 2005).

B. p38

P38 is a member of the mitogen-activated protein (MAP) kinase superfamily. Protein kinases are critical players in a variety of signaling events. Studies showed that the p38 MAP kinase is activated in cells treated with IFNα

and that inhibitors of the p38 MAP kinase signaling pathway block IFN-inducible transcription in luciferase promoter assays. Binding of IFNα to the type I IFN receptor triggers a cascade that leads to activation of the p38 MAP kinase which in turn, activates downstream substrates that play important roles in transcription of genes that mediate the IFN response (reviewed in Katsoulidis et al., 2005).

C. RNA helicases

The RNA helicases RIG-I and MDA-5 are cytoplasmic sensors of viral RNA (Yoneyama et al., 2004; Yoneyama et al., 2005). It has been shown that RIG-I binds to 5'-triphosphate RNA (Hornung et al., 2006). Many of the RNA species in the cytosol of eukaryotic cells do not have a free 5'-triphosphate group. Hence, RIG-I is able to distinguish viral RNA from self RNA. RIG-I and MDA-5 are members of the DexD/H box-containing RNA helicase family. They share a conserved structure consisting of two N-terminal caspase recruitment domains (CARD) and a C-terminal DexD/H box helicase domain. The CARD is responsible for activation of downstream signaling and the helicase domain regulates CARD. It is believed that the helicase domain is activated upon binding to dsRNA, resulting in a conformational change that exposes CARD to downstream effectors.

Studies using knock-out mice lacking either RIG-I or MDA-5 demonstrated that these helicases are not redundant in their ability to recognize viral RNA. RIG-I is essential for the recognition of negative-strand RNA viruses such as Sendai virus, vesicular stomatitis virus and influenza A virus, and the positive-

strand Japanese encephalitis virus (Kato et al., 2006). The absence of MDA-5 did not affect the IFN response to these viruses. MDA-5 is required for the recognition of picornaviruses such as encephalomyocarditis virus, Mengo virus and Theiler's virus (Gitlin et al., 2006; Kato et al., 2006). The basis for the differential detection of these viruses is poorly understood, although some evidence suggests that it is due to differences in viral RNA. Uncapped 5'-triphosphate RNA is present in viruses recognized by RIG-I, but absent in viruses recognized by MDA-5 (Sumpter et al., 2005; Neumann et al., 2004; Lee et al., 1977).

D. Interferon regulatory factors (IRFs)

IRFs are a family of transcription factors that play important roles in host defense, immune cell development, cell cycle regulation, apoptosis and oncogenesis (Taniguchi et al., 2001; Barnes et al., 2002). The mammalian IRF family consists of nine members (IRF-1 to IRF-9) (Taniguchi et al., 2001). They share a conserved DNA-binding domain located at the N-terminus. This domain recognizes a consensus DNA sequence known as the IFN-stimulated response element (ISRE) which was first identified in the promoters of genes that are induced by type I IFN (reviewed in Honda and Taniguchi, 2006). The carboxy terminal regions of IRFs, except IRF-1 and IRF-2, contain an IRF-associated domain (IAD) that mediates homomeric and heteromeric interactions with other IRFs or transcription factors such as STATs (Taniguchi et al., 2001).

IRF-3 and IRF-7 have gained much attention as the key regulators of type I IFN gene expression induced by viruses. IRF-3 is expressed constitutively in most cell types and resides in the cytoplasm in an inactive form (Au et al., 1995). Viral infection leads to phosphorylation of specific serine residues in its C-terminal region (Lin et al., 1998). Two kinases involved in activation of IRF-3 are IκB kinase-ε (IKK-ε) and TBK1 (Fitzgerald et al., 2003; Sharma et al., 2003). Phosphorylated IRF-3 forms a homodimer or a heterodimer with IRF-7, and interacts with the co-activators CBP (cyclic-AMP-responsive-element-binding protein (CREB)-binding protein) or p300 (Weaver et al., 1998; Yoneyama et al., 1998). This complex translocates to the nucleus and binds its target DNA sequence in type I IFN genes and presumably alters the local chromatin structure through the histone acetyltransferase activity of the coactivators. Other transcription factors are recruited to initiate transcription of target genes. Unlike IRF-3, IRF-7 is produced in small amounts in most cells but is strongly induced by type I IFN signaling, making it a key factor in the positive feedback loop that enhances IFNα/β expression (Au et al., 1998; Marie et al., 1998; Sato et al., 1998). Similar to IRF-3, IRF-7 resides in the cytoplasm and is phosphorylated at serine residues in the C-terminal region following viral infection (Marie et al., 1998). IRF-7 is also activated by kinases TBK1 and IKK-ε (Fitzgerald et al., 2003; Sharma et al., 2003).

1.3.2   Virus strategies for evading the interferon response

Viruses have acquired a variety of strategies to evade the interferon response. These strategies fall into three categories: 1) suppression of IFN production, 2) interference with IFN signaling, and 3) inhibition of IFN-induced antiviral proteins (reviewed in Gotoh et al., 2002). Several IFN antagonists have been identified in both DNA and RNA viruses. The large T antigen of murine polyoma virus binds to Jak1 and renders it inactive (Weihua et al., 1998). The E6 oncoprotein of human papilloma virus (HPV)-18 interacts with Tyk2 and impairs its activation (Li et al., 1999). The immediate early protein BZLF-1 of Epstein-Barr virus associates with IRF-7 and prevents it from activating type I IFN promoters (Hahn et al., 2005). The NS1 protein of influenza virus suppresses IFN production by inhibiting activation of IRF-3 and blocks activation of the IFN-induced antiviral proteins PKR and OAS (Li et al., 2006; Min and Krug, 2006; Talon et al., 2000). The VP35 protein of Ebola virus inhibits activation of IRF-3 and PKR (Basler et al., 2003; Feng et al., 2007). The E3L protein of vaccinia virus blocks phosphorylation of IRF-3 and IRF-7 and inhibits activation of PKR (Smith et al., 2001; Chang et al., 1992). The NSP1 protein of rotavirus induces degradation of IRF-3, IRF-5 and IRF-7 (Barro and Patton, 2005; Barro and Patton, 2007).

Several paramyxoviruses encode IFN antagonists.   The nonstructural proteins, NS1 and NS2, of respiratory syncytial virus cooperatively counteract the antiviral effects of IFN (Bossert and Conzelmann, 2002; Shlender and

Conzelmann, 2000). The C proteins of Sendai virus inhibit IFN signaling by blocking phosphorylation of STAT2 and dephosphorylation of STAT1 (Gotoh et al., 2003; Komatsu et al., 2002; Saito et al., 2002). The V proteins of SV5, SV41, NDV and mumps virus block IFN signaling by targeting STAT1 for degradation (Didcock et al., 1999; Nishio et al., 2001; Huang et al., 2003; Kubota et al., 2001), whereas the V protein of hPIV2 targets STAT2 for degradation (Nishio et al., 2001; Parisien et al., 2001). The V proteins of measles virus, Nipah virus and Hendra virus inhibit IFN signaling by blocking STAT1 and STAT2 nuclear accumulation (Palosaari et al., 2003; Rodriguez et al., 2002; Rodriguez et al., 2003; Takeuchi et al., 2003). The V protein of SV5 also blocks nuclear translocation of IRF-3 (He et al., 2002). The V proteins of 13 paramyxoviruses (SV5, hPIV2, SV, mumps virus, Hendra virus, measles virus, NDV, Menangle, Mapuera, Salem, Tioman, Nipah and Porcine rubulavirus) bind to MDA-5 and block its activation of the IFNβ promoter in response to dsRNA (Andrejeva et al., 2004; Childs et al., 2007).

1.3.2.1 Mechanisms by which SV5 V protein counteracts the IFN response

The V protein of SV5 is one of the most well studied IFN antagonists. Unlike the NDV V protein, the crystal structure of the SV5 V protein in complex with the UV-damaged DNA binding protein 1 (DDB1) has been solved (Li et al., 2006). DDB1 is a cellular protein that was first identified as a subunit of a protein complex that recognizes the UV-induced DNA lesions in the nucleotide excision

repair pathway (Chu and Chang, 1988). DDB1 is also a subunit of the Cul4A-based ubiquitin ligase complex. Several studies show that paramyxoviruses such as SV5, hPIV2 and mumps virus are able to hijack the DDB1-Cul4A-Roc1 E3 complex to promote degradation of STATs (Didcock et al., 1999; Parisien et al., 2001; Ulane and Horvath, 2002). The V proteins of these viruses bind to DDB1 and recruit STATs to the Cul4A-based ubiquitin ligase complex (Andrejeva et al., 2002; Lin et al., 1998; Ulane and Horvath, 2002). DDB1 has a multidomain structure consisting of three β propellers (BPA, BPB and BPC) and a helical C-terminal domain (Figure 8A). The SV5 V protein binds to DDB1 using both its N-terminal extension and the globular core domain. It inserts its N-terminal helix into the pocket formed between BPA and BPC while the core domain interacts extensively with the BPC domain. By this interaction, the V protein is able to hijack the DDB1-Cul4A E3 complex to promote degradation of STAT proteins and block IFN signaling.

The V protein of SV5, as well as that of hPIV2 and mumps virus, facilitate degradation of STAT proteins by coordinating the assembly of a ubiquitin ligase (E3) complex consisting of cellular factors such as DDB1, cullin 4A and both target and nontarget STATs (Andrejeva et al., 2002; Ulane et al., 2003). This complex, with the components V, DDB1 and Cul4A is called V-dependent degradation complex (VDC). It has been shown that the V proteins of SV5, hPIV2, mumps, measles, Nipah and Hendra viruses can oligomerize homotypically and heterotypically. Oligomerization is mediated by the conserved
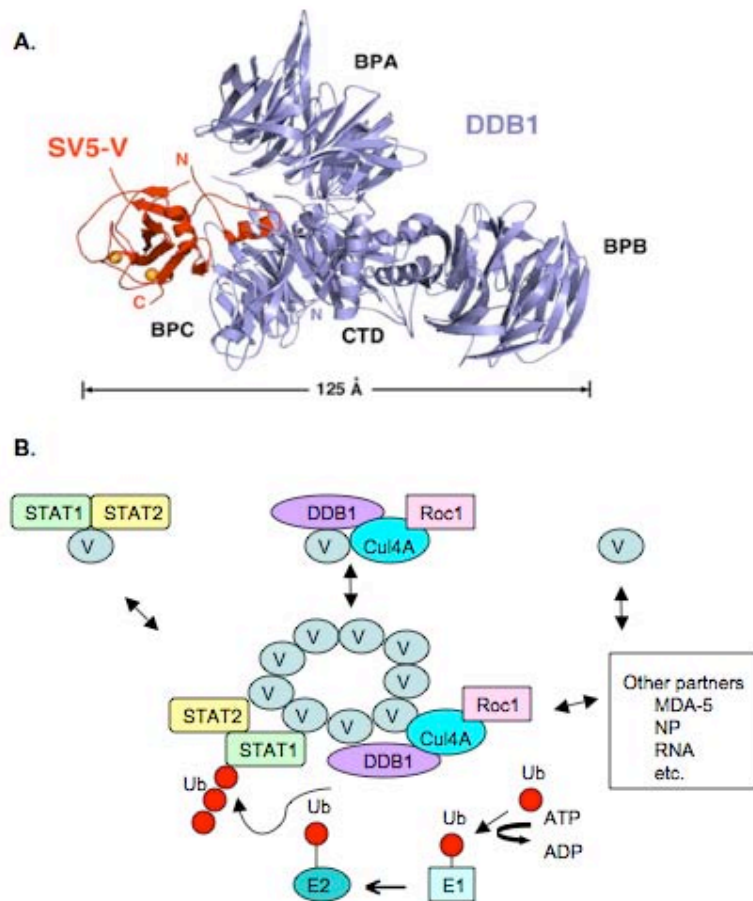
Figure 8. Paramyxovirus V proteins recruit components of the ubiquitin ligase complex.
(A) Schematic representation of the crystal structure of SV5 V protein in complex with the UV-damaged DNA binding protein 1 (DDB1). Taken from Li et al., 2006. (B) V-dependent degradation complex (VDC) structure. V proteins oligomerize to form spherical particles that act as a scaffold for E3 ubiquitin ligase activity. Adapted from Ulane et al., 2005.

C-terminal domain of the V protein (Ulane et al., 2005). In addition, the purified V protein of SV5, can self-assemble to form large spherical particles. These particles were also found in VDC preparations isolated from transfected cells. Based on these studies, Ulane et al. (2005) proposed a model for VDC assembly and function (Figure 8B). V proteins can interact with STATs or with components of the ubiquitylation machinery (DDB1, Cul4A and Roc1). These subcomplexes are brought together through V protein oligomerization via the C-terminal domain to form spherical particles that act as a scaffold for E3 ubiquitin ligase activity.

1.3.2.2 Mechanisms by which NDV V protein counteracts the IFN response

The mechanism by which the NDV V protein antagonizes IFN has been studied by two groups. Huang et al. (2003) created a recombinant virus with a carboxyl-terminus truncated V protein (rBC/V-stop) and a recombinant virus which is deficient in expression of both the V and W proteins (rBC/edit). Human 2fTGH cells were infected with these mutant viruses and the parental virus (rBC). By Western blot of cell lysates, they showed that infection with rBC resulted in degradation of STAT1, whereas infection with either rBC/V-stop or rBC/edit failed to degrade STAT1. In addition, transfection of a plasmid encoding the C-terminus of the V protein resulted in degradation of STAT1 in 2fTGH cells. From these results, the authors concluded that the C-terminus of the V protein inhibits IFN signaling by targeting STAT1 for degradation. Childs et al. (2007) showed that

the V protein of the La Sota strain of NDV, as well as 12 other paramyxoviruses, interacts with the RNA helicase MDA-5 via the conserved cysteine-rich C-terminal domain. To test whether this interaction correlated with inhibition of IFNβ induction, plasmids encoding each of the V proteins were co-transfected with an IFNβ promoter reporter plasmid in cells that were stimulated with the synthetic dsRNA poly(rI)-poly(rC). Their results demonstrated that all the V proteins were able to inhibit IFNβ induction.

The exact mechanism by which the NDV V protein achieves degradation of STAT1 has not been studied. The crystal structure of the NDV V protein also has not been solved. To date, MDA-5 is the only cellular factor known to interact directly with the NDV V protein. Although it is clear from pathogenicity studies that the V protein plays a role in NDV virulence, its role in the differential virulence exhibited by different pathotypes of the virus has not been studied.

1.4   Objectives of dissertation

The mechanisms by which HN and V proteins contribute to NDV virulence have not been completely elucidated. Therefore, the goal of this dissertation is to further investigate this process. The first project involves comparison of the IFN antagonistic activity of the V protein from a lentogenic and a mesogenic strain of the virus. The second project involves characterization of an HN-specific monoclonal antibody called AVS-I. Data from Srinivasappa et al. (1986) suggested that AVS-I recognizes an epitope that is conserved in lentogenic strains and raises the possibility that this epitope may colocalize with a

determinant of virulence in HN. A more complete understanding of these mechanisms will contribute to a greater understanding of the molecular basis for NDV virulence and may aid in development of antiviral strategies and generation of recombinant NDVs suitable for use in cancer and gene therapy.

CHAPTER II

Materials and Methods

2.1 Comparison of the IFN antagonistic activity of V proteins from two different pathotypes of NDV

2.1.1 Plasmid constructs and site-directed mutagenesis

The P genes of La Sota and Beaudette C (BC) were gifts from Dr. Siba Samal (University of Maryland). The La Sota and BC viruses were obtained from Veterinary Services Laboratory in Ames, Iowa. The P genes were cloned from these viruses within a few egg passages of the original stock (personal communication, Dr. Siba Samal). The La Sota P gene was released from the vector in which it was supplied by *Kpn*I/*Spe*I digestion and ligated into pBluescript SK(+) (pBSK) (Stratagene, La Jolla, CA). The BC P gene was released by *Nco*I/*Stu*I digestion and ligated into pBSK, which was previously modified to contain *Nco*I and *Stu*I sites. pBSK-La Sota P and pBSK-BC P constructs were transformed into *Escherichia coli* strain CJ236 (New England Biolabs, Beverly, MA) and single-stranded DNA (ssDNA) was rescued by R408 helper phage (Stratagene). To generate the V gene from the P gene, pBSK-La Sota P ssDNA template was mutated using primer 5'CGTCCAATGCTAAAAA GGG<u>G</u>CCCATGGTCGAG3' (Integrated DNA Technologies, Coralville, IA), while pBSK-BC P ssDNA template was mutated using primer 5'CGTCCAATGCT AAAAAGGG<u>G</u>CCTATGGTCGAG 3' (Integrated DNA Technologies). This allows insertion of a G nucleotide into the editing site. The mutagenic primers were

phosphorylated with T4 polynucleotide kinase (New England Biolabs), annealed to the ssDNA template, extended with T4 DNA polymerase and ligated with T4 DNA ligase (Roche, Indianapolis, IN). The mutagenesis reactions were transformed into *E. coli* strain MV1190 (Bio-Rad, Hercules, CA) and plated onto LB/Ampicillin plates. Several colonies were picked and DNA was prepared using the QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). The presence of the desired mutation was identified by DNA sequencing. To generate mutant V proteins, pBSK-La Sota V and pBSK-BC V ssDNA templates were prepared and mutagenesis was performed using mutagenic primers (see appendix for list of primers). Mutagenic primers were designed to either introduce or delete a restriction site to facilitate screening for mutants. DNA sequencing was performed to confirm the presence of the desired mutation(s). The wild type (wt) La Sota and BC V genes, and their mutants were subcloned into pCAGGS (a gift from Dr. Anne Moscona, Weill Medical College, Cornell University) by blunt-end ligation. Briefly, La Sota wt and mutant V genes were excised from pBSK by *Kpn*I/*Spe*I digestion, and BC wt and mutant V genes were excised by *Nco*I/*Stu*I digestion. These genes were blunt-ended by treatment with DNA polymerase I, large (Klenow) fragment (New England Biolabs) and T4 DNA polymerase (Roche), and ligated into pCAGGS at *Sma*I. The wt La Sota and BC V genes were also subcloned by blunt-end ligation into the bicistronic pIRES2-AcGFP1 vector (Clontech Laboratories, Inc., Mountain View, CA) to make it possible to sort untransfected (GFP-) and transfected (GFP+) cells by flow cytometry.

2.1.2 Preparation of chimeric V genes

Chimeric La Sota/BC V proteins were created by taking advantage of a unique *NgoM*IV restriction site. This made it possible to swap the N-terminal and C-terminal regions of the V protein. The pCAGGS-La Sota V and pCAGGS-BC V plasmids were digested with *Sac* I/*NgoM*IV and *NgoM*IV/*Xho* I to excise the N-terminal and C-terminal regions, respectively. The resulting fragments were run on a 1% agarose gel and purified using QIAquick gel extraction kit (Qiagen Inc.). The purified fragments were ligated to pCAGGS at *Sac*I and *Xho*I sites using T4 DNA ligase and transformed into *E. coli* strain DH5α. The colonies were selected by ampicillin resistance and chimeric DNA constructs were confirmed by DNA sequencing.

2.1.3 Cell culture

DF1 cells (chicken embryo fibroblast cell line) (a gift from Dr. Siba Samal) were maintained in Dulbecco's Modified Eagle medium (DMEM) with high glucose and L-glutamine supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 4 U/ml penicillin and 4 μg/ml streptomycin. The tissue culture reagents were obtained from Invitrogen.

2.1.4 Transient Expression

DF1 cells were seeded in 6-well plates at 3×10^5 cells per well one day prior to transfection. Two μg of each plasmid construct were transfected into DF1 cells using 5 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or 7 μl of Genejuice Transfection Reagent (Novagen, Madison, WI) according to the

protocols provided by the manufacturers. Assays were performed at 24 or 48 hours post-transfection.

2.1.5   Western blots

Western blots were performed to check expression of wt, mutant and chimeric V proteins in DF1 cells. Lysates were prepared from DF1 cells 24 hours post-transfection. Briefly, transfected cells were washed once with cold phosphate buffered saline (PBS). After adding 1 ml cold PBS, the cells were scraped on ice using a cell scraper. The cells were centrifuged for 5 min at 13,000 rpm and the pellet was lysed on ice for 30 minutes using 50 μl NP-40 cell lysis buffer (Biosource International, Inc., Camarillo, CA) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). The lysate was centrifuged for 5 min at 13,000 rpm and 20 μl of the supernatant was loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen). The gel was run under reducing conditions using the Xcell SureLock Mini-Cell Electrophoresis System (Invitrogen). Proteins were transferred onto an Immobilon-P transfer membrane (Millipore, Bedford, MA) and blocked overnight at 4°C with Detector Block (Kirkegaard and Perry Laboratories, Gaithersburg, MD). A polyclonal antibody (a gift from Dr. Siba Samal) raised against the C-terminal 18 amino acids of the V protein of BC was used as primary antibody to detect La Sota and BC wt and mutant V proteins. This antibody was diluted at 1:480 in Detector Block. Horseradish peroxidase-conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories) was used as the secondary

antibody at a dilution of 1:10,000 in Detector Block. Proteins were visualized using the Amersham ECL Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK). The membrane was stripped using Re-Blot Plus Strong Solution (Chemicon International, Temecula, CA) and blocked overnight at 4°C with 5% nonfat milk in PBS-Tween 20. As a loading control, the membrane was re-probed with anti-actin (Sigma) at a 1:1000 dilution in 0.5% nonfat milk in PBS-Tween 20.

2.1.6   NDV-GFP assay

The IFN antagonistic activity of the wt, mutant and chimeric V proteins were tested by their ability to rescue growth of an IFN-sensitive recombinant NDV (strain BC) tagged with green fluorescent protein (NDV-GFP) (a gift from Dr. Siba Samal). The enhanced GFP gene was inserted between the P and M genes of the BC cDNA and the NDV-GFP virus was rescued from cDNA (Elankumaran et al., 2006). The assay was done using the protocol described by Park et al. (2003) with some modifications. Briefly, DF1 cells were seeded in 6-well plates and transfected at 80% confluence with 2 μg plasmid construct and 5 μl Lipofectamine 2000. Each plasmid was transfected in triplicates. After 24 hours, cells were washed three times with warm PBS and infected with NDV-GFP at an moi of 0.001. The growth of the virus was monitored at 24 hours post-infection by counting the number of fluorescent cells in 3-5 fields at 20x magnification. The medium was removed and cells were lysed in 300 μl NP40 lysis buffer supplemented with 1 mM PMSF and protease inhibitor cocktail.

Lysates were loaded onto a NuPAGE 4-12% Bis-Tris gel which was run under reducing conditions using the Xcell SureLock Mini-Cell Electrophoresis System. Proteins were transferred onto an Immobilon-P membrane. Western blot was performed to monitor levels of different components of the IFN response. Proteins probed for include STAT1, STAT2, p38, IRF-3 and IRF-7. Primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and were used at the concentrations recommended by the manufacturer. These include STAT1α p91 (sc-345), STAT2 (sc-476), p38 (sc-535), IRF-3 (sc-9082) and IRF-7 (sc-9083). Horseradish peroxidase-conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories) was used as the secondary antibody at a dilution of 1:10,000. For some blots, Detector Block and/or Amplicruz Western Blot Signal Enhancement System (Santa Cruz Biotechnology, Inc.) were used as blocking reagents and dilution buffers. Densitometry was performed to quantitate the protein levels.

2.1.7 Flow cytometry

Flow cytometry was performed to sort untransfected (GFP-) and transfected (GFP+) DF1 cells. At 24 or 48 hours post-transfection, cells were washed with 2 ml DMEM supplemented with 5% FCS. The cells were incubated for 4 minutes at room temperature with 1 ml of 0.0625 mM EDTA in PBS and detached from the plate by pipetting several times and transferred to a tube. The remaining cells were washed off the plate by using 400 μl DMEM supplemented with 5% FCS and transferred to the same tube. The cells were centrifuged for 2

minutes at 13,000 rpm and the pellet was washed with DMEM supplemented with 5% FCS. The cells were resuspended in 500 µl DMEM supplemented with 1% FCS and sorted using the FACSVantage SE flow cytometry system (Becton Dickinson, Franklin Lakes, NJ). The GFP- and GFP+ cell populations were collected, washed with cold PBS and lysed using 50 µl NP40 lysis buffer supplemented with 1 mM PMSF and protease inhibitor cocktail. The lysates were run on a NuPAGE 4-12% Bis-Tris gel under reducing conditions. The proteins were transferred onto an Immobilon-P membrane and Western blot was performed as described before to monitor levels of STAT1 and IRF-7.

2.2 Characterization of AVS-I antibody

2.2.1 Enzyme-linked immunosorbent assay (ELISA)

To test the avirulent specificity of AVS-I, an ELISA was performed using intact virions of several additional strains of the virus not tested previously with this MAb. These include lentogenic, mesogenic, and velogenic strains. The two lentogenic strains are B1-Hitchner/48 (B1) and Ulster/64 (U). The mesogenic strains include NJ-Roakin/FRB/46 (F) and Massachusetts-4F/46 (M). The velogenic strains include Australia-Victoria/32 (AV), Texas-GB/48 (GB), Iowa-Salsbury/49 (IS), Kansas-Leavenworth/48 (L), and California-RO/44 (RO). Each purified virus stock was diluted to 20 µg/ml with phosphate-buffered saline (PBS), and 50 µl of this solution were placed into each well of a 96-well microtiter plate. The wells were allowed to dry overnight and blocked with 2% agamma horse serum. Fifty microliters of a 1:2,700 dilution of AVS-I-containing ascites fluid were

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added to each well and incubated at 37°C for 1 h. Horseradish peroxidase-conjugated goat anti-mouse antibody (Kirkegaard and Perry Laboratories) was added at a 1:1,000 dilution and incubated at 37°C for 1 h. Following the addition of SureBlue TMB microwell peroxidase substrate (Kirkegaard and Perry Laboratories), the absorbance was measured at 650 nm. Antibody to site 1 was used as a positive control. This antibody has previously been shown to have broad specificity for a variety of NDV strains (Iorio et al., 1984). Antibody to site 23, previously shown to be highly specific for the AV strain (Iorio et al., 1989), was used as a negative control.

2.2.2   Hemagglutination Inhibition (HI)

The ability of AVS-I to inhibit the hemagglutinating (HA) activity of our panel of NDV strains was also determined as described previously (Iorio and Bratt, 1984) with some modifications. Serial two-fold dilutions of ascites fluid (25 μl) were incubated with four HA units of purified virus in 25 μl PBS with Ca2+ and Mg2+ at 37°C for 30 min. After adding chicken erythrocytes (Biolink Inc., Liverpool, NY), plates were incubated at 4°C for 1 h. An antibody to site 1, which exhibits hemagglutination inhibition (HI) activity (Iorio and Bratt, 1984), was used as a positive control, while antibody to site 4, which has no detectable HI activity (Iorio and Bratt, 1984), served as a negative control.

2.2.3   Neutralization assay

To test the ability of AVS-I to neutralize our panel of NDV strains, 10^7 PFU/ml of each virus was incubated at 25°C for 1 h with an equal volume of

AVS-I ascites fluid diluted to an antibody concentration of 50 μg/ml in Hanks' balanced salt solution (Invitrogen). Aliquots of this mixture were plated on chicken embryo cells and plaque assays were performed as described previously (Iorio and Bratt, 1985). For avirulent strains that require trypsin to form plaques, cells were overlaid with medium 199 (Invitrogen) supplemented with 0.1% NaHCO3, 2.5% tryptose phosphate broth, 0.9% agar, and 10 μg/ml trypsin. Treatment with rabbit anti-mouse immunoglobulin (RAM Ig) (Litton Bionetics, Kensington, MD) was carried out to reduce the persistent fraction of non-neutralized virus.

2.2.4   Competition antibody binding assay

To map the binding site of AVS-I on HN relative to our panel of MAbs, reciprocal competition antibody binding assays were performed using B1 virus. By necessity, competing MAbs in this experiment are restricted to those members of our panel that recognize this strain. These include antibodies to sites 1, 14, 4, and 2, but not those to sites 12, 23, and 3. All antibodies were adjusted to an initial concentration of 2.5 mg/ml. In the first set of experiments, microtiter plates were coated with 1 μg/well of B1 virus and blocked with 2% agamma horse serum. A serial dilution of the first antibody (unlabeled AVS-I) was added and the plate was incubated at 37°C for 1 h. After several washes with PBS, the second antibody (biotinylated AVS-I or biotinylated antibody to either site 1, 14, 4, or 2) was added at a 1:2048 or 1:4096 dilution and again incubated at 37°C for 1 h. Finally, streptavidin-horseradish peroxidase (Zymed Laboratories Inc., South

San Francisco, CA) was added at a 1:5,000 dilution for 1 h at 37°C and absorbance measured as described before.

2.2.5   Neuraminidase (NA) inhibition

To further characterize the functional inhibition profile of AVS-I, its ability to inhibit the NA activity of avirulent strain B1 was determined. Virus was diluted to 0.35 mg/ml in 0.1 M sodium acetate (pH 6.0). Forty microliters of this solution was incubated at 37°C for 1 h with an equal volume of 0.2 mg/ml affinity-purified AVS-I antibody. Twenty microliters of this mixture was incubated with 0.5 ml of 625 μg/ml of neuraminlactose (Sigma) at 37°C for 1 h. The amount of N-acetyl-neuraminic acid released was determined by the method of Aminoff (Aminoff, 1961). The antibody to site 2 was used as a positive control. This antibody has previously been shown to inhibit the NA activity of the AV strain. A MAb specific for the nucleocapsid protein (NP) was used as a negative control.

2.2.6   Western blot

To determine whether the epitope recognized by AVS-I is linear or conformational, a Western blot was performed. Polyacrylamide gel electrophoresis was carried out under reducing conditions using 20 μg of viral proteins from either the B1 or U strain. The proteins were transferred to a nitrocellulose membrane and probed with MAbs to site 14 or 4 or AVS-I. Antibody to site 14 (positive control) recognizes a conserved, linear epitope, while antibody to site 4 (negative control) recognizes a conserved, conformation-dependent epitope (Iorio et al., 1984; Iorio et al., 1986; Iorio et al., 1991).

2.2.7 Chimera construction and functional assays

To begin to map the epitope recognized by AVS-I, HN chimeras consisting of domains from the velogenic strain AV and the lentogenic strain B1 were constructed by taking advantage of a unique *Nsp*I restriction site. This made it possible to exchange amino acids 344-571 of AV HN with amino acids 344-577 of B1 HN, creating the HN chimeras AV-B1 and B1-AV. These chimeras were expressed in BHK-21 cells (American Type Culture Collection, Manassas, VA) using the T7 RNA polymerase expression system (Fuerst et al., 1986). To determine whether the chimeras are functional, a hemadsorption (HAd) assay was performed. Briefly, HN chimera-expressing monolayers were incubated for 30 minutes with a 2% suspension of guinea pig erythrocytes (Biolink, Inc.) in PBS supplemented with 1% $CaCl_2$ and 1% $MgCl_2$ (PBS+). The cells were washed 3-5 times with PBS+ and adsorbed erythrocytes were lysed in 50 mM NH_4Cl. Lysates were cleared by centrifugation and HAd activity was quantitated by measuring the absorbance at 540 nm. To assess binding of AVS-I to the HN chimeras, flow cytometry was performed. A cocktail of antibodies to sites 1, 14, and 2 was used to confirm that the chimeras are, indeed, expressed at the cell surface.

2.2.8 Isolation and characterization of an escape mutant virus to AVS-I

To begin to identify the HN amino acid residues in the epitope recognized by AVS-I, an escape mutant virus was isolated. Virus (strain B1) was passaged three times in eggs. The allantoic fluid was treated with AVS-I and plated on chicken embryo cells in the presence of trypsin. A plaque assay was performed

as described previously (Iorio and Bratt, 1985). Treatment with AVS-I resulted in a 5-10% persistent fraction of non-neutralized virus. The neutralization studies with this strain suggest that, despite its escape from neutralization, this persistent fraction does have antibody bound to it. Thus, to make variant selection possible, AVS-I-neutralized virus was treated with RAM Ig. This reduces the persistent fraction to 0.1% and makes possible the isolation of escape mutants. One escape mutant, AVS-B1, was isolated and plaque purified. Genomic RNA sequencing of the HN gene of escape mutant AVS-B1 revealed the presence of a single mutation, G570R. The presence of this mutation, and only this mutation, in AVS-B1 HN was confirmed by reverse transcription-PCR. To establish that residue G570 is important for the binding of AVS-I, the G570R mutation was introduced into B1 HN through site-directed mutagenesis using the mutagenic primer 1696-CTCAAAGATGAT**AGG**GTT<u>CGCGA</u>GGCCAGGTC-1727 with the mutated codon in bold. Screening for the mutant was facilitated by the introduction of an *Nru*I site (underlined). DNA sequencing was performed to confirm the presence of the G570R mutation. The G570R-mutated HN was expressed in BHK cells using the vaccinia T7 RNA polymerase expression system (Fuerst et al., 1986) and HAd inhibition and flow cytometric analyses were performed to assess the ability of AVS-I to recognize it. HAd inhibition was performed using 300 μl of AVS-I ascites fluid with the amount of HAd quantitated by lysing of the adsorbed guinea pig erythrocytes in ammonium chloride and measurement of absorbance at 540 nm. For flow cytometry, a cocktail of

antibodies to sites 1, 14, and 2 was used to confirm that the G570R-mutated HN

is expressed at a level comparable to that of wild-type HN.

CHAPTER III

Comparison of the IFN antagonistic activity of the V proteins from a

lentogenic and a mesogenic NDV strain

Introduction

The evidence that the V protein plays a role in the virulence of NDV comes from several studies. Mebatsion et al. (2001) used the lentogenic NDV Clone-30 as the parent virus to create recombinant viruses lacking V protein expression. By deleting 6 nucleotides in the editing site and creating a stop codon in the V open reading frame (ORF), they were able to generate recombinant V-deficient viruses, which they called NDV-Δ6 and NDV-Vstop, respectively. To be able to grow these mutants in vitro without the addition of proteases, the F cleavage site was modified to that of a virulent strain (GGRQGR to GRRQKR). They found that NDV-Δ6 and NDV-Vstop viruses had impaired growth in cell cultures and 6-day-old chicken embryos and no growth in 9- to 11-day-old embryonated chicken eggs. Subsequently, Huang et al. (2003) used the mesogenic strain BC as the parent virus to create recombinant viruses lacking expression of the V protein or both the V and W proteins. By introducing a stop codon in the V frame, they were able to generate a recombinant virus with a carboxyl-terminus truncated V protein which they called rBC/V-stop. By introducing two silent nucleotide mutations in the editing site of the P gene, they were able to create a recombinant virus, which is deficient in expression of both

the V and W proteins, which they called rBC/edit. These mutant viruses showed impaired growth in cell cultures, but not in Vero cells, which lack an intact IFNα/β system. In addition, their pathogenicity studies showed that these mutant viruses were highly attenuated in 1-day-old and 6-week-old chickens.

Although these studies show that the V protein contributes to virulence, its role in the differential virulence patterns exhibited by the pathotypes of NDV has not been studied. Therefore, the **aim** of this chapter is to compare the IFN antagonistic activity of the V proteins of avirulent and virulent strains of NDV. The **hypothesis** to be tested is that the IFN antagonistic activity of the V protein of a virulent strain is greater than that of an avirulent strain. The **rationale** for this hypothesis is that if the V protein plays a role in the virulence of NDV, then its ability to antagonize IFN should be greater in virulent strains than in avirulent strains. The **approach** is to compare the IFN antagonistic activity of the V protein of avirulent or lentogenic strain La Sota and mesogenic strain BC using a modification of the NDV-GFP assay developed by Park et al. (2003). Ideally, the V protein of a lentogenic strain should be compared with that of a velogenic strain. However, these studies will eventually lead to pathogenicity studies in animals. Some virulent strains of NDV are classified as select agents. They cannot be used in animal studies at BL-3. Thus, the mesogenic strain BC was used instead.

Results

3.1 La Sota and BC V proteins are expressed in DF1 cells

The V genes of La Sota and BC were generated from the P genes by site-directed mutagenesis using primers that insert one G nucleotide into the editing site. The V genes were subcloned into pCAGGS vector and expression of the V proteins was tested by transient transfection of the plasmid constructs into DF1 cells. Lysates were prepared from transfected cells and a Western blot was performed using a rabbit antipeptide serum specific for the C-terminal 18 amino acids (RLCASDDVYDGGNITESK) of the BC V protein (V18 Ab). Figure 9A shows that both La Sota and BC V proteins are expressed, although the intensity of the band corresponding to the V protein is less for La Sota as compared to BC. Densitometric analysis showed that the intensity or density of the BC V protein band is 1.4 times greater than that of La Sota (Figure 9B). There are two possibilities to explain this result. Either La Sota V is expressed at a lower level than BC V or the V18 Ab recognizes La Sota V less efficiently than it does BC V. Comparison of the C-terminal 18 amino acids of the V proteins reveals an amino acid difference between La Sota and BC at position 234. La Sota V has an aspartic acid (D), whereas BC V has an asparagine (N) at this position. It is possible that this amino acid difference affects the ability of the serum to recognize the V protein, so that the V18 Ab is not able to bind La Sota V as well as it does BC V. To test this possibility, La Sota V was mutated at position 234 to the corresponding residue in BC V. Likewise, BC V was mutated at position 234

A.

B.
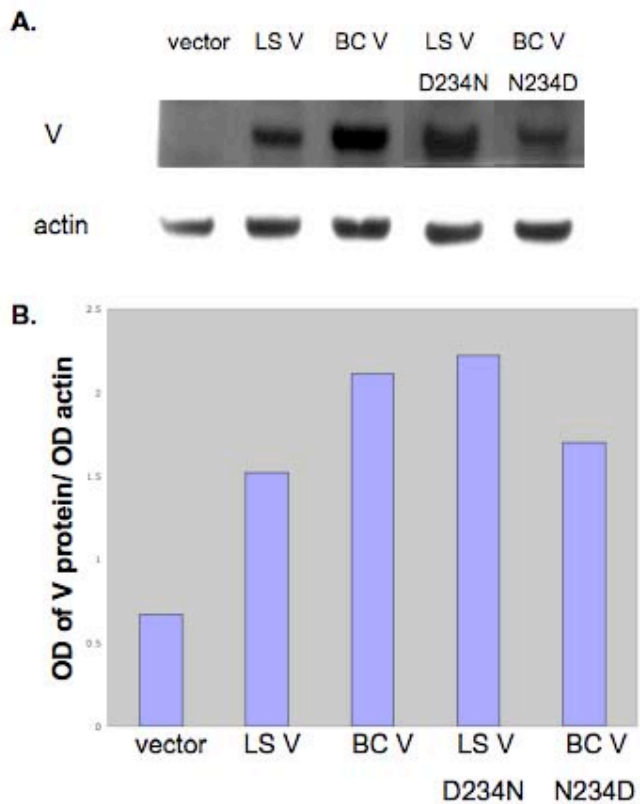
Figure 9. The V18 antibody recognizes La Sota and BC V proteins at different levels.
DF1 cells were transfected with La Sota and BC wt and mutant V plasmids to test expression of the V protein. At 24 hours post-transfection, cell lysates were prepared and Western blot (A) was performed using a rabbit antipeptide serum specific for the C-terminal 18 amino acids of the BC V protein (V18 Ab). To quantitate the protein levels, densitometry was performed using the Biorad Fluor-S MultiImager system. Values shown are the ratios of the optical density (OD) of the V protein over that of actin (B). This data represents one experiment, out of at least 2 experiments.

to the corresponding residue in La Sota V. Figure 9 shows that the La Sota V D234N mutant was recognized by the V18 Ab at a level similar to wild type BC V. Conversely, the BC V N234D mutant was recognized by the V18 Ab at a reduced level, similar to wild type La Sota V. Hence, the amino acid difference at position 234 accounts for the differential recognition of the two V proteins by the V18 Ab.

3.2 The IFN antagonistic activity of the V protein of BC is greater than that of La Sota

The IFN antagonistic activity of the La Sota and BC V proteins were tested by their ability to rescue growth of an IFN-sensitive recombinant NDV-GFP. The assay was done using the protocol described by Park et al. (2003) with some modifications. The chicken embryo fibroblast cell line DF1 was used instead of chicken embryo fibroblasts (CEFs). In addition, the recombinant NDV-GFP virus was derived from mesogenic strain BC instead of lentogenic strain B1-Hitchner. It should also be noted that both of these NDV-GFP viruses have an intact V ORF. However, the IFN-induced inhibition of NDV-GFP growth occurs before infection, so within the time frame of the assay, an antiviral state has already been established before the V protein is expressed from the virus. Thus, the V protein expressed by the virus is a minimal factor.

Figure 10A shows uninfected DF1 cells. Figure 10B shows that the NDV-GFP virus grows very well in these cells. However, treating the cells with chicken IFNα (1000 U/ml) 24 hours before infection inhibits growth of the virus (Figure 10C). This result shows that the NDV-GFP virus (strain BC) is susceptible to the

A) Uninfected cells
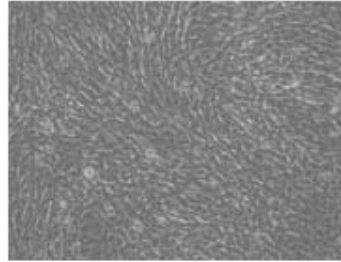
B) Infected cells
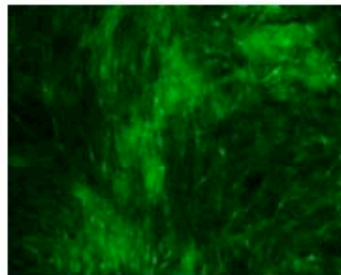
C) IFN 1000 U/ml
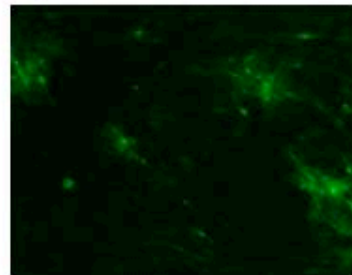
Figure 10. Chicken IFN-α inhibits growth of the NDV-GFP virus in DF1 cells.
DF1 cells were left uninfected (A), or infected with NDV-GFP (rBC-GFP) at an moi of 0.001 (B), or treated with chicken IFN-α at 1000 U/ml prior to infection with NDV-GFP at an moi of 0.001 (C). Images were taken at 20x magnification using a Carl Zeiss Axiovert 200 microscope.

antiviral effects of IFN within the time frame of the assay, consistent with the results of Park et al. (2003) for NDV-GFP virus (strain B1-Hitchner). Hence, this system is useful for screening proteins that exhibit IFN antagonistic activity. In addition, using the cell line DF1 instead of isolating CEFs is a cheaper and easier way to do the assay. Figure 11 shows that the virus also grows well in mock-transfected cells. However, transfection with the empty vector pCAGGS inhibits growth of the virus, consistent with the results of Park et al. (2003). Based on the experiments done by Park et al. (2003), the proposed explanation for this inhibition of viral growth is that transcription from the expression plasmid, in this case pCAGGS, can lead to generation of double-stranded RNA which induces the production of IFN and establishment of an antiviral state. Another possible explanation is that plasmid DNA is recognized by a cytosolic DNA sensor (i.e. DAI), which triggers IFN production. Transfection with either La Sota V or BC V plasmid construct rescues growth of the NDV-GFP virus, but at different efficiencies. As shown in Figure 11, the V protein of the mesogenic strain BC rescues growth of the NDV-GFP virus more efficiently than that of the avirulent strain La Sota. To quantitate this result, the number of fluorescent cells from 3 different fields (each field contains approximately 3000 cells) was counted. As shown in Figure 12, the ability of the BC V protein to rescue growth of the NDV-GFP virus is four times greater than that of the La Sota V protein. One caveat is that this difference in rescue of viral growth could be due only to differences in expression levels of these two V proteins. However, this possibility is unlikely

A) Mock **B) pCAGGS**

C) La Sota V (wt) **D) BC V (wt)**
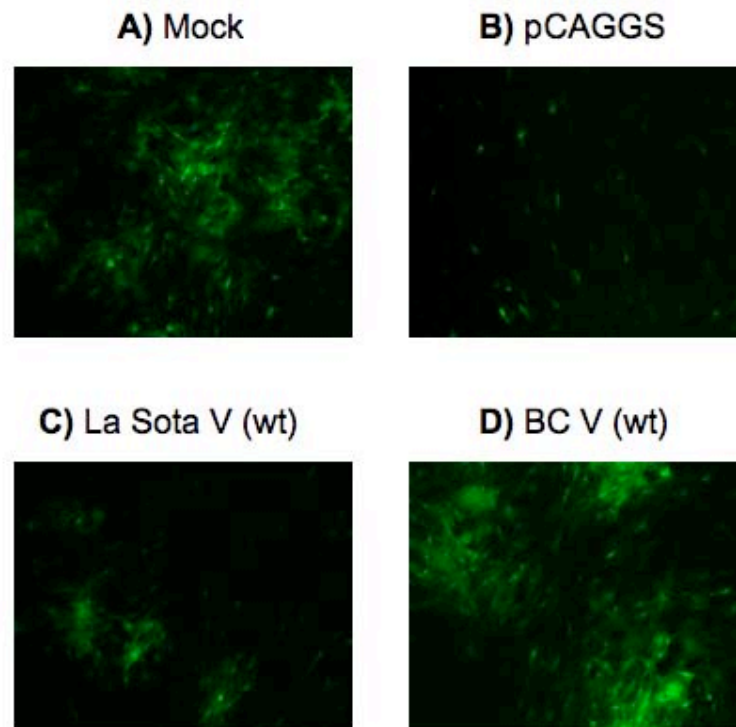
Figure 11. The V protein of BC rescues growth of the NDV-GFP virus more efficiently than that of La Sota.
DF1 cells were mock-transfected (A), or transfected with empty vector pCAGGS (B), or La Sota V (C), or BC V (D). Cells were infected with NDV-GFP virus at an moi of 0.001, 24 hours post-transfection and examined for green fluorescence 24 hours post-infection. Images were taken at 20x magnification using a Carl Zeiss Axiovert 200 microscope.
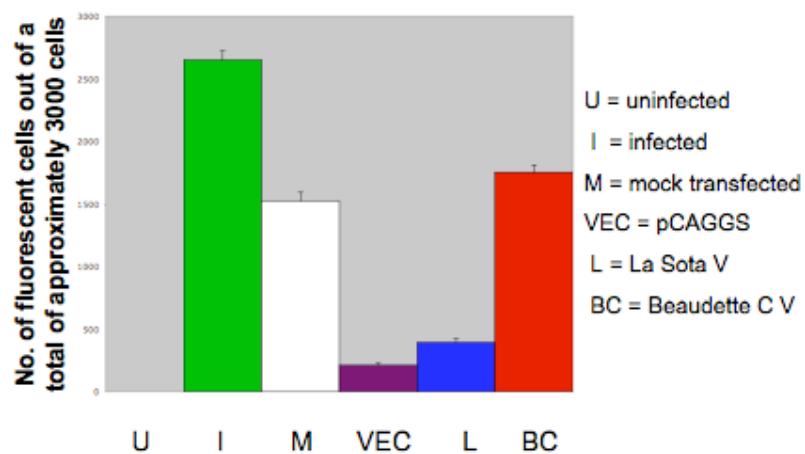
Figure 12. The BC V protein exhibits a 4-fold greater ability than the La Sota V protein to rescue growth of the NDV-GFP virus.
The experiments were performed as described in the legend to Figure 11. The growth of the NDV-GFP virus was quantitated by counting the number of fluorescent cells from 3 different fields. Values shown in the y axis are the average of the number of fluorescent cells from 3 different fields. Each field contains approximately 3000 cells and represents one well of DF1 cells. Error bars represent the standard deviations. This data represents one experiment, out of a total of four experiments. The absolute numbers of fluorescent cells vary from one experiment to another but the relative activity of the various transfected proteins is consistent between experiments.

because the expression levels, as determined by Western blot using the V18 Ab, do not correlate with the ability of La Sota and BC V mutants and chimeras to rescue viral growth, as will be discussed in the next chapter. Overall, these results are consistent with the V protein having a role in NDV virulence and that the mechanism by which it does so, is due, at least in part, to its IFN antagonistic activity.

CHAPTER IV

Identification of amino acid residues responsible for the difference in IFN antagonistic activity between the La Sota and BC V proteins

Introduction

In the previous chapter, it was shown that the IFN antagonistic activity of the V protein of mesogenic strain BC is significantly greater than that of lentogenic strain La Sota. However, the basis for this difference in IFN antagonistic activity is not known. Therefore, the **aim** of this chapter is to identify amino acid residues in the V protein that will account for the difference between the IFN antagonistic activity of the La Sota and BC V proteins. The **hypothesis** to be tested is that amino acid differences in the C-terminal region of La Sota V and BC V account for their difference in IFN antagonistic activity. The **rationale** for this hypothesis is that the C-terminal region of the NDV V protein is necessary and sufficient for its IFN antagonistic activity. Evidence to support this comes from a study done by Park et al. (2003). Plasmids encoding either the full-length V protein, or the amino-terminal region of V (amino acid residues 1-135), or the carboxy-terminal region of V (amino acid residues 136-239) were transfected into chicken embryo fibroblasts and their IFN antagonistic activities were determined using the NDV-GFP assay. The results showed that the carboxy-terminal region of V was able to rescue growth of the NDV-GFP virus as efficiently as the full-length V protein. The amino-terminal region did not rescue growth of the virus.

This indicates that the IFN antagonistic activity of the NDV V protein resides in its carboxy-terminal region. To test the hypothesis, the **approach** is to compare the C-terminal regions of the La Sota and BC V proteins, identify amino acid differences in this region, mutate the two V proteins at these positions, and test the IFN antagonistic activity of the mutated proteins using the NDV-GFP assay.

Results

4.1 La Sota and BC V mutated proteins are recognized by the V18 antibody at different efficiencies

Comparison of the C-terminal regions of La Sota and BC V proteins revealed four amino acid differences at positions 144, 153, 161 and 234 (Figure 13). La Sota V has a serine (S), glutamic acid (E), serine and aspartic acid (D) at positions 144, 153, 161 and 234, respectively, whereas BC V has a proline (P), lysine (K), proline and asparagine (N) at these positions. To test the contribution of each of these amino acid differences to the difference in the IFN antagonistic activity of the two V proteins, La Sota V was mutated at each of these positions to the corresponding residue in BC V. Reciprocal changes were also made in BC V. In addition, double, triple and quadruple mutants of La Sota and BC V proteins were also created. To test expression of these mutated proteins, the plasmid constructs were transfected into DF1 cells and Western blots were performed using the V18 antibody as previously described for the wt V proteins.

Figure 14 shows that La Sota V carrying a mutation of S144P, E153K or S161P are expressed at levels similar to wt La Sota V (90%, 90% and 110% of
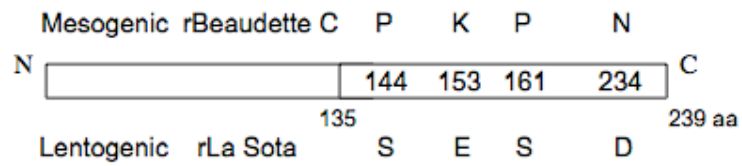
Figure 13. BC and La Sota V proteins differ at four positions in their C-terminal regions.
BC V has a proline (P), lysine (K), proline and asparagine (N) at positions 144, 153, 161 and 234, respectively. La Sota V has a serine (S), glutamic acid (E), serine and aspartic acid (D) at positions 144, 153, 161 and 234, respectively.
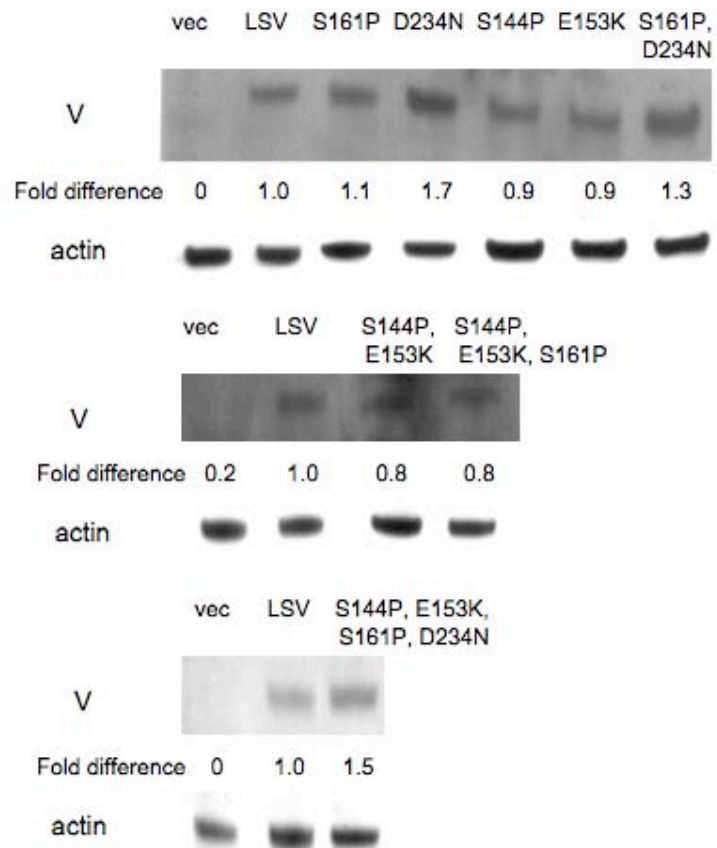
Figure 14. La Sota V mutants are expressed in DF1 cells.
DF1 cells were transfected with La Sota wt and mutant V plasmids to test expression of the V protein. The transfection efficiency of DF1 cells was approximately 40%. At 24 hours post-transfection, cell lysates were prepared and Western blot was performed using a rabbit antipeptide serum specific for the C-terminal 18 amino acids of the BC V protein (V18 Ab). Densitometry was performed to quantitate the protein levels. Fold difference is the amount of the V protein standardized to that of actin. La Sota V (wt) is set as 1.0. This data represents one experiment, out of at least 2 experiments.

La Sota V, respectively). The D234N-mutated La Sota V protein was recognized more efficiently by the V18 Ab as compared to wt La Sota V (170% of La Sota V). The S161P/D234N-doubly mutated La Sota V protein was also recognized more efficiently by the V18 antibody as compared to wt (130% of La Sota V). The La Sota V double mutant S144P/E153K was expressed at 80% of La Sota V. The La Sota V triple mutant S144P/E153K/S161P was expressed at 80% of La Sota V. The La Sota V quadruple mutant S144P/E153K/S161P/D234N was expressed at 150% of La Sota V.

Figure 15 shows that the BC V single mutants P144S, K153E and P161S are expressed at levels similar to wt BC V (110%, 100% and 100% of BC V, respectively). The BC V N234D mutant was recognized by the V18 Ab at 80% of BC V. The BC V double mutant P161S/ N234D was recognized by the V18 Ab at a lower level as compared to wt BC V (40% of BC V). The BC V double mutant P144S/K153E was expressed at 90% of BC V. The BC V triple mutant P144S/K153E/P161S was expressed at 110% of BC V. The BC V quadruple mutant P144S/K153E/P161S/N234D was recognized by the V18 Ab at 20% of BC V.

There are two possibilities to explain the differences in the intensity of the band corresponding to the V protein in the Western blots. For mutated V proteins that show an increased signal as compared to wt, one possibility is that they are expressed at a higher level than wt. Another possibility is that the V18 antibody recognizes the V protein more efficiently when N is present at position 234.
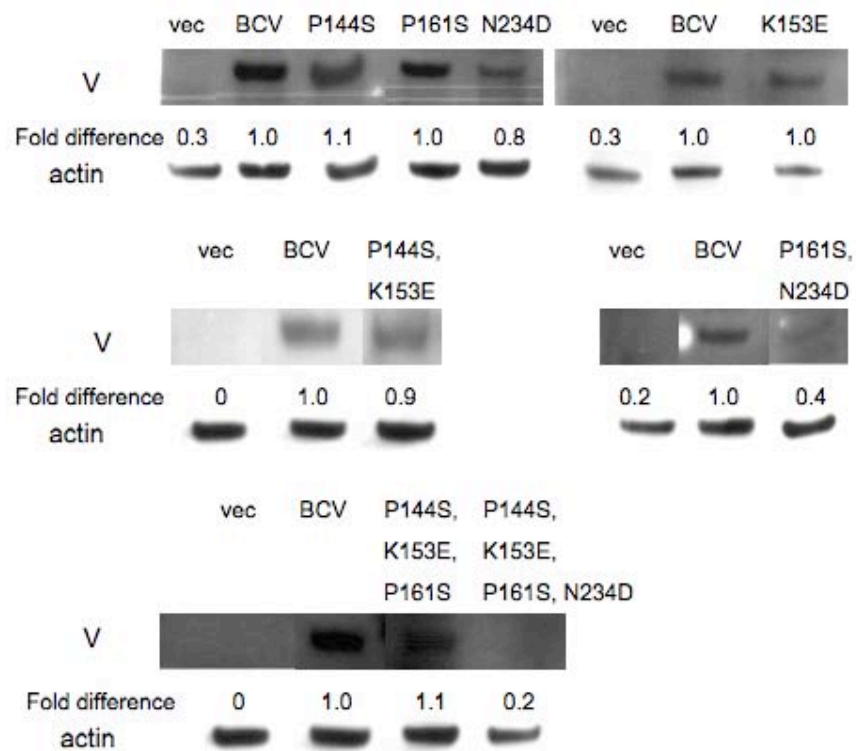
Figure 15. BC V mutants are expressed in DF1 cells.
DF1 cells were transfected with BC wt and mutant V plasmids to test expression of the V protein. The transfection efficiency of DF1 cells was approximately 40%. At 24 hours post-transfection, cell lysates were prepared and Western blot was performed using a rabbit antipeptide serum specific for the C-terminal 18 amino acids of the BC V protein (V18 Ab). Densitometry was performed to quantitate the protein levels. Fold difference is the amount of the V protein standardized to that of actin. BC V (wt) is set as 1.0. This data represents one experiment, out of at least 2 experiments.

Conversely, for mutant V proteins that show a decreased signal as compared to wt, one possibility is that they are expressed at a lower level than wt. Another possibility is that the V18 antibody recognizes the V protein less efficiently when D is present at position 234.

4.2   No single amino acid substitution accounts for the difference in IFN antagonistic activity between the La Sota and BC V proteins.

The IFN antagonistic activities of the La Sota and BC V mutated proteins were tested using the NDV-GFP assay. Figures 16 and 18A show that all of the individually mutated La Sota V proteins have an increased ability to rescue growth of the NDV-GFP virus as compared to wt. The number of fluorescent cells for La Sota V was 393. The number of fluorescent cells for La Sota V carrying S144P, E153K, S161P or D234N mutation increased to 657, 761, 620 and 511, respectively. Figures 17 and 18B show that all of the individually mutated BC V proteins have a decreased ability to rescue growth of the virus as compared to wt. The number of fluorescent cells for BC V was 1753. The number of fluorescent cells for BC V P144S, K153E, P161S and N234D mutants decreased to 861, 1329, 569 and 713, respectively. From these data, it can be seen that no single mutation of La Sota V results in rescue of viral growth at the same level as BC V, and no single mutation of BC V results in rescue of viral growth at the same level as La Sota V. These results indicate that no single residue accounts for the difference in IFN antagonistic activity between the La Sota V and BC V proteins.
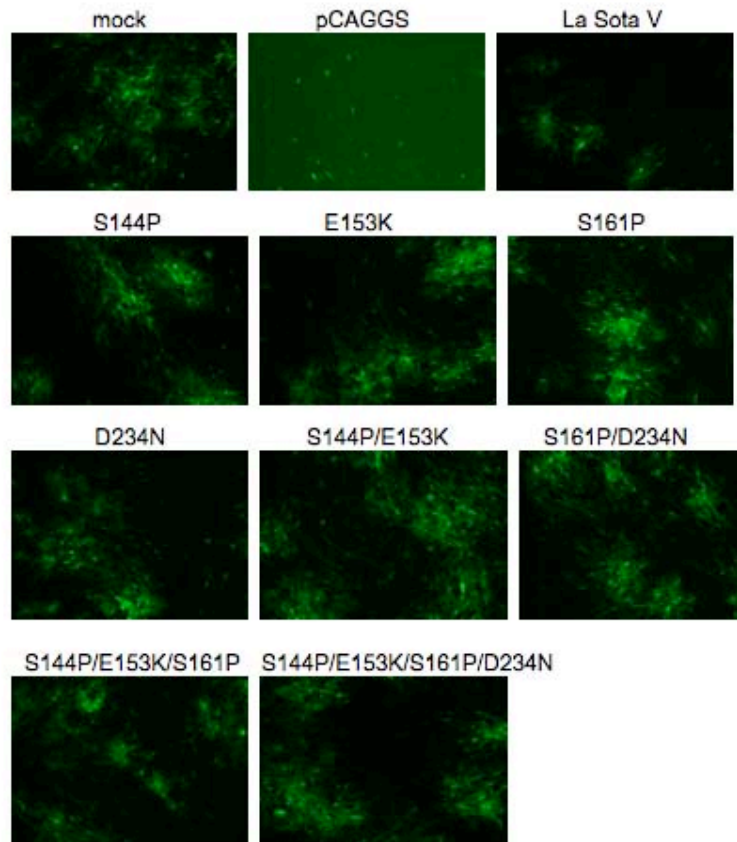
Figure 16. La Sota V mutants exhibit increased ability to rescue growth of the NDV-GFP virus.
DF1 cells were mock-transfected, or transfected with empty vector pCAGGS, or wt La Sota V or La Sota V mutants. Cells were infected with NDV-GFP virus at an moi of 0.001 at 24 hours post-transfection and examined for green fluorescence at 24 hours post-infection. The number of fluorescent cells was counted. For cell clusters, the number of nuclei was counted (one nucleus equals one cell). Each image was taken from 1 well of cells (3 wells were transfected for each plasmid) at 20x magnification using a Carl Zeiss Axiovert 200 microscope.
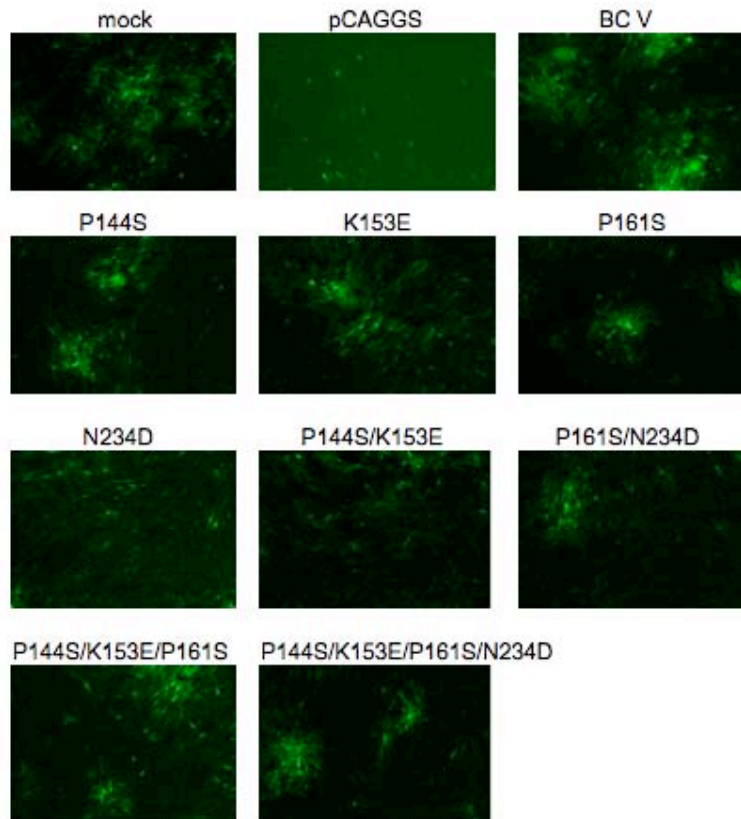
Figure 17. BC V mutants exhibit decreased ability to rescue growth of the NDV-GFP virus.
DF1 cells were mock-transfected, or transfected with empty vector pCAGGS, or wt BC V or BC V mutants. Cells were infected with NDV-GFP virus at an moi of 0.001 at 24 hours post-transfection and examined for green fluorescence at 24 hours post-infection. The number of fluorescent cells was counted. For cell clusters, the number of nuclei was counted (one nucleus equals one cell). Each image was taken from 1 well of cells (3 wells were transfected for each plasmid) at 20x magnification using a Carl Zeiss Axiovert 200 microscope.

A. La Sota wt and single mutant V proteins
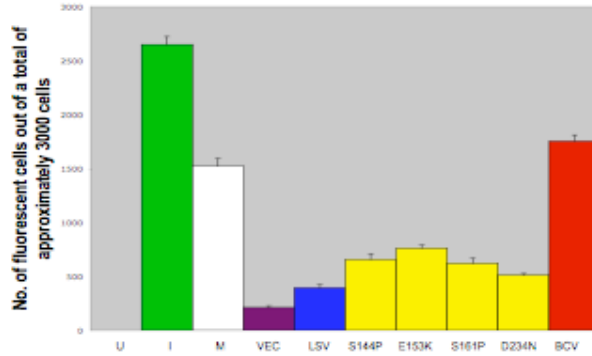
B. BC wt and single mutant V proteins
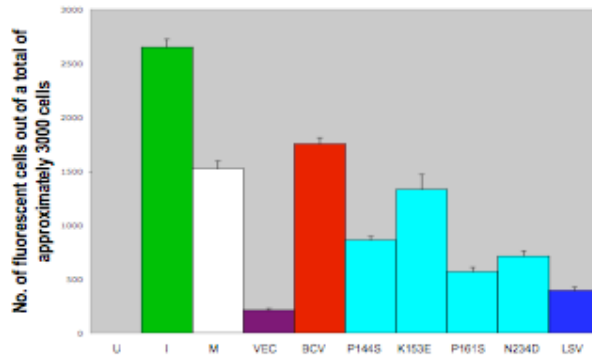
Figure 18. Effect of wild type and single mutant V proteins on growth of NDV-GFP virus

DF1 cells were mock-transfected (M), or transfected with empty vector pCAGGS (vec), or La Sota wt and single mutant V plasmids (panel A) or BC wt and single mutant V plasmids (panel B). Cells were infected with NDV-GFP virus at an moi of 0.001 24 hours post-transfection and examined for green fluorescence 24 hours post-infection. As additional controls, cells were left uninfected (U) and infected (I) with the NDV-GFP virus at the same moi. Values shown in the y axis are the average of the number of fluorescent cells from 3 different fields. Each field contains approximately 3000 cells and represents one well of cells. Error bars represent the standard deviations. This data represents one experiment, out of a total of two experiments. The absolute numbers of fluorescent cells vary from one experiment to another but the relative activity of the various transfected proteins is consistent between experiments.

70

To test whether the presence of multiple mutations has an additive effect, the IFN antagonistic activities of the La Sota and BC V double, triple and quadruple mutated proteins were tested using the NDV-GFP assay. Figures 16 and 19A show that the double mutated proteins of La Sota V are better than the single mutated proteins in rescue of viral growth. The number of fluorescent cells for La Sota V S144P/E153K (D1) and S161P/D234N (D2) mutated proteins was 1144 and 896, respectively. However, the triple and quadruple mutated proteins of La Sota V are not better than the double mutated proteins. The number of fluorescent cells for La Sota V S144P/E153K/S161P and S144P/ E153K/ S161P/ D234N mutated proteins was 877 and 859, respectively. Figures 17 and 19B show that the BC V P144S/K153E double mutated protein has a decreased ability to rescue the virus as compared to the proteins carrying individual P144S and K153E mutations. The number of fluorescent cells for BC V P144S/K153E (D1) mutated protein was 645. The BC V P161S/N234D double mutated protein rescues viral growth at almost the same level as the P161S and N234D mutated proteins. The number of fluorescent cells for BC V P161S/N234D (D2) mutated protein was 615. Surprisingly, the triple and quadruple mutated proteins of BC V are better than the double mutated proteins. The number of fluorescent cells for BC V P144S/K153E/P161S and P144S/K153E/P161S/N234D mutated proteins was 999 and 850, respectively.

It is important to note that the expression levels as determined by Western

A. La Sota wt and mutant V proteins

D1= S144P, E153K
D2= S161P, D234N
T = S144P, E153K,
    S161P
Q = S144P, E153K,
    S161P, D234N

B. BC wt and mutant V proteins

D1= P144S, K153E
D2= P161S, N234D
T = P144S, K153E,
    P161S
Q = P144S, K153E,
    P161S, N234D
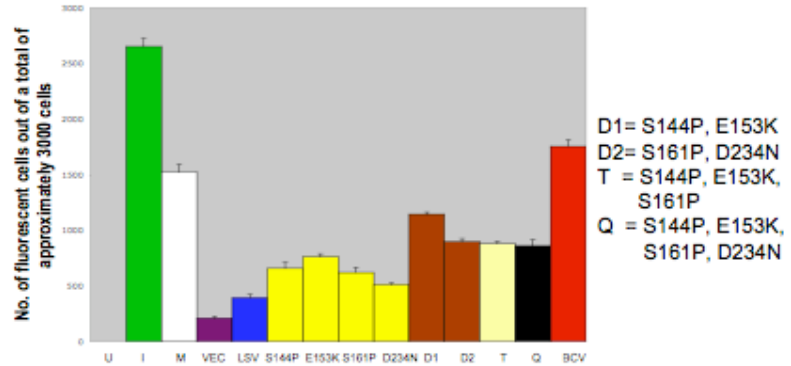
Figure 19. Effect of wild type and mutant V proteins on growth of NDV-GFP virus

DF1 cells were mock-transfected (M), or transfected with empty vector pCAGGS (vec), or La Sota wt and mutant V plasmids (panel A) or BC wt and mutant V plasmids (panel B). Cells were infected with NDV-GFP virus at an moi of 0.001 24 hours post-transfection and examined for green fluorescence 24 hours post-infection. As additional controls, cells were left uninfected (U) and infected (I) with the NDV-GFP virus at the same moi. This figure includes data presented in figure 18.

blot using the V18 antibody do not correlate with the ability of the V mutants to rescue growth of the NDV-GFP virus. For instance, even though the BC V P144S/K153E/P161S/N234D quadruple mutated protein was recognized by the V18 antibody at only 20% of BC V, it rescues growth of the virus at a greater level than that of other mutated proteins that are recognized more by the V18 Ab (i.e. BC V P161S mutated protein which is recognized at 100% of BC V).

As seen from these data, the La Sota V S144P/E153K/S161P/D234N quadruple mutated protein failed to rescue viral growth at the same level as BC V. The BC V P144S/K153E/P161S/N234D quadruple mutated protein also failed to rescue viral growth at the same level as La Sota V. This indicates that the four amino acid differences in the C-terminal region of V do not account entirely for the difference in IFN antagonistic activity of the La Sota and BC V proteins. These results suggest that amino acid residues in the N-terminal region of the V protein may be important.

4.3 The N-terminal region of the BC V protein contributes to its IFN antagonistic activity

To test the possibility that the N-terminal region of the V protein contributes to its IFN antagonistic activity, the N- and C-terminal regions of La Sota V and BC V were exchanged to create chimeric V proteins (Figure 20). The LS-BC chimera contains the N-terminal region of La Sota V and the C-terminal region of BC V. The BC-LS chimera contains the N-terminal region of BC V and the C-terminal region of La Sota V. In addition, as a control, the N- and C-
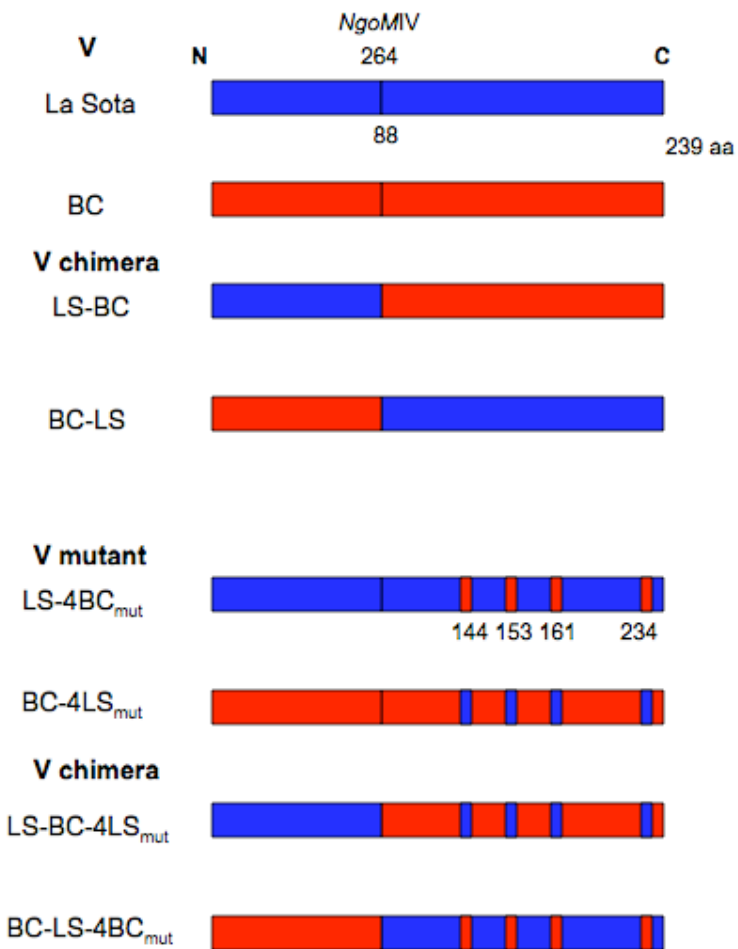
Figure 20. Diagram of La Sota/BC V chimeras

The N- and C-terminal regions of La Sota V and BC V proteins were exchanged by taking advantage of a unique *NgoM*IV restriction site. The N- and C-terminal regions of the La Sota V S144P/E153K/S161P/ D234N mutant (LS-4BCmut) and BC V P144S/K153E/ P161S/N234D mutant (BC-4LSmut) were also exchanged in the same way.

terminal regions of the La Sota V S144P/E153K/S161P/D234N quadruple mutated protein (LS-4BC$_{mut}$) and the BC V P144S/K153E/P161S/N234D quadruple mutated protein (BC-4LS$_{mut}$) were also exchanged. The LS-BC-4LS$_{mut}$ chimera contains the N-terminal region of LS-4BC$_{mut}$ and the C-terminal region of BC-4LS$_{mut}$ and thus, has the same amino acid sequence as La Sota V. The BC-LS-4BC$_{mut}$ chimera contains the N-terminal region of BC-4LS$_{mut}$ and the C-terminal region of LS-4BC$_{mut}$ and has the same amino acid sequence as BC V. The chimeric V plasmid constructs were transfected into DF1 cells and Western blot was performed using the V18 antibody to test expression. Figure 21 shows that the LS-BC and BC-LS-4BC$_{mut}$ chimeras were recognized by the V18 antibody at a level similar to that of BC V. The BC-LS and LS-BC-4LS$_{mut}$ chimeras were recognized by the V18 antibody at a level similar to that of La Sota V. Again, this is consistent with previous results indicating that the V18 antibody recognizes the V protein better when N is present at position 234. The IFN antagonistic activity of the chimeric V proteins was tested using the NDV-GFP assay. Figure 22 shows that the LS-BC chimera (172 fluorescent cells) has a decreased ability to rescue growth of the virus as compared to BC V (556 fluorescent cells). The BC-LS chimera rescues viral growth at the same level as La Sota V (both 222 fluorescent cells). The LS-BC-4LS$_{mut}$ chimera (283 fluorescent cells) rescues viral growth at a level similar to La Sota V, as expected. The BC-LS-4BC$_{mut}$ chimera (479 fluorescent cells) rescues viral growth at a level similar to BC V, as expected. All together, these results suggest
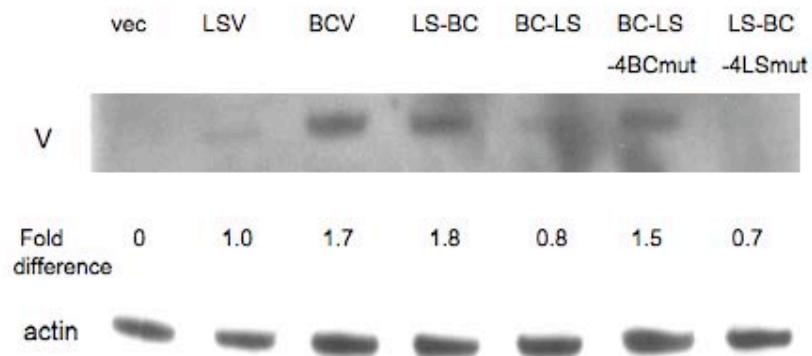
Figure 21. The La Sota/BC V chimeras are expressed in DF1 cells.
DF1 cells were transfected with empty vector pCAGGS (vec), or wt La Sota
V, or wt BC V or La Sota/BC V chimeras to test expression of the V protein.
At 24 hours post-transfection, cell lysates were prepared and Western blot
was performed using a rabbit antipeptide serum specific for the C-terminal
18 amino acids of the BC V protein (V18 Ab). Densitometry was performed
to quantitate the protein levels. Fold difference is the amount of the V protein
standardized to that of actin. La Sota V (wt) is set as 1.0.

A.

mock | pCAGGS | La Sota V | BC V

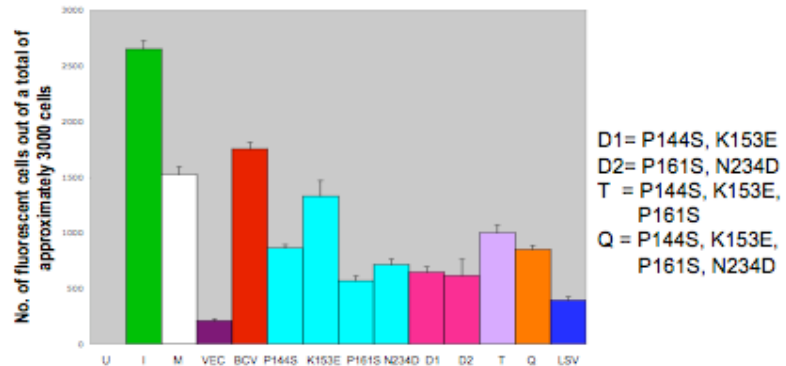LS-BC | BC-LS | LS-BC-4LS$_{mut}$ | BC-LS-4BC$_{mut}$

B.
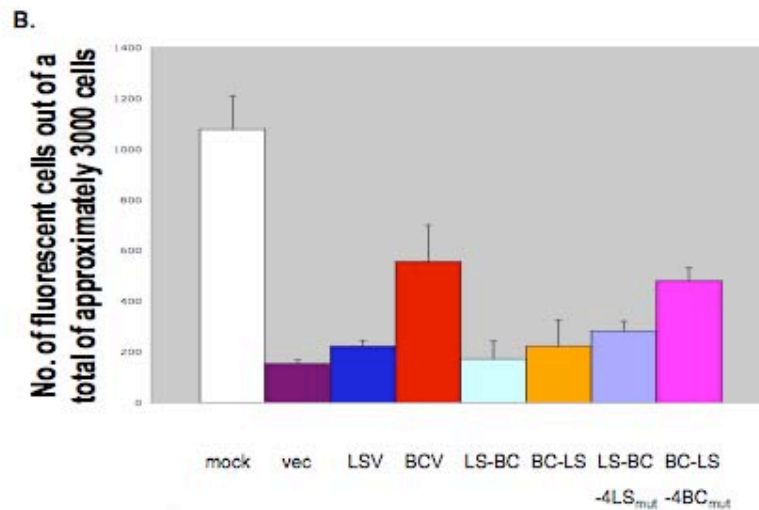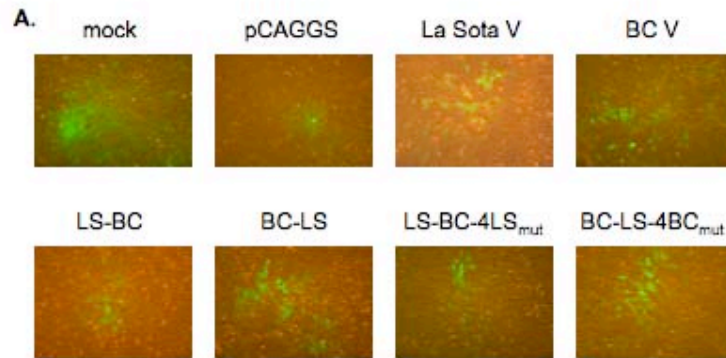
Figure 22. Effect of wild type and chimeric V proteins on growth of NDV-GFP virus.
DF1 cells were mock transfected, or transfected with empty vector pCAGGS (vec), or wt La Sota V, or wt BC V, or chimeric V plasmids. Cells were infected with NDV-GFP virus at an moi of 0.001 at 24 hours post-transfection and examined for green fluorescence 24 hours post-infection (A). The growth of the NDV-GFP virus was quantitated by counting the number of fluorescent cells from 3 different fields (each field contains approximately 3000 cells) (B).

that the N-terminal region contributes to the IFN antagonistic activity of the BC V protein.

Overall, the results indicate that amino acid residues 144, 153, 161 and 234 modulate the IFN antagonistic activity of the V protein of NDV. However, these four residues in the C-terminal region do not account completely for the difference in IFN antagonistic activity of the La Sota and BC V proteins. The decreased ability of the LS-BC chimera, relative to BC V, to rescue growth of the NDV-GFP virus suggests that the N-terminal region contributes to the IFN antagonistic activity of the BC V protein. There are five amino acid differences between the La Sota and BC V proteins in the N-terminal region (Figure 23). Further studies need to be done to determine which of these residues are important for the IFN antagonistic activity of the V protein. This is the first evidence that the N-terminus of NDV V contributes to its IFN antagonistic activity.
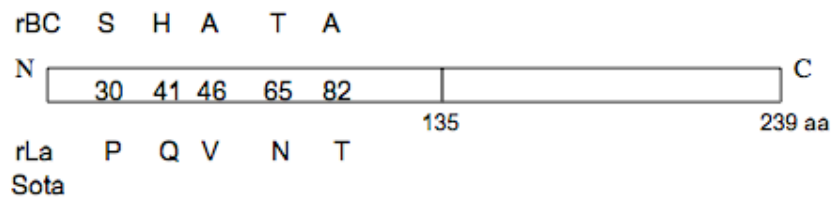
Figure 23. BC and La Sota V proteins differ at five positions in their N-terminal regions.

BC V has a serine (S), histidine (H), alanine (A), threonine (T) and alanine at positions 30, 41, 46, 65 and 82, respectively. La Sota V has a proline (P), glutamine (Q), valine (V), asparagine (N) and threonine at positions 30, 41, 46, 65 and 82, respectively.

CHAPTER V

Mechanism for the difference in IFN antagonistic activity of the La Sota and

BC V proteins

Introduction

The V protein of NDV has been shown to inhibit the IFN response in two ways. Huang et al. (2003) showed that the level of STAT1 was significantly lower in human 2fTGH cells infected with a recombinant BC (rBC) virus as compared to mock-infected cells. However, mutant viruses deficient in the expression of the V protein failed to decrease the level of STAT1. In addition, transient transfection of a plasmid encoding the C-terminus of the V protein (amino acid residues 136-239) resulted in STAT1 degradation in 2fTGH cells at 20 hours post-transfection. These results led the authors to conclude that the C-terminus of the V protein inhibits IFN signaling by targeting STAT1 for degradation. Another study showed that the V protein of La Sota interacts with MDA-5 via the C-terminal domain and that this interaction correlates with inhibition of IFNβ induction (Childs et al., 2007). In both studies, only one strain of NDV was used. Hence, it is not clear whether different strains of the virus affect components of the IFN response to varying extents. Therefore, the **aim** of this chapter is to determine the molecular basis for the difference in IFN antagonistic activity of the La Sota V and BC V proteins. The **hypothesis** to be tested is that the La Sota V and BC V proteins differ in their ability to degrade one or more components of the IFN response.

The **rationale** for this hypothesis is that the NDV V protein has been shown to antagonize IFN by affecting some components of the IFN response such as MDA-5 and STAT1. The **approach** is to determine the levels of components of the IFN response by Western blot using lysates from cells expressing the La Sota or BC V protein.

Results

5.1   The NDV V protein does not target STAT1 for degradation.

Huang et al. (2003) previously showed that the V protein of BC targets STAT1 for degradation in human 2fTGH cells. As a prelude to comparing the activities of BC V and La Sota V, a plasmid encoding BC V was transfected into 2fTGH cells. Lysates were prepared 20 hours post-transfection and Western blot was performed to determine the level of STAT1. As a control, a plasmid encoding BC P was also transfected into these cells. Figure 24A confirms that BC V is expressed in 2fTGH cells. In addition, the level of STAT1 in BC P-transfected cells was equal to vector-transfected cells (100% of vector), whereas the level of STAT1 in BC V-transfected cells was slightly reduced (70% of vector). The decrease in the level of STAT1 is not nearly as significant as that demonstrated by Huang et al. (2003) who showed that transfection of the C-terminus of BC V reduced the level of STAT1 to below the detection limits of the assay (Western blot). To test whether detection of the V protein-induced degradation of STAT1 is temporally dependent, cells were lysed at various times post-transfection (12, 14,

A.

	VEC	BCP	BCV
STAT1			
Fold difference	1.0	1.0	0.7
actin			
V			

LEGEND
VEC = pCAGGS
BCP = Beaudette C P
BCV = Beaudette C V

B.

Experiment 1	U	I	M	VEC	LSV	BCV
STAT1						
Fold difference	0.7	0.9	0.6	1.0	0.8	0.7
actin						

Experiment 2	U	I	M	VEC	LSV	BCV
STAT1						
Fold difference	1.1	1.2	1.4	1.0	0.9	0.8
actin						

LEGEND
U = untreated
I = infected
M = mock-transfected, then infected
VEC = pCAGGS-transfected, then infected
LSV = La Sota V-transfected, then infected
BCV = Beaudette C V-transfected, then infected

Figure 24. Western blots for STAT1
Human 2fTGH cells were transfected with plasmids encoding either BC P or V protein. Lysates were prepared 20 hours post-transfection and Western blot was performed to determine the levels of STAT1. Lysates were also probed with the V18 antibody to confirm that the V protein is expressed in 2fTGH cells (A). After performing the NDV-GFP assay, lysates were prepared from the DF1 cells and probed for STAT1 (B). The transfection efficiency of DF1 cells was approximately 40%. Densitometry was performed to quantitate the protein levels. Fold difference is the amount of STAT1 standardized to that of actin. Vector is set as 1.0.

16, 36, 42, 48 and 60 hours post-transfection). In addition, 293 cells were used because of their relatively high transfection efficiency. Despite these efforts, the results obtained by Huang et al. (2003) could not be reproduced at any of these time points (data not shown).

The IFN antagonistic activities of La Sota V and BC V were tested in DF1 cells using the NDV-GFP assay as described in Chapter 3. In addition to quantitating the fluorescent cells, lysates were also prepared and Western blots were performed to further examine whether the NDV V protein targets STAT1 for degradation. Figure 24B shows that the level of STAT1 in La Sota V-transfected cells is similar to that in vector-transfected cells (average of 85% of vector for two experiments). The level of STAT1 in BC V-transfected cells is slightly reduced (average of 75% of vector for two experiments), consistent with the results in 2fTGH cells (Figure 24A). Although there is a small difference in the levels of STAT1 between the La Sota V- and BC V- transfected cells, this difference is insufficient to account for the difference in IFN antagonistic activity between the two V proteins.

Thus, several different approaches were used to attempt to reproduce the results of Huang et al. (2003). These include: 1) transfection of BC V plasmid into 2fTGH cells and preparation of lysates at different times post-transfection, 2) transfection of BC V plasmid into 293 cells that have a relatively high transfection efficiency, and 3) use of lysates that were prepared from the NDV-GFP assay.

Despite these efforts, the level of STAT1 degradation obtained by Huang et al. could not be reproduced.

As a final attempt to examine whether the NDV V protein targets STAT1 for degradation, the La Sota V and BC V genes were subcloned into the bicistronic vector pIRES2-AcGFP1, which contains an internal ribosome entry site (IRES), and expresses the green fluorescent protein (AcGFP1) and the protein of interest from the same transcript in transfected cells. The plasmids encoding the V proteins were transfected into DF1 cells. At 24 hours post-transfection, cells expressing the V protein were identified by screening for green fluorescence by flow cytometry. Cells that do not express the V protein (GFP-negative cells) were also collected as controls. Lysates were prepared from the GFP-negative and GFP-positive cells and Western blot was done to determine the level of STAT1. This method is more stringent because it enables one to focus exclusively on those cells that express the V protein. In addition, chicken cells (DF1), which are the natural host of NDV, were used. Figure 25 shows that the level of STAT1 in La Sota V-GFP+ cells (lane 4) is actually slightly increased (120%) relative to that of vector-GFP+ cells. The level of STAT1 in BC V-GFP+ cells (lane 6) is equal to that of vector-GFP+ cells (100% of vector). These results confirm that neither La Sota V nor BC V targets STAT1 for degradation.

Taken together, these results show that the STAT1 levels in La Sota V- and BC V-transfected cells are not significantly different and cannot account for the difference in IFN antagonistic activity between the two V proteins. This
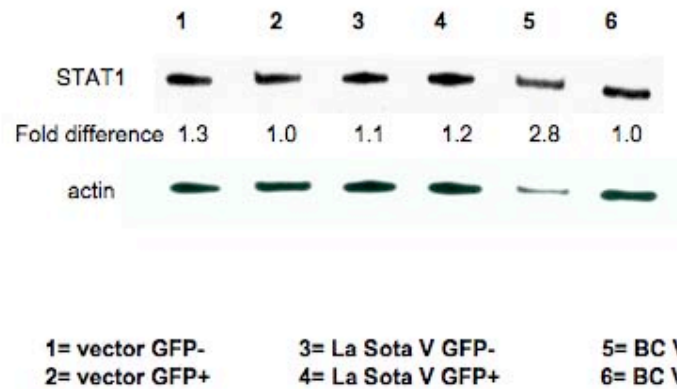
Figure 25. Western blot for STAT1 in GFP-sorted cells.
The La Sota and BC V genes were subcloned into the bicistronic vector pIRES2-AcGFP1 and transfected into DF1 cells. At 24 hours post-transfection, cells expressing the V protein were collected by screening for green fluorescence by flow cytometry. Cells that do not express the V protein (GFP-negative cells) were also collected as controls. Lysates were prepared from the GFP-negative and GFP-positive cells and Western blot was done to determine the level of STAT1. Densitometry was performed to quantitate the protein levels. Fold difference is the amount of STAT1 standardized to that of actin. Vector GFP+ is set as 1.0.

suggests that the difference in IFN antagonistic activity of the two V proteins involves other component(s) of the IFN response.

5.2 The levels of STAT2, p38 and IRF-3 in vector-, La Sota V- and BC V-transfected DF1 cells are not significantly different.

To determine whether the NDV V protein affects other components of the IFN response and whether La Sota V and BC V differ in this regard, Western blots were performed using cell lysates collected from the NDV-GFP assay to determine the levels of STAT2, p38 and IRF-3 in DF1 cells. Figure 26 shows that the levels of STAT2 in La Sota V- and BC V-transfected cells are similar to that in vector-transfected cells (both are 90% of vector). The level of p38 in La Sota V-transfected cells is equal to that in vector-transfected cells (100% of vector), whereas the level of p38 in BC V-transfected cells is slightly reduced (80% of vector). The level of IRF-3 is slightly increased in La Sota V-transfected cells (120% of vector), whereas the level of IRF-3 in BC V-transfected cells is equal to vector-transfected cells (100% of vector). Thus, the levels of STAT2, p38 and IRF-3 are not significantly different between the La Sota V- and BC V-transfected cells. This indicates that neither STAT2, nor p38, nor IRF-3 contributes to the difference in IFN antagonistic activity of the two V proteins.

5.3 The levels of IRF-7 in La Sota V- and BC V-transfected DF1 cells are slightly reduced as compared to vector-transfected cells

To determine whether La Sota V and BC V differ in their ability to degrade IRF-7, Western blots were performed using cell lysates collected from the

	U	I	M	VEC	LSV	BCV	LEGEND

STAT2

Fold difference 1.4   1.0   1.1   1.0   0.9   0.9

actin

p38

Fold difference 0.8   0.9   0.9   1.0   1.0   0.8

actin

IRF-3

Fold difference 0.7   0.9   1.2   1.0   1.2   1.0

actin

IRF-7   ←IRF-7

Fold difference 0   1.7   1.1   1.0   0.8   0.8

actin

LEGEND

U = untreated

I = infected

M = mock-transfected,
    then infected

VEC = pCAGGS-transfected,
    then infected

LSV = La Sota V-transfected,
    then infected

BCV = Beaudette C V-
    transfected, then
    infected
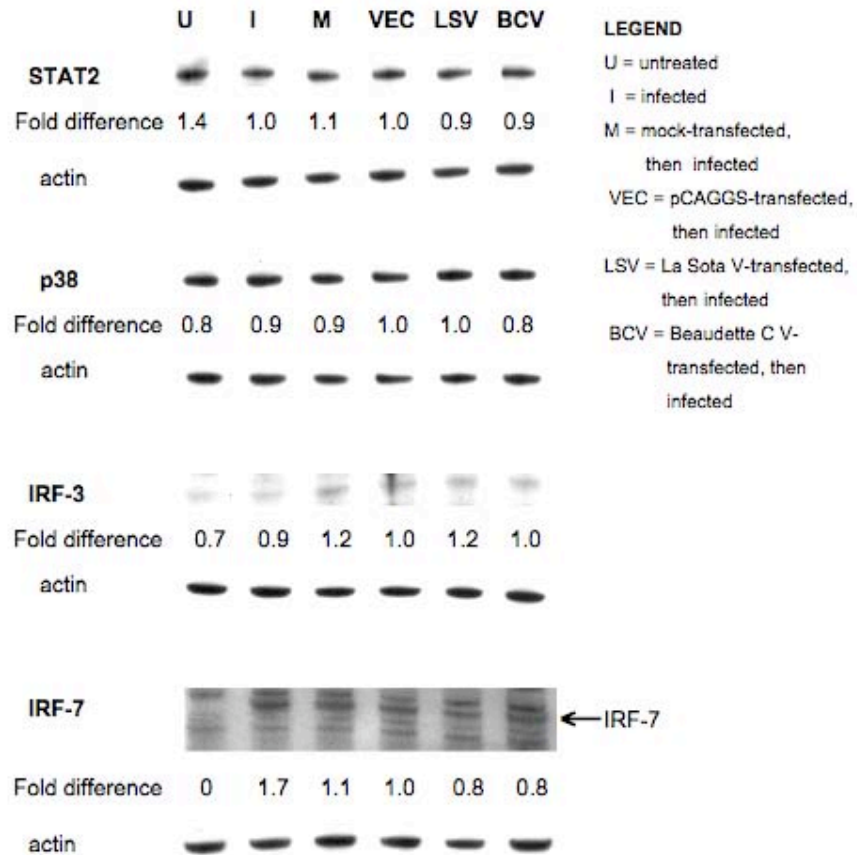
Figure 26. Western blots for STAT2, p38, IRF-3 and IRF-7
After performing the NDV-GFP assay, lysates were prepared from the DF1 cells and Western blot was performed to determine the levels of STAT2, p38, IRF-3 and IRF-7. Densitometry was performed to quantitate the protein levels. Fold difference is the amount of either STAT2, p38, IRF-3 or IRF-7 standardized to that of actin. Vector is set as 1.0.

NDV-GFP assay. There was a noticeable change in the level of IRF-7 among the samples (Figure 26). In untreated DF1 cells, IRF-7 was not detectable. However, when cells were infected with the NDV-GFP virus, IRF-7 was increased significantly. This is consistent with IRF-7 being an inducible protein. When cells were transfected with either La Sota V or BC V, and then infected with the NDV-GFP virus, the level of IRF-7 is slightly decreased as compared to vector-transfected cells (80% of vector for both La Sota V-transfected and BC V-transfected cells).

5.4   Both La Sota and BC V proteins target IRF-7 for degradation.

In the previous section, it was shown that expression of either NDV V protein resulted in a reduction in the level of IRF-7 relative to the control. Encouraged by this result, another strategy was used to compare the effects of La Sota V and BC V on the level of IRF-7. Because the transfection efficiency of DF1 cells is not high (approximately 40% transfection efficiency), it is possible that dramatic changes in IRF-7 levels may not be detectable when using lysates prepared from a mixture of transfected and untransfected cells. To overcome this problem, the La Sota V and BC V genes were expressed in DF1 cells from the bicistronic vector pIRES2-AcGFP1. At 24 hours post-transfection, GFP-negative and GFP-positive cells were sorted by flow cytometry. Lysates were prepared from the GFP-negative and GFP-positive cells and Western blot was performed to determine the levels of IRF-7. Figure 27A shows that there is a remarkable change in IRF-7 levels among the samples at 24 hours post-transfection. Again,

A. 24 hours post-transfection

	1	2	3	4	5	6
IRF-7 →						← IRF-7*
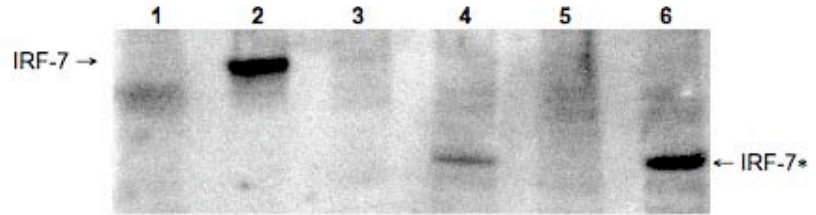
Fold difference

	1	2	3	4	5	6
IRF-7	0	1.0	0	0.1	0	0.1
IRF-7*	0.9	1.0	1.3	123	0.9	255

actin

B. 48 hours post-transfection

	1	2	3	4	5	6
IRF-7 →						← IRF-7*
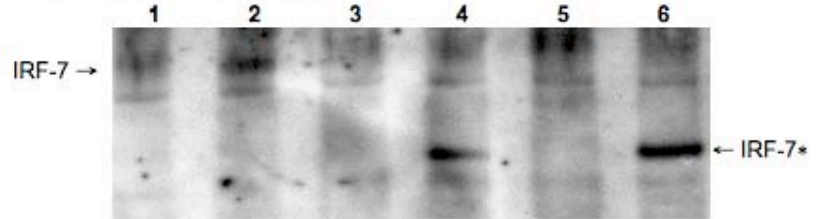
Fold difference

	1	2	3	4	5	6
IRF-7	0	1.0	0	0	0	0
IRF-7*	0	1.0	0.3	70	0.3	121

actin
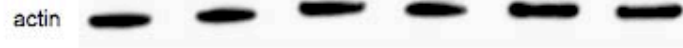
1= vector GFP- 3= La Sota V GFP- 5= BC V GFP-
2= vector GFP+ 4= La Sota V GFP+ 6= BC V GFP+

Figure 27. The La Sota and BC V proteins target IRF-7 for degradation.
The La Sota and BC V genes were subcloned into the bicistronic vector
pIRES2-AcGFP1 and transfected into DF1 cells. At 24 and 48 hours post-
transfection, cells expressing the V protein were collected by screening for
green fluorescence by flow cytometry. Cells that do not express the V protein
(GFP-negative cells) were also collected as controls. Lysates were prepared
from the GFP-negative and GFP-positive cells and Western blot was done to
determine the level of IRF-7. Densitometry was performed to quantitate the
protein levels. Fold difference is the amount of IRF-7 or IRF-7* standardized
to that of actin. Vector GFP+ is set as 1.0.

IRF-7 is not detectable in any of the untransfected samples (GFP-negative cells) (lanes 1, 3, 5). However, the level of IRF-7 is significantly increased in cells transfected with the vector (lane 2). This is consistent with the earlier results indicating that IRF-7 is an inducible protein. It is also consistent with the idea that transcription from a plasmid generates dsRNA that triggers induction of IRF-7.

Most importantly, IRF-7 is significantly decreased in cells transfected with either La Sota V (lane 4) or BC V (lane 6) relative to vector-GFP+ (lane 2) (Figure 27A). These results suggest that both La Sota V and BC V may target IRF-7 for degradation. Indeed, the levels of IRF-7 in lanes 4 and 6 are below the detection limits of the system. This is the first evidence that IRF-7 is targeted for degradation by a paramyxovirus V protein.

5.5   Expression of NDV V converts IRF-7 to a faster migrating form.

Interestingly, Figure 27A also shows that a protein with a lower molecular weight (IRF-7∗ is approximately 39 kDa) is present in cells transfected with either La Sota V (lane 4) or BC V (lane 6), but not in cells transfected with the vector (lane 2) at 24 hours post-transfection. To try to determine the identity of IRF-7∗, the Western blot was aligned with a Coomassie stained gel and the band corresponding to IRF-7∗ was excised and sent for mass spectrometric analysis. The results showed that there are three possible peptides that could be from IRF-7. Two peptides (residues 63-70 and 104-122) lie in the N-terminal region and one peptide (residues 352-363) lies in the C-terminal region of the protein. However, these peptides have weak spectra so it is hard to make a definite

conclusion from these results. Nevertheless, these results are consistent with IRF-7∗ being a degradation product of IRF-7. This suggests that both the La Sota and BC V proteins promote conversion of IRF-7 to the lower molecular weight form. It is also interesting to note that the amount of IRF-7∗ is 2.1-fold greater in cells expressing BC V (lane 6) as compared to cells expressing La Sota V (lane 4), indicating that the extent of conversion of IRF-7 to IRF-7∗ correlates with the extent of IFN antagonistic activity of the V protein.

To investigate if the conversion of IRF-7 to IRF-7∗ changes over time, the cells were also sorted at 48 hours post-transfection. The amount of IRF-7 at 48 hours post-transfection (Figure 27B) shows a similar pattern to that at 24 hours post-transfection (Figure 27A). Again, the levels of IRF-7 in La Sota V-transfected cells (lane 4) and BC V-transfected cells (lane 6) are below the detection limits of the system. Similar to the results at 24 hours, the amount of IRF-7∗ is 1.7-fold greater in cells transfected with BC V (lane 6) as compared to cells transfected with La Sota V (lane 4).

At both time points, the amount of IRF-7∗ in BC V-transfected cells (lane 6) is similar to the amount of full-length IRF-7 in vector-transfected cells (lane 2), whereas the amount of IRF-7∗ in La Sota V-transfected cells (lane 4) is significantly less than the amount of full-length IRF-7 in vector-transfected cells (lane 2). This suggests that BC V promotes complete conversion of IRF-7 to IRF-7∗, whereas La Sota V does so less efficiently.

5.6 The amount of IRF-7∗ decreases in a dose-dependent manner in the presence of a proteasome inhibitor.

To examine whether IRF-7∗ is a degradation product of IRF-7, DF1 cells were transfected with BC V in the absence or presence of the proteasome inhibitor clasto-lactacystin-β-lactone. Previously, it was found that clasto-lactacystin-β-lactone inhibits the chymotryptic, tryptic and peptidylglutamyl activities of the 20S and 26S proteasomes (Craiu et al., 1997). Clasto-lactacystin-β-lactone is a derivative of lactacystin which inhibits the proteasome by acting as a pseudosubstrate that becomes covalently linked to the hydroxyl groups on the active site threonine of the β subunits (Fenteany et al., 1995). Unlike peptide aldehyde proteasome inhibitors (i.e. MG132), clasto-lactacystin-β-lactone does not inhibit lysosomal proteases, making it a more specific inhibitor of proteasomes (Craiu et al., 1997).

DF1 cells were treated with clasto-lactacystin-β-lactone for 2 hours prior to transfection with the BC V plasmid. At 24 hours post-transfection, GFP-negative and GFP-positive cells were again sorted by flow cytometry. As a control, cells were also treated with only the solvent, dimethyl sulfoxide (DMSO). Figure 28A shows that, in cells transfected with BC V, the amount of IRF-7∗ is decreased by 61% in the presence of 2 μM inhibitor compared to the amount of IRF-7∗ in the untreated control (compare lanes 6 and 8). Surprisingly, the amount of full-length IRF-7 did not increase in the presence of the inhibitor. Figure 28B shows that, in cells transfected with BC V, the amount of IRF-7∗ is decreased by 87% relative

A. 2 μM clasto-lactacystin-β-lactone

	1	2	3	4	5	6	7	8
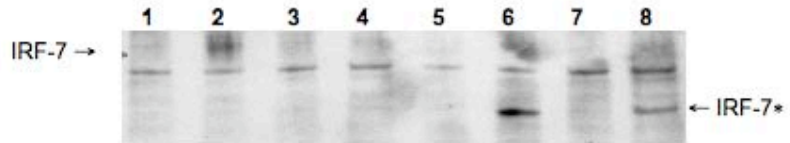
IRF-7 →

← IRF-7*

Fold difference

	1	2	3	4	5	6	7	8
IRF-7	0.1	1.0	0.2	0.1	0	0.1	0	0
IRF-7*	6.4	1.0	10	0	7.5	218	13	85

actin

B. 5 μM clasto-lactacystin-β-lactone

	1	2	3	4	5	6	7	8
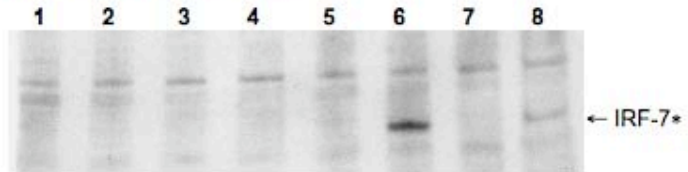
← IRF-7*

Fold difference

	1	2	3	4	5	6	7	8
IRF-7*	0.8	1.0	1.2	1.3	0.3	16	0.2	2.1

actin
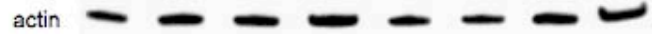
1= vector GFP- (- β-lactone)   5= BC V GFP- (- β-lactone)
2= vector GFP+(- β-lactone)   6= BC V GFP+(- β-lactone)
3= vector GFP- (+ β-lactone)   7= BC V GFP- (+ β-lactone)
4= vector GFP+(+ β-lactone)   8= BC V GFP+(+ β-lactone)

Figure 28. The amount of IRF-7* decreases in the presence of the proteasome inhibitor clasto-lactacystin-β-lactone.
The BC V gene was subcloned into the bicistronic vector pIRES2-AcGFP1 and transfected into DF1 cells in the absence or presence of the proteasome inhibitor clasto-lactacystin-β-lactone (2 μM or 5 μM). At 24 hours post-transfection, cells expressing the V protein were collected by screening for green fluorescence by flow cytometry. Cells that do not express the V protein (GFP-negative cells) were also collected as controls. Lysates were prepared from the GFP-negative and GFP-positive cells and Western blot was done to determine the level of IRF-7. Densitometry was performed to quantitate the protein levels. Fold difference is the amount of IRF-7 or IRF-7* standardized to that of actin. Vector GFP+(-β-lactone) is set as 1.0.

to the untreated control in the presence of a higher concentration (5 μM) of the inhibitor (compare lanes 6 and 8). Hence, the amount of IRF-7∗ decreases in a dose-dependent manner in the presence of the proteasome inhibitor. This result is consistent with IRF-7∗ being a degradation product of IRF-7.

Overall, the results indicate that the V proteins of La Sota and BC target IRF-7 for degradation, and demonstrate an additional mechanism by which the NDV V protein antagonizes the IFN response. Both La Sota and BC V degrade IRF-7 to undetectable levels. In addition, the results indicate that BC V promotes complete conversion of IRF-7 to the lower molecular weight form (IRF-7∗), whereas La Sota V does so less efficiently. These findings correlate with the difference in IFN antagonistic activities of the two V proteins and in turn with the virulence of the viruses from which they were derived.

CHAPTER VI

Characterization of AVS-I monoclonal antibody

(Alamares et al., 2005)

Introduction

The evidence that the HN protein plays a role in virulence of NDV comes from a study done by Huang et al. (2004). The HN gene of the mesogenic recombinant Beaudette C (BC) strain was exchanged with that of the lentogenic recombinant La Sota strain, creating a BC virus having the HN of La Sota (rBC LaSoHN), and a La Sota virus having the HN of BC (rLaso BCHN). Pathogenicity studies were done in chicken embryos and chickens. They found that the mean embryo death time (MDT) for rLaSo BCHN virus was decreased (84 hours) as compared to the parental rLaSota virus (96 hours), indicating that the virulence of the chimeric virus was increased. The MDT for rBC LaSoHN virus was increased (72 hours) as compared to the parental rBC virus (62 hours), indicating that the virulence of the chimeric virus was decreased. In chickens, the rLaso BCHN virus had an intracerebral pathogenicity index (ICPI) of 0.75, whereas the rLaSota virus had an ICPI of 0.0, indicating increased virulence of the chimeric virus. The rBC LaSoHN virus had an ICPI of 1.02, whereas the rBC virus had an ICPI of 1.58, indicating decreased virulence of the chimeric virus. The rest of the difference in virulence between La Sota and BC is thought to be contributed by

the F and V proteins. Although these results indicate that HN plays a role in virulence, the amino acid residues involved have not been identified.

The monoclonal antibody AVS-I recognizes lentogenic strains of NDV (B1-Hitchner, La Sota, Queensland V4, and Ulster) and ten commonly employed commercial B1-Hitchner and La Sota vaccine strains, as determined by hemagglutination inhibition (HI) assay. However, AVS-I did not react with three mesogenic (ENG P3R10, Roakin and Kimber) or six velogenic (GB Texas, Largo, Calif 1083, KM, P1307, and P5658) strains tested (Srinivasappa et al., 1986). These results suggested that AVS-I recognizes an epitope that is conserved in lentogenic strains and have led to its use in identifying strains of NDV belonging to this pathotype. These findings also raise the possibility that AVS-I may identify a domain on HN that contributes to virulence. Therefore, the **aim** of this chapter is to further characterize AVS-I and the epitope it recognizes. The **hypothesis** to be tested is that characterization of AVS-I and its epitope may identify a determinant of virulence in HN. The **rationale** for this hypothesis is that AVS-I recognizes only lentogenic strains but not mesogenic or velogenic strains. The **approaches** are to: 1) determine the specificity of AVS-I for several additional strains of NDV; 2) map its binding to HN in competition with a panel of monoclonal antibodies; 3) determine its functional inhibition profile; and, 4) isolate and characterize an AVS-I escape mutant virus.

Results

6.1 AVS-I recognizes several lentogenic strains, as well as one mesogenic and one velogenic strain of NDV.

The specificity of AVS-I for avirulent strains of NDV was tested by ELISA using intact virions of several additional strains of the virus not tested previously. These include lentogenic, mesogenic, and velogenic strains. The two lentogenic strains are B1-Hitchner/48 (B1) and Ulster/64 (U). The mesogenic strains include NJ-Roakin/FRB/46 (F) and Massachusetts-4F/46 (M). The velogenic strains include Australia-Victoria/32 (AV), Texas-GB/48 (GB), Iowa-Salsbury/49 (IS), Kansas-Leavenworth/48 (L), and California-RO/44 (RO). Table 3 shows the ELISA results. Antibody to site 1 was used as a positive control. This antibody has previously been shown to have broad specificity for a variety of NDV strains. Antibody to site 23, previously shown to be highly specific for the AV strain, was used as a negative control. Consistent with the results of the original study (Srinivasappa et al., 1986), AVS-I recognizes the lentogenic strains B1 and U and does not recognize mesogenic strain F or velogenic strains GB, IS, L, and AV. However, most unexpectedly, AVS-I does recognize mesogenic strain M and velogenic strain RO. Hence, these strains are exceptions to the specificity of AVS-I for avirulent strains. This finding calls into question the suitability of AVS-I as a marker for avirulent strains of the virus.

NDV strains (pathotype)		ELISA reactivity [a]			HI titer [b]			Neutralization [c]	
								AVS-I	
		Site 1	Site 23	AVS-I	Site 1	Site 4	AVS-I	- RAM	+RAM
B1-Hitchner/48 [B1]	(L)	++	-	++	4,096	4	1,024	+	+
Ulster/64 [U]	(L)	++	-	++	4,096	32	512	-	+
NJ-Roakin/FRB/46 [F]	(M)	++	-	-	2,048	8	<2	-	-
Massachusetts-4F/46 [M]	(M)	++	-	++	4,096	<2	4,096	-	+
Australia-Victoria/32 [AV]	(V)	++	+	-	2,048	8	<2	-	-
Texas-GB/48 [GB]	(V)	++	+	-	8,192	32	16	ND	ND
Iowa-Salsbury/49 [IS]	(V)	++	-	-	16,384	8	8	ND	ND
Kansas-Leavenworth/48 [L]	(V)	++	-	-	16,384	64	8	-	-
California-RO/44 [RO]	(V)	+	-	++	4,096	8	64	+	+

Table 3. Functional inhibition properties of AVS-I

The specificity of AVS-I for several NDV strains was tested by ELISA, HI and neutralization. These include lentogenic (L), mesogenic (M) and velogenic (V) strains.

a: Data represent the means of absorbance values at 650 nm of two to four determinations.
++, an optical density at 650 nm (OD 650) of >0.200
+, an OD 650 between 0.020 and 0.200
-, an OD 650 of <0.020

b: The reciprocal of the dilution of ascites fluid inhibiting four HA units of virus. Data represent the means of two to seven determinations.

c: Data represent three independent determinations.
 The concentration of AVS-I antibody is 50 μg/ml.
-RAM, without RAM Ig
+RAM, with RAM Ig (25 μg/ml)
+, neutralized (<10% persistent fraction)
 -, not neutralized (>80% persistent fraction)
ND, not determined

98

6.2 Hemagglutination inhibition (HI)

The ability of AVS-I to inhibit the hemagglutinating (HA) activity of our panel of NDV strains was determined (Table 3). An antibody to site 1 was used as a positive control, while antibody to site 4 served as a negative control (Iorio and Bratt, 1984). AVS-I inhibits the HA activity of avirulent strains B1 and U but not that of strain F or AV and only marginally that of GB, IS, or L, consistent with the ELISA results. AVS-I recognizes the virulent strain RO only at a low level, whereas it had bound to this strain quite efficiently in the ELISA. However, AVS-I does inhibit the HA activity of strain M at a very high titer.

6.3 Neutralization

The ability of AVS-I to neutralize our panel of NDV strains was tested. Table 3 shows that there are three categories of neutralization by AVS-I. Members of the first group are neutralized to a significant extent by AVS-I alone. This group includes strains B1 and RO, treatment of which with AVS-I results in persistent fractions of 5 to 10%. Addition of rabbit anti-mouse immunoglobulin (RAM Ig) results in complete neutralization of both strains. The addition of a second antibody specific for a nonneutralizing first antibody has long been known to result in an enhanced level of neutralization. This phenomenon has previously been demonstrated with antibodies to NDV (Iorio and Bratt, 1985).

Members of the second group, which includes strains AV, F, L (Table 3), and Eng F (data not shown), are not neutralized by AVS-I, either alone or with RAM Ig, suggesting that the antibody does not bind at all to these strains. These

results are consistent with the ELISA data in Table 3 and the original HI data for Eng F (Srinivasappa et al., 1986).

Members of the third group exhibit an apparently paradoxical neutralization profile. This group, which includes strains U and M (Table 3), as well as La Sota (data not shown), are not neutralized by AVS-I alone but are neutralized upon the addition of RAM Ig. NDV-AV mutants of this type selected with antibodies to HN sites 1, 2, and 4 have previously been characterized in our lab (Iorio and Bratt, 1985). A likely explanation for this is that AVS-I binds to these strains but the binding is not of sufficient avidity to result in neutralization. The RAM Ig second antibody stabilizes its binding, possibly by binding bivalently to two AVS-I molecules. Consistent with this, binding of AVS-I to both strain U and strain M can be detected by ELISA (Table 3).

6.4   Neuraminidase (NA) inhibition

The ability of AVS-I to inhibit the NA activity of avirulent strain B1 was determined (Table 4). The antibody to site 2 was used as a positive control. This antibody has previously been shown to inhibit the NA activity of the AV strain. An MAb specific for the nucleocapsid protein (NP) was used as a negative control. For the avirulent strain B1, the percentages of NA activity remaining are 97% ± 7%, 25% ± 3%, and 68% ± 10% for the NP, site 2, and AVS-I antibodies, respectively. This indicates that AVS-I partially inhibits the NA activity of strain B1. To be certain that the effect is not dose dependent, a threefold higher concentration of each antibody was also tested. The percentages of NA activity

Antibody	Percent of NA activity remaining	
	1X	3X
NP	97 ± 7	102 ± 5
Site 2	25 ± 3	23 ± 3
AVS-I	68 ± 10	72 ± 8

Table 4. Neuraminidase (NA) inhibition by AVS-I antibody
The ability of AVS-I to inhibit the NA activity of avirulent NDV strain B1 was
determined using two concentrations of the antibody. An antibody to NP was
used as a negative control, whereas an antibody to site 2 was used as a positive
control. Data shown is the average of three determinations. ± refer to the
standard deviations.

remaining were not significantly different from those obtained with the lower antibody concentration, i.e., 102% ± 5%, 23% ± 3%, and 72% ± 8% for the NP, site 2, and AVS-I antibodies, respectively. As a control, the AVS-I antibody does not inhibit the NA activity of the virulent strain AV (106% ± 26% NA activity remaining), consistent with its inability to recognize this strain. The results with B1 suggest that, although the AVS-I epitope maps close to the epitope recognized by the site 2 MAb, it maps further from the NA active site than site 2.

6.5   Mapping the binding site of AVS-I on HN relative to our panel of MAbs

To map the binding site of AVS-I on HN relative to our panel of MAbs, reciprocal competition antibody binding assays were performed using B1 virus. By necessity, competing MAbs in this experiment are restricted to those members of our panel that recognize this strain. These include antibodies to sites 1, 14, 4, and 2 but not those to sites 12, 23, and 3 (Iorio et al., 1984; Iorio et al., 1986). As shown in Figure 29A, AVS-I blocks itself and antibodies to sites 1 and 2 at the same concentrations but does not block antibodies to either site 14 or site 4. Figure 29B shows the results of the reciprocal experiment in which the second antibody is biotinylated AVS-I. Again, AVS-I blocks itself and the antibody to site 2 also blocks AVS-I. The antibody to site 1 blocks AVS-I, but only at higher antibody concentrations. The antibodies to sites 14 and 4 do not block AVS-I. Thus, AVS-I and the antibody to site 2 compete with each other reciprocally. AVS-I efficiently blocks the antibody to site 1, but the antibody to site 1 blocks
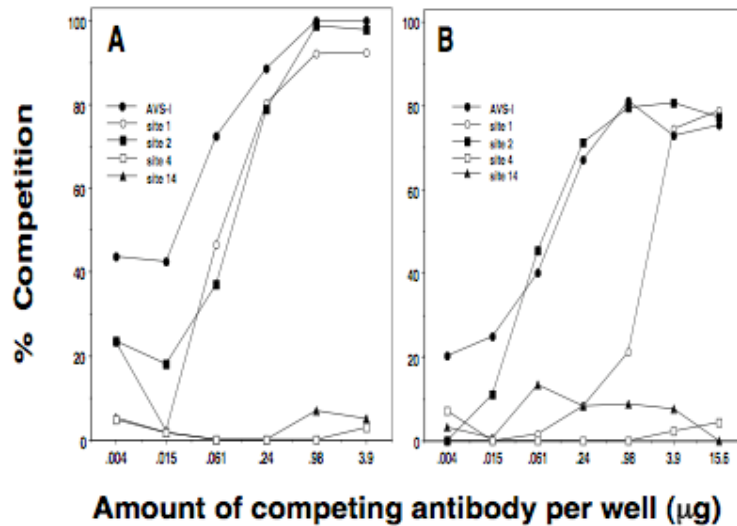
Figure 29. Competition antibody binding assays with AVS-I and antibody to sites 1, 14, 4 and 2.
Microtiter wells coated with intact B1 virions were incubated with serial 4-fold dilutions of an ascites fluid preparation of each antibody. This was followed by the appropriate biotinylated affinity-purified antibody. Streptavidin-horseradish peroxidase was added at a 1:5000 dilution and the absorbance was measured at 650 nm after addition of substrate. The data represent the percentages of competition by increasing concentrations of unlabeled antibodies for the binding of biotinylated antibodies. (A) Inhibition of the binding of biotinylated antibody to sites 1, 14, 4 and 2 by unlabeled AVS-I. The data shown are representative of three independent experiments, each done in duplicate. (B) Inhibition of the binding of biotinylated AVS-I by unlabeled antibody to sites 1, 14, 4 and 2. The data shown represent three independent experiments, each done in duplicate.

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AVS-I only at very high concentrations. These data suggest that the binding site of AVS-I is located closest to antigenic site 2 and also partially overlaps with site 1. One possibility that could account for the nonreciprocal competition between AVS-I and the site 1 antibody is that the binding of AVS-I may induce a nonreciprocal conformational change in the protein that alters site 1.

6.6   AVS-I recognizes a conformation-dependent epitope.

To determine whether the epitope recognized by AVS-I is linear or conformational, a Western blot was performed. Viral proteins from either the B1 or U strain were run on a polyacrylamide gel under reducing conditions. The proteins were transferred to a nitrocellulose membrane and probed with MAbs to site 14 or 4 or AVS-I. Antibody to site 14 recognizes a conserved, linear epitope, while antibody to site 4 recognizes a conserved, conformation-dependent epitope (Iorio et al., 1984; Iorio et al., 1986; Iorio et al., 1991). As expected, the site 14 antibody, but not the site 4 antibody, binds to HN in the Western blot (Figure 30). AVS-I does not bind to HN, which suggests that it recognizes a conformation-dependent epitope.

6.7   AVS-I binds to an epitope in the C-terminal region of B1 HN.

To begin to map the epitope recognized by AVS-I, HN chimeras consisting of domains from the virulent strain AV and the avirulent strain B1 were constructed. This made it possible to exchange amino acids 344 through 571 of AV HN with amino acids 344 through 577 of B1 HN, creating the HN chimeras AV-B1 and B1-AV (Figure 31). These chimeras were expressed in BHK-21 cells.
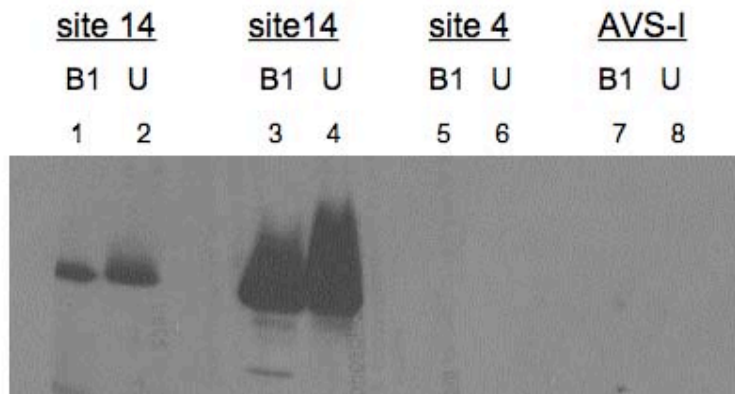
Figure 30. AVS-I recognizes a conformation-dependent epitope.
Viral proteins from either the B1 or U strain were run on a polyacrylamide
gel under reducing conditions. The proteins were transferred to a nitrocellulose
membrane and probed with MAbs to site 14 or 4 or AVS-I. Antibody to site 14
recognizes a conserved, linear epitope, while antibody to site 4 recognizes a
conserved, conformation-dependent epitope (Iorio et al., 1984; Iorio et al.,
1986; Iorio et al., 1991). Lanes 1 and 2 were probed with a site 14-containing
ascites fluid. Lanes 3 and 4 were probed with a site 14 hybridoma supernate.
Lanes 5 and 6 were probed with a site 4 hybridoma supernate. Lanes 7 and 8
were probed with an AVS-I-containing ascites fluid.

Binding (% B1 HN)

		HN mix	AVS-I
AV	571	68.5	4.7
B1	577	100	100
AV-B1	577	104.7	105.4
B1-AV	571	79.2	5.8
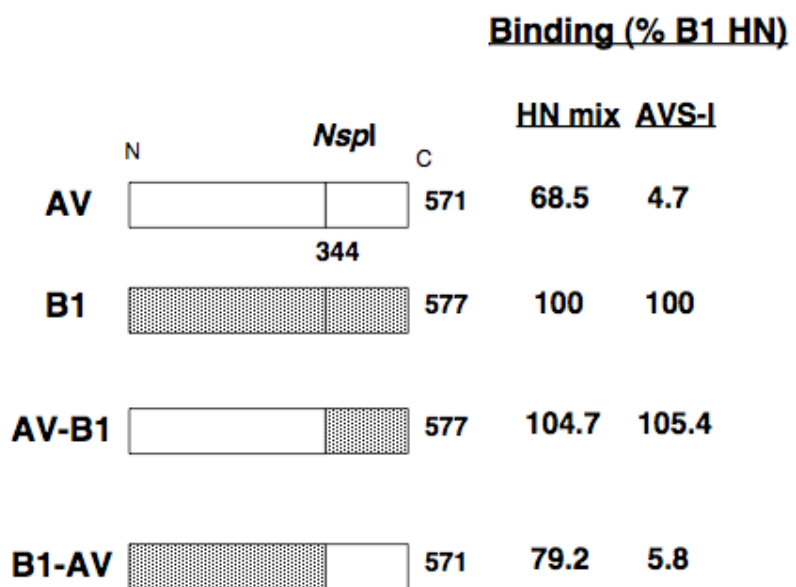
Figure 31. Binding of AVS-I to HN chimeras and wild type HN
The HN chimeras and wild type HN were expressed in BHK-21 cells using
the vaccinia virus T7 RNA polymerase expression system. Flow cytometric
analysis was performed to assess binding of AVS-I to HN chimeras and wt
HN. A cocktail of antibodies to sites 1, 14, and 2 (HN mix) was used to confirm
that the chimeras are expressed at the cell surface. Binding is expressed as
a percentage of that of wt B1 HN. Values shown are based on mean
fluorescence intensity (MFI).

To determine whether the chimeras are functional, a hemadsorption (HAd) assay was performed. Each of the HN chimeras hemadsorbs at a level comparable to that of wild-type (wt) HN (data not shown), indicating that they are expressed and are functional. To assess binding of AVS-I to the HN chimeras, flow cytometric analysis was performed. A cocktail of antibodies to sites 1, 14, and 2 was used to confirm that the chimeras are indeed expressed at the cell surface. Binding is expressed as a percentage of that of wild-type B1 HN. As shown in Figure 31, AVS-I binds to B1 HN and the AV-B1 chimera, whereas it does not bind to either AV HN or the B1-AV chimera. This suggests that AVS-I binds to an epitope in the C-terminal region of B1 HN between amino acids 344 and 577.

6.8   An escape mutant virus is not recognized by AVS-I.

To begin to identify the HN amino acid residues in the epitope recognized by AVS-I, an escape mutant virus was isolated. B1 virus was passaged three times in eggs. The allantoic fluid was treated with AVS-I and plated on chicken embryo cells in the presence of trypsin. A plaque assay was performed as described previously. Treatment with AVS-I resulted in the usual persistent fraction of nonneutralized virus. The neutralization studies with this strain (Table 3) suggest that, despite its escape from neutralization, this persistent fraction does have antibody bound to it. Thus, to make variant selection possible, the passaged pool of B1 virus was treated with AVS-I antibody to sensitize the virus. Then, the antibody-sensitized virus was treated with RAM Ig. This reduces the persistent fraction to 0.1% and makes possible the isolation of escape mutants.

One escape mutant, AVS-B1, was isolated and plaque purified. To test whether antibody AVS-I recognizes the AVS-B1 virus, an ELISA was performed. Figure 32 shows that the AVS-B1 virus is not recognized by AVS-I, consistent with its ability to escape neutralization by the antibody. Antibodies to sites 1 and 14 serve as positive controls, and antibody to site 23 serves as a negative control.

6.9   Amino acid residue G570 is important for binding of AVS-I.

Genomic RNA sequencing of the HN gene of escape mutant AVS-B1 revealed the presence of a G570R mutation. The presence of this mutation, and only this mutation, in AVS-B1 HN was confirmed by reverse transcription-PCR. To establish that residue G570 is important for the binding of AVS-I, the G570R mutation was introduced into B1 HN through site-directed mutagenesis. The G570R-mutated HN was expressed in BHK cells and HAd inhibition and flow cytometric analyses were performed to assess the ability of AVS-I to recognize it. Antibody AVS-I did not inhibit the HAd activity of G570R-mutated B1-HN, resulting in an absorbance of 104% of that obtained with untreated monolayers. As controls, AVS-I completely blocked the HAd activity of wt B1-HN and antibody to site 14 completely inhibited that of both the wt and mutated proteins.

For flow cytometry, a cocktail of antibodies to sites 1, 14, and 2 was used to confirm that the G570R-mutated HN is expressed at a level comparable to that of wild-type HN (Figure 33). Unlike the HAd inhibition results, an interaction between AVS-I and G570R-mutated B1-HN was detected, although significantly decreased compared to the wt protein (39 ± 3%) (Figure 33). This finding, along
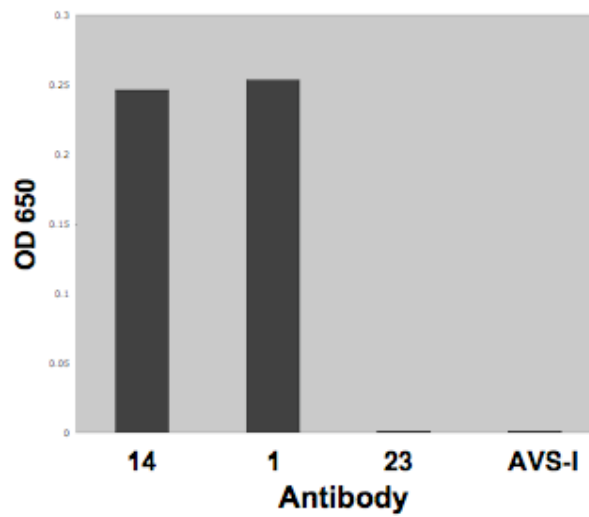
Figure 32. AVS-I does not recognize the B1 escape mutant virus.
Microtiter wells coated with intact virions of the B1 escape mutant to AVS-I were incubated with a 1:2700 dilution of AVS-I. Horseradish peroxidase-conjugated goat anti-mouse antibody was added at a 1:1000 dilution and the absorbance was measured at 650 nm after addition of substrate. Antibodies to sites 1 and 14 serve as positive controls, and antibody to site 23 serves as a negative control.

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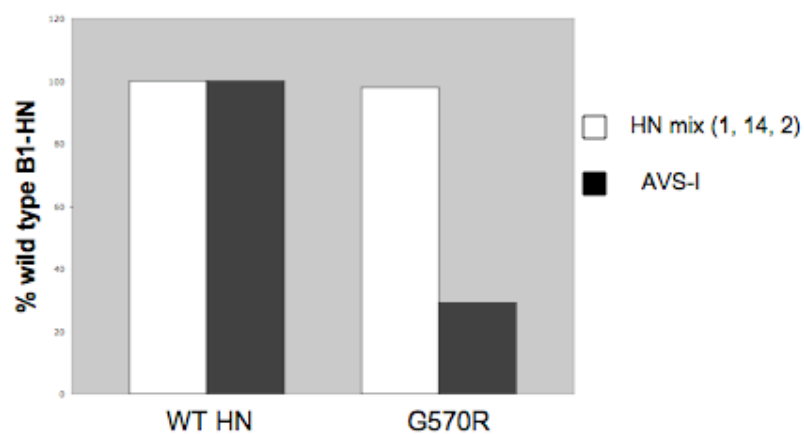
Figure 33. Binding of AVS-I to G570R HN mutant
The G570R-mutated HN was expressed in BHK-21 cells using the vaccinia virus T7 RNA polymerase expression system. Flow cytometric analysis was performed to assess binding of AVS-I to the G570R HN mutant. A cocktail of antibodies to sites 1, 14 and 2 (HN mix) was used to confirm that the G570R-mutated HN was expressed at a level comparable to that of wt HN.

with our inability to identify another mutation in the mutant virus, suggests that the G570R mutation does not completely eliminate the ability of AVS-I to recognize the virus but does reduce the avidity of the interaction to a level insufficient to block infectivity or attachment to receptors on red blood cells.

CHAPTER VII

Discussion

The pathogenesis of a virus is a complex process that involves a variety of viral and host factors. With NDV, it is clear that several viral proteins play important roles in this process. It has been established that cleavage of the fusion protein precursor (F_0) is the major determinant of virulence. Virulent strains of the virus have two pairs of basic amino acids at the cleavage site, allowing host proteases, which are found in a wide range of tissues, to cleave F_0 and enable the virus to spread to various organs. Avirulent strains of the virus possess only two basic amino acids at the cleavage site. Hence, their F_0 is cleaved only by trypsin-like proteases secreted by a limited number of tissues, resulting in localized infections (Nagai et al., 1976; Glickman et al., 1988; Toyoda et al., 1989).

Although cleavage of the F protein is the primary determinant of virulence, several studies have shown that it is not the sole determinant. Modification of a lentogenic F cleavage site to a velogenic one increased virulence, but not to the level of velogenic strains, indicating that other viral proteins also contribute to virulence (Panda et al., 2004; Peeters et al., 1999). Two such proteins are the HN and V proteins. The evidence that the HN protein plays a role in virulence comes from a study done by Huang et al. (2004). The HN gene of the mesogenic recombinant BC strain was exchanged with that of the lentogenic recombinant strain La Sota. Pathogenicity studies demonstrated that the BC virus, having the

HN of La Sota, decreased in virulence, whereas the La Sota virus having the HN of BC increased in virulence, indicating that HN plays a role in this process. The evidence that the V protein also contributes to virulence comes from several studies. Recombinant viruses lacking the V protein have impaired growth in cell cultures and chicken embryos (Mebatsion et al., 2001; Huang et al., 2003). In addition, pathogenicity studies showed that V-deficient recombinant viruses are highly attenuated in chickens, indicating that the V protein plays an important role in virulence (Huang et al., 2003).

The contributions of the HN and V proteins in NDV virulence are only beginning to be elucidated. First, the roles of HN and V in the differential virulence patterns exhibited by virulent and avirulent members of the NDV serotype have not been examined in detail. Second, the specific amino acid residues in the two proteins that contribute to virulence have not been identified. Third, the specific mechanisms by which these two proteins contribute to virulence have not been completely elucidated. A better understanding of these aspects will help in the development of antiviral strategies and production of recombinant viruses suitable for use in gene therapy.

7.1 The IFN antagonistic activity of the V protein from the mesogenic strain BC is greater than that from the lentogenic strain La Sota

The importance of IFN antagonists in virulence has been demonstrated for several viruses. It was found that viruses with mutations in genes encoding HSV type 1 ICP34.5, vaccinia virus E3L, and influenza virus NS1 proteins have altered

pathogenicity in mice (Chou et al., 1990; Brandt and Jacobs, 2001; Talon et al., 2000). Similarly, a mutant Sendai virus with a single mutation in the C protein is avirulent in mice (Garcin et al., 1997). The role of the V protein in virulence has also been demonstrated for several paramyxoviruses. A V-deficient recombinant Sendai virus was found to be remarkably attenuated in mice (Kato et al., 1997). Recombinant measles virus lacking the V protein undergoes limited replication and fails to spread in the brain of mice. In addition, this mutant virus causes lower mortality in mice relative to the parental virus (Patterson et al., 2000). Studies have also shown that NDV V-deficient recombinant viruses have impaired growth in cell culture and decreased pathogenicity in chickens (Mebatsion et al., 2001; Huang et al., 2003). In addition, these mutant viruses have increased sensitivity to the antiviral effects of exogenous interferon (Huang et al., 2003). Using the NDV-GFP-based assay, it was shown that the NDV V protein exhibits IFN antagonistic activity (Park et al., 2003). Hence, the mechanism by which the V protein plays a role in virulence is due, at least in part, to its IFN antagonistic activity.

To begin to understand the role of the V protein in the differential virulence patterns exhibited by different pathotypes of NDV, the IFN antagonistic activity of the V protein from the lentogenic strain La Sota was compared with that of the mesogenic strain BC. Ideally, one would like to compare the V protein of a lentogenic strain with that of a velogenic strain. However, these studies will eventually lead to pathogenicity studies in animals. All velogenic NDV strains are

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classified as select agents and cannot be used in animal studies in BL-3. Hence, the mesogenic strain BC was used.

Using the NDV-GFP-based assay, the V protein of the lentogenic NDV strain B1-Hitchner has been shown to antagonize IFN. In this assay, the ability of a protein to antagonize IFN is measured by its ability to rescue growth of the NDV-GFP virus. This assay has proven to be useful for screening proteins with IFN antagonistic activity. Other known IFN antagonists such as the influenza virus NS1 protein and the Ebola virus VP35 protein have been shown to rescue growth of the NDV-GFP virus in this assay. In addition, the Nipah virus V, W and C proteins also rescue growth of the virus. However, the Nipah C protein rescues viral growth less efficiently than either the Nipah V or W protein (Park et al., 2003).

To compare the IFN antagonistic activity of the V proteins of the lentogenic strain La Sota and the mesogenic strain BC, I modified the NDV-GFP assay developed by Park et al. (2003). The chicken embryo fibroblast cell line DF1 was used instead of chicken embryo fibroblasts (CEFs). In addition, the NDV-GFP virus was derived from strain BC instead of B1-Hitchner and was used at a lower moi (0.001 instead of 1). Using this assay, we showed that the BC V protein exhibits a 4-fold greater ability than the La Sota V protein to rescue growth of the NDV-GFP virus. This is consistent with the known virulence properties of these strains.

One caveat is that this difference in rescue of viral growth could be due only to differences in expression levels of these two V proteins. However, this possibility is unlikely because the expression levels, as determined by Western blot using the V18 antibody, do not correlate with the ability of the La Sota V and BC V mutated proteins and chimeras to rescue growth of the NDV-GFP virus. Our results demonstrate that the V protein of the mesogenic strain BC exhibits greater IFN antagonistic activity than that of the lentogenic strain La Sota. Hence, the IFN antagonistic activities of the two V proteins correlate with the known virulence properties of these strains. Taken together, our results are consistent with the V protein having a role in the virulence of NDV and that the mechanism by which it does so is due, at least in part, to its IFN antagonistic activity.

7.2 Identification of amino acid residues responsible for the difference in IFN antagonistic activity between the La Sota and BC V proteins

7.2.1 Amino acid residues in the C-terminal region modulate the IFN antagonistic activity of the NDV V protein

The V proteins of paramyxoviruses show an overall homology of 44%. The region of highest homology is found in the C-terminus, which is approximately 50% identical among all known paramyxovirus V proteins (Southern et al., 1990). The C-terminal region contains seven conserved cysteine residues capable of binding two zinc atoms. The contribution of this region to the IFN antagonistic activity of the V protein and virulence is well documented. A recombinant Sendai virus, which has a truncated V protein lacking the C-terminal region was shown

to be attenuated in mice (Kato et al., 1997). Similarly, recombinant Sendai viruses with mutations in the C-terminus of the V protein showed impaired replication in mouse lungs and attenuated virulence in mice (Fukuhara et al., 2002; Huang et al., 2000). A recombinant hPIV2 that expresses a truncated V protein lacking its C-terminal region was sensitive to inhibition by IFN (Kawano et al., 2001). In addition, it was demonstrated that cells expressing hPIV2 V protein are resistant to the antiviral effects of IFNα/β and that the cysteine-rich domain of the V protein is necessary for resistance to IFN (Nishio et al., 2001). Likewise, a recombinant SV5 that lacks the V protein C-terminal domain also failed to block IFN signaling (He et al., 2002). All of these findings point to the importance of the C-terminus of the paramyxovirus V protein in virulence.

Using the NDV-GFP assay, it was also shown that the C-terminus of NDV V (amino acid residues 136-239) is necessary and sufficient to antagonize IFN (Park et al., 2003). However, the specific amino acid residues involved have not been identified. Comparison of the C-terminal regions of the La Sota and BC V proteins revealed four amino acid differences at positions 144, 153, 161 and 234. In our study, it was shown that the IFN antagonistic activity of La Sota V increases when any of these residues is mutated to the corresponding residue in BC V. Conversely, the IFN antagonistic activity of BC V decreases when any of these residues is mutated to the corresponding residue in La Sota V. However, no single mutated La Sota V protein rescues viral growth at the same level as BC V, and no single mutated BC V protein rescues viral growth at the same level as

La Sota V. These results indicate that, no single residue accounts for the difference in IFN antagonistic activity between the La Sota V and BC V proteins.

Having shown that single mutations in residues 144, 153, 161 and 234 alter the ability of La Sota and BC V to antagonize IFN, we investigated whether the introduction of multiple mutations in these two V proteins has an additive effect. The double mutated proteins of La Sota V (S144P/E153K and S161P/D234N) are better than the single mutated proteins in rescue of viral growth. However, the triple (S144P/E153K/S161P) and quadruple (S144P/ E153K/S161P/D234N) mutated proteins of La Sota V are not better than the double mutated proteins. One of the double mutated proteins of BC V (P144S/K153E) has a decreased ability to rescue the virus as compared to the single mutated proteins. However, the other double mutated protein of BC V (P161S/N234D) rescues viral growth at almost the same level as the P161S and N234D mutated proteins. Surprisingly, the triple (P144S/K153E/P161S) and quadruple (P144S/K153E/P161S/N234D) mutated proteins of BC V are better than the double mutated proteins. These results demonstrate that the presence of multiple mutations in the V protein has a partial additive effect. It is not clear why the triple and quadruple mutated proteins of La Sota V do not rescue viral growth better than the double mutated proteins, or why the triple and quadruple mutated proteins of BC V do not exhibit decreased rescue of viral growth than the double mutated proteins. I speculate that different combinations of amino acid residues would have different effects on the conformation of the V protein,

which in turn affect the ability of the V protein to interact with other proteins involved in the IFN response. Taken together, the results of this study indicate that the amino acid residues 144, 153, 161 and 234 are collectively responsible for the difference in the IFN antagonistic activity of the two V proteins.

The crystal structure of the NDV V protein has not been solved. To gain some insight as to the location of NDV V residues 144, 153, 161 and 234 in the three dimensional structure of the protein, the sequences of the La Sota and BC V proteins were aligned with that of the SV5 V protein for which the crystal structure is known (Li et al., 2006). Sequence alignment reveals that NDV V residues 144, 153 and 161 correspond to SV5 V residues 138, 147 and 155, respectively. NDV V residue 234 does not have a corresponding residue because SV5 V consists of only 222 amino acids. The crystal structure of SV5 V protein in complex with the UV damaged DNA binding protein 1 (DDB1) is shown in Figure 34. DDB1 has a multidomain structure consisting of three β propellers (BPA, B and C) and a helical C-terminal domain. The SV5 V protein binds to DDB1 using both its N-terminal extension and the core domain. It inserts its N-terminal helix into the pocket formed between BPA and BPC while the core domain interacts extensively with the BPC domain. By this interaction, the SV5 V protein is able to hijack the DDB1-Cul4A E3 complex to promote degradation of STAT proteins and block IFN signaling. Another relevant finding is that paramyxovirus V proteins can oligomerize and that the cysteine-rich zinc-binding C-terminal domain is necessary and sufficient for oligomerization, allowing the V

proteins to form spherical particles that act as a scaffold for E3 ubiquitin ligase activity (Ulane et al., 2005).

The location of residues 138, 147 and 155 in the crystal structure of SV5 V was determined (Figure 34). Analysis reveals that residue 138 is close to the pocket formed between BPA and BPC. Residue 147 is close to the conserved seven cysteines in the C-terminal domain of paramyxovirus V proteins. Residue 155 is missing in the crystal structure. Given this, it is tempting to speculate that amino acids at these positions could modulate the ability of the V protein to interact with DDB1 and/or to oligomerize, either of which would result in a decreased ability to recruit components of the ubiquitin ligase complex and, in turn, to mediate the degradation of components of the IFN response.

To determine whether the identity of NDV V residues 144, 153, 161 and 234 correlates with the known virulence properties of different NDV strains, the nucleotide sequences of the P gene of 12 strains were obtained from the GenBank. One G nucleotide was inserted into the RNA editing site and the amino acid sequences of the putative V proteins were aligned (Figure 35). The V proteins of these strains are approximately 59% identical. Surprisingly, analysis revealed that lentogenic strains are more similar to velogenic strains than they are to mesogenic strains at positions 144, 153 and 234 (Table 5). There is no pattern for the identity of residue 161 for these strains. In addition, there are no amino acids that are specific for any of the three pathotypes. Given this, it would be informative to compare the V proteins of velogenic and lentogenic   strains as
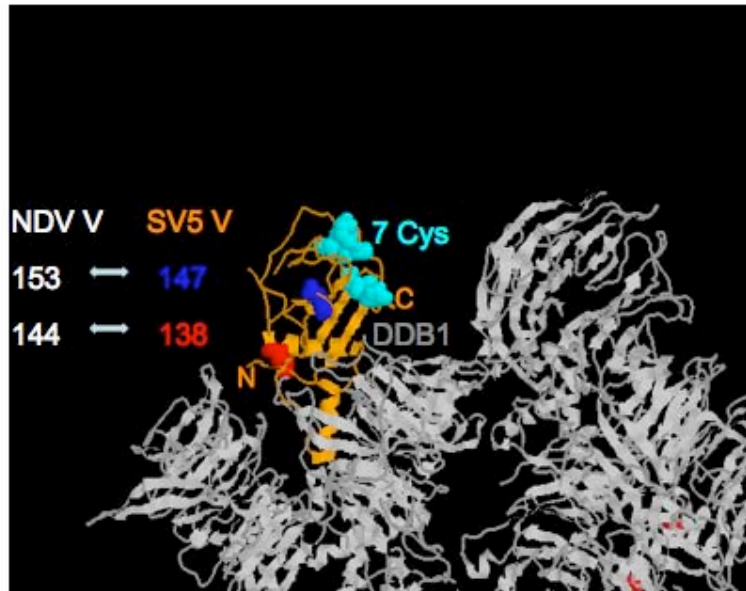
Figure 34. NDV V residues 144 and 153 correspond to SV5 V residues 138 and 147, respectively.
The amino acid sequence of NDV V was aligned with that of SV5 V. NDV V residues 144, 153 and 161 correspond to SV5 V residues 138, 147 and 155, respectively. NDV V residue 234 does not have a corresponding residue because SV5 V consists of only 222 amino acids. SV5 V residue 138 is close to the DDB1 double propeller pocket and residue 147 is close to the conserved seven cysteines. SV5 V residue 155 is missing in the crystal structure (adapted from Li et al., 2006).

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Strain                              V protein amino acid sequence

LaSota   (L)   MATFTDAEIDELFETSGTVIDNIITAQGKPAETVGRSAIPQGKTKVLSAAWEKHGSIQPP  60
B1/48    (L)   MATFTDAEIDELFETSGTVIDNIITAQGKPAETVGRSAIPQGKTKVLSAAWEKHGSIQPP  60
Kimber47 (M)   MATFTDAEIDELFETSGTVIDNIITAQGKPAETAGRSAIPHGKTKALSAAWEKHGSIQPP  60
Roakin48 (M)   MATFTDAEIDELFETSGTVIDNIITAQGKPAETVGRSAIPHGKTKALSAAWEKHGSIQPP  60
BC       (M)   MATFTDAEIDELFETSGTVIDNIITAQGKSAETVGRSAIPHGKTKALSAAWEKHGSIQPP  60
TXGB/48  (V)   MATFTDAEIDELFETSGTVIDSIITAQGKPAETVGRSAIPQGKTKALSAAWEKHGSIQPP  60
Ulster   (L)   MATFTDAEIDELFETSGTVIDSIITAQGKPVETVGRSAIPRGKTKALSSAWEKHGSVQSP  60
QV4/66   (L)   MATFTDAEIDDIFETSGTVIDSIITVQGKSVETFGKSATPQGKTKAPSTTWEKHGSIQPP  60
AV       (V)   MATFTDAEIDDLFETSGTIIDSIITAQGKPVETVGRSAIPQGKTKALSAAWEKHESIQPP  60
Herts/33 (V)   MATFTDAEIDELFETSGTVIDNIITAQGKPAETVGRSAIPQGKTKVLSAAWEKHGSIQPP  60
Largo    (V)   MATFTDAEIDELFETSGTVIDSIITAQGKSAETVGRSAIPQGKTKALSTAWEKHGSIQPS  60
Fontana  (V)   MATFTDAEIDELFETSGTVIDSIITAQGKSAETVGRSAIPQGKTKVLSTAWEKHGSIQPP  60


LaSota         ASQDNPDRQDRSDKQPSTPEQTTPHDSPPATSADQPPTQATDEAVDTQLRTGASNSLLLM  120
B1/48          ASQDNPDRQDRSDKQPSTPEQRTPHDSPPATSADQPPTQATDEAVDTQLRTGASNSLLLM  120
Kimber47       ASQDTPDRQDRSDKQPSTPEQATRHDSPPATPADQPPTQATDEAVDTQLRTGASNSLLLM  120
Roakin48       ASQDTPDRQDRSDKQPSTPEQATPHDSPPATSADQPPTQATDEAVDTQLRTGASNSLLLM  120
BC             ASQDTPDRQDRSDKQPSTPEQATPHDSPPATSADQPPTQATDEAVDTQLRTGASNSLLLM  120
TXGB/48        ASQDTPDRQDRSDKQPSTPEQATPHDSPPATSADQPPAQATDEVVDTQLKTGASNSLLLM  120
Ulster         ASQDTPDRQDRSDKQLSTPEQVTPHDSPPATSTDQPPTQAADEAGDTQLKTGASNSLLSM  120
QV4/66         ASQDTPDRQDRSDKQLSTPEQASPNDSPPATSTDQPPTQAADEAGDTQLKTGASNSLLSM  120
AV             ASQDTPDRQNRSDKQPSTPEQMTPQNSPPATSTDQPPTQAAGEAGDTQLKTGASNSLLSM  120
Herts/33       ASQDNPDRQDRSDKQPSTPEQVTPHNSPPATSTDQPPTQAAGEAGDTQLKTGASNSLLSM  120
Largo          ASQDTPDQQDRSDKQPSTPEQATPHNSPPATPTEPPPTQAAGEAGDAQLKTGASNSLLSM  120
Fontana        ASQDTPDQQDRSDKQPSTPEQATPHNSPPATSTEPPPTQAAGEAGDTQLKTGASNSLLSM  120


LaSota         LDKLSNKSSNAKKGPMVEPPRGESPTSDSTAGESTQPRKQSGKTAEPSQGRPWKPGHRRE  180
B1/48          LDKLSNKSSNAKKGPMVEPPRGESPTSDSTAGESTQSRKQSGKTAEPSQGRPWKPGHRRE  180
Kimber47       LDKLSNKSSNAKKGPMVEPPRGEPPTSDPTAGKSTQPRKQPGKTAEPSQGRPWKPGHRPE  180
Roakin48       LDKLSNKSSNAKKGPMVEPPRGEPPTSDSTAGKSTQPRKQSGKTAEPSQGRPWKPGHRRE  180
BC             LDKLSNKSSNAKKGPMVEPPRGEPPTSDSTAGKSTQPRKQSGKTAEPSQGRPWKPGHRRE  180
TXGB/48        LDKLSNKSSNAKKGPMVEPPRGESPTSDSTAGESTQPRKQPGKTAEPSQGRPWKPGHRRE  180
Ulster         LDKLSNKSSNAKKGPMVKPPGRASSTSDSTAGESTKPRKQSRETAEPGQGRPWKPGHRRE  180
QV4/66         LDKLSNKSSNAKKGPMVEPSRKASSTSDSTTGESTKPRKQPRETAEPGQGHPWKPGHRRE  180
AV             LDKLSNKSSNAKKGPMVESPGRTSSTSDPTAGESTEPRKQSGETAEPGQCRPWRPGHRRE  180
Herts/33       LDKLSNKSSNAKKGPMVESPGRASSTSAPTAGESTEPREQSGKTAKPGQGRPWRPGHRRE  180
Largo          LDKLSNKSSNTKKGPMVKSSRRTSPTSGQTTWESAEPRKQPGKIPAPGQGRPWKPGHRRE  180
Fontana        LDKLSNKSSNAKKGPMVEPPGRTSPTSGQTTGETAEPRKQPGKTAEPGQGRPWKPGHRRE  180


LaSota         HSISWTMGGVTTISWCNPSCSPIKAEPRQYPCICGSCPATCRLCASDDVYDGGDITESKZ  240
B1/48          HSISWTMGGVTTISWYNPSWSPVMAKPCHNPGFCGSFPHTSWLWECEDVFVGGDFTESNZ  240
Kimber47       HSISWTMGGFTTISWCNPSCSPIKAKPKQYPCFCGSFPPTCKLCATDDVYDGGNITESKZ  240
Roakin48       HSISWTMGGVTTISWCNPSCSPIKAKPKQYPCICGSFPATCRLCASDDVYDGGNITESKZ  240
BC             HSISWTMGGVTTISWCNPSCSPIKAEPRQYPCICGSCPATCRLCASDDVYDGGNITESKZ  240
TXGB/48        HSISWTMGGVTTISWCNPSCSPIKAEPRQYPCICGSCPATCRLCASDDVYDGGSVTESKZ  240
Ulster         HSISWTMGGVTTISWCNPSCSPIRAEPRQYSCTCGSCPATCRLCASDDVYDGGDITEGKZ  240
QV4/66         HSISWTMGGVTTISWCNPSCSPIRAEPRQYSCTCGSCPVTCRLCAGDDVYDGGDITEGKZ  240
AV             HSISWTMGGVTTISWCNPSCSPVRAEPRQYSCICGSCPATCRLCAGDDVYDGGDITEGKZ  240
Herts/33       HSISWTMEGVTTISWCNPSCPPVRAKPKQYSCTCGSFPPTCQLCAGDDVYDGGVITEGKZ  240
Largo          HSISWTTEGVTTISWCNPSCSPIRAEPRQYSCICGSCPATCRLCAGDDVYDGGVIAEGKZ  240
Fontana        HSISWTTEGVTTISWCNPSRSPIRAEPRQYSCTCGSCPATCRLCAGDDVYDGGDITEGKZ  240
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Figure 35. Alignment of the V proteins of several NDV strains

The nucleotide sequences of the P gene of 12 NDV strains were obtained from the GenBank. These include lentogenic (L), mesogenic (M) and velogenic (V) strains. One G nucleotide was inserted into the RNA editing site and the amino acid sequences of the putative V proteins were aligned.

NDV strain	Pathotype	Amino acid residue			
		144	153	161	234
La Sota	L	S	E	S	D
B1/48	L	S	E	S	D
Ulster	L	S	E	S	D
Queensland V4/66	L	S	E	P	D
Beaudette C	M	P	K	P	N
Kimber/47	M	P	K	P	N
Roakin/48	M	P	K	S	N
Australia-Victoria/32	V	S	E	S	D
Texas GB/48	V	S	E	P	S
Herts/33	V	S	E	S	V
Largo/71	V	S	E	P	V
CA 1083 (Fontana)/72	V	S	E	P	D

Table 5. Comparison of residues 144, 153, 161 and 234 of the V protein of some NDV strains.
The amino acid sequences of the V proteins of several NDV strains were aligned as shown in Figure 35. These include lentogenic (L), mesogenic (M) and velogenic (V) strains.

I have done here. There may be other amino acid differences between the V proteins of these strains that could account, at least in part, for their wide difference in virulence.

7.2.2 The N-terminal region of the BC V protein also contributes to its IFN antagonistic activity

The results of the NDV-GFP assay also showed that the La Sota V S144P/E153K/S161P/D234N quadruple mutated protein failed to rescue viral growth at the same level as BC V. Similarly, the BC V P144S/K153E/ P161S/N234D quadruple mutated protein also failed to rescue viral growth at the same level as La Sota V. These results suggest that amino acid residues in the N-terminal region of the V protein may be important. Indeed, the decreased ability of the LS-BC chimera, relative to BC V, to rescue growth of the NDV-GFP virus suggests that the N-terminal region contributes to the IFN antagonistic activity of the BC V protein.

These results are consistent with those obtained with other paramyxoviruses. A spontaneous SV5 mutant with mutations in the P/V N-terminal common domain showed no anti-IFN activity, indicating that this domain contributes to its IFN antagonistic activity (Chatziandreou et al., 2002; Wansley and Parks, 2002; Young et al., 2001). Another study showed that tyrosine 110, which is located in the P/V N-terminal common domain, is important for inhibition of IFNα/β and IFNγ signaling by the V protein of measles virus (Fontana et al., 2008). The amino acid sequence of the LS-BC chimera differs from that of BC V

at five positions in the N-terminal region. It is conceivable that these changes in the N-terminus could affect the three dimensional structure of the V protein, making the C-terminus less accessible to interacting proteins. Structure-function studies are required to verify this theory, but this awaits determination of the crystal structure of the NDV V protein. It is also plausible that residues in the N-terminal region could contribute directly to the IFN antagonistic activity of the V protein.

7.3   Mechanism for the difference in IFN antagonistic activity of the La Sota and BC V proteins

7.3.1   Contrary to an earlier report, NDV V does not target STAT1 for degradation

Huang et al. (2003) showed that infection of human 2fTGH cells with a recombinant BC virus results in degradation of STAT1. Moreover, they showed that transient transfection of a plasmid encoding the C-terminus of BC V also results in STAT1 degradation. In both experiments, endogenous STAT1 was degraded to a level below the detection limits of the system.

The results of our study show that only 30% of endogenous STAT1 is degraded when 2fTGH cells are transiently transfected with BC V. This decrease in the level of STAT1 is not nearly as significant as that demonstrated by Huang et al. (2003). Thus, I was unable to reproduce the findings of Huang et al., using a transient transfection system. To be certain, I tried several other approaches to try to confirm these results. First, the levels of STAT1 at different times post-

transfection were determined and total degradation could not be demonstrated at 12, 14, 16, 20, 36, 42, 48 or 60 hours post-transfection. I also examined the level of STAT1 in 293 cells transfected with the NDV V protein. Though these cells have a much higher transfection efficiency than 2fTGH cells, I was still unable to reproduce the results of Huang et al. (2003).

The results of our NDV-GFP assay showed that both La Sota and BC V exhibit IFN antagonistic activities, although at different extents. Thus, we wanted to examine further whether STAT1 is degraded under these conditions. Our results show that the level of STAT1 in La Sota V-transfected DF1 cells is similar to that in vector-transfected cells (average of 85% of vector), whereas the level of STAT1 in BC V-transfected cells is only slightly reduced (average of 75% of vector). Most importantly, the decrease in STAT1 under these conditions is not nearly as significant as that shown by Huang et al. (2003).

As a final attempt to determine whether the NDV V protein targets STAT1 for degradation, DF1 cells were transfected with La Sota or BC V that were subcloned in a bicistronic vector that expresses GFP. This strategy allowed us to sort cells that express either of the two V proteins by using GFP as a marker. This approach is more stringent because it enables one to focus exclusively on those cells that express the V protein. In addition, chicken cells, which are the natural host of NDV, were used. The results showed that the level of STAT1 did not decrease in cells expressing either of the two V proteins, again confirming that neither La Sota V nor BC V targets STAT1 for degradation.

Hence, despite considerable efforts to reproduce the results of Huang et al. (2003), complete degradation of STAT1 could not be achieved. In retrospect, the fact that human 2fTGH cells do not have a high transfection efficiency would seem to make it unlikely that STAT1 could be degraded completely in a culture transiently transfected with the V protein. Therefore, these results call into question the previous finding that the NDV V protein targets STAT1 for degradation.

7.3.2   NDV V promotes the conversion of IRF-7 to a lower molecular weight form   (IRF-7*)

A novel and interesting finding in our study is that the NDV V protein targets IRF-7 for degradation. Transfection of either La Sota V or BC V in DF1 cells and examination of the levels of IRF-7 in GFP-sorted cells by Western blot reveal that IRF-7 is reduced to undetectable levels. Hence, both V proteins apparently target IRF-7 for degradation. This demonstrates an additional mechanism by which the NDV V protein antagonizes the IFN response. This is also the first evidence that IRF-7 is targeted by a paramyxovirus V protein.

IRF-7 is a very important player in the IFN response. Studies using IRF-7 knockout mice showed that transcription of both IFNα and IFNβ is dependent on IRF-7, indicating that IRF-7 is the master regulator of type I IFN-dependent responses (Honda et al., 2005). Hence, it is advantageous for NDV to target IRF-7. This phenomenon has also been observed in several other viruses. The NSP1 protein of rotavirus induces degradation of IRF-7 (Barro and Patton, 2007).

The immediate early protein BZLF-1 of Epstein-Barr virus associates with IRF-7 and prevents it from activating type I IFN promoters (Hahn et al., 2005). The E3L protein of vaccinia virus blocks phosphorylation of IRF-7 (Smith et al., 2001). It would be interesting to determine the exact mechanism by which the NDV V protein targets IRF-7 for degradation.

Because the IRF-7 levels are undetectable in both La Sota V- and BC V-transfected cells at 24 and 48 hours post-transfection, comparison of the ability of the La Sota and BC V proteins to target IRF-7 for degradation is difficult. It is possible that differences in the levels of IRF-7 are detectable earlier to which end sorting the cells at earlier time points might be informative. However, one limitation of our method is that GFP is expressed at 24 hours post-transfection, so it would be difficult to identify GFP-positive cells at earlier time points. Hence, an alternative system is necessary to further examine whether La Sota V and BC V differ in their ability to degrade IRF-7.

A novel observation from our studies is that a protein of lower molecular weight (IRF-7∗) is detected by the IRF-7 antibody in both La Sota V- and BC V-transfected DF1 cells, but not in vector-transfected cells. The antibody used in this experiment was raised against residues 1-126 of human IRF-7. In our experiments using chicken DF1 cells, this antibody recognizes full-length IRF-7, which migrates at approximately 66 kDa and IRF-7∗, which is approximately 39 kDa. Mass spectrometric analysis of a band excised from a Coomassie stained gel that aligned with IRF-7∗ on the Western blot revealed that there are three

possible peptides that could be from IRF-7. However, this result is not definitive because the peptides have weak spectra. One possibility that could account for the inability to identify IRF-7∗ as being a fragment of IRF-7 is the presence of very small amounts of IRF-7∗ in the excised gel band. Nevertheless, these results are consistent with IRF-7∗ being a degradation product of IRF-7. Furthermore, this suggests that both La Sota and BC V promote conversion of IRF-7 to IRF-7∗.

7.3.3   The extent of conversion of IRF-7 to IRF-7∗ correlates with both IFN antagonistic activity and virulence

Our studies demonstrate that the amount of IRF-7∗ is significantly greater in BC V-transfected cells as compared to La Sota V-transfected cells. This indicates that the extent of conversion of IRF-7 to IRF-7∗ correlates with the extent of IFN antagonistic activity of the V protein and, in turn, with the level of virulence. This is consistent with the conversion of IRF-7 to IRF-7∗ playing a role in both IFN antagonistic activity and virulence.

Indeed, most notably, the amount of IRF-7∗ in BC V-transfected cells is similar, while the amount in La Sota V-transfected cells is considerably less, than the amount of full-length IRF-7 in vector-transfected cells. This suggests that BC V promotes complete conversion of IRF-7 to the lower molecular weight form, whereas La Sota V does so less efficiently.

7.3.4 Formation of IRF-7∗ is blocked by a proteasome inhibitor

To examine whether IRF-7∗ is a degradation product of IRF-7, DF1 cells were transiently transfected with BC V expressed from the bicistronic vector pIRES2-AcGFP1 in the absence or presence of the proteasome inhibitor clasto-lactacystin-β-lactone. At 24 hours post-transfection, GFP-negative and GFP-positive cells were sorted by flow cytometry. Our results show that, in cells expressing BC V, the amount of IRF-7∗ is significantly decreased in the presence of 2 μM inhibitor relative to the untreated control. A further reduction in the amount of IRF-7∗ was observed when 5 μM inhibitor was used. Thus, the amount of IRF-7∗ decreases in a dose-dependent manner in the presence of a proteasome inhibitor. This is consistent with IRF-7∗ being a degradation product of IRF-7, whose formation is mediated by a proteasome-dependent pathway.

Surprisingly, the amount of full-length IRF-7 did not increase in the presence of the inhibitor. It is possible that the amount of full-length IRF-7 protected by treatment with the proteasome inhibitor is below the detection limits of the system. Another possible explanation for this result is that the steady-state synthesis of IRF-7 is insufficient to restore the level of IRF-7. A similar result was obtained by Andrejeva et al. (2002) who found that treatment of 2fTGH cells stably expressing SV5 V or PIV2 V with the proteasome inhibitor MG132 resulted in very little increase in the levels of either STAT1 or STAT2 in the respective cells. Nevertheless, these results provide support that IRF-7∗ is a degradation product of IRF-7. It is possible that IRF-7 is degraded in a progressive manner by

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the proteasome and reaches a point where it can no longer proceed. A precedent for this is the role of the proteasome in the proteolytic processing of the transcription factor NF-κB. Palombella et al. (1994) demonstrated that the p50 subunit of NF-κB is generated from the p105 precursor through a proteasome-dependent process, wherein the C-terminal region of p105 is rapidly degraded, leaving the N-terminal p50 fragment intact.   This demonstrates that the proteasome functions both in the partial and complete degradation of proteins.

There are several studies that demonstrate that viruses target IRFs for proteasomal degradation. The Npro protein of classical swine fever virus, ICPO protein of bovine herpesvirus 1 and NSP1 protein of rotavirus all induce proteasomal degradation of IRF-3 (Bauhofer et al., 2007; Saira et al., 2007; Barro and Patton, 2005). The RTA protein of Kaposi's sarcoma herpesvirus and NSP1 protein of rotavirus target IRF-7 for proteasomal degradation (Yu et al., 2005; Barro and Patton, 2007). However, the targeting of IRF-7 for proteasomal degradation by a paramyxovirus V protein has not been described previously. Hence, our results provide the first evidence for this as a mechanism for modulating the IFN response by a paramyxovirus.

However, it cannot be ruled out that IRF-7∗ is a cleaved form of IRF-7 whose formation is mediated by a proteasome-independent degradation pathway. There is evidence for the existence of IRF-1 species that have greater electrophoretic mobilities than the full-length protein in monocytic cell lines. These smaller IRF-1 species were recognized by a rabbit polyclonal antiserum

against human IRF-1 in a Western blot. In addition, the results suggested that these smaller IRF-1 species are generated by cleavage of intact IRF-1 by a serine protease in monocytes (Qiao et al., 2002). Given this, it is possible that La Sota V and BC V are promoting the cleavage of IRF-7 at one site and converting it to a lower molecular weight protein. There is no evidence that the NDV V protein possesses proteolytic activity so it might be recruiting a protease to cleave IRF-7. Since the antibody was raised against the N-terminal 246 residues, it seems likely that IRF-7 is being degraded at the C-terminal end of the protein. Additional experiments need to be done to confirm this.

IRF-7 is activated by phosphorylation of specific amino acid residues in its C-terminal region. Marie et al. (1998) previously demonstrated that IRF-7 containing mutations in serine 425 and serine 426 was not phosphorylated and did not activate IFNα gene expression. In addition, Sato et al. (1998) showed that a mutated IRF-7 with a deletion of the region containing the potential sites of phosphorylation (amino acids 411-453) no longer translocated to the nucleus after viral infection. Another study showed that serine 437 and serine 438 are the primary targets for virally induced phosphorylation (Caillaud et al., 2005). Our results demonstrate that the NDV V protein targets IRF-7 for degradation and promotes conversion of full-length IRF-7 to a lower molecular weight form (IRF-7*). Based on its apparent molecular weight of 39 kDa, IRF-7* is approximately 354 amino acids in length. Given that the antibody that we used was raised against the N-terminal 246 amino acids of IRF7, it seems likely that

IRF-7∗ no longer contains the sites of phosphorylation, which are important for IRF-7 activation and translocation to the nucleus to promote induction of IFN. Therefore, it is possible that the NDV V protein inhibits the IFN response by converting IRF-7 to a form that lacks the critical phosphorylation sites. In this regard, it would be interesting to determine if IRF-7∗ is: a) phosphorylated and b) translocated to the nucleus.

It would be worthwhile to examine the effect of the NDV V protein on the functional activity of IRF-7. One way to do this is to co-transfect the V protein with IRF-7 and examine the following: 1) phosphorylation status of IRF-7 by mobility shift assay; 2) dimerization of IRF-7 by non-denaturing PAGE; 3) nuclear translocation of IRF-7 by immunofluorescence assay; 4) DNA binding ability of IRF-7 by electrophoretic mobility shift assay; and 5) induction of IFN by real-time RT-PCR (to measure IFN mRNA levels) and luciferase reporter gene assays.

7.3.4   Proposed model for inhibition of the IFN response by the NDV V protein

A model for the induction of the type I IFN response has been described by Honda and Taniguchi (2006). In the early phase of the response, double-stranded RNA is generated upon virus infection, and recognized by pattern recognition receptors such as MDA-5 and RIG-I. Recognition of dsRNA triggers a cascade that leads to phosphorylation of IRF-3 and IRF-7. Activated IRF-3 and IRF-7 homodimerize or heterodimerize and translocate to the nucleus and induce expression of small amounts of type I IFN. In the late phase of the response,

secreted IFNα/β binds to its receptor and activates the Jak/STAT pathway leading to induction of transcription of more IRF-7. Following dsRNA recognition, the newly synthesized IRF-7 is again activated and leads to induction of large amounts of type I IFN, resulting in a positive feedback loop.

Previously, the NDV V protein has been shown to bind MDA-5 and inhibit IFNβ induction (Childs et al., 2007). This indicates that NDV can act at an early step in the pathway to inhibit the IFN response. Our results demonstrate that the NDV V protein also acts at another step in the pathway. I propose that the NDV V protein inhibits the IFN response by targeting IRF-7 for degradation and converting it to a lower molecular weight form (IRF-7∗). The consequence of this is that the amount of IRF-7 available for activation is decreased. Additionally, IRF-7 is converted to a form that lacks the critical phosphorylation sites. Hence, IRF-7 is not activated and cannot translocate to the nucleus to induce IFN genes. As mentioned previously, IRF-7 is important at both the early and late phase of the IFN response. Additionally, IRF-7 is the master regulator of type I IFN-dependent responses. Hence, targeting IRF-7 through the action of the V protein provides a major advantage for NDV. Furthermore, this finding demonstrates that the NDV V protein is an IFN antagonist that acts at multiple levels of the IFN pathway.

7.4   Characterization of AVS-I antibody

The results of our functional inhibition studies with AVS-I are reminiscent of previous data obtained with antibody to site 2 (Iorio et al., 1984). Even though

the antibody to this site was made against the virulent strain AV, it has the same strain preference as AVS-I. The antibody to site 2 is better able to neutralize avirulent strains than virulent ones: the persistent fractions of avirulent strains B1, La Sota, and W are lower than those of virulent strains Italy-Milano/45, Israel-HP/53, L, and F. A distinct exception to this is the virulent strain RO, which is neutralized to a threefold-greater extent than AV. Hence, the strain specificity of AVS-I is similar to that of antibody to site 2. Also, this represents another example of the HN of virulent strain RO being more similar to the HN proteins of avirulent strains. It also points to a relationship between the C-terminal end of HN and virulence.

Given all of this, it would not be surprising for residue 570 to be part of a determinant of virulence in HN. Consistent with this possibility is the fact that it is situated close to the sialic acid binding site (Iorio et al., 2001). Furthermore, a cold-adapted temperature-sensitive B1 virus, which does not bind AVS-I (Gelb et al., 1996), has the identical G570R mutation (personal communication, Dr. Bruce Seal). However, the escape mutant, AVS-B1, does not exhibit an altered mean embryo death time relative to the wt virus, suggesting that the mutation does not affect virulence (data not shown). There is also not a consistent relationship between the nature of residue 570 and virulence. Though virulent strains Chiba/85 and Ibaragi/85 have an arginine at position 570, most virulent strains, including Beaudette C/45, Texas GB/48, Australia-Victoria/32, Herts/33, and Italien/45, have a glycine at this position (Sakaguchi et al., 1989). Thus, despite

being part of the AVS-I epitope, residue G570 appears not to be a major determinant of virulence. However, given the complexity of antibody-binding epitopes, other residues that make up the AVS-I epitope could certainly contribute to virulence. A complete understanding of the relationship between AVS-I recognition of a virus and virulence will require a complete fine mapping of each of the amino acids that contributes to the epitope.

Primers used for site-directed mutagenesis

Table 6.   Primers for generation of mutated La Sota V proteins

Mutation/s	Enzyme	Primer Sequence (5' to 3')
S144P	SpeI	GAGCCACCAAGAGGGGAA**CCA**CCA<u>ACTAGT</u>GACTCAAC
E153K	None	GACTCAACAGCAGGA**AAG**TCAACCCAGCCGCGCAAAC
S161P	None	CAGCCGCGAAAACAG**CCA**GGAAAGACCGCAGAACCAAG
D234N	None	GATGTCTATGACGGAGGC**AAT**ATCACAGAGAGTAAG
S144P, E153K	SpeI	GAGCCACCAAGAGGGGAA**CCA**CCA<u>ACTAGT</u>GATTCAACAGCAGGA**AAG**TCAACCCAGCCACGCAAAC
S161P, D234N	None	Sequential (S161P and D234N primers)
S144P, E153K, S161P	SpeI	Sequential (S161P then S144P, E153K primer)
S144P, E153K, S161P, D234N	SpeI	Sequential (D234N primer then GAGCCACCAAGAGGGGAA**CCA**CCA<u>ACTAGT</u>GATTCAACAGCAGGA**AAG**TCAACCCAGCCACGCAAACAG**CCA**GGAAAGACCGCAGAAC)

Mutation/s indicate the amino acid residues introduced into the La Sota V protein. Mutated codon(s) are highlighted in bold. Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

Table 7.   Primers for generation of mutated BC V proteins

Mutation/s	Enzyme	Primer Sequence (5' to 3')
P144S	SpeI	CCAAGAGGGGAA**TCT**CCA<u>ACTAGT</u>GACTCAACAGCAG
K153E	None	GACTCAACAGCAGGG**GAA**TCAACACAGCCGCGCAAACAG
P161S	None	CAGCCGCGAAACAG**TCA**GGAAAGACCGCAGAACCAAG
N234D	EcoRV	GATGTCTACGATGGAGGC**<u>GAT</u>**<u>ATC</u>ACAGAGAGTAAG
P144S, K153E	SpeI	CCAAGAGGGGAA**TCT**CCA<u>ACTAGT</u>GACTCAACAGCAGGT**GAA**TCAACACAGCCACGGAAACAGCCAG
P161S, N234D	EcoRV	Sequential (P61S primer and then N234D primer)
P144S, K153E, P161S	SpeI	CCAAGAGGGGAA**TCT**CCA<u>ACTAGT</u>GACTCAACAGCAGGT**GAA**TCAACACAGCCACGCAAACAG**TCA**GGTAAGACCGCAGAAC
P144S, K153E, P161S, N234D	EcoRV and SpeI	Sequential (N234D primer then CCAAGAGGGGAA**TCT**CCA<u>ACTAGT</u>GACTCAACAGCAGGT**GAA**TCAACACAGCCACGCAAACAG**TCA**GGTAAGACCGCAGAAC)

Mutation/s indicate the amino acid residues introduced into the BC V protein. Mutated codon(s) are highlighted in bold. Enzyme indicates the restriction enzyme site introduced into the gene. Restriction enzyme site in the primer sequence is underlined.

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