Characterization of Ebola virus VP35 first basic patch as a therapeutic target

Jennifer Binning

Iowa State University

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Characterization of Ebola virus VP35 first basic patch as a therapeutic target

by

Jennifer M. Binning

A Thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biochemistry

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Iowa State University

Ames, Iowa

2014

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<tr>
<td>5'-PPP</td>
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<td>Ψ</td>
<td>psi</td>
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<td>AZT</td>
<td>3'-azido-3'-deoxythymidine</td>
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<td>BDBV</td>
<td>Bundibugyo virus</td>
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<td>bp</td>
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<td>CARD</td>
<td>caspase activation and recruitment domains</td>
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<td>central basic patch</td>
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<td>CCR5</td>
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<td>CREB</td>
<td>Cyclic AMP-response element binding protein</td>
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<td>dsRNAs</td>
<td>double-stranded RNA</td>
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<td>DMSO</td>
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<td>EBOV</td>
<td>Ebola virus</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>eVP35 IID</td>
<td>EBOV VP35 IID</td>
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<td>FBP</td>
<td>first basic patch</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>Fpg</td>
<td>formamidopyrimidine glycosylase</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>HA</td>
<td>hemagglutinin</td>
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<td>hepatitis B virus</td>
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<td>hepatitis B virus surface antigen</td>
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<td>human immunodeficiency virus</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
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<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IID</td>
<td>IFN inhibitory domain</td>
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<tr>
<td>IKKε</td>
<td>IkappaB kinase epsilon</td>
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<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
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<tr>
<td>IRF</td>
<td>interferon regulatory transcription factors</td>
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<td>ISGs</td>
<td>interferon stimulated genes</td>
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</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<tr>
<td>KPNA</td>
<td>karyopherin-α</td>
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<tr>
<td>L</td>
<td>large protein, the viral RNA-dependent RNA polymerase</td>
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<td>LLOV</td>
<td>Lloviu virus</td>
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<td>Acronym</td>
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<td>LPG-2</td>
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<td>MARV</td>
<td>Marburg virus</td>
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<td>MAVS</td>
<td>mitochondrial antiviral signaling</td>
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<tr>
<td>MDA-5</td>
<td>melanoma differentiation associated gene 5</td>
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<td>MFE</td>
<td>minimum free energy</td>
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<td>MG</td>
<td>minigenome assay</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NA</td>
<td>neuraminidase</td>
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<tr>
<td>NC</td>
<td>nucleocapsid</td>
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<td>NF-kappaB</td>
<td>Nuclear factor kappa B</td>
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<td>NLRs</td>
<td>NOD-like receptors</td>
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<tr>
<td>NP</td>
<td>nucleoprotein</td>
<td></td>
</tr>
<tr>
<td>NS5B</td>
<td>nonstructural protein 5B</td>
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<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
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<tr>
<td>PKR</td>
<td>RNA-dependent protein kinase</td>
<td></td>
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<td>PRRs</td>
<td>pattern recognition receptors</td>
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<td>RAVV</td>
<td>Ravn virus</td>
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<tr>
<td>RESTV</td>
<td>Reston virus</td>
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<tr>
<td>RLRs.</td>
<td>RIG-I like receptors</td>
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<tr>
<td>RMSD</td>
<td>root-mean-square deviation</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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</tr>
<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
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<tr>
<td>RT</td>
<td>reverse transcriptase</td>
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SELEX  Systematic Evolution of Ligands by EXponential enrichment
SeV   Sendai virus
SRF   Serum response factor
siRNA short interfering RNA
shRNA short-hairpin RNA
SUDV  Sudan virus
TAFV  Taï Forest virus
TBK-1 TANK-binding kinase
TCEP  tris(2-carboxyethyl)phosphine
TEV   tobacco etch virus
TLRs  Toll-like receptors
tRNA  transfer RNA
VLP   virus-like particles
VP24  viral protein 24
VP30  viral protein 30
VP35  viral protein 35
VP40  viral protein 40
WB    Western blot
ACKNOWLEDGEMENTS

The enclosed thesis was made possible by the collective efforts and support of colleagues, friends, and family. I would like to start by thanking my thesis advisor Dr. Gaya Amarasinghe, as he has been an invaluable mentor at every step of my graduate studies, and I am grateful for the opportunity to work in his laboratory. Throughout my time in Gaya's lab, he has provided guidance and support, and his general enthusiasm for science is contagious and fosters a stimulating learning environment which has helped me developed as an independent scientist. I would also like to give a special thanks to Dr. Daisy Leung. Through the help and mentorship of both Gaya and Daisy, I have grown as a student, a scientist, a colleague, and a person, and will forever be grateful for the guidance they have given me.

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Lastly, I would like to thank my family and friends for providing an invaluable support network. Through their continuous love and support, I had the courage to follow my dreams.
Viral protein 35 (VP35), encoded by filoviruses, are multifunctional dsRNA binding proteins that play important roles in viral replication, innate immune evasion and pathogenesis. The multifunctional nature of these proteins also presents opportunities to develop countermeasures that target distinct functional regions. However, functional validation and the establishment of therapeutic approaches toward such multifunctional proteins, particularly for non-enzymatic targets, are often challenging. With the current lack of approved vaccines or therapeutic options available to target filoviral infections, work that lends itself to the development of such inhibitors will be instrumental in countering this highly pathogenic virus. Our previous work on filoviral VP35 proteins defined two highly conserved conserved basic patches, the first basic patch (FBP) and the central basic patch (CBP), located within the C-terminal dsRNA binding interferon (IFN) inhibitory domain (IID). This work went on to show that the CBP is important for VP35 mediated IFN antagonism, in part through its ability to bind dsRNA. The goal of my thesis work was to investigate the functional importance of the FBP through a combination of structural and biochemical studies, and validate VP35 as a potential therapeutic target. These efforts established that residues within the FBP are functionally distinct from the CBP, but are important for VP35 polymerase co-factor function. Although the exact role of these VP35 residues in replication is poorly defined, the replication defective FBP mutants lost their ability to interact with the viral nucleoprotein, indicating that the VP35 FBP provides critical contacts which establish the VP35-NP interaction. In order to therapeutically target these functional regions of
VP35 and validate VP35 as a potential and promising antiviral target, we targeted Ebola virus (EBOV) VP35 (eVP35) for aptamer selection using SELEX. Select aptamers, representing two distinct classes, were further characterized based on their interaction properties to eVP35 IID. These results revealed that the aptamers bind to distinct regions of eVP35 IID with high affinity (10-50 nM) and specificity. These aptamers can compete with dsRNA for binding to eVP35 and disrupt the eVP35-NP interaction. Consistent with the ability to antagonize eVP35-NP interaction, select aptamers can inhibit the function of the EBOV polymerase complex reconstituted by expression of select viral proteins. Taken together, our results serve as an initial step in enhancing our understanding of the protein-protein interactions that establish the EBOV polymerase complex, and support the identification of two aptamers that bind filoviral VP35 proteins with high affinity and specificity and have the capacity to potentially target filoviral VP35 proteins as a therapeutic target.
CHAPTER 1. INTRODUCTION

1.1 Introduction

In order to protect against pathogens, host immune systems must recognize, counter, and purge themselves of foreign invaders. Distilled down to its most basic concept, the immune system must distinguish differences between self and non-self in order to effectively clear an infection. Many viral pathogens have co-evolved with their host organisms and developed ways to replicate and sustain themselves despite an onslaught of host immune defenses. Deconstructing the viral-host interface at a molecular level will be crucial to understanding of both host immunity and viral pathogenesis. Research designed to elucidate the intermolecular interactions between viral immunomodulatory proteins and host proteins will therefore be critical to the development of new and innovative therapeutic agents to counter viral infections.

The host immune system responds to pathogens through highly tailored, intricate pathways, which can integrate various signals and generate systematic cellular changes to counter and clear the invading pathogens. Host immune responses can be divided into two branches: innate and adaptive immunity, the latter which will not be discussed in the context of this thesis. Innate immunity provides the first line of defense, allowing the detection of a wide range of pathogens. The broad response elicited by the innate immune system is achieved through cellular surveillance proteins called pattern recognition receptors (PRRs) which recognize molecular markers characteristic to pathogens, termed pathogen-associated molecular patterns (PAMPs) (Akira et al. 2006) (Figure 1.1A). PRRs can be categorized into four classes: C-type lectin receptors (CLR), Toll-like receptors (TLR), NOD-like receptors (NLR), and Retinoic acid-
inducible gene I (RIG-I)-like receptors (RLRs) (Figure 1.1B). These PRRs are located to various cellular compartments, including the cell surface, the endosome, and the cytoplasm providing surveillance for a diverse range of PAMPs. Engagement of PAMPs by select PRRs results in signal transduction cascades which produce interferons, chemokines and cytokines, ultimately leading to the establishment of an inflammatory response, activation of adaptive immunity, and clearance of the pathogen. The pathways and signaling events involved in these processes are highly regulated with distinct and overlapping characteristics and varying degrees of cross-talk to provide the fullest protective coverage (Crozat et al. 2009).

Filoviruses potently antagonize the Type-I interferon pathway, in particularly RLR-dependent activation of IFN-β signaling (Figure 1.2). RLRs are PRRs localized to the cytoplasm that play a critical role in detecting viral PAMPs, especially those derived from RNA viruses. In an inactivated state, RLRs are found in an auto-inhibited conformation, but undergo conformational rearrangement upon association with viral RNA. The rearrangement allows their caspase activation and recruitment domains (CARDs) to interact with the CARD domains of the mitochondrial antiviral signaling (MAVS) protein, an essential mitochondrial anchored adapter protein in the Type-I IFN signaling cascade (Hou et al. 2011). Notably, both the RLRs and the TLRs signal through MAVS and therefore converge at this point. Association of the RLR and MAVS CARD domains activates the Type-I IFN response which mediates downstream signaling and leads to the phosphorylation and translocation of interferon regulatory transcription factors (IRF)-3/7 to the nucleus. IRF3/7 then promote the induction of Type-I IFNs (IFN-α and IFN-β). Interferons produced by an infected cell are secreted
and can act in an autocrine or paracrine fashion to activate the JAK/STAT pathway and promote the induction of a number of interferon stimulated genes, or ISGs, which work in concert to establish an antiviral state within the cell (Zuniga et al. 2007; Bonjardim et al. 2009).

The RLR family contains three proteins: RIG-I, melanoma differentiation associated gene 5 (MDA-5), and laboratory of genetics and physiology 2 (LGP-2) (Figure 1.3). Both RIG-I and MDA-5 have two N-terminal CARD domains, whereas LGP-2 has none (Fig 1.3A). The CARD domains are essential for RIG-I mediated signaling, and it has been shown that expression of the CARD domains alone results in constitutive activation of the Type-I signaling pathway (Yoneyama et al. 2004). Due to the lack of CARD domains in LGP-2, it has been speculated that LGP-2 serves as a negative regulator for RIG-I and MDA-5 (Fig 1.3B). However, a recent report by Satoh et al. (Satoh et al. 2010) suggests LGP-2 serves as an activator of RLR-dependent signaling. Though there have been a number of recent discoveries that have shaped our understanding of these RLRs, how these proteins are regulated and the exact role of LGP-2 remains poorly understood.

Recent structural work with both RIG-I and MDA-5 has provided much needed insight into the activation mechanism of RLRs, as well as clarified how RIG-I and MDA-5 discriminate between their respective PAMPs (Kolakofsky et al. 2012; Leung et al. 2012; Beckham et al. 2013; Feng et al. 2013; Wu et al. 2013). Despite their structural similarities, RIG-I and MDA-5 recognize both common and distinct viral species (Figure 1.3C). This is likely due to their recognition of overlapping (double-strandedness), but distinct (blunt-ends), viral PAMPs: RIG-I is preferentially activated by 5'-triprophosphate (5'—

Recent work from our lab has investigated the mechanism by which two different filoviruses, *Ebolaviruses* and *Marburgviruses*, preferentially antagonize RIG-I and MDA-5. Despite their similar genome organization and replication strategies, ebolaviruses encodes a protein, VP35, that inhibits both RIG-I and MDA-5 recognition of viral RNA. However, marburgvirus VP35 only prevents viral RNA recognition by MDA-5 (Ramanan et al. 2012). These findings exemplify the distinct, but overlapping, viral recognition of RIG-I and MDA-5 and suggest that even closely related viruses generate distinct PAMPs which are differentially recognized by these RLRs. Additionally, these studies raise the question of what are the true physiological ligands of RIG-I and MDA-5? Such a point has been heavily addressed in the literature but has yielded no definitive answers.

### 1.2 Filoviral pathogenesis and epidemiology

Viruses, as well as other pathogens, have developed sophisticated means to counteract host immune defenses, and filoviruses are capable of an early and potent inhibition of host innate immunity. Filoviruses are single-stranded, non-segmented, negative sense RNA viruses that cause severe hemorrhagic fever in humans and non-human primates (Bray et al. 2007), and exhibit fatality rates as high as 90% during reported outbreaks. The high lethality associated with filoviral infection is in large part
due to an early and potent inhibition of host immune responses. In addition to the
dysregulation of the host innate and adaptive immune responses, the pathology
associated with filoviral infection is also, in part, attributed to broad tissue tropism
allowing the virus to infect and kill a wide variety of cell types and inducing of a strong
inflammatory response. Though filoviruses can infect a number of different cell types,
the primary site of infection often occurs in monocytes, macrophages, dendritic cells,
hepatocytes, and endothelial cells. The induction period for filoviral infection ranges
between 2 days and 2-3 weeks. The induction period is followed by an onset of non-
specific symptoms including fever, soreness, diarrhea, nausea, and in some cases, a
maculopapular rash (Bwaka et al. 1999; Feldmann et al. 2011). The disease
progression of filoviral infection occurs rapidly and after ~2 weeks, the initial non-
specific symptoms can manifest into fatal hemorrhagic fever which is characterized by
organ failure and severe internal hemorrhaging. Despite the high levels of viral
replication, potent inhibition of host immune responses, and rapid disease progression,
there are still no approved vaccines or therapeutic options available to treat filoviral
infection. The severe pathology exhibited by these viruses and the lack of treatment
options underscores the potential public health risk posed by filoviruses and the need to
develop innovative countermeasures.

The Filoviridae family consists of two genera, *Ebolaviruses* and *Marburgviruses*. However, a new genus, *Cuevavirus* (Negredo et al. 2011; Bukreyev et al. 2013), has been proposed. There are two species of *Marburgvirus*, Marburg virus (MARV) and Ravn virus (RAVV) (Kuhn et al. 2010), and five species of *Ebolavirus*, Ebola virus (EBOV), Sudan virus (SUDV), Taï Forest virus (TAFV), Reston virus (RESTV), and
Bundibugyo virus (BDBV), all of which are named after the geographical location where they were first discovered. The first reported filoviral outbreak occurred in 1967 in Marburg, Germany and Yugoslavia when laboratory workers contracted MARV and developed a hemorrhagic fever after handling infected tissue samples from monkeys imported from Uganda. Since these initial occurrences of marburgvirus, there have been other fatal, but sporadic, outbreaks in Kenya and Zimbabwe, and more severe outbreaks in the Democratic Republic of Congo and Angola. Within a decade of the first reported cases of MARV, ebolavirus emerged with two outbreaks occurring in 1976 in the African countries of Zaire and Sudan. Unlike marburgvirus, the initial outbreaks of ebolaviruses were not restricted to a small subset of animal handlers, and, in total, ebolaviruses has claimed approximately four times the number of lives than marburgviruses. The 1976 outbreaks of Ebola virus and Sudan virus resulted in a combined 431 deaths and identified the Zaire strain of ebolavirus as one of the most lethal viruses ever isolated, with nearly a 90% fatality rate. At present there are two ongoing outbreaks of ebolavirus in Uganda and in the Democratic Republic of Congo highlighting the current and ongoing threat these viruses pose to world health.

Among the five species of ebolaviruses, RESTV is the only one not of African origin and does not cause disease in humans; however, RESTV is highly pathogenic in non-human primates (1990; Jahrling et al. 1990; Hayes et al. 1992; Groseth et al. 2002). Moreover, the recent isolation of RESTV from swine populations in the Philippines, suggests the potential zoonotic nature of filoviruses (Jahrling et al. 1990; Barrette et al. 2009). In addition to primates and swine, ebolaviral RNA has been identified in rodents and shrews from central Africa (Morvan et al. 1999), and bats have
been shown to be able to support ebolaviral replication at high viral titers without apparent pathological characteristics (Swanepoel et al. 1996). Furthermore, a distinct filovirus, Lloviu virus, was recently isolated from insectivorous bats in Cueva del Lloviu, Spain (Negredo et al. 2011). These findings have provided insight into the possible natural reservoir of filoviruses, with current opinions in the field strongly favoring fruit bats, though no definitive conclusion has been agreed upon (Leroy et al. 2005; Swanepoel et al. 2007; Pourrut et al. 2009; Towner et al. 2009). Together, these observations highlight the zoonotic nature of filoviruses and the importance of identifying their elusive natural reservoir, as well as emphasize the need to improve our understanding of animal-to-animal transmission in order to better predict and/or contain filoviral outbreaks.

1.3 Filoviral viral replication

Filoviruses are recognized by their pleomorphic, filamentous morphology and have an approximately 19-kilobase genome which encodes seven genes: the viral nucleoprotein (NP), viral protein 35 (VP35), VP40, glycoprotein (GP), VP30, VP24, and L, the viral RNA-dependent RNA polymerase (Figure 1.4). Filoviurses are enveloped viruses with their ribonucleoprotein (RNP)-complex enclosed by a host-derived cell membrane. NP, VP35, VP30, and L, along with the viral genomic RNA, comprise the RNP-complex. Beneath the viral envelope are viral proteins VP40 and VP24, the major and minor matrix proteins, respectively, and embedded in the membrane is the GP trimeric spike protein. Viral entry is thought to occur in a GP-dependent manner primarily through macropinocytosis, though alternative mechanism such as caveolin-
dependent and clathrin-dependent endocytosis have been proposed (Nanbo et al. 2010; Saeed et al. 2010; Aleksandrowicz et al. 2011; Hunt et al. 2011). Given the broad tissue tropism exhibited by filoviruses, the cellular receptors involved in filoviral entry are poorly understood.

Once in the cytoplasm, ebolavirus, like all negative-stranded RNA viruses, must carry out de novo synthesis of viral mRNA (Knipe 2001). Due to the fact that ebolavirus replicates through RNA intermediates, the virus must encode its own RNA-dependent RNA polymerase and package the polymerase into the virion in order to carry out transcription once inside newly infected cells. The ebolavirus RNA-dependent RNA polymerase complex is responsible for both viral transcription and replication, and consists of four viral proteins: NP, VP35, VP30, and the polymerase, L (Figure 1.5). The molecular basis for protein-protein and protein-RNA interactions that establish the polymerase complex, as well as the molecular switch between replication and transcription are currently unknown. Initial studies with marburgvirus, and subsequent studies with ebolavirus, established that NP, VP35, and L are the minimal components required for viral replication (Becker et al. 1998; Muhlberger et al. 1998; Muhlberger et al. 1999). Though VP30 is not required for replication, it has been shown to be important for transcription initiation within the context of ebolaviral infection, but not marburgviral infection (Muhlberger et al. 1998; Muhlberger et al. 1999; Weik et al. 2002). Following viral entry, the viral polymerase complex transcribes polyadenylated, monocistronic viral mRNAs which contain a 5'-cap allowing for cap-dependent translation of the viral mRNA (Shabman et al. 2013). The filoviral genome is arranged in a 3'-5' orientation, with the general abundance of each viral proteins correlating to the
proximity of its gene to the 3' end. This is due to a gradual decrease in the levels of mRNA of the NP gene at the 3' end relative to the viral polymerase gene, L, at the 5' end (Knipe 2001). It is tempting to speculate that the decrease in mRNA levels moving from the 3' to 5' direction of the genome results from the polymerase complex dissociating from the genomic viral RNA template, but the mechanism(s) by which filoviruses carry out viral transcription and regulate the levels of their transcripts are currently not understood.

The viral mRNA generated during viral transcriptions is then translated by host cell machinery to produce viral proteins. An accumulation of viral proteins, particularly NP, within the cell triggers the RNA-dependent polymerase complex to switch from transcription to replication. In the context of viral replication, the viral polymerase complex first generates a positive sense anti-genome, which like the negative sense genome, is encapsidated by NP. The resulting anti-genome intermediate then serves as the template for synthesis of the negative sense genome. The newly synthesized viral genome can then serve as a template for the transcription of additional viral mRNA and/or be packaged into a budding virion. Little is known about how the polymerase complex switches between transcription and replication, but the phosphorylation state of VP30 plays a critical role in determining whether the polymerase complex supports replication or transcription (Biedenkopf et al. 2013). Additionally, a recently published study has identified regulatory roles for VP40 and VP24 in viral transcription (Hoenen et al. 2010), suggesting that the dynamics between viral replication and transcription are tightly regulated.
Viral assembly, budding, and egress are mediated through the viral matrix proteins, VP40 and VP24. VP40 is the more abundant of the two proteins and alone is sufficient for the formation of virus-like particles (VLP) that resemble authentic virions (Harty et al. 2000; Yasuda et al. 2003). Though VP40 can mediate the budding process independently, in the context of an infection it supports a number of critical interactions with viral and host components to properly coordinate assembly and budding. Once the virion egresses from the infected host cell, it can infect a new cell where the replication cycle can be carried out again.

1.4 Filoviruses encode multiple virulence factors

Of the seven proteins encoded by ebolavirus, VP24, GP, and VP35 are known virulence factors. These three proteins function to subvert and inhibit host immune defenses permitting the virus to antagonize both innate and adaptive immune responses (Figure 1.6). Attesting to the potent ability of these proteins to counter host immunity, fatal cases of filoviral infection often indicate no activation of adaptive immunity though these individuals were immunocompetent.

Filovirus infection potently inhibits the innate immune system by stunting host secretion of IFN-β. However, in instances where IFN-β is produced, the EBOV minor matrix protein VP24 is able to antagonize downstream IFN-β signaling molecules (Figure 1.6). IFN-β can act in an autocrine or paracrine fashion to activate the JAK/STAT pathway, a pathway which allows for the phosphorylation and translocation of STAT transcription factors into the nucleus where they stimulate the production of a number of anti-viral ISGs. Ebolaviral VP24 inhibits this pathway by interacting with
karyopherin-α (KPNA), a protein involved in the importin complex which helps shuttle STAT into the nucleus (Reid et al. 2006; Mateo et al. 2010; Shabman et al. 2011; Zhang et al. 2012). One important aspect of VP24-mediated inhibition is the potential to disrupt KPNA-dependent translocation of many other transcription factors to the nucleus, thus drastically changing the expression profile within an infected cell.

The fourth gene encoded by ebolavirus is the viral glycoprotein, GP, which plays a role in antagonizing the adaptive immune response. GP is the only viral protein located on the surface of the virion, and therefore the immunodominant antigen targeted by host antibodies. The GP gene encodes for two proteins as a result of transcriptional editing (Feldmann et al. 1999). A secreted form of GP, soluble GP (sGP), represents the unedited form of the gene and accounts for approximately 80% of the transcribed mRNA (Feldmann et al. 2001). Given the unbalanced ratio of sGP to membrane bound GP, it is believed that the high levels of circulating sGP can act as a decoy by serving as a “sink” for neutralizing antibodies, preventing them from binding to cell-surface GP (Mohamadzadeh et al. 2007).

Ebolaviral VP35 is a multifunctional immune antagonist that functions at several stages in the viral replication cycle (Basler et al. 2000; Basler et al. 2003; Cardenas et al. 2006; Hartman et al. 2006; Leung et al. 2010). VP35 is known to antagonize numerous components in the IFN induction and signaling pathway (Basler et al. 2000; Basler et al. 2003; Hartman et al. 2004; Cardenas et al. 2006; Enterlein et al. 2006; Hartman et al. 2006; Feng et al. 2007; Haasnoot et al. 2007; Hartman et al. 2008; Leung et al. 2010), including RLRs (Cardenas et al. 2006; Prins et al. 2010; Luthra et al. 2013), IFN kinases IKKε and TBK-1 (Prins et al. 2009), RNA-dependent protein kinase
PKR) (Feng et al. 2007; Schumann et al. 2009), and PKR activator (PACT) (Luthra et al. 2013). VP35 also functions as an RNAi silencing suppressor (Haasnoot et al. 2007; Fabozzi et al. 2011), a co-factor for the viral polymerase (Becker et al. 1998; Muhlberger et al. 1998; Muhlberger et al. 1999; Prins et al. 2010), and a structural component of the viral nucleocapsid (Huang et al. 2002; Noda et al. 2002; Johnson et al. 2006; Shi et al. 2008).

In an effort to better understand VP35 and VP35 mediated functions, our lab has taken both a structural and functional approach to define the critical intermolecular interaction involving VP35. These efforts resulted in the crystal structure of the C-terminal domain of VP35, termed the interferon inhibitory domain (IID) (Leung et al. 2009; Leung et al. 2010). The VP35 structure revealed two basic patches, termed the first basic patch (FBP) and the central basic patch (CBP), which are highly conserved among ebolaviral species (Figure 2.1). Using the structural data in conjunction with previously published biochemical data (Hartman et al. 2004), residues known to be important for interferon inhibition were mapped to the CBP of VP35 IID. More recently, our lab has determined the crystal structure of the VP35 IID bound to dsRNA (Leung et al. 2010), and in doing so, clarified the mechanism by which the VP35 CBP interacts with dsRNA and inhibits host immune responses. In the crystal structure four VP35 IID molecules interact with 8 base pair (bp) dsRNA through direct contacts with the phosphodiester backbone and blunt ends (Figure 1.7A). Residues R312, K319, R322, and K339 are critical CBP residues responsible for interacting with the RNA backbone, whereas hydrophobic residue F239 is responsible for 'end-capping' the blunt ends. Alanine substitution mutations to either the CBP residues or the 'end-capping' residues
disrupt the VP35 IID-dsRNA interaction and correspondingly inhibit the induction of IFN-β.

The strong correlation between dsRNA binding and inhibition of IFN-β production suggests a mechanism by which VP35 sequesters dsRNA and prevents detection by RLRs. Work from our lab has established that VP35 can out-compete RIG-I for dsRNA binding, further supporting a model in which VP35 antagonizes host immune responses, at least in part, by preventing activation of RLRs. Moreover, recent structural studies revealed an interaction between RIG-I and the blunt ends of dsRNA, indicating that double-strandedness and the blunt ends are common ligands recognized by both RIG-I and VP35 (Figure 1.7B and C) (Leung et al. 2010; Ramanan et al. 2012). Further supporting the critical role of the VP35 CBP in viral pathogenesis, a guinea pig adapted ebolavirus was engineered with alanine mutations to CBP residues K319 and R322. The CBP mutant virus was severely attenuated despite being replication competent. Prior infection with the mutant virus conferred protection against subsequent infection by wild-type (WT) ebolavirus, suggesting that if the guinea pig can mount an effective adaptive immune response these animals are immunized against ebolavirus (Prins et al. 2010). In addition to the dsRNA-dependent mechanism of inhibition of IFN-β production, VP35 is also able to inhibit in an RNA-independent manner. While the exact mechanism by which this occurs is not clear, VP35 appears to serve as a pseudosubstrate for IKKε and TBK-1, two kinases which phosphorylate IRF-3/7 (Prins et al. 2009). Direct inhibition of these kinases would provide a possible explanation for RNA-independent inhibition of IFN-β production downstream of RLRs. Altogether, these studies highlight the importance of VP35 proteins in viral-mediated host immune suppression and
support a model in which VP35 binds dsRNA preventing its recognition by RLRs. Additionally, this work establishes a framework for targeting VP35 with potential therapeutics.

1.5 VP35 as a therapeutic target

Despite a substantial body of literature describing the molecular mechanisms that drive filoviral pathogenesis, there are still no FDA-approved treatments for filoviral infections (Wilson et al. 2001; Geisbert et al. 2003; Hensley et al. 2005; Bray et al. 2007; Geisbert et al. 2010; Hartman et al. 2010; Feldmann et al. 2011). Though filoviral outbreaks are sporadic, and thus far restricted to rural regions of Africa, the high fatality rates, zoonotic nature, and threat of exploitation by bioterrorists underscore the public health threat these viruses pose.

The relevance of VP35 to host immune inhibition and viral replication make it an attractive target for therapeutic development. Previous studies have demonstrated that antisense oligonucleotides and small interfering RNAs can interfere with EBOV replication by targeting VP35 (Enterlein et al. 2006; Geisbert et al. 2006; Warfield et al. 2006; Groseth et al. 2007). Additional studies indicate that ebolaviruses containing mutant VP35 are highly attenuated and guinea pigs infected with these mutant ebolaviruses are protected against challenges with wildtype ebolavirus (Prins et al. 2010).

My thesis will focus on deconstructing the many roles of the multifunctional VP35 protein, assessing the potential of VP35 as a therapeutic target, and developing VP35 inhibitors. Specifically, I have investigated the role of the VP35 C-terminal domain
termed the interferon inhibitory domain (IID) in viral replication through its interaction with NP. In an effort to target the two functionally relevant basic patches of VP35 with inhibitory molecules, we have identified and characterized anti-VP35 RNA aptamers selected against Ebola virus (EBOV) VP35 (eVP35) IID WT and mutant proteins. Through these studies we have determined that the aptamers bind with high affinity and specificity to eVP35 IID and disrupt the eVP35-NP interaction. Correspondingly, aptamers that disrupt the eVP35-NP interaction inhibit the functionality of the viral polymerase complex in a reconstituted minigenome (MG) assay. As viral replication is a critical step in viral propagation, an enhanced understanding of this process at the molecular level will undoubtedly aid in the development of therapeutics. My thesis provides much needed biochemical insight into the formation of the ebolaviral RNA-dependent RNA polymerase complex by analyzing the critical residues within eVP35 IID at the VP35-NP interface, further implicating VP35 as a promising target for antivirals.
1.6 Figures

Figure 1.1. Pattern recognition receptors allow for early detection of invading pathogens. (A) Pathogen associated molecular patterns (PAMPs) are detected by cellular proteins termed pattern recognition receptors (PRRs). Once the PRRs detect a PAMP, in this case a viral PAMP, the PRR elicits an innate immune response to counter and clear the invading pathogen. (B) Four classes of PRR, C-type lectin receptors (CLR), Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I like receptors (RLRs), detect pathogens at the cell surface, in the endosomal compartment, and within the cytoplasm.
Figure 1.2. Type I IFN pathway can be divided into the IFN production and IFN response pathways. Cytosolic RLRs detect viral PAMPs, including dsRNA, and trigger a signal transduction cascade which results in the phosphorylation and translocation of IRF-3/7 to the nucleus where it stimulated the production of IFN-β. IFN-β can act in an autocrine or paracrine fashion to activate the JAK/STAT pathway and upregulate IFN-stimulated genes (ISGs) ultimately establishing an anti-viral state within the cell. Adapted from (Leung et al. 2010).
**Figure 1.3. RIG-I like receptors detect RNA viruses.** (A) RLRs include RIG-I, MDA-5, and LGP2. Both RIG-I and MDA-5 both contain two tandem CARD domains at their N-termini, this is followed by a helicase domain followed by a C-terminal domain (CTD). LGP2 contains the helicase and CTD similar to RIG-I and MDA-5 but is lacking the N-terminal CARD domains. (B) RIG-I and MDA-5 can recognize viral dsRNA and activate the Type-I IFN signalling pathway through the mitochondrial anchored adapter protein and (B) (C) Viral activators of RIG-I (top, white box), both RIG-I and MDA-5 (middle, light grey box), and MDA-5 (bottom, dark grey box) (adapted from Loo et al. (Loo et al. 2008)).
Figure 1.4. The basic architecture of the filoviral virion. (A) Electron micrograph of the *ebolavirus* virion displays the characteristic filamentous appearance from which filoviruses derive their name. (Centers for Disease Control and Prevention; http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/Fact_Sheets/Ebola_Fact_Booklet.pdf). (B) Filoviruses are enveloped, single-stranded, non-segmented, negative sense RNA viruses (-ssRNA). The viral envelope is embedded with the viral glycoprotein, GP, and beneath the membrane is the viral major and minor matrix proteins, VP40 and VP24, respectively. Within the virion is the ribonucleoprotein (RNP) complex which is made up of NP, VP35, VP30, and L which is associated with the viral genomic RNA. (C) The viral genome is oriented in the 3'-5' orientation and encodes seven genes.
Figure 1.5. Ebolavirus replication complex is responsible for both viral transcription and replication. (A) The minimal ebolavirus RNA-dependent RNA polymerase complex is comprised of viral proteins NP, VP35, and L, with the addition of VP30 being required for viral transcription (Becker et al. 1998; Muhlberger et al. 1998). (B) The ebolavirus polymerase complex is required for both viral transcription and replication. (1) Upon viral entry, the nucleocapsid is released into the cytoplasm. (2) The ebolavirus polymerase complex then transcribes viral mRNA. Translation of viral mRNA by host cell machinery results in an abundance of viral protein within the cell, triggering the ebolavirus polymerase complex to switch from transcription replication. (4) The negative sense genomic viral RNA is synthesized through a positive sense RNA intermediate and (5,6) subsequently packaged into a budding virion. Adapted from Fields Virology (Knipe 2001).
**Figure 1.6. Ebolaviruses antagonize multiple host immune pathways.** Ebolaviral encoded VP35, VP24, and GP antagonize host immune responses. VP35 inhibits the production of IFN-β by binding to and sequestering dsRNA, thus preventing its detection by RLRs (left). VP24 inhibits the IFN-β response pathway by engaging KPNA and preventing the translocation of STAT to the nucleus (middle). The mechanism(s) of GP inhibition are less well defined, but the most prevalent models suggest sGP and 'dispatched' GP can bind to antibodies and prevent their association with cell-surface GPs (right).
Figure 1.7. eVP35 IID binds to dsRNA and compared with RIG-I for dsRNA binding. (A) The crystal structure of eVP35 IID bound to 8-base pair dsRNA (PDB ID: 3L25) reveals that four VP35 IID molecules associate with both the blunt ends of the dsRNA (VP35 IIDs shown in blue) and the phosphodiester backbone (VP35 IIDs shown in teal). The dsRNA is shown in pink. (B) 90° rotation of (A) showing only two VP35 molecules highlights the ability of VP35 to bind the blunt- ends and backbone of dsRNA. (C) Pymol alignment of VP35 IID and RIG-I dsRNA bound structures, with the RNA molecules aligned. Based on the alignment, VP35 and RIG-I sterically clash, suggesting that in vivo VP35 and RIG-I compete for the same ligand. C-terminal domain of RIG-I bound dsRNA (PDB ID: 3OG8) (Lu et al. 2011).
1.8 References


CHAPTER 2. FUNCTIONAL VALIDATION OF THE VP35 IID FIRST BASIC PATCH

The research within this chapter consists of data that are published in the Journal of Virology.


This work was a collaboration between the Basler and Amarasinghe laboratories. My contributions to this manuscript included the expression and purification of VP35 IID WT and mutant proteins for the NMR and ITC experiments, carrying out the NMR and ITC experiments, as well as purifying all of the MBP-fusion VP35 IID proteins used in the NP pulldown assays. Additionally, I cloned the lysine-to-arginine and arginine-to-lysine mutations to address reviewer’s comments during the submission process. In addition to data analysis, I drafted and edited then methods, results and discussion sections pertaining to my work.

2.1 Introduction

Filoviral VP35 is a well known immune antagonist as described in Chapter 1. In addition to being vital for immune suppression, VP35 is a critical component of the viral polymerase complex. The multifunctional nature of VP35 provides opportunities to investigate host immune suppression mechanisms, negative-stranded viral RNA
synthesis, and the basis for one protein to mediate multiple functions and support multiple binding partners. EBOV VP35 engages in a number of intermolecular interactions with viral proteins such as nucleoprotein (NP) (Becker et al. 1998), the large protein (L) (Moller et al. 2005), and VP40 (Johnson et al. 2006), and cellular components such as dsRNA (Cardenas et al. 2006; Haasnoot et al. 2007; Leung et al. 2010; Leung et al. 2010), IKKɛ (Prins et al. 2009), TBK-1 (Prins et al. 2009), IRF-7 (Chang et al. 2009), and PKR (Feng et al. 2007; Schumann et al. 2009). VP35 is composed of an N-terminal oligomerization domain and a C-terminal interferon inhibitory domain (IID). To better understand VP35 and VP35 mediated functions, our lab has taken both a structural and functional approach which has resulted in a crystal structures of VP35 IID alone and bound to dsRNA revealing two highly conserved basic patches, the FBP and the CBP. Biochemical data implicated a number of basic residues within IID as important for dsRNA binding (Hartman et al. 2004; Leung et al. 2010), but the availability of the VP35 IID-dsRNA structure provided important insight in to how these residues formed a basic patch, the CBP, and how this basic patch binds to viral dsRNA and prevents its detection by RLRs. In contrast to the CBP, the FBP was not required for inhibiting the production of IFN-β. Some, but not all VP35 mediated functions depend on the ability of VP35 to bind dsRNA. In addition to its role in host immune inhibition, VP35 is an essential component of the filoviral replication complex. Co-expression and immunofluorescence studies by Becker et al. revealed cellular localization patterns for filoviral proteins. In the context of viral infection, NP, VP30 and VP35 all co-localized within inclusion bodies. When expressed individually, NP was found to localize within inclusion bodies, while VP30 and VP35 were distributed
throughout the cell. When VP30 or VP35 were co-expressed with NP they went from being homogenously dispersed to being found in NP-induced inclusion bodies. These studies established interactions between NP-VP35, NP-VP30, VP35-L, and NP-VP35-L, and support a model in which VP35 interacts with both NP and L, thus tethering the polymerase and its template together. Here we biochemically characterize the eVP35 FBP residues and demonstrate that the FBP is not involved in dsRNA binding, but is critical for the polymerase cofactor function of VP35 (Prins et al. 2010). Although the exact role of these VP35 residues in replication is poorly defined, we show that the replication defective FBP mutations loss their ability to interact with NP, but maintain the VP35-L interaction. Together these results further our understanding of the protein-protein interactions that establish the EBOV polymerase complex and our data supports a model where two distinct regions of VP35 contribute to pathogenesis through polymerase co-factor function and IFN inhibition/dsRNA binding.

2.2 Materials and methods

**Antibodies.** Monoclonal antibodies against Zaire EBOV VP35 and against Zaire EBOV NP were generated in collaboration with the Mount Sinai Hybridoma Center and have been described previously (Cardenas et al. 2006; Martinez et al. 2007). A monoclonal antihemagglutinin (anti-HA) antibody was purchased from Sigma (St. Louis, MO). A monoclonal anti-maltose binding protein (anti-MBP) antibody was purchased from New England Biolabs.
**Cell lines and viruses.** HEK293T cells and Vero cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Sendai virus (SeV) strain Cantell was grown in 10-day-old embryonated chicken eggs for 2 days at 37°C.

**Plasmids.** The Zaire EBOV VP35 expression plasmid pCAGGS-VP35 and an IFN-promoter reporter plasmid were described previously (Basler et al. 2000; Basler et al. 2003). VP35 point mutations were generated by standard PCR-based methods and cloned into the mammalian expression plasmid pCAGGS (Niwa et al. 1991). Firefly luciferase was expressed from the pCAGGS plasmid in the minigenome assays (see below). The pRL-TK Renilla luciferase expression plasmid was purchased from Promega (Madison, WI). Sequences encoding Zaire EBOV L amino acids 1 to 505 (HA-L amino acids 1 to 505) were amplified from plasmid pTM1-L by PCR (Prins et al. 2010), cloned with an amino-terminal HA tag, and cloned into pCAGGS. The expression plasmid for Zaire EBOV NP, pcDNA3 EBOV NP, has been described previously (Martinez et al. 2007).

**MBP-fusion VP35 IID and VP35 IID protein expression and purification.** MBP-fusion VP35 IID proteins were expressed and purified as described previously (Leung et al. 2009; Leung et al. 2009). Briefly, BL21(DE3) cells expressing MBP-fusion VP35 IID proteins were lysed using an EmulsiFlex-C5 homogenizer (Avestin) and clarified by centrifugation at 30,000 g at 4°C for 30 min. The supernatant was purified by affinity and cation-exchange chromatography, prior to final purification by size exclusion
chromatography. For VP35 IID proteins, the fusion tags were removed prior to final purification by cleavage with tobacco etch virus (TEV) protease followed by size-exclusion chromatography. The purity of the protein samples was assessed by SDS-PAGE.

**Nuclear Magnetic Resonance (NMR).** 300μl samples were prepared at 100μM ¹⁵N labeled VP35 IID in 10mM HEPES (pH 7), 150mM NaCl, 2mM Tris(2-carboxyethyl)phosphine (TCEP) and 5% D₂O buffer. ¹H¹⁵N heteronuclear single quantum coherence (HSQC) experiments were collected at 25°C on a Bruker Avance II spectrometer [700.13MHz]. Data were processed with NMRPipe/NMRDraw and analyzed in NMRView.

**Isothermal titration calorimetry (ITC) assays.** Quantitative analysis of VP35 IID protein binding interactions with dsRNA was performed on a microcalorimeter (VP-ITC; Microcal, North Hampton, MA) by isothermal titration calorimetry (ITC), using protein samples dialyzed against 500 ml of dialysis buffer [10 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM MgCl₂, 2 mM TCEP] for 12 h. The resulting raw microcalorimeter data were processed and analyzed to determine n (number of binding sites) and K_D (binding constant) using ORIGIN software. Protein concentrations in the syringe were between 130 to 150 µM and dsRNA 8mer in the cell was at 4 to 6 µM, using 5 μcal/sec as the reference power.
**IFN-β–luciferase reporter assay.** HEK293T cells were transfected by using Lipofectamine 2000 (Invitrogen) with the indicated amounts of expression plasmid, an IFN-β–firefly luciferase reporter plasmid (400 ng), and a constitutively expressed Renilla luciferase reporter plasmid (pRLTK, 200 ng). To induce reporter gene expression, cells were infected with SeV at a multiplicity of infection of 10. Twenty-four hours posttransfection, the cell lysates were assayed with the dual luciferase reporter assay (Promega), and firefly luciferase activity was normalized to Renilla luciferase activity. The results are presented as percent induction of the positive control (SeV infected, empty vector transfected [no VP35]), the value for which was set equal to 100%.

**EBOV transcription/replication assay.** The EBOV transcription/replication assay was based on a previously described system (Muhlberger et al. 1999). HEK293T cells were cotransfected by the calcium phosphate precipitation method with phage T7-driven expression plasmids encoding the Zaire EBOV NP, L, and VP30 and VP35 proteins. Also transfected were a T7 RNA polymerase expression plasmid, a plasmid that expresses from a T7 promoter a Zaire EBOV minigenome which encodes a fused green fluorescent protein (GFP)-chloramphenicol acetyltransferase (CAT) reporter gene. This is flanked by the cis-acting sequences necessary for replication and transcription of the RNA by a reconstituted EBOV polymerase complex. Also transfected was a constitutively expressing luciferase expression plasmid that served as a transfection control. At 36 h posttransfection, cells were lysed with reporter lysis buffer (Promega) and both CAT and luciferase reporter activities were determined. CAT activity was normalized to luciferase activity. Minigenome reporter activation is presented as percent
activity relative to that of the positive-control reaction (250 ng of wild-type (Emsley et al.) VP35 plasmid), which was set equal to 100%. Error bars represent the standard deviation (SD) from at least three experiments.

**Immunoprecipitations (IPs).** To immunoprecipitate full-length VP35 proteins, lysates from transfected cells were incubated with 1 µg of anti-VP35 monoclonal antibody overnight at 4°C, followed by 1 h incubation with protein G-Sepharose beads (Roche). The beads were washed five times with lysis buffer. After the beads were washed, they were resuspended in SDS-PAGE sample loading buffer, separated by 10% SDS-PAGE, and analyzed by Western blotting, as indicated.

**MBP-VP35 IID fusion protein interactions with NP.** Forty-five micrograms of pcDNA3 EBOV NP was transfected into 3 x 10^7 HEK293T cells. At 24 h posttransfection, the cells were lysed in 4 ml NP-40 lysis buffer (50 mM Tris-HCl [pH 8], 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, and 10% glycerol with protease inhibitors). 350µl of clarified lysate was incubated with the VP35 IID domain fused with MBP or equivalent amounts of the MBP-mutant IID fusion proteins at 4°C. Twenty-four hours later, MBP fusion proteins were bound with 40 ml of amylose resin for 30 min. The resin was washed three times, and MBP fusion proteins were eluted with 100 ml of 10 mM maltose. A fraction of the elution was analyzed by Western blotting. MBP was detected with an anti-MBP antibody (New England Biolabs), and NP was detected with the previously described (see above) anti-NP mouse monoclonal antibody. To determine if the MBP-VP35 IID fusion proteins could interact with NP in the presence of dsRNA,
lysates expressing NP were generated as described above. Before addition of the MBP-VP35 IID protein to the cellular lysate, increasing amounts of poly(I:C) (Invitrogen) were first added to the lysate. Poly(I:C) was added at a concentration of either 16 or 98 nM, while either WT or mutant VP35 IID was used at a concentration of 2.4 µM. Following a 24-h incubation period, samples were processed as described above.

2.3 Results

2.3.1 FBP is not important for dsRNA binding or immune inhibition

The structure of eVP35 IID was solved to 1.4 Å and revealed a novel RNA binding fold comprised of an alpha helical and beta sheet sub-domain (Leung et al. 2009). Residues K222, R225, K248, and K251 form the FBP, which is located on the alpha helical sub-domain, and residues R312, K319, and R322 are three primary residues that form the CBP, which is located on the beta sheet sub-domain (Figure 2.1). The CBP residues had previously been shown to be important for dsRNA binding and host immune inhibition (Leung et al. 2010), but it was not clear what role, if any, the FBP played in VP35 mediated functions. In order to assess the functional relevance of the FBP and determine whether FBP mutations (R225A, K248A, and K251A) affected the structural integrity of eVP35 IID or its ability to bind dsRNA, we carried out NMR and ITC studies. ¹H/¹⁵N HSQC spectra were collected for each FBP mutant and compared to the corresponding eVP35 IID WT spectra. Figure 2.2 displays the 2-D HSQC spectra for the FBP mutants alone and overlaid with eVP35 IID WT. Each chemical shift in a ¹H/¹⁵N HSQC spectra arises from a non-proline amide group and is sensitive to its immediate surroundings or local environment. As expected, the alanine substitutions
gave rise to small chemical shift perturbations due to the changes in local environment at the site of mutation, but overall no global changes to the protein fold were observed. Consistent with these structural studies, our ITC data shows that the FBP mutants retain their ability to bind dsRNA. Alanine substitution mutants R225A, K248A, and K251A bound 8 base pair (bp) dsRNA comparable to wild-type eVP35 IID with $K_D$ values of 0.7 µM, 1.0 µM, 2.4 µM, 0.8 µM, for eVP35 IID WT, R225A, K248A, and K251A, respectively (Figure 2.3). Together, these data clearly show that mutations to FBP residues cause minimal perturbations to the overall structure of eVP35 IID and have no effect on dsRNA binding.

### 2.3.2 FBP residues are involved in EBOV replication

In addition to its role in host innate immune suppression, VP35 is a critical component of the filoviral polymerase complex. In contrast to the CBP, the FBP was shown to be not important for binding dsRNA or inhibiting the production of IFN-β. In order to determine the role, if any, of the FBP in viral replication, we employed an EBOV minigenome assay (Figure 2.4). In this assay, an EBOV minigenome is constructed by flanking a reporter gene (GFP-CAT or luciferase) with the 3’ and 5’ EBOV leader and trailer sequences which contain the necessary cis-acting elements required by the EBOV replication complex. The viral polymerase complex is reconstituted by the expression of viral proteins NP, VP35, VP30, and L, and if these proteins come together to form a functional polymerase complex, then the minigenome can serve as the template for RNA synthesis ultimately resulting in the expression of the reported gene
As has previously been shown, the presence of VP35 was required for minigenome activity. FBP mutants R225A, K248A, and K251A completely abolished minigenome activity, whereas the mutant K222A retained its polymerase co-factor function similar to WT (Figure 2.5). These results indicate that three out of the four FBP residues, R225, K248, and K251, are important for the VP35 polymerase cofactor function.

In addition to the alanine substitution mutants, we also generated glutamic acid substitution mutants and lysine-to-arginine or arginine-to-lysine mutants. All of the eVP35 IID glutamic acid substitution mutants expressed to comparable levels, and similar to the alanine substitution mutants, lost their ability to support minigenome activity (Figure 2.6A). In contrast to the alanine and glutamic acid substitution mutants, lysine-to-arginine or arginine-to-lysine mutations, in which lysine residues were mutated to arginine and vice versa, showed that the FBP basic residues are interchangeable as these mutation have little to no effect on minigenome activity (Figure 2.6B). Therefore, the basic charge of the FBP residues, and not the identity of the residues, is the important factor in determining eVP35 IID functionality in the minigenome assay.

2.3.3 FBP residues are involved in NP interaction

Previous work by Becker et al. has established an interaction between NP and VP35 as indicated by the co-localization of both proteins into NP induced inclusion bodies (Becker et al. 1998). To determine whether the inability of the VP35 FBP mutants to function in the minigenome assay could be explained by the loss of interaction with NP, we employed a combination of co-immunoprecipitation (Co-IP) and
pulldown assays. For the co-immunoprecipitation assays, NP was co-transfected into HEK293T cells in the absence or presence of WT or mutant eVP35s. FBP mutants R225A and K248A displayed no detectable interaction with NP, whereas mutant K251A displays a significantly diminished ability to interact with NP (Figure 2.7A). However, mutants R225A and K248A expressed at a lower level compared to WT and displayed an unusual migration pattern, running unexpectedly high on the SDS-PAGE gel. In addition to the FBP mutants, CBP residues, 'end-capping' residues, and conserved basic residues bordering the CBP (border basic residues) were assessed for their ability to interact with NP. With the exception of H240A, all of these additional mutants maintained the ability to bind NP. Similar to R225A and K248A, H240A displayed an unusual migration pattern on the SDS-PAGE gel. Follow up experiments investigating the H240A mutation in the context of eVP35 IID revealed stability issues, possibly explaining its inability to bind NP in the present assay. Consistent with the Co-IP results, a MBP pulldown assay showed that MBP fused eVP35 IID (MBP-eVP35 IID) protein was able to interact with NP whereas MBP alone cannot, indicating that the IID alone is sufficient to interact with NP (Figure 2.7B). Moreover, the MBP-eVP35 IID R225A and K248A mutants were expressed to comparable levels relative to WT yet still displayed no detectable interaction with NP, therefore allowing us to more accurately conclude that the loss of minigenome activity exhibited by eVP35 mutants R225A, K248A, and K251A is due to loss of critical contacts with NP.

VP35 establishes multiple interactions in the context of both host immune evasion and viral replication. It is unknown whether VP35 can carry out both functions simultaneously or if it partitions between the two functions. In order to address this
question, we tested the ability of VP35 IID to bind NP the absence or presence of dsRNA (Figure 2.7C). To this end, MBP-eVP35 IID was bound to NP, and incubated with increasing amounts of poly I:C. MBP-eVP35 IID WT displayed a diminished ability to bind NP in the presence of poly I:C, but dsRNA binding mutants F239A, R312A, and R322A were unaffected. These results suggest that the dsRNA binding function of VP35 can influence the VP35-NP interaction in a way where both of these binding interactions may not be able to occur concurrently. More work is needed to understand mechanistically how VP35 carries out its multiple function. Specifically the use of RNA ligands which better mimic the physiological dsRNA recognized by VP35 would benefit these studies as it has been shown that small dsRNA (18mers) cannot replicate the results presented here for poly I:C (data shown in Figure 4.16).

2.3.4 VP35 FBP and CBP residues are not important for VP35-L interaction

Initial studies with MARV, and subsequent studies with EBOV, have established that NP, VP35, and L are the minimal components required for viral replication (Muhlberger et al. 1998). These studies have clearly demonstrated interactions between NP-VP35, NP-VP30, VP35-L, and NP-VP35-L, supporting a model in which VP35 interacts with both NP and L, thus tethering the polymerase and its template together. Due in part to its large size, studies with the viral polymerase have been limited. However, it was been shown for MARV L that the N-terminal 1-503 residues are sufficient to interact with MARV VP35. Translating these findings from MARV to EBOV, we were able to show that the first 1-505 residues from EBOV L are sufficient to interact with eVP35. Using our panel of eVP35 mutants we assessed the importance of these
residues in the VP35-L interaction. All the tested mutants maintained the ability to interact with L (Figure 2.8) indicating that FBP residues, CBP residues, border basic residues, and 'end-capping' residues are not important for the VP35-L interaction. Furthermore, it has been shown that homo-oligomerization of MARV VP35 through its N-terminus is required for the MARV VP35-L interaction, hence the use of full-length eVP35 in the present studies (Moller et al. 2005). These data together with the pulldown results strongly suggest that the VP35-L interaction may be mediated through the N-terminus of VP35 with little to no contribution from the C-terminus.

2.4 Conclusions

The molecular basis for protein-protein and protein-RNA interactions that establish the polymerase complex, as well as the molecular switch between replication and transcription are currently unknown. Work described here begins to shed light on the critical VP35-NP interaction, and identifies the importance of the VP35 FBP residues in viral replication. The EBOV RNA-dependent RNA polymerase consist of four viral proteins: NP, VP35, VP30, and the polymerase, L. Characterization of the eVP35 IID FBP revealed that unlike the CBP, the FBP is not required for dsRNA binding or inhibition of IFN-β production. Instead, the present work demonstrates a critical function for the FBP residues in viral replication/transcription, particularly in establishing an essential interaction with NP. Mutagenesis studies showed that either alanine or glutamic acid substitution mutants to the FBP residues R225, K248, and K251 resulted in the loss of minigenome activity. In contrast, mutations from either lysine to arginine or arginine to lysine had no effect on minigenome activity indicating that the basic nature of
these residues is sufficient to support the polymerase cofactor function of eVP35. Consistent with these findings, the minigenome defective eVP35 mutants lost their ability to interact with NP. It has been well established in the literature that VP35 and NP form a critical interaction (Becker et al. 1998; Muhlberger et al. 1998; Watanabe et al. 2006; Shi et al. 2008), resulting in a current working model that assumes VP35 binds to both NP and L, and in doing so, structurally bridges the polymerase and its template together. However, additional studies are required to pinpoint the determinant factors in the VP35-NP interaction and to resolve the NP residues involved in the VP35-NP interaction. Together these results further our understanding of the protein-protein interactions that establish the EBOV polymerase complex, in particularly the eVP35-NP interaction, and our data supports the current model in that the loss of the VP35-NP interaction exhibited by the FBP mutants results in loss of minigenome activity.
2.5 Figures

Figure 2.1. Highly conserved basic residues within eVP35 IID are localized to the FBP and the CBP. (A) Ribbon representation of eVP35 IID WT (PDB ID: 3FKE) highlighting FBP residues (left) and CBP residues (right). (B) Corresponding electrostatic surface representation of the FBP and the CBP (scale, -10 kT/e to +10 kT/e).
Figure 2.2. Mutations to the FBP residues do not significantly perturb the overall structure of eVP35 IID. $^{1}$H/$^{15}$N HSQC spectra were collected for eVP35 IID (A) WT (black), and FBP mutants proteins (B) R225A (blue), (C) K248A (red), and (D) K251A (green) on a Bruker Avance II spectrometer [700.13MHz]. Spectra were overlaid with VP35 IID WT in NMRView and revealed minimal chemical shift perturbations.
Figure 2.2 continued.

Figure 2.2. Mutations to the FBP residues do not significantly perturb the overall structure of eVP35 IID. $^1$H/$^{15}$N HSQC spectra were collected for eVP35 IID (A) WT (black), and FBP mutants proteins (B) R225A (blue), (C) K248A (red), and (D) K251A (green) on a Bruker Avance II spectrometer [700.13MHz]. Spectra were overlaid with VP35 IID WT in NMRView and revealed minimal chemical shift perturbations.
Figure 2.3. eVP35 IID FBP mutants retain ability to bind dsRNA. ITC binding isotherms and corresponding raw data for 8-bp dsRNA binding to VP35 IID (A) WT, (B) R225A, (C) K248A, and (D) K251A. The corresponding average K_D values for WT, R225A, K248A, and K251A are 0.7, 1.0, 2.4, and 0.8 µM, respectively.
Figure 2.4. Schematic model for the EBOV minigenome assay. The EBOV minigenome is constructed by flanking a reporter gene (GFP-CAT or luciferase, shown in yellow) with the 3' and 5' EBOV leader and trailer sequences (dark grey) which contain the necessary cis-acting elements required by the EBOV replication complex. The EBOV polymerase complex is reconstituted by transfecting of plasmids for viral proteins NP, VP35, VP30 and L in the presence of the minigenome. Co-transfected is a plasmid that expresses firefly luciferase from an RNA polymerase II promoter, and the reporter gene activity is normalized to the firefly luciferase activity. If the EBOV proteins are able to come together and form a functional polymerase complex, the minigenome serves as a template for both replication and transcription ultimately resulting in the expression of luciferase, which provides the readout for minigenome activity.
Figure 2.5. Three out of four eVP35 FBP residues are critical for polymerase cofactor function. Alanine substitution mutants were generated for the FBP residues K222, R225, K248, and K251A and tested in the EBOV minigenome assay. In the absence of VP35 (empty vector, -) no minigenome activity was observed. Increasing concentration (63ng, 125ng, and 250ng) of VP35 WT or mutant plasmids were transfected into cells, and 250ng was identified as the optimal concentration and set to 100% minigenome activity. Alanine substitution mutants for eVP35 R225, K248, and K251 resulted in loss of minigenome activity. Error bars represent 1 standard deviation for three experiments. Cell lysates were Western blotted (WB) for VP35 expression (lower panel).
Figure 2.6. The basic charge of the eVP35 FBP residues is the determinant for functionality in minigenome assay. In the absence of VP35 (empty vector, -) no minigenome activity is observed. (A) Glutamic acid substitution mutants for FBP residues R225, K248, and K251 lost their polymerase cofactor function. Increasing concentration (63ng, 125ng, 250ng, and 500ng) of VP35 WT or mutant plasmids were transfected into cells, and 250ng was identified as the optimal concentration and set to 100% minigenome activity. (B) eVP35 mutants R225K, K248R, and K251R maintained the ability to support minigenome activity. 125ng, 250ng, 500ng of eVP35 WT or mutant plasmids were transfected into cells, and data were expressed as the fold induction over empty vector. Error bars represent standard deviations, and the lower panel for both (A) and (B) show the corresponding Western blots (WB) for eVP35.
Figure 2.7. Replication defective eVP35 FBP mutants lose their ability to interact with NP. (A) Cells were transfected with NP in the absence (-) or presence of full-length eVP35 WT or mutant proteins. Cell lysate was immunoprecipitated using anti-VP35 antibody and proteins were detected using anti-MBP (α-MBP) and anti-NP (α-NP) antibodies. (B) MBP alone or MBP-tagged eVP35 IID WT or mutant proteins were immobilized on amylose resin and assessed for their ability to pull down NP from clarified lysate of NP expressing cells. Proteins were detected as describe in (A). (C) MBP-eVP35 IID WT or mutant proteins were incubated with lysate of NP expressing cells in the absence (-) or presence of increasing amounts of poly I:C (16nM, 98nM). MBP-eVP35 IID was immobilized on amylose resin and proteins were detected as described in (A).
Figure 2.8 eVP35 IID mutants retain ability to interact with EBOV L. (A) Cells were transfected with HA-tagged EBOV L 1-505 in the absence (−) or presence of full-length eVP35 WT or mutant proteins. Cell lysate was immunoprecipitated using anti-VP35 antibody and proteins were detected using anti-MBP (α-MBP) and anti-HA (α-HA) antibodies. Whole cell extract (WC).
2.6 References


CHAPTER 3. APTAMERS IN VIROLOGY

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I wrote the initial draft of the review and figures with input and advice from Dr. Gaya Amarasinghe and Dr. Daisy Leung.

3.1 Introduction

Owing to it multiple functions and interaction partners necessary to carry out innate immune suppression and viral replication, VP35 is a potential, and promising, therapeutic target. In order to target VP35 and inhibit known VP35 functions, our lab has employed two approaches using both small molecules and RNA aptamers. In the context of this dissertation, I will focus on the later, and in this chapter highlight the utility and advances of aptamers in virology. Aptamers are *in vitro* selected nucleic acid molecules that bind to a broad range of targets with high affinity and specificity. The basic isolation process, termed 'systematic evolution of ligands by exponential enrichment' or SELEX (Ellington et al. 1990; Robertson et al. 1990; Tuerk et al. 1990), utilizes a large initial pool of randomized nucleic acids from which high affinity binders are isolated and enriched through subsequent rounds of selection (Figure 3.1). Most aptamers characterized to date are single stranded DNA or RNA molecules ranging from 20-90 bases that can bind targets ranging from a few atoms (small molecules) to
many atoms (macromolecules) to intact cells and viruses. These single stranded polynucleotide sequences can fold into a variety of secondary structural elements, including double stranded RNA (dsRNA) or dsDNA, stem loops, pseudoknots, kinks, and bulges, providing multiple recognition surfaces for target binding.

Aptamers are often compared to antibodies, due to the high specificity for their targets. However, aptamers have notable advantages over antibodies, including low immunogenicity and the potential for chemical derivatization to enhance in vivo stability and bioavailability (Jellinek et al. 1995; Chelliserrykattil et al. 2004; Kato et al. 2005). The aptamer selection process can be carried out in as little as 2-4 weeks, which is significantly shorter than antibody development, which can take on the order of months. The general SELEX principles guiding the selection process have remained largely unaltered. However, identified aptamers can be further optimized using biased libraries and counter selection strategies that result in aptamer subpopulations with higher specificity and/or affinity and other desirable characteristics, such as enhanced stability or membrane permeability. In addition, facile generation of modified aptamers allows for coupling of diagnostic tags, which can extend the use of aptamers through prolonged lifetimes in serum. Given the traditional comparisons with antibodies, the utility of aptamers in the clinic is taking longer than anticipated from the outset of discovery. Nonetheless, with the 2004 FDA approval of pegaptanib, an aptamer-based drug used to treat wet age-related muscular degeneration, and others in the pipeline, it is clear that aptamers will continue to contribute to our understanding of normal and aberrant cellular processes as well as disease treatment. Recent comprehensive reviews highlight advances in basic research (James 2001; James 2007; Syed et al. 2010; Sun et al.
2011) and clinical settings (Bunka et al. 2006; Gopinath 2007; Gopinath 2008; Meyer et al. 2011). These developments have positioned aptamers to make a significant impact in many areas.

3.2 Aptamers in therapeutics

The host-viral interface has become an increasingly popular target for aptamers because interactions between nucleic acids and proteins are critical to viral replication (Figure 3.2). Although not a prerequisite, targeting nucleic acid binding proteins by aptamers has a high probability of success due to the presence of basic surface charges on most nucleic acid binding proteins. The remaining sections in this chapter summarize select studies in order to highlight the use of aptamers against viral components and discuss challenges and future prospects of the field.

By targeting essential viral proteins, aptamers provide a means for developing therapeutic agents to counter viral infections. Aptamers can potentially inhibit viral infectivity at any stage in the viral replication cycle (Figure 3.2). Attempts to target human immunodeficiency virus (HIV) through the use of aptamers has been of interest for a number of groups, and has yielded multiple aptamers which target various HIV proteins and various stages within the viral replication cycle (Held et al., 2006). In order to target HIV entry into helper T-cells, aptamers were generated against the HIV glycoprotein 120 (gp120). The anti-gp120 aptamers, B40, binds gp120 and in doing so competes with the HIV co-receptor, CCR5. The aptamer binding site is located in a highly conserved region of gp120, thereby, allowing neutralization of a broad range of HIV isolates by disrupting the gp120-CCR5 interaction (Dey et al. 2005; Dey et al.
Additionally, binding to a conserved region of gp120 is critical due to the propensity of HIV to undergo mutagenesis that results in drug resistance. The anti-gp120 aptamer has not been approved for clinical trials, but has been used as a basis for developing chimeric aptamers (Neff et al. 2011; Wheeler et al. 2011; Zhou et al. 2011). Coupling of siRNA and the anti-gp120 aptamer through phi29 packaging RNA allows incorporation of two functions into one molecule (Zhou et al. 2011). The anti-gp120 aptamer has an inhibitory effect, while the siRNA downregulates the expression of its target.

Viral entry of human cytomegalovirus (HCMV) has also been targeted via aptamers (Wang et al. 2000). Aptamers generated against HCMV virus particles was carried out without prior information on specific targets. Two aptamers, L13 and L19, were isolated which inhibited HCMV plaque formation and growth. The antiviral activity of L13 and L19 aptamers identified HCMV glycoproteins B and H as targets, respectively, by binding to the glycoproteins and blocking viral entry (Wang et al. 2000).

Viral RNA-dependent RNA polymerases have been a common target because viral polymerases use RNA as the template during genome replication. For example, the Hepatitis C virus (HCV) polymerase nonstructural protein 5B (NS5B), is required for transcribing the HCV genome and has been used to generate both anti-NS5B RNA and DNA aptamers (Biroccio et al. 2002; Bellecave et al. 2003; Bellecave et al. 2008). Both sets of aptamers have been shown to bind to NS5B with high affinity and inhibit polymerase activity. The mechanism of inhibition of the anti-NS5B RNA aptamer is non-competitive with regards to the template RNA, and mutagenesis studies revealed that the aptamer binding site is localized to a basic patch within the thumb domain of NS5B.
(Biroccio et al. 2002). On the other hand, two DNA aptamers were identified with one anti-NS5B DNA aptamer inhibiting replication by competing with the RNA template; the other inhibiting through a non-competitive mechanism in which initiation and post initiation events are disrupted. RNA aptamers capable of inhibiting NS5B polymerase activity were further optimized through the use of deletion clones and point mutagenesis, which identified a GC-rich motif and a stem-loop with a bulge as important features for aptamer binding to NS5B (Kanamori et al. 2009).

In the context of HIV pathogenesis, the viral reverse transcriptase (RT) converts the single stranded RNA (ssRNA) viral genome into dsDNA. tRNA aptamers that target HIV-RT effectively inhibit the synthesis of cDNA (Tuerk et al. 1992). The ligand 1.1 aptamer targets the HIV-RT, and is postulated to reduce adverse side effects, a main concern with current HIV-RT drugs, such as 3'-azido-3'-deoxythymidine (AZT) and dideoxyinosine. Moreover, by targeting an enzyme early in the HIV-1 replication cycle, the likelihood that mutations will arise resulting in drug-resistance should diminish. These factors make aptamers generated against HIV-RT promising candidates for drug development.

Inhibition of protein translation is a key host defense mechanism against viral infections since viruses are completely dependent on the host machinery for protein synthesis. However, viruses have developed various means to evade host defenses and hijack the host translational machinery. For example, the HCV mRNA has an internal ribosome entry site (IRES), a structured region within the mRNA that binds the ribosome and initiates cap-independent translation, allowing HCV to circumvent the requirement for host initiation and elongation factors. Aptamers targeting the HCV IRES
inhibit IRES-dependent translation of HCV proteins (Aldaz-Carroll et al. 2002; Kikuchi et al. 2003; Da Rocha Gomes et al. 2004; Kikuchi et al. 2005). Furthermore, many of these HCV IRES aptamers inhibit translation both in vitro and in vivo, highlighting the potential therapeutic value of these aptamers since they target RNA secondary structures that are characteristic of select viral mRNAs but not host mRNA. Therefore, such selection may result in a reduction of negative side effects. The viral inhibitory properties of HCV anti-IRES aptamers can be enhanced through conjugation to other aptamers, which target different binding sites within the HCV IRES. This results in a conjugated anti-IRES aptamer that binds the HCV IRES with a higher affinity and is functionally more effective than the unconjugated aptamers (Kikuchi et al. 2009). Alternatively, HCV anti-IRES aptamers can be coupled to the hammerhead ribozyme to prevent translation. The chimeric RNA molecule, HH363-50, inhibits IRES-dependent translation in vitro likely through disruption of 80S complex formation with no effect on IRES-independent translation. HH363-50 also reduced the level of HCV RNA due to the ribozyme activity of the molecule (Romero-Lopez et al. 2009).

3.3 Aptamers in viral diagnostics

Early and reliable detection of pathogens is a critical step in the successful treatment of infection. Due to many potential advantages aptamers provide, aptamers are ideal tools for diagnostics. Many examples of aptamers as a detection tool have been described. These studies include simple modifications to the enzyme linked immunosorbent assay (ELISA) as well as more complex diagnostic systems, such as
those that use inhibitory aptamers to suppress multiturnover enzymes (Zhou et al. 2010).

The Influenza A virus hemagglutinin (HA) and neuraminidase (NA) antigens are glycoproteins found on the surface of the viral particle, and serve important roles in host membrane fusion. A number of aptamers have been isolated against Influenza A HA that inhibit viral infectivity (Jeon et al. 2004; Misono et al. 2005; Gopinath et al. 2006; Dhar et al. 2009; Park et al. 2011). In addition to disrupting viral HA-mediated membrane fusion, the P30-10-16 aptamer can distinguish between closely related Influenza A strains. This aptamer property is novel, considering that most monoclonal antibodies against HA have been unsuccessful at differentiating among influenza subtypes. Development of these aptamers for viral subtype diagnostics will provide a significant advancement in our ability to differentiate highly pathogenic influenza strains from those that are less virulent. Further biophysical characterization to examine how this aptamer is able to differentiate between closely related influenza strains will likely yield important insights into its mechanism of action.

During hepatitis B viral (HBV) infection, the hepatitis B virus surface antigen (HBsAg) is found on the membrane of HBV-infected hepatocytes. An aptamer targeting HBsAg, HBs-A22, was fluorescently labeled and used to isolate cells expressing HBsAg. Use of fluorescence microscopy showed that that anti-HBsAg aptamer bound to the HBsAg-positive cell line (HepG2.2.15) but not to the HBsAg-negative cell line (HepG2). These results establish the use of aptamers for imaging, but also provide the first HBV specific antigen aptamer that could be used for early detection and treatment of HBV-infected cells (Liu et al. 2010).
Aptamers can also function as diagnostic tools when coupled to existing biosensors (Davis et al. 1998; Jhaveri et al. 2000; Fang et al. 2001; O'Sullivan 2002). Biosensors utilize a biological recognition element, such as aptamers, and a physiochemical transducer for easier detection and quantification. An aptamer based biosensor, or aptasensor, was isolated against the trans-activator of transcription (Tat) of HIV-1, which is important for regulating the early phases of HIV-1 infection (Chang et al. 1997; Mucha et al. 2002). The Tat aptasensor was generated by immobilizing the aptamer on a piezoelectric quartz-crystal, the physiochemical transduction component of the aptasensor. Comparison of the Tat aptasensor to a corresponding immunosensor that uses an anti-Tat monoclonal antibody showed that the two biosensors are similar in terms of sensitivity and reproducibility. Although the sensitivity of the aptasensor does not allow for detection of Tat in sera and cell culture supernatants, this methodology further establishes the potential use of aptamers in the generation of biosensors (Minunni et al. 2004; Tombelli et al. 2006). Further development of the aptasensors incorporating more stable chemical derivatives will likely overcome some of the current limitations of the aptasensors.

A multicomponent reporter system consisting of an inhibitory aptamer bound to a restriction endonuclease and a target complement/trigger system was successful in differentiating Dengue virus serotypes (Fletcher et al. 2010). One of the key advantages of this method is that the same aptamer/enzyme complex can be used since only the nucleic acid complement/trigger needs modification to detect new targets. Such a system can be more sensitive due to the multiturnover nature of the endonuclease.
Further development of these aptasensors and incorporating more stable chemical derivatives will likely overcome some of the limitations of current aptasensors.

### 3.4 Aptamers in basic research

In addition to diagnostic and therapeutic applications, aptamers can be used as laboratory reagents in a number of biochemical and cell-based assays, similar to antibodies. Aptamers have key advantages over antibodies in that they often have higher affinity and specificity for their targets with some binding constants ($K_D$) $<$1 nM, which results in an enhanced signal-to-noise ratio. Additionally, aptamers can be generated against almost any target ranging from small organic molecules to proteins to whole cells (Jayasena 1999), and they are often times smaller than antibodies (7-30 KDa for aptamers vs. ~150 KDa for antibodies) allowing them to bind regions inaccessible to antibodies.

Utilizing aptamers that disrupt protein-protein interactions, one can begin to dissect and characterize cellular pathways. The human T-cell leukemia virus type 1 Tax protein is a trans-activator that regulates the expression of various viral and cellular genes. Tax cannot directly bind DNA in the absence of certain host transcription factors, suggesting that protein-protein interactions are essential for Tax function. The YT1 aptamer disrupts Tax interactions with the cyclic AMP-response element binding protein (CREB) and NF-kappaB, but not serum response factor (SRF), all of which are known to interact with Tax proteins (Tian et al. 1995). These results suggest that the CREB and Nf-kappaB binding interfaces on Tax may potentially overlap or are in close
proximity, whereas the SRF binding site occupies a different surface on Tax. Therefore, aptamers provide a potential tool to map interaction surfaces between binding partners.

Assembly of the viral nucleocapsid (NC) requires multiple protein-protein and protein-nucleic acid interactions. The HIV NC is a highly conserved protein that plays a key role in RNA encapsidation and viral replication, and specifically binds viral genomic RNA through a unique sequence termed the psi (Ψ). An aptamer generated against NC competes with the psi sequence and prevents proper encapsidation of the viral genome, which likely inhibits proper packaging of the genomic RNA (Kim et al. 2002). Other HIV-1 NC aptamers against the HIV-1 Gag protein bind the matrix and the NC domains of Gag and reduced HIV replication in cultured cells. The inhibitory effect of these aptamers was not due to a defect in virion release but to the downregulation of intracellular Gag protein and mRNA. A subset of aptamers which specifically bound NC competes with the psi packing signal of HIV-1, and disrupts interactions between Gag and viral RNA (Ramalingam et al. 2011). Unlike other aptamers that inhibit early stages in viral infection, the NC aptamer targets events that occur after viral entry, and therefore, allows one to study the intracellular effects of viral infection.

Using aptamers to specifically target and modulate functions of proteins within cellular pathways or the viral replication cycle can provide invaluable information about these respective systems. Nonetheless, an additional advantage of using aptamers to investigate cellular signaling is the ability to selectively turn the aptamers “on” and “off” with effectors. Further advances in the spatial and temporal regulation of aptamers will be important for enhancing our understanding of viral/host interactions. The potential regulation of aptamers with effector molecules is highlighted by Vuyisich et al. in the
regulation of formamidopyrimidine glycosylase (Fpg), a bacterial enzyme involved in DNA repair (Vuyisich et al. 2002). A modified SELEX protocol was used where RNA-bound Fpg was treated with neomycin in order to elute the bound RNA. The Fpg aptamer inhibited Fpg activity, but the functionality was restored in the presence of neomycin. Rusconi et al. utilized anti-sense RNA in order to regulate the function of an aptamer targeted against the blood coagulation factor IXa (Rusconi et al. 2002). The anti-sense RNA base pairs to complementary regions within the aptamer and disrupts aptamer function. The factor IXa aptamer and anti-sense RNA antidote pair was an effective, reversible anticoagulant and shows potential for clinical application.

### 3.5 Conclusions

Many viral proteins, in particularly filoviral proteins, bind nucleic acids, which makes them ideal targets for aptamer development. As outlined above, aptamers have been used in various studies to characterize and counter host-viral interactions. For example, an aptamer targeting the HIV-1 Tax protein was able to differentiate Tax binding to specific transcription factors. Similarly, aptamers can be used to dissect protein-protein interfaces and to validate targets for small molecules. An advantage aptamers have over small molecules is their ability to be identified against a target without a priori knowledge of the exact site or target. Similarly, aptamers can be used in target validation, a particularly important area where aptamers can potentially provide a distinct advantage. Current selection of small molecules often depend on the availability of validating functional assays, structural and biochemical data, prior to initiating drug discovery efforts. Such efforts inherently consume large amounts of time and resource.
In contrast, one can develop aptamers towards a target with a fraction of the cost and time. However, many of the above examples have limited knowledge on the structural and biochemical aspects of these aptamer/target interactions, and available data are primarily restricted to secondary structure predictions or mapping. The addition of structural and biochemical studies of aptamer/target pairs can enhance the utility of these aptamers by providing new information on the binding interfaces and by providing information for structure-based optimization of aptamers. Continuing development and optimization of multifunctional and chimeric aptamers, and development of aptamer libraries for strain specific viral identification and coupling of aptamers to more sensitive detection methods will lead to more broadly applicable therapeutic and diagnostic applications in the future.
Figure 3.1 The SELEX process. An initial pool of dsDNA is in vitro transcribed into RNA. The resulting pool of RNA is subject to (A) counter selection to remove non-specific binders. (B) RNAs that binds to the target are selected by filter binding. (C) RNA bound to the membrane is recovered, reverse transcribed and amplified by PCR. (D) The resulting DNA pool is further subject to another round of SELEX for enrichment or cloned and sequenced.
**Figure 3.2** Key steps in the viral replication cycle are potential targets for aptamer development. Aptamers have been generated to target viral proteins found in both the extracellular and intracellular space. Examples of viral proteins with existing aptamers are identified in red type.
3.7 References


CHAPTER 4. DEVELOPMENT OF RNA APTAMERS TARGETING EBOLAVIRUS VP35

The research within this chapter consists of data that are published in *Biochemistry*.

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Dr. Tianjiao Wang and I wrote the initial draft of the manuscript, with extensive editing and final manuscript preparation from Dr. Daisy Leung. I was responsible for biochemically characterizing the binding of 1G8-14 and 2F11-14 to VP35 IID WT and mutant proteins, developing the NP pulldown assay to test the functionality of these aptamers *in vitro*, and cloning a series of aptamers for the minigenome studies which were conducted in the laboratory of Dr. Christopher Basler. Tianjiao Wang and I crystallized eVP35 IID FBP4mut and CBP3mut, respectively, and I solved the structures of these two proteins with help from Dr. Wei Xu.
4.1 Introduction

Negative stranded RNA viruses possess a limited number of open reading frames (ORFs), such that many of the encoded proteins function at multiple stages of the viral replication cycle. The multifunctional nature of these viral proteins potentially provides several opportunities to develop antiviral drugs. However, target validation of non-enzymatic viral proteins is particularly challenging as inhibitors must be identified de novo. Innovative methods that combine structure/function information can provide critical target validation prior to initiation of more effective small molecule-based methods.

Development of inhibitors that target key components in pathogens often requires detailed knowledge of structure and function or screening of large compound libraries with highly sensitive reporter(s). Nucleic acid aptamers provide an alternative method for inhibitor development as well as target validation (Lee et al. 2006; Kang et al. 2012). Aptamer identification and characterization does not require detailed knowledge of the target (Wang 2009; Bunka et al. 2010; Soontornworajit et al. 2011; Binning et al. 2012). However, in situations where detailed structural and functional information are available, aptamer development can be tailored to enhance the impact of aptamer binding by targeting aptamer interactions to functionally important regions. Using such an approach, here we report the development of aptamers against eVP35 IID. Previous structural and functional results were utilized to identify functionally relevant regions of VP35 to target for aptamer development. Aptamers were identified using SELEX (Ellington et al. 1990; Tuerk et al. 1990) methods by targeting WT eVP35
IID and a mutant form of the protein. Two aptamers, representing two distinct binding modes, were selected for further characterization. These studies revealed that one aptamer (1G8-14) preferentially binds EBOV and RESTV VP35 IID proteins, while the other (2F11-14) binds both EBOV and MARV VP35 IID proteins. Using in vitro studies, we demonstrate the utility of our selected aptamers as potential inhibitors, which can disrupt a critical protein-protein interaction in the filoviral replication complex and the activity of the minigenome system in cells. Taken together, our results suggest that aptamers identified here are valuable reagents for basic research and target validation of filoviral VP35 proteins for antiviral development.

4.2 Materials and Methods

Protein expression and purification. Mutant eVP35 IID proteins were generated by overlap PCR and the plasmids were sequenced to confirm the mutation(s). Recombinant eVP35 IID WT and mutant proteins were expressed as previously described (Leung et al. 2010; Ramanan et al. 2012). The integrity of the proteins was assessed by $^1$H/$^{15}$N-HSQC NMR spectra.

Crystallization and structure determination. Initial crystallization conditions for eVP35 IID CBP3mut (where CBP residues R312, K319, and R322 are mutated to alanine) and FBP4mut (where FBP residues K222, R225, K248, and K251 were mutated to alanine) were identified using commercially available crystallization screens (Hampton Research), and in-house optimized native crystals were grown at 25 °C using
the hanging-drop vapor diffusion method. eVP35 IID CBP3mut (16.4 mg/mL) was diluted in a 1:1 ratio with reservoir solution containing 2.2 M Na/K phosphate (pH 4.5). eVP35 IID FBP4mut (24 mg/mL) was diluted in a 1:1 ratio with reservoir solution containing 0.2 M MgCl₂, 0.1 M HEPES (pH 7.5), 30% PEG 400. Crystals were soaked in reservoir solution containing 25% glycerol and vitrified in liquid nitrogen. Diffraction data was collected at Advanced Photon Source (Sector 19) at 100 K (Table 1). One hundred and eighty frames of data were collected with a frame width of 1.0° and detector-to-crystal distance of 300 mm for eVP35 IID FBP4mut. 450 frames of data were collected with a frame width of 0.2° and detector-to-crystal distance of 250 mm for eVP35 IID CBP3mut. Diffraction data were indexed, scaled and merged using HKL-3000 (Otwinowski et al. 1997). Intensities were converted to structure factors using CCP4 (Collaborative Computational Project 1994). The structures were solved using molecular replacement, with chain B from eVP35 IID crystal structure (PDB ID 3FKE) as the search model using MOLREP (Collaborative Computational Project 1994). The structures were refined using REFMAC5 (Murshudov et al. 1997) and PHENIX (Adams et al. 2010). Addition of solvent molecules and manual model building was performed using Coot (Emsley et al. 2004). TLS parameters were refined using the TLMSD server (Painter et al. 2006). Final validation was performed using MOLPROBITY server (Chen et al. 2010).

**In vitro selection of aptamers.** *In vitro* SELEX selection of aptamers was carried out using eVP35 IID wildtype (WT) and eVP35 IID CBP3mut as targets (Fitzwater et al. 1996; Jhaveri et al. 2001). Oligonucleotide templates and primers were chemically
synthesized with standard desalting by Integrated DNA Technology (Coralville, IA). The dsDNA library was generated from a ssDNA library (5`-GCCTGTGTGAGCCTCCTGTGCGAA(N45)TTGAGCGTTATTTCTGCTCCC-3`). Using the dsDNA library as a template, RNAs were in vitro transcribed following established protocols. The transcribed RNA was purified by phenol/chloroform extraction followed by ethanol precipitation and separated out on a 7M urea-8% PAGE. Purified RNAs were incubated with either eVP35 IID WT or eVP35 IID CBP3mut at 37°C for 0.5 to 1 hr in 10 mM HEPES (pH 7.0), 150 mM NaCl, 2 mM TCEP (tris(2-carboxyethyl)phosphine), and 1 mM MgCl₂ and filtered through nitrocellulose membrane (Millipore). The protein-bound RNA was eluted with 7M urea, precipitated by ethanol, and reverse transcribed using SuperScript™ reverse transcriptase (Invitrogen). The reverse transcribed cDNAs were amplified by polymerase chain reaction (PCR) using oligo-003 (5`-TAATACGACTCACTATAGGGAGACAAGAATAAACGCTCAA-3`) and oligo-004 (5`-GCCTGTGTGAGCCTCCTGTGCGAA-3`). In vitro transcription, RNA-protein binding, partition, and RT-PCR were repeated for 14 rounds and the resulting binders at rounds 10 and 14 were subcloned into the pCR-XL-TOPO vector (Invitrogen) for sequencing. Negative selection was carried out against the nitrocellulose membrane at rounds 3, 6, 9, and 12 to reduce non-specific interactions. The protein concentration for eVP35 IID WT was decreased from 0.4 µM at round 1 to 0.025 µM by round 14 in order to enhance selection stringency. For eVP35 IID CBP3mut, the protein concentration was decreased from 4 µM at round 1 to 0.05 µM by round 14. The concentration of protein used in each selection was adjusted so that we can obtain about 1% retention of aptamers during each selection. Thus, the initial values of 0.4 µM
for selection against eVP35 IID WT and 4 µM for selection against eVP35 IID CBP3mut were determined through this process.

**End labeling of dsRNA and aptamers.** $^{32}$P 5'-'end labeling of RNAs was carried out at 37°C for 1.5 hrs in a 20 µl reaction containing 70 mM Tris (pH 7.6 at 25°C), 10 mM MgCl$_2$, 5 mM DTT, 1U/µl T4 polynucleotide kinase (New England Biolabs), 1 µM γ-ATP (Perkin Elmer), 5 µM RNA. $^{32}$P 3'-end labeling of RNA was carried out at 16°C for 18 hr in a 30 µl reaction of 50 mM Tris-HCl (pH 7.8 at 25°C), 10 mM MgCl$_2$, 10 mM DTT, 1 mM ATP, 10% DMSO, 0.67 U/µl T4 RNA ligase 1 (New England Biolabs), 2 µM $^{32}$P-cytidine-3',5'-bis-phosphate (32pCp), 5 µM RNA (dsRNA or aptamer) (Peters et al. 2006). Labeled RNAs were separated on a 7M urea-8% PAGE and eluted by crush soak method.

**Filter binding assays for binding and competition assays for eVP35 IID-aptamer interaction.** Filter binding assays were carried out as reported previously (Ramanan et al. 2012). For dissociation constant (K$_D$) measurements, the reaction mixtures contained 1-2 nM $^{32}$P-aptamer or $^{32}$P-dsRNA and eVP35 IID protein ranging from 0-100 µM. For single binding point assays, the reaction mixtures contained 10 nM $^{32}$P-aptamer and 0.5 µM eVP35 IID proteins. In the competition assay, the reaction mixtures included 10 nM $^{32}$P-aptamer, 0.5 µM eVP35 IID or 10 nM $^{32}$P-dsRNA, 10 µM eVP35 IID and unlabeled aptamer or dsRNA ranging from 0-20 µM. All reaction mixtures were incubated at 25°C for 15 min. Binding experiments were carried out in buffer containing 10 mM HEPES (pH 7.0), 150 mM NaCl, 2mM TCEP, 1 mM MgCl$_2$ in 50 µl reactions in a 96-well
vacuum filtration apparatus assembled with a nitrocellulose membrane on top of a positively charged nylon membrane. Membranes were washed and air-dried before exposure for 12-16 hrs along with a calibration standard, scanned on a Typhoon 9410 Variable Mode I Imager in phosphor storage mode, and quantified by ImageQuant (GE Healthcare). The ratio of the intensity on nitrocellulose membrane to that of the sum on nitrocellulose membrane plus nylon membrane was expressed as fractional binding. The binding curves were fit to the equation \( B = \frac{B_{\text{max}}}{1 + \frac{[M]}{K_D}} \) using ORIGIN 7.0 software (OriginLab), where \( B \) is the fraction bound, \( B_{\text{max}} \) is the maximum fractional binding, \([M]\) is the protein concentration, and \( K_D \) is the dissociation constant. Competition curves were fitted to the equation \( \frac{B}{B_0} = \frac{1}{1 + \frac{[M]}{IC_{50}}} \)), where \( B \) is the fraction of \( ^{32}\)P-labeled aptamers bound to nitrocellulose membrane at different concentrations of competitors (aptamer or dsRNA), \( B_0 \) is fraction of \( ^{32}\)P-labeled aptamers bound to nitrocellulose membrane without any competitors, \([M]\) is the competitor concentration, \( IC_{50} \) is the concentration where 50% competition occurs, and \( n \) is a constant.

**Isothermal titration calorimetry (ITC).** Quantitative analysis of eVP35 IID protein-aptamer interactions were performed on a VP-isothermal titration calorimeter (VP-ITC) (Microcal). Protein samples were dialyzed against 500 mL of buffer containing 10 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM MgCl₂, and 2 mM TCEP for 12 h at 25 °C. Aptamer stocks were diluted >200-fold with dialysis buffer. The syringe contained eVP35 IID proteins at 100 to 150 µM, and the cell contained aptamers (1G8-14, 2F11-14) at 3 to 5 µM. ITC titrations were carried out using a reference power of 5 µcal/sec.
The resulting ITC data were processed and fit to a one-site binding model or a two-site binding model to determine n (number of binding sites) and $K_D$ (dissociation constant) using ORIGIN 7.0 software. Both one and two site binding models were considered for each data set, but selection of the model was based on the quality of the fits. For two site binding, both sequential and independent binding modes were also considered. The two-site binding model used in this study assumes two thermodynamically independent binding events.

**Pulldown assays.** Pulldown assays were performed in buffer containing 10 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM MgCl$_2$, and 5 mM 2-mercaptoethanol at 25 °C. MBP-His tagged eVP35 IID WT or mutant proteins were immobilized on amylose resin. Resin bound MBP-His tagged eVP35 IID was incubated with purified His-tagged NP protein and subsequently washed. For aptamer competition assays, either 100 µM aptamer or 300 µM dsRNA was incubated with MBP-His tagged eVP35 IID prior to incubation with NP. The level of protein used in the assay reflects our efforts to ensure proper detection of the pulldown experiment. Pulldown samples were separated on SDS-PAGE and analyzed by Western blot using mouse anti-His antibody (Santa Cruz biotechnology), followed by horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (Bio-Rad). Membranes were developed using Millipore Immobilon Western Chemiluminescence HRP substrate and recorded on a ChemiDoc (Bio-Rad).

**EBOV transcription/replication assays.** The plasmids used in the EBOV transcription/replication assays were described previously (Muhlberger et al. 1999;
Leung et al. 2010; Prins et al. 2010). NP, VP35, VP30 and L proteins were cloned into pTM1 with only the cDNA sequence (no virus-derived UTRs were present). Aptamers were cloned into a modified pSilencer plasmid (Ramalingam et al. 2011) and the corresponding plasmids were sequenced to verify the accuracy of the aptamers.

**Quantitation of total aptamer copy number in transfected cells by qRT-PCR.** Genome equivalent (GEq) copy numbers (total copies,) of aptamers in the minigenome assay were measured using primer/probes targeting the aptamers. Briefly, real-time PCR (RT-PCR) was performed using Superscript II RT-PCR kits (Invitrogen). All RT-PCR mixtures contained 5 µl of RNA eluate and master mixes were set up following the manufacturer’s protocols. Standards and test samples were assayed in triplicate using the CFX96 detection system (Bio-Rad) with the following cycle conditions: 50°C for 10 min, 95°C for 5 min, and 40 cycles of 95°C for 10 sec and 59°C for 30 sec. Threshold cycle (CT) values representing aptamers were used to determine Total copies. To create the GEq standard, DNA from aptamers stocks (100 ng) was used and the number of genomes was calculated using Avogadro’s number and the total molecular weight of the plasmid with aptamer.

**Aptamer secondary structure prediction.** The computational program RNAfold from Vienna package v.2.0 (Gruber et al. 2008) was used to predict the secondary structures for RNA aptamers 1G8-14 and 2F11-14. Default parameters on the RNAfold server were used except for the Turner Model 2004 (Mathews et al. 1999), which was used as
the RNA parameter source. Results were assessed for the minimum free energy (MFE) structure prediction as well as thermodynamic ensemble predictions, including centroid structure (Ding et al. 2005) and base-pairing probabilities using dot plot analysis (McCaskill 1990) as implemented in RNAfold. Resulting secondary structures for the MFE and the centroid were visualized with the RNA graphics program VARNA (Darty et al. 2009).

**Circular dichroism (CD).** CD data were collected for 1G8-14 and 2F11-14 at 10 µM in a quartz cuvette with a 1 mm path-length using a Chirascan™ CD spectrometer. CD spectra were acquired from 200 to 320 nm at 5 and 95 °C at an interval of 1 nm and time-per-point of 2 sec. Temperature melting of aptamers was performed by monitoring the ellipticity at 260 nm from 5 to 95 °C with a 2 °C increment at a 1 °C/min gradient. ORIGIN 7.0 software was used to plot CD spectra and to calculate first derivatives from the temperature melting plots.

### 4.3 Results

**4.3.1 Mutation to FBP and CBP residues does not perturb the overall fold of eVP35 IID**

The CBP and FBP contain highly conserved basic residues in VP35 IID that are functionally important as mutations of either the CBP or the FBP impair several VP35 mediated functions (Leung et al. 2010). Therefore, a series of structural studies were
carried out to characterize the impact of these mutations on the integrity of the structure of eVP35 IID. The eVP35 IID FBP4mut and CBP3mut were expressed, purified, and concentrated to 24 mg/ml and 16.4 mg/ml, respectively, and subsequently used for crystallographic studies. Commercially available Hampton crystal screens were used to identify initial crystallization conditions using the hanging-drop vapor diffusion method. eVP35 IID FBP4mut initially crystallized in 0.2 M MgCl₂, 0.1 M HEPES (pH 7.5), 30% PEG 400, yielding crystals suitable for data collection. eVP35 IID CBP3mut initially crystallized in 0.1 M HEPES sodium (pH 7.5), 0.8 M sodium phosphate monobasic monohydrate, 0.8 M potassium phosphate monobasic, and after several optimization steps, crystals were obtained in 2.2 M Na/K phosphate (pH 4.5), which yielded diffraction data suitable for structure determination of the protein (Figure 4.1). These studies resulted in crystal structures of eVP35 IID CBP3mut (PDB code 4IJE) and FBP4mut (PDB code 4IJF), which were solved to a resolution of 1.9 and 2.5 Å, respectively using the eVP35 IID WT (PDB code 3FKE) structure as the search model for molecular replacement (Table 4.1). These structures revealed minimal changes in the overall protein fold when compared to eVP35 IID (Figure 4.2). The most notable difference between the structures was observed in a short helix (α5) located within the β-sheet subdomain, in which the CBP3mut and FBP4mut structures make one turn of the helix instead of two. Comparison of the eVP35 IID WT, CBP3mut, and FBP4mut structures revealed that eVP35 IID CBP3mut and eVP35 IID FBP4mut structures display RMSD values of 0.68 and 0.46 Å, respectively, which further suggest minimal structural changes upon mutating out the CBP and FBP. Analysis of surface electrostatics, however, showed significant differences among the three structures
(Figure 4.3). As expected, the CBP3mut resulted in a loss of the basic charge at the CBP, but the FBP was relatively unaffected, while the structure of FBP4mut revealed that the basic charge at the FBP was lost with the CBP unaffected.

4.3.2 SELEX identifies RNA aptamers that bind eVP35 IID

In order to generate different classes of aptamers for eVP35 IID, aptamer selection was carried out against two targets, eVP35 IID WT and eVP35 IID CBP3mut proteins using SELEX (Figure 4.7A). By using eVP35 IID WT and CBP3mut proteins, we biased the aptamer selection to different conserved regions of eVP35 IID. We expected that the dsRNA binding site formed by CBP residues would dominate aptamer selection using eVP35 IID WT as a target due to the highly basic charge and dsRNA binding properties in this region. In contrast, by mutating the CBP (in eVP35 IID CBP3mut), we reduced the basic nature of the CBP region with the intent that other charged regions, preferably the FBP, could compete during aptamer selection. The initial RNA pool contained RNA species of approximately 90 bases, with 45 nucleotides in the variable region flanked on either side by a 5` and a 3` common region. This initial pool bound eVP35 IID WT with a \( K_D \) of 2900 ± 200 nM and bound eVP35 IID CBP3mut with a \( K_D \) of 36000 ± 8000 nM. After several rounds of SELEX, each aptamer pool displayed enhanced affinity and specificity for eVP35 IID WT and eVP35 IID CBP3mut proteins. Our progress curves show >10-fold affinity enhancement for the final aptamer pool, with an affinity of 300 ± 100 nM at round 14 for eVP35 IID WT (Figure 4.4A) and 2800 ± 600 nM at round 10 for eVP35 IID CBP3mut (Figure 4.4B). We also extended our selection against eVP35 IID CBP3mut up to round 14. Although this pool displayed
a high degree of non-specific binding to the nitrocellulose membrane, we were able to identify select individual aptamers from rounds 10 and 14. As discussed below, we were able to further characterize these select aptamers for affinity measurements and ranking.

For our initial SELEX studies, we observed significant enrichment of select sequences from a total of 96 clones. Using ClustalW multiple sequence alignments, sequences with variations less than 5% of the total aptamer length (i.e. 4 nucleotides or less that includes the constant/primer regions and the variable region) were grouped together and represented by a single member. We first selected 9 aptamers from the 1-series and 3 aptamers from the 2-series (Figure 4.4C). Subsequently, we identified representative members from each group based on their affinity for eVP35 IID WT and their ability to inhibit the interaction between VP35 IID and NP (see below). These selected aptamers were subjected to further characterization and ranking based upon affinity measurements with the top binders from each series selected for further analysis. Aptamer 1G8-14 was chosen to represent the 1-series aptamers, which were selected against eVP35 IID WT, and aptamer 2F11-14 was chosen to represent the 2-series aptamers, which were selected against eVP35 IID CBP3mut. In order to further examine RNA structural determinants, we used ViennaRNA structural prediction software to predict the RNA secondary structure of 1G8-14 and 2F11-14. The resulting secondary structure predictions for the MFE and the centroid were visualized with the RNA graphics program VARNA (Darty et al. 2009), and base-pairing probabilities for the MFE and ensemble are shown using dot plot analysis (Figure 4.5) (Murshudov et al. 1997). Based on the structure predictions, the 1G8-14 MFE and centroid structure
displayed 15.12 ensemble diversity and the MFE structure represented 5.12% of the ensemble compared to the 2F11-14 MFE and centroid structure with 13.46 ensemble diversity and 4.23% of the ensemble represented by the MFE. However, the free energy of the ensemble for 1G8-14 was -22.63 kcal/mol compared to -12.75 kcal/mol for 2F11-14 suggesting that the 1G8-14 aptamer may adopt a more stable structure. Our CD data show differences in the corresponding spectra providing some experimental evidence that indicate structural differences between 1G8-14 and 2F11-14 aptamers (Figure 4.6). However, additional studies are required to appropriately characterize the actual structural differences between 1G8-14 and 2F11-14 aptamers. As an initial step in our efforts to define the optimum aptamer, we carried out boundary determination studies and tested several shorter aptamers based on the results of our boundary determination experiments. These results are described in the section entitled "Primer binding region of 1G8-14 is important for high affinity VP35 IID binding" (see below). These studies supported the use of full length aptamers in our present studies as discussed below.

Aptamers 1G8-14 and 2F11-14 bound eVP35 IID WT with affinities higher than dsRNA. At 1µM protein, the fractional binding for both 1G8-14 and 2F11-14 was >0.5, while the fractional binding for dsRNA was <0.3 (Figure 4.7B). eVP35 IID CBP3mut bound to 2F11-14 with high affinity and to 1G8-14 only at higher protein concentrations (10 µM protein) (Figure 4.7C). These binding studies provided an initial indication that we had identified aptamers that bind to eVP35 IID differently, with the CBP residues contributing to 1G8-14 aptamer binding. The ability of eVP35 IID WT and eVP35 IID CBP3mut to bind ssRNA and dsRNA was also assessed. As previously shown, eVP35
IID WT binds to dsRNA, but not ssRNA (Figure 4.7C). eVP35 IID CBP3mut does not bind to dsRNA due to mutations at key dsRNA binding residues, R312, K319 and R322 (Figure 4.7C) (Hartman et al. 2004; Prins et al. 2010). Previous studies have shown that eVP35 binds dsRNA containing a 5`ppp moiety with higher affinity than blunt end dsRNA (Kimberlin et al. 2010; Leung et al. 2010). Therefore, we tested if a 5`ppp or 5`OH affected aptamer binding to eVP35 IID. The resulting data suggest that the 5` phosphorylation state of the aptamer does not influence eVP35 IID binding (Figure 4.8). Additionally, this observation supports the notion that the eVP35-aptamer interactions are distinct from those observed between eVP35 IID and dsRNA (Cardenas et al. 2006; Leung et al. 2010).

4.3.3 Aptamers recognize partially overlapping sites in eVP35 IID

We and others have shown that eVP35-dsRNA interactions are mediated through the CBP region of eVP35 IID (Hartman et al. 2004; Cardenas et al. 2006; Leung et al. 2009; Kimberlin et al. 2010; Leung et al. 2010). Therefore, a series of basic residues in eVP35 IID were mutated to determine the contribution of these residues, if any, to 1G8-14 and 2F11-14 aptamer binding. Representative single point binding data for 1G8-14 and 2F11-14 aptamers revealed distinct binding patterns. 1G8-14 aptamer binding primarily depends on a subset of residues that are also important for dsRNA binding and IFN inhibition. Single mutation of residues F239, R305, K309, R312, R322, and K339 to alanine had little effect on the 1G8-14 aptamer binding (<30% relative to WT) whereas double/triple mutations (K309A/R312A, K319A/R322A, CBP3mut) significantly diminished binding to 1G8-14 (>70% relative to WT) (Figure 4.9). Mutations
of the FBP residues (eVP35 IID FBP4mut) also impair binding to 1G8-14; however, further experiments revealed that this decrease in 1G8-14 binding to eVP35 IID FBP4mut is largely due to a decrease in the maximum fractional binding and not due to a substantial change in binding affinity (data not shown). Moreover, these aptamers readily competed with dsRNA in competition assays (Figure 4.10). These results are different from those observed for dsRNA binding, where select single point mutation of residues in the CBP abolished dsRNA binding (Leung et al. 2010). Collectively, these results suggest that aptamer binding involves multiple residues within a given eVP35 IID surface, with the CBP residues providing the largest contribution towards 1G8-14 aptamer binding.

For the 2F11-14 aptamer, we observe a set of residues different from those important for 1G8-14 binding, including R225, K251, and F239, as mutation of these residues to alanine result in some loss of aptamer binding (<40% relative to WT) (Figure 4.9B). Most significantly, R305A mutant alone can drastically decrease binding to 2F11-14 (>70% relative to WT). In contrast to 1G8-14, eVP35 IID CBP3mut had limited impact on 2F11-14 binding, consistent with the single point data (Figure 4.7C). Residues R305 and K309 are important for both aptamers, suggesting that the aptamer binding sites are partially overlapping at or near these residues.

4.3.4 Residues in eVP35 IID CBP and FBP contribute differentially to aptamer binding

To further characterize eVP35-aptamer interactions, we measured the binding affinities of aptamer to eVP35 IID WT and mutant proteins using ITC. eVP35 IID WT
One- and two-site binding models were considered, ultimately resulting in the ITC data being fit using a two-site binding model, indicating that there are two independent binding modes for eVP35 IID on 1G8-14eVP35. The first binding mode is of high affinity ($K_{D,1} = 3.7 \pm 0.2$ nM, $n_1 = 1.1 \pm 0.1$), which we term the "aptamer binding mode", whereas the second binding mode showed moderate affinity ($K_{D,2} = 1400\pm 500$ nM , $n_2 = 2.7 \pm 0.4$) (Figure 4.11A, Table 4.2). The affinity and the multiple bindings displayed by the second binding mode suggest that this is the dsRNA binding mode. Mutation of residues in the FBP (e.g. K248A and FBP4mut) had little effect on binding (Figure 4.11B-C). When we test binding of aptamer to eVP35 IID proteins containing mutations of residues important for dsRNA binding (e.g. R312A and F239A), we find that the second binding mode is eliminated and the data can be fit to a one-site binding model ($K_{D,1}$ values of $83 \pm 10$ nM and $85 \pm 50$ nM, respectively) (Figure 4.11D-E). Furthermore, mutation of three CBP residues together (i.e R312A/K319A/R322A triple mutant or CBP3mut) or in combination with mutations of the four FBP residues (i.e. FBP4mut/R312A and FBP4mut/F239A) in eVP35 IID results in complete loss of 1G8-14 aptamer binding (Figure 4.11F-H). For the second binding mode, these data are consistent with previously reported descriptions of the dsRNA-eVP35 interaction (Leung et al. 2010). Altogether, our data suggest that the first binding mode is the high affinity aptamer-eVP35 IID interaction (aptamer mode) and the second binding mode is eVP35 IID binding to double stranded regions (dsRNA mode) in the aptamer (Table 4.3).
The ITC binding data for 2F11-14 aptamer to eVP35 IID were fit to a two-site binding model, where $K_{D,1} = 7.1 \pm 0.1$ nM ($n_1 = 1.2 \pm 0.0$) and $K_{D,2} = 380 \pm 40$ nM ($n_2 = 5.1 \pm 0.1$) (Figure 4.12A, Table 4.2). A single point mutation in the FBP, K248A, leads to slightly diminished binding with an approximately 5-fold increase in $K_{D,1}$ and a 2.5-fold increase in $K_{D,2}$ (Figure 4.12B). Mutation of four residues in the FBP (eVP35 IID FBP4mut) leads to near complete loss of 2F11-14 binding (Figure 4.12C), emphasizing a critical role for the FBP in 2F11-14 aptamer binding. Mutation of residues in the CBP eliminates the second binding event with $K_{D,1} = 56 \pm 7$ nM, $K_{D,1} = 100 \pm 8$ nM, and $K_{D,1} = 52 \pm 6$ nM for eVP35 IID CBP3mut, F239A, and R312A, respectively (Figure 4.12D-F). Interestingly, mutation of K248A in combination with the CBP3mut leads to a ~25-fold increase in $K_{D,1}$ compared to eVP35 IID WT (Figure 4.12G), but does not abolish binding altogether. Thus, in contrast to 1G8-14, residues in the FBP are critical for high affinity 2F11-14 binding. Similar to the results observed for 1G8-14 binding, residues in the CBP contribute to the second binding event, presumably through contacts with double stranded regions of the 2F11-14 aptamer. Our results are summarized in Table 4.3.

In order to better define the binding interface for eVP35 IID-1G8-14 and eVP35 IID/2F11-14, and how these aptamers are oriented with respect to eVP35 IID, we used a filter binding competition assay to see if 1G8-14 could compete with 2F11-14 for eVP35 IID binding and vice versa. As expected, unlabeled 1G8-14 was able to compete off $^{32}$P-labeled 1G8-14. Unlabeled 1G8-14 can also compete off $^{32}$P-labeled 2F11-14 (Figure 4.10B-C). However, unlabeled 2F11-14 could only compete off $^{32}$P-labeled 2F11-14, but not $^{32}$P-labeled 1G8-14. Since 1G8-14 was selected against WT eVP35
IID, it is conceivable that 1G8-14 aptamer would make contacts with both basic patches. This notion is supported by our ITC data (described above), showing that the CBP is the primary binding site for 1G8-14, but the FBP also contributes to 1G8-14 binding, and mutations to a CBP residue in combination with mutations to the FBP region (FBP4mut + R312A) results in loss of interaction. In contrast, 2F11-14 aptamer, which was selected against a mutant protein with only the FBP present, may predominantly interact with the FBP surface. Altogether, these data are consistent with the notion that distinct classes of high affinity aptamers can be developed using eVP35 WT and mutant eVP35 IID proteins as SELEX targets.

4.3.5 Primer binding region of 1G8-14 is important for high affinity VP35 IID binding

The structure predictions for the 1G8-14 aptamer (Figure 4.5) suggested that the primer binding regions may be important for secondary structure. To further evaluate the contributions from secondary structure of the 1G8-14 primer regions, we performed binding site boundary measurements to identify the nucleotides of 1G8-14 important for binding to eVP35 IID (Figure 4.13). The resulting data suggests that the region between nucleotides 36 and 65 is important. However, given the nature of the experiment, it was not clear if this is due to binding of the structural element shown in Figure 4.13B or if binding was primarily driven by the sequence between nucleotides 36 to 65 (indicated by arrows in Figure 13C). In this assay, we used partially digested aptamers under native conditions and isolated aptamer fragments that bound eVP35 IID binding. RNA bound eVP35 IID proteins were subsequently subjected to denaturing gel
electrophoresis for sequence mapping. In order to differentiate between the two potential aptamer fragments that form the high affinity binding, we generated the minimum RNA sequence (nucleotides 36-65) and subjected that sequence to structure predictions and binding studies. In addition, we also tested RNA fragments corresponding to 1-65 and 36-90 nucleotides. These predictions revealed that bases 36-65 forms a stem-loop, which is not a sub-structure predicted for full length 1G8-14 structure (compare Figure 4.14A with Figure 4.14B). We performed ITC experiments in order to compare the full-length and truncated 1G8-14 constructs in their ability to bind eVP35 IID as well as mutant eVP35 IID proteins. Truncated 1G8-14 36-65 bound to eVP35 IID WT with a two-site binding mode similar to full-length 1G8-14. However, the truncated 1G8-14 36-65 aptamer was unable to bind eVP35 IID R312A (Figure 4.14; see Figure 4.14F vs Figure 4.14C). The sensitivity to the eVP35 IID R312A mutation was also observed previously for dsRNA binding. We also conducted ITC experiments for truncated 1G8-14 1-65 and 1G8-14 36-90 to assess the importance of the 5' constant region and 3' constant region, respectively (Figure 4.15). Truncated 1G8-14 1-65 bound eVP35 IID WT with a two-site binding mode, but similar to truncated 1G8-14 36-65 was unable to bind eVP35 IID R312A. Additionally, truncated 1G8-14 1-65 binding to eVP35 IID WT appear to be cooperative and this observation will require additional experiments in order to fully explore this behavior (Figure 4.15D-F). However, it is evident that there are significant differences between WT and the 1-65 truncated aptamer. In contrast, truncated 1G8-14 36-90 bound eVP35 IID WT with a two-site binding mode similar to full length aptamer and more importantly, this truncated aptamer bound eVP35 IID R312A with high affinity (Figure 4.15G-I). Together these
data suggest that the variable region of 1G8-14 is important for binding to eVP35 IID, but additional contributions from the constant regions are required for the optimal aptamer formation. Based on these observations, we used full length aptamers in our studies described here.

**4.3.6 Aptamers disrupt eVP35 IID-NP interactions and inhibit EBOV replication/transcription activity**

In order to test the utility of the 1G8-14 and 2F11-14 aptamers, we assessed their ability to inhibit interactions between eVP35 IID and NP proteins. This interaction is important for the function of the filoviral replication complex (Muhlberger et al. 1999; Prins et al. 2010). Specifically, residues in the FBP of eVP35 IID are important for eVP35 IID-NP interactions and minigenome activity (Figure 4.16A) (Prins et al. 2010). Mutation of all four residues in the FBP (eVP35 IID FBP4mut) leads to loss of eVP35 IID-NP binding in a pulldown assay. In contrast, mutation of residues in the CBP (eVP35 IID R305A/K309A and eVP35 IID CBP3mut) does not impact eVP35-NP binding. Since the FBP appears to be important for both 1G8-14 and 2F11-14 binding, we tested the ability of these aptamers to compete with NP for binding to eVP35. Both 1G8-14 and 2F11-14 aptamers were able to inhibit eVP35 IID-NP complex formation, while dsRNA and aptamers 2B3-10 and 2D1-10 are unable to disrupt eVP35 IID-NP interaction (Figure 4.16B). Aptamers 2B3-10 and 2D1-10, selected against eVP35 IID CBP3mut, are of similar length to 1G8-14 and 2F11-14 aptamers, and they display similar high affinity binding to eVP35 IID (Figure 4.16C). Together, our data demonstrate the ability of 1G8-14 and 2F11-14 aptamers to specifically disrupt the eVP35-NP interaction. We also tested the ability of aptamers 1G8-14 and 2F11-14 to inhibit RNA synthesis in a
replication/transcription assay, where the viral replication complex was reconstituted by expression of select viral proteins (Muhlberger et al. 1999). Resulting data show that 1G8-14 and 2F11-14 can antagonize the replication/transcription activity in a dose dependent manner (Figure 4.17A). In contrast to these two specific aptamers, additional aptamers with similar length and affinity show little to no inhibition, despite all six aptamers being present at comparable levels as judged by qRT-PCR (Figure 4.17B). Altogether, these results demonstrate that 1G8-14 and 2F11-14 aptamers can potentially function as competitive inhibitors of the filoviral replication complex by disrupting eVP35 IID-NP interactions. However, it is worth noting that the aptamers can interact with NP at high protein concentrations (>100 µM); thus, it is not clear if additional rounds of aptamer optimization with negative selection against NP will be necessary prior to use of these aptamers in virally infected cells.

4.3.7 Apatmers differentially recognize closely related filoviral VP35 IID proteins.

Filoviral VP35 proteins share high sequence similarity in the IID region (97.6% similar, 88.9% identical for RESTV aligned with EBOV; 81.7% similar, 22.9% identical for MARV aligned with EBOV) (Figure 4.18C). Therefore, we tested the ability to the 1G8-14 and 2F11-14 aptamers to differentiate between closely related VP35 proteins. The resulting data showed that 1G8-14 aptamer preferentially binds to eVP35 IID proteins (EBOV and RESTV VP35 IID proteins) (Figure 4.18A). When binding was tested at protein concentrations <1 µM, EBOV and RESTV showed a fractional binding >0.6, while <0.1 fractional binding was observed for MARV. In contrast, 2F11-14 aptamer bound to all three filoviral VP35 IID proteins with >0.5 fractional binding at
protein concentrations > 1µM. (Figure 4.18B). The ability of 1G8-14 to differentiate between eVP35 IID and MARV VP35 IID proteins is likely due to sequence differences between EBOV and MARV VP35 proteins near the CBP. For example, in EBOV VP35 IID positions 319 and 322 are K319 and R322, respectively, while corresponding positions in MARV VP35 IID are T309 and K312 (Figure 4.18C). Thus, it appears that the relative sensitivity to different filoviral species is entirely dependent on the relative contributions of the different basic patches.

4.4 Conclusions

In this study, we have identified and characterized two aptamers against eVP35 IID that bind with low nanomolar affinity using SELEX technology. These aptamers were generated by using two targets, eVP35 IID WT and eVP35 IID CBP3mut, with the goal of identifying different classes of aptamers that interact with distinct sets of residues within filoviral VP35 proteins. Target selection was in large part motivated by available structure-function data and our goal was to drive the selection process to different functionally important regions of this multifunctional non-enzymatic protein. Based on the data described above, our selected aptamers display binding affinities that are 20-100 fold higher than dsRNA and the 92 nucleotide long random RNA pool that was used in the initial SELEX, supporting the specific enrichment of aptamers. From the initial groups of aptamers, 1G8-14 and 2F11-14 aptamers were chosen to represent two aptamer classes as they bound eVP35 IID with high affinity, disrupted the eVP35IID-NP complex, and displayed significant differences at the nucleotide level (~40% difference in the aptamer variable region). Biochemical and functional mapping of select aptamers
revealed that 1G8-14 and 2F11-14 aptamers bind eVP35 IID primarily through interacting with residues from the CBP and the FBP, respectively, although it is apparent that additional interactions between eVP35 IID and aptamers are also formed due to the highly basic nature of the eVP35 IID surface (Leung et al. 2009). Our ITC data revealed two independent binding modes: a high affinity aptamer binding mode and a the dsRNA binding mode (Figure 4.9). Each aptamer has a single high affinity binding site and multiple dsRNA binding sites. These two binding events are energetically independent, at least in the context of eVP35 IID. Interestingly, mutations to CBP residues, or key residues for dsRNA binding resulted in the presumable loss of the dsRNA binding mode. Therefore, at low eVP35 IID concentrations, we observe the high affinity aptamer binding event with a eVP35 IID:aptamer ratio of 1:1. As we increase eVP35 IID concentrations, eVP35 IID is able to make additional interactions with double stranded regions of the aptamers due to its inherent dsRNA binding function, therefore, allowing multiple eVP35 IID proteins to interact with a single aptamer.

This work demonstrates how integrating available structural and functional data to tailor SELEX selection can result in aptamers that target different functional regions and potentially generate multivalent aptamers. 1G8-14 aptamer was selected against eVP35 IID WT and binds primarily to residues in the CBP with additional contacts to the FBP region. Despite the fact that the CBP is important for both 1G8-14 aptamer and dsRNA binding, there are significant differences in how eVP35 IID binds these two ligands. Most notably, a single point mutation within the CBP completely abolishes dsRNA binding (Hartman et al. 2004; Leung et al. 2010; Prins et al. 2010). However,
single point mutations have only marginal effects on 1G8-14 aptamer binding (Figure 4.9). In contrast, double and triple mutation of CBP residues significantly affect the ability of 1G8-14 aptamer to bind eVP35 IID, which supports the notion that the aptamers establish multiple contact sites at the binding interface(s). In addition to the CBP, our data shows that mutations within the FBP affect 1G8-14 aptamer binding (increases $K_{D,1}$). Mutation of the FBP along with a single mutation in the CBP region abolishes aptamer binding altogether. These results further support the view that 1G8-14 aptamer recognizes several surfaces across eVP35 IID. In contrast, the main site of interaction on eVP35 IID for the 2F11-14 aptamer appears to be near the FBP, with the CBP providing additional contact points. Our results clearly suggest that both 1G8-14 and 2F11-14 aptamers interact with multiple copies of eVP35 IID and therefore there is a high potential for these aptamers to inhibit multiple VP35-mediated functions in vivo.

In this work, we were able to target the CBP or the FBP of eVP35 IID and show that 1G8-14 and 2F11-14 aptamers have distinct binding footprints through biochemical mapping. Moreover, we are able to distinguish that the CBP is the primary binding site for 1G8-14, while 2F11-14 uses the FBP as its primary binding site. As a multifunctional protein, eVP35 IID inhibits IFN responses to viral infection, in part through dsRNA sequestration, and supports RNA viral synthesis through structural bridging of NP and the viral polymerase L. We have shown that aptamers bind eVP35 IID using multiple points of contact, a mode that is distinct from dsRNA binding. Furthermore, aptamers can compete with dsRNA for eVP35 IID binding, indicating that aptamers have the potential to disrupt the IFN inhibitory function of eVP35. Here we show that aptamers disrupt the eVP35-NP interaction and replication/transcription activity, indicating their
potential to inhibit eVP35 polymerase cofactor function. It is interesting to note that selected aptamers can potentially inhibit both the eVP35 immune suppressor function and the polymerase cofactor function.

Aptamers are well known for their high affinity and specificity against their targets. The high specificity of aptamers can be used to distinguish among viral subtypes, which is relevant for the development of aptamers against viral proteins. On the other hand, aptamers with broad specificity can potentially serve as pan-antivirals. One of our selected aptamers, 1G8-14, can distinguish between EBOV and MARV VP35 IIDs, binding to EBOV and RESTV VP35 IIDs with $K_D$’s 20-fold lower (at 1 mM concentration of protein, see Figure 4.6A) than MARV VP35 IID. The other selected aptamer, 2F11-14, can bind EBOV, RESTV, and MARV VP35 IIDs with comparable affinities. Thus, we have demonstrated that it is feasible to develop both highly specific and pan-antifiloviral aptamers using VP35 as the SELEX target molecule, and our success in selecting both highly specific and pan-antifiloviral aptamers indicates that there are highly conserved regions of VP35 which are recognized by 2F11-14, as well as species unique regions in VP35, which allows differential binding of 1G8-14 to EBOV and MARV VP35 IIDs.
### 4.5 Tables

**Table 4.1.** Data collection, structure solution, and refinement statistics.

<table>
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<th>eVP35 IID CBP3mut</th>
<th>eVP35 IID FBP4mut</th>
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<td><strong>PDB</strong></td>
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<td>4IJF</td>
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<td>59.73, 59.73, 67.78</td>
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<tr>
<td>α, β, γ (°)</td>
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<td>90, 90, 120</td>
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<td>Resolution range (Å)*</td>
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<td>50 – 2.5</td>
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<td>(1.93 – 1.90)</td>
<td>(2.54 – 2.50)</td>
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<tr>
<td>Unique reflections</td>
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<td>5063 (226)</td>
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<td>Redundancy</td>
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<td>4.0 (3.9)</td>
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<tr>
<td>Completeness (%)</td>
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<td>99.3 (100.0)</td>
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<tr>
<td>Rmerge (%)</td>
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<td>7.5 (77.1)</td>
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<td>I / Iσ</td>
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<td>16.3 (2.3)</td>
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<td><strong>Structure solution and refinement</strong></td>
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<td>R.m.s. deviations</td>
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<td></td>
<td>Bond angles (°)</td>
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<tr>
<td></td>
<td>(chain B)</td>
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<td></td>
<td>(chain C)</td>
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<td>Molprobity clashscore</td>
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<td>7.97</td>
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*Values in parentheses are for the highest resolution shell.*
Table 4.2. Measured binding affinities between aptamers and WT or mutant eVP35 IID proteins by ITC.

<table>
<thead>
<tr>
<th></th>
<th>1G8-14</th>
<th></th>
<th>2F11-14</th>
<th></th>
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<tr>
<td></td>
<td>n&lt;sub&gt;1&lt;/sub&gt;</td>
<td>K&lt;sub&gt;D,1&lt;/sub&gt;(nM)</td>
<td>n&lt;sub&gt;2&lt;/sub&gt;</td>
<td>K&lt;sub&gt;D,2&lt;/sub&gt;(nM)</td>
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<tr>
<td>WT</td>
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<td>3.7±0.2</td>
<td>2.7±0.4</td>
<td>1400±500</td>
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<tr>
<td>K248A</td>
<td>1.1±0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13±1</td>
<td>3.3±0.4</td>
<td>1600±90</td>
</tr>
<tr>
<td>FBP4&lt;sub&gt;mut&lt;/sub&gt;</td>
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<td>48±30</td>
<td>3.2±0.6</td>
<td>1200±800</td>
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<td>83±10</td>
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<td>-</td>
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<tr>
<td>F239A</td>
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<td>85±50</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBP3&lt;sub&gt;mut&lt;/sub&gt;/K248A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
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</table>

<sup>a</sup> = 1.2 ± 0.04  <sup>e</sup> = 1.1 ± 0.02  
<sup>b</sup> = 1.1 ± 0.03  <sup>f</sup> = 1.4 ± 0.04  
<sup>c</sup> = 1.7 ± 0.03  
<sup>d</sup> = 1.4 ± 0.01

-: no binding  n.d: not determined
Table 4.3. Summary of ITC results for aptamers binding to WT or mutant eVP35 IID proteins.

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>1G8-14</th>
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<tr>
<td></td>
<td>$K_{D,1}$</td>
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<tr>
<td>WT</td>
<td>++ + + +</td>
<td>+++ + +</td>
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<td>K248A</td>
<td>++ + + +</td>
<td>+++ + +</td>
</tr>
<tr>
<td>FBP4mut</td>
<td>n.d.</td>
<td>++ + + +</td>
</tr>
<tr>
<td>R312A</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F239A</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CBP3mut</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBP3mut+ K248A</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

++ + + + = WT

+++ = 5-9 fold decrease in $K_D$

++ = 10-19 fold decrease in $K_D$

+ = >20 fold decrease in $K_D$

- = no binding

n.d: not determined
Figure 4.1 Crystallization optimization of eVP35 IID CBP3mut crystals. (A) Representative crystals of eVP35 IID CBP3mut at various stages of crystal optimization. eVP35 IID CBP3mut crystals suitable for data collection were eventually obtained by hanging-drop vapor diffusion method in 2.2M Na/K phosphate (pH 4.5). (B) Representative diffraction pattern of the crystals which diffracted to 1.9Å resolution. (C) 2Fo-Fc density map at 1σ showing the experimental electron density (blue mesh) for eVP35 IID CBP3mut centered at residues I320 (yellow sticks).
Figure 4.2. Mutations to the FBP and CBP do not significantly perturb the overall structure of eVP35 IID. Ribbon representation of eVP35 IID WT (PDB: 3FKE) highlighting residues from the FBP (left) and CBP (right). Ribbon representation of eVP35 IID (B) CBP3mut (PDB: 4IJE) and (C) FBP4mut (PDB: 4IJF). (D) PyMol alignment of eVP35 IID WT (yellow), CBP3mut (purple), and FBP4mut (cyan) with display RMSD values of 0.68 and 0.46 Å, respectively.
Figure 4.3. Surface electrostatic analysis of eVP35 IID FBP4mut and CBP3mut.

Electrostatic surface representation of (A) eVP35IID WT (PDB: 3FKE), (B) CBP3mut (PDB: 4IJE), and (C) FBP4mut (PDB: 4IJF) (scale, -10 kT/e to +10 kT/e).
Figure 4.4. Enrichment of aptamer populations using SELEX. The progress of in vitro selection of aptamers against eVP35 IID WT and eVP35 IID CBP3mut was measured by the filter binding assay. The binding curves of RNA populations against (A) eVP35 IID WT from rounds 1 and 14 and populations against (B) eVP35 IID CBP3mut from rounds 1 and 10 are shown. (C) Representative 1-series aptamers from (A) and 2-series aptamers from (B) are shown. Frequency was determined by sequencing 96 clones at round 10 and round 14 of SELEX. The number following the dash line in the aptamer name (e.g. -10, -14) indicate the round when sequencing was done and the aptamers were identified.
Figure 4.5. Secondary structure predictions for aptamers 1G8-14 and 2F11-14.

RNAfold from Vienna package v.2.0 was used to predict the secondary structure for 1G8-14 and 2F11-14. Results for 1G8-14 are shown as the (A) minimum free energy (MFE) structure prediction and (B) base-pairing probabilities using dot plot analysis. Results for 2F11-14 are shown as the (A) minimum free energy (MFE) structure prediction and (B) base-pairing probabilities using dot plot analysis. Resulting secondary structures for the MFE were visualized with the RNA graphics program VARNA. Bases depicted in grey represent the 5' and 3' common region of the aptamers.
Figure 4.6. CD spectra show that 1G8-14 and 2F11-14 have different secondary structures. Left. Ellipicity of 260nm for (A) 1G8-14 and (B) 2F11-14 were collected at 5°C (solid line) and 95°C (dashed line) from 200 to 320 nm. Right. Temperature melting of 1G8-14 (black) and 2F11-14 (red) were performed by monitoring the ellipticity at 260 nm from 5°C to 95°C with a 2°C increment using a 1°C/min gradient. (C) First derivatives of the temperature melts in (A) and (B).
Figure 4.7. 1G8-14 and 2F11-14 aptamers bind eVP35 IID WT with high affinity. (A) eVP35 IID contains two basic patches. The first basic patch (FBP) contains residues K222, R225, K251, and K248 (purple). The central basic patch (CBP) contains residues R312, K319, and R322 (cyan). eVP35 IID WT and eVP35 IID CBP3mut were used as targets for in vitro selection of 1G8-14 and 2F11-14 aptamers, respectively. Filter binding assays were used to assess the ability of (B) eVP35 IID WT and (C) eVP35 IID CBP3mut proteins to bind ssRNA, dsRNA, 1G8-14 aptamer, and 2F11-14 aptamer at protein concentrations of 0.1 µM (gray), 1 µM (green), and 10 µM (blue).
Figure 4.8. Aptamer binding is not affected by the 5'-ppp moeity. ITC raw data and corresponding binding isotherms for eVP35 IID WT binding to (A) 5'-PPP 1G8-14 and (B) 5'-OH 1G8-14.
Figure 4.9. Mutational analysis reveals differences in eVP35 IID-aptamer binding sites. Fractional binding of aptamers (A) 1G8-14 and (B) 2F11-14 to eVP35 IID WT and mutant proteins measured by filter binding assay. Average fractional binding normalized to eVP35 IID WT are from two independent experiments, each with four repeats for individual eVP35 IID variants.
Figure 4.10. Aptamers compete with dsRNA for eVP35 IID binding, and 1G8-14, but not 2F11-14, can compete with 1G8-14 for eVP35 IID binding. Filter binding assays were carried out to determine the competition of aptamers and dsRNA for binding to eVP35 IID. (A) Binding of $^{32}$P-dsRNA to eVP35 IID was competed by increasing concentrations of unlabeled 92 nt RNA (■), dsRNA (○), 1G8-14 (▲), or 2F11-14 (●). (B) Binding of $^{32}$P-1G8-14 to eVP35 IID was competed by increasing concentrations of dsRNA (○), 1G8-14 (▲), or 2F11-14 (●). (C) Binding of $^{32}$P-2F11-14 and eVP35 IID is competed by increasing concentrations of dsRNA (○), 1G8-14 (▲), or 2F11-14 (●).
Figure 4.11. The CBP is important for high affinity binding of 1G8-14 aptamer to eVP35 IID. ITC raw data and corresponding binding isotherms for 1G8-14 binding to eVP35 IID (A) WT, (B) K248A, (C) FBP4mut, (D) R312A, (E) F239A, (F) CBP3mut, (G) FBP4mut+R312A, and (H) FBP4mut+F239A.
Figure 4.12. The FBP is important for high affinity binding of 2F11-14 aptamer to eVP35 IID. ITC raw data and corresponding binding isotherms for 2F11-14 binding to eVP35 IID (A) WT, (B) K248A, (C) FBP4mut, (D) CBP3mut, (E) F239A, (F) R312A, and (G) CBP3mut+K248A.
Figure 4.13. The minimal binding region of 1G8-14 with eVP35 IID. (A) Partially digested 5' or 3' labeled 1G8-14 were selected by filter binding for interactions with eVP35 IID. The 5' boundary (left) was determined to start at base 36, and the 3' boundary (right) was determined to end at base 65. (B) Secondary structure of the protected region, based on the predicted MFE structure (see Figure S4A). Although constant regions may be part of the secondary structure under native conditions, these additional oligonucleotides are separated under denaturing conditions. (C) The nucleotide sequence for 1G8-14 is shown with the constant regions highlighted in grey and the variable region highlighted in blue. The location of the minimal sequence of 1G8-14, nucleotides 36-65, are indicated by the arrows.
Figure 4.14. eVP35 IID WT and eVP35 IID R312A show differential binding to 1G8-14 full length and 1G8-14 truncations. Minimum free energy (MFE) structure prediction for (A) 1G8-14 and (D) 1G8-14 36-65, and a schematic of (G) dsRNA. ITC raw data and corresponding binding isotherms show that aptamers 1G8-14, 1G8-14 36-65, and dsRNA binding to eVP35 IID WT (B, E, and H, respectively) or R312A (C, F, and I, respectively). Protein concentrations in the syringe were between 90 to 110 µM for binding to 1G8-14, and at 200µM for binding to dsRNA. Aptamer/dsRNA concentrations in the cell were at 3 to 5 µM, using 5 µcal/sec as the reference power.
Figure 4.15. Both the 5' and 3' constant regions of 1G8-14 contributes to high affinity binding to eVP35 IID. Minimum free energy (MFE) structure prediction for (A) 1G8-14, (D) 1G8-14 1-65, and (G) 1G8-14 36-65. ITC raw data and corresponding binding isotherms show that aptamers 1G8-14, 1G8-14 1-65, and 1G8-14 36-65 binding to eVP35 IID WT (B, E, and H, respectively) or R312A (C, F, and I, respectively). Binding to eVP35 IID WT by 1G8-14 1-65 displayed cooperativity (circled in red) which we cannot explain.
Figure 4.16. **1G8-14 and 2F11-14 aptamers disrupt eVP35 IID-NP interaction.** MBP-His tagged WT or mutant eVP35 IIDs were immobilized on amylose resin and incubated with His-tagged NP proteins in the (A) absence or (B) presence of dsRNA or aptamer. (A) Lanes 1, 3, 5, 7 are input (I) samples for MBP-His eVP35 IID WT, CBP3mut, 305A/309A, and FBP4mut, respectively. Lanes 2, 4, 6, and 8 are final bead (FB) samples for MBP-His eVP35 IID WT, CBP3mut, 305A/309A, and FBP4mut, respectively. (B) Lane 1 shows MBP-His tagged eVP35 IID WT bound to the amylose resin. Lane 2 shows the presence of both His-NP and MBP-His eVP35 IID WT, and lanes 3-8 are final bead samples for pulldowns done in the absence (lane 3) or presence of dsRNA (lanes 4) and aptamers 1G8-14 (lane 5), 2B3-10 (lane 6), 2D1-10 (lane7) and 2F11-14 (lane 8). (C) eVP35 IID WT binds aptamers 1G8-14, 2B3-10, 2D1-10, and 2F11-14 at protein concentrations of 1µM (gray), 10µM (black).
**Figure 4.17.** 1G8-14 and 2F11-14 aptamers inhibit EBOV replication/transcription activity in a dose dependent manner. (A) A replication/transcription assay was performed in which plasmids encoding the different aptamers (250, 500 ng, left and right, respectively) were co-transfected with the plasmids required for minigenome replication and transcription. Minigenome reporter activation was expressed as relative activity by setting the negative control (without VP35) to a value of 1. The error bars indicate standard deviation of three independent replicates, *p=0.001, **p=0.0005. (B) The western blot shows expression of NP and eVP35. (C) Quantitative RT-PCR for aptamers mRNA levels using aptamer specific primers was performed. Genome equivalent (GEq) copy numbers (Total copies) of aptamers in minigenome assay were measured using primer/probes targeting the aptamers. Standards and test samples were assayed in triplicate using the CFX96 detection system. Threshold cycle (C_T) values representing aptamers were used to determine Total Copies. The error bars indicate the standard deviation of three independent replicates.
Figure 4.18. Aptamers differentially bind to filoviral VP35 IID proteins. eVP35 IID from EBOV, RESTV, and MARV were tested in their ability to bind (A) 1G8-14 and (B) 2F11-14 aptamers at varying protein concentrations shown as fractional binding. (C) Sequence alignment of the IID region of VP35 proteins from EBOV, RESTV, and MARV. VP35 IID sequences from EBOV (accession no AAD14582), RESTV (accession no BAB69004.1), and MARV (accession no CAA78115.1) were aligned using ClustalW. Residues highlighted in black are conserved among all three VP35 IID species. Residues highlighted in gray are only conserved between two VP35 IID species.
Figure 4.19. 1G8-14 and 2F11-14 aptamers have multiple binding modes. VP35 aptamers display two binding modes that include a single high affinity binding site and multiple dsRNA binding sites. At low eVP35 IID WT concentrations or when binding to key dsRNA binding mutant R312A, only the high affinity site is occupied. At higher concentrations of eVP35 IID WT additional binding events occur via the dsRNA binding mode.
4.7 References


CHAPTER 5. CONCLUSIONS

Ebolaviruses are highly pathogenic filoviruses, which infect human and non-human primates, and are characterized by severe hemorrhagic fever resulting in fatality rates as high as 90%. Although filoviral vaccine options are under investigation, no approved vaccines are available at this time. Furthermore, other therapeutics such as small molecule or drug-like molecules are largely unavailable making the need for antivirals that counter filoviral infection a pressing concern. Though multiple factors contribute to disease progression, the ability of filoviruses to severely antagonize early host innate immune responses while proliferating at high levels in many cell types plays an essential role in the high fatality rates associated with filoviral infections.

The multifunctional VP35 plays important roles in antagonizing host immune responses and in viral replication. Given the essential nature of VP35 to filoviral pathogenesis, VP35 is a potentially promising target for antiviral development. However, in order to effectively target VP35 it is imperative to biochemically and structurally define functionally relevant regions of this protein. Recent structural and functional studies from our lab have identified the mechanism by which VP35 binds dsRNA and inhibits host immune responses (Leung, Prins et al. 2010). Additionally, these studies revealed that there are two highly conserved basic patches termed the first basic patch (FBP) and the central basic patch (CBP) located within the C-terminal interferon inhibitory domain (IID) of VP35. In order to assess the role of these two basic patches, we conducted a series of structural, biochemical, and functional studies. The crystal structure of VP35 IID complexed with dsRNA revealed that residues within the CBP
make direct contacts with dsRNA, and mutating CBP residues to alanine resulted in loss of RNA binding and inhibition of IFN production. Furthermore, these alanine substitution mutants resulted in an attenuated recombinant guinea pig adapted ebolavirus that conferred protection against WT ebolaviral infection. Work presented in this thesis investigates the functionality of the VP35 FBP and demonstrates that, unlike the CBP, the FBP is not important for RNA binding or IFN inhibition, but is important for VP35's polymerase co-factor function. Current literature in the field has established interactions among NP-VP35, NP-VP30, VP35-L, and NP-VP35-L, and these known interactions support a model in which VP35 interacts with both NP and L, thus tethering the polymerase and its template together (Becker, Rinne et al. 1998; Muhlberger, Lotfering et al. 1998; Muhlberger, Weik et al. 1999). Although the exact role of VP35 in replication is not well understood, alanine substitution mutations show that the FBP is important for the VP35-NP interaction, but not the VP35-L interaction. Furthermore, this work provides the first detailed report of critical residues within VP35 that contribute to its polymerase co-factor function and defines VP35 residues at the VP35-NP interaction. Aside from structurally bridging NP and L together, it is unclear if VP35 performs additional functions in the context of the polymerase complex. At present, the molecular basis for protein-protein and protein-RNA interactions that establish the polymerase complex are poorly defined, but the work presented here, along with additional studies on VP35 and the other viral proteins, will be instrumental in developing a comprehensive model of the polymerase complex, and provide a framework for understanding how the polymerase complex switches between viral transcription and replication.
With the functional validation of the FBP and the CBP, and their respective roles in viral replication and host immune antagonism, inhibition of VP35 functions through targeting these basic patches should not only aid in the development of antivirals, but may also provide novel target sites and mechanistic insight into critical VP35-mediated functions. In an effort to selectively target the FBP and CBP, we generated RNA aptamers targeted against eVP35 IID WT and CBP3mut proteins using SELEX technology. Though there are a number of advantages to using aptamers over antibodies or small-molecule based strategies, one main advantage is that no prior structural information is required for aptamer development. However, in cases such as ours where there are available structural and functional data, the SELEX selection strategies can be tailored to enhance aptamer selection to desired functional regions. Using eVP35 IID WT and CBP3mut protein as two independent SELEX targets, we aimed, and succeeded, in identifying two classes of aptamers which bound to different surfaces of VP35. Through these studies we have demonstrated that the aptamers bind with high affinity and specificity to eVP35 IID, with aptamer 1G8-14 primarily binding to the CBP and aptamer 2F11-14 primarily binding to the FBP. Additional studies revealed that selected aptamers, 1G8-14 and 2F11-14, can disrupt the VP35-NP interaction, and correspondingly inhibit the functionality of the viral polymerase complex in a reconstituted minigenome (MG) assay. The work presented in this thesis, supports the development of VP35 specific aptamers, demonstrates how aptamers can be used to facilitate the validation of VP35 as an antiviral target, and provides a previously unavailable avenue for the development of filoviral antivirals.
In order to consider these aptamers as a serious filoviral therapeutic option, additional studies need to be done. In our present work, much effort has been put forth to characterize the regions of VP35 which interact with the selected aptamers, 1G8-14 and 2F11-14, but in moving forward, we need to characterize the aptamers themselves and, if need be, improve the pharmacological properties of these aptamers. In contrast to antibodies, aptamers often exhibit low immunogenicity and have the ability to be chemically modified which allows for enhanced in vivo stability and bioavailability. To improve the stability of aptamers, particularly RNA aptamers, in vivo, the aptamers can be synthesized with modified bases such as 2'-amino-modified or 2'-fluoro-modified pyrimidines, and these modified bases enhance the stability of the RNA aptamers by replacing the highly reactive 2'-OH with a less reactive substitute. This in turn increases the aptamers half-life and reduces sensitivity to nucleases in serum, and is highly beneficial in the development of effective therapeutic aptamers.

In addition to enhanced biostability, improving bioavailability is another important step in developing these aptamers as therapeutics. In instances such as ours, the aptamers need to bind an intracellular viral protein, and therefore, must be taken up within an infected cell. Due to their size and negative charge, aptamers have difficulty crossing the lipid bilayer. In cultured cells, this dilemma can be overcome by standard transfection methods or by expressing the aptamers in the cells. As a therapeutic, however, alternative strategies are needed to administer the aptamers and target them to the site of infection. Two potential ways to address this are to generate chimeric aptamers or to employ an adenoviral expression system. In recent years, aptamers have become an increasingly useful tool for delivering RNA molecules such as siRNA to
their desired target cells (McNamara, Andrechek et al. 2006). In these experiments, RNA aptamers are generated against cell specific markers, and these aptamers are used to target their "cargo" to these cells. The feasibility of conjugating aptamers together has been investigated in a number of studies, some which are outlined in Chapter 3. To adapt this methodology for our purposes, we could fuse 1G8-14 or 2F11-14 to another aptamer which would be responsible for targeting the chimeric construct to desired cells and enhancing its uptake into these cells. A different approach would be to use an adenoviral system to express the aptamers within cells. Recombinant adenoviral expression systems have become increasingly popular tools to achieve transient gene expression in cell based assays and in vivo, and more recently it has been shown that adenoviral vectors can effectively deliver shRNA to cells (Crowther, Ely et al. 2008). Additionally, an adenoviral expression system provides a key advantage in that after systemic administration, adenoviruses exhibit a preferential tropism for hepatocytes, a primary location for filoviral replication. In moving forward, there are a number of steps that can, and need, to be taken to improve our aptamers and provide the filoviral community with the necessary information and reagents to accelerate the development of effective therapeutic agents and basic research tools to counter filoviral infections.

Alternatively, evaluation of the VP35 IID structure revealed a hydrophobic pocket near the FBP which could be used as a target for small molecule development. In comparison to aptamers, small molecules provide a more traditional approach to therapeutic development, and the pharmacological properties which make for ideal drug-like molecules are more well defined. Nevertheless, targeting viral proteins from
RNA viruses is often challenging in part due to the propensity of these viruses to generate escape mutants. The RNA-dependent polymerases encoded by these viruses lacks the proofreading ability of DNA polymerases, therefore, leading to higher error rates and the likely hood that a resulting mutation will provide the virus with a selective advantage. Escape mutants become especially problematic when applying a small molecule approach to drug development. This is in large part due to the fact that small molecule-protein interactions are often supported by few direct contacts and have little buried surface area at the binding interface. In contrast, aptamers can establish multiple points of contact with their target, and in our work, we have shown that 1G8-14 and 2F11-14 binding is largely insensitive to single point mutations. Additionally, the greater buried surface area located at the aptamer-protein interface may be advantageous when targeting a protein-protein interaction, as small molecules may be unable to disrupt enough protein-protein contacts to be effective. Although aptamers and small molecules have their own advantages and disadvantages, both are valuable reagents that will lead to new and improved therapeutics. In light of the research provided here, which establishes VP35 as a promising therapeutic target and identifies the FBP and the VP35-NP interface as a region for antiviral development, targeting a pocket near the FBP using a small molecule approach may provide an alternative way to therapeutically target VP35 and potentially disrupt its role in viral replication.

In summary, these studies supports a model where two distinct regions of VP35 contribute to viral pathogenesis through their polymerase co-factor function and IFN inhibition function. Together, this work has further probed the multifunctional VP35, providing structural and biochemical insight into the role of the FBP residues, and has
identified a potentially new region, or surface, of VP35 to therapeutically target. Through the selection and characterization of anti-eVP35 IID aptamers, we have identified aptamers which bound with high affinity to VP35, therefore, allowing us to dissect the VP35-NP interaction, further confirming the importance of the VP35-NP interaction in viral replication, and to provide the initial development of inhibitors targeted against VP35. Moreover, these studies demonstrate the feasibility, and potential advantage, of using available structural and functional data to design SELEX selection strategies which promote aptamer generation against preferred functional regions of a protein. These results serve as an initial step in enhancing our understanding of the protein-protein interactions that establish the EBOV polymerase complex, and establishes a framework for targeting VP35 as a potential and effective therapeutic target.
5.1 References


