Role of Disulfide Bond Rearrangement in Newcastle Disease Virus Entry: A Dissertation

Surbhi Jain
University of Massachusetts Medical School

Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss

Part of the Amino Acids, Peptides, and Proteins Commons, Chemical Actions and Uses Commons, Inorganic Chemicals Commons, Organic Chemicals Commons, and the Viruses Commons

Repository Citation

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
ROLE OF DISULFIDE BOND REARRANGEMENT IN NEWCASTLE DISEASE VIRUS ENTRY

A Dissertation Presented

By

Surbhi Jain

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

June 26, 2008

Molecular Genetics and Microbiology
ROLE OF DISULFIDE BOND REARRANGEMENT IN NEWCASTLE DISEASE VIRUS ENTRY

A Dissertation Presented
By
Surbhi Jain

____________________
Trudy Morrison, Ph.D., Thesis Advisor

____________________
Dennis Brown, Ph.D., Member of Committee

____________________
Heinrich Gottlinger, Ph.D., M.D., Member of Committee

____________________
Raymond Welsh, Ph.D., Member of Committee

____________________
Robert Woodland, Ph.D., Member of Committee

____________________
Paul Clapham, Ph.D., Chair of Committee

____________________
Anthony Carruthers, Ph.D.
Dean of Graduate School of Biomedical Sciences

Molecular Genetics and Microbiology

June 26, 2008
COPYRIGHT NOTICE

Parts of this dissertation have appeared in following publication:


*Journal of Virology, March 2007, Volume 81, No.5*
ACKNOWLEDGEMENTS

There are a lot of people who guided, encouraged, inspired, and helped me throughout the time in the graduate school. I thank all of them for making it possible for me to complete the work presented in this thesis.

First, I would like to thank my mentor, Dr. Trudy Morrison, for providing me with the opportunity to work on and training me for my thesis work. She has taught me a great deal about all the aspects of scientific research. There could not have been a better teacher or a role model for me to follow.

Next, I would like to thank the members of my thesis research committee, Dr. Paul Clapham, Dr. Raymond Welsh, and Dr. Robert Woodland for their support and guidance provided during the thesis work. I would also like to thank Dr. Heinrich Gottlinger and Dr. Dennis Brown for their advice and support during final stage of my thesis work.

I would also like to thank all the members of Morrison laboratory, Lori McGinnes for teaching me to design and perform experiments, Dr. Julie Reitter and Kathy Gravel for the encouragement provided by them, Dr. Homer Pantua and Dr. Jason Laliberte for being my friends besides helping me as co-workers.

Lastly, I want to thank my family, my Mom and my brother (and his wife and sons), for all the love and support provided by them. My work would not have been possible without their support. I also want to thank all my friends for being my second family away from home.
ABSTRACT

Newcastle disease virus (NDV), an avian paramyxovirus, enters the host cell by fusion of viral and host cell membranes. The fusion of two membranes is mediated by the viral fusion (F) protein. The F protein, like other class I fusion proteins, is thought to undergo major conformational changes during the fusion process. The exact mechanism that leads to major refolding of F protein is not clear. Recently, it has been proposed that disulfide bond reduction in the fusion protein of some viruses may be involved in the conformational changes in fusion proteins. In some viruses, the reduction of disulfide bonds in the fusion protein is mediated by host cell disulfide isomerases belonging to the protein disulfide isomerase (PDI) family. In this study, the role of disulfide bond isomerization in the entry of NDV was analyzed. Using inhibitors of thiol-disulfide isomerases, we found that blocking the reduction of disulfide bonds in the fusion protein inhibited cell-cell fusion as well as virus entry into the host cell. Also, over-expression of isomerases belonging to the PDI family significantly enhanced cell-cell fusion. Taken together, these results suggest that free thiols play an important role in fusion mediated by NDV glycoproteins.

Using a thiol specific, membrane impermeable biotin, MPB, we found that free thiols are produced in cell surface-expressed NDV F protein. The production of free thiols was inhibited by inhibitors of thiol-disulfide isomerases. Over-expression of isomerases belonging to the PDI family enhanced detection of free thiols in F protein. In F protein, present in virions or in virus-like particles, free thiols were detected only after the particles were attached to target cells. Taken together, these results suggest that free
thiols are produced in F protein and the production of free thiols is mediated by host cell thiol-disulfide isomerases.

Using conformation sensitive antibodies, we also studied the conformation of cell surface-expressed F protein in the presence of thiol-disulfide isomerase inhibitors or in cells over-expressing thiol-disulfide isomerases. In the presence of thiol-disulfide isomerase inhibitors, the cell surface-expressed F protein was in a prefusion conformation while in cells over-expressing thiol-disulfide isomerases the F protein was in a post-fusion conformation.

We also correlated the production of free thiols to the conformational changes in F protein. Using temperature-arrested intermediates or F protein with mutations in heptad repeat domains, which are defective in attaining intermediate conformations, we found that free thiols are produced before any of the proposed conformational changes in F protein. Also, the production of free thiols in F protein was found to be independent of its activation by hemagglutinin-neuraminidase (HN) protein. These results suggest that free thiols are probably required for the activation of F protein during membrane fusion.
TABLE OF CONTENTS

COPYRIGHT NOTICE ......................................................................................... iii

ACKNOWLEDGEMENTS ..................................................................................... iv

ABSTRACT ........................................................................................................ v

TABLE OF CONTENTS ....................................................................................... vii

LIST OF FIGURES ............................................................................................. x

LIST OF TABLES ................................................................................................. xii

LIST OF ABBREVIATIONS .................................................................................. xiii

CHAPTER I: INTRODUCTION

A. General statement of topic ................................................................. 1

B. Paramyxoviruses—general properties and classification .............. 1

C. Newcastle disease virus—structure, genome and proteins .......... 3

D. NDV-entry into the host cell ............................................................... 5

E. NDV F protein, a class I fusion protein—structure and conformational changes .... 7

F. Proposed role of thiol-disulfide isomerization in other viruses ......... 13

G. Protein disulfide isomerase family ..................................................... 15

H. Contents of this dissertation ............................................................. 18

CHAPTER II: THIOL-DISULFIDE EXCHANGE IS REQUIRED FOR MEMBRANE FUSION DIRECTED BY THE NEWCASTLE DISEASE VIRUS FUSION PROTEIN

A. Introduction ............................................................................................... 23

B. Materials and Methods ........................................................................... 27
CHAPTER III: OVER-EXPRESSION OF THIOL-DISULFIDE ISOMERASES ENHANCES MEMBRANE FUSION DIRECTED BY THE NEWCASTLE DISEASE VIRUS FUSION PROTEIN

A. Introduction..........................................................................................60
B. Materials and Methods.........................................................................64
C. Results...................................................................................................70
D. Discussion..............................................................................................82

CHAPTER IV: ROLE OF THIOL-DISULFIDE ISOMERIZATION IN NEWCASTLE DISEASE VIRUS ENTRY

A. Introduction..........................................................................................88
B. Materials and Methods.........................................................................92
C. Results...................................................................................................97
D. Discussion..............................................................................................113

CHAPTER V: GENERAL DISCUSSION

A. Free thiols in F protein expressed on cell surfaces and virus or VLPs........120
B. Free thiols in F protein are required for membrane fusion....................121
C. Disulfide bond reduction in F protein is mediated by the PDI Family of isomterases.................................................................123
D. Free thiols are produced in F protein at a very early stage during membrane fusion
   (i) Free thiols are produced before hemifusion of the membranes.......124
(ii) Free thiols are produced before any major conformational changes in F protein.................................................................124

(iii) Free thiols are detected in a prefusion form of F protein and not in a post-fusion form.................................................................125

(iv) Free thiols are produced after attachment of HN protein to the cell surface receptor.................................................................127

(v) Free thiols are probably required for F protein activation...........127

E. Possible role of free thiols in membrane fusion.................................128

REFERENCES.........................................................................................132
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Newcastle disease virus</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Model for steps in membrane fusion</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Schematic of three prototypical class I viral fusion proteins</td>
<td>8</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Structure of F proteins from different paramyxoviruses</td>
<td>10</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Model for proposed conformational changes in F protein</td>
<td>12</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Schematic of domain composition of PDI</td>
<td>16</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Mechanism of PDI-catalyzed disulfide bond oxidation, reduction or isomerization</td>
<td>17</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Effect of PDI inhibitors on cell viability and protein expression</td>
<td>37</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Effect of PDI inhibitors on HN protein activities</td>
<td>40</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Effect of PDI inhibitors on cell-cell fusion</td>
<td>42</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Effect of PDI inhibitors on virus entry</td>
<td>46</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Biotinylation using a thiol specific biotin, MPB</td>
<td>49</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Effect of MPB on cell-cell fusion</td>
<td>51</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Binding of different conformation sensitive antibodies to surface-expressed F protein in presence of inhibitors</td>
<td>53</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Over-expression of ERdj5 and PDI and its effect on HN and F protein expression and cell viability</td>
<td>71</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Effect of ERdj5 and PDI over-expression on cell-cell fusion</td>
<td>74</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Effect of inhibitors of PDI isomerases on cell-cell fusion in cells over-expressing ERdj5 or PDI</td>
<td>75</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Effect of ERdj5 and PDI over-expression on production of free thiols in F protein</td>
<td>77</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Binding of conformation sensitive antibodies to F protein on cell surfaces in the presence of MPB or neuraminidase</td>
<td>79</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Effect of ERdj5 and PDI over-expression on binding of conformation sensitive antibodies to F protein on cell surfaces</td>
<td>81</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Model for the role of production of free thiols in cell surface-expressed NDV F protein</td>
<td>86</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Detection of free thiols in virions</td>
<td>98</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Free thiols in virus-like particles (VLPs)</td>
<td>102</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>MPB labeling of temperature arrested intermediates of F protein in VLPs</td>
<td>104</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>MPB labeling of temperature-arrested intermediates of F protein in virions</td>
<td>106</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>MPB labeling of F mutants in VLPs</td>
<td>108</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>MPB labeling of F protein in VLPs with HN mutants</td>
<td>110</td>
</tr>
<tr>
<td>Figure 4.7</td>
<td>Model for production of free thiols in F protein in virions</td>
<td>115</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Predicted model for production of free thiols in NDV F protein</td>
<td>129</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Table 1.1</td>
<td>Examples of members of the Family Paramyxoviridae</td>
<td>2</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Effect of MPB treatment on infectivity of NDV</td>
<td>100</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>AV</td>
<td>Australia-Victoria strain</td>
<td></td>
</tr>
<tr>
<td>BSL</td>
<td>Bio-Safety Level</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycholate</td>
<td></td>
</tr>
<tr>
<td>DTNB</td>
<td>5’5-dithio-bis(2-nitrobenzoic acid)</td>
<td></td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
<td></td>
</tr>
<tr>
<td>ELL-0</td>
<td>East Lansing Strain</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
<td></td>
</tr>
<tr>
<td>Env</td>
<td>Envelope glycoprotein</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Fusion protein</td>
<td></td>
</tr>
<tr>
<td>FP</td>
<td>Fusion Peptide</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
<td></td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin protein</td>
<td></td>
</tr>
<tr>
<td>HN</td>
<td>Hemagglutinin-Neuraminidase protein</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>Heptad Repeat</td>
<td></td>
</tr>
<tr>
<td>HDV</td>
<td>Hepatitis Delta Virus</td>
<td></td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Virus type 1</td>
<td></td>
</tr>
<tr>
<td>hPIV-3</td>
<td>Human Parainfluenza Virus type 3</td>
<td></td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>IF</td>
<td></td>
</tr>
<tr>
<td>Immunoglobulin G</td>
<td>IgG</td>
<td></td>
</tr>
<tr>
<td>Kilodalton</td>
<td>kDa</td>
<td></td>
</tr>
<tr>
<td>Matrix protein</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>3-(N-maleimidyl propionyl) biocytin</td>
<td>MPB</td>
<td></td>
</tr>
<tr>
<td>3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide</td>
<td>MTT</td>
<td></td>
</tr>
<tr>
<td>Murine Leukemia Virus</td>
<td>MLV</td>
<td></td>
</tr>
<tr>
<td>N-ethyl maleimide</td>
<td>NEM</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Neutravidin-Agarose</td>
<td>N-A</td>
<td></td>
</tr>
<tr>
<td>Newcastle Disease Virus</td>
<td>NDV</td>
<td></td>
</tr>
<tr>
<td>Nucleocapsid Protein</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>Phosphoprotein</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Phosphate-Buffered Saline</td>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>Propidium Iodide</td>
<td>PI</td>
<td></td>
</tr>
<tr>
<td>Phenyl methane sulfonyl fluoride</td>
<td>PMSF</td>
<td></td>
</tr>
<tr>
<td>Protein Disulfide Isomerase</td>
<td>PDI</td>
<td></td>
</tr>
<tr>
<td>Red Blood Cells</td>
<td>RBCs</td>
<td></td>
</tr>
<tr>
<td>Ribonucleoprotein</td>
<td>RNP</td>
<td></td>
</tr>
<tr>
<td>Octadecyl rhodamine B chloride</td>
<td>R18</td>
<td></td>
</tr>
<tr>
<td>Sendai Virus</td>
<td>SeV</td>
<td></td>
</tr>
<tr>
<td>Simian Virus 5 (also called parainfluenza virus 5)</td>
<td>SV5/PIV5</td>
<td></td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>Severe Acute Respiratory Syndrome</td>
<td>SARS</td>
<td></td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>SDS</td>
<td></td>
</tr>
<tr>
<td>Surface subunit</td>
<td>SU</td>
<td></td>
</tr>
<tr>
<td>Tris-HCL, NaCl, EDTA</td>
<td>TNE</td>
<td></td>
</tr>
<tr>
<td>Transmembrane domain</td>
<td>TM</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>UT</td>
<td></td>
</tr>
<tr>
<td>Untranslated region</td>
<td>UTR</td>
<td></td>
</tr>
<tr>
<td>Virus-like particles</td>
<td>VLPs</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

A. General statement of topic

The first step in the initiation of infection by paramyxoviruses is entry of virus into the host cell by fusion of the viral membrane with the cell membrane. Entry is mediated by viral fusion glycoproteins, but details of the mechanism by which fusion proteins accomplish membrane fusion is poorly understood. The aim of the work in this thesis is to understand the mechanism by which paramyxovirus F protein mediates the membrane fusion, using Newcastle disease virus (NDV) as a prototype. The results in this thesis have established that host cell proteins belonging to the protein disulfide isomerase family play an important role in membrane fusion directed by NDV F protein. This step of infection provides a potential target for developing antiviral agents that inhibit virus entry.

B. Paramyxoviruses-general properties and classification

Paramyxoviruses belong to the family *Paramyxoviridae* within the order *Mononegavirales*, which consist of enveloped, negative sense, single stranded RNA viruses. *Paramyxoviridae* includes many significant human and animal pathogens encompassing many recently identified viruses. Some of these viruses cause fatal diseases (Hendra and Nipah viruses) in humans. Newcastle disease virus (NDV), classified as the only member of Avulavirus genus within the subfamily *Paramyxovirinae* (Table 1.1), is an economically important avian pathogen. Because of its potential as an
### TABLE 1.1 Examples of Members of the Family *Paramyxoviridae*

**Family *Paramyxoviridae***

**Subfamily *Paramyxovirinae***

**Genus *Rubulavirus***
- Mumps virus (MuV)
- Parainfluenza virus 5 (previously called simian virus 5 [SV5]) (PIV5)
- Human parainfluenza virus type 2, type 4a and 4b (hPIV2/4a/4b)
- Mapuera virus

**Genus *Avulavirus***
- Newcastle disease virus (avian paramyxovirus 1) (NDV)

**Genus *Respirovirus***
- Sendai virus (mouse parainfluenza virus type 1) (SeV)
- Human parainfluenza virus type 1 and type 3 (hPIV1/3)
- Bovine parainfluenza virus type 3 (bPIV3)

**Genus *Henipaviruses***
- Hendra virus (HeV)
- Nipah virus (NiV)

**Genus *Morbillivirus***
- Measles virus (MeV)
- Canine distemper virus (CDV)
- Rinderpest virus

**Subfamily *Pneumovirinae***

**Genus *Pneumovirus***
- Human respiratory syncytial virus A2, B1, S2 (hRSV)
- Bovine respiratory syncytial virus (bRSV)
- Pneumonia virus of mice (PVM)

**Genus *Metapneumovirus***
- Human metapneumovirus (hMPV)
- Avian metapneumovirus

**Unclassified paramyxoviruses***
- Fer-de-Lance virus (FDLV)
- *Tupaia* paramyxovirus (TPMV)
- Menangle virus (MenV)
- Tioman virus (TiV)
- Beilong virus
- J virus
- Mossman virus (MoV)
- Salem virus (SaV)
- Nariva virus
agent of agro-bioterrorism, the government of United States has classified the virulent strains of NDV as select agents under the Patriot Act (Swayne and King 2003).

Paramyxoviruses replicate in the cytoplasm of host cells. The life cycle of paramyxoviruses begins by entry of virus into host cells by fusion of viral membranes to cell membranes. The genomic RNA released into the cytoplasm serves two functions: (a) as a template for synthesis of mRNAs and (b) as a template for synthesis of the antigenome (+) strand. Viral replication occurs after synthesis of the mRNAs by the viral encoded RNA polymerase and requires the continuous synthesis of viral proteins. The newly synthesized antigenome (+) strand serves as the template for further copies of the (-) strand genomic RNA. Assembly of paramyxoviruses is believed to require coordinated localization of multiple virus components. Assembly complexes bud from sites on the plasma membrane, then pinch off resulting in the release of particles (reviewed in (Lamb 2007)).

C. Newcastle disease virus—structure, genome and proteins

NDV virions are pleomorphic in size, ranging from 150-300nm in diameter and are usually spherical in shape. Virions are enveloped in a lipid bilayer that is derived from host cell membranes during budding of progeny virus (Klenk and Choppin 1970). The NDV genome contains 15, 186 nucleotides and encodes 6 genes, positioned 3’-NP-P-M-F-HN-L-5’, which are separated, in the positive sense, by short untranslated regions (UTRs) (Figure 1.1, panel A). These 6 genes encode the viral proteins.

The membrane-associated glycoproteins, hemagglutinin-neuraminidase (HN) protein and fusion (F) protein, are involved in attachment and penetration of virus into
Figure 1.1: Newcastle disease virus.

Panel A: Gene order of NDV. The schematic shows NDV genes from 3’ end to 5’ end. NP, nucleocapsid protein gene; P, phosphoprotein gene (also encodes V and W accessory proteins); M, matrix protein gene; F, fusion protein gene; HN, hemagglutinin-neuraminidase protein gene; L, large polymerase gene. Grey bars between genes represent intergenic regions.

Panel B: Schematic of NDV showing membrane components (F, HN and M proteins) and RNP complex.
the host cell. The matrix (M) protein lines the inner surface of virions and plays an important role in assembly and budding of progeny virions. The core proteins associate with the RNA genome and form a ribonucleoprotein (RNP) complex, which consists of nucleoprotein (NP) and the polymerase complex composed of the phosphoprotein (P) and the large (L) protein (Figure 1.1, panel B) (Lamb 2007). Two additional proteins, V and W, are derived from the P gene via RNA editing (Steward, Vipond et al. 1993). V protein has been shown to antagonize the host interferon response (Park, Garcia-Sastre et al. 2003), while the role of W proteins is not yet known.

D. NDV-entry into the host cell

NDV entry into host cells occurs at the plasma membrane in a pH independent manner. Entry begins with the attachment of HN protein to a sialic acid containing receptor on the target cell surface (Figure 1.2, panel A). The actual fusion of membranes is mediated by F protein. However, HN protein provides more than a docking function, as F protein cannot mediate membrane fusion without a homotypic HN protein (Hu, Ray et al. 1992; Deng, Wang et al. 1999). Moreover, some mutations in HN protein have been shown to eliminate its fusion promotion activity without affecting attachment or neuraminidase activity (Stone-Hulslander and Morrison 1999; Takimoto, Taylor et al. 2002; Corey, Mirza et al. 2003; Gravel and Morrison 2003). It has been proposed that HN protein binding to its receptor stimulates conformational changes in the HN protein, releasing F protein from the HN-F complex to refold into a more stable conformation mediating membrane fusion in the process (McGinnes, Gravel et al. 2002; Takimoto,
Figure 1.2: Model for steps in membrane fusion.
Panel A: Attachment of HN protein to its sialic acid containing receptor on the host cell membrane.
Panel B: Hemifusion, fusion of the outer lipid layer of viral and host cell membranes.
Panel C: Pore formation, fusion of both the lipid layers of viral and host cell membranes, leading to cytoplasmic content mixing.
Panel D: Pore expansion leading to release of genetic material into the host cell cytoplasm.
(McGinnes, Gravel et al. 2002; Takimoto, Taylor et al. 2002; Zaitsev, von Itzstein et al. 2004; McGinnes and Morrison 2006). Whether other factors are involved in the activation of F protein has not been explored.

After activation, F protein undergoes refolding, which pulls the target and viral membranes together. Mechanistic details of subsequent steps in membrane fusion are less well defined. The first step is hemifusion in which only the outer lipid layers of the two membranes have fused (Figure 1.2, panel B). The next step is pore formation where both the lipid layers fuse to form a pore and the cytoplasmic contents can be transferred (Figure 1.2, panel C). Pore formation leads to the final stage in virus entry, pore expansion, where the RNP core is released into the host cell cytoplasm (Figure 1.2, panel D). In the case of cell-cell fusion, pore expansion leads to formation of multinucleated cells or syncytia (Cleverley and Lenard 1998; Melikyan, Lin et al. 1999; Li, Han et al. 2005).

E. **NDV F protein, a class I fusion protein- Structure and conformational changes**

Viral fusion proteins have been categorized into two and possibly three groups based on their structure and mechanisms for mediating fusion (Weissenhorn, Dessen et al. 1999; Heinz and Allison 2001; Roche, Bressanelli et al. 2006). Class 1 fusion proteins, which fold as trimers, include paramyxovirus F proteins, influenza HA proteins, and retrovirus envelope (env) proteins. These proteins, synthesized as inactive precursors, are cleaved into two subunits, F₁ and F₂, in the case of paramyxoviruses. The sequence at the new amino terminus, generated by this cleavage, is the fusion peptide (FP), which inserts into the target membrane upon fusion activation (reviewed in
Figure 1.3: Schematic of three prototypical class I viral fusion proteins. Schematics of the paramyxovirus F, influenza virus HA, and HIV env glycoproteins are shown. All three proteins are produced as precursor polypeptide chains, which form trimers that are subsequently cleaved (represented by gap) post-translationally. Paramyxovirus F protein is cleaved into two subunits, F1 and F2, HA protein into HA1 and HA2, and HIV env protein into gp120 and gp41. Adjacent to the cleavage site is a hydrophobic stretch of amino acids known as the fusion peptide (FP) followed by an N-terminal heptad repeat sequence (HRA/HR1). In paramyxovirus F and HIV env proteins, a separate C-terminal heptad repeat sequence is easily identifiable (HRB/HR2). In the influenza HA protein sequence, HRB/HR2 is used to indicate the C-terminal portion of the primary heptad repeat that is observed to reverse its orientation during the low-pH conformational change. Transmembrane anchors (TM) are also indicated and these are proximal to HRB/HR2.
These proteins also contain two important heptad repeat (HR) domains. The F protein HR domains are located just carboxyl terminal to the fusion peptide (HR1) and adjacent to the transmembrane (TM) domain (HR2) (Figure 1.3).

HR1 and HR2 peptides have a strong affinity and form a very stable six-stranded, coil-coil with the HR1 forming an interior trimer and the HR2 binding in the grooves of the trimer in an anti-parallel orientation (Baker, Dutch et al. 1999). Inhibition of fusion with either HR1 or HR2 peptides suggest that HR1 and HR2 domains, in the intact protein, are not associated prior to F protein activation, while the two domains are complexed in the post-fusion F protein (Young, Hicks et al. 1997; Joshi, Dutch et al. 1998; Russell, Jardetzky et al. 2001).

Changes in F protein conformation were further studied by using HR1 and HR2 peptides for inhibition of cell-cell fusion mediated by SV5 glycoproteins at different temperatures (Russell, Jardetzky et al. 2001). The first intermediate, susceptible to HR1 peptide binding, was proposed to exist at lower temperatures while another intermediate form, susceptible to both HR1 and HR2 peptides, was shown to exist at higher temperature. Based on these observations, it was proposed that paramyxovirus F proteins undergo a series of conformational changes leading to formation of the final six helical bundle (6HB).

Subsequent studies of structures of F protein from different paramyxoviruses confirmed that F protein may exist in at least two different forms. One form, exemplified by the structures of parainfluenza virus 3 F protein (Yin, Paterson et al. 2005) and NDV F
Figure 1.4: Structure of F proteins from different paramyxoviruses.
Structures shown are generated using coordinates deposited in the protein database and are presented using Protein Explorer. F2 domain is shown in green, HR1 is shown in brown, HR2 is shown in blue, and residues adjacent to fusion peptide are shown in magenta.

Panel B: Structure of hPIV3 F protein monomer in the post-fusion form (Yin, HS et al, 2006).
protein (Chen, Gorman et al. 2001) (Figure 1.4, panels B and C), was proposed to be in post-fusion conformation and contains the two HR domains complexed in 6HB form. Another structure, derived from SV5 F protein (Yin, Wen et al. 2006), was stably trimerized in soluble form by fusing the carboxyl terminus of the HR2 domain to the yeast GCN4 sequence. This sequence prevented 6HB formation between HR2 and HR1 domains. The resulting structure was proposed to be in a prefusion form (Figure 1.4, panel A).

Based on these results, a model for conformational changes in F protein during membrane fusion was proposed (Russell and Luque 2006; Yin, Wen et al. 2006). According to this model, the first change in the initial metastable F protein produces an early fusion intermediate in which the HR2 strands open up. The next stage is a pre-hairpin intermediate in which HR1 forms a triple-stranded, coiled-coil and the fusion peptide, at the end of this structure, inserts into the target membrane. The final stage is the formation of the fusogenic hairpin structure (6HB) that actively brings together the viral and cellular membranes (Figure 1.5).

Further evidence for the importance of HR domains was provided by studies of HR1 and HR2 mutants. Mutations in both HR1 and HR2 domains in F protein in different paramyxoviruses have been shown to variably affect membrane fusion activity (McGinnes, Sergel et al. 2001; Sergel, McGinnes et al. 2001; West, Sheehan et al. 2005). The SV5 F protein with a point mutation in HR2 domain has been proposed to be defective in formation of an early F protein intermediate, arrested in a conformation prior to prehairpin formation (Paterson, Russell et al. 2000; Russell, Kantor et al. 2003). F
Figure 1.5: Model for proposed conformational changes in F protein.

Panel A: Structure of the prefusion conformation. HR2 is shown in blue, HR1 is shown in green, respectively.
Panel B: ‘Open stalk’ conformation, in which the HR2 stalk melts and separates from the prefusion head region. This conformation is consistent with a low-temperature intermediate that is inhibited by HR1 peptides, but not HR2 peptides.
Panel C: A pre-hairpin intermediate generated by formation of the HR1 coiled coil and insertion of the fusion peptide into the target cell membrane. This intermediate can be inhibited by peptides derived from both HR1 and HR2 regions.
Panel D: Before formation of the final 6HB, folding of the HR2 linker may stabilize the juxtaposition of viral and cellular membranes.
Panel E: The formation of the post-fusion 6HB is tightly linked to membrane fusion and pore formation, juxtaposing the membrane-interacting fusion peptides and transmembrane domains.

protein with mutations in the HR1 domain is proposed to be defective in the formation of
the HR1 helix. Some mutations in the SV5 and Sendai virus F protein HR1 domains
result in hyperfusogenic F protein. It has been proposed that these mutations destabilize
the spring loaded conformation and the mutations may lower the activation energy for
refolding, after activation by HN protein, probably at a stage where HR1 domains refold
to form helices (prehairpin intermediate) (Ito, Komada et al. 1992; West, Sheehan et al.
2005; Luque and Russell 2007). How F protein accomplishes this major refolding during
membrane fusion is not known.

F. Proposed role of thiol-disulfide isomerization in other viruses

In various cell entry proteins, including diphtheria toxin and fusion proteins of
some animal viruses, it has been shown that disulfide bonds undergo reduction at the time
of membrane fusion and this cleavage of disulfide bonds is necessary for fusion of
membranes (Sanders 2000; Hogg 2003; Wouters, Lau et al. 2004). During vaccinia virus
infection, the disulfide bonds in core proteins are reduced during entry into the host cell
(Locker and Griffiths 1999). Disulfide bonds in the envelope protein in Sindbis virus are
reduced during cell entry (Abell and Brown 1993). Disulfide bond rearrangement is
involved in forming the fusogenic complex of the baculovirus gp64 (Markovic, Pulyaeva
et al. 1998). Recently, entry of hepatitis delta virus (HDV) has been shown to be blocked
by membrane impermeable inhibitors of thiol-disulfide exchange. It was proposed that
the conserved cysteine residues of hepatitis B virus envelope protein in HDV undergo
rearrangement at the time of virus entry and are required for the disassembly of the virion
(Abou-Jaoude and Sureau 2007).
The surface subunit (SU) of the env protein in Moloney murine leukemia virus has a CXXC motif that leads to isomerization of a disulfide bond between surface (SU) and transmembrane (TM) subunits of env proteins. This isomerization is triggered by binding of SU to the receptor and is required for fusion (Pinter, Kopelman et al. 1997; Ferrari and Soling 1999; Wallin, Ekstrom et al. 2004). Some other retroviruses, such as the avian leukosis viruses (ALV) do not contain the CXXC motif in their SU subunits. In avian leukosis virus-A (ALV-A), reactive cysteine thiols are produced in the surface subunit of env protein after it binds to its receptor and the free thiols are essential for infection (Smith and Cunningham 2007). It was proposed that these viruses are dependent on cellular factors for reduction of disulfide bonds in the fusion proteins.

Studies of the HIV-1 env protein have shown that a plasma membrane-associated oxido-reductase, protein disulfide isomerase (PDI) or a related protein, is required for fusion of membranes mediated by HIV-1 env protein (Ryser, Levy et al. 1994; Fenouillet, Barbouche et al. 2001; Markovic, Stantchev et al. 2004). It was proposed that, upon gp120 binding to receptors, thiol-disulfide isomerase activity cleaves disulfide bonds in env protein, thereby facilitating its refolding required for membrane fusion. Down regulation of PDI has also been shown to inhibit infection by mouse polyoma virus (Gilbert, Ou et al. 2006).

In the SARS coronavirus, the cysteine-rich spike protein is another example of a class I fusion protein. In contrast to HIV-1 env protein, inhibitors of thiol-disulfide exchange were shown to have no effect on membrane fusion mediated by the spike protein (Lavillette, Barbouche et al. 2006).
In paramyxoviruses, cysteine residues in the F protein are highly conserved. In Sendai virus F protein there are ten cysteine residues all of which are involved in disulfide bonds (Iwata, Schmidt et al. 1994). NDV F protein has two additional cysteine residues. Whether all the cysteines in NDV F protein are involved in disulfide bonds has not been studied. Whether the cysteine residues in paramyxovirus F proteins play a role in membrane fusion is not known.

G. Protein disulfide isomerase family

The enzymes of the protein disulfide isomerase (PDI) family are thiol-disulfide oxidoreductases and belong to the thioredoxin superfamily (Ellgaard and Ruddock 2005). In recent years, an increasing number of enzymes have been added to the PDI family based on similar structures and enzymatic properties. The family comprises 19 published members and all of them contain a predicted signal sequence and at least one thioredoxin-like domain (reviewed in (Appenzeller-Herzog and Ellgaard 2008)). The thioredoxin-like domains can be either catalytic or non-catalytic. By convention, these two types of domains are called a and b-type domains, respectively. The a-type domains usually contain two cysteines in a CXXC active-site motif with an intervening GH sequence being the most common in the PDIs. The b-type domains do not have cysteines in the active site and are, therefore, not redox active. The domain structure of PDI, the founding member of the family, is shown in Figure 1.6. The members of the PDI family are thought to have isomerase activity as implied by family name, but this activity has not
Figure 1.6: Schematic of domain composition of PDI.
Thioredoxin-like domains are shown as rectangles. Grey rectangles represent catalytic domains (a and a’) with active site sequence shown in the rectangle. White rectangles represent non-catalytic domains (b and b’).
Figure 1.7: Mechanism of PDI catalyzed disulfide bond oxidation, reduction or isomerization.

**Oxidation:** PDI is in oxidized form and catalyzes the formation of disulfide bond in substrate proteins.

**Reduction:** PDI is in reduced state and catalyzes the cleavage of disulfide bonds in substrate proteins.

**Isomerization:** PDI catalyzes isomerization of disulfide bonds in substrate proteins.
been demonstrated experimentally for all family members. Although ER resident proteins, these proteins are also present in other cellular locations including plasma membranes, associating with integral plasma membrane proteins through noncovalent interactions (Turano, Coppari et al. 2002; Jordan and Gibbins 2006).

These enzymes catalyze the reduction, formation, and isomerization of disulfide bonds in proteins in the endoplasmic reticulum (ER) as shown in Figure 1.7 (Pirneskoski 2003; Wilkinson and Gilbert 2004). The redox function of PDI-like proteins is based on one or more catalytic domains bearing a CXXC motif (Luz and Lennarz 1996; Gilbert 1997). In the ER, these isomerases act mainly as oxidases. Cysteines in their CXXC motifs are oxidized leading to formation of disulfide bonds in interacting proteins. At the cell surface, these isomerases predominantly act as reductases and cysteines in their CXXC motifs are in the form of free thiols. These cell surface forms of the proteins lead to cleavage of disulfide bonds and production of free thiols in interacting proteins (Noiva 1999).

H. Contents of this dissertation

Results in this thesis demonstrate the following:

(i) Free thiols are produced in cell surface-expressed NDV F protein and are not detected in NDV HN protein.

(ii) Production of free thiols in F protein is required for fusion mediated by NDV F protein.
(iii) In absence of free thiols, F protein is in a prefusion conformation while enhanced production of free thiols favors a post-fusion conformation of F protein.

(iv) All the cysteine residues in F protein in virions or virus-like particles are involved in disulfide bonds.

(v) Free thiols are produced in F protein in virions or virus-like particles after binding of the particles to target cells.

(vi) Free thiols are produced at a very early stage in fusion, even before any major conformational changes occur in F protein.

(vii) Reduction of disulfide bonds in F protein is required for its activation and this activation of F protein is independent of its activation by HN protein.

Evidence supporting the above conclusion is presented in a series of papers, one published and two in submission. The abstracts of each paper are shown below.

**Chapter II: Thiol-Disulfide exchange is required for membrane fusion directed by the Newcastle disease virus fusion protein** (Published in the Journal of Virology, March 2007, Volume 81:5, 2328-2339)

Newcastle disease virus (NDV), an avian paramyxovirus, initiates infection with attachment of the viral hemagglutinin-neuraminidase (HN) protein to sialic acid containing receptors followed by fusion of viral and cell membranes, which is mediated by the fusion (F) protein. Like all class 1 viral fusion proteins, the paramyxovirus F protein is thought to undergo dramatic conformational changes upon activation. How the F protein accomplishes extensive conformational rearrangements is unclear. Since
several viral fusion proteins undergo disulfide bond rearrangement during entry, we asked if similar rearrangements occur in NDV proteins during entry. We found that inhibitors of cell surface thiol-disulfide isomerase activity, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), bacitracin, and anti-protein disulfide isomerase antibody inhibited cell-cell fusion and virus entry but had no effect on cell viability, glycoprotein surface expression, or HN protein attachment or neuraminidase activities. These inhibitors altered the conformation of surface-expressed F protein as detected by conformation sensitive antibodies. Using biotin maleimide (MPB), a reagent that binds to free thiols, free thiols were detected on surface-expressed F protein but not HN protein. The inhibitors DTNB and bacitracin blocked the detection of these free thiols. Furthermore, MPB binding inhibited cell-cell fusion. Taken together, our results suggest that one or several disulfide bonds in cell surface F protein are reduced by PDI family of isomerases and F protein exists as a mixture of both oxidized and reduced forms. In the presence of HN protein, only the reduced form may proceed to refold to additional intermediates leading to fusion of membranes.

Chapter III: Over-expression of thiol-disulfide isomerases enhances membrane fusion directed by the Newcastle disease virus fusion protein (Submitted to the Journal of Virology)

Newcastle disease virus (NDV) fusion (F) protein directs membrane fusion required for virus entry and cell-cell fusion. We have previously shown that free thiols are present in cell surface expressed NDV F protein and that blocking the production of free thiols by thiol-disulfide exchange inhibitors inhibited the membrane fusion mediated
by F protein (Jain, McGinnes et al. 2007). Extending these observations, we evaluated the role of over-expression of two disulfide bond isomerases, PDI and ERdj5, in cell-cell fusion mediated by NDV glycoproteins. Over-expression of these isomerases resulted in significantly increased membrane fusion, as measured by syncytia formation and content mixing. Over-expression of these isomerases enhanced production of free thiols in F protein when expressed without HN protein but decreased free thiols in F protein expressed with HN protein. By evaluating the binding of conformation sensitive antibodies, we found that over-expression of these isomerases favored a post-fusion conformation of surface-expressed F protein in the presence of HN protein. These results indicate that isomerases belonging to the PDI family catalyze the production of free thiols in F protein and free thiols in F protein facilitate membrane fusion mediated by F protein.

**Chapter IV: Role of thiol-disulfide isomerization in Newcastle disease virus entry**

(Submitted to the Journal of Virology)

Newcastle disease virus (NDV) entry into host cells is mediated by the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. We previously showed that production of free thiols in F protein is required for membrane fusion directed by F protein (Jain, McGinnes et al. 2007). In this study we evaluated the oxidation state of F protein in virions and virus-like particles and its relationship to activation of F protein by HN protein, F protein conformational intermediates, and virus-cell fusion. F protein, in particles, does not have free thiols but free thiols were produced upon binding of particles to target cells. Free thiols were produced at 16ºC in F protein in virions or virus-like particles bound to the target cells. They also appeared in different fusion defective
mutant F proteins. Free thiols were produced in the presence of mutant HN proteins that are defective in F protein activation but are attachment competent. These results suggest that free thiols appear prior to any of the proposed major conformational changes in F protein which accompany fusion activation. These results also indicate that HN protein binding to its receptor likely facilitates the interaction between F protein and host cell isomerasers leading to reduction of disulfide bonds in F protein. Taken together, these results show that free thiols are produced in F protein at a very early stage during the onset of fusion and that production of free thiols is required for fusion in addition to activation by HN protein.
CHAPTER II

THIOL-DISULFIDE EXCHANGE IS REQUIRED FOR MEMBRANE FUSION
DIRECTED BY THE NEWCASTLE DISEASE VIRUS FUSION PROTEIN

A. Introduction

Cell entry of enveloped viruses requires fusion of the viral envelope with host cell membranes, a step in infection that is mediated by viral fusion proteins. Viral fusion proteins have been categorized into two and possibly three groups based on their structure and mechanisms for mediating fusion (Weissenhorn, Dessen et al. 1999; Heinz and Allison 2001; Roche, Bressanelli et al. 2006). Class 1 fusion proteins, which fold as trimers, include paramyxovirus F proteins, influenza HA proteins, and retrovirus envelope (env) proteins. These proteins, synthesized as inactive precursors, are cleaved into two subunits, F1 and F2 in the case of paramyxoviruses. The sequence at the new amino terminus, generated by this cleavage, is the fusion peptide (FP), which inserts into the target membrane upon fusion activation (reviewed in ((Hernandez, Hoffman et al. 1996; Weissenhorn, Dessen et al. 1999; Morrison 2003; Earp, Delos et al. 2005)). These proteins also contain two important heptad repeat (HR) domains. The F protein HR domains are located just carboxyl terminal to the fusion peptide (HR1) and adjacent to the transmembrane (TM) domain (HR2). HR1 and HR2 peptides have a strong affinity and form a very stable six-stranded, coil-coil with the HR1 forming an interior trimer and the HR2, binding in the grooves of the trimer in an anti-parallel orientation (Baker, Dutch et al. 1999). Inhibition of fusion with either HR1 or HR2 peptides suggest that HR1 and
HR2 domains in the intact protein are not associated prior to F protein activation while the two domains are complexed in the post-fusion F protein (Young, Hicks et al. 1997; Joshi, Dutch et al. 1998; Russell, Jardetzky et al. 2001).

Current models for class 1 fusion proteins propose that fusion activation, by receptor binding or acid pH (reviewed in ((Lamb 1993; Hernandez, Peters et al. 1997; Colman and Lawrence 2003; Earp, Delos et al. 2005)), results in dramatic conformational changes in these proteins. First, the FP is exposed for insertion into a target membrane, anchoring the protein in that membrane. It is then proposed that the protein proceeds to refold forming a complex between heptad repeat domains, which pulls the target and the effector membranes together (reviewed in ((Colman and Lawrence 2003; Jardetzky and Lamb 2004; Russell and Luque 2006))). Models for the mechanistic details of the subsequent hemi-fusion and pore formation are less well defined, although there may be additional conformational changes in the F protein during these stages of fusion (Cleverley and Lenard 1998; Melikyan, Lin et al. 1999; Li, Han et al. 2005). How fusion proteins accomplish these extensive conformational rearrangements is not clear.

Thiol-disulfide exchange in various cell entry proteins including diphtheria toxin and fusion proteins of some animal viruses has been shown to be necessary for fusion of membranes (Hogg 2003; Wouters, Lau et al. 2004). During vaccinia virus infection, the disulfide bonds in core proteins are reduced during entry into the host cell (Locker and Griffiths 1999). Disulfide bonds in the envelope protein in Sindbis virus are reduced during cell entry (Abell and Brown 1993). Disulfide bond rearrangement is involved in forming the fusogenic complex of the baculovirus gp64 (Markovic, Pulyaeva et al. 1998).
The surface subunit (SU) of the env protein in Moloney murine leukemia virus, has a CXXC motif that leads to isomerization of a disulfide bond between SU and TM proteins, which is required for fusion (Pinter, Kopelman et al. 1997; Ferrari and Soling 1999; Wallin, Ekstrom et al. 2004). Recent studies of the HIV-1 env protein have shown that a plasma membrane associated oxido-reductase, protein disulfide isomerase (PDI) or a related protein, is required for fusion of membranes mediated by HIV-1 env protein (Ryser, Levy et al. 1994; Fenouillet, Barbouche et al. 2001; Markovic, Stantchev et al. 2004). It was proposed that, upon gp120 binding to receptors, thiol-disulfide isomerase activity cleaves disulfide bonds in env protein, thereby facilitating its refolding required for membrane fusion. Down regulation of PDI has also been shown to inhibit infection by mouse polyoma virus (Gilbert, Ou et al. 2006).

PDI and other PDI-like isomerases belong to the thioredoxin superfamily (Ellgaard and Ruddock 2005). These enzymes catalyze the reduction, formation, and isomerization of disulfide bonds in proteins in the endoplasmic reticulum (ER) (Wilkinson and Gilbert 2004). Although ER resident proteins, these proteins are also present in other cellular locations, including plasma membranes, associating with integral plasma membrane proteins through noncovalent interactions (Turano, Coppari et al. 2002; Jordan and Gibbins 2006). The redox function of PDI-like proteins is based on one or more catalytic domains bearing a CXXC motif (Luz and Lennarz 1996; Gilbert 1997). In the ER, these isomerases act mainly as oxidases. Cysteines in their CXXC motifs are oxidized leading to formation of disulfide bonds in interacting proteins. At the cell surface, these isomerases predominantly act as reductases and cysteines in their CXXC
motifs are in the form of free thiols. These forms of the proteins lead to cleavage of disulfide bonds and production of free thiols in interacting proteins (Noiva 1999).

It is unknown if disulfide bond rearrangement has any role in paramyxovirus attachment and fusion mediated by HN and F proteins, respectively. We found that free thiols can be detected in surface-expressed Newcastle disease virus (NDV) F protein but not in HN protein. Furthermore, we found that the membrane impermeable inhibitors of disulfide bond isomerases, 5′5-dithio(2-bis-nitrobenzoic acid) (DTNB) and bacitracin, as well as anti-PDI antibodies inhibited cell-cell fusion and virus entry mediated by NDV glycoproteins. DTNB and bacitracin also inhibited the formation of free thiols in the F protein. These inhibitors also altered the conformation of cell surface-expressed F protein as detected by conformation sensitive antibodies. Furthermore, blocking the free thiols in F protein by covalent addition of a thiol specific biotin inhibited cell-cell fusion. Our results suggest that disulfide bond reduction in cell surface NDV F protein is mediated by PDI-like enzymes and that disulfide bond isomerization may be required for conformational changes in F protein that are essential for membrane fusion.
B. Materials and Methods

**Cells, virus and plasmids:** COS-7 cells, obtained from American Type Culture Collection, were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with nonessential amino acids, vitamins, penicillin, and streptomycin, and 10% fetal calf serum (FCS). Stocks of NDV B1, an avirulent strain, were grown in embryonated chicken eggs and purified by standard protocols (Mc Ginnes, Reiter et al. 2006).

NDV F and HN genes were inserted into pSVL and pCAGGS expression vectors, as previously described (McGinnes, Gravel et al. 2002; McGinnes and Morrison 2006).

**Transfections:** Transfections were accomplished using Lipofectin or Lipofectamine (Invitrogen). For lipofectamine transfection, a mixture of DNA (pSVL 1µg/35mm plate or pCAGGS 0.5µg/35mm plate) and lipofectamine (7µl/35mm plate) in OptiMEM media (Gibco) was incubated at room temperature for 45 minutes, and added to COS-7 cells grown in 35mm plates and previously washed with OptiMEM. For lipofectin transfection, DNA (pSVL 1µg/35mm plate) and lipofectin (10µl/35mm plate) were incubated with OptiMEM separately at room temperature for 40 minutes. Both DNA and lipofectin were mixed and incubated further for 15 minutes and added to OptiMEM washed cells grown in 35mm plates. Cells were incubated for 5h at 37°C, OptiMEM was removed, and 2ml of supplemented DMEM were added.

**Antibodies and PDI inhibitors:** Anti-NDV antibody was raised in rabbits against UV-inactivated stocks of NDV, strain AV, by standard protocols as previously described (McGinnes, Gravel et al. 2002). Rabbit anti-HR1 and anti-HR2 antibodies
were raised against peptides with the HR1 and HR2 sequences from NDV F protein as
described previously (McGinnes, Gravel et al. 2002; Dolganiuc, McGinnes et al. 2003).
Anti-Fu1a is a mouse monoclonal antibody specific for NDV F protein and was obtained
from M. Peeples (Morrison, Peeples et al. 1987). Anti-AS antibody was raised against a
peptide with a sequence from the NDV HN protein as previously described (McGinnes
and Morrison 1994). Secondary antibodies used were anti-rabbit IgG coupled to
horseradish peroxidase (Amersham biosciences), goat anti-rabbit IgG Alexa fluor 488
(Molecular probes), and anti-mouse IgG Alexa fluor 568 (Molecular probes).

PDI inhibitors, 5’5-Dithio-bis(2-nitrobenzoic acid) (DTNB) and bacitracin, were
purchased from Sigma. Bacitracin was used with protease inhibitor,
phenylmethanesulfonyl fluoride (PMSF) (0.2mg per ml of 7.5mM bacitracin), to prevent
degradation of proteins by contaminating proteases (Vitkovic and Sadoff 1977).
Polyclonal rabbit anti-PDI antibody was purchased from Stressgen. Anti-CD71 antibody
(Santa Cruz Biotechnology) was used as control IgG.

**Cell viability:** COS-7 cell monolayers grown in 35mm plates were incubated
overnight with DTNB (2.5mM, 5mM, and 7.5mM) or bacitracin (2.5mM, 5mM, and
7.5mM) or anti-PDI antibodies (3µl and 6µl per ml) or left untreated. Cells were then
incubated with 2ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide] (Promega corporation) (0.5mg/ml in PBS) for 3h at 37º C. After removing the
MTT solution, cells were treated with 2ml acidified isopropanol (0.04M HCl in absolute
isopropanol) to solubilize blue formazan crystals. Blue color was quantified by
measuring absorbance at test wavelength of 570nm and reference wavelength of 650nm as described previously (Mosmann 1983).

Annexin V-FITC and propidium iodide (PI) (Biovision) double staining was performed as described previously (Abdel-Latif, Murray et al. 2006). As a positive control for annexin V binding and PI staining by fluorescence microscopy, cells were treated with 0.05% Triton X-100 for 30 min on ice to allow access to the interior of the cells. Incubation for 6h with staurosporine (Sigma Co.) (1µM), which induces apoptosis (Knerr, Schnare et al. 2006), and 0.05% Triton X-100 (30 min on ice) were used as a positive control for flow cytometry analysis (FACS). Addition of Triton X-100 enhanced PI staining. COS-7 cell monolayers were either incubated overnight with PDI inhibitors or left untreated. Cells were then detached for FACS or left on coverslips for fluorescence microscopy and incubated with Annexin V-FITC (5µl) and PI (5µl) in annexin binding buffer (Biovision inc.) for 10 minutes in dark at room temperature. Cells were washed twice with PBS, fixed with 2% paraformaldehyde, and analyzed by visualizing by microscopy or FACS.

**Hemadsorption:** COS-7 cells grown in 35mm plates were transfected with pSVL HN. Transfected cells, incubated overnight with DTNB (7.5mM), bacitracin (7.5mM) or anti-PDI antibodies (6µl per ml), were then incubated with 0.4% suspension of washed guinea pig RBCs (Biolink inc.) in PBS for 30 minutes at 4°C. Unbound RBCs were removed by washing three times with cold PBS. Bound RBCs were lysed with 0.75ml of 50mM NH₄Cl and hemoglobin released was quantified by measuring absorbance at 540nm.
**Neuraminidase:** Cells grown in 35mm plates, transfected with pSVL HN, were incubated overnight with PDI inhibitors. The cells were then analyzed for neuraminidase activity as previously described (Morrison and McGinnes 1989). Briefly, cells were washed twice with PBS and once with 0.1M sodium acetate (pH 6) and incubated for 4h at 37°C with N-acetylneuraminyl lactose (Sigma co.) (0.25mg in 0.5ml sodium acetate [0.1M, pH 6]). Supernatant was collected and incubated with 0.5ml of 25mM periodic acid for 30 minutes at 37°C. 0.4ml of 2% sodium arsenite was then added to tubes and mixed. The mix was then boiled with thiobarbituric acid (14.2mg/ml, pH 9) for 15 minutes followed by addition of 4ml acid-butanol (5% HCl in butanol). The tubes were centrifuged for 15 minutes and O.D. of the top layer was determined.

**Surface Biotinylation:** COS-7 monolayers grown in 35mm plates and transfected with pSVL HN and pSVL F were incubated with PDI inhibitors overnight and washed three times with PBS-CM (PBS with 0.1mM CaCl$_2$ and 1mM MgCl$_2$). PBS-CM containing 0.5mg/ml sulfo-NHS-SS-biotin (Pierce) was added and cells were incubated for 40 minutes at 4°C. Unbound biotin was absorbed with 2ml DMEM and cells were washed three times with PBS and lysed with RSB buffer (0.01M Tris-HCl [pH 7.4], 0.01M NaCl, 1.5mM MgCl$_2$) containing 1% Triton X-100, 0.5% sodium deoxycholate, 2.5mg of N-ethyl maleimide per ml, and 0.2mg of DNase per ml. Lysates were incubated for 1h at room temperature with 0.3% SDS containing neutravidin-agarose (Pierce) that had been washed with PBS containing 0.5% tween-20, and 5mg/ml BSA and then with PBS containing 0.5% Tween-20 and 1mg/ml BSA. Precipitates were washed three times with PBS containing 0.5% Tween-20 and 0.4% SDS, resuspended in gel sample buffer
(125mM Tris-HCl, pH 6.8, 2% SDS and 10% glycerol) with 0.7M β-mercaptoethanol and resolved by polyacrylamide gel electrophoresis (10%). The gels were equilibrated in transfer buffer (25mM Tris, pH 8.2, 192mM glycine, 12.5% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes and probed with anti-HR2 antibody (1:1000) or anti-AS antibody (1:1000) (primary) and goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase (secondary). Bound antibodies were detected using ECL Western blotting detection reagent (Amersham Biosciences).

**Syncytia formation:** COS-7 cells grown in 35mM plates were cotransfected with pSVL HN (1µg/plate) and pSVL F (1µg/plate) using lipofectin (Invitrogen). At 24h posttransfection, DTNB (2.5mM, 5mM, and 7.5mM), bacitracin (2.5mM, 5mM, and 7.5mM), or anti-PDI antibody (6µl per ml) were added. At 48h post transfection, the numbers of nuclei in 20 fusion areas were counted to determine the average size of the syncytia as previously described (Sergel, McGinnes et al. 1993). Values obtained after transfection with the empty vector were subtracted.

**Content mixing:** Content mixing was measured by using a modification of a previously described protocol (McGinnes, Sergel et al. 2001). Briefly, cells grown in 35mm plates were transfected with pCAGGS-HN (0.5µg/plate), pCAGGS-F (0.5µg/plate) DNAs and a plasmid encoding a tetracycline-responsive transcriptional activator, tTA (Clontech) (1µg/plate). A separate population of cells was transfected with a plasmid encoding the β-galactosidase protein under the control of the tetracycline-responsive transcriptional activator (pB1-G) (Clontech) (1µg/plate). After 36h, PDI inhibitors were added to respective plates and after 40h, the cells transfected with the
pB1-G were removed from the plates with trypsin and placed on top of the HN and F protein-expressing cells. Cells were further incubated with inhibitors for 6h, washed twice with PBS, lysed (Promega cell lysis buffer), and extracts were assayed for β-galactosidase activity (Promega protocols). Activity due to background fusion of COS-7 cells was measured after mixing cells cotransfected with pTA and empty vector with cells transfected with pB1-G. Values obtained were subtracted from values obtained with cells expressing HN and F proteins.

**Lipid mixing:** The protocol used was similar to that previously described (Kemble, Henis et al. 1993). Briefly, guinea pig red blood cells (RBCs) (Bio Link) were washed in PBS and resuspended to give 0.4% suspension and were incubated with 15µg/ml of R18 (octadecyl rhodamine B chloride) (Molecular Probes) for 30 minutes at room temperature in the dark. Three volumes of complete medium (DMEM with 10% fetal calf serum) were added, and incubation was continued for 30 minutes. The RBCs were then washed four times in ice-cold PBS, resuspended to give 0.4% suspension of RBCs in PBS containing CaCl₂ (0.01%). These RBCs were added to cells that had been grown on coverslips in 35mm plates and transfected with pSVL HN and pSVL F and washed in PBS-CaCl₂. Cells were incubated with labeled RBCs for 30 minutes on ice. Cells were then washed with ice-cold PBS-CaCl₂ and incubated at 37°C for 40 minutes. After incubation, cells were washed in cold PBS-CaCl₂ and immediately visualized and photographed with Nikon Diaphot 300 fluorescence microscope.

**Virus infection:** COS-7 monolayers grown in 35mm plates were incubated with egg grown NDV (strain B1) (moi of 10) at 37°C for 40 minutes in presence or absence of
inhibitors. Monolayers were washed three times with DMEM and further incubated at 37°C for 9h with or without inhibitors. Cells were then washed and lysed. Proteins in lysates were resolved by SDS-PAGE and analyzed by Western blot using a mix of anti-NDV (1:1250), anti-HN protein (anti-AS) (1:1650), and anti-F protein (anti-HR2) (1:2500) antibodies described above.

For analysis of infection by immunofluorescence, COS-7 cells were grown in 35mm plates containing glass coverslips and infected in presence or absence of inhibitors as described above. After 40 minutes, cells were washed three times with DMEM and further incubated for 2h with or without inhibitors. Inhibitors were removed and surface bound virus was neutralized by anti-NDV antibody (1:40 dilution). After 6h of incubation at 37°C cells were washed twice with PBS, fixed in 1% paraformaldehyde, and incubated at 4°C in IF buffer (PBS containing 1% bovine serum albumin, 0.02% sodium azide, and 0.01% CaCl₂) for 1h. Cells were incubated with primary antibody (anti-NDV) diluted in IF buffer (1:200) at 4°C for 1h and washed three times with IF buffer and incubated for 1h with anti-rabbit IgG coupled to Alexa-488 diluted in IF buffer (1:200). Cells were washed in ice-cold IF buffer. Coverslips were mounted on slides, and cells were photographed immediately with a Nikon Diaphot 300 fluorescence microscope.

**Biotinylation with MPB:** MPB (3-(N-maleimidylpropionyl) biocytin) (Molecular Probes) was used to biotinylate free thiols in cell surface proteins. Transfected cells grown in 35mm plate were washed with PBS-CM (PBS with 0.1mM CaCl₂ and 1mM MgCl₂) and incubated with MPB (0.5mM in PBS) at 25°C for 40 minutes in presence or absence of
DTNB (7.5mM) or bacitracin (7.5mM). Cells were then washed once with DMEM and twice with PBS and lysed using RSB lysis buffer as described above. Lysates were precipitated with 0.3% SDS containing neutravidin-agarose that had been washed sequentially with PBS containing 0.5% tween-20, and 5mg/ml BSA and PBS containing 0.5% tween-20 and 1mg/ml BSA. Precipitates were washed three times with PBS containing 0.5% tween-20 and 0.4% SDS and resolved by SDS-PAGE and analyzed by western blot using anti-F (anti-HR2) and anti-HN (anti-AS) antibodies as described previously.

**Immunofluorescence:** COS-7 cells were grown in 35mm plates containing glass coverslips and transfected with pSVL HN and pSVL F as described above. After 36h, DTNB (7.5mM) or bacitracin (7.5mM) were added to the cells and incubated overnight. After 48h the cells were washed twice with ice-cold IF buffer (PBS containing 1% bovine serum albumin, 0.02% sodium azide, and 0.01% CaCl₂) and incubated for 1h at 4°C in IF buffer with or without inhibitors. Cells were then incubated at 4°C for 1h with IF buffer containing antibody (diluted 1:200) and inhibitors. The cells were washed three times with ice-cold IF buffer and incubated for 1h on ice with IF buffer containing Alexa 488-labeled anti-rabbit IgG or Alexa 570-labeled anti-mouse IgG (diluted 1:200) as well as inhibitors. The cells were washed with ice-cold IF buffer, fixed with 2% paraformaldehyde, and mounted for microscope.

Quantification of surface immunofluorescence was accomplished by determining the mean fluorescence intensities for cells, using Adobe Photoshop as described previously (Takizawa, Smith et al. 2006). Individual cells were outlined manually using
lasso tool in immunofluorescence pictures opened in Photoshop. Mean fluorescence of selected areas were determined with the histogram submenu. Background fluorescence values were obtained from empty vector transfected cells and were subtracted from the values obtained from cells expressing HN and F proteins.
C. Results

**PDI inhibitors did not affect cell viability or viral glycoprotein surface expression.** To explore the role of disulfide bond rearrangement in the function of NDV glycoproteins, we determined the effects of inhibitors of disulfide bond exchange, DTNB, bacitracin and anti-PDI antibodies, on virus attachment and membrane fusion.

For these studies, it was first necessary to determine the effect of these compounds on cell viability and on glycoprotein surface expression. Cell viability was determined using the compound MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is metabolized to form blue formazan crystals in live cells (Mosmann 1983). COS-7 cells, treated overnight with increasing concentrations of DTNB, bacitracin, and anti-PDI antibodies, were incubated with MTT and the development of blue color in these cells was compared to untreated controls. As shown in Figure 2.1, panel A, there were no differences in viability between untreated cells and cells subjected to prolonged incubation with the inhibitors.

The effect of inhibitors on cell viability was further explored using annexin V-FITC and PI double staining. Annexin V binds to cell surfaces and propidium iodide stains nuclei only in cells undergoing apoptosis (Zelenkov, Baumgartner et al. 2006). Annexin V-FITC and PI double staining of control and inhibitor treated cells were determined by both fluorescence microscopy and FACS and the results are shown in Figure 2.1, panels B and C, respectively. These results confirm that PDI inhibitors do not affect cell viability.

To determine the effects of PDI inhibitors on total expression of HN and F glycoproteins or on their cell surface expression, intact COS-7 cells expressing the NDV HN and F proteins
Figure 2.1: Effect of PDI inhibitors on cell viability and protein expression.

Panel A: Cells incubated overnight with DTNB (2.5mM, 5mM, 7.5mM), bacitracin (BACI) (2.5mM, 5mM, 7.5mM), or anti-PDI antibody (3µl or 6µl/ml) were incubated with MTT for 3h at 37°C. The blue metabolic product of MTT, measured by OD570, is represented as percent of the value obtained for untreated cells (No Tt). The result shown is the average of three independent experiments and bars denote the standard deviation.

Panels B and C: Cells were treated with Triton X-100 (0.05%) (panel B) or with Triton X-100 and staurosporine (1µM) (panel C) as positive control or with DTNB (7.5mM), bacitracin (BACI) (7.5mM), anti-PDI antibody (6µl/ml) or left untreated (No Tt). Cells were stained with Annexin V-FITC and PI and
were analyzed for double staining by immunofluorescence (panel B) and FACS (panel C). Numbers in each quadrant indicate percent of total cells. Panels D and E: Cells, transfected with empty vector (lanes 2 and 7) or HN and F-protein cDNAs (lanes 3-6 and 8-11), were incubated with DTNB (7.5mM) (lanes 4 and 9), bacitracin (BACI) (7.5mM) (lanes 5 and 10) or anti-PDI antibody (6µl/ml) (lanes 6 and 11) or without inhibitor (No Tt) (lanes 3 and 8). Surface F protein (panel D) or HN protein (panel E), biotinylated using sulfo-NHS-S-S-biotin, were precipitated with neutravidin-agarose (lanes 3-6), and total F or HN protein in the extracts (lanes 8-11) were resolved by SDS-PAGE and analyzed by Western blot using anti-F protein antibody (anti-HR2) or anti-HN protein antibody (anti-AS). The amount of the total extract loaded represents one third of the amount of extract used to precipitate biotinylated surface proteins. Lane 1 shows infected cell extract used as a marker.
were either treated with PDI inhibitors overnight or left untreated, and the surface proteins were biotinylated with sulfo-NHS-SS-biotin. Incubation with inhibitors had no effect on total expression of F protein (Figure 2.1, panel D, lanes 8-11) or HN protein (Figure 2.1, panel E, lanes 8-11). Biotinylated surface proteins in these lysates were precipitated with neutravidin and F and HN proteins in the precipitates are shown in Figure 2.1, panels D and E, lanes 3-6, respectively. Clearly cell surface expression of both F protein and HN protein was unaffected by prolonged incubation with inhibitors.

**PDI inhibitors did not affect receptor binding or neuraminidase activity of HN protein.** The effect of inhibitors on the receptor binding activity of NDV HN protein expressed on cell surfaces was quantified by measuring the ability of these cells to bind red blood cells (RBCs). COS-7 cells expressing NDV HN protein were treated overnight with DTNB, bacitracin, or anti-PDI antibodies and then incubated with RBCs. Bound RBCs were quantified as described in Materials and Methods. As shown in Figure 2.2, Panel A, PDI inhibitors did not affect binding of RBCs to surface-expressed HN protein.

To determine if PDI inhibitors affected neuraminidase activity of HN protein expressed on cell surfaces, cells expressing NDV HN protein were treated overnight with PDI inhibitors or left untreated. Cell surface neuraminidase activity was quantified as described in Materials and Methods. Figure 2.2, Panel B shows that PDI inhibitors did not affect neuraminidase activity of cell surface HN protein. These results also verify that the levels of HN protein expressed on surfaces of inhibitor treated cells were similar to levels on untreated cells.
Figure 2.2: Effect of PDI inhibitors on HN protein activities.

Panel A: Effects of overnight incubation in DTNB (7.5mM), bacitracin (BACI) (7.5mM), or anti-PDI antibody (6µl/ml) on attachment activity of cell surface HN protein were analyzed by binding of RBCs. Cells were incubated with RBCs for 30 minutes at 4ºC, bound RBCs were lysed, and released hemoglobin was quantified as described in Materials and Methods. RBC binding to inhibitor treated cells is expressed as percent of binding obtained using untreated cells (No Tt). The results are average of three independent experiments and bars denote standard deviations.

Panel B: Effects of overnight incubation in DTNB (7.5mM), bacitracin (BACI) (7.5mM), or anti-PDI antibody (6µl/ml) on neuraminidase activity of cell surface HN protein were analyzed as described in Materials and Methods. Results are expressed as percent of values obtained for untreated cells (No Tt) and are average of three independent experiments.
**PDI inhibitors inhibited cell-cell fusion.** We next asked if PDI inhibitors blocked F protein directed cell-cell fusion. Cell-cell fusion was measured in three separate assays, syncytia formation, content mixing between effector and target cells, and hemifusion between HN and F protein expressing cells and red blood cells. As shown in Figure 2.3, panel A, increasing concentrations of both DTNB and bacitracin increasingly blocked syncytia formation. At the highest concentrations, DTNB (7.5 mM) inhibited syncytia formation by 83% and bacitracin (7.5mM) by 71%. Anti-PDI antibodies inhibited syncytia formation by 42%. The effects of PDI inhibitors on pore formation were determined by measuring the extent of content mixing between effector and target cells using a reporter assay described in Materials and Methods. As showed in Figure 2.3, panel B, DTNB inhibited content mixing by 95%, bacitracin by 77% and anti-PDI antibodies by 51%.

Effects of PDI inhibitors on hemifusion were determined by assessing their effects on lipid mixing between HN and F protein expressing cells and RBCs labeled with the fluorescence labeled lipid R18. The extent of lipid dye transfer from RBC membranes to COS-7 cell membranes, which occurs during initial stages of fusion, was determined by fluorescence microscopy and representative results are shown in Figure 2.3, panel C. Fluorescent dye transfer occurred between RBCs and untreated cells or cells treated with anti-PDI antibodies (indicated by arrows), while DTNB and bacitracin significantly decreased the number of cells with positive dye transfer. The number of cells with positive dye transfer per field was expressed as percent of the numbers of positive, untreated cells. As shown in Figure 2.3, Panel D, DTNB inhibited hemifusion by 75%, bacitracin by 60%
Figure 2.3: Effect of PDI inhibitors on cell-cell fusion.
Panel A: Syncytia formation in presence of DTNB (2.5mM, 5mM, 7.5mM), bacitracin (BACI) (2.5mM, 5mM, 7.5mM), or anti-PDI antibody (6µl/ml), was quantified as described in Materials and Methods. Inhibitors were added 24h post transfection, and at 48h post transfection the average size of syncytia was determined. Results are expressed as percent of values obtained for untreated cells (No Tt) and are the average of three independent experiments with bars denoting standard deviation.
Panel B: Effects of DTNB (7.5mM), bacitracin (BACI) (7.5mM), or anti-PDI antibody (6µl/ml) on content mixing were assayed using a β-galactosidase reporter assay. Effector cells co-transfected with HN and F protein cDNAs as well as pTA were treated with inhibitors or left untreated. Treated or untreated target cells, transfected with pB1-G only, were overlaid onto HN and F protein expressing cells. Content mixing between effector and target cells was quantified by measuring β-galactosidase in cell extracts and is shown as percent of activity detected in untreated cells (No Tt). The results are the
average of three independent experiments and bars denote standard deviations. Panels C and D: Effects of DTNB (7.5mM), bacitracin (BACI) (7.5mM), or anti-PDI antibody (6µl/ml), on lipid mixing, were assayed in COS-7 cells transfected with HN and F protein cDNAs. At 24h post transfection inhibitors were added to cells and at 48h posttransfection cells were incubated with R18 labeled RBCs on ice followed by incubation at 37°C for 40 minutes. Cells were visualized with a fluorescent microscope for dye transfer (Panel C). Cells with positive dye transfer are indicated by the arrows. Average numbers of cells with positive dye transfer per field were determined and are represented as percent of values determined for untreated cells (No Tt) (D). Results are the average of three different experiments and bars represent standard deviation.
and anti-PDI antibodies by 20%. These results suggested that PDI inhibitors inhibited cell-cell fusion and that the inhibition occurred prior to the onset of fusion, before hemifusion.

**PDI inhibitors inhibited virus entry.** To determine if PDI inhibitors can inhibit virus entry, COS-7 cells were infected with egg grown NDV, B1 strain, in presence or absence of inhibitors. The extents of infection were measured by the levels of newly made proteins in infected cells. NDV protein expression at 9h in untreated cells is shown in Figure 2.4, panel A, lane 3. Incubation with increasing concentrations of DTNB (lanes 4-6) and bacitracin (lanes 7-9) significantly decreased NDV protein expression compared to untreated cells (lane 3) while anti-PDI antibody (lanes 10 and 11) was not as effective. Levels of NDV protein expression were quantified using M protein (Figure 2.4, panel B). Higher concentrations of DTNB and bacitracin inhibited M protein expression by 95%, and anti-PDI antibody inhibited only by 20%.

To determine if the slight inhibition of virus infection by anti-PDI antibody was a specific effect of this antibody, cells were infected in presence of anti-CD71 antibody, a rabbit polyclonal IgG. As shown in Figure 2.4, panel C, anti-CD71 antibody did not affect the NDV protein expression (lane 5) while anti-PDI antibody slightly decreased the protein expression (lane 4).

To ensure that the inhibition of infection by PDI inhibitors was not due to effects on viral replication after virus entry, cells were incubated with the inhibitors for only 2h after virus adsorption. To prevent entry of bound virus after removal of inhibitors, anti-NDV antibody was added after removal of inhibitors. The efficiency of anti-NDV antibody in neutralizing virus already bound to the target cells is shown in Figure 2.4, panel D.
Infected cells were incubated at 4°C for 2h (lane 5). At 4°C virus particles bind to cells but are unable to proceed to membrane fusion. After 2h cells were washed and anti-NDV antibody was added. Cells were further incubated and analyzed for NDV protein expression. The protein expression is decreased and is comparable to lane 2, which shows cell lysates infected with virus pre-incubated with anti-NDV antibody. This result indicates that anti-NDV antibody can neutralize NDV bound to cells.

Panel E in Figure 2.4 shows that removal of inhibitors at 2h post infection still inhibited virus infection, results consistent with effects on virus entry. Cells were infected for 2h in the presence of inhibitors, the inhibitors were removed, and anti-NDV antibody was added. Cells were incubated for 6h and then analyzed for NDV protein expression by cell surface immunofluorescence. Cells infected in presence of DTNB (panel E, III) and bacitracin (panel E, IV) showed significantly decreased surface expression of NDV proteins compared to cells infected without any inhibitor (panel E, II). These results are consistent with an inhibition of virus entry.

To further confirm that PDI inhibitors inhibit a very early step in infection, DTNB was added at different time points after the start of the infection. As shown in Figure 2.4, panel F, adding the inhibitor as early as 15 minutes (lane 5) and 30 minutes (lane 6) after infection resulted in significant levels of virus infection while adding the inhibitor with the virus blocked infection (lane 4). These results are consistent with effects of DTNB on virus entry.

**Free thiols were detected in F protein but not in HN protein.** Cell surface PDI molecules are thought to primarily reduce disulfide bonds of surface-expressed proteins
Figure 2.4: Effect of PDI inhibitors on virus entry.
Panels A and B: COS-7 cells, incubated with DTNB (2.5mM, 5mM, 7.5mM) (lanes 4-6), bacitracin (BACI) (2.5mM, 5mM, 7.5mM) (lanes 7-9), or anti-PDI antibody (3µl or 6µl/ml) (lanes 10-11) were infected with NDV, strain B1, (moi 10) in presence (lanes 4-11) or absence (lane 3) of inhibitors (No Tt). Cells were further incubated with or without inhibitors for 9h at 37°C. Proteins in the resulting cell lysates were resolved by SDS-PAGE (10%) and analyzed for NDV protein expression by Western blot using a mix of anti-NDV, anti-HN, and anti-F protein antibodies as described in Materials and Methods (Panel A). Lane 2 shows mock infected cell extract and lane 1 shows infected
cell extract used as marker. The M protein bands were quantified by
densitometry and values are expressed as percent of values for cells infected
in absence of inhibitors (Panel B). Results are average of three different
experiments.
Panel C: Cells were incubated overnight with anti-PDI antibody (lane 4) or
anti-CD71 antibody (lane 5) or left untreated (lane 3) and infected with NDV,
B1 strain in presence or absence of antibodies and analyzed for NDV protein
expression as described above.
Panel D: Lane 1 shows cells pre-incubated with anti-NDV antibody, washed,
and then infected with virus (negative control). Lane 2 shows cells infected
with virus pre-incubated with antibody (positive control). Lanes 3 and 4 show
infected cells with antibody added at the time of infection and 2h after the
infection respectively. Lane 5 shows cells to which anti-NDV antibody was
added after 2h of virus adsorption at 4ºC. Lane 6 shows cells infected in the
presence of DTNB and incubated with anti-NDV antibody after 2h.
Panel E: Cells plated on coverslips were incubated with inhibitors and
infected with NDV as described above. After 2h of incubation, inhibitors were
removed and bound virus was neutralized by anti-NDV antibody. Cells were
further incubated for 6h and surface-expressed NDV proteins were detected
by immunofluorescence using anti-NDV antibody. Top panels show nuclei of
cells stained with Hoechst stain and bottom panels show cells incubated with
anti-NDV antibody. First column (I) shows mock infected cells, second
column (II) shows cells infected without any inhibitor treatment (No Ti), third
column (III) shows cells infected in presence of DTNB (7.5mM), and fourth
column (IV) shows cells infected in the presence of bacitracin (BACI)
(7.5mM).
Panel F: DTNB (7.5mM) was added either at the time of infection (as
described above) (lane 4) or was added 15 minutes (lane 5), 30 minutes (lane
6), 45 minutes (lane 7) or 60 minutes (lane 8) after the infection of cells.
(Mandel, Ryser et al. 1993; Matthias and Hogg 2003). Thus it seemed likely that these inhibitors blocked fusion by preventing the formation of free thiols. It follows that either surface-expressed HN or F proteins may have free thiols. To detect free thiols on cell surface proteins, we used MPB (3-(N-maleimidylpropionyl) biocytin), a membrane impermeable, thiol specific biotin that binds to and biotinylates only free thiol containing proteins.

COS-7 cells expressing either HN or F or co-expressing both proteins were incubated with MPB. MPB labeled proteins present in lysates of these cells were precipitated under stringent conditions with neutravidin, resolved by SDS-PAGE, and HN and F proteins in the polyacrylamide gels were detected by Western analysis. SDS was included in the precipitation with neutravidin to eliminate any association of host proteins with either viral protein. As a positive control, cells cultured in parallel were incubated with the reducing agent, DTT (dithiothreitol), in order to generate free thiols. Figure 2.5, panel A, shows detection of HN protein in the precipitates. HN protein expressed alone or with F protein was not biotinylated by MPB (lanes 3 and 4) while HN protein expressed on cell surfaces treated with DTT, prior to incubation with MPB, was biotinylated (lanes 6 and 7). This result indicates that HN protein at the cell surface does not have free thiols. Figure 2.5, panel B, shows that F protein was biotinylated by MPB when expressed alone or with HN protein (lanes 3 and 4). This result suggests that surface-expressed F protein has free thiols. The slightly lower levels of HN protein in lane 7 or F protein in lane 4 can be accounted for by a slight reduction in recovery of total HN or F protein when co-expressed (data not shown) as has been previously noted (Bousse, Takimoto et al. 1997).
**Figure 2.5: Biotinylation using a thiol specific biotin, MPB.**

Panels A and B: COS-7 monolayers were incubated with MPB (0.5mM in PBS) at 25ºC for 40 minutes. Proteins in resulting cell lysates were precipitated with neutravidin as described in Materials and Methods and precipitated proteins were resolved by SDS-PAGE (10%) and analyzed by Western blot using anti-HN protein antibody (A) and anti-F protein antibody (B). Lane 1 contains infected cell extract used as marker, lanes 2 and 5 contain empty vector transfected cell extracts, lanes 3 and 6 contain HN protein (A) or F protein (B) transfected cell extracts, and lanes 4 and 7 contain HN and F protein co-transfected cell extracts. Lanes 2-4 show cells incubated with MPB and lanes 5-7 are cells incubated with DTT (Dithiothreitol) prior to biotinylation with MPB.

Panels C and D: Cells transfected with empty vector (lanes 2 and 5), F protein cDNA (lanes 3 and 6) or HN and F protein cDNAs (lanes 4 and 7) were incubated with MPB in presence (lanes 5-7) or absence (lanes 2-4) of DTNB (C) or bacitracin (D). Proteins in resulting cell lysates were precipitated with neutravidin. Precipitated proteins were resolved by SDS-PAGE (10%) and analyzed by western blot using anti-F protein antibody.
**PDI inhibitors blocked detection of free thiols in F protein.** To determine if PDI inhibitors block the formation of free thiols in surface-expressed F protein, F protein expressing cells were incubated with MPB in the presence of DTNB or bacitracin. Figure 2.5, panel C shows that both DTNB (Figure 2.5, panel C, lanes 6 and 7) and bacitracin (Figure 2.5, panel D, lanes 6 and 7) inhibited biotinylation of F protein by MPB. These results indicate that the PDI inhibitors effectively blocked the formation of free thiols in the F protein, which correlated with their ability to block membrane fusion.

**MPB binding to F protein blocked cell-cell fusion.** We also asked if MPB binding to free thiols in F protein could block cell-cell fusion, using the content mixing assay. Effector cells were overlaid with target cells in presence or absence of MPB and the extent of fusion between two cell populations was determined by measuring the levels of β-galactosidase activity as described in Materials and Methods. Figure 2.6 shows that MPB binding inhibited fusion by 60-70%, indicating that blocking the free thiols in the F protein inhibited cell-cell fusion. This result also shows that F protein with free thiols is a biologically functional form of the protein.

**DTNB and bacitracin altered antibody binding to surface F protein.** If disulfide bond reduction has a role in conformational changes associated with the onset of fusion, then inhibition of the formation of free thiols in F protein may alter the conformation of F protein on the cell surfaces. Since antibodies are often used to detect conformation differences in proteins, two different conformation sensitive anti-F protein antibodies, anti-Fu1a (Morrison, Peeples et al. 1987) and anti HR1 antibody (McGinnes, Gravel et al. 2002), were used to determine if incubation with disulfide bond isomerase
Figure 2.6: Effect of MPB on cell-cell fusion.
Effect of MPB on cell-cell fusion was assayed using a β-galactosidase reporter assay. Cells, co-transfected with HN and F protein cDNAs as well as pTA, were overlaid with target cells, transfected with pB1-G, in presence of MPB (0.5mM in PBS or serum free medium) for 1h at 25°C. Cells were further incubated with supplemented DMEM for 5h at 37°C. Cells were washed and content mixing between HN and F protein expressing cells and target cells was quantified by measuring β-galactosidase as described in Materials and Methods and is shown as percent of activity detected in cell mixtures treated with PBS without MPB (No Tt). The results are the average of three independent experiments and bars denote standard deviations.
inhibitors affected the binding of either of these antibodies to F protein. Anti-NDV antibody was used as a control and, as shown in Figure 2.7, panel A, anti-NDV antibody binding to cells was unaffected by overnight incubation in DTNB or bacitracin. By contrast, incubation with inhibitors significantly increased the binding of anti-Fu1a (Figure 2.7, panels B). Furthermore, incubation with inhibitors eliminated the binding of anti-HR1 antibody (Figure 2.7, panels C). Mean fluorescence intensities were quantified for cells shown in panels A, B and C and is shown in panels D (using anti-NDV antibody), E (using anti-Fu1a antibody) and F (using HR1 antibody). These results indicate that inhibitors of disulfide bond isomerases altered the conformation of the F protein expressed on cell surfaces.
Figure 2.7: Binding of different conformation sensitive antibodies to surface-expressed F protein in presence of inhibitors.

Panels A, B, and C: show cell surface immunofluorescence using anti-NDV antibody, anti-Fu1a antibody, and anti-HR1 antibody, respectively. Column 1 shows cells transfected with empty vector without any treatment (No Tt). Columns 2 and 3 show cells transfected with empty vector and treated with DTNB (7.5mM) and bacitracin (BACI) (7.5mM) respectively. Column 4 shows the cells transfected with HN and F protein cDNAs without any treatment (No Tt) and columns 5 and 6 show cells transfected with HN and F protein cDNAs and treated with DTNB (7.5mM) and bacitracin (BACI) (7.5mM), respectively.

Panels D, E and F: show the quantification of fluorescence intensity of the cells shown in panels A, B and C respectively. The results shown are average of mean fluorescence of 10 different cells and are normalized for background fluorescence by subtracting mean fluorescence of vector alone transfected cells.
D. Discussion

Fusion proteins are proposed to undergo extensive conformational changes with the activation of fusion. Evidence for these changes include alterations in antibody reactivities upon activation (Edwards, Mann et al. 1983; Kielian and Helenius 1985; Kostolansky, Russ et al. 1988; Wahlberg and Garoff 1992; Earl, Broder et al. 1994; Finnegan, Berg et al. 2002), alterations in protease sensitivity (Kielian, Jungerwirth et al. 1990; Puri, Booy et al. 1990; Meyer, Gidwitz et al. 1992; Wahlberg and Garoff 1992), changes in oligomeric status (Wahlberg and Garoff 1992; Locker and Griffiths 1999), and differential binding of peptides with sequences from one or the other HR domains (Russell, Jardetzky et al. 2001). Structural analyses of the pre-fusion and post-fusion forms of the influenza HA (Wilson, Skehel et al. 1981; Skehel, Bayley et al. 1982; Bullough, Hughson et al. 1994; Tamm, Han et al. 2002) are quite consistent with these proposed conformational shifts. In addition, significant conformational differences have been observed between two crystallized forms of the paramyxovirus F protein. One form, exemplified by the structures of the parainfluenza virus 3 F protein (Yin, Paterson et al. 2005) and NDV F protein (Chen, Gorman et al. 2001), contain a six-stranded, coiled coil composed of the two HR domains of the proteins. Although these crystals were derived from uncleaved forms of the F proteins, they are proposed to be characteristic of the post-fusion form of the paramyxovirus F protein (Yin, Wen et al. 2006). Another F protein structure (Yin, Wen et al. 2006), derived from a crystal formed by the ectodomain of an uncleaved SV5 F protein fused at the carboxyl terminus of the HR2 domain with the yeast GCN4 sequence, was significantly different. The GCN4 sequence forces the
HR2 domain to trimerize preventing the formation of the HR1-HR2 complex. It has been proposed that this alternative structure represents the conformation of the pre-fusion F protein (Russell and Luque 2006; Yin, Wen et al. 2006). Based on these two different structures, a model for the conformational changes in the paramyxovirus F protein has been presented (Russell and Luque 2006; Yin, Wen et al. 2006), changes that involve significant rearrangement of the molecule.

Disulfide bond isomerization in the HIV env protein is proposed to accompany and facilitate conformational shifts in that molecule during fusion. Evidence for this idea includes observations that inhibitors such as DTNB, bacitracin, and anti-PDI antibody, which block the activities of PDI and related isomerases, inhibited HIV entry and HIV cell-cell fusion (Ryser, Levy et al. 1994; Fenouillet, Barbouche et al. 2001; Markovic, Stantchev et al. 2004). Second, free thiols have been detected in gp120 subunit of the env protein (Gallina, Hanley et al. 2002; Barbouche, Miquelis et al. 2003; Markovic, Stantchev et al. 2004). These free thiols are reported to form upon CD4 binding (Gallina, Hanley et al. 2002; Barbouche, Miquelis et al. 2003; Markovic, Stantchev et al. 2004) or upon binding to CXCR4 (Barbouche, Miquelis et al. 2003). Third, other retroviruses, including MuLV, have, within the env protein sequence, CXXC motifs which are used during fusion to isomerize the disulfide bond between the TM and SU subunits (Pinter, Kopelman et al. 1997; Ferrari and Soling 1999; Wallin, Ekstrom et al. 2004). It has been suggested that HIV relies on host isomerases while other retroviruses have evolved to encode their own isomerase activity (Ryser, Levy et al. 1994; Wallin, Ekstrom et al. 2004).
Additionally, supporting the idea that disulfide bond isomerization may be involved in membrane fusion is a theoretical analysis of disulfide bonds in proteins involved in cell entry (Wouters, Lau et al. 2004). This study described some unusual disulfide bonds present in these proteins. These bonds, called cross strand disulfides (CSDs), link cysteine residues in adjacent strands in same β-sheet and have high potential energy stored in them (Matthias and Hogg 2003; Wouters, Lau et al. 2004). CSDs are identified by higher dihedral strain energy in these bonds due to torsional strain produced by linking adjacent strands (Hogg 2003; Matthias and Hogg 2003; Wouters, Lau et al. 2004). Also CSDs are more readily cleaved than other disulfide bonds and are more susceptible to reducing agents, thioredoxin or PDI (Matthias and Hogg 2003; Wouters, Lau et al. 2004). These bonds were found at higher frequency in proteins involved in cell entry including bacterial toxins and the viral fusion proteins, HIV gp120, influenza HA, and NDV F protein. CSD in NDV fusion protein links Cys338 and Cys347, which raises the possibility that cleavage of this disulfide bond, may be involved in cell entry by NDV (Wouters, Lau et al. 2004).

Results presented here are consistent with the idea that thiol-disulfide exchange in NDV F protein, mediated by a PDI or PDI-like isomerases, is required for virus entry or cell-cell fusion mediated by F protein. Inhibitors of PDI, DTNB and bacitracin inhibited cell-cell fusion. The results showing an inhibition of virus protein expression are consistent with effects on virus entry. Furthermore, inhibition of cell-cell fusion occurred at an early stage in the onset of fusion since these inhibitors blocked hemifusion, a result consistent with effects on fusion activation. These results are less likely to be due to
nonspecific effects of these inhibitors since the inhibitors had no effect on cell viability, glycoprotein surface expression, or the attachment and neuraminidase activities of the HN protein.

Anti-PDI antibody was less effective in inhibiting cell-cell fusion as well as virus entry. DTNB and bacitracin are not specific inhibitors of PDI. DTNB is a non-specific inhibitor of free thiols (Feener, Shen et al. 1990) and bacitracin binds to CXXC motif in catalytic domains that are present in all the members of thioredoxin family (Mou, Ni et al. 1998). Lower efficiency of anti-PDI antibody in fusion inhibition suggests that isomerization of disulfide bonds in F protein may be due to one or several other thiol reactive proteins that are found in cells (Wilkinson and Gilbert 2004; Ellgaard and Ruddock 2005). Indeed, it has been shown that down regulation of PDI using siRNA had only a small effect on infection or cell fusion mediated by HIV-1 (Ou and Silver 2006) suggesting that other thiol active enzymes at the cell surfaces are involved in reduction of the HIV envelope glycoprotein.

Since cell surface PDI and related proteins usually serve to reduce bonds in interacting proteins, it seemed likely that the inhibitors blocked the reduction of one or more disulfide bonds in F protein and that surface F protein may have free thiols. Indeed, results showed that MPB, a membrane impermeable thiol specific reagent, binds to the F protein. This binding was inhibited by DTNB and bacitracin suggesting that the free thiols in F protein are due to reduction by a PDI-like isomerase. The result that inhibitors blocked both fusion and detection of the free thiols is consistent with the idea that only F protein with free thiols can proceed to direct fusion. Interestingly, MPB also inhibited
fusion. This result may indicate that completion of the fusion process requires re-oxidation of the free thiols in F protein, perhaps as the protein refolds during onset of fusion. Alternatively, addition of MPB to the F protein may interfere with subsequent conformational changes necessary for fusion. Inhibition of fusion by MPB indicates that F protein with free thiols is not an aberrant, biologically irrelevant form nor is it in a post-fusion conformation.

The current model for proposed conformational changes in F protein during membrane fusion shows that F protein undergoes a series of conformational changes from spring loaded prefusion form to a six helical bundle containing post-fusion form (Russell and Luque 2006; Yin, Wen et al. 2006). The existence of these predicted conformational forms is supported by changes in reactivity to the conformation sensitive anti-F protein antibodies, anti-Fu1a and anti-HR1 antibodies, after incubation in inhibitors of disulfide bond isomerases (depicted in revised model, Figure 3.7). Fu1a antibody binding is significantly enhanced in the presence of DTNB and bacitracin. By contrast, reactivity to antibodies specific for the HR1 domain was lost in the presence of these inhibitors. Both results are consistent with the suggestion that a significant population of F protein on cell surfaces contains free thiols and that the reduction alters the conformation of the protein decreasing reactivity to anti-Fu1a and increasing accessibility of the HR1 domain.

Free thiols were detected on both F protein expressed alone as well as F protein co-expressed with HN protein. This result may indicate that binding of the attachment protein to receptors is not required for formation of free thiols in the F protein. Rather,
the results are consistent with the possibility that F protein may exist as a mixture of both oxidized and reduced forms on cell surfaces. In the presence of HN protein only the reduced form may proceed to refold into additional intermediates. The result that covalent addition of MPB blocks fusion provides additional evidence for further conformational changes that are required for fusion.
CHAPTER III
OVER-EXPRESSION OF THIOL-DISULFIDE ISOMERASES ENHANCES MEMBRANE FUSION DIRECTED BY THE NEWCASTLE DISEASE VIRUS FUSION PROTEIN

B. Introduction

Newcastle disease virus (NDV), like other paramyxoviruses, enters host cells by fusion of the viral membrane with host cell plasma membranes. This fusion is triggered by attachment of the hemagglutination-neuraminidase (HN) protein to the sialic acid containing host cell receptors and is mediated by the fusion (F) protein. Based on similarities in protein structure and fusion mechanisms, paramyxovirus fusion proteins, influenza hemagglutinin (HA) proteins and retroviral envelope (env) proteins have been categorized as class I fusion proteins (as reviewed in (Baker, Dutch et al. 1999; Weissenhorn, Dessen et al. 1999; Skehel and Wiley 2000)).

Class I fusion proteins are synthesized as single polypeptides (F₀ in paramyxoviruses), which form homotrimers and are cleaved into two subunits, a membrane distal (F₂ in paramyxoviruses) and a membrane anchored subunit (F₁ in paramyxoviruses). At the amino terminus of the membrane anchored subunit is a fusion peptide, which inserts into the target membranes upon fusion activation. Adjacent to the fusion peptide is a conserved heptad repeat, HR₁, and another conserved heptad repeat, HR₂, is located next to the transmembrane domain (as reviewed in (Baker, Dutch et al. 1999; Morrison 2003)). The F protein, in a metastable, cleaved form on the virus or cell surface, can be triggered to undergo conformational changes, which result in membrane
fusion. These conformational changes are triggered by binding of HN protein to receptors (Lamb 1993; Sergel, McGinnes et al. 1993; McGinnes and Morrison 2006).

The conformational changes proposed to take place in F protein during activation and onset of fusion (Yin, Wen et al. 2006) are significant, but how this refolding is accomplished is unclear.

A potential mechanism to facilitate these conformational changes is suggested by a number of studies of different viruses, which have shown that, during membrane fusion, fusion glycoproteins undergo thiol/disulfide isomerization leading to reduction of disulfide bonds and production of free thiols in fusion glycoproteins (Ryser, Levy et al. 1994; Wallin, Ekstrom et al. 2004; Gilbert, Ou et al. 2006; Schelhaas, Malmstrom et al. 2007; Delos, Brecher et al. 2008). The production of free thiols in these glycoproteins is essential for membrane fusion and may facilitate conformational changes required for fusion. In some viruses, like murine leukemia virus (MLV), the thiol/disulfide isomerization is thought to be mediated by an isomerase motif, Cys-X-X-Cys (CXXC), in the env glycoprotein and this isomerization is triggered by binding of glycoprotein to its receptor (Pinter, Kopelman et al. 1997; Wallin, Ekstrom et al. 2004; Wallin, Loving et al. 2005). In other viruses, like HIV-1, the thiol/disulfide isomerization is thought to be catalyzed by a host cell protein, protein disulfide isomerase (PDI) or a related protein. This conclusion is based on the studies showing inhibition of HIV-1 entry and cell-cell fusion by inhibitors of the PDI family of isomerases (Ryser, Levy et al. 1994; Fenouillet, Barbouche et al. 2001; Gallina, Hanley et al. 2002; Barbouche, Miquelis et al. 2003; Markovic, Stantchev et al. 2004).
In another study, contribution of PDI in HIV-1 env protein mediated membrane fusion was evaluated by decreasing expression of endogenous PDI protein using siRNA (Ou and Silver 2006). It was shown that down regulation of PDI did not significantly inhibit the membrane fusion mediated by HIV-1 env protein. The authors suggested that other isomerases of PDI family may also be involved in disulfide bond reduction and that this function might be redundant as many of the members of PDI isomerase family have similar catalytic domains and can catalyze reduction of disulfide bonds (as reviewed in (Appenzeller-Herzog and Ellgaard 2008)).

PDI is a member of a family of 19 structurally related isomerases with a thioredoxin like domain (as reviewed in (Appenzeller-Herzog and Ellgaard 2008)). Most of the isomerases in PDI family have CXXC motif that catalyzes formation, reduction and rearrangement of disulfide bonds in proteins (Noiva 1999; Wilkinson and Gilbert 2004; Ellgaard and Ruddock 2005; Appenzeller-Herzog and Ellgaard 2008). These isomerases are primarily involved in folding of proteins in endoplasmic reticulum (ER), catalyzing formation of disulfide bonds. In recent years, isomerases from PDI family have also been shown to be present on cell surfaces. Cell surface isomerases have been proposed to be involved in processes such as cell adhesion, nitric oxide signaling, and reduction of disulfide bonds in cell entry proteins of viruses (Turano, Coppari et al. 2002; Jordan and Gibbins 2006).

Recently we reported that free thiols can be detected in cell surface-expressed NDV fusion protein and these free thiols are required for cell-cell membrane fusion (Jain, McGinnes et al. 2007). We showed that cell-cell membrane fusion as well as viral entry
was inhibited by DTNB, a nonspecific inhibitor of free thiols, bacitracin, an inhibitor of PDI family of isomerases, and anti-PDI antibodies. However anti-PDI antibodies inhibited membrane fusion less efficiently than the two inhibitors. This result suggested that reduction of disulfide bonds in the F proteins may be accomplished by other members of PDI isomerase family.

To explore the role of PDI and other PDI-like isomerases on F protein function, we studied the effects of over-expression of two different thiol isomerases, PDI and ERdj5, on F protein mediated fusion and on the conformation of surface expressed F protein. This approach was pursued since inhibition of all the PDI-like isomerases using siRNA was not feasible. The results showed that over-expression of either of the isomerases led to enhanced cell-cell fusion and favored a post-fusion conformation of F protein. These results are consistent with the conclusion that free thiols have an important role in NDV membrane fusion and suggest that other thiol isomerases with a CXXC motif can be involved in disulfide bond reduction in viral fusion glycoproteins and in virus entry.
B. Materials and Methods

**Cells, plasmids and antibodies:** COS-7 cells, obtained from American Type Culture Collection, were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with nonessential amino acids, vitamins, penicillin, and streptomycin, and 10% fetal calf serum.

ERdj5 and PDI genes were purchased from American Type Culture Collection in pCMV-Sport6ccdB and pBluescript SK- vectors, respectively. PDI gene was subcloned in pCMV-Sport6ccdB vectors using NotI and KpnI restriction sites. NDV F and HN genes were inserted into pSVL and pCAGGS expression vectors as previously described (McGinnes, Gravel et al. 2002; McGinnes and Morrison 2006).

Anti-Fu1a is a mouse monoclonal antibody specific for NDV F protein and was obtained from M. Peeples. Anti-HR1 and anti-HR2 antibodies were raised against peptides with sequences from the NDV F protein HR1 and HR2 domains, respectively, and have been previously described (McGinnes and Morrison 2006). Anti-AS antibody was raised against the peptide from the NDV HN protein and has been previously described (McGinnes and Morrison 2006). Anti-NDV antibody was raised in rabbits against UV-inactivated stocks of NDV, strain AV, by standard protocols as previously described (McGinnes, Gravel et al. 2002). Mouse monoclonal Anti-PDI and Anti-ERdj5 antibodies were purchased from Abnova Corporation.

**Transfection:** Transfections of COS-7 cells were accomplished using Lipofectamine or Lipofectin (Invitrogen) as recommended by the manufacturer. For each transfection, a mixture of DNA (0.5µg/35-mm plate) and 7µl of Lipofectamine or
Lipofectin in OptiMEM media (Gibco/Invitrogen) was incubated at room temperature and added to cells previously washed with OptiMEM. The cells were incubated for 5h and OptiMEM was replaced with 2ml of supplemented DMEM.

**Surface Biotinylation:** COS-7 monolayers grown in 35mm plates and transfected with cDNAs encoding the HN and F proteins were incubated with PDI inhibitors overnight and washed three times with PBS-CM (PBS with 0.1mM CaCl₂ and 1mM MgCl₂). PBS-CM containing 0.5mg/ml sulfo-NHS-SS-biotin (Pierce) was added and cells were incubated for 40 minutes at 4°C. Unbound biotin was absorbed with 2ml DMEM and cells were washed three times with PBS and lysed with RSB lysis buffer (0.01M Tris-HCl [pH 7.4], 0.01M NaCl, 1.5mM MgCl₂) containing 1% Triton X-100, 0.5% sodium deoxycholate, 2.5mg of N-ethyl maleimide per ml, and 0.2mg of DNase per ml. Lysates were incubated for 1h at room temperature with neutravidin-agarose (Pierce), containing 0.3% SDS, that had been washed with PBS containing 0.5% tween-20, and 5mg/ml BSA and then with PBS containing 0.5% Tween-20 and 1mg/ml BSA. Precipitates were washed three times with PBS containing 0.5% Tween-20 and 0.4% SDS, resuspended in gel sample buffer (125mM Tris-HCl, pH 6.8, 2% SDS and 10% glycerol) with 0.7M β-mercaptoethanol and resolved by polyacrylamide gel electrophoresis.

**Biotinylation with MPB:** MPB (3-(N-maleimidylpropionyl) biocytin) (Molecular Probes) was used to biotinylate free thiols in cell surface proteins. Transfected cells grown in 35mm plate were washed with PBS-CM (PBS with 0.1mM CaCl₂ and 1mM MgCl₂) and incubated with MPB (0.5mM in PBS) at 25°C for 30 minutes. Cells were then washed once
with DMEM and twice with PBS and lysed using RSB lysis buffer as described above. Proteins in extracts were precipitated with neutravidin-agarose, containing 0.3% SDS, which had been washed sequentially with PBS containing 0.5% tween-20, and 5mg/ml BSA and PBS containing 0.5% tween-20 and 1mg/ml BSA. Precipitates were washed three times with PBS containing 0.5% tween-20 and 0.4% SDS and resolved by SDS-PAGE and analyzed by Western blot using anti-F (anti-HR2) antibody.

**Polyacrylamide gel electrophoresis and Western blot analysis:** Proteins in cell extracts or immunoprecipitates, diluted in gel sample buffer (125mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) with 0.7M β-mercaptoethanol, were resolved on 10% polyacrylamide gels. After electrophoresis, gels were equilibrated in transfer buffer (25mM Tris, pH 8.2, 192mM glycine, 15% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes. The membranes were blocked overnight at 4°C in PBS containing 0.5% Tween 20 and 10% nonfat milk, washed with PBS-Tween 20, and incubated for 1h at room temperature with primary antibody diluted to 1:1,000 in PBS-Tween 20. Membranes were then washed, incubated for 1h at room temperature with secondary antibody (goat anti-rabbit IgG or anti-mouse IgG coupled to horseradish peroxidase) (Amersham Biosciences) diluted to 1:40,000 in PBS-Tween 20, and then washed extensively in PBS-Tween 20. Bound antibody was detected using the ECL Western blotting detection reagent system (Amersham Biosciences). Quantification of the signal was accomplished using a Fluor-S imager (Bio-Rad).

**Cell viability:** COS-7 cell monolayers plated on a 12-well plate were transfected with empty vector or ERdj5 or PDI cDNAs with or without HN and F cDNAs. Cells
were then incubated with 1ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide] (Promega) (0.5mg/ml in PBS) for 3h at 37º C. After
removing MTT, cells were treated with 1ml acidified isopropanol (0.04M HCl in absolute
isopropanol) to solubilize blue formazan crystals and development of blue color was
quantitated by measuring absorbance at 570nm and subtracting background at 650nm
(Mosmann 1983; Jain, McGinnes et al. 2007).

**Syncytia formation:** COS-7 cells, grown in 35mm plates, were transfected with
HN and F cDNAs with or without ERdj5 or PDI cDNAs (0.5µg/plate) using lipofectin as
described above. Average number of nuclei per syncytium was counted in 20 fusion
areas for each plate at 24, 48 and 72 hrs respectively. Values obtained after transfection
with empty vector were subtracted.

**Content mixing:** Content mixing was measured by using a modification of a
previously described protocol (McGinnes, Sergel et al. 2001). Briefly, cells plated on 12
well plates were transfected with pCAGGS-HN (0.04µg/well), pCAGGS-F (0.04µg/well)
cDNAs and a plasmid encoding a tetracycline-responsive transcriptional activator, tTA
(Clontech) (0.4µg/well). A separate population of cells was transfected with a plasmid,
encoding the β-galactosidase protein under the control of the tetracycline-responsive
transcriptional activator, pB1-G (Clontech) (0.4µg/well) with or without ERdj5 or PDI
cDNAs (0.2µg/well). After 40h, the cells transfected with the pB1-G were removed from
the plate with trypsin and placed on top of the HN protein and F protein expressing cells.
Cells were further incubated for 6h, washed twice with PBS, lysed (Promega cell lysis
buffer), and extracts were assayed for β-galactosidase activity as described in Promega
protocols. Activity due to background fusion of COS-7 cells was measured after mixing cells cotransfected with pTA and vector alone with cells transfected with pB1-G and values obtained were subtracted from values obtained with cells expressing HN and F proteins.

**Immunofluorescence:** COS-7 cells were grown in 35mm plates containing glass coverslips and transfected with cDNAs encoding HN and F proteins with or without ERdj5 or PDI cDNAs. After 36h one set of cells was treated with neuraminidase (0.25U/ml). After 48h one set of cells was treated with MPB for 30min as described above and further incubated for 3h at 37°C. The cells were washed twice with ice-cold IF buffer (PBS containing 1% bovine serum albumin, 0.02% sodium azide, and 0.01% CaCl₂) and incubated for 1h at 4°C in IF buffer. Cells were then incubated at 4°C for 1h with IF buffer containing antibody (diluted 1:200). The cells were washed three times with ice-cold IF buffer and incubated for 1h on ice with IF buffer containing Alexa 488-labeled anti-rabbit IgG or Alexa 570-labeled anti-mouse IgG (diluted 1:200). The cells were washed with ice-cold IF buffer, fixed with 2% paraformaldehyde, and mounted for microscopy (Nikon Diaphot 300 fluorescence microscope).

Quantification of surface immunofluorescence was accomplished by determining the mean fluorescence intensities for cells, using Adobe Photoshop as described previously (Takizawa, Smith et al. 2006). Individual cells were outlined manually using lasso tool in immunofluorescence pictures opened in Photoshop. Mean fluorescence of selected areas were determined with the histogram submenu. Background fluorescence values were
obtained from empty vector transfected cells and were subtracted from the values obtained from cells expressing HN and F proteins.
C. Results

Over-expression of ERdj5 and PDI in COS-7 cells and effects on cell viability and NDV glycoprotein expression. To explore effects of over-expression of PDI-like isomerases on membrane fusion, we first examined the expression of two different members of the PDI isomerase family, PDI and ERdj5, after transfection of cells with cDNAs encoding these isomerases. As shown in Figure 3.1, panel A, COS-7 cells transfected with ERdj5 or PDI cDNAs expressed the respective proteins at 1.5 and 1.8 fold higher levels compared to untransfected cells.

To determine if increased expression of these isomerases affected cell viability, cell viability was determined using MTT, a compound that is metabolized to form blue formazan crystals in live cells (Mosmann 1983). COS-7 cells transfected with cDNAs encoding ERdj5 or PDI with or without HN and F protein cDNAs, were incubated with MTT and development of blue color in these cells was compared to untransfected cells or cells transfected with HN and F cDNAs only. As shown in Figure 3.1, panel B, over-expression of ERdj5 or PDI did not affect cell viability.

To determine the effect of over-expression of these isomerases on surface and total expression of HN or F proteins, surfaces of COS-7 cells expressing HN and F proteins with or without ERdj5 or PDI were biotinylated using sulfo-NHS-SS-biotin. Lysates of these cells were precipitated with neutravidin-agarose to detect the biotinylated surface proteins. The total HN and F proteins in the resulting cell lysates were also determined by Western analysis. The results in Figure 3.1, panel C show that
Figure 3.1: Over-expression of ERdj5 and PDI and its effect on HN and F protein expression and cell viability.

Panel A: Proteins in extracts of COS-7 cells, transfected with empty vector or cDNAs encoding ERdj5 (top) or PDI (bottom) were resolved by SDS-PAGE and analyzed by Western blots. The blots detected for actin as a loading control are shown below each panel. Numbers at bottom of each panel represent expression of the respective proteins in transfected cells as a percent of expression in untransfected cells. The quantification was performed in films with protein bands in linear range.

Panel B: Cells, untransfected (UT) or transfected with ERdj5 or PDI with or without F and HN protein cDNAs were incubated with MTT for 3h at 37°C. The blue metabolic product of MTT, measured by optical density at 570nm, is represented as a percentage of the value obtained for untransfected cells. The values represented are average of three different experiments and error bars represent the range.

Panel C: Cells were transfected with empty vector (lanes 2 and 6) or co-transfected with HN and F protein cDNAs (lanes 3 and 7) or HN and F protein cDNAs with ERdj5 cDNA (lanes 4 and 8) or with PDI cDNA (lanes 5 and 9). Surface HN protein (top) or F protein (bottom), biotinylated using
sulfo-NHS-SS-biotin, were precipitated with neutravidin-agarose (lanes 6 to 9), and total F or HN protein in the extracts (lanes 2 to 5) were resolved by SDS-PAGE and analyzed by Western blots using anti-F protein antibody (anti-HR2) or anti-HN protein antibody (anti-AS). The amount of the total extract loaded represents one-third of the amount of extract used to precipitate biotinylated surface proteins. Lane 1 shows infected-cell extract used as a marker.
neither total expression of HN or F protein (lanes 3-5) or surface expression of HN or F protein (lanes 7-9) was affected by over-expression of ERdj5 or PDI.

**ERdj5 or PDI over-expression in cells expressing HN and F proteins enhances cell-cell fusion.** To determine the effect of over-expression of these thiol isomerases on cell-cell fusion, we compared the sizes of syncytia in cells co-transfected with cDNAs encoding HN, F, and ERdj5 or PDI proteins to those in cells transfected only with cDNAs encoding HN and F proteins. Figure 3.2, panel A, shows representative cell monolayers visualized by immunofluorescence using anti-NDV antibody. Syncytia in monolayers over-expressing ERdj5 or PDI isomerases were two to three times larger than syncytia in monolayers expressing HN and F proteins only. This result was quantified by determining the average number of nuclei per syncytium in cells expressing HN and F proteins with or without isomerases. As shown in Figure 3.2, panel B, the average number of nuclei per syncytium, determined at different time points after transfection, was two fold higher in cells over-expressing ERdj5 or PDI compared to cells expressing HN and F proteins only.

Cell-cell fusion was also measured by a cytoplasmic content mixing assay as described in Materials and Methods. Results in Figure 3.2, panel C, and Figure 3.3, panel A, show that over-expression of ERdj5 or PDI increased content mixing by two to four fold.

To determine if this increase in cell-cell fusion in cells over-expressing ERdj5 or PDI protein was due directly to increased production of free thiols, we characterized the effect of an inhibitor of free thiols on cytoplasmic content mixing in cells over-expressing
Figure 3.2: Effect of ERdj5 and PDI over-expression on cell-cell fusion.
Panel A: Syncytia were visualized by immunofluorescence of cells using anti-NDV antibody. Cells transfected with empty vector or co-transfected with HN and F protein cDNAs with or without ERdj5 or PDI cDNAs, were processed for immunofluorescence at 48 h posttransfection.
Panel B: Average sizes of syncytia after transfection with HN and F protein cDNAs (HN+F) or HN, F, and ERdj5 cDNAs (HN+F+E) or HN, F, and PDI cDNAs (HN+F+P), were determined by counting number of nuclei per syncytium in 20 different fusion areas at 24, 48, and 72h post transfection. The results shown are the averages of three independent experiments, with the error bars indicating standard deviations.
Panel C: Effects of over-expression of ERdj5 and PDI on content mixing were assayed using a β-galactosidase reporter assay. Effector cells co-transfected with HN and F protein cDNAs, as well as pTA were mixed with target cells, transfected with pB1-G with or without ERdj5 or PDI. Content mixing between effector and target cells was quantified by measuring β-galactosidase in cell extracts and is shown as a percentage of the activity detected in cells transfected with HN and F cDNAs (HN+F). The results shown are the averages of three independent experiments, and the error bars indicate the range.
Figure 3.3: Effect of inhibitors of PDI isomerases on cell-cell fusion in cells over-expressing ERdj5 or PDI.

Panel A: Effects of DTNB on content mixing between effector cells cotransfected with HN and F protein cDNAs, as well as pTA, were mixed with target cells, transfected with pB1-G alone (HN+F) or along with PDI (HN+F+PDI) or ERdj5 (HN+F+ERdj5). The results represent the average of three independent experiments with the error bars representing the range.

Panel B: Effects of anti-PDI antibody on content mixing between effector cells and target cells. The results represent the average of three independent experiments with the error bars representing the range.

Panel C: Effects of anti-ERdj5 antibody on content mixing between effector cells and target cells. β-galactosidase activity in cell extracts represents content mixing and is shown as a percentage of the activity detected in cells transfected only with HN and F cDNAs (HN+F).
ERdj5 or PDI proteins. DTNB, a nonspecific inhibitor of free thiols (Feener, Shen et al. 1990), efficiently inhibited the content mixing in cells over-expressing ERdj5 or PDI proteins as well as in cells expressing NDV glycoproteins alone (Figure 3.3, panel A).

To determine if the increased fusion was directly due to over-expression of the isomerases, we determined the effect of antibodies specific to the PDI protein (Figure 3.3, panel B) or ERdj5 protein (Figure 3.3, panel C) on the enhanced cell-cell fusion. In the presence of either antibody, content mixing in cells over-expressing the isomerases was significantly reduced, and reduced to levels approaching that seen in cells not over-expressing the isomerases.

**Effect of over-expression of ERdj5 and PDI on detection of free thiols in F protein expressed without or with HN protein.** To determine directly if over-expression of ERdj5 or PDI proteins leads to alteration in detection of free thiols in F protein, surfaces of cells expressing F protein with either of the isomerases were labeled with MPB. MPB binds the free thiols in proteins on cell surfaces and thus biotinylates those proteins (Hotchkiss, Matthias et al. 1998). We have previously reported that the surface-expressed F protein, but not the HN protein, is labeled with this reagent (Jain, McGinnes et al. 2007). The cell surface proteins labeled with MPB were precipitated with neutravidin-agarose and the amount of protein precipitated was normalized to the total F protein in the extracts. Figure 3.4, panel A, shows that detection of free thiols in both F₀ and F₁ proteins, expressed in the absence of HN protein, was increased in cells co-transfected with cDNAs encoding PDI or ERdj5. The labeling of F₁ was increased by slightly over two fold in cells over-expressing the isomerases (Figure 3.4, panel A).
Figure 3.4: Effect of ERdj5 and PDI over-expression on production of free thiols in F protein.

Panel A: MPB labeling of F protein when expressed without HN protein. Panel B: MPB labeling of F protein when expressed with HN protein. Cells, transfected with empty vector or F protein cDNA or HN and F protein cDNAs with or without ERdj5 or PDI cDNAs were labeled with MPB as described in materials and methods. Proteins in resulting cell extracts were precipitated with neutravidin-agarose (lanes 1-4) and resolved by SDS-PAGE. Total proteins in cell extracts (lanes 5-8) were resolved as a control for expression of the proteins. Quantification of F1 labeled by MPB is shown at the bottom of each panel and was performed in films with protein bands in linear range.
We also determined the effects of over-expression of the isomerases on detection of free thiols in F protein co-expressed with HN protein. We previously reported that levels of MPB binding to F protein were only slightly affected by co-expression with HN protein (Jain, McGinnes et al. 2007). Interestingly, over-expression of ERdj5 or PDI proteins resulted in significantly decreased detection of free thiols in F protein in the presence of HN protein (Figure 3.4, panel B). This result is consistent with the proposal that, due to the increased fusion in cells over-expressing the isomerases, more of the steady state cell surface F protein is in a post-fusion conformation and that this conformation may not be a substrate for thiol isomerases.

**Over-expression of ERdj5 or PDI in cells expressing HN and F proteins favors post-fusion conformation of F protein.** Previously we showed that binding of two different conformation-sensitive anti-F protein antibodies, anti-Fu1a and anti HR1 antibodies, to cell surface F protein expressed with HN protein was altered in presence of inhibitors of free thiols (Jain, McGinnes et al. 2007). Anti-Fu1a binding was increased and binding of anti-HR1 was decreased by inhibition of isomerases and fusion. This result suggested that these antibodies may differentially bind to pre and post-fusion conformations of F protein. We further analyzed the binding of these antibodies to cell surface F protein expressed with HN protein (Figure 3.5) in order to define further the form of F protein recognized by these antibodies. In cells incubated overnight with neuraminidase, the majority of cell surface F protein is likely in a pre fusion conformation since the absence of cell receptors for HN protein will inhibit HN protein binding and F protein activation. In cells grown in the presence of neuraminidase,
Figure 3.5: Binding of conformation sensitive antibodies to F protein on cell surfaces in presence of MPB or neuraminidase.

Panels A, C, and E: Cells transfected with pSVL vector (top panels) or with pSVL-HN and pSVL-F (bottom panels) were processed for surface immunofluorescence using anti-Fu1a antibody, anti-HR1 antibody, and anti-NDV antibody, respectively (indicated on left). Column 1 shows cells without any treatment and Columns 2 and 3 show cells treated with MPB and neuraminidase respectively, as explained in materials and methods.

Panels B, D, and F: show quantification of the fluorescence intensities of the cells shown in panels A, C, and E, respectively. The results shown are averages of the mean fluorescence intensity of 10 different cells and are normalized for background fluorescence by subtracting the mean fluorescence of cells transfected with empty vector. Error bars represent the range of fluorescence intensity of 10 different cells within one experiment and the experiment shown is representative of three similar experiments.
binding of anti-Fu1a antibody (Figure 3.5, panels A and B) was increased 5-6 fold compared to untreated cells (first lane) and binding of anti-HR1 antibody (Figure 3.5, panels C and D) was inhibited. The binding of anti-NDV antibody, a control antibody, (Figure 3.5, panels E and F) was not affected. These results suggest that anti-Fu1a antibody recognizes primarily F protein in prefusion conformation and anti HR1 antibody binds better to F protein in an intermediate conformation or a post-fusion state. Similar results were seen when fusion was inhibited by MBP binding (Jain, McGinnes et al. 2007) (middle lane). These results are consistent with our previous results that binding of anti-Fu1a antibody is increased and binding of anti-HR1 antibody is inhibited when cells are treated with inhibitors of free thiols, DTNB and bacitracin, conditions that also inhibit fusion. Taken together, these results argue that anti-Fu1a detects preferentially prefusion F protein while anti-HR1 detects F protein after its activation.

Next we determined the effects of over-expression of ERdj5 and PDI proteins on binding of these conformation sensitive anti-F protein antibodies (Figure 3.6). As shown in Figure 3.6, panels A and B, binding of anti-Fu1a antibody was decreased and binding of anti-HR1 antibody (Figure 3.6, panels C and D) was increased by over-expression of isomerases. Binding of anti-NDV antibody (Figure 3.6, panels E and F) was not affected. These results suggested that the majority of F protein in these cells is in a post-fusion conformation when fusion is stimulated by increased expression of isomerases.
Figure 3.6: Effect of ERdj5 and PDI over-expression on binding of conformation sensitive antibodies to F protein on cell surfaces.

Panels A, C, and E: Cells transfected with pSVL vector (top panels) or with pSVL-HN and pSVL-F (bottom panels) were processed for surface immunofluorescence using anti-Fu1a antibody, anti-HR1 antibody, and anti-NDV antibody respectively (indicated on left). Column 2 shows cells transfected with ERdj5 cDNA along with above cDNAs and column 3 shows cells transfected with PDI cDNA along with above cDNAs.

Panels B, D, and F: show quantification of mean fluorescence intensities of the cells shown in panels A, C, and E, respectively. The results shown are averages of the mean fluorescence intensity of 10 different cells and are normalized for background fluorescence by subtracting the mean fluorescence of cells transfected with empty vector. Error bars represent the range of fluorescence intensity of 10 different cells with in one experiment and the experiment shown is representative of three similar experiments.
D. Discussion

In this study we evaluated the effect of over-expression of the thiol isomerases, PDI and ERdj5, on the activity and conformation of the NDV F protein. Over-expression of either isomerase resulted in significantly increased cell-cell membrane fusion mediated by NDV glycoproteins. Over-expression of either isomerase also resulted in increased detection of free thiols in F protein expressed in the absence of HN protein and a decreased detection of free thiols in F protein expressed with HN protein. Over-expression of these isomerases also altered the binding of two different conformation sensitive antibodies in a manner consistent with an enhanced population of surface expressed F protein in a post-fusion conformation in the presence of HN protein. These results, together with our previous results showing inhibition of membrane fusion by inhibitors of free thiols (Jain, McGinnes et al. 2007), indicated that production of free thiols in the NDV fusion protein has a role in membrane fusion.

Increase in membrane fusion by over-expression of PDI-like isomerases is likely to be a direct effect of increased isomerase activity rather than a nonspecific or indirect effect as DTNB, an inhibitor of isomerases, inhibited cell-cell fusion even in the cells over-expressing PDI or ERdj5. Furthermore, the increased fusion, resulting from over-expression of either isomerase, was significantly decreased by antibodies specific for each isomerase. Anti-PDI antibody was less efficient in inhibiting the enhanced cell-cell fusion than the anti-ERdj5 antibody for reasons that are not clear. It is possible that anti-ERdj5 antibody is a better inhibitor than anti-PDI antibody due to a higher affinity for its target than anti-PDI antibody. Alternatively, anti-ERdj5 antibody may cross-react
with other isomerases. In any event, both antibodies significantly decreased the enhanced fusion activity suggesting that the enhanced fusion is directly due to the increased expression of the isomerases.

That enhanced fusion observed after over-expression of the isomerases is a direct effect of enhanced isomerase activity was also indicated by alterations in the levels of free cysteines detected in the F protein. Over-expression of PDI-like isomerases increased production of free thiols in F protein expressed in the absence of HN protein as indicated by a two fold increase in labeling of F protein by MPB. We have previously shown that a population of F protein expressed in the absence of HN protein contains free cysteines. The results here indicate that increased expression of isomerases increases the population of surface F protein with free thiols. These combined results suggest that F protein, in the cell membrane, may exist in several forms which may be in equilibrium and the concentration of each form may reflect the concentration of surface isomerases. These results, as well as previous results which showed that binding of the thiol reactive MPB blocked fusion (Jain, McGinnes et al. 2007), suggest that it is the form containing free thiols that may proceed through the conformational changes which result in cell-cell fusion.

When HN protein was co-expressed with F protein and the isomerases, we found that MPB labeling of F protein was reduced to about 50% of MPB labeling of F protein when expressed with HN protein in the absence of enhanced expression of an isomerase. One possibility for the decrease in MPB labeling of F protein seen in this case is that, in presence of HN and the isomerases, cell-cell fusion is enhanced and more of steady state
F protein is in post-fusion conformation. Post-fusion F protein is thought to be a very stable structure and, therefore, may not be a substrate for thiol isomerases and, therefore, is not labeled by MPB.

We utilized conformation sensitive antibodies to explore this hypothesis. According to current models proposed for conformational changes in F protein during membrane fusion (Russell, Jardetzky et al. 2001; Yin, Wen et al. 2006) the first conformational change in F protein is the disassociation of HR2 domains in the trimer, followed by the unfolding of the HR1 domain. The HR1 domain refolds into an extended trimeric helix and the fusion peptide inserts into the target cell. F protein in this state is referred to as pre-hairpin intermediate. After insertion of fusion peptide into the target membrane, the next proposed major conformational change involves refolding of F protein into a stable trimer of hairpins formed by HR1 and HR2 domains complexed in a six helical bundle (6HB). The folding of F protein into this post-fusion form leads to the close approach and fusion of the target and effector membranes.

Anti-Fu1a antibody is thought to bind the fusion peptide (Morrison, Ward et al. 1985) and anti HR1 antibody, raised against an HR1 peptide, binds the HR1 domain in F protein. In the model discussed above, the first major conformational change in F protein leads to pre-hairpin intermediate in which the fusion peptide is inserted into the target membrane and HR1 domain is organized into a helical trimer. Therefore, it is likely that the anti-Fu1a antibody will bind preferentially to a prefusion conformation of F protein before any conformational change. In contrast, it is likely that anti-HR1 antibody will bind preferentially to the HR1 trimer in the pre-hairpin conformation or subsequent forms
of the F protein. The results of antibody binding in cells treated with neuraminidase, which prevents receptor binding and consequently fusion, were consistent with the predicted binding for these antibodies. Inhibition of F protein activation resulted in increased binding of anti-Fu1a and decreased anti-HR1 binding. Our previous findings that inhibitors of thiol isomerases blocked fusion and increased binding of anti-Fu1a and decreased anti-HR1 binding are also consistent with this conclusion.

We have previously shown that MPB binding to free thiols also blocks fusion. Here we showed that after MPB binding, the anti-Fu1a antibody binding increases and anti-HR1 antibody binding decreases. These combined results suggest that binding of MPB leads to the presence of more F protein in the prefusion state. In addition, MPB may also bind to and inactivate cell surface thiol isomerases and this inactivation results in decrease in disulfide bond reduction in F protein, inhibition of fusion, and presence of more F protein in prefusion form. In either case, this result further confirmed that, when free thiols are blocked, F protein is in prefusion conformation.

In contrast, when isomerases were over-expressed, fusion was significantly enhanced and the binding of anti-Fu1a antibody decreased and anti-HR1 antibody increased. These results suggested that over-expression of the isomerases significantly changed the conformation of the F protein and it is likely that the majority of F protein, under these conditions, is in a post-fusion conformation.

The overall implication from these results is that, in absence of free thiols, F protein is in prefusion conformation. However, when disulfide bonds are reduced, F protein, in presence of HN protein may move into conformations that may reflect the
Figure 3.7: Model for role of production of free thiols in cell surface-expressed NDV F protein.

Panel A: F protein is present in prefusion, metastable conformation with intact disulfide bonds.

Panel B: Disulfide bonds are cleaved by PDI-like isomerase resulting in free thiols in F protein. MPB binds to the free thiols in F protein. Anti-Fu1a antibody binds to F protein in prefusion state.

Panel C: Production of free thiols is followed by the conformational changes in F protein required for fusion. Binding of MPB prevents further conformational changes and fusion of membranes. Anti-HR1 antibody binds preferentially to activated or post-fusion form of F protein. Enhanced expression of PDI or ERdj5 favors conformation C at steady state.
post-fusion form or intermediate forms. The reduction of disulfide bonds in F protein may be facilitating the conformational changes that F protein undergoes during membrane fusion.
CHAPTER IV
ROLE OF THIOL/DISULFIDE ISOMERIZATION IN NEWCASTLE DISEASE VIRUS ENTRY

C. Introduction

Newcastle disease virus (NDV), an avian paramyxovirus, enters the host cell by fusion of the viral membrane to the plasma membrane. Two virion-associated glycoproteins, the haemagglutinin-neuraminidase (HN) and fusion (F) proteins, are responsible for virion attachment to the target cell receptor and fusion of viral and host cell membranes, respectively. F protein, a trimer, is synthesized as a precursor, F0, which is cleaved into two disulfide linked subunits F1 and F2 (as reviewed in (Morrison 2003; Earp, Delos et al. 2005; Lamb and Jardetzky 2007)). The new amino terminus, generated by cleavage of the precursor, is the fusion peptide (FP). The fusion protein also contains two important heptad repeat (HR) domains (reviewed in (Colman and Lawrence 2003)). One HR domain (HR1 or HRA) is located just carboxyl terminal to the fusion peptide and another (HR2 or HRB) is located adjacent to transmembrane (TM) domain. HR1 and HR2 peptides have strong affinity and form a very stable six helical bundle (6HB) (Baker, Dutch et al. 1999). Based on studies showing inhibition of cell-cell fusion by each of these peptides, it is thought that HR1 and HR2 domains do not form the coil-coil, six helical bundle prior to fusion activation and are complexed only in the post-fusion form (Lambert, Barney et al. 1996; Young, Li et al. 1999; Russell, Jardetzky et al. 2001).

Subsequent studies of structures of F protein from different paramyxoviruses showed that F protein may exist in two different forms. One form, exemplified by the
structures of parainfluenza virus 3 F protein (Yin, Paterson et al. 2005) and NDV F protein (Chen, Gorman et al. 2001), is proposed to be in post-fusion conformation because the structures contain the two HR domains complexed in the 6HB form. Another structure was derived from a soluble form of SV5 F protein (Yin, Wen et al. 2006), which was stably trimerized by fusing the carboxyl terminus of the HR2 domain to the yeast GCN4 sequence, preventing 6HB formation between HR2 and HR1 domains. This structure was proposed to be in prefusion form. Changes in F protein conformation were also explored by defining the effects of HR1 and HR2 peptides (Russell, Jardetzky et al. 2001) and mutations in HR1 and HR2 domains (Ito, Komada et al. 1992; Paterson, Russell et al. 2000; McGinnes, Sergel et al. 2001; Sergel, McGinnes et al. 2001; Russell, Kantor et al. 2003; West, Sheehan et al. 2005; Luque and Russell 2007) on cell-cell fusion at different temperatures. Based on these studies, it was proposed that paramyxovirus F protein undergoes a series of major conformational changes leading to final 6HB formation (Russell, Jardetzky et al. 2001).

The trigger for this major refolding of F protein is thought to be binding of the HN protein to its receptor. Whether other factors besides interaction of F protein with HN protein play a role in activation of F protein has not been explored. Nor is it clear how the F protein accomplishes this major refolding. One potential mechanism is suggested by studies in HIV-1 Env protein. It has been shown that one or multiple disulfide bonds in Env are reduced, at the time of membrane fusion, by host cell protein disulfide isomerases (PDI) facilitating refolding of Env (Ryser, Levy et al. 1994; Fenouillet, Barbouche et al. 2001; Gallina, Hanley et al. 2002; Barbouche, Miquelis et al.
2003; Markovic, Stantchev et al. 2004). In some other retroviruses, such as murine leukemia virus (MLV), the thiol/disulfide isomerization is thought to be mediated by an isomerase motif, Cys-X-X-Cys (CXXC), in the Env glycoprotein, which is triggered by binding of glycoprotein to its receptor (Pinter, Kopelman et al. 1997; Wallin, Ekstrom et al. 2004; Wallin, Loving et al. 2005). Recently entry of many other viruses, for example Sindbis virus (Abell and Brown 1993) and avian leukosis virus-A (ALV-A) (Smith and Cunningham 2007), has been shown to be dependent on free thiols in fusion proteins. It has been shown that the conserved cysteine residues of hepatitis B virus envelope protein in hepatitis delta virus (HDV) undergo rearrangement at the time of virus entry, are required for the disassembly of the virion, and are susceptible to inhibition by inhibitors of thiol-disulfide isomerases (Abou-Jaoude and Sureau 2007).

We have reported previously that free thiols are detected in cell surface expressed NDV F protein and that the presence of free thiols is required for virus entry into cells as well as for cell-cell fusion in cells expressing NDV glycoproteins (Jain, McGinnes et al. 2007). We also reported that the fusion of membranes in cells expressing NDV glycoproteins is inhibited by free thiol blockers at a very early step, before hemifusion (Jain, McGinnes et al. 2007). How the appearance of free thiols correlates with F protein activation or with conformational changes during membrane fusion is unknown.

In this report we show that free thiols are not present in F protein on the surface of virions or virus-like particles (VLPs) but are produced after binding of virus or VLPs to the target cells. We also correlated the appearance of free thiols in F protein in virus and virus-like particles to the conformational changes proposed to occur in F protein and to
the activation of F protein by HN protein. Our results suggest that free thiols are produced in F protein on surfaces of virus or virus-like particles only after attachment to cells but before any major proposed conformational changes in F protein and that the appearance of free thiols is independent of activation of F protein by HN protein.
B. Materials and Methods

Cells, virus, and antibodies: COS-7 cells, obtained from American Type Culture Collection, were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with nonessential amino acids, vitamins, penicillin, and streptomycin, and 10% fetal calf serum (FCS). East Lansing Line (ELL-0) avian fibroblasts, obtained from American Type Culture Collection, were maintained in DMEM supplemented with penicillin-streptomycin and 10% FCS. Guinea pig red blood cells (RBCs) were obtained from Bio-Link, Inc.

NDV, strain Australia-Victoria (AV), was propagated in embryonated chicken eggs by standard protocols (Mc Ginnes, Reiter et al. 2006) in BCL-3 containment. Anti-HR2 antibody was raised against peptide with sequence from the NDV F protein HR2 domain (McGinnes and Morrison 2006). Anti-NDV antibody was raised in rabbits against UV-inactivated stocks of NDV (strain AV), by standard protocols as previously described (McGinnes, Gravel et al. 2002). Mouse monoclonal Anti-PDI antibody was purchased from Abnova Corporation.

Plasmids: NDV HN, F, M and NP cDNAs, subcloned in pCAGGS vector as described previously (Pantua, McGinnes et al. 2005; McGinnes and Morrison 2006), were used for transfection. F protein gene with mutations in the HR1 domain (N147K) and the HR2 domain (L45K) regions have been described previously (McGinnes, Sergel et al. 2001; Sergel, McGinnes et al. 2001). HN protein gene mutants, (HN I133L and HN V81A/L110A), have been described previously (Stone-Hulslander and Morrison 1999; Gravel and Morrison 2003).
**Transfections:** Transfection of cells was accomplished using Lipofectamine (Invitrogen) as recommended by the manufacturer. For each transfection, a mixture of DNA (0.5µg/35mm plate) and 7µl of Lipofectamine in OptiMEM media (Gibco/Invitrogen) was incubated at room temperature and added to cells previously washed with OptiMEM. The cells were incubated for 5h and OptiMEM was replaced with 2ml of supplemented DMEM.

**Production and purification of virus-like particles (VLPs):** VLPs were generated from avian cells and purified as described previously (Pantua, McGinnes et al. 2005). Briefly, cells were transfected with cDNAs (4µg each/100mm plate) encoding NDV HN, F, M and NP proteins. Supernatant from the cells was collected at 24h, 48h, and 72h and was clarified by centrifugation at 5,000 rpm for 5min at 4°C. The clarified supernatant was overlaid on top of a step gradient consisting of 1ml 20% and 0.5ml 65% sucrose solutions in TNE buffer (25mM Tris-HCl, pH 7.4, 150mM NaCl, 5mM EDTA), and centrifuged at 24,000 rpm for 12h at 4°C using an SW50.1 rotor (Beckman). The interface (containing concentrated particles) was collected in a 1ml volume using a polystaltic pump, mixed with 1ml of 80% sucrose, and overlaid on top of a 1ml 80% sucrose cushion. Additional layers of sucrose (1ml of 50% and 0.5ml of 10% sucrose) were layered on top of the particle containing interface. The gradient was centrifuged at 38,000 rpm for 16h at 4°C. The top 2ml of the gradient containing particles was collected using a polystaltic pump, mixed with 3ml of TNE buffer, and pelleted by centrifuging at 38,000 rpm for 6h at 4°C. The pellet was dissolved in 100µl of TNE and used as VLP stock.
**MPB labeling of virions and VLPs:** Equal quantities of NDV (AV strain) virions or VLPs were aliquoted into three separate tubes. Set 1 was treated with 5mM dithiothreitol (DTT) (Sigma-Aldrich, Inc) for 1h at 37°C while other two sets received no treatment. To remove DTT, virions or VLPs were purified by centrifugation on a step gradient consisting of 1ml 20% and 0.5ml 65% sucrose solutions in TNE buffer at 24,000 rpm for 8h at 4°C. The interface containing purified particles was collected and set 1 and 2 particles were incubated with MPB (3-(N-maleimidylpropionyl) biocytin) (Molecular Probes) at room temperature for 30min. Set 3 particles were negative controls. To remove unbound MPD, virions or VLPs were purified by centrifugation through layer of 20% sucrose solution in TNE buffer at 24,000 rpm for 8h at 4°C. The pellet was dissolved in 100µl of RSB lysis buffer (0.01M Tris-HCl [pH 7.4], 0.01M NaCl, 1.5mM MgCl₂) containing 1% Triton X-100, 0.5% sodium deoxycholate, 2.5mg of N-ethyl maleimide per ml, and 0.2mg of DNase per ml and particles were precipitated with neutravidin-agarose that had been washed sequentially with PBS containing 0.5% tween-20, and 5mg/ml BSA and PBS containing 0.5% tween-20 and 1mg/ml BSA and contained 0.3% SDS. Precipitates were washed three times with PBS containing 0.5% tween-20 and 0.4% SDS and resolved by SDS-PAGE.

**Labeling of virions and VLPs with MPB in presence of Red Blood Cells (RBCs):** Guinea pig RBCs were washed in PBS containing 0.1mM CaCl₂ and 1mM MgCl₂ (PBS-CM) and resuspended in PBS-CM to give a 0.4% solution by volume. RBCs were counted and nearly 10⁶ cells were incubated with NDV (AV strain) (MOI~100) or VLPs in presence or absence of MPB (0.5mM) for 1h. Unbound virions
or VLPs along with free MPB were removed by washing RBCs three times with PBS-CM. Washed RBC pellet was lysed with RSB lysis buffer and analyzed for labeling by MPB as described above.

**Polyacrylamide gel electrophoresis and Western blot analysis:** Proteins in extracts of cells, virions or VLPs lysed with RSB buffer or immunoprecipitates, diluted in gel sample buffer (125mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) with 0.7M β-mercaptoethanol, were resolved on 10% polyacrylamide gels. After electrophoresis, gels were equilibrated in transfer buffer (25mM Tris, pH 8.2, 192mM glycine, 15% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes. The membranes were blocked overnight at 4°C in PBS containing 0.5% Tween 20 and 10% nonfat milk, washed with PBS-Tween 20, and incubated for 1h at room temperature with primary antibody diluted to 1:1,000 in PBS-Tween 20. Membranes were then washed, incubated for 1h at room temperature with secondary antibody (goat anti-rabbit IgG or anti-mouse IgG coupled to horseradish peroxidase) (Amersham Biosciences) diluted to 1:40,000 in PBS-Tween 20, and then washed extensively in PBS-Tween 20. Bound antibody was detected using the ECL Western blotting detection reagent system (Amersham Biosciences). Quantification of the signal was accomplished using a Fluor-S imager (Bio-Rad).

**Plaque assay:** NDV virus (AV strain) was labeled with MPB and purified by centrifugation through sucrose gradient, as described above. Purified virus was serially diluted in Ca^{2+}-rich medium and then added to confluent avian cells. As a control, unlabeled virus was similarly purified, serially diluted and added to the cells with or
without MPB (0.5mM). After adsorption for 45 min at 37°C, unbound virus was removed and agar diluted to 1% in DMEM and supplemented with nonessential amino acids, vitamins, penicillin-streptomycin, sodium bicarbonate, and 10% FCS was then placed over the monolayers. After 48 h of incubation, plaques were counted.
C. Results

Detection of free thiols in F protein in virions: To determine whether free thiols are present in F protein in virions, virions were incubated with MPB, a maleimidyly compound that binds to free thiols and biotinylates surface-expressed proteins with free thiols. Virions were then repurified to remove unbound MPB and lysed. Proteins in lysed virions were analyzed for MPB labeling by precipitating with neutravidin-agarose. As shown in Figure 4.1, panel A (lane 6), F protein in virions was not labeled by MPB while in virions treated with DTT, a reducing agent, (lane 7) prior to incubation with MPB, F protein was labeled with MPB. This result indicated that F protein in virions does not have free thiols.

We have previously shown that F protein on transfected cell surfaces has free thiols which are labeled by MPB. The detection of free thiols was repressed by inhibitors of isomerases belonging to PDI family (Jain, McGinnes et al. 2007). One possibility for the absence of free thiols in F protein in virions is that PDI-like isomerases are not packaged into the virions. To determine whether PDI isomerase is present in virions, we analyzed virion-associated proteins by Western analysis using anti-PDI antibody. As shown in Figure 4.1, panel B (lanes 4-6), PDI was not detected in virion extracts while PDI was detected in extracts from infected (lanes 7-9) or uninfected (1-3) COS-7 cells. Similarly, we also detected PDI in extracts from avian cells and guinea pig red blood cells (Figure 4.1, panel C). We have previously reported that isomerase inhibitors block virus infection. Thus failure to detect free thiols in virion-associated F protein suggested that free thiols may be produced in virion-associated F protein only after binding to target
Figure 4.1: Detection of free thiols and PDI in virions.
Panel A: Virions were incubated with MPB (lanes 3 and 6), analyzed for MPB labeling of F protein by precipitating with neutravidin-agarose (lanes 5-7) and detected by Western blot using anti-HR2 antibody. Untreated virions (No Tt, lanes 2 and 5) and virions treated with DTT prior to incubation with MPB (lanes 4 and 7) were used as controls. Total F protein in extracts from virions (lanes 2-4) was resolved as a control for the amounts of virions.
Panel B: Increasing amounts of extracts from virions (lanes 4-6) and COS-7 cells either uninfected (lanes 1-3) or infected with NDV (lanes 7-9) were resolved by SDS-PAGE and detected for PDI (top) and NP protein (bottom).
Panel C: Increasing amounts of extracts from avian cells and guinea pig red blood cells were resolved by SDS-PAGE and detected for PDI.
Panel D: Virions were incubated with RBCs and MPB at 4°C for 1h followed by 22°C for 15 min, shown in lanes 4 and 7. RBCs incubated with MPB alone (lanes 2 and 5) or virus alone (lanes 3 and 6) were used as controls. Unbound virions and MPB were removed and F protein in bound virions was analyzed for MPB labeling by precipitation with neutravidin-agarose (lanes 2-4) followed by detection with anti-HR2 antibody. Extracts from RBCs (lanes 5-7) were resolved as control for the amount of virus bound to RBCs.
cells. To test this possibility, we incubated virions with guinea pig RBCs in presence of MPB. RBCs were washed to remove unbound virus as well as soluble MPB. F protein in bound virions present in the RBCs extract was then analyzed for MPB labeling. As shown in Figure 4.1, panel D (lane 4), virion F protein was labeled by MPB in presence of RBCs. This result suggested that free thiols are produced in F protein only after binding to target cells.

**Effect of MPB labeling of virions on infectivity:** We have previously shown that MBP binding inhibits cell-cell fusion (Jain, McGinnes et al. 2007). If free thiols in virion F protein are produced only after adding target cells, then incubation of virions with MPB prior to adding target cells should not have any effect on their infectivity, while incubation of virus with MPB virus attachment to cells should inhibit infection. To test this proposal, we incubated virions with MPB and then repurified virions to remove unbound MPB. Infectivity of repurified virions was compared to infectivity of virions not previously treated with MPB and to infectivity of virus added to target cells along in presence of MPB. Results in Table 4.1 showed that infectivity of virions pre-treated with MPB was not significantly different from that of unlabeled virions consistent with absence of MPB labeling. In contrast, infectivity of virions added to target cells along with MPB was about 10 fold lower than that of unlabeled virions, a result consistent with the generation of free thiols upon virus binding.

**Detection of free thiols in F protein in VLPs:** We have previously reported that avian cells expressing the NDV NP, M, HN and F proteins release virus-like particles that
Table 4.1: Effect of MPB treatment on infectivity of NDV.

<table>
<thead>
<tr>
<th>Virus treatment</th>
<th>PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>1.45±0.2E+9</td>
</tr>
<tr>
<td>aMPB-before infection</td>
<td>1.17±0.14E+9</td>
</tr>
<tr>
<td>bMPB-during infection</td>
<td>1.07±0.83E+8</td>
</tr>
</tbody>
</table>

a Virions were treated with MPB and purified before adding to the cells.
b MPB was added to the cells at the time of infection and unbound virions as well as MPB were removed before adding agar overlay.
resemble virus both biochemically and morphologically (Pantua, McGinnes et al. 2005). Because we wished to explore MPB labeling of different F protein mutants and F protein in the presence of HN protein mutants in particles, we determined whether free thiols are present in F protein in VLPs. NDV VLPs were incubated with MPB and VLPs were then re-purified to remove unbound MPB. Proteins in VLP extracts were analyzed for MPB binding by precipitating with neutravidin-agarose. Results in Figure 4.2, panel A (lane 3), showed that, similar to F protein in virions, F protein in VLPs was not labeled by MPB while F protein in VLPs treated with DTT (lane 4) prior to incubation was labeled with MPB.

To determine whether free thiols are produced in F protein in VLPs after adding target cells, VLPs were incubated with RBCs in presence of MPB. RBCs were washed to remove unbound VLPs as well as soluble MPB. F protein in bound VLPs present in RBC extracts was then analyzed for MPB labeling. As shown in Figure 4.2, panel B (lane 3), F protein in VLPs was labeled by MPB in presence of RBCs. These results suggested that, similar to virions, free thiols are produced in F protein in VLPs only after binding to target cells.

**Production of free thiols in F protein in different temperature arrested conformations:** It has been previously documented that conformational changes in paramyxovirus F protein upon activation can be arrested at low temperatures (described in (Russell, Jardetzky et al. 2001)). The first intermediate, susceptible to HR1 peptide binding, was proposed to exist at 15°C while another intermediate form, susceptible to both HR1 and HR2 peptides, was shown to exist only at 37°C.
Figure 4.2: Free thiols in virus like particles (VLPs).
Panel A: VLPs were incubated with MPB (lanes 3 and 6) and analyzed for MPB labeling of F protein by precipitating with neutravidin-agarose (lanes 2-4) followed by Western analysis using anti-HR2 antibody. Untreated VLPs (No Tt, lanes 2 and 5) and VLPs treated with DTT prior to incubation with MPB (lanes 4 and 7) were used as controls. Total F protein in extracts from VLPs (lanes 5-7) was resolved as a control for the amounts of VLPs.
Panel B: VLPs were incubated with RBCs and MPB at 4ºC for 1h followed by 22 ºC for 15 min, shown in lanes 3 and 6. RBCs incubated with MPB alone (lanes 1 and 4) or VLPs without MPB (lanes 2 and 5) were used as controls. Unbound VLPs and MPB were removed and F protein in bound VLPs was analyzed for MPB labeling by precipitation with neutravidin-agarose (lanes 1-3). Extracts from RBCs (lanes 4-6) were resolved as control for the amount of VLPs bound to RBCs.
To determine the earliest form of F protein with detectable free thiols, we characterized MPB labeling of temperature arrested intermediates of F protein in VLPs and in virions. For these studies, we first determined the efficiency of MPB labeling at different temperatures by incubating DTT treated VLPs with MPB at 4°C, 16°C, 22°C, and 37°C. As shown in Figure 4.3, panel A (lanes 2-5), labeling of F protein in VLPs by MPB was comparable at all the temperatures.

Next, we assessed MPB labeling of F proteins in VLPs when incubated with RBCs at 4°C, 16°C, 22°C, 37°C. RBCs were washed to remove unbound VLPs as well as MPB. F protein in bound VLPs present in RBC extracts was then analyzed for MPB labeling. A representative result is shown in Figure 4.3, panel B, and quantification of three similar experiments is shown in Figure 4.3, panel C. As shown in Figure 4.3, panel B (lanes 7-10), F protein in VLPs was labeled by MPB at all the incubation temperatures. Labeling of F protein in VLPs incubated at 4°C was significantly decreased while labeling at other temperatures was comparable when normalized to VLP binding to RBCs (Figure 4.3, panel C).

Similar results were seen after binding of virions to RBCs at different temperatures. As shown in Figure 4.4, panel A (lanes 4-7), F protein in virions was labeled by MPB at all the incubation temperatures. Labeling of F protein in RBC bound virions incubated at 4°C was significantly decreased while labeling at other temperatures was comparable when normalized to virus binding (Figure 4.4, panel B). These results suggest that free thiols are produced in F protein before the major proposed conformational changes in F protein.
Figure 4.3: MPB labeling of temperature arrested intermediates of F protein in VLPs.
Panel A: VLPs were treated with DTT and labeled with MPB at 4°C (lanes 2 and 6), 16°C (lanes 3 and 7), 22°C (lanes 4 and 8), or 37°C (lanes 5 and 9). F protein in VLPs was analyzed for labeling with MPB by precipitating with neutravidin agarose (lanes 2-5) and extracts from VLPs (lanes 6-9) were resolved as a control for amount of VLPs.
Panel B: VLPs were incubated with RBCs with (lanes 7-10 and 16-19) or without MPB (lanes 3-6 and 12-15) at indicated temperatures. RBCs incubated with MPB in absence of VLPs (lanes 2 and 11) were used as control. Unbound VLPs and MPB were removed and F protein in bound VLPs was analyzed for MPB labeling by precipitation with neutravidin-agarose (lanes 2-10) followed by Western analysis using anti-HR2 antibody.
Extracts from RBCs (lanes 11-19) were resolved as control for the amount of VLPs bound to RBCs.

Panel C: Quantification of MPB labeling of temperature arrested intermediates of F protein in VLPs. F protein bands in Western blot (B) were quantified by densitometer, the values for MPB labeling (lanes 2-10) were normalized for binding (lanes 11-19) at respective temperatures and expressed as percent of labeling at 22°C. Error bars indicate the range obtained in three independent experiments. Quantification was accomplished using blots exposed in linear range of film.
Figure 4.4: MPB labeling of temperature arrested intermediates of F protein in virions.

Panel A: Virions were incubated with RBCs and MPB (lanes 4-7 and 10-13) at indicated temperatures. RBCs incubated with MPB in absence of virions (lanes 2 and 8) and virions without MPB at 22ºC (lanes 3 and 9) were used as control. Unbound virions and MPB were removed and F protein in bound virions was analyzed for MPB labeling by precipitation with neutravidin-agarose (lanes 2-7). Extracts from RBCs (lanes 8-13) were resolved as control for the amount of virions bound to RBCs.

Panel B: Quantification of MPB labeling of temperature arrested intermediates of F protein in virions. F protein bands in Western blot (A) were quantified by densitometer, the values for MPB labeling (lanes 2-7) were normalized for binding (lanes 8-13) at respective temperatures and expressed as percent of labeling at 22ºC. Error bars indicate the range obtained in three independent experiments. Quantification was accomplished using blots exposed in linear range of film.
**Production of free thiols in HR1 and HR2 mutant F proteins:** HR2 and HR1 mutant F proteins are thought to be defective in attaining the pre-hairpin (Figure 4.7, panel C) intermediate conformation (Paterson, Russell et al. 2000; McGinnes, Sergel et al. 2001; Sergel, McGinnes et al. 2001; Russell, Kantor et al. 2003; West, Sheehan et al. 2005). To determine whether these mutant proteins can form free thiols, we generated VLPs containing wild type F protein or HR1 (N147K) or HR2 (L45K) mutant F proteins. Proteins in VLPs containing wild type F protein or F protein with mutations in HR1 (N147K) or HR2 (L45K) domain are shown in Figure 4.5, panel A. The amounts of VLPs were equalized by normalizing to NP protein. Equivalent amounts of VLPs were labeled by MPB in presence of RBCs and analyzed. That the amounts of VLPs used were comparable is confirmed by comparable amounts of F protein in bound VLPs containing wild type F protein (Figure 4.5, panel B, lane 6) or HR1 or HR2 mutant F proteins (lanes 7 or 8). As shown in Figure 4.5, panel B, HR1 mutant F protein (lane 3) was labeled by MPB as well as wild type F protein (lane 2) while HR2 mutant F protein (lane 4) was labeled at significantly higher levels compared to the wild type F protein. This result suggested that free thiols are produced before any major conformational change in F protein. These results also suggest that F protein with an HR2 mutation may be more accessible to isomerases than wild type or HR1 mutant proteins.

**Role of HN protein in production of free thiols in F protein:** We have previously reported that, in transfected cells, free thiols were detected in F protein when it was expressed alone, without HN protein (Jain, McGinnes et al. 2007). This result suggested that production of free thiols in F protein is independent of HN protein in
Figure 4.5: MPB labeling of F mutants in VLPs.

Panel A: VLPs were generated using Fwt (lane 1) or HR1 mutant, F (N147K) (lane 2) or HR2 mutant, F (L45K) (lanes 3 and 4). The amounts of VLPs generated in each case were estimated by detecting particle associated proteins by a Western blot using a mix of anti-NDV antibody, anti-F antibody and anti-HN antibody. The protein band below NP protein (*) is a degradation product of NP variably seen in different preparations of virus and VLPs (unpublished observation). The protein band below HN protein (*) is a nonspecific protein band variably detected by anti-AS antibody.

Panel B: Equivalent amounts of VLPs (normalized to NP protein shown in A) with Fwt (lanes 2 and 6) or HR1 mutant F (N147K) (lanes 3 and 7) or HR2 mutant F (L45K) (lanes 4 and 8) were incubated with RBCs and MPB at 4°C for 1h followed by 22°C for 15min. RBCs incubated with MPB in absence of VLPs (lanes 1 and 5) were used as control. Unbound VLPs and MPB were removed and F protein in bound VLPs was analyzed for MPB labeling by precipitation with neutravidin-agarose (lanes 1-4) followed by Western analysis using anti-HR2 antibody. Extracts from RBCs (lanes 5-8) were
resolved as control for the amount of VLPs bound to RBCs.

Panel C: Quantification of MPB labeling in (B). The results are representative of two similar experiments. Quantification was accomplished using blots exposed in linear range of film.
Figure 4.6: MPB labeling of F protein in VLPs with HN mutants.

Panel A: MPB labeling of F protein in VLPs with HN mutants. (A) VLPs were generated using HNwt (lane 1) or HN (I133L) (lanes 2 and 3) or HN (V81A/L110A) (lane 5) separately. The amounts of VLPs generated in each case were estimated by detecting proteins associated to VLPs by SDS-PAGE and analyzing by Western blot using a mix of anti-NDV antibody, anti-F antibody and anti-HN antibody. The protein band below NP protein (*) is a degradation product of NP variably seen in different preparations of virus and VLPs (unpublished observation). The protein band below HN protein (*) is a nonspecific protein band variably detected by anti-AS antibody.

Panel B: Equivalent amounts of VLPs (normalized to NP protein shown in A) with HNwt (lanes 3 and 6) or HN mutant HN I133L (top panel)) or HN V81A/L110A (bottom panel) (lanes 4 and 7) were incubated with RBCs and MPB at 4°C for 1h followed by 22°C for 15min. RBCs incubated with MPB in absence of VLPs (lanes 1 and 5) were used as control. Unbound VLPs and MPB were removed and F protein in bound VLPs was analyzed for MPB labeling by precipitation with neutravidin-agarose (lanes 2-4) followed by Western analysis using anti-HR2 antibody. Extracts from RBCs (lanes 5-7)
were resolved as control for the amount of VLPs bound to RBCs.  
**Panel C:** Quantification of MPB labeling in (B). The results are representative of two similar experiments. Quantification was accomplished using blots exposed in linear range of film.
transfected cells. To determine the role of HN protein in production of free thiols in F protein in VLPs, we analyzed VLPs containing mutant HN protein (HN I133L and HN V81A/L110A) for labeling of F protein by MPB. These mutant HN proteins have alterations in the stalk domain and have been shown to be defective in fusion promotion but are comparable to wild type HN protein in attachment activity (Stone-Hulslander and Morrison 1997; Gravel and Morrison 2003). Figure 4.6, panel A, shows the proteins incorporated in VLPs containing wild type or mutant HN proteins. The amounts of VLPs were equalized by normalizing to NP protein. Equivalent amounts of VLPs were labeled by MPB in presence of RBCs and analyzed. Figure 4.6, panel B (lanes 6 and 7), show that the amounts of VLPs used were comparable as the amounts of F protein in bound VLPs were similar in VLPs containing wild type or mutant HN protein. The F protein in VLPs containing mutant HN protein migrates slightly faster on the gel than F protein in VLPs with wild type HN protein (Figure 4.6, panel B, lanes 6 and 7). This difference may be due to the treatment of cells with neuraminidase during production of VLPs containing mutant HN proteins as these mutants are defective in neuraminidase activity (Stone-Hulslander and Morrison 1997; Gravel and Morrison 2003). Lower neuraminidase activity inhibits release of VLPs from cells (unpublished observation). As shown in Figure 4.6, panel B (lane 4), F protein in VLPs containing these mutant HN proteins was labeled by MPB at levels similar to VLPs containing wild type HN protein. This result suggested that production of free thiols in F protein in VLPs is independent of HN protein activation.
D. Discussion

Paramyxovirus F proteins are thought to undergo major conformational changes during membrane fusion. These changes are required for the fusion process and are triggered by HN protein binding to the host cell receptors. How F protein accomplishes this refolding is not clear. One possible mechanism proposed to facilitate refolding of F protein is reduction of disulfide bond in F protein by host cell thiol/disulfide isomerases (Jain, McGinnes et al. 2007). We have previously reported that free thiols are present in cell surface expressed F protein and that these free thiols are required for fusion mediated by F protein. In this study we evaluated F protein in virions and VLPs for the presence of free thiols and the relationship of reduced forms of F protein to F protein activation by HN protein and the conformational changes in F protein.

Cysteine residues are highly conserved among the F proteins in paramyxoviruses. In Sendai virus there are 10 cysteine residues and it has been shown that all the cysteines are involved in disulfide bonds (Iwata, Schmidt et al. 1994). NDV F protein has 2 additional cysteine residues. Our results showing lack of MPB labeling of F protein in purified virions suggest that there are no free thiols in virion-associated F protein and all the 12 cysteines are involved in disulfide bonds. Consistent with this observation is the absence of PDI isomerase in virus membranes. It has been reported that cell surface PDI is not associated with lipid raft domains (Markovic, Stantchev et al. 2004). We have previously reported that NDV is assembled in lipid raft domains (Laliberte, McGinnes et al. 2006), thus failure to package PDI into virions is not surprising.
Free thiols were, however, detected in virions and VLPs in the presence of cells and incubation with MPB could only inhibit plaque formation if it was present during particle binding to cell surfaces. These results suggested particles must bind to cell membranes to be exposed to the cell surface host cell isomerases and only then are F protein disulfide bonds reduced.

Conformational intermediates in F protein refolding have been defined previously by evaluating inhibition of cell-cell fusion after HR1 or HR2 peptide binding at temperatures lower than 37ºC, reasoning that, at lower temperatures, the energy available for F protein refolding is suboptimal (Russell, Jardetzky et al. 2001). Based on their results, Russell, et al (Russell, Jardetzky et al. 2001) have proposed that F protein at 4ºC or 15ºC is arrested in a prefusion or open stalk state as shown in Figure 4.7. Our results that F protein in virions or VLPs is labeled by MPB at 16ºC, at levels comparable to those at 22ºC or 37ºC, suggest that free thiols are produced in F protein before any major conformational changes in F protein take place. The decrease in MPB labeling seen at 4ºC compared to 16ºC could be due to lower activity of host cell isomerases at this temperature.

We also observed a decrease in binding of virions to RBCs at 37ºC in presence of MPB. One possibility for this decrease in binding is that neuraminidase activity of HN protein at this temperature leads to disassociation of bound virions. Since MPB inhibits fusion (Jain, McGinnes et al. 2007), the F protein with bound MPB may not insert its fusion peptide into the target membranes. In this case, the F protein may not anchor the virus to target cells facilitating release of the particles from the cells by the HN protein.
Figure 4.7: Model for the role of free thiols virion-associated F protein.
Panel A: F protein in a prefusion conformation. HN protein binds to the receptor and facilitates interaction between F protein and target cell isomerases.
Panel B: This binding leads to cleavage of disulfide bond in F protein in prefusion or open stalk conformation which has been shown to be present at lower temperatures.
Panel C: F protein in a pre-hairpin conformation which has been shown to require 37°C. HR1 and HR2 mutants are proposed to be defective in attaining form C and are arrested in form B, an open stalk conformation.
Panel D: F protein in post-fusion conformation.
neuraminidase (Porotto, Murrell et al. 2005). A failure of F protein to anchor to target cells in presence of MPB is consistent with the proposal that free thiols are produced before the pre-hairpin intermediate of F protein is formed. It is this form of F protein, with the FP domain extended towards the target membrane, which is thought to insert into target membranes.

Studies of HR1 and HR2 F protein mutants from different paramyxoviruses have shown that mutations in these domains destabilize the spring loaded F protein and affect its fusion activity (Ito, Komada et al. 1992; Paterson, Russell et al. 2000; McGinnes, Sergel et al. 2001; Sergel, McGinnes et al. 2001; Russell, Kantor et al. 2003; West, Sheehan et al. 2005; Luque and Russell 2007). Mutations in HR2 domain were proposed to be arrested in an early intermediate form, before prehairpin formation (Russell, Kantor et al. 2003) and mutations in HR1 domain were proposed to be defective in attaining prehairpin conformation (Russell, Kantor et al. 2003; West, Sheehan et al. 2005; Luque and Russell 2007). Our results showed that both HR1 as well as HR2 mutant F protein have free thiols suggesting that free thiols are produced in either prefusion form or a form with open HR2 stalk domain of F protein. Increase in labeling of HR2 mutant F protein suggests that a larger percent of the total HR2 mutant F protein contains free thiols. It is possible that this mutant F protein may be more accessible to isomerases or that this mutant protein is arrested in a reduced form.

For paramyxovirus F protein activity, binding of HN protein to its receptor has been shown to be essential (Moscona and Peluso 1991; Porotto, Murrell et al. 2003). This requirement for HN protein for fusion is virus specific suggesting that there is an
essential interaction between HN and F proteins (Hu, Ray et al. 1992). The details of this interaction are not clear and whether other factors are involved in F protein activation is not yet known. We have shown previously that while production of free thiols in F protein is required for cell-cell and virus-cell fusion, free thiols can be detected in F protein expressed on cell surfaces without HN. These results suggest that reduction of disulfides and interactions with HN protein are independently required for fusion activation. This conclusion is supported by our finding that MPB binding to F protein was unaffected by the presence of HN proteins which were attachment competent but defective in fusion activation. This finding suggests that during virus entry, HN protein binding to its receptor facilitates production of free thiols in F protein by bringing the F protein to proximity of cell surface isomerases and that interaction between HN and F proteins leading to activation of F protein is not required for production of free thiols in F protein. Upon making contact with cell surfaces or in transfected cell membranes, the prefusion F protein may be in equilibrium between oxidized and reduced forms. If HN protein is present, the F protein with free thiols may proceed to refold resulting in membrane fusion. Refolding is blocked if the free cysteines are blocked with MPB (Figure 4.7), and fusion is inhibited as shown in previous results (Jain, McGinnes et al. 2007) and in Table1.

It has been recently reported that F protein in Sendai virus virions is a mixed population of prefusion and post-fusion forms (Ludwig, Schade et al. 2008). If the NDV F protein in particles exists in both forms, then our results indicate that neither the pre or post-fusion virion-associated F protein has free thiols. We have found that over-
expression of PDI-like isomerases in cells expressing both HN and F proteins leads to enhanced cell-cell fusion and significantly decreased MPB labeling of F protein (unpublished observations). Thus the post-fusion form of F protein may not be a substrate for isomerases.

Taken together, our results show that free thiols are produced in virion-associated F protein only after binding of virus to cells. These free thiols appear in F protein prior to any major conformational changes in the protein upon fusion activation and are independent of the activation of F protein by HN protein.
CHAPTER V
GENERAL DISCUSSION

In this dissertation, I have studied the mechanism of viral and host cell membrane fusion mediated by NDV F protein. The results presented above have shown that:

(a) Free thiols are produced in cell surface-expressed NDV F protein and production of free thiols in F protein is required for fusion mediated by the NDV F protein.

(b) The cleavage of disulfide bonds in F protein is mediated by host cell isomerases belonging to PDI family.

(c) All the cysteine residues in F protein in virions or virus-like particles are involved in disulfide bonds. Free thiols are produced in F protein in virions or virus-like particles after binding of the particles to the target cells.

(d) Free thiols are produced at a very early stage in fusion, before any major conformational change occurs in F protein.

(e) In absence of free thiols, cell surface-expressed F protein is in a prefusion conformation, while enhanced production of free thiols favors a post-fusion conformation of F protein.

These results suggest a mechanism by which F protein undergoes major refolding which leads to membrane fusion. Also these results suggest that host cell isomerases can be used as target for development of new antiviral drugs that can inhibit paramyxovirus entry.

Since an in depth discussion of the results has been provided in Chapters II, III, and IV, the discussion here is a brief overview of the major points made in the thesis,
their significance, and relevant questions regarding these points that need to be explored further.

**A. Free thiols in F protein expressed on cell surfaces and virus or VLPs**

Free thiols were detected in cell surface-expressed F protein but not in F protein in virions or virus-like particles. However, F protein in virus or virus-like particles had detectable free thiols after the particles were bound to the target cells. This result suggested that free thiols are generated in F protein at the time of virus attachment and that production of free thiols is mediated by host cell isomerases.

Based on theoretical analysis of the disulfide bonds, it was proposed that NDV F protein has one disulfide bond, linking Cys338 and Cys347, that is more susceptible to the reducing agents, thioredoxin or PDI, and is likely to be the disulfide bond that is involved in cell entry by NDV (Wouters, Lau et al. 2004). Wouters, et al, described some unusual disulfide bonds present in the proteins involved in cell entry. These bonds, called cross strand disulfides (CSDs), link cysteine residues in adjacent strands in same β-sheet and have high potential energy stored in them (Matthias and Hogg 2003; Wouters, Lau et al. 2004). CSDs are identified by higher dihedral strain energy in these bonds due to torsional strain produced by linking adjacent strands (Hogg 2003; Matthias and Hogg 2003; Wouters, Lau et al. 2004) and are proposed to be more susceptible to reducing agents, thioredoxin or PDI than other disulfide bonds (Matthias and Hogg 2003; Wouters, Lau et al. 2004).

However, it remains to be determined which of the six disulfide bonds in NDV F protein is cleaved at cell surfaces. One of the approaches, used in exploring contributions
of cysteines in fusion proteins in hepatitis delta virus (Abou-Jaoude and Sureau 2007),
human respiratory syncytial virus (Day, Branigan et al. 2006) and avian leukosis virus
(Smith and Cunningham 2007; Delos, Brecher et al. 2008), is to generate cysteine mutant
fusion proteins and characterize their fusion activity. However, mutations in cysteine
residues usually abolish or greatly reduce the expression of mutant proteins. NDV F
protein with mutations of cysteine residues at positions 338 and 347 did not express on
surfaces and, therefore, could not be analyzed for their fusion efficiency (unpublished
observations). Further analysis of mutations in the other cysteine residues in F protein
may still be useful in future studies.

Another approach to the analysis of disulfide bonds in viral fusion proteins, used
in Sindbis virus (Whitehurst, Soderblom et al. 2007), is to label free cysteines by thiol-
reactive reagents and to identify the labeled cysteine residue by mass spectroscopy.
Identification of free cysteine residues in NDV F protein using this approach is one of the
future goals as identification of these residues can provide some insight into the role of
free thiols in fusion activity of F protein.

B. Free thiols in F protein are required for membrane fusion

I have shown that the membrane impermeable inhibitors of disulfide bond
isomerase, 5′5-dithio(2-bis-nitrobenzoic acid) (DTNB), and bacitracin, as well as anti-
PDI antibodies, inhibited cell-cell fusion and virus entry mediated by NDV
glycoproteins. In addition, over expression of isomerase belonging to the PDI family
significantly enhanced fusion mediated by NDV glycoproteins. These results suggest
that free thiols in F protein play a significant role in the fusion process. Also, inhibition
of fusion by MPB, a membrane impermeable, thiol specific biotin which binds to free thiols in F protein, indicates that F protein with free thiols is not an aberrant, biologically irrelevant form and is in a prefusion conformation.

A number of recent studies of many different viruses have shown that, during virus entry, viral fusion glycoproteins undergo thiol-disulfide isomerization leading to reduction of disulfide bonds and production of free thiols. The pioneering studies in HIV-1 env protein have shown that one or multiple disulfide bonds are reduced at the time of membrane fusion by host cell protein disulfide isomerases (PDI) (Ryser, Levy et al. 1994; Fenouillet, Barbouche et al. 2001; Gallina, Hanley et al. 2002; Barbouche, Miquelis et al. 2003). While in another retrovirus, murine leukemia virus (MLV), env protein itself has an isomerase motif, Cys-X-X-Cys (CXXC). Binding of env glycoprotein to its receptor triggers the thiol-disulfide isomerization in env protein (Pinter, Kopelman et al. 1997; Wallin, Ekstrom et al. 2004; Wallin, Ekstrom et al. 2006).

Entry of many other viruses, for example Sindbis virus (Abell and Brown 1993) and avian leukosis virus (ALV-) (Smith and Cunningham 2007), is blocked by inhibitors of thiol-disulfide isomerases. A recent study of hepatitis delta virus (HDV) has shown that the conserved cysteine residues in the virus envelope protein (derived from hepatitis B virus) undergo thiol-disulfide exchange at the time of virus entry. The reduction of disulfide bonds in the virus envelope protein was proposed to be required for the disassembly of the virion and was shown to be susceptible to inhibitors of thiol-disulfide isomerases (Abou-Jaoude and Sureau 2007).
These results all indicate that cleavage of disulfide bonds in fusion glycoproteins play an important role in membrane fusion during virus entry into the host cell but the exact role of free thiols in the fusion process is not yet clear.

C. Disulfide bond reduction in F protein is mediated by PDI Family of isomerases

As with many viruses, the thiol-disulfide isomerization of the HIV-1 env protein is thought to be catalyzed by a host cell protein, protein disulfide isomerase (PDI) or a PDI-like isomerase (Gallina, Hanley et al. 2002). This conclusion is based on the studies showing inhibition of HIV-1 entry and cell-cell fusion by inhibitors of the PDI family of isomerases.

The results presented here have shown that the inhibitors of disulfide bond isomerases, 5'5-dithio(2-bis-nitrobenzoic acid) (DTNB), bacitracin, and anti-PDI antibodies inhibited NDV cell-cell fusion and virus entry and these inhibitors decreased the production of free thiols in cell surface-expressed NDV F protein. Also, over-expression of the isomerases belonging to the PDI family of isomerases enhanced cell-cell fusion. Over-expression of these isomerases also increased the production of free thiols in cell surface-expressed F protein in absence of HN protein. These results indicate that disulfide bond cleavage in NDV F protein is likely to be mediated PDI family of isomerases.

In another study, effects of down regulation of PDI, using siRNA, on HIV-1 env protein mediated membrane fusion was evaluated (Ou and Silver 2006). It was shown that down regulation of PDI did not significantly inhibit the membrane fusion mediated by HIV-1 env protein. One possible reason for this observation, suggested by the
authors, was that the reduction of disulfide bonds in env protein may be catalyzed by other isomerases of the PDI family as many of the members of the PDI isomerase family have similar catalytic domains (as reviewed in (Appenzeller-Herzog and Ellgaard 2008)).

The results in Chapter III that either of the PDI-like isomerases, PDI or ERdj5, when over-expressed, enhanced cell-cell fusion to comparable levels are consistent with multiple members of the PDI family being involved in disulfide bond reduction in NDV F protein. These results also explain the less effective fusion inhibition by anti-PDI specific antibody described in results presented in Chapter II.

D. Free thiols are produced in F protein at a very early stage during membrane fusion

(i) Free thiols are produced before hemifusion of the membranes: Hemifusion is the first stage in membrane fusion where only the outer layers of the lipid bilayer in two membranes have fused. The results showing inhibition of R18 lipid mixing by inhibitors of free thiols, DTNB and bacitracin, indicate that free thiols are produced before the stage of hemifusion.

(ii) Free thiols are produced before any major conformational changes in F protein: Analysis of free thiols in F protein arrested in different intermediate conformations at lower temperatures showed that free thiols are produced in F protein even at lower temperatures, 16°C, a temperature at which only prefusion or open stalk form of protein is thought to exist (Russell, Jardetzky et al. 2001). This result indicated that free thiols are produced in prefusion or open stalk form of F protein and, therefore, before any major refolding of F protein.
Analysis of F protein with mutations in HR1 or HR2 domains showed that free thiols are produced in both the mutant F proteins. Mutations in the HR2 domain have been proposed to be arrested in an early intermediate form, before pre-hairpin formation (Russell, Kantor et al. 2003) and mutations in HR1 domain have been proposed to be defective in attaining the pre-hairpin conformation (Russell, Kantor et al. 2003; West, Sheehan et al. 2005; Luque and Russell 2007). The results showing presence of free thiols in F protein with HR1 or HR2 mutations also indicate that free thiols are produced before any major conformational change occurs in F protein, probably when F protein is in prefusion state.

(iii) **Free thiols are detected in a prefusion form of F protein and not in a post-fusion form:** Using electron cryomicrography, it has been shown that, in intact PIV5 (previously known as SV5) virions, F protein exists in two different structural forms, prefusion as well as post-fusion (Ludwig, Schade et al. 2008). How F protein in the prefusion form converts to the post-fusion form while embedded in the virus membrane is not yet clear. This result suggested that wild type F protein exists as a mix of both prefusion and post-fusion forms in virus or VLPs. The structure of F protein with mutations in the HR2 domain has not been studied but, as described above, these mutant F proteins are thought to be defective in attaining a pre-hairpin conformation (Russell, Kantor et al. 2003) and are likely to be in a prefusion form. The results showing that labeling of F protein with mutations in the HR2 domain by MPB was increased by two-three fold over the wild type F protein are consistent with the proposal that F protein in
the prefusion form has significantly more free thiols than F protein in the post-fusion form.

In the case of cell surface-expressed F protein, when co-expressed with HN protein, F protein is likely to exist as a mixed population of prefusion and post-fusion forms similar to virions (Ludwig, Schade et al. 2008). In the presence of increased levels of isomerases, PDI or ERdj5, the production of free thiols in F protein and consequently membrane fusion is increased. The increase in membrane fusion may shift the equilibrium between prefusion and post-fusion forms of F protein so that more steady state F protein is in a post-fusion form. When expressed without HN protein, F protein is not activated and is unlikely to undergo conformational changes. The predominant steady state form of F protein in this case is likely to be the prefusion form.

The results showed that, when expressed alone, MPB labeling of F protein is enhanced by over-expression of isomerases and, when co-expressed with HN protein, the labeling of F protein is decreased in presence of isomerases. This result indicates that free thiols are present in prefusion form of F protein (expressed alone) and production of free thiols can be enhanced in this form by over-expression of isomerases. In contrast, in the post-fusion form (co-expressed with HN protein and isomerases), free thiols are not present and steady state levels of free thiols cannot be enhanced by over-expression of isomerases.

Both of these results suggest that free thiols are present in the prefusion form of F protein and are not present in subsequent forms of F protein. The presence of free thiols
in the prefusion form of F protein is also consistent with their significance in the fusion of membranes.

(iv) **Free thiols are produced after attachment of HN protein to the cell surface receptor:** In studies of HIV-1, it has been shown that PDI coprecipitates with soluble as well as cell surface CD4 (Gallina, Hanley et al. 2002). It was proposed that PDI-CD4 association at cell surfaces enables the interaction between PDI and CD4 bound virus. Such an association of NDV proteins has not been explored, but our results showing that free thiols are present in F protein expressed on cell surfaces without HN protein and that free thiols are generated in F protein in virions or VLPs only after addition of target cells suggest that HN protein binding to its receptors is required and binding probably facilitates interaction between F protein and host cell isomerases.

(v) **Free thiols are probably required for F protein activation:** For paramyxovirus F protein activation, binding of HN protein to its receptor has been shown to be essential (Moscona and Peluso 1991; Porotto, Fornabaio et al. 2006). Also, it has been shown that HN protein binding to its receptor is not just providing a docking function as co-expression of a heterologous attachment protein with the fusion protein did not result in membrane fusion (Moscona and Peluso 1991; Hu, Ray et al. 1992; Porotto, Fornabaio et al. 2006). This result suggested that there is an essential interaction between HN and F proteins. The detailed mechanism of F protein activation by HN protein is not yet known but it is thought that upon binding to its receptor, HN protein undergoes a conformational change and this change releases the F protein from the HN and F protein complex allowing the F protein to refold into a more stable conformation (McGinnes, Gravel et al.
2002; Takimoto, Taylor et al. 2002; McGinnes and Morrison 2006). Also, whether other factors play a role in F protein activation has not been explored.

The results showing MPB labeling of F protein in VLPs with HN protein mutants, which are competent in attachment but are defective in fusion promotion, suggest that the production of free thiols is independent of HN protein activation of F protein. Probably both, interaction with HN protein and production of free thiols, are independently required for the activation of F protein. Taken together, these results suggest that one or several disulfide bonds in cell surface F protein are reduced and F protein exists as a mixture of both oxidized and reduced forms. In the presence of HN protein, only the reduced form may proceed to refold into additional intermediates leading to fusion of membranes as shown in Figure 5.1. In absence of HN protein, F protein is likely to exist as a mix of prefusion forms with intact disulfide bonds (Figure 5.1, Panel A) and with free thiols (Figure 5.1, Panel B).

E. Possible role of free thiols in membrane fusion

One possible mechanism, proposed as a result of studies of HIV-1 env protein, is that cleavage of disulfide bonds in fusion proteins may facilitate conformational changes in the fusion glycoprotein that are required for membrane fusion (Ryser, Levy et al. 1994). Class I fusion proteins undergo major refolding during membrane fusion and the mechanism of refolding is not yet clear.

Disulfide bonds are critical for the integrity of stable secondary structures in proteins. The cleavage of structure-stabilizing disulfide bonds in proteins is well
**Figure 5.1: Predicted model for production of free thiols in NDV F protein.**

**Panel A:** F protein in a prefusion conformation. HN protein binds to the receptor and facilitates interaction between F protein and target cell isomerases.

**Panel B:** Isomerases lead to cleavage of disulfide bonds in F protein in a prefusion conformation. Inhibitors of thiol-disulfide exchange inhibit production of free thiols. MPB binds to free thiols and prevents further conformational changes and membrane fusion. PDI and ERdj5 (shown in magenta) enhance production of free thiols and consequently increase membrane fusion.

**Panel C:** Open stalk conformation which has been shown to be present at lower temperatures.

**Panel D:** F protein in a pre-hairpin conformation which has been shown to require 37°C. F proteins with mutations in HR1 domain (shown in green) or HR2 domain (shown in blue) are thought to be defective in attaining pre-hairpin conformation.

**Panel E:** F protein in a post-fusion conformation.
accepted as a cause of conformational changes (Hughson and Hazelbauer 1996; Gallina, Hanley et al. 2002). The evidence for conformational changes in viral fusion glycoproteins after reduction by thiol-disulfide isomerase was provided by studies of HIV-1 env protein, which showed that PDI-induced reduction of gp120 disulfide bonds exposed new proteolytic sites in the gp120 protein (Gallina, Hanley et al. 2002).

Based on the background evidence, it is conceivable that cleavage of disulfide bonds in NDV F protein may facilitate conformational changes required for the fusion of membranes. Using conformation sensitive anti-F protein antibodies, we have shown that, in presence of inhibitors of free thiols, F protein is in prefusion conformation. When isomerases are over-expressed, most of surface-expressed F protein, in presence of HN protein, is in post-fusion conformation. Also, the results showing that free thiols are produced in the F protein before any major conformational changes occur in F protein are consistent with the idea that reduction of disulfide bonds in NDV F protein may facilitate conformational changes.

**Summary**

In summary, the work in this thesis indicates that one or many of the disulfide bonds in NDV F protein undergo reduction during the membrane fusion process. The production of free thiols is mediated by host cell isomerases belonging to the PDI family and is essential for membrane fusion and virus entry into the host cells. Cleavage of disulfide bonds in F protein may facilitate the conformational changes required for the fusion of membranes mediated by F protein.
Taken together these results suggest that production of free thiols is required for activation of F protein and, therefore, have identified new factors involved in activation of F protein in addition to its interaction with HN protein. Also this work suggests that host cell isomerases may be a potential target for developing antivirals that inhibit virus entry.
References


