A Crucial Epitope in the Influenza A and B Viral Neuraminidase and its Broad Inhibition by a Universal Antibody

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ABSTRACT

The antigenic variability of the Influenza virus hinders our ability to develop new therapeutic and vaccine strategies which provide a broad protection against all influenza strains. It has been previously suggested that a means to approach this challenge is to identify conserved sequences within viral proteins and use these for future therapeutic targets. Although such conserved sequences are plentiful amongst the internal viral proteins, their lack of exposure to the host immune system makes mounting an immune response against these regions difficult. Alternatively, the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) have been shown to provide host protection against a limited number of influenza strains when used as vaccine targets; however conserved regions within these proteins which are also antibody accessible are extremely rare. My Ph.D. thesis project is focused on investigating the functional role of a conserved region within the NA protein and to further determine the protection afforded by a monoclonal antibody to this region.

In a comprehensive bioinformatics analysis, the only universally conserved sequence amongst all influenza A and B viral NA has been previously identified as being located between amino acids (a.a.) 222-230 (dubbed the HCA-2 region). However, the potential role of this region remains largely unknown. Through an array of experimental approaches including mutagenesis, reverse genetics and growth kinetics, I have found that substitutions in this sequence significantly affect viral replication by impairing the catalytic activity, substrate-binding and thermostability of NA. These findings prompted me to further investigate if antibody to this region may provide protection against influenza infection.

Indeed, universal monoclonal antibody (HCA-2 MAb) against this peptide provided broad inhibition against all nine subtypes of NA *in vitro* and heterosubtypic protection in mice challenged with lethal doses of mouse-adapted viruses. I further demonstrated that residues within this peptide that are exposed on the surface of NA and located in close proximity to the active site, I222 and E227, are indispensable for antibody-mediated inhibition. These data are the first to demonstrate a monoclonal antibody against the NA protein which provides heterosubtypic protection.

Since I observed that the HCA-2 antibody provided a broad inhibition against all nine subtypes of influenza A NA, I decided to investigate whether this inhibitory effect could be extended against Influenza B. Here, I have further reported that HCA-2 MAb provides a broad inhibition against various strains of influenza B viruses of both Victoria and Yamagata genetic lineage. I also demonstrate that the growth and NA enzymatic activity of two drug resistant influenza B strains are also inhibited by the HCA-2 antibody.

The findings of my Ph.D. thesis project have thus demonstrated that the HCA-2 region is paramount to optimal viral function. Additionally, my data show that antibodies generated against this region provide heterosubtypic protection both *in vitro* and *in vivo* and against drug resistant strains. These results indicate that this universally conserved epitope should be further explored as a potential target for future antiviral intervention and vaccine-induced immune responses.

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DEDICATION

To my wife, Melissa Bahm-Doyle

I love you more than hydrogen loves oxygen.

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LIST OF ABBREVIATIONS

AA	Amino Acid
ANOVA	Analysis of variances
BSA	Bovine serum albumin
cDNA	Complementary DNA
CTLs	Cytotoxic T-cells
DNA	Dexoyribonucleic acid
DMEM	Dulbecco's modified Eagle Medium
dsRNA	Double stranded RNA
E. coli	Escherichia coli
ELISA	Enzyme linked immunosorbent assay
ELLA	Enzyme linked lectin assay
FBS	Fetal Bovine Serum
FcR	Fragment crystallisable receptor
g	gram
HA	Hemagglutinin
HA0	Hemagglutinin precursor protein
HA1	Hemagglutinin subunit 1
HA2	Hemagglutinin subunit 2
HEK-293	Human Embryonic Kidney-293 cells
HI	Hemagglutinin Inhibition
IC_{50}	Median inhibitory concentration
Ig	Immunoglobulin
IgG	Immunoglobulin G
Kb	Kilobase
Kg	Kilogram
LAIV	Live-attenuated influenza vaccine
LD_{50}	Median lethal dose
M	Molar
MAb	Monoclonal Antibody
μg	Microgram
mg	Milligram
μl	Microliter
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of Infection
mRNA	Messenger RNA
M1	Matrix 1 protein
M2	Matrix 2 protein
M2e	Matrix 2 protein extracellular domain
NA	Neuraminidase Protein
NEP	Nuclear Export Protein
NP	Nucleoprotein
NS1	Nonstructrual protein 1
NS2	Nonstrucural protein 2

N-terminus	Amino terminus
PA	Acidic polymerase protein
PB1	Basic polymerase 1 protein
PB2	Basic polymerase 2 protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
pH1N1	Pandemic H1N1
rAd	Recombinant adenovirus
RNA	Ribonucleic acid
rNA	Recombinant neuraminidase
RT	Room Temperature
SA	Sialic Acid
SAα2,6 linked gal	Sialic Acid attached to the galactose by an α 2,6 linkage
SAα2,3 linked gal	Sialic Acid attached to the galactose by an α 2,3 linkage
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBS	Tris buffered saline
TBS-T	Tris buffered saline with 0.1% Tween-20
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
vRNA	Viral RNA
vRNPs	Viral ribonucleoprotein complexes
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Influenza Viruses

Influenza viruses are highly contagious respiratory viruses which are abundant in nature and affect a number of mammalian and avian species. Each year, seasonal influenza epidemics affect up to 500 million people worldwide and can have resulted in the deaths of up to 500 000 individuals (Smith et al., 2006;Poland et al., 2001). Morbidity and mortality are often caused by complications with pneumonia, either from opportunistic infection or from the influenza virus itself (Simonsen et al., 2007). Seasonal epidemics due to antigenically novel strains can also increase disease severity and viral spread which can result in global pandemics, the most recent of which was the 2009 H1N1pandemic (pH1N1). Although strides have been made in anti-influenza prophylactic and therapeutic treatments, the virus still poses a significant disease burden upon society.

1.1.1 Viral Classifications

Influenza viruses comprise three of the five different genuses included within the *Orthomyxoviridae* family which in total encompass *Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus and Isovirus* (Kawaoka et al., 2005). The three types of influenza (A, B and C) are classified according to serological responses to their nucleoprotein (NP) and the matrix 1 protein (M1) (Zambon, 1999). The first isolation of influenza occurred in 1931 when Richard Shope isolated the virus from infected pigs (Shope, 1931) and Wilson Smith later making an isolation in humans (Smith et al., 1933). Subsequent isolation of influenza viruses which were antigenically distinct from the primary isolation were made soon after the 1931 discovery and these distinct discoveries became dubbed influenza A, B and C respectively

(Francis, 1940;Briody, 1948). As more Influenza A strains were identified, it became apparent that the virus could be further characterized by subtypes which are distinguished by differences within the HA and NA proteins and to date there have been 16 different undisputed HA subtypes identified within the HA and 9 of NA. There has been a newly identified influenza A virus H17N10 in small yellow shoulder bats, however this virus has yet to grow in mammalian cells and the HA and NA of this virus have yet to be confirmed as true hemagglutinin or neuraminidase proteins (Tong et al., 2012).

Phylogenic analysis has been used to classify the HA and NA of influenza A virus is into two groups within their respective subtypes. HA group 1 consists of H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16 while HA group 2 is composed of H3, H4, H7, H10, H14 and H15 (Sui et al., 2009). NA is also divided into two groups, with group 1 including N1, N4 N5 and N8 and group 2 containing N2, N3, N6, N7 and N9 (Nabel and Fauci, 2010).

1.1.2 Influenza Virion Morphology

The influenza virus is an enveloped negative sense strand RNA virus which is divided into eight separate segments. The envelope is composed of a lipid bilayer from the host cellular membrane which contains lipid raft associated and non-associated proteins and on its surface is embedded two spike glycoproteins HA and NA; these proteins are present in an approximate 10:1 ratio respectively. Also on the surface is the ion channel matrix 2 (M2) which is only present in a proportion of about 16-20 proteins per virion (Naffakh et al., 2008). Underlying the lipid bilayer is the matrix 1 (M1) protein to which the viral ribonucleoproteins (vRNP) is attached. Internally lies the eight segmented viral genome (vRNA) which are coated by

nucleoprotein (NP) and a small amount of nuclear export protein (NEP) which was originally termed non-structural protein 2 (NS2). The vRNA are also associated with the RNA polymerase which is composed of three separate proteins; polymerase basic 1 (PB1) polymerase basic 2 (PB2) and polymerase acid (PA) (Compans et al., 1972;Steinhauer and Skehel, 2002). Together these polymerase proteins and the NP form the viral ribonucleoprotein complexes (vRNPs). The morphology of the virion is generally spherical however mutations which affect lipid raft location and virus budding can have an effect on virus shape and result in a filamentous morphology. These particles can range from 100nm to 2μm in diameter (Choppin et al., 1960;Nakajima et al., 2010).

1.1.3 Influenza Genome Organization and Viral Proteins

The genome of the influenza virus is composed of 8 separate segments of negative strand RNA. These range between 890-2341 base pairs and code for eleven separate proteins (Chan et al. 2006). Each segment of influenza A virus has a 13 and 12 nucleotide conserved sequence at both the 5' and 3' end of the genome which are partially complementary to each other (Desselberger et al. 1980). Together these form a "panhandle structure" which has been shown to be essential for RNA polymerase binding and virus packaging (Pritlove et al. 1999; Leahy et al. 2001; Bae et al. 2001).

1.1.3.1 RNA Polymerase Proteins

The first 3 segments of the influenza genome code for PB2, PB1 and PA proteins respectively, all of which are necessary components of the RNA polymerase and essential for viral replication. PB2 is involved in cap-snatching the 5' mRNA cap from host cells and using these as primers to start mRNA synthesis (Bouloy et al., 1978). The PB1 subunit has binding sites for both PB2 and PA, it is the backbone of the polymerase complex (Digard et al., 1989) and is further responsible for elongation during negative and positive sense RNA synthesis. It has also been noted that the second segment of the genome further encodes for two non-structural proteins, PB1-F2 (Chen et al., 2001) and PB1-N40 (Wise et al., 2009). Whereas the role of PB1-N40 has yet to be determined, PB1-F2 has been shown to be a pro-apoptotic gene which interacts with the mitochondrial membrane and increase viral virulence. The final protein of the polymerase complex PA is encoded within the third segment of the genome. Although the primary role of PA is endonuclease activity, it has further been suggested that this protein plays an essential role in polymerase stability and promoter binding (Li et al., 2001;Shi et al., 1995).

1.1.3.2 Hemagglutinin

The fourth segment of the viral genome codes for HA, the most abundant glycoprotein on the surface of the influenza virus. The functional roles of HA have been thoroughly investigated and the main role of the protein has been identified as viral attachment and fusion to host cells through the sialic-acid receptor (Skehel and Wiley, 2000). At neutral pH, HA is a homotrimer which is synthesized as a naïve protein "H0" but is later cleaved by host-cell proteases into two subunits, HA1 and HA2 that are linked by a disulfide bond. In order for HA to functionally

preform it must be cleaved (Choppin et al., 1960). After the cleavage, The HA1 subunit becomes available for sialic acid binding, thus mitigating viral attachment to host-cells and the N-terminus of HA2 (dubbed the fusion peptide) becomes fusion competent. Upon internalization of the virus and acidification of the endosome, HA2 undergoes a conformational change triggering release of the fusion peptide into the host cell membrane.

1.1.3.3 Nucleoprotein

Segment number 5 codes for NP which coats the negative stranded vRNA, and positive stranded complementary RNA (Portela and Digard, 2002;Kobayashi et al., 1994). NP is a trimeric internal protein of the influenza A virus and each subunit is 498 amino acids that are predominantly arginine and glycine rich (Londo et al., 1983;Gorman et al., 1990). The recently identified crystal structure of NP reveals that each subunit is composed of two helical domains which are fold in a banana shape. The subunits are then further connected in a tail to head fashion and the heads of each subunit can oligermize with one another (Ng et al., 2008;Elton et al., 1999). Although one of the primary functions of NP is the binding of ssRNA, the RNA binding motif has yet to be identified. However, crystal structure observations suggest that the tail head binding capacity of these subunits may enable the forming of a ring structure which is necessary for RNA binding (Martin-Benito et al., 2001;Portela and Digard, 2002).

NP is also required to condense the segmented genomic RNA into a helical nucleocapsid and, with the polymerase subunits PB1, PB2 and PA, form a ribonucleoprotein (RNP) complex which is necessary for RNA transcription, replication and packaging (Kawaguchi and Nagata,

2006). The RNP lies underneath the shell of the M1 protein to which NP also has been shown to bind and disassociates from during exposure to an acidic environment.

Although NP is not thought to have enzymatic activity, or protect ssRNA from RNase, it does interact with a variety of cellular host factors, including nuclear import factors (Portela and Digard, 2002). In addition to nuclear import NP has also been shown to bind nuclear export factors such as CRM-1, suggesting it to be an essential protein involved in RNP transport across the nuclear membrane (Neumann et al., 1997). In support of this hypothesis, at early times of viral infection, NP is abundantly found in the nucleus, but in the cytosol during later times of infection (Elton et al., 2001).

In addition to contribution to RNP transport and structural support for ssRNA, NP also has an obvious role in vRNA synthesis (Bishop et al., 1971). It has been shown that during both viral replication and virion packaging NP is necessary to structurally maintain the template in an ordered conformation which is necessary for these processes. In addition NP has been suggested to have a role in the switch from mRNA transcription to genome replication but the mechanism of this still remains elusive.

1.1.3.4 Neuraminidase

Segment 6 of the influenza genome encodes the NA protein which encompasses approximately 17% of the surface glycoproteins. NA is a tetramer with four identical subunits, each of which is composed of six, four stranded anti-parallel β-sheets where the enzymatic site is located within a pocket on the surface of each protein (Varghese et al., 1988;Varghese et al., 1983). Within this pocket NA binds sialic acid, or *N*-acetylneuraminic acid, and cleaves the ketosidic linkage between a terminal sialic acid and an adjacent sugar residue, often galactose (Colman, 1994).

Previous reports suggest that the breaking of this bond manifests the predominant role of NA, which is to facilitate the release of the virus particles by catalyzing the cleavage of sialic acid from infected cells and permitting the spread of the virus to neighboring cells (Liu et al., 1995;Griffin et al., 1983;Varghese et al., 1983;Varghese et al., 1988;Air and Laver, 1989;Palese et al., 1974)) . Indeed this role is supported by the observation that NA-defective virus, or wild type viruses in the presence of NA inhibitors, form aggregations on the apical surface of the cells (Griffin et al., 1983;Liu et al., 1995;Hashem et al., 2009). Furthermore, NA has been suggested to play additional roles in viral infection including mucus breakdown, which allows for increased viral diffusion throughout the respiratory tract during infection (Colman et al., 1983;Klenk and Rott, 1988). More recently NA has been shown to contribute to the entry and fusion of the influenza virus into host cells (Ohuchi et al., 2006;Su et al., 2009). It is also noteworthy that the viral neuraminidase may increase bacterial adherence after viral infection, predisposing the patients to secondary bacterial infection, which is one of the most common causes of fatality in influenza patients (McCullers, 2011;Govorkova and McCullers, 2012).

1.1.3.5 Matrix One and Two Proteins

Segment seven has been shown to code for two proteins, M1 and M2. M1 is the most abundant viral protein in the virion, and lies underneath the lipid bilayer of the envelope, where it binds both the vRNPs and the bilayer. Furthermore it anchors the surface proteins HA, NA, and M2 and regulates the transport of vRNPs transport. Is also embedded on the surface of the influenza virus, but unlike HA and NA which are predominantly exposed on the surface of the virus, the majority of M2 is either spans the transmembrane or is internalized as a cytoplasmic

tail, thus very little of it is surface exposed. The M2 protein is a type III glycoprotein which is 97 amino acids (aa) long and embedded in the influenza A viral envelope (Lamb and Lai, 1981). It has three segments: an extracellular N-terminal segment (aa 1-23), a transmembrane segment (aa 24-46) and an intracellular C-terminal segment (aa47-97) (Holsinger et al., 1995). It functions as a selective proton ion channel which is triggered by an acidic pH and is the target for the anti-viral drugs amantadine and rimantadine (Pinto et al., 1992). Mutagenesis studies have identified both His37 and Trp41 are residues within the channel lining which are important for the protein function as these have been shown to determine proton selectivity and ensure unidirectional conductance of the channel (Chizhmakov et al., 1996;Tang et al., 2002).

During viral entry through receptor mediated endocytosis, the influenza virus experiences two changes in pH which contribute to the viral propagation. The first, a decrease from the external pH to the endosomal environment activates the M2 protein to begin transport of protons across the viral membrane from the endosome (Lakadamyali et al., 2003;Hay et al., 1985). This transport eventually results in the second decrease of pH between the endosomal environment and the virus interior (Helenius, 1992). This acidification induces a conformational change in the HA protein which exposes the fusion peptide necessary for viral fusion with the cellular membrane (White et al., 2008). It has been suggested that this decrease in pH may also result in a weakening of electrostatic interaction between the M1 protein and RNP complexes. This weakening thereby enables the uncoated RNPs to be released into the cell cytosol upon viral fusion (Helenius, 1992).

Once in the cytosol, the viral RNPs are transported to the nucleus and the mRNA and vRNA are synthesized (Martin and Helenius, 1991). After the HA, NA and M2 are proteins are translated they are transported to the cell surface by the trans-Golgi network. Here, another role

of M2 has been identified as it prevents the Golgi lumen pH from becoming too low as this would induce the HA protein to undergo a premature conformational change resulting in its inability to eventually fuse with newly infected host cells (Ciampor et al., 1992).

1.1.3.6 Non-structural Proteins One and Two

The final eighth segment of the influenza genome codes for NS1 and NS2 which has been identified as nuclear export protein (NEP). NS1 is essential for viral replication as it is integral in regulating vRNA synthesis and controls vRNA splicing (Hale et al., 2008). NS1 has also been demonstrated to suppress type I interferon (IFN) antiviral responses, thus allowing for increased viral infection (Garcia-Sastre et al., 1998;Katze et al., 2002). The functional role of NEP/NS2 is to export vRNPs from the nucleus to the cytoplasm where viral assembly and budding may begin. This protein is also responsible for the regulation of influenza virus transcription and replication (Robb et al., 2009).

1.3.1.7 Antigenic Drift and Shift

The influenza genome is highly prone to genetic variation, and this is described in terms of antigenic drift and shift. Drift refers to point mutations which gradually accumulate within the viral genome which are due to the error prone viral polymerase. Often these mutations do not have an effect on protein function, but sometimes these point mutations can result in a novel strain which may vary in degree of pathogenicity. Antigenic shift refers to the reassortment of the viral genome segments within a host infected with one or more strains of influenza. Since the viral genome is segmented, if a host cell is infected with more than one strain, it is possible for

there to be mixing of these segments. The most recent pandemic resulting from such reassortment was the pH1N1influenza virus in 2009 which contained genes from human, avian and swine viruses.

1.1.4 Influenza Viral Cycle

The influenza viral cycle can be divided into five separate stages and these include: viral attachment and entry into a host cell; entry of vRNPs into the host nucleus; transcription and replication of the viral genome; export of the vRNPs from the nucleus; and assembly and budding at the host plasma membrane.

Viral attachment to the host cell membrane begins with HA which, as mentioned in section 1.1.3, binds to the host cell sialic acid receptor. Two major classifications of this receptor are either α(2,3) or α(2,6). This is determined by the linkage between the sialic acid and the carbohydrates to which they are bound (White et al., 2008). The α(2,3) and α(2,6) linkages are also important in determining the specificity of HA in binding in different species, for example influenza virus which effectively infect humans recognize the α(2,6) linkage whereas those from avian or equine preferentially bind to the α(2,3) linkage and those from swine recognize both. Binding the host cell sialic acid receptor triggers both clatherin dependent and independent pathways resulting in the endocytosis of the virus (Lakadamyali et al., 2003). The low pH of the endosome initiates an irreversible conformational change exposing the HA2 portion of the HA protein allowing fusion of the viral and endosomal membranes (White et al., 2008).

The acidic environment of the endosome also opens the M2 ion channel allowing protons to be pumped into the viral core; it is this internal acidification of the virion which releases the vRNP from M1 allowing it to be released into the host cell cytoplasm (Shimbo et al., 1996). The vRNPs (NP, PA, PB1, PB2) contain nuclear location signals (NLS) which bind to cellular import machinery such as importin α and β; thus, after these are released into the cytoplasm, they are then transported into the cell nucleus where transcription and replication occur (Samjim, 2009).

Since the influenza viral genome is composed of negative sense strand RNA, it requires a conversion into positive strand RNA before it can be translated into viral protein. As mentioned in section 1.1.3, the 5' and 3' ends of the vRNA contain partially inverted sequences, when these ends bind to each other they create a 'cork-screw' confirmation to which the RNA polymerase (composed of PB1, PB2 and PA) binds to initiate transcription (Samjim, 2009). In order for mRNA to be translated by host cell machinery it requires both a 5' methylated cap and a 3' polyadenylated tail. The poly(A) tail is produced by a 'stuttering' step where the viral polymerase continuously replicates a short run of 5-7 uradines at the end of the vRNA (Digard et al., 1989) . The 5'cap is 'snatched' from host mRNAs. The endonuclease activity of the PB2 protein is essential to this process and as it cleaves host methylated cap from host mRNA and uses it to prime host transcription of the viral mRNA.

In the early stages of infection, the newly transcribed mRNA is then exported from the nucleus to the host cytoplasm where the translation of early infection proteins, PA, PB1,PB2 and NP occur. The viral polymerase complex and the NP are then transported back into the nucleus to allow for replication of uncapped RNA, which serves as the template for new vRNA (Wise et al., 2009). Later in the viral infection, newly synthesized vRNA are encapsidated with NP and these vRNPs are translocated to the host cytoplasm where they associate with NS2/NEP for

transportation to the apical surface and eventually with M1 for viral packaging. Also later in the viral cycle, the mRNA transcripts for the viral surface proteins M2, HA and NA are transported to the host cytosol, translated and transported through to the Golgi apparatus to the apical membrane (Johansson and Cox, 2011). It has been previously demonstrated that the transport of these proteins to the cell membrane is dependent upon lipid raft formation and localization and if this association is disrupted, then there is non-differential transport to the membrane (Nayak et al., 2009).

Once vRNPs and viral proteins accumulate at the apical cell membrane, viral packaging and budding occur (Barman et al., 2001;Barman et al., 2004). All three viral surface proteins associate with M1 and once this protein begins to accumulate, it allows protrusion from the host membrane, thus initiating budding of the virion. After the virus has budded, it is released through the cleavage of host sialic acid (which is bound to the HA), by the NA protein (Air, 2011).

1.2 Antiviral Strategies

The influenza virus presents a large disease burden upon society as a whole and as such has necessitated the development of several anti-influenza strategies to combat the disease. These strategies fall within two broad groups: vaccine therapy and antiviral drugs. While anti-viral drugs aim to treat an individual already infected with influenza, vaccine therapies have a prophylactic objective. Regardless, developing different treatment regimens for the influenza virus poses multiple challenges as the variable antigenic nature of the virus produces resistant variants to both drug and vaccination strategies.

1.2.1 Antiviral Drugs

Although there have been several possible target regions identified as potential therapeutic drug targets, to date there are only two broad classes of influenza inhibitors which are marketed.

These are ion pump inhibitors which target the M2 protein and sialidase inhibitors which target NA.

1.2.1.1 Ion Pump Inhibitors

As mentioned previously, M2 is a 97 residue protein (109 residue in influenza B), and the majority of these residues are either within the transmembrane domain (residues 24-46) or intracellular (47-97). The protein has two states, open or closed, by which it regulates the transportation of protons into the core of the virion (Pielak and Chou, 2011). The transmembrane domain of the M2 protein has been demonstrated to be essential for this proton selection process, particularly His37 and Trp41. Mutation studies with both amino acids have indicated that protonation of the histidine may lead to the opening of the ion channel and the tryptophan is necessary for a uni-directional transportation of ions into the virion (Tang et al., 2002). Likewise, the necessity of the histidine/tryptophan motif is evidenced by its conservation amongst all influenza A subtypes as well as being present in influenza B (Holsinger et al., 1995).

The transport of ions into the virion is necessary for viral propagation as it is this acidification which allows the disassociation of vRNPs from M1, mitigating their release into the cell cytosol and initiating replication of the viral genome. The inhibition of this acidification can have a drastic effect upon viral growth (Pielak and Chou, 2011). To this end, the M2 protein was

targeted as a means of anti-viral intervention and both amantadine hydrochloride and its derivative rimantadine are molecules which inhibit this acidification in influenza A strains (Ciampor et al., 1992). Despite their chemical similarities, it has been suggested that both of these compounds appear to have different mechanisms of blocking the M2 protein. Crystal structure has revealed that amantadine directly binds internal residues within the pore channel, inhibiting the proton transport. However, NMR solution of rimantadine and M2 suggests that this derivative does not bind internally, but rather it binds on the outside of the pore and stabilizes the closed pore formation thus inhibiting proton transport (Hay et al., 1985). The initial effectiveness of both amantadine and rimantadine at inhibiting viral growth has since been eclipsed as the ability of the influenza virus to rapidly adapt has resulted in multiple mutations conferring resistance to these drugs. Thus far it has been reported that mutations in amino acids 26, 27, 30, 31and 34 allow the virus to be resistant to both M2 inhibitors (Pielak and Chou, 2011). The emergence of resistant strains greatly increased during the 2005/2006 influenza season, prompting warnings from the WHO to restrict the usage of these inhibitors as a viable source of treatment against influenza infection.

1.2.1.2 Sialidase Inhibitors

The second class of influenza inhibitors target the sialidase activity of the neuraminidase protein (NA). As mentioned in section 1.1.3.4, NA is a tetramer composed of approximately 418 a.a. and its primary role is to mitigate the release of virus from cells and prevent viral aggregation on the cell surface by cleaving the sialic acid receptor (McKimm-Breschkin, 2013;Hashem et al., 2009). To date there are four separate neuraminidase inhibitors (NAI) (zanamivir, oseltamivir,

peramivir and laninamivir) with the two licensed inhibitors being zanamivir and oseltamivir (Kim et al., 2013). These inhibitors vary in chemical composition but use similar mechanisms to blockade the activity of NA by binding to the enzymatic active sight more strongly than the host sialic acid substrate. The successful inhibition of NA can significantly inhibit the spread of influenza virus and these NAIs have been effective when given either prophylactically during an influenza outbreak, or as treatment to those already infected with the virus (Air, 2011;Johansson and Cox, 2011).

As with M2 inhibitors, there has been increasing resistance by the influenza virus against this class of drugs. The first emergence was observed with oseltamivir and observed in both challenge studies and naturally occurring infections with 1-4% of individuals treated with oseltamivir infected with a resistant strain (McKimm-Breschkin, 2013). It has been subsequently demonstrated that mutations within the highly conserved active site or amino acids which provide structural stability to this site, can confer resistance against the NAI and 2008-2009, more than 90% of isolates collected from H1N1 infected patients were resistant against oseltamivir (McKimm-Breschkin et al., 2003). Specifically H274Y (which provides resistance only in N1 subtypes against oseltamivir) and E119V and R292K (which confers resistance only in N2 subtypes in oseltamivir) are the primary resistant mutations, however mutations in I222R or I222V have also been reported. Although resistance against zanamivir is not as common as that against oseltamivir, many of the resistant mutations are similar (Cheam et al., 2004;Hurt et al., 2006). Specifically, R292K and Q136K also decrease sensitivity to zanamivir while conferring complete resistance against oseltamivir.

Since NAI resistant mutations confer resistance by preventing the NAI from binding to the enzymatic site, it has been further noted that these resistant mutations can also reduce viral

fitness and transmissibility. To accommodate for this decrease in NA function, various compensatory mutations have developed within resistant strains in the HA protein. These mutations decrease the sialic acid binding affinity of HA thus matching the lack of NA enzymatic activity and increasing the overall robustness of the virus (von Itzstein et al., 1993).

1.2.2 Current Anti-influenza Vaccine strategies

Although there has been reasonable success with anti-influenza treatment, vaccine development against influenza is the primary means of prophylactic therapy and preventing disease spread. To this end several different vaccine strategies have been employed, with the most commonly used strategy being injection of inactivated virus to provide immunity against potential infection. Additionally live attenuated influenza vaccine (LAIV) has been used in various countries as well as alternative strategies such as DNA or peptide vaccinations which are currently used in experimental approaches and clinical trials.

1.2.2.1 Inactivated Vaccines

As previously mentioned, the most commonly used vaccine strategies incorporate inactivated strains of influenza which, when exposed to the immune system, provide both antibody and cyto-toxic T-cell mediated responses. Currently these types of vaccines typically combine three different strains, one from H1N1, H3N2 and B, and thus are referred to as trivalent inactivated vaccines (or TIV) (Hardy et al., 2011). Generally, TIV are primarily aimed towards inducing immune protection against HA and NA, however, the antigenic nature of these proteins

necessitates annual reformulation of the TIV (Poland et al., 2001). This is completed by utilizing strain surveillance programs which identify upcoming antigenic variants using the serum hemagglutinin-inhibition (HI) assay (Carrat and Flahault, 2007). This strategy can predict the circulating strains up to 9-12 months in advance, thus allowing for the development of new vaccines before the predicted strains can infect a population.

Inactivated vaccines are produced by first growing the selected strain in embryonated hens' eggs and then treating virus harvested in the allantoic fluid with either formalin or β-propiolactone (BPL). This treatment directly inactivates the virus, but allows for the preservation of viral proteins, which when exposed to the immune system can induce a protective response to future infection (Goldstein and Tauraso, 1970;Poland et al., 2001). However, many factors can affect the effectiveness of TIV, including the antigenic match between circulating and vaccine strains, the age of the recipients and their history of influenza exposure (Ambrose et al., 2011).

1.2.2.2 Live Attenuated Vaccines

As an alternative to TIV, live attenuated influenza vaccines (LAIV) have been developed by generating reassortant virus containing the HA and NA from epidemic strains, but the remainder of the genome and proteins from attenuated cold adapted virus (Belshe et al., 1998). These temperature sensitive viruses cannot replicate in the upper and lower respiratory tract, but they still provide immune system exposure the targeted NA and HA proteins. LAIV are delivered by intranasal spray and are typically used for children (over the age of 24 months) and pregnant woman (Ambrose et al., 2011).

LAIV can have several advantages to using a TIV. Primarily, since LAIV are delivered directly to the respiratory in addition to inducing cell-mediated responses, they can also induce mucosal immunity. LAIV has also been shown to be more protective in children when compared to the TIV and LAIV has a better efficacy rate when the predictive strains are unmatched (Ambrose et al., 2011).

1.3 Heterosubtypic protection against influenza virus

Despite the relative effectiveness of both TIV and LAIV, there have been several limitations to this vaccine approach. As mentioned above one of the largest obstacles is the variable nature of the influenza proteins resulting in the necessity of the pre-selection of vaccine strains to match the circulating strains (Johansson and Brett, 2007;Widjaja et al., 2006); (Ebrahimi and Tebianian, 2010). Also, given the mutagenic nature of the influenza glycoproteins HA and NA, vaccines which are directed towards eliciting antibody response to these antigens provide protection against homologous but not heterologous virus (Liang et al., 1994). Therefore development of a vaccine which induces a broad cross-protection between viral strains would be an advantage. To this end, many groups have explored the antigenicity of highly conserved regions within the NP, M2, HA and NA influenza proteins and used alternative methods for eliciting an immune response only to these conserved regions.

1.3.1 Heterosubtypic Protection Afforded by Vaccines Targeting NP

It has been previously demonstrated that NP is a highly conserved internal protein between subsets of Influenza A and B (Heiny et al., 2007;ElHefnawi et al., 2011a). Unlike HA and NA based vaccines, which exploit the epitope accessibility of these surface glycoproteins to induce neutralizing IgG antibodies and mediate T-cell responses, the inaccessibility of NP surface expression makes a robust humoral to response to either of these proteins unlikely in a whole virus vaccine model. However, using various methods to express only the protein of interest both *in vitro* and *in vivo* several studies have reported the ability of NP to illicit not only a humoral immune response, but also a robust cellular response which can confer protective heterosubtypic immunity (Liang et al., 1994).

Since NP is internalized, it was originally assumed that vaccines directed towards exploiting the conserved nature of NP may only provoke a cellular immune response but surprisingly, recent research has demonstrated that anti-NP IgG may also contribute to heterosubtypic immunity against Influenza A. This hypothesis is further supported by studies which show that after NP DNA vaccination, cross-subtype protection was not maintained in B-cell deficient mice (Rangel-Moreno et al., 2008).

Attempts have been further made to increase the protection of NP DNA vaccine by boosting with recombinant adenovirus vector (rADV) containing the NP insert. This prime-boost immunization further induces T-cell responses and demonstrated heterosubypic protection in mice were then challenged with a sublethal doses of either H1N1 or H3N2 (Epstein, 2003;Epstein et al., 2005).

1.3.2 M2 as a Heterosubtypic Vaccine Candidate

Unlike NP, M2 is not an internal protein but has a 24 amino acid stretch which is external to the viral envelope (Holsinger et al., 1995). However, this extracellular domain is not as antigenic as the other influenza surface glycoproteins as it is shielded by both HA and NA. In whole viral vaccines, this shielding may greatly prevent an effective antibody response to M2 (Johansson and Brett, 2007). Interestingly, M2 is still abundantly expressed on the surface of infected cells leaving it susceptible to complement activation and opsonizing antibodies (Lamb et al., 1985). DNA vaccines which solely express M2 can elicit an IgG response which may confer protective immunity through this complement pathway (Jegerlehner et al., 2004).

It has also recently been shown that alveolar macrophages and the IgG Fc-gRIII receptor also play a key role in immunity against the M2 (El Bakkouri et al., 2011). In addition to humoral immunity, like NP, epitopes from the highly conserved ectodomain of M2 (M2e) have also been shown to bind in both the MHC-I and MHC-II pockets permitting antigen presentation and inducing cellular immunity. In this way the M2e epitope regions are effective in inducing both the humoral and cellular arms of the immune system (ElHefnawi et al., 2011b).

Rather than rely on current TIV and LAIV vaccine strategies, many groups have opted to examine the protective effect of vaccines which only induce an immune response to the highly conserved ectodomain of the M2 protein (M2e). To this end viral like particles (VLP) are used which express the M2e peptide in insect cells which are then used for intranasal vaccination. These have been shown to induce high titres of anti-M2e IgG and provided cross protection against different subtypes of H3N2, H1N1 and H5N1 in mice, suggesting that this may be an alternative method for universal vaccine development (Song et al., 2011; Fiers et al., 2009).

1.3.2 Heterosubtypic Protection Afforded by Hemagglutinin Protein

As mentioned above, current vaccination strategies primarily rely upon neutralizing antibodies generated against the HA protein, as anti-HA antibodies can directly block virus attachment to the target cells by interfering with virus-receptor interaction and prevent influenza infection. However, it has been long believed that, during natural infection and vaccination with killed or live-attenuated viruses, the host immune system mainly recognizes the bulky and highly variable-immunodominant globular head domains in HA which shield the more conserved regions such as the stem region of HA (Nabel and Fauci, 2010) thus, it does not lead to the development of universal protection or heterosubtypic immunity, against drifting strains. Such immunity has always been thought to mainly mediated by cross-reactive cytotoxic T lymphocytes (CTLs) directed against the highly conserved internal proteins but not the surface glycoproteins (Rimmelzwaan et al., 2007). To solve this problem, multiple groups have taken to identifying conserved regions within the HA protein, and then using DNA vaccine techniques to promote heterosubtypic immunity (Wei et al., 2012).

Cross protective antibodies against HA have been long known (Sanchez-Fauquier et al., 1987;Nabel and Fauci, 2010) although most of these antibodies lacked neutralization activity. Recently, two independent research teams identified a panel of broadly neutralizing antibodies through screening of such libraries from healthy immunized volunteers. These antibodies bind to a highly conserved conformational dependent pocket-like epitope in the stem region of HA protein, which is formed by two elements critical for the pH-induced conformational change, the fusion peptide and the αA-helix (Ekiert et al., 2009). The epitope is highly conserved among all group 1 influenza A viruses. Thus, these cross-neutralizing antibodies, CR6261 (Ekiert et al., 2011) and F10 (Sui et al., 2009), inhibited all group 1 influenza A viruses by blocking the release

of the fusion peptide, the first step in membrane fusion, and protected mice from lethal H5N1 or H1N1 challenges. However, they do not neutralize group 2 influenza A viruses, which appear to be structurally different from group 1 viruses.

Although, the degree of sequence diversity between HA subtypes is great, particularly in the HA1 subunit, different reports showed the existence of some conserved sequences within HA1(Veljkovic et al., 2009). Additionally, the HA2 polypeptide is known to be relatively more conserved across influenza A virus subtypes compared to HA1; particularly the HA1–HA2 joint region, which includes the N-terminal fusion peptide of HA2 (Okuno et al., 1993;Horvath et al., 1998). Indeed, our recent comprehensive analysis of all available HA sequences from the NCBI influenza virus resource from all hosts (3896 strains of influenza) showed that the HA2 N-terminal 14 amino acids (GLFGAIAGFIEGGW) of the fusion peptide (donated Uni-1 peptide) are conserved among all known 16 subtypes of influenza A viruses as well as influenza B viruses with only minor substitutions (Chun et al., 2008). Thus, such highly conserved protein sequences could serve as targets for vaccine, anti-virals or diagnostic tools development, particularly when used in DNA plasmid vaccines.

1.3.4 Neuraminidase as a Heterosubytic Vaccine Antigen

Although the main target of current vaccines is the HA, it has been previously demonstrated that antibodies to the NA protein can interfere with viral spread and contribute to protection against influenza infection (Johansson et al., 1989;Qiu et al., 2006;Chen et al., 2000;Chen et al., 2005;Hashem et al., 2011). Since both, HA and NA proteins are highly variable (Johansson and Brett, 2007;Hashem et al., 2011), heterosubtypic protection against influenza A

viruses is largely believed to be mediated by cross-reactive cytotoxic T cells against the highly conserved internal proteins rather than by antibodies against HA or NA (Epstein et al., 2005;Takada et al., 2003;Rimmelzwaan et al., 2007). However, recent reports from Sandbulte *et al.* (Sandbulte et al., 2007) and Marcelin *et al.* (Marcelin et al., 2011) have shown that cross-reactive anti-NA polyclonal antibodies elicited by H1N1 could not only contribute to protection against H1N1, but also provide cross-protection against H5N1 and 2009 pandemic H1N1 viruses, respectively. Yet, an antigenically conserved sequence affording heterosubtypic inhibition across all subtypes of NA has not been reported (Marcelin et al., 2012;Johansson and Brett, 2007).

In an attempt to identify future vaccine targets, several groups have been able to apply different bioinformatics approaches to identify conserved peptides or epitopes within the neuraminidase protein. In identifying such epitopes in H5N1 subtype, Somvanshi *et al* computationally analyzed the NA sequences of Influenza A H5N1 variants and identified 13 NA conserved major histocompatibility complex (MHC) class I and II epitopes between chicken and duck H5N1 isolates, but not human isolates (Somvanshi et al., 2008). Similarly, *in silico* analysis by Parida *et al*. revealed two conserved peptides within the NA of H5N1 viruses, IRIGKGDV and DRSPHRTLM, which were predicted to be putative B-cell and T-cell epitopes (Parida et al., 2007).

Similarly to the conserved sequences identified in H5N1, Gupta *et al*. compared the H1N1 neuraminidase amino acid sequences from isolates taken from 86 different countries and obtained from the EpiFlu database (Gupta et al., 2010). The group analyzed these conserved sequences using resources available from the immune epitope database, to predict 9mers and 13mers for MHC class I and HC class II epitopes; respectively. Thus, 15 conserved epitopes

between H1N1 variants were predicted to elicit robust T-helper, cyotoxic T-cell and B-cell responses (Gupta SK, 2010).

Although identification of potential epitopes within viral strains is useful to generating cross-protective antibodies, there has yet to be an antibody generated against a universally conserved sequence which confers heterosubtypic protection against NA. To this end, my lab has recently completed a comprehensive bioinformatics analyses of all NA sequences in the GenBank which has identified a novel conserved peptide region in the NA protein (denoted HCA-2), with a conservation rate being nearly 100% (Gravel et al., 2010;Varghese et al., 1983). This epitope, comprised of "ILRTQESEC", constitutes part of the enzymatic active site. Monoclonal antibody (MAb) (denoted as HCA-2 antibody) generated against this linear epitope was able to quantify the NA component in vaccine preparations.

While the HCA-2 MAb has been used only as a reagent for NA quantification, the virological importance of this unique epitope, or the inhibition capacity of the MAb remains essentially unknown. To address these questions, I aimed to dissect the functional roles of this universal epitope in viral replication and determined the potential of the HCA-2 antibody in inhibiting viral growth across viruses representing all influenza A NA subtypes and influenza B NA. These studies revealed that this universally conserved region contributes significantly to efficient viral replication through maintaining favourable NA protein structure and substrate binding to maximize enzymatic activities (Doyle et al., 2013). I further report that the HCA-2 antibody is the first to significantly inhibit all 9 NA subtypes and NB *in vitro* and provide heterosubtypic protection in mice that were passively transferred with the antibody and then challenged with lethal doses of either H1N1 or H3N2 virus.

1.4 Objectives:

1. To use a reverse genetics approach to generate alanine scanning virus within the HCA-2 region.
2. To analyze the function of these viral variants and determine the contribution of the HCA-2 region to viral fitness.
3. To determine the heterosubtypic inhibitory capacity of the HCA-2 MAb both *in vitro* and *in vivo*.
4. To deduce if this HCA-2 MAb can inhibit different lineages of influenza B and drug resistant strains of influenza B

Chapter 2

Materials and Methods

2.1 Cells and Viruses

Madin-Darby Canine Kidney (MDCK) cells and Human Embryonic Kidney 293-T (HEK 293-T) cells were obtained from the American Type Culture Collection. Cells were grown and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, as previously described (Hashem et al., 2012). Influenza A strains used to investigate the heterosubtypic inhibition effect of the HCA-2 antibody are listed in Table 2.1. Virus stocks were propagated either at or 37°C in the allantoic cavities of 10-day-old embryonated hen eggs for 24 hrs or, in the case of influenza B, at 35°C in MDCK cells for 24hrs. Allantoic fluid was clarified by centrifugation, aliquoted and stored at -80 °C until used. Viruses were titred in MDCK cells as previously described (Hashem et al., 2009).

Table 2.1: Viral Stains Used for Monoclonal *in vitro* and *in vivo* Inhibition

Virus Used	Type of Experiment used
A/FM/1/47/MA (H1N1)	*In vivo*
A/HK/1/68/MA (H3N2).	*In vivo*
A/PR8/34 (H1N1)	*In vitro*
A/Turkey/Mass/3740/65 (H6N2)	*In vitro*
A/Turkey/Oregon/71 (H7N3)	*In vitro*
A/Turkey/Ont/6118/68 (H8N4)	*In vitro*
A/Shearwater/Australia/72 (H6N5)	*In vitro*
A/DK/Czechoslovakia/56 (H4N6)	*In vitro*
A/EQ/Paragues/1/56 (H7N7)	*In vitro*
A/Quail/Italy/1117/65/(H10N8)	*In vitro*
A/Turkey/Wisconsin/68/(H5N9).	*In vitro*

2.2 Mutagenesis and Reverse Genetics

To determine the effect of each individual a.a. within the HCA-2 region on antibody binding or viral growth, substitution of each with an alanine was performed. The influenza A/Puerto Rico/8/34(H1N1) virus 8 plasmid reverse genetics system was kindly provided by Dr. Richard Webby (St. Jude Children's Research Hospital, Memphis, TN). The Stratagene Quick change II mutagenesis kit was used to generate these substitution mutations within the NA plasmid. Primers were then designed to insert either substitution mutations or the deletion mutation. Mutagenesis was then performed as per the manufacturer's instructions and plasmids were sequenced to ensure the presence of each mutation. Viruses containing the WT NA sequence or each of the mutated NA were then generated using eight-plasmid reverse genetics system (Hoffmann et al., 2000), thus WT NA containing plasmid or plasmids with the mutated NA sequence were used to transfect HEK 293-T cells with backbone plasmids from influenza A/Puerto Rico/8/34(H1N1) virus (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW197-M, pHW198-NS).

For transfection, HEK 293-T cells were seeded onto 6 well plates in regular DMEM + 10% FBS media until a monolayer of cells were confluent. These cells were then washed and 2ml of OPTI-MEM media (Invitrogen) was added. Each plasmid was then added to an eppendorf tube containing 270µl of Lipofectamine (which was brought to room temperature) at a concentration of 0.65µg/ml. The Lipofectamine and plasmid mixture was incubated at RT for 30 minutes. The mixture was then slowly added to the monolayer of HEK-293T cells. The cells were incubated for 48hrs at 37^{o}C, after which the OPTI-MEM media was changed and 1ml of media was added. After an additional 24hr incubation with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) trypsin was added to the cells at a concentration of 2µg/ml.

Between 24hr-48hrs later, the cells were lifted and 300ul of the cell mixture was injected into each 10 day embryonated chicken egg. The eggs were incubated at 37^{o}C for 72hrs and the allantoic fluid was then harvested and tested for a positive HA test with 0.5% chicken red blood cells.

After a positive HA test was detected from harvested samples, virus samples were amplified by additional passaging in 10 day old embryonated chicken eggs, purified and concentrated over a 30% sucrose cushion. To ensure that no additional mutations occurred in the NA or HA during generation or passaging, vRNA was extracted from E3 viruses and sequenced (see below).

2.3 Sequencing Analysis

vRNA from rescued virus was extracted using vRNA extraction kit from Qiagen and cDNA was generated using the Superscript III kit (Invitrogen) and reverse universal primers for both NA and HA. Samples were then incubated for 20 minutes with RNase H at 37^{o}C. The samples were then PCR amplified using platinum Taq (Invitrogen) and universal forward and reverse primers for either HA or NA. PCR products were then gel purified using 0.8% Ultra-pure agarose and extracted using Qiagen gel extraction kit. Extracted products were then PCR amplified using ABI big dye kit along with one of six separate primers for either HA or NA which ensured ample coverage of the entire gene. After amplification the samples were cleaned with Millipore filtered plates and sequenced using H.A.L sequencer. Chromatograms were analyzed using SequencherTM software and protein translation and blastN and blastP using ExPasy and the NCBI data base.

2.4 Plaque Assay

Viruses were prepared in 10 fold dilutions in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.2% bovine serum albumin (BSA) and 2μg/ml of TPCK treated trypsin. Confluent monolayers of canine kidney cells (MDCK) were inoculated with 1ml of virus/media mixture and incubated at 37^{o}C for 1hr at which point the inoculums were removed and the cells were washed with phosphate buffered saline (PBS) and overlaid with DMEM containing 1.6% agarose, 0.2% BSA and 2μg/ml of TPCK-treated trypsin. After 3 days of incubation at either 37^{o}C (for influenza A virus) or 35^{o}C (for influenza B virus) cells were fixed with 10% formaldehyde followed by staining with 0.5% of crystal violet and plaques were counted and (in some cases) photographed.

2.5 Growth Curves

MDCK cells in a 12 well plate were inoculated with viral samples in serum free DMEM at a high multiplicity of infection (MOI) of 5 pfu per cell or a low MOI of 0.001 for 1 hr in the presence of 2 μg of TPCK-treated trypsin. This MOI was determined by first lifting a monolayer of MDCK cells in a test well and counting them, and then determining the appropriate amount of virus to add based upon the pfu titre of each viral sample which was previously determined (Table 2.5). The inoculum was removed by extensive washing with 0.9% NaCl in citric acid buffer and PBS thereafter. Next, DMEM serum-free media with 2 μg/ml TPCK-treated trypsin was then added to the inoculated cell monolayers. For the high MOI, supernatants were harvested every 1.5 hrs from time 0 hrs until time 9 hrs. For the low MOI, supernatant was harvested over a course of 72 hrs at time points 0 hrs, 6 hrs, 12 hrs, 18 hrs, 24 hrs, 36 hrs, 48 hrs

and 72 hrs. Growth curve analysis was performed in triplicates. Harvested media was then titred using plaque assay as previously described (Hashem et al., 2009).

Table 2.5: Example of an MOI calculation to determine the appropriate amount of virus to inoculate for growth curve.

Number of cells determined in a 12 well plate: 5 x 105/ml

MOI required: 5

Calculation:

Amount of virus required = 5 ($5x\ 10^5$/ml)
= 2.5×10^6/ml virus

2.6 NA Enzymatic Activity

NA enzymatic activity was measured by standardizing the samples with pfu as described (Ilyushina et al., 2012). Virus was aliquoted at a titer of 1 x 10^7 pfu and then incubated at 37^{o}C with MUNANA [2-(4-methylumberlliferyl)-a-D-*N*--acetylneuraminic acid] in black walled clear bottom 96 well pates. After 20 min the reaction was stopped with 0.14M NaOH and 83% ethanol. The signal was then measured with the SynergyTM 2 Multi-Mode Microplate Reader with an excitation of 360 nm and emission of 448 nm (Potier et al., 1979). The analysis was performed in triplicate where the average activity of the WT was set as 100% and the average activity of the other variants is expressed as percentage of the WT. To assess *Km* and *Vmax*, virus samples were incubated with concentrations of MUNANA ranging from 0 to 1200 μM. Fluorescence was monitored every 60s for 60 min. The *Km* and *Vmax* were calculated with GraphPad Prism software (GraphPad version 5), by fitting the data to Michaelis-Menten equation using non-linear regression.

2.7 Thermostability of NA

The effect of temperature on NA function was determined by incubating the NA proteins for 15 min at various temperatures ranging from 36 – 54^{o}C (Palese et al., 1974). After incubation, NA activity was immediately determined using MUNANA assay and, as described above. With all variants and WT virus, 100% NA enzymatic activity was set as that determined by MUNANA assay at 37oC. Readings generated at higher temperatures were referring to the percentage of the activity at this optimum temperature for enzymatic activity.

2.8 Protein Modeling

Mutations were homology modelled against influenza N1 crystal structure. Models were prepared for each mutation using 'automodel' module from the Modeller software version 9.8 (Eswar et al., 2006) and were superimposed with the WT NA and inspected for impact on enzyme structure and sialic acid binding. Visualization and analysis were performed using the software UCSF Chimera with the protein data base (pdb) code I33K (for N1) and R36K (for NB) (Pettersen et al., 2004).

2.9 Antibodies Against the Universally Conserved Epitope in NA

A computational analysis was performed previously to identify the HCA-2 region and rabbit mono-specific polyclonal antibodies were generated to this region (Gravel et al., 2011;Chun et al., 2008;Gravel et al., 2010). Hybridoma cell lines were generated from rabbit spleens at Epitomics and these cell lines were used to inoculate CellLine bioreactor flasks in hybridoma media (Gibco). The antibody was harvested from these flasks once per week and dialyzed with tubing (Sigma) for 48 hrs with three changes of BuPh buffer provided by Thermo Scientific. After 48hrs the samples were purified using protein A/G columns (Thermo Scientific) and again dialysed in phosphate buffered saline (PBS) with three buffer changes. After 48hrs of dialysis, the antibody was removed from the tubing and concentrated using amplicon 15-centrifugation units (Millipore). The antibody was then filtered in 0.22μm filters and the concentration of MAb was then determined by measuring absorbency using Nano-drop.

To sequence the immunoglobulin genes encoding the antibodies against the HCA-2 epitope, the hybridoma cells were lysed and mRNA was extracted using Qiagen mRNA extraction kit. The mRNA was then amplified and sequenced using previously reported primers

to conserved regions on the rabbit V_H and V_L chain genes (Foti et al., 1998). In addition, to confirm sequencing data, purified MAb sample was analyzed by ultra performance liquid chromatography coupled with mass spectrometry. Samples were digested with trypsin or chymotrypsin then analyzed using both quadruple-time of flight (QTOF) and ion trap Fourier transform ion cyclotron resonance (IT-FTICR) MS/MS systems. Both MS/MS systems were coupled to a Waters NanoAcquity UPLC, which separated peptides on a reverse phase (C18) analytical column. A custom database was generated by appending the translated gene sequencing results to the SwissProt protein database (updated 2012-07) and data were searched there against using Mascot server version 2.3 (Matrix Science). Positive control (polyclonal anti-N1 sera) was obtained from National Institute for Biological Standards and Control, Potters Bar, U.K. Normal rabbit IgG (negative control) was purchased from Sigma, St Louis, MO.

2.10 Virus Inhibition Assay

The assays were carried out as described previously (Hashem et al., 2010). Unless specified, antibodies were serially diluted and mixed with equal volume of each virus (100 pfu) in a total volume of 60 µl of DMEM containing 0.2% BSA and 2 µg of trypsin and were incubated at 37^{o}C for 1 hr. The antibody-virus mixture was transferred to confluent MDCK cells in 96 well plates and incubated at 37^{o}C for 20 hrs. To determine whether the antibody could have inhibitory effect on the viral entry, the antibody-virus mixture was incubated on MDCK cells for 1 hr and then removed. The cells were then washed with PBS three times and covered with 50 µl of DMEM and incubated at 37^{o}C for an additional 19 hrs. Afterwards, the media was removed, cells were washed with PBS and fixed with 80% cold acetone for 10 min and washed in PBS and 0.5% Tween. Viral NP antigen was then detected by indirect ELISA with anti-NP monoclonal

antibody. Fixed cells were washed three times with PBS/Tween and incubated with mouse anti-NP antibody (Sigma), diluted 1:4000 in PBS-T containing 5% skim milk at 37^{o}C for 1hr. Cells were then washed again for four times and incubated with 1:2000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma) at 37^{o}C for 1hr. After 6 washes 100µl of TMB substrate was added to wells at RT for 30 minutes. The reaction was then stopped with 100µl of stop solution and absorbance was read at 450nm using SynergyTM 2 Multi-Mode Microplate Reader. Inhibition was calculated as a percent of the average absorbance from triplicate wells of virus-antibody mixture relative to no antibody (uninfected) control.

2.11 Enzyme Linked Lectin Assay (ELLA)

Ninety six well flat bottom plates were coated with 25 µg/ml of fetuin (Sigma) and stored at 4^{o}C for at least 18 hrs before use. Viruses were previously titrated using the ELLA analysis for optimal signal and this amount of virus was then incubated for 2 hrs with serial dilutions of purified monoclonal HCA-2 antibody. The fetuin coated plates were then washed in PBS - tween and virus-antibody mixture was transferred to the wells and incubated at 37^{o}C for 20 hrs. The plates were again washed and incubated with peanut lectin (Sigma) for 2 hrs at room temperature in the dark and, after washing, were then incubated with Avedin Peroxidase for 1 hr in the dark at room temperature. After washing the substrate, o-phenylenediamine dihydrochloride (OPD) (Sigma) dissolved in citrate buffer was then added to the wells for 10 min when the reaction was stopped with 1N Sulfuric acid and plates were read with SynergyTM 2 Multi-Mode Microplate Reader at 490 nm. Inhibition was calculated as a percent of the average absorbance from triplicate wells of virus-antibody mixture relative to no antibody (uninfected) control.

2.12 Mice

Female, 19-21 gram, CD-1 mice were purchased from Charles River Laboratories International (Wilmington, MA). The animals were maintained in the University of Ottawa animal facility (Biochemistry, Microbiology and Immunology department, University of Ottawa, Ottawa, ON). All *in vivo* research was performed in accordance with the guidelines of the Canadian Council on Animal Care. The animal study protocol was approved by the University of Ottawa Animal Care Committee (Protocol No. BMI-85). All efforts were made to minimize suffering, and mice were humanely euthanized upon experimental endpoint (when infection resulted in greater than 25% body weight loss accompanied by respiratory distress).

2.13 Passive Transfer and Influenza Challenge Studies

Naïve CD-1 mice (n =5) were injected intraperitoneally with either 60 mg/kg of HCA-2 MAb or normal rabbit IgG for 3 days; day -2, -1 and 0 relative to challenge. On day 0, mice were intra-nasally challenged with 25µl (5xLD50) 5 x 10^3 pfu of influenza A/FM/1/47/MA (H1N1) or 2 x 10^3 pfu of influenza A/HK/1/68-MA (H3N2). Mice were weighed daily and clinically monitored for signs of illness for 14 days.

Statistical Analyses

Two-way Analysis of Variance (ANOVA) with the Bonferroni post-test was used to compare data between variants. All statistical analysis was conducted using GraphPad Prism

software (San Diego, CA). P-values <0.05 were considered statistically significant. Results are presented as the mean ± SEM.

Chapter 3

Results

3.1 Alanine Scanning and Viral Rescue

In order to determine the contribution of the universally conserved HCA-2 region to NA function, I substituted each individual amino acid (a.a.) with alanine in the HCA-2 domain of the NA protein and analyzed the viability of the mutant viruses using the 8-plasmid reverse genetics system (Hoffmann et al., 2000), (Liu et al., 2009). The successful rescue of virus was first determined by positive hemagglutination assay, followed by plaque assay in conjugation with vRNA detection. These experiments suggest that substitution of L223 or C230 (Table 3.1) with alanine may be lethal to viral survival, thus revealing potential critical roles for these a.a. to viral replication. Moreover, transfectant viruses missing the entire HCA-2 region were also found to be not rescuable, further confirming that this region is necessary for the production of infectious virus. The other mutants I222A, T225A, Q226A, E227A, S228A, and E229A substitutions were generated. Sequencing of vRNA extracted from these variants confirmed the presence of the substitutions and the absence of any additional mutations in other regions of the NA or HA after passaging.

Table 3.1: The Rescue of Viral Variants of the HCA-2 Region

Variant	Rescued
WT	+
I222A	+
L223A	-
R224A	+
T225A	+
Q226A	+
E227A	+
S228A	+
E229A	+
C230A	-

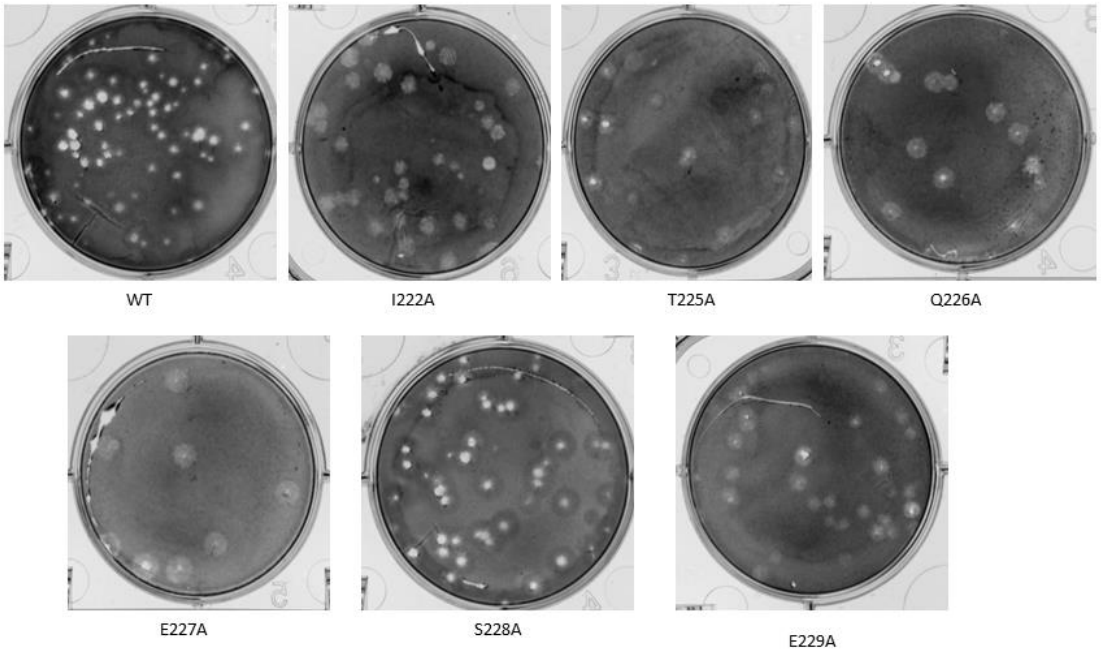
Figure 3.1

Plaque Morphology of Alanine Scanning Mutants:

After rescuing a proportion of the alanine scanning variants, we next explored the effect of these mutations on plaque morphology. As seen in Figure 3.1, when compared to the WT, all of the rescued variants display a difference in plaque size. Although the plaques of some variants appear to be larger in size when compared to the WT, all of these demonstrate a halo effect surrounding the plaque. This indicated an incomplete lysis of the surrounding cells and further suggested that mutations within the HCA-2 region may impede viral spread. We noted that the plaques which appeared to most resemble that of the WT, was S228A, as, although the halo effect was still present with this variant, there was also a clear lysis pattern within the center of the plaque; a characteristic not observed amongst the other variants.

Figure 3.1

Plaque Morphology: Cell monolayers were infected with purified virus (either WT or an alanine scanning mutant) for 72 hours with agarose. Cell layers were then stained with 1% crystal violet and images of plaques were taken.



3.2 The HCA-2 Region Affects Viral Spread and Growth Kinetics

In order to compare the growth kinetics of these viral variants, I infected MDCK cells with viruses at high and low multiplicity of infection (MOI) to determine the effect of these substitutions on a single-round of viral replication and viral spread, respectively. As shown in the high MOI growth curve (Fig.3.2.1), the wild-type (WT) virus grew significantly better than all other NA variants. Although the T225A variant appeared to be the most robust in growth of all the variants during the 6hr and 7.5hr time points, S228A produced the highest viral titer and grew significantly better than any of the mutants at the 9hr time point. In addition, variation in viral growth was observed between the remainder of the variants, and I222A, E227A and E229A appeared to be the most suppressed in growth. These high MOI results further confirm the importance of the HCA-2 region as these mutations significantly hindered the growth of influenza virus.

Next, I aimed to determine the effect the HCA-2 mutations on viral spread. To this end I examined the low MOI growth curve of the variants (Fig.3.2.2). The significant difference in viral growth was again demonstrated between the WT virus and all the other HCA-2 variants, particularly at the 24 - 72hr time points. Consistent with the High MOI growth, S228A grew relatively better than the other mutants at the time points between 24 – 72hrs, but significantly less so than WT virus. These data show that all of the NA variants grew much less effectively than the WT and that this impaired growth differed in magnitude between them.

Figure 3.2.1

High MOI Growth Curve: To determine single step virus growth, MDCK cells were infected with WT or mutants viruses at a high MOI (5) and at various times post infection up to 9hrs the supernatants were collected for virus titration in a plaque assay. Error bars represent s.e.m. Two-way Analysis of Variance (ANOVA) was used for significance comparison, using bonferoni posthoc tests with p-value <0.05 being considered significant. * denotes significant difference found between WT and the remainder of the variants.

Figure 3.2.1

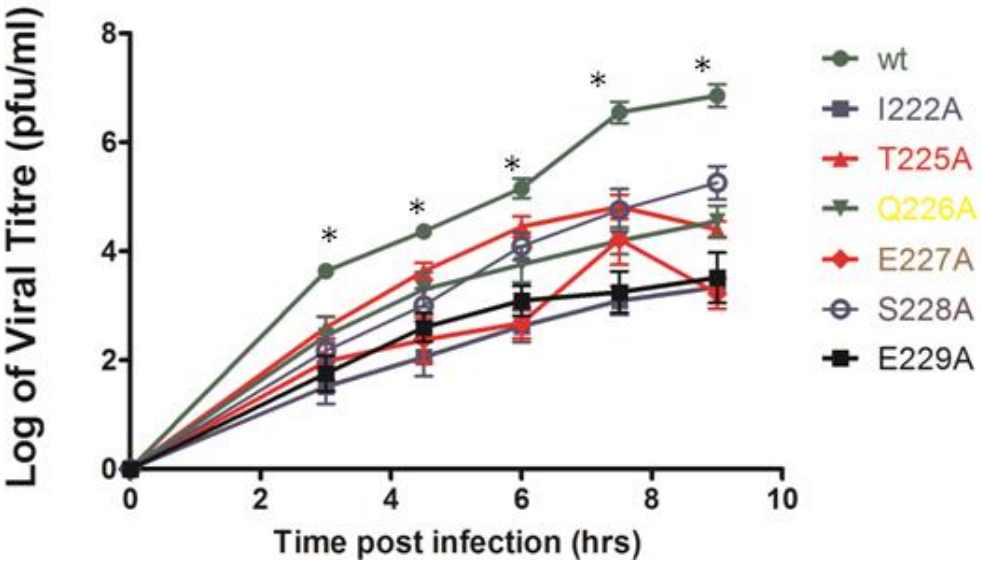
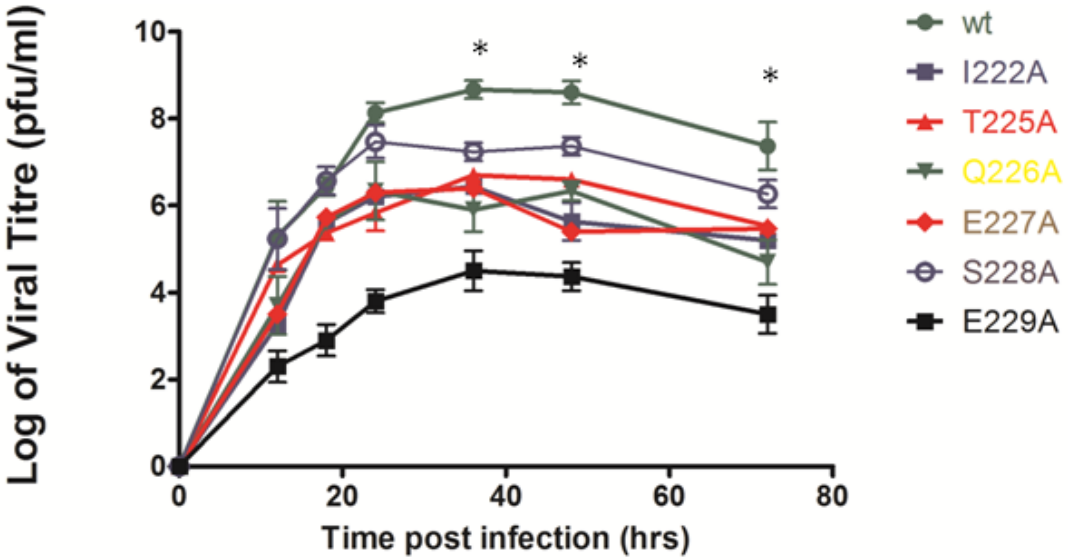

Log of Viral Titre (pfu/ml)
Time post infection (hrs)
0
2
4
6
8
10
wt
I222A
T225A
Q226A
E227A
S228A
E229A
*
*
*
*
*

Figure 3.2.2

Low MOI Growth Curve: To determine multiple steps of virus growth, MDCK cells were infected with WT or mutants viruses at a low MOI (0.001) and at various times post infection up to 72hrs the supernatants were collected for virus titration in a plaque assay. Error bars represent s.e.m. Two-way Analysis of Variance (ANOVA) was used for significance comparison, using bonferoni posthoc tests with p-value <0.05 being considered significant. * denotes significant difference found between WT and the remainder of the variants.

Figure 3.2.2


Log of Viral Titre (pfu/ml)
10
8
6
4
2
0
0
20
40
60
80
Time post infection (hrs)
*
*
*
wt
I222A
T225A
Q226A
E227A
S228A
E229A

3.3 The HCA-2 region significantly alters NA enzymatic activity and substrate binding

I next determined whether the decreased levels of viral replication of these mutant viruses were associated with altered enzymatic activities. The NA enzymatic activities of all mutants showed significantly lower activities compared with the WT virus (Fig. 3.3.1). It is also of note that the observed low NA enzymatic activities in the mutants were largely correlated with the viral growth data shown in Fig. 3.2.1 and 3.2.2.

To shed light on the molecular mechanism underlying the altered enzymatic activities in the various mutants, I determined the *Km* and *Vmax*, which reflect the enzyme's affinity for the substrate and the maximum rate a reaction can occur when fully saturated with substrate. As shown in Fig. 3.3.2, the WT enzyme has a higher rate of converting the substrate to the final products. Here it is shown that the WT had a significantly smaller *Km* and larger *Vmax* values compared with all of the mutants. Specifically, the WT *Km* value is approximately 40 uM compared with 122 – 253 uM for the mutants, suggesting that mutation in this region resulted in a decreased affinity for sialic acid substrate. Moreover, the smaller *Vmax* values of the variants represent the diminished rates of enzyme catalysis as a result of mutations in HCA-2 region. This difference in the *Km* and *Vmax* between the mutants is also noted, although not as marked as that compared with the WT. Indeed, the conversion rate was higher in the WT, being 7.6 times more effective, when *Vmax*/*Km* ratio is considered, than S228A, a mutant which possessed the best viral replication among all HCA-2 region variants (Figure 3.3.2 and Table 3.3). Apparently, the difference in the viral growth and Michaelis-Menten kinetics amongst the viral mutants may be due to varying degree of structuring changes in the NA proteins induced by the individual a.a substitution (see below for more discussion).

Figure 3.3.1

Measurements of NA enzymatic activities. Variants containing alanine mutations were analyzed based upon the amount of MUNANA metabolized, experiments were performed in triplicates with error bars representing s.e.m. Two-way Analysis of Variance (ANOVA) was used for significance comparison, with Bonferoni post-hoc tests, with p-value <0.05 being considered significant.

Figure 3.3.1

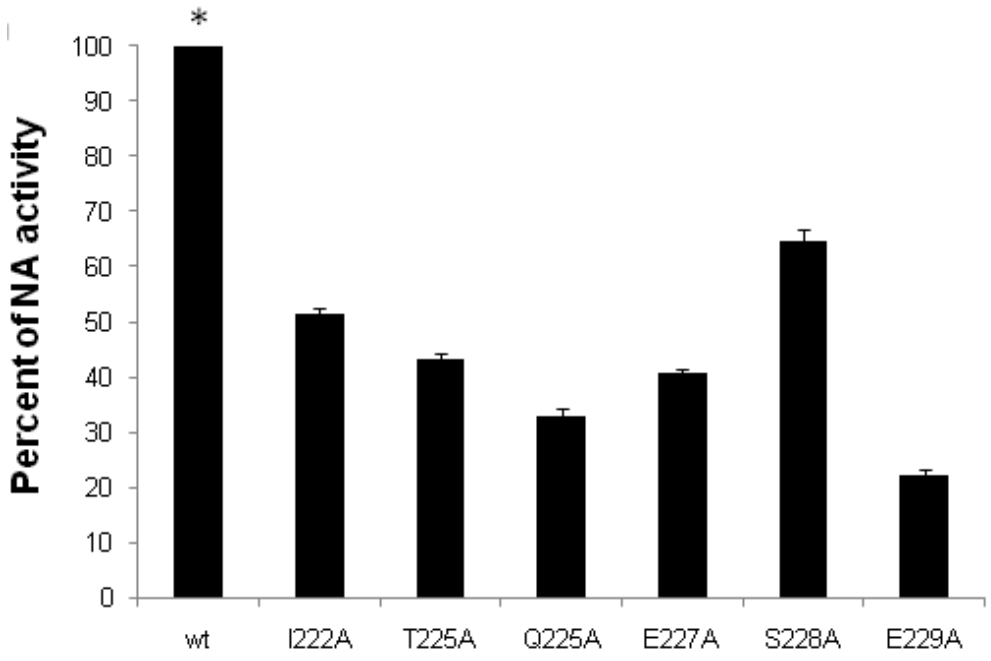

*
Percent of NA activity
100
90
80
70
60
50
40
30
20
10
0
wt
I222A
T225A
Q225A
E227A
S228A
E229A

Figure 3.3.2

Enzymatic kinetics study on WT and variants.

Fluorescence of MUNANA was measured at concentrations of 0 – 1200 mM every 60 sec for 60 min. Velocity is expressed in terms of flurometric units/sec. Data shown represents one of three experiments conducted on three different occasions.

Figure 3.3.2

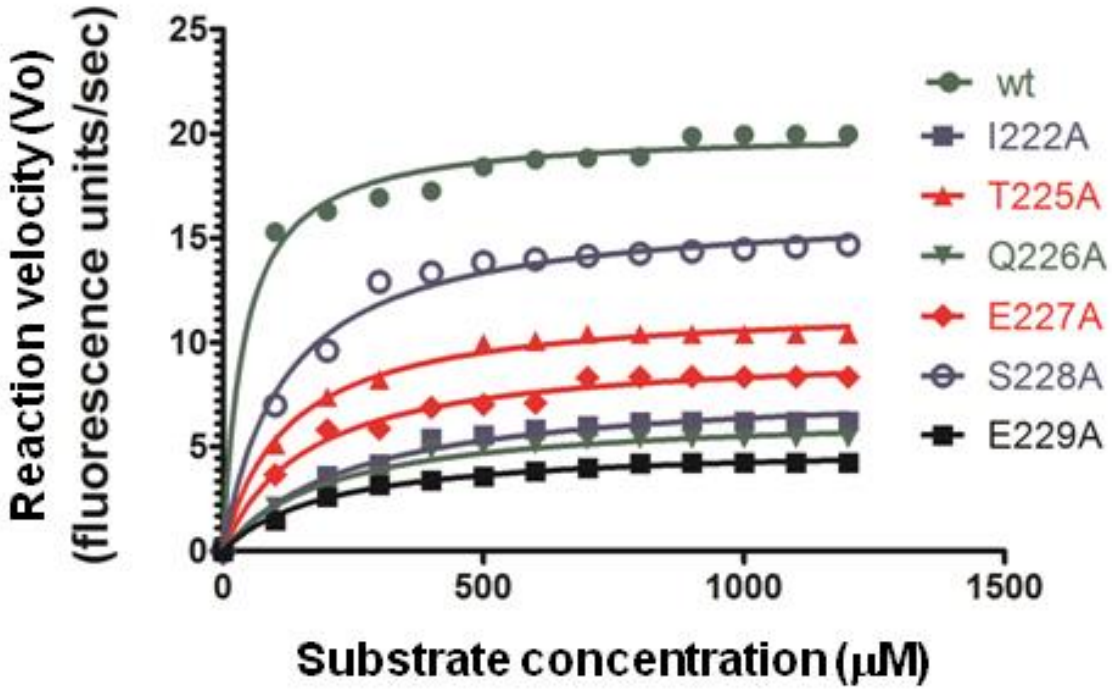

Reaction velocity (Vo)
(fluorescence units/sec)
25
20
15
10
5
0
0
500
1000
1500
Substrate concentration (μM)
wt
I222A
T225A
Q226A
E227A
S228A
E229A

Table 3.3

***Km* and *Vmax* of Viral Variants**

Variant	*Vmax* (U/sec)	*Km* (μM)	*Vmax/Km*	Fold¶
WT	20.85 +/- 0.3213	40.88 +/- 6.550	0.510	1
I222A	7.975 +/- 0.3889	253.2 +/- 41.95	0.031	0.06
T225A	11.85/- 0.1925	122.4 +/- 10.22	0.097	0.09
Q226A	6.656 +/- 0.2726	209.8 +/- 32.26	0.031	0.03
E227A	9.658 +/- 0.2727	162.2 +/- 19.78	0.052	0.05
S228A	16.48 +/- 0.3859	117.8 +/- 14.26	0.13	0.13
E229A	5.097 +/- 0.1001	201.3 +/- 15.18	0.024	0.02

Values are derived from three separate runs of experiments; +/- represent standard deviation.

¶ the ratio of *Vmax*/*Km is* arbitrarily set as 1, with the values of the mutants being expressed against that of the WT.

3.4 Mutations in the HCA-2 region affect thermostability

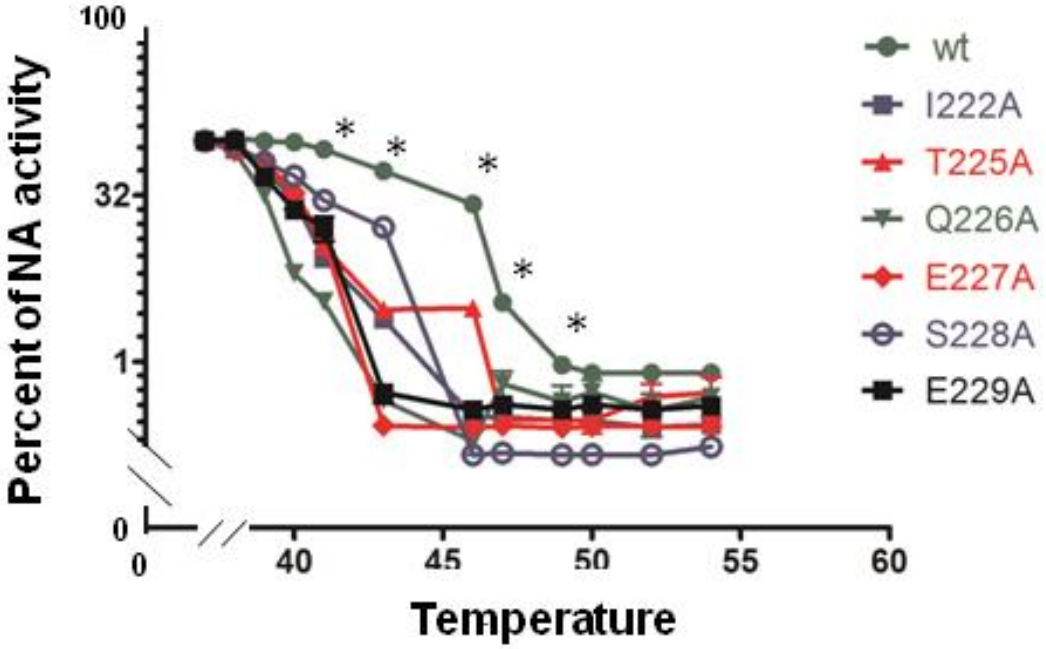
Having observed that decreased substrate binding and enzyme activities in the mutants, I then assessed how these mutants altered the protein stability. To this end, we investigated the thermostability of these mutants by heating the proteins at various temperatures and measuring the activity in a previously described procedure (Palese et al., 1974). As shown in Figure 3.4, all the mutated NA proteins were significantly more thermolabile than the WT protein as measured as a function of enzymatic activity following heat treatment. These data suggest that the structures of these mutants were less stable, constituting a suboptimal environment for NA protein function.

Figure 3.4

Thermostability of viral variants.

WT and mutant virus were incubated at various temperatures for 15 min and the samples were then used in a standard MUNANA assay to measure NA activity. 100% of NA activity for each variant was determined by using samples which were not incubated for the 15 min time frame. Experiments were performed in triplicate, * signify p-value <0.05 and error bars represent s.e.m.

Figure 3.4


100
32
1
0
0
40
45
50
55
60
Percent of NA activity
Temperature
wt
I222A
T225A
Q226A
E227A
S228A
E229A
*
*
*
*
*

3.5 Protein modeling

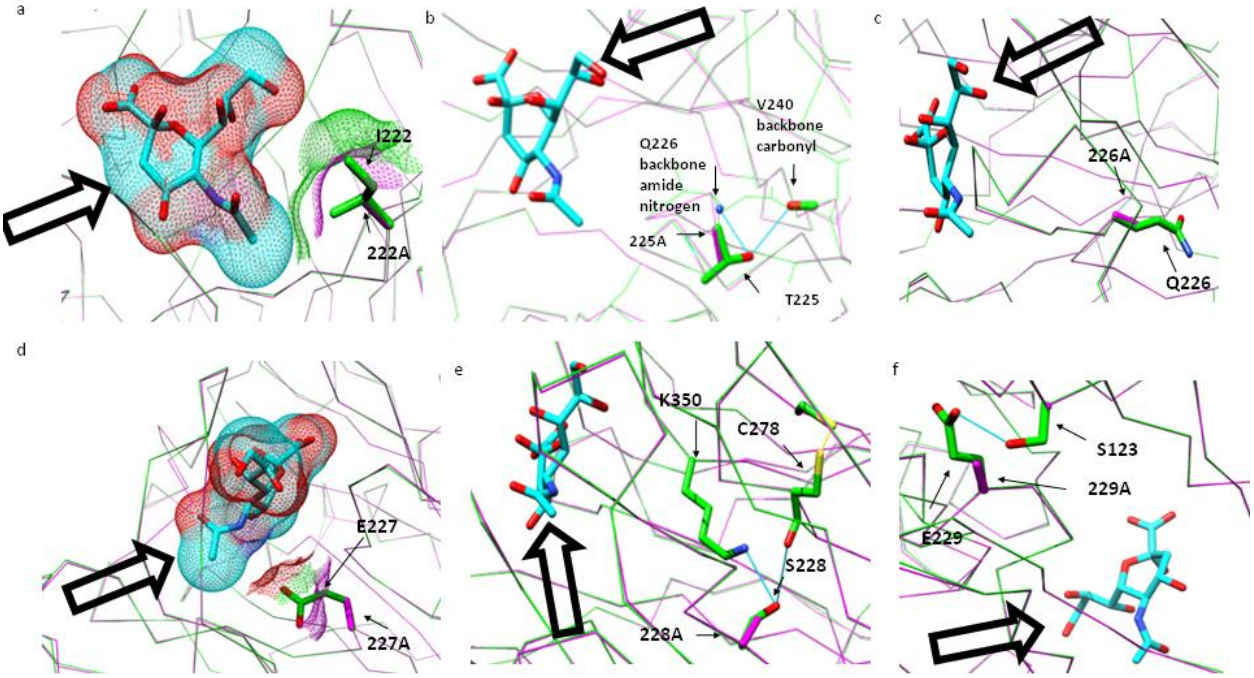
Given that previous studies on crystal structure of NA were focused on the amino acids within the enzymatic region and substrate binding pocket (Colman et al., 1987;Colman et al., 1989;Colman et al., 1983), I next used protein modelling to analyze how each of the a.a contributed to NA structural integrity. Importantly, our analyses revealed that substitutions of the a.a in this highly conserved region could have profound effects on the structural integrity of substrate enzyme binding. Specifically, as shown in Fig. 3.5, I222 (green) directly binds to the substrate (indicated by open arrow, same below) by creating a hydrophobic contact with the methyl group on the sialic acid and substitution of the isoleucine residue with an alanine (magenta) reduces the hydrophobic contact area available for binding with the sialic acid. The side chain of T225 directs toward the neuraminidase core and participates in stabilizing a β-sheet by making a hydrogen (H)-bond with the V240 backbone carboxyl. The T225A mutation abolishes these H-bonds, possibly destabilizing the β-sheet. Q226 (panel C) directly constitutes part of a β-sheet. It is likely that the Q226A mutation results in a reduced side chain volume, generating a large internal cavity and potentially destabilizing the internal structure. As far as E227 is concerned, it is known to directly bind to the substrate; specifically, its side chain carboxyl group makes a polar contact with the sialic acid. Mutation of this residue to alanine reduces the available binding surface area to sialic acid and abolishes the polar contact area, thus weakening the binding with the substrate. S228 resides in the center of the protein and mutation of this residue abolishes hydrogen bonds from the serine side chain hydroxyl oxygen to the K350 and the C278, thus disrupting the internal structure needed for protein stability. Finally, E229 forms hydrogen bonds with S123 and E229A mutation could result in the loss of one hydrogen bond from the E229 side chain carboxyl oxygen to S123 side chain hydroxyl group. Taken

together, these modelling analyses on NA structure indicate that the universally conserved sequence is either directly involved in binding to the substrate or contributing to the inter-molecule bonds necessary for optimal catalytic reaction by the NA protein.

Figure 3.5

The effects of substitution on protein structure. In all panels of this figure, the open arrows indicate the sialic acid substrates. **(A)** I222 (green) creates a hydrophobic contact with the methyl group on the sialic acid and substitution of the isoleucine residue with an alanine (magenta) reduces the hydrophobic contact area available for binding with the sialic acid. **(B)** The T225 side chain (green) directs toward the neuraminidase core and participates in stabilizing a β-sheet by making a hydrogen bond with the V240 backbone carboxyl. The T225A mutation abolishes these h-bonds, possibly destabilizing the β-sheet. **(C)** The Q226A mutation results in a reduced side chain volume. **(D)** The E227 side chain carboxyl group makes apolar contact with the sialic acid and mutation of this residue to alanine reduces the available binding surface area to sialic acid, and abolishes the polar contact area. **(E)** S228 resides in the center of the protein and mutation of this residue abolishes hydrogen bonds from the serine side chain hydroxyl oxygen to the K350 and the C278. **(F)** E229A could result in the loss of one hydrogen bond from the E229 side chain carboxyl oxygen to S123 side chain hydroxyl group.

Figure 3.5


a
I222
222A
b
V240
backbone
carbonyl
Q226
backbone
amide
nitrogen
225A
T225
c
226A
Q226
d
E227
227A
e
K350
C278
S228
228A
f
S123
229A
E229

3.6 HCA-2 MAb Provides Broad-Inhibition Against all Influenza A Subtypes in vitro

Having observed that HCA-2 region was essential for viral function, particularly in maintaining structural integrity and NA enzymatic activity, I set out to determine whether MAb to this region would significantly inhibit viral growth. Previously, we used the HCA-2 peptide to generate mono-specific polyclonal and subsequently monoclonal antibodies (MAb) in rabbits (Gravel et al., 2010) and these antibodies were found to bind to all nine subtypes of influenza NA protein. In an immunoblot assay adapted from HA quantification (Li et al., 2010), these antibodies were capable of quantifying NA in vaccine samples (Gravel et al., 2010). However, the potential of this MAb in inhibiting virus replication remained completely unknown.

To determine whether all nine NA-subtype of influenza a virus could be inhibited, I pre-incubated HCA-2 MAb with viruses containing one of the nine NA subtypes. As shown in Fig. 3.6.1, the growth of all 9 NA subtypes was substantially inhibited by HCA-2 MAb compared to normal IgG control. It is of note that for the majority of the subtypes, this inhibition was found to be between 80-90% in viral replication. On the other hand, a slightly lower percentage of inhibition (~ 70%) against N4 and N5 strains was detected. The reason for the slightly lower rate of inhibition remains to be fully understood but is likely to be multi-factorial such as the balanced action between HA and NA, known to affect viral replication, might vary between different strains used in the study (Wagner et al., 2002), amino acid substitutions within the HCA-2 region ie: for H8N4 which has L223M and H6N5 which has E229S and sequences outside the universal epitopes (In supplementary information, Table S1) was also detected (Appendix Table 1). This was further reflected in the IC50 values of the HCA-2 MAb required to inhibit viral growth which ranged between 2.6 – 18.2 µg/ml depending on the subtype (Table 3.6).

As HCA-2 MAb was present throughout the entire procedure of the virus inhibition assay, I next incubated the antibody-virus mixture with the cells for only 1 hour and removed the inoculum by washing with PBS to determine whether the HCA-2 MAb could still inhibit the viral propagation, given that some recent reports suggest that NA could contribute to entry and fusion of the influenza virus into host cells (Su et al., 2009;Matrosovich et al., 2004). Here, we found that the inhibitory effect of these antibodies was drastically decreased to about 20% even at the highest concentration, suggesting that the HCA-2 MAb mainly exerts its inhibitory activity on viral release, a late stage in the viral life cycle facilitated by the NA (Fig. 3.6.2)(Webster and Bean, 1978;Air and Laver, 1989).

Figure 3.6.1.

HCA-2 MAb Shows Broad Inhibitory Effect Against all Influenza NA Subtypes.

HCA-2 MAb or normal rabbit IgG control were tested for their inhibitory effect against H1N1, H6N2, H7N3, H8N4, H6N5, H11N6, H7N7, H10N8 or H5N9. Data are presented as percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

Figure 3.6.1

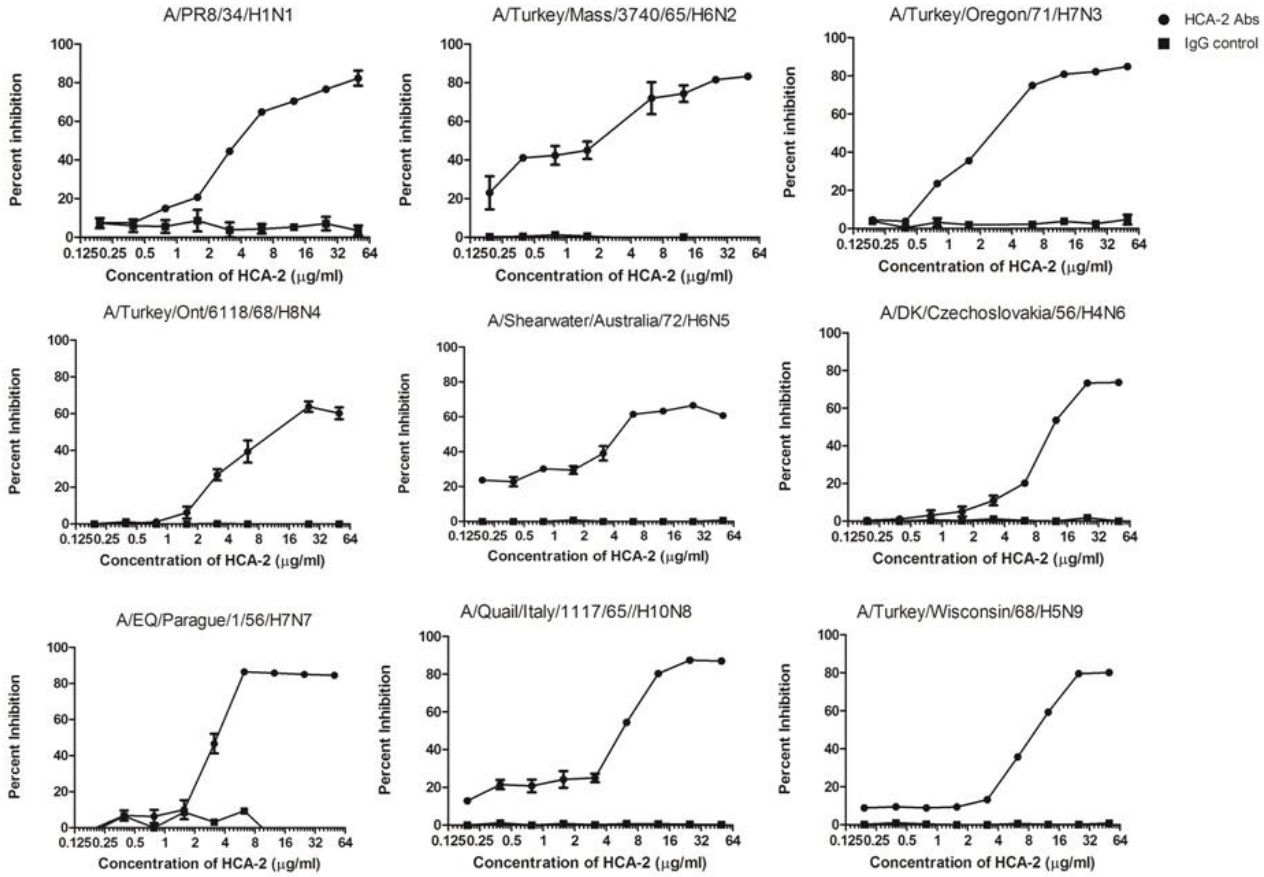

A/PR8/34/H1N1
A/Turkey/Mass/3740/65/H6N2
A/Turkey/Oregon/71/H7N3
HCA-2 Abs
IgG control
A/Turkey/Ont/6118/68/H8N4
A/Shearwater/Australia/72/H6N5
A/DK/Czechoslovakia/56/H4N6
A/EQ/Parague/1/56/H7N7
A/Quail/Italy/1117/65//H10N8
A/Turkey/Wisconsin/68/H5N9
Percent inhibition
Percent Inhibition
Concentration of HCA-2 (μg/ml)
0.125 0.25 0.5 1 2 4 8 16 32 64
0 20 40 60 80 100

Table 3.6

IC50 of HCA-2 MAb in Inhibiting Influenza Viral Growth. IC50s were calculated using GraphPad Prism software; samples were repeated in triplicates.

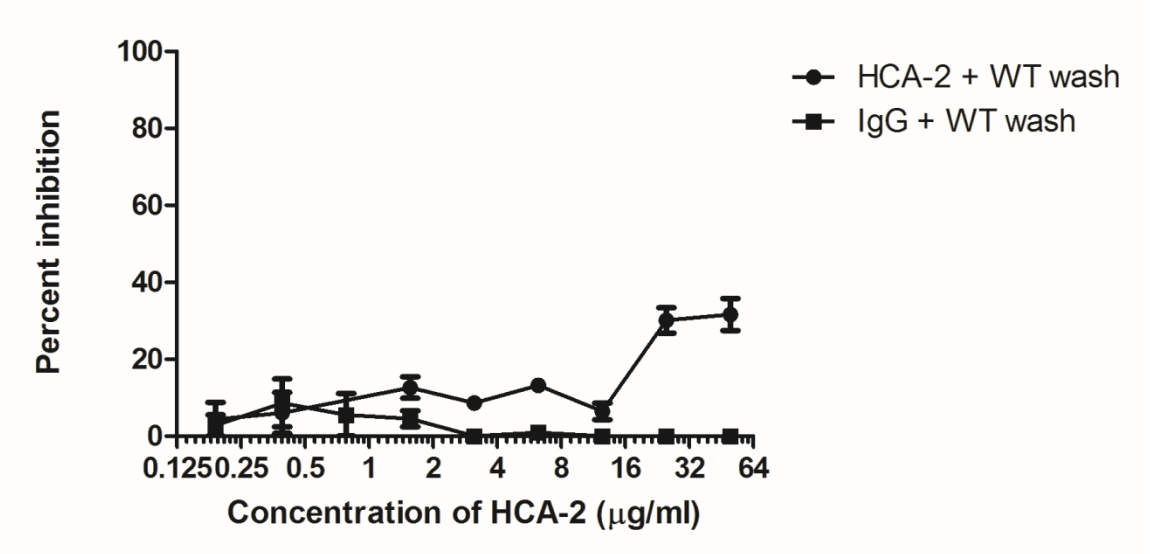
Virus	IC50
A/PR8/34/H1N1	5.30 μg/ml
A/Turkey/Mass/3740/65/H6N2	8.49 μg/ml
A/Turkey/Oregon/71/H7N3	2.56 μg/ml
A/Turkey/Ont/6118/68/H8N4	16.03 μg/ml
A/Shearwater/Australia/72/H6N5	18.2 μg/ml
A/DK/Czechoslovakia/56/H4N6	12.68 μg/ml
A/EQ/Parague/1/56/H7N7	2.67 μg/ml
A/Quail/Italy/1117/65/H10N8	5.43 μg/ml
A/Turkey/Wisconsin/68/H5N9	10.16 μg/ml

Figure 3.6.2

HCA-2 Appears to Only Inhibit by Preventing Exit of Virus from Cells

In order to determine if HCA-2 MAb might prevent viral growth by inhibiting absorption to cells, virus was pre-incubated with the MAb for 1hr and then the antibody/virus inoculum was put onto cell monolayers. After 1hr, the antibody/virus inoculum was removed and cells were incubated over night before conducting the NP ELISA. Data are presented as percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

Figure 3.6.2


Percent inhibition
100
80
60
40
20
0
0.125
0.25
0.5
1
2
4
8
16
32
64
Concentration of HCA-2 (μg/ml)
HCA-2 + WT wash
IgG + WT wash

3.7 HCA-2 MAb Inhibits NA activity as Determined by ELLA

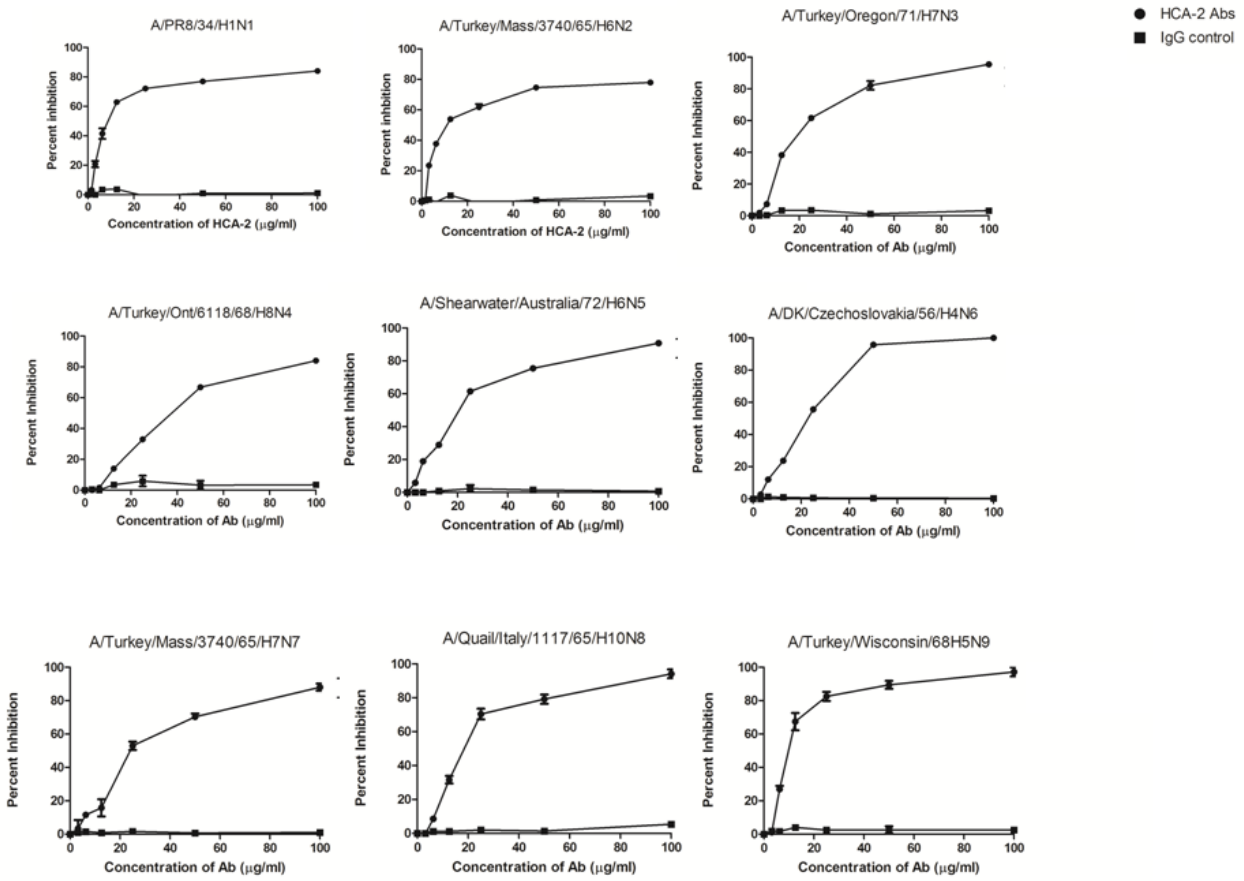
Given the polyvalent nature of sialic acid *in vivo,* I sought to assess the effect of HCA-2 MAb on the NA enzymatic activity of virus representing 9 NA subtypes by using an Enzyme Linked Lectin Assay (ELLA), which analyzes the enzymatic activity of NA in cleaving polyvalent sialic acid on Fetuin coated plates (Lambre et al., 1991). Here, we observed a drastic decrease in NA enzymatic activity in all NA subtypes when these viruses were pre-incubated with serial dilutions of MAb HCA-2 (Fig. 6). These data further confirm our mutagenesis data and suggest that the HCA-2 antibody specifically inhibits viral replication by interfering with NA enzymatic activity.

Figure 3.7.1

HCA-2 MAb Inhibits NA Activity Across All Nine NA Subtypes.

Fetuin coated plates were incubated with HCA-2 MAb and virus containing one of the nine NA subtypes. Data are presented as a percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

Figure 3.7.1


HCA-2 Abs
IgG control
A/PR8/34/H1N1
A/Turkey/Mass/3740/65/H6N2
A/Turkey/Oregon/71/H7N3
A/Turkey/Ont/6118/68/H8N4
A/Shearwater/Australia/72/H6N5
A/DK/Czechoslovakia/56/H4N6
A/Turkey/Mass/3740/65/H7N7
A/Quail/Italy/1117/65/H10N8
A/Turkey/Wisconsin/68H5N9
Percent inhbition
Percent Inhibition
Concentration of HCA-2 (μg/ml)
Concentration of Ab (μg/ml)

3.8 Identification of Key a.a. in the NA Epitope Involved in HCA-2 MAb-Mediated Viral Inhibition

In order to determine the key a.a. residues within the HCA-2 region which contribute to the observed antibody inhibitory effects, we tested the MAb against the reassortant viruses. Interestingly, HCA-2 MAb inhibited variants T225A, Q226A, S228A and E229A to levels that were similar to the WT virus, however, these inhibitory effects were largely abolished against I222A and E227A mutants (Fig 3.8.1). The fact that both of these two amino acids (I222 and E227), were exposed on the protein surface may explain their critical roles in antibody binding (Figure 3.8.2)(Colman et al., 1983). It is therefore possible that mutations at these two locations in the NA resulted in a structural change that interfered with the interaction to the complementarity determining region of the antibody and were thus unfavorable for the MAb to bind the NA protein .

Figure 3.8.1

Critical Amino Acid Residues for HCA-2 MAb Inhibitory Effect. HCA-2 MAb or normal rabbit IgG were tested for their inhibition against viruses containing the WT NA sequence or any of the mutated NA sequences in an influenza A/Puerto Rico/8/34 (H1N1) backbone. Data are presented as percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

Figure 3.8.1

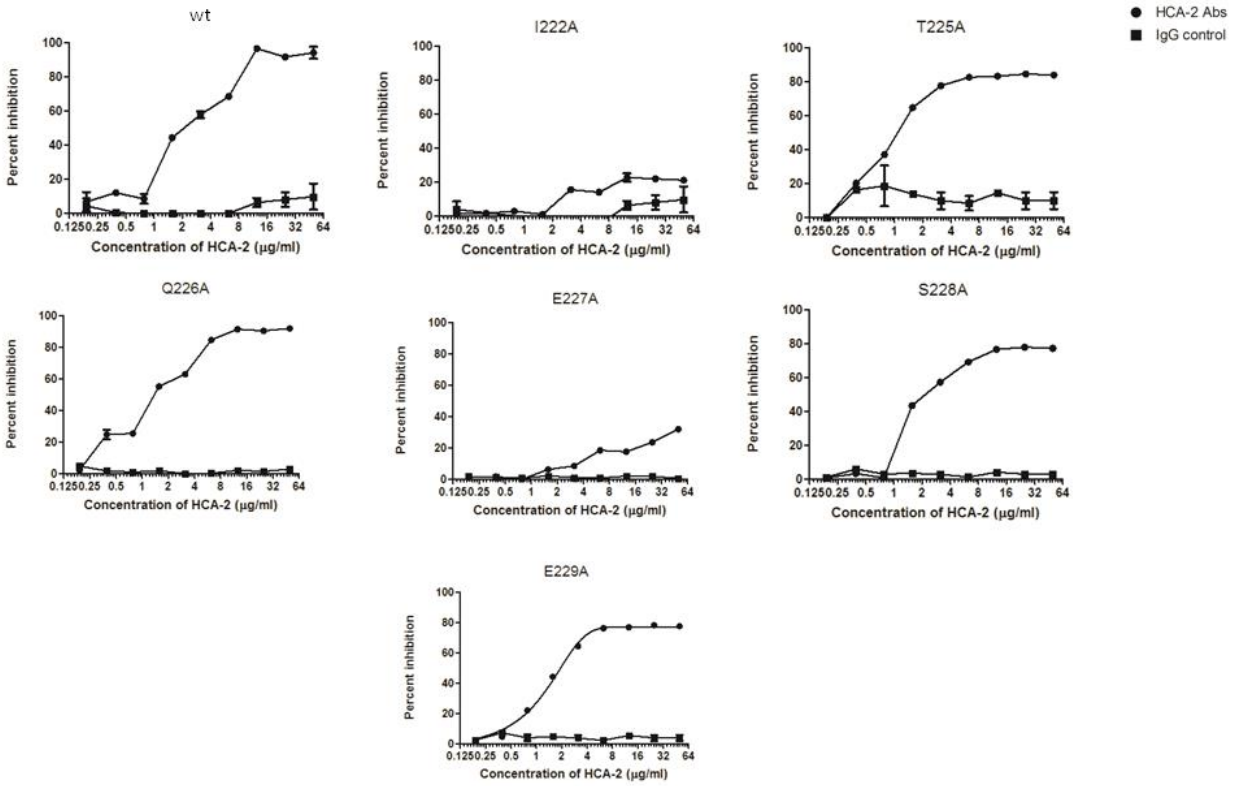

wt
I222A
T225A
Q226A
E227A
S228A
E229A
HCA-2 Abs
IgG control
Percent inhibition
Concentration of HCA-2 (µg/ml)
0.125 0.25 0.5 1 2 4 8 16 32 64
0 20 40 60 80 100

Table 3.8.1

The IC50s of Antibody Inhibition of NA Enzymatic Activity

IC50 of HCA-2 MAb against viral variants determined by the inhibition against the NA enzymatic activity. Experiments were conducted in triplicates.

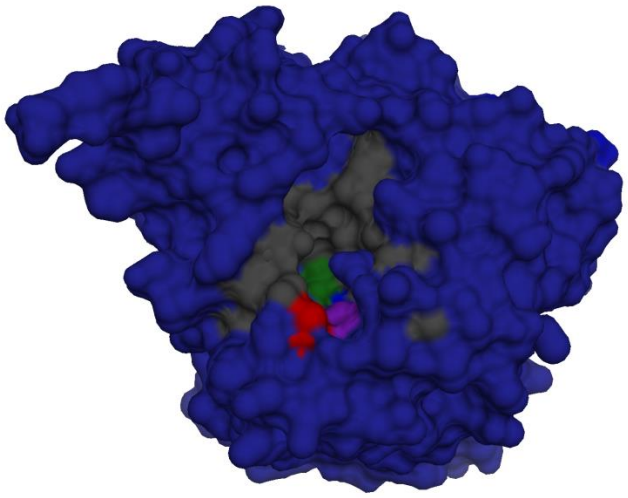
Variant	IC50
WT	1.82 μg/ml
I222A	181.93 μg/ml
T225A	0.95 μg/ml
Q226A	1.60 μg/ml
E227A	106.26 μg/ml
S228A	2.27 μg/ml
E229A	1.87 μg/ml

Figure 3.8.2

Location of Critical Amino Acids Required for Antibody Binding

The top view of neuraminidase (N2) protein with highlighted enzymatic region in grey and the exposed HCA-2 a.a. are highlighted in red (I222), green (E227) and purple (R224) while the remainder of the HCA-2 region is buried within the protein. Visualization and analysis were performed using the software UCSF Chimera with the protein identification number 1NN2.

Figure 3.8.2



3.9 HCA-2 MAb Reduces Influenza A Viral Burden in Mice

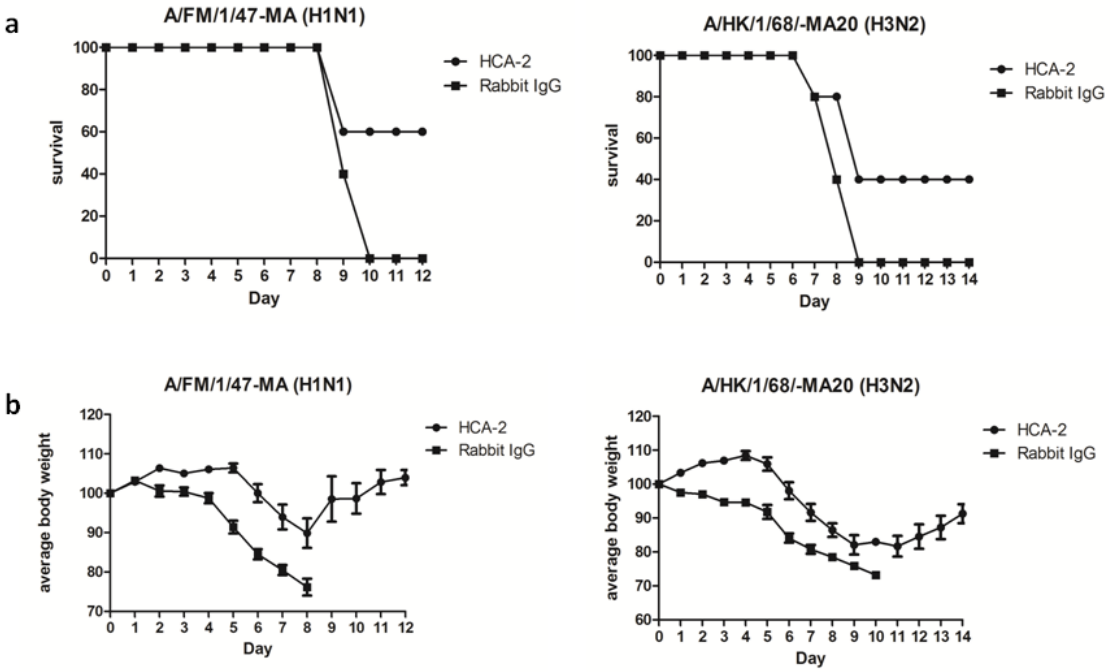
I further investigated whether the MAb could inhibit viral growth in a murine model. To this end, HCA-2 MAb was passively transferred intraperitoneally into naïve mice 24 hours before challenge with two mouse-adapted viruses; we observed that the MAb substantially reduced viral replication in mice treated with the MAb, compared to the control group which received normal IgG control. Specifically, animals passively transferred with HCA-2 MAb and challenged with either H1N1 or H3N2 exhibited decreased weight loss (Fig. 3.9B) as well as 60% and 40% more protection respectively when compared to IgG controls (Fig. 3.9A). This data confirms our *in vitro* findings, indicating that the universal MAb HCA-2 antibody provides heterosubtypic protection *in vivo*.

Figure 3.9

***In vivo* effect of HCA-2 MAb.**

HCA-2 MAb or normal rabbit IgG (60 mg/kg) were passively transferred to naïve (n=5) mice via intra-peritoneal injection on days -2, -1 and 0 relative to intranasal challenge with either 5 x 103 pfu of influenza A/FM/1/47/MA (H1N1) or 2 x 103 pfu of influenza A/HK/1/68-MA20(H3N2). Mice were weighed and monitored daily for signs of illness for 14 days. Panels depict (A) survival curves of mice post-challenge with both influenza viruses. and (B) body weight loss

Figure 3.9


a
A/FM/1/47-MA (H1N1)
survival
Day
HCA-2
Rabbit IgG
A/HK/1/68/-MA20 (H3N2)
survival
Day
HCA-2
Rabbit IgG
b
A/FM/1/47-MA (H1N1)
average body weight
Day
HCA-2
Rabbit IgG
A/HK/1/68/-MA20 (H3N2)
average body weight
Day
HCA-2
Rabbit IgG

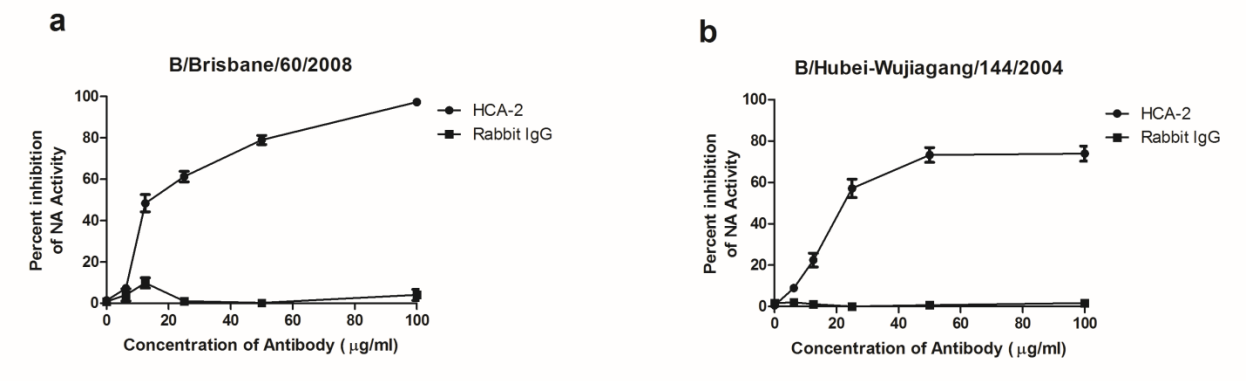
3.10 HCA-2 MAb Provides Inhibition of NA Enzymatic Activity of Various Influenza B Strains

Considering I previously observed that the HCA-2 region was essential for optimal viral function and that MAb against this region significantly inhibited enzymatic activity of influenza A subtypes, we next set out to determine whether MAb to this region would significantly inhibit enzymatic activity of various strains of influenza B. To this end, I treated B/Brisbane/60/2008 or B/Hubei-Wujigang/144/2004 with HCA-2 antibody and then measured the degree of enzyme inhibition with an ELLA assay. As shown in Fig. 3.10.1, both strains which are representative of the two influenza B lineages were substantially inhibited by HCA-2 MAb compared to normal rabbit IgG control. These data suggest that the MAb antibody not only inhibits the NA activity of Influenza A, but also Influenza B.

Figure 3.10.1

HCA-2 MAb Inhibits NA Activity of Influenza B Strains Representative of Both Lineages. Fetuin coated plates were incubated with HCA-2 MAb or normal rabbit IgG control and virus containing either B/Brisbane/60/2008 or B/Hubei-Wujigang/144/2004. Data are presented as a percentage of inhibition compared to virus control ± s.e.m from three independent experiments

Figure 3.10.1


a
B/Brisbane/60/2008
Percent inhibition of NA Activity
Concentration of Antibody (μg/ml)
HCA-2
Rabbit IgG
b
B/Hubei-Wujiagang/144/2004
Percent inhibition of NA Activity
Concentration of Antibody (μg/ml)
HCA-2
Rabbit IgG

3.11 HCA-2 MAb Inhibits Influenza B Viral Growth

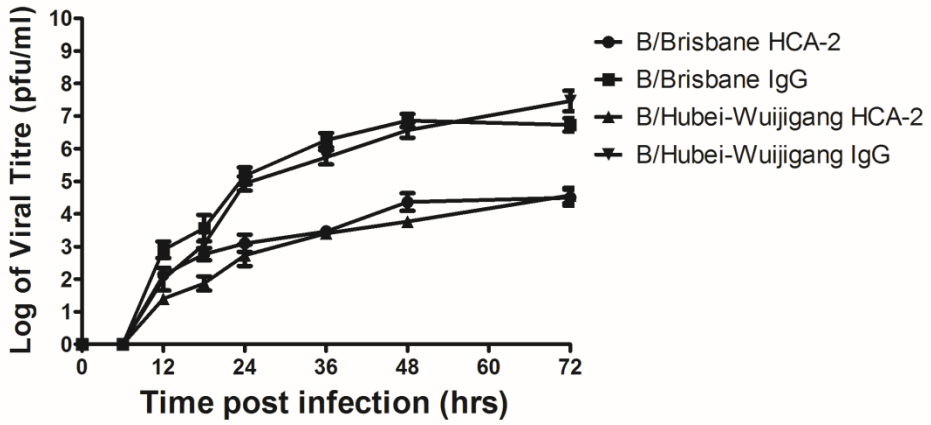
After observing that HCA-2 MAb effectively inhibited the NA activity from influenza derived from both B strain lineages, I hypothesized that this antibody may also effectively inhibit viral growth of these strains. Thus, I infected MDCK cells with either B/Brisbane/60/2008 or B/Hubei-Wujigang/144/2004 in the presence of HCA-2 antibody or IgG control. These cells were infected at a low MOI of 0.001 and samples were harvested at various time points through until 72hrs post infection. Here the data show observed that indeed the HCA-2 MAb does significantly inhibit viral growth of both influenza B strains when compared to IgG control (Figure 3.11).

Figure 3.11

HCA-2 MAb Inhibits Growth of Both Influenza B Lineages.

HCA-2 MAb or normal rabbit IgG control were tested for their inhibitory effect against B/Brisbane/60/2008 or B/Hubei-Wujigang/144/2004. Data are presented as percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

Figure 3.11


Log of Viral Titre (pfu/ml)
10
9
8
7
6
5
4
3
2
1
0
0
12
24
36
48
60
72
Time post infection (hrs)
B/Brisbane HCA-2
B/Brisbane IgG
B/Hubei-Wuijigang HCA-2
B/Hubei-Wuijigang IgG

3.12 HCA-2 MAb Inhibits Enzymatic Activity and Viral Growth of Drug Resistant Influenza B

Given that we observed the HCA-2 MAb inhibits both the NA activity and the viral growth of influenza B strains, I next aimed to determine whether this antibody could also be effective at inhibiting drug resistant strains of influenza B. To do this we used two influenza B resistant strains, B/Perth/211/2001 with a mutation E197D and B/Taiwan/4/2002 with mutation E117N, both of which have shown decreased sensitivity to both oseltamivir and zanamivir. Here I observe that the HCA-2 antibody significantly inhibits the enzymatic activity (Fig. 3.12.1) and the *in vitro* growth of both resistant strains as well as the wild type B/Perth/211/2001 (Fig. 3.12.2). These levels of inhibition are further comparable to the antibody effect on WT influenza B virus suggesting that the mutations conferring drug resistance did not affect antibody inhibition. This is in spite of the drug resistant mutations being in close proximity to the HCA-2 region (Figure 3.12.3).

Figure 3 12.1

HCA-2 MAb Inhibits NA enzymatic Activity of Drug Resistant Strains of Influenza B

HCA-2 MAb or normal rabbit IgG control were tested for their inhibitory effect of NA activity. HCA-2 antibody or IgG control was incubated with either wilde type drug sensitive B/Perth/211/2001 or drug resistant strains B/Perth/211/2001 (E197D) B/Taiwan/4/2002 (E117N) and then transferred to fetuin coated plates for ELLA analysis. Data are presented as percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

Figure 3.12.1

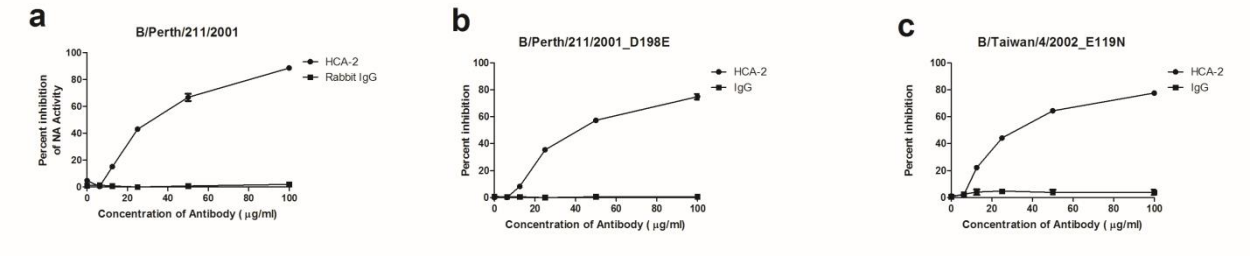

a
B/Perth/211/2001
Percent inhibition of NA Activity
Concentration of Antibody (µg/ml)
HCA-2
Rabbit IgG
b
B/Perth/211/2001_D198E
Percent inhibition
Concentration of Antibody (µg/ml)
HCA-2
IgG
c
B/Taiwan/4/2002_E119N
Percent inhibition
Concentration of Antibody (µg/ml)
HCA-2
IgG

Figure 3.12.2

HCA-2 MAb Inhibits Growth of Drug Resistant Strains.

HCA-2 MAb or normal rabbit IgG control were tested for their inhibitory effect against the drug resistant variants B/Perth/211/2001 (E197D) B/Taiwan/4/2002 (E117N) or wild-type control B/Perth/211/2001. Data was assessed by viral titre as determined by pfu. Data are compared to virus control ± s.e.m from three independent experiments.

Figure 3.12.2

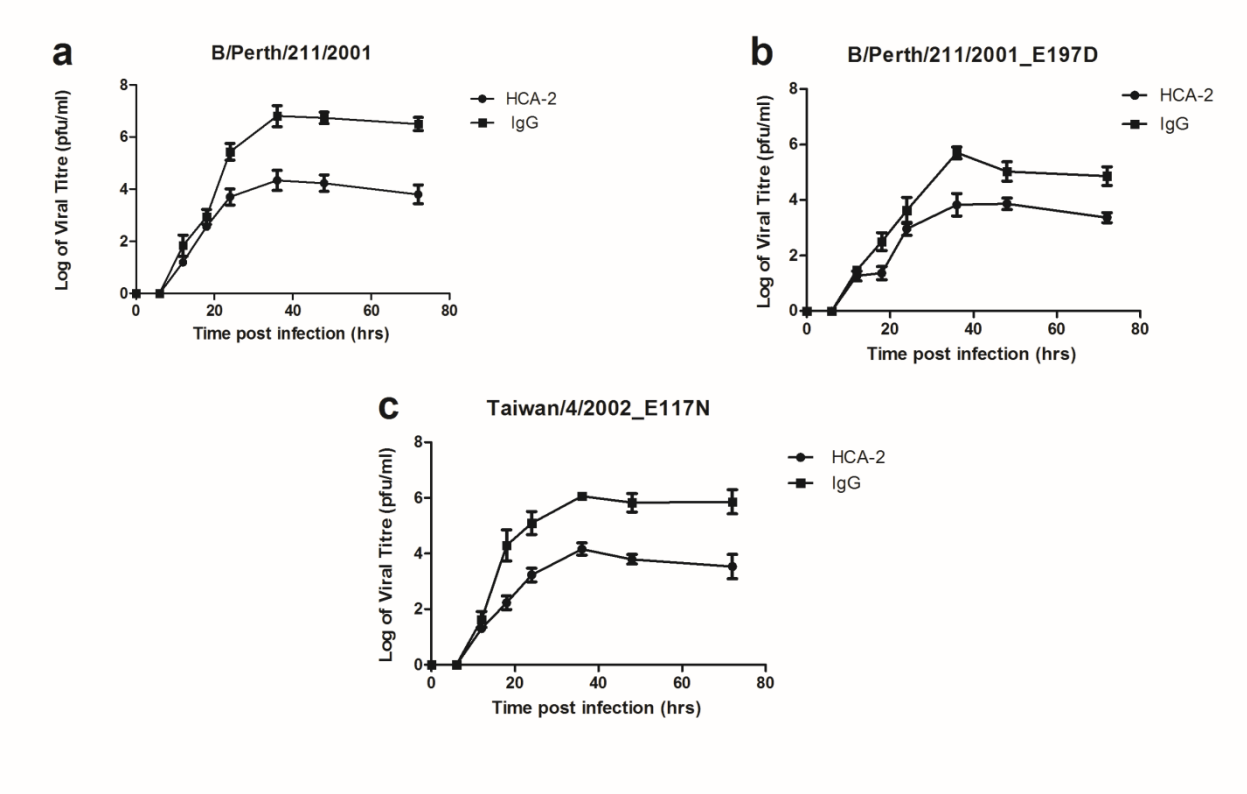
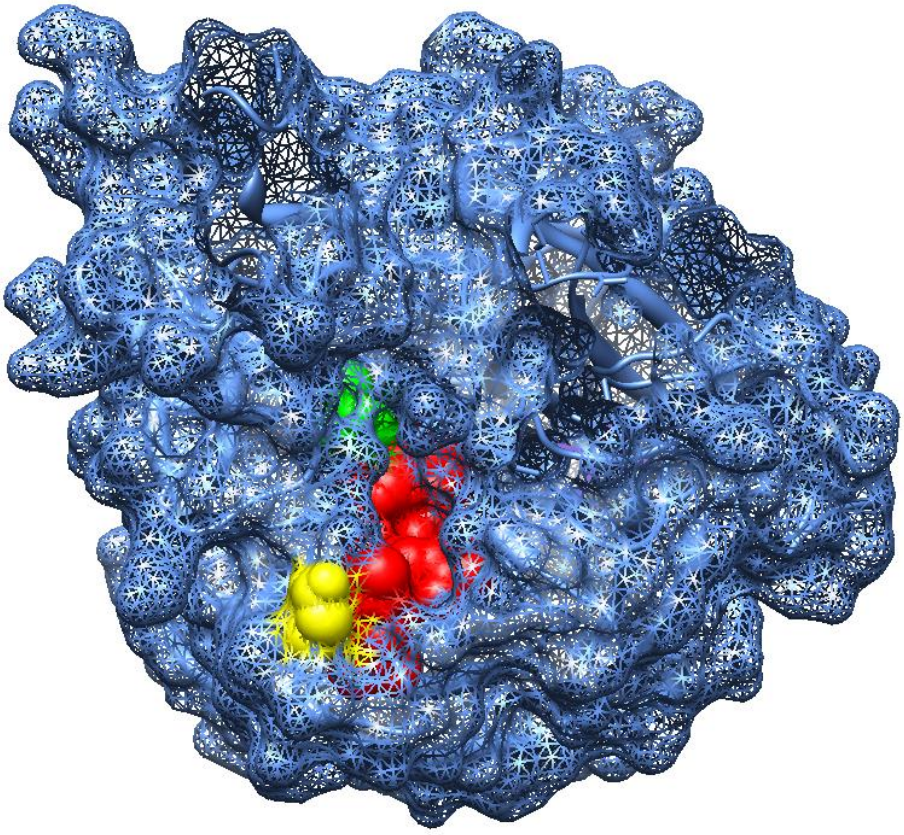

a
B/Perth/211/2001
Log of Viral Titre (pfu/ml)
Time post infection (hrs)
HCA-2
IgG
b
B/Perth/211/2001_E197D
Log of Viral Titre (pfu/ml)
Time post infection (hrs)
HCA-2
IgG
c
Taiwan/4/2002_E117N
Log of Viral Titre (pfu/ml)
Time post infection (hrs)
HCA-2
IgG

Figure 3.12.3:

The Location of Resistance Mutations in Relation to the HCA-2 Region.

Amino acid 197 (yellow) and E117 (green) are demonstrated through modelling of NA using Chimera (protein database ID: 3K36). The HCA-2 region (red) lies in close proximity to these amino acids but the resistance mutation do not interfere with antibody inhibition.

Figure 3.12.3



Chapter 4

Discussion

The ever-evolving nature of influenza viral HA and NA is well known and presents daunting challenges for scientific and medical communities in achieving the objective to prevent and treat influenza disease. Identifying conserved sequences in HA and NA and investigating their potential roles in viral pathogenesis are of critical importance to explore new antiviral and vaccine targets. However, since these two surface proteins are antigenically variable, identifying conserved regions within them and has proven difficult. Although there has been quite a lot of studies on identifying target regions within the HA there has been much less so on NA in this context. Specifically, the only universally conserved sequence of HA was found to be the fusion peptide (Chun et al., 2008) and antibodies against this peptide demonstrated neutralizing activities against diverse strains of the virus (Hashem et al., 2010). There are also reports showing antibodies with wide range of cross-neutralizing activity against conformational epitopes in HA (Corti et al., 2011;Ekiert et al., 2009;Ekiert et al., 2011;Sui et al., 2009;Wei et al., 2012). However, studies on NA in these areas have been very limited, with no report on the potential role of highly or universally conserved sequence of NA in virus replication and its potential as a novel vaccine candidate. In my Ph.D. thesis I have explored the functional role of the only universally conserved region in the NA protein. I have further demonstrated that MAb to this region are the first against NA to provide heterosubtypic protection *in vitro* and *in vivo.* Finally, this work shows that HCA-2 MAb can also inhibit strains representing the two lineages of influenza B and drug resistant influenza B strains. These findings are not only the first of its kind, but also suggest that the HCA-2 region may be a viable target for new vaccine therapies.

4.1 The Functional Role of the HCA-2 Region in Viral Replication

Our previous studies have revealed that a.a. 222-230 is the only universally conserved sequence in NA, with conservation rate being close to 100%, with the exception of the recently reported extraordinarily divergent influenza virus in bats (bat/Guatemala/164/2009), which cannot be grown in culture or chicken embryos (Tong et al., 2012). Although antibodies targeting the HCA-2 region have proven to be a useful tool for NA quantification in vaccines (Gravel et al., 2010), the potential role of this universal epitope in viral replication as well as efficacy of the MAb targeting this sequence in inhibiting viral replication remained completely unknown. Clearly, investigation of functional role of this only universally conserved sequence in the viral NA would be of unquestionable importance for better understanding of influenza viral pathogenicity and formulation of new preventative and therapeutic strategy.

Given the proximity of this sequence to enzyme active site, I hypothesized that the HCA-2 region could be critically important for efficient virus replication through maintaining structural integrity of the enzyme. Indeed, the experimental results show that among the ten mutations, three of them (L223A, R224A, C230A) as well as the deletion of this entire region, were found to be lethal, thus making it impossible for me to conduct comprehensive analyses of the three a.a. (L223, R224 and C230) in subsequent studies. However the demonstrated lethality as a result of mutations in these three positions suggesting that these three amino acids might be critical for enzyme functions. Specifically, R224 lies directly in the enzymatic pocket and has been previously been shown to be quintessential for substrate binding as it provides a polar contact for the sialic acid (Colman et al., 1983;Varghese et al., 1983). Additionally C230 is involved in salt bridge formation, thus contributing to the structural stability of the protein. Finally it is also possible that L223, although not directly in contact with sialic acid substrate,

might assist I222 in providing a hydrophobic region within the protein which is necessary for the cleavage of substrate (Colman, 1994).

Furthermore, the remaining six mutants, even though they could be rescued by reverse genetics were found to possess significantly altered plaque morphology (Figure 3.1) and impaired replication capability in single and multiple rounds of viral growth (Figure 3.2.1 and 3.2.2). This decrease in viral growth and spread were largely in agreement with the decreased levels of enzymatic activity (Fig. 3.3.1). While these data reinforce the notion that enzymatic activity is positively associated with viral growth, my subsequent enzymatic analyses shed light on how the a.a. in this region are needed for NA protein to maintain its structural conformation necessary for maximal enzymatic activity (Fig. 3.3.2). Specifically, in the enzymatic kinetics studies, the larger *Km* values in the mutants indicate their decreased affinity in NA binding to the substrate, which may translate into much less effective conversion of substrates into final products as suggested by substantially reduced ratio of *Vmax*/*Km* (Table 3.3). Furthermore, the thermolabile properties of these mutants yield additional evidence that a.a. in this region are indispensable to constitute an optimal tertiary environment needed for maximal enzymatic activities (Fig. 3.4).

The data from biological and biochemical analyses are consistent with the findings of protein modelling, which helped to understand the molecular mechanisms underlying the contribution of the universal sequence to viral propagation. Specifically, it explains the mechanism for the loss of replication capabilities in I222A and E227A viruses since both I222 and I227 are directly interacting with the sialic acid substrate. Furthermore, even though T225, Q226, S228 and E229 do not directly interact with the sialic acid substrate, substitution of these amino acids to alanine resulted in significant loss of NA activity and decreased viral growth. As

protein modelling suggested, it is likely that the primary effect of these substitutions is to abolish internal hydrogen bonding, thereby generating cavities and destabilizing the tertiary structure of the NA protein. It is possible that the introduction of these cavities by alanine substitution may result in a structural re-configuration to reduce the size of this internal cavity and thus distort the active site similar to observations made in other protein studies (Lim et al., 1992;Fuchs et al., 2012;Monera et al., 1996;Xu B et al., 2002).

4.2 A Broadly Neutralizing Antibody Against the HCA-2 region of the Neuraminidase Protein of Influenza A

The importance of NA in inducing protective antibodies has been well documented (Marcelin et al., 2012). Recent publications suggest that polyclonal antibodies against NA can induce cross-protection against diverse strains within the same subtype (Marcelin et al., 2011;Sandbulte et al., 2007), but NA heterosubtypic protection, ie., antibody generated by immunization of one NA subtype capable of protecting against a different NA subtypes, has not yet been reported (Johansson and Cox, 2011). This lack of NA specific heterosubtypic protection is mainly attributed to genetic variation combined with immune pressure resulting in marked structural variability (Colman, 1992;Colman et al., 1983). Thus there is significant interest in searching for universally conserved epitopes which can be accessed by antibody.

After I confirmed the biological importance of the HCA-2 universal epitope, I further sought to investigate if antibodies against this region could inhibit viral growth and potentially provide heterosubtypic protection across influenza strains. Indeed, the HCA-2 MAb significantly inhibited all nine NA subtypes of influenza A viruses used in the *in vitro* study, with the inhibition of most viruses being around 90% (Fig. 3.6.1). The slightly lower inhibition

rates (~ 70%) against N4 (H8N4) and N5 (H6N5) viruses compared to the others remain to be fully explained, but is likely due to structural differences around and within the epitope between proteins (Table 1 Appendix A). In addition, through ELLA analysis, we demonstrated that the HCA-2 MAb further inhibited the NA activity across all nine NA subtypes, suggesting that HCA-2 MAb inhibits viral replication mainly through interfering with NA enzymatic activity (Fig. 3.7). This hypothesis was further supported when I exposed virus infected cells to MAb for the only the first hour of infection. Theoretically, viral absorption and entry occur during this hour and viral release between 4.5-6 hours into the virus cycle. When the HCA-2 antibody was removed from the cells, the viral growth was not inhibited, further suggesting that the MAb decreases NA activity and inhibits viral spread by preventing release from the cell.

While the *in vitro* inhibition of virus propagation by this MAb could reach 90%, the *in vivo* protection by passively transferring the MAb to naïve mice prior to viral challenge, although still conferring heterosubtypic protection, was not as large (Figure 3.9). It is not completely unexpected that there was less potent protection against the virus infection in the mouse than that observed in *in vitro* cultures, given that the MAb is of rabbit origin. It is possible that the *in vivo* efficacy of HCA-2 MAb could be improved by combination therapy with NA inhibitors such as oseltamivir or zanamivir. Alternatively a higher dose of the MAb or intranasal versus systemic intraperitoneal administration could also potentially increase the protection demonstrated by HCA-2 (Weltzin and Monath, 1999; Seiler et al., 2000). Furthermore, through sequencing the VH and VL regions of the HCA-2 antibody (Table 4.1), this *in vivo* protection may be further increased through optimization or humanization of the MAb allowing for effective protection across the species barrier.

Interestingly, similar *in vivo* protection rates (40-60 %) of NA-specific antibodies has

been reported by others using polyclonal subtype-specific antibodies (Sandbulte et al., 2007;Marcelin et al., 2011). In contrast to the HCA-2 antibody, these investigators used polyclonal antisera obtained from animals vaccinated with whole NA proteins. Noticeably, such polyclonal antisera were reported to inhibit multiple strains only within the same subtype. The protection afforded by HCA-2 MAb is distinguished from any previously-reported studies in that this MAb is the first antibody capable of inhibiting all 9 subtypes of NA viruses *in vitro* and, when passively transferred, showed protection of mice infected with two mouse-adapted strains representative of the two different groups of NA of which the subtypes are broadly classified. Thus it is the first MAb against NA which provides heterosubtypic protection.

After demonstrating this heterosubtypic protection, I aimed to use the alanine scanning mutants to identify key amino acids within the epitope for antibody binding. Here, these experiments revealed that at least two a.a. (I222 and E227) could be directly involved in the MAb-mediated protection as either the growth or NA enzymatic activity (although decreased from the WT) of both of these variants were not affected by antibody treatment. Due to the lethality of alanine substitution at residues L223, R224 or C230, it was impossible for us to determine whether these three a.a. were involved in the MAb-mediated inhibition or not. Of the 6 rescued viruses, only the I222A and E227 mutants were found to be resistant to the MAb treatment (Fig. 3.8.1). Based on published crystal structures (Varghese et al., 1983), these two a.a. are exposed on the surface, situated in the enzymatic active site and likely make direct contact with sialic acid (Fig. 3.8.3). Therefore, mutation of these two a.a. resulted in decreased levels of enzymatic activity and viral growth, while at the same time making the two viruses resistant to MAb treatment. As shown in Fig. 3.8.3 six of the nine a.a. in the HCA-2 region are buried, making this epitope less optimal to elicit strong specific antibodies if the whole viral NA

protein is used, such as in current vaccine preparations where the entire protein is used as an immunogen. Nevertheless, these data suggest that to exploit this epitope as a potential vaccination target, alternative designs of NA-based immunogens for the induction of strong antibody responses to this conserved epitope may need to be considered.

Table 4.1 (a) Sequence of the V_H with highlighted CDR regions as determined by alignment with the most homologous germline gene – S76746. (b) Sequence of the V_L chain with highlighted CDR regions as determined by alignment with the most homologous germline gene K01358.

(a)

```
S7676            13 --------GAGTCCGGGGGTCGCCTGGTCACGCCTGGGACACCCCTGACA    54
                            |||||||||||||||||||||||||||||||||||||||||||
HCA-2 HC        251 TGTGCCCCGAGTCCGGGGGTCGCCTGGTCACGCCTGGGACACCCCTGACA   300
S7676            55 CTCACCTGCACAGTCTCTGGATTCTCCCTCAGTAGCTATGCAATGAGCTG    104
                    |||||||||||||||||.||.||..||||||.|.||||.|||||.||||
HCA-2 HC        301 CTCACCTGCACAGTCTCTAGAATCGACCTCAGAATCTATTCAATGGGCTG   350

S7676           105 GGTCCGCCAGGCTCCAGGGAAGGGGCTGGAATGGATCGGAATCATTAGTA   154
                    |||||||||||||||||||||||||||||.|||||||||.|||||||||
HCA-2 HC        351 GGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGATCGGATTCATTAATA   400
                                      CDR 2
S7676           155 GTAGTGGTAGCACATACTACGCGAGCTGGGCGAAAGGCCGATTCACCATC   204
                    .|..||||||||||||.|||||||||||||||||||||||.|||||||||
HCA-2 HC        401 ATGATGGTAGCACATATTACGCGAGCTGGGCGAAAGGCCGGTTCACCATC   450

S7676           205 TCCAAAACC---TCGACCACGGTGGATCTGAAAATCACCAGTCCGACAAC   251
                    |||||||||   |||||||||||||||||.|||||.|||.|||.||||||
HCA-2 HC        451 TCCAAAACCTCGTCGACCACGGTGGATCTAAAAATGACCCGTCTGACAAC   500
                                                      CDR 3
S7676           252 CGAGGACACGGCCACCTATTTCTGTGCCAGAG---TG-CTGGTAGTAGT-   296
                    |||||||||||||||||||||||||||||||   || ||..||.||.|
HCA-2 HC        501 CGAGGACACGGCCACCTATTTCTGTGCCAGAGGATTGTCTAATAATAATA   550

S7676           297 -TGACTGGTTGGATCTCTGGGGCCAGGGCACCCTGGTCACCGTCTCCT--   343
                     |.||          .|||||||||..|||||||||||||||||||
HCA-2 HC        551 ATAAC---------ATCTGGGGCCCAGGCACCCTGGTCACCGTCTCCTTG   591
```

(b)

```
K01358 28    ATGACCCAGACTCCAGCCTCTGTGGAGGTAGCTGTGGGAGGCACAGTCACCATCAAGTGC  87

             |||||||||||||||  ||  |||||    | ||||||||||||||||||||||||||| |||
HCA-2 530    ATGACCCAGACTCCATCCCCTGTGTCTGCAGCTGTGGGAGGCACAGTCACCATCAACTGC 589
                          CDR1
K01358 88    CAGGCCAGTCAGAG-CATTA-G-T-AC--CTACTTATCCTGGTATCAGCAGAAACCAGGG  141
              ||||||||||||||    ||| |  | ||   | |||||  ||||||||||||||||||||||
HCA-2 590    CAGGCCAGTCAGAGTGTTTATGATAACAACAACTTAGCCTGGTATCAGCAGAAACCAGGG  649
                                                CDR2
Query 142    CAGCGTCCCAAGCTCCTGATCTACAGGGCATCCACTCTGGCATCTGGGGTCTCATCGCGG  201
              |||| ||||||||||||||||     |||||  ||||||||||||||| ||||||||
Sbjct 650    CAGCCTCCCAAGCTCCTGATCTATTATACATCCTATCTGGCATCTGGGGTCCCATCGCGG  709

Query 202    TTCAAAGGCAGTGGATCTGGGACAGAGTTCACTCTCACCATCAGCGGCGTGGAGTGTGCC  261
             |||||||||||||||||||||||||| |||||||||||||||||||| |||||| |
Sbjct 710    TTCAAAGGCAGTGGATCTGGGACAGAGTACACTCTCACCATCAGCGGCGTGCAGTGTGAC  769
                                                CDR3
Query 262    GATGCTGCCACTTACTACTGTCAACAGG-G-TTGGAGTAGTAGTAATGTTGA--G-AATG  316
             ||||||||||||||||||||    ||| |   |  | | ||| || ||   ||  |   ||
Sbjct 770    GATGCTGCCACTTACTACTGTC--TAGGCGAATTTACTTGTACTACTGCGGATTGTTTTG  827
```

4.3 The inhibitory effect of the HCA-2 MAb against the Neuraminidase of Influenza B

After demonstrating that HCA-2 region was critical for optimal viral function and that MAb to this region can inhibit growth of all nine influenza A subtypes *in vitro* (Doyle et al., 2013) I decided to examine if this inhibitory capacity could be potentially extended to influenza B strains. Indeed my results demonstrated that the HCA-2 MAb could inhibit the NA enzyme activity and replication of influenza B viruses from either the Victoria or Yamagata lineages (Figure 3.10 and 3.11). Additionally I also demonstrated that strains containing NA mutations conferring NAI resistance could also be inhibited by the antibody further suggesting that this epitope maybe a valuable therapeutic target. These data suggest that similar to Influenza A, the broad protection afforded by the MAb can be extended to Influenza B (Figure 3.12 .1and 3.12.2).

Given that the MAb is targeted towards the NA protein there was potential that NAI resistant strains with NA mutations may have been more poorly inhibited than wild type strains. Interestingly, the two amino acid mutations in the NAI resistant strains tested here (E117D and D197E) are conformationally proximal to the HCA-2 region (Figure 3.12.3) (Hurt et al., 2004;Cheam et al., 2004;Hurt et al., 2006). Although both D197 and E117 are not in direct contact with the substrate, they are located closely to surface-exposed amino acids such as I222, R224 or E227 which are known to be in direct contact with the sialic acid substrate (Colman et al., 1983) and also found to be necessary for the MAb to inhibit the enzymatic activity of the influenza A neuraminidases (Doyle et al., 2013). Importantly, the E117D or D198E mutations did not alter the interaction between the antibody and the NA protein.

Clearly more studies are needed to determine whether Abs targeting the universally conserved epitope in viral neuraminidase could protect animals from influenza in a suitable animal model, an issue which is not addressed in this study. Nonetheless, it could be envisaged

that during the course of infection in tissue cultures, the viral peptide is sufficiently exposed to allow access by Abs, making it an attractive target for antiviral intervention and vaccine development.

Overall Conclusions and Future Directions

In short, my Ph.D. thesis has reported that the HCA-2 universal epitope in NA is indispensable to constitute an optimal tertiary environment needed for maximal enzymatic activities. Additionally, during the course of infection in tissue cultures and viral replication *in vivo*, this viral peptide is sufficiently exposed to allow access by attacking antibodies, making it an attractive target for antiviral intervention and vaccine development, particularly given that it is universally conserved among all viral NA. To this end potential future directions include developing branch peptide vaccines. These experiments could include combining HCA-2 peptides and conserved HA peptides. Recent studies have consistently shown the benefits of such vaccines and merit investigating the protective effects of these conserved regions.

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Collaborators Contributions

- Dr. Gary Van Domselaar did all computational analyses and protein modelling in Figure 3.5.
- Dr. Yi-Min She performed the LC MS/MS analysis and protein identification of the V_H and V_L regions of MAb in the laboratory of Dr. Terry Cyr
- Bozena Jaentschke assisted with alanine scanning mutagenesis and growth curve work (Section 3.1 and 3.2)
- Dr. Earl Brown performed all animal experiments and provided mouse adapted H1N1 and H3N2 strains (Section 3.9)
- Dr. Richard Webby provided all eight plasmids used in the reverse genetics system to generate for PR8/H1N1 virus.
- Primers were synthesized by Bio S&T

APPENDIX 1

Tables and Figures

Figure S1. Comparison of HCA-2 inhibition of enzymatic activity and in vitro growth across all subtypes and variants. The HCA-2 antibody inhibition of growth and NA activity at a concentration of 50mg of MAb. For alanine scanning mutants, inhibition of viral growth of the variants is not shown. Data are presented as percentage of inhibition compared to virus control ± s.e.m from three independent experiments.

Fig. S1

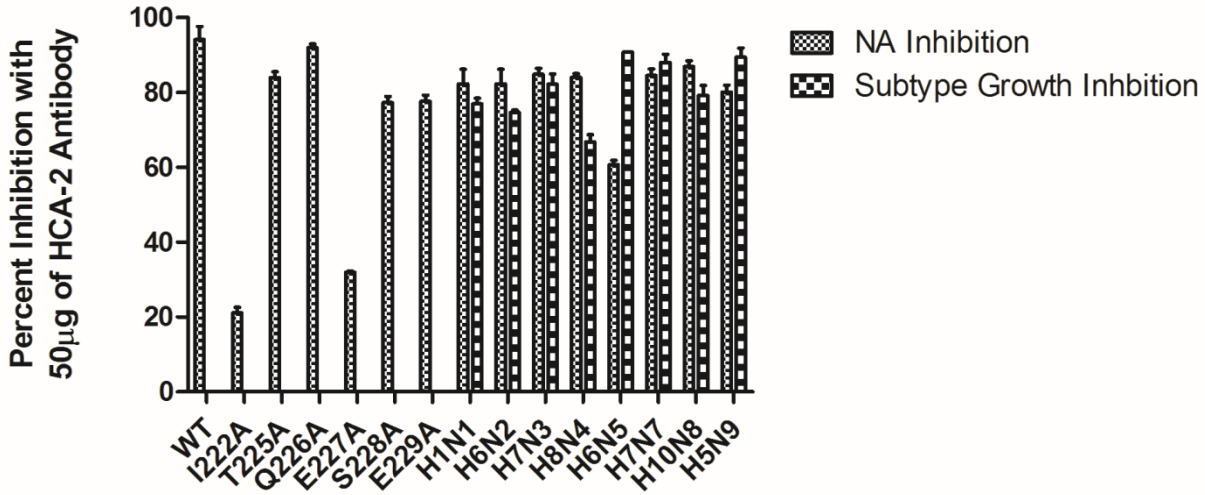
Percent Inhibition with 50μg of HCA-2 Antibody
100
80
60
40
20
0
WT
I222A
T225A
Q226A
E227A
S228A
E229A
H1N1
H6N2
H7N3
H8N4
H6N5
H7N7
H10N8
H5N9
NA Inhibition
Subtype Growth Inhbition

Table S1: Protein sequencing alignment between A/PR8/34/H1N1 and the remainder of the strains used in the inhibition studies (a-h) (a) A/Turkey/Mass.3740/65/H6N2 (accession # AJ574906.1) (b) A/Turkey/Oregon/71/H7N3 (accession # DQ870896.1), (c) A/Turkey/Ont/6118/68/H8N4 (accession EU429793.1), (d) A/Shearwater/Australia/72/H6N5 (AB278601.1) (e) A/DK/Czechoslovakia/56/H4N6 (GU052383.1), (f) A/EQ/Parague/1/56/H7N7 (U85989.1), (g) A/Quail/Italy/1117/65/H10N8 (L06587.1), (h) A/Turkey/Wisconsin/68/H5N9 (CY080509.1). All strains had between 44%-55% homology with the H1N1 strain and all but two had a 100% homology within the HCA-2 region. As can be observed from the alignment the strains representing the N4 and N5 had substitutions within the region, namely L223M and E229 respectively.

(a)

```
H1N1 1    MNPNQKIITIGSVCMTIGMANLILQIGNIISIWISHSIQ----LGNQNQIETCNQSVITY  56
          MNPNQKIITIGSV +TI     ++QI  + +    H  Q    +   NQ+  C + +I
H6N2 1    MNPNQKIITIGSVSLTIATVCFLMQIAILATTVTLHFKQNECSIPANNQVVPC-EPIIIE  59

H1N1 57   ENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGDVFV  116
           N T +   Y+N +         VV  +   +   C ++G+A +SKDNS+R+ + G+++V
H6N2 60   RNITEI--VYLNSTTIEKEICPGVVEYR-NWSKPQCQITGFAPFSKDNSIRLSAGGNIWV  116

H1N1 117  IRKPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFES  176
           R+P++SC P +C  F L QG  L++KHSNGTI DR P+RTL+   +G VP    ++
H6N2 117  TREPYVSCDPGKCYQFALGQGTTLDNKHSNGTIHDRIPHRTLLMNELG-VPFHLGTKQVC  175

H1N1 177  VAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNG  236
          +AWS+S+CHDG  WL + I+G D  A A   Y+G++ D+I SW  NILRTQESEC C+NG
H6N2 176  IAWSSSSCHDGKAWLHVCITGDDRNATASFIYDGMLVDSIGSWSQNILRTQESECVCING  235

H1N1 237  SCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNW  296
          +C  VMTDG ++G+A  KI  + +GKIV    ++    H EECSCYP    + CVCRDNW
H6N2 236  TCTVVMTDGSASGRADTKILFVREGKIVHISPLSGSAQHIEECSCYPRYPNVRCVCRDNW  295

H1N1 297  HGSNRPWVSFNQNLEYQI--GYICSGIFGDNPRPNDKTGSC---GPVSSNGANGVKGFSF  351
           GSNRP +  +   +Y I   Y+CSG+ GD PR +D + S     P +  G  GVKG++F
H6N2 296  KGSNRPVIDISM-ADYSIDSSYVCSGLVGDTPRNDDSSSSSNCKDPNNERGNPGVKGWAF  354

H1N1 352  KYGNGVWIGRTKSISSRNGFEMIWDPNGWTGTDNNFSI-KQDIVGINEWSGYSGSFVQHP  410
            GN VW+GRT S  SR+G+E      GWT  ++   + +Q IV  N WSGYSG F
H6N2 355  DNGNDVWMGRTISKDSRSGYETFRVIGGWTTANSKSQVNRQVIVDNNNWSGYSGIF----  410

H1N1 411  ELTGLDCIRPCFWVELIRGRPKENTI-WTSGSSISFCGVNSDTVGWSWPDGAELPF  465
           + G  CI  CF+VELIRGRP+E  + WTS S + FCG +      SWPDGA + F
H6N2 411  SVEGKSCINRCFYVELIRGRPQETRVWWTSNSIVVFCGTSGTYGTGSWPDGANINF  466
```

(b)

```
H1N1   1   MNPNQKIITIGSVCMTIGMANLILQIGNIISIWISHSIQLGNQN----------QIETCN  50
           MNPNQKIITIG V  T+    L++ +GN++   + H  ++GN            +  C+
H7N3   1   MNPNQKIITIGVVNTTLSTIALLIGVGNLVFNTVIHE-KIGNHQTVIHPTITTPAVPNCS  59

H1N1  60   QSVITYENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGS  110
            ++ITY N         NI+ T     + +    L     LCP  G+  + KDN++R+G
H7N3  60   DTIITYNN-----TVINNITTTIITEAERLFKPPLP----LCPFRGFFPFHKDNAIRLGE  110

H1N1 111   KGDVFVIRKPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPY  170
             DV V R+P++SC    C +F L QGALL  KHSNGTIKDR+PYR+L+  PIG  P
H7N3 111   NKDVIVTREPYVSCDNDNCWSFALAQGALLGTKHSNGTIKDRTPYRSLIRFPIGTAPVLG  170

H1N1 171   NSRFESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESE  230
           N +   +AWS+S+C DG  W+ + ++G DN A A + Y G +TD+IKSWR +ILRTQESE
H7N3 171   NYKEICIAWSSSSCFDGKEWMHVCMTGNDNDASAQIIYAGRMTDSIKSWRKDILRTQESE  230

H1N1 231   CACVNGSCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPN---YHYEECSCYPDSSE  287
           C  C+ G+C   +TDGP+   A ++++ I +G+IVK    N P     H EECSCY D  +
H7N3 231   CQCIGGTCVVAVTDGPAANSADHRVYWIREGRIVK--YENVPKTKIQHLEECSCYVD-ID  287

H1N1 288   ITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIFGDNPRPND-KTGSC-GPVSSNGANG  345
           + C+CRDNW GSNRPW+  N     + GY+CS    D PRP D  T SC  P + NG  G
H7N3 288   VYCICRDNWKGSNRPWMRINNETILETGYVCSKFHSDTPRPADPSTVSCDSPSNINGGPG  347

H1N1 346   VKGFSFKYGNGVWIGRTKSISSRNGFEMIWDPNGWTGTDNNF-SIKQDIVGINEWSGYSG  404
           VKGF FK GN VW+GRT S S R+GFE+I   +GW  + N+  S+ Q +V  N+WSGYSG
H7N3 348   VKGFGFKAGNDVWLGRTVSTSGRSGFEIIKVTDGWINSPNHAKSVTQTLVSNNDWSGYSG  407

H1N1 405   SFVQHPELTGLDCIRPCFWVELIRGRPKEN--TIWTSGSSISFCGVNSDTVGWSWPDGAE  462
           SF+    +    C +PCF+VELIRGRP +N    WTS S ++FCG++++    +WPDG+
H7N3 408   SFI----VKTKGCFQPCFYVELIRGRPNKNDDVSWTSNSIVTFCGLDNEPGSGNWPDGSN  463

H1N1 463   LPF  465
           + F
H7N3 464   IGF  466
```

(c)

```
H1N1  1    MNPNQKIITIGSVCMTIGMANLILQIGNIISIWISHSIQLGNQNQIETCNQSVITYENNT  60
           MNPNQKIITIGS  + +    L+LQI ++ SIW SH  Q+  Q   + C+ +   Y N T
H8N4  1    MNPNQKIITIGSASIVLTTIGLLLQITSLCSIWFSHYNQV-TQPHEQACSNNTTNYYNET  59

H1N1  61   WVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGDVFVIRKP  120
           +VN T V  + T      +   V  +    LCPV GWA  SKDN +RIGS+G+VFVIR+P
H8N4  60   FVNVTNVQNNYTTIIEPSAPNVVHYSSGRDLCPVKGWAPLSKDNGIRIGSRGEVFVIREP  119

H1N1  121  FISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVAWS  180
           FISCS  ECRTFFLTQGALLNDKHSNGT+KDRSP+RTLMSCP+G  PSP NSRFESVAWS
H8N4  120  FISCSISECRTFFLTQGALLNDKHSNGTVKDRSPFRTLMSCPMGVAPSPSNSRFESVAWS  179

H1N1  181  ASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFT  240
           A+AC DG  WLT+GI+GPD  AVAVLKYNGIITDT+KSW+ NI+RTQESEC C +  C+T
H8N4  180  ATACSDGPGWLTLGITGPDATAVAVLKYNGIITDTLKSWKGNIMRTQESECVCQDEFCYT  239

H1N1  241  VMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNWHGSN  300
           ++TDGPSN QA YKI +I KGKIV   ++NA  +H+EECSCYP  +++ CVCRDNW GSN
H8N4  240  LITDGPSNAQAFYKILKIRKGKIVSVKDVNATGFHFEECSCYPSGTDVECVCRDNWRGSN  299

H1N1  301  RPWVSFNQNLEYQIGYICSGIFGDNPRPNDKTGSCGPVSSNGAN--GVKGFSFKYGNGVW  358
            RPW+ FN +L+YQIGY+CSGIFGDNPRP D  GSC    +NG    GVKGFSF+YG+GVW
H8N4  300  RPWIRFNSDLDYQIGYVCSGIFGDNPRPVDGIGSCNSPVNNGKGRYGVKGFSFRYGDGVW  359

H1N1  359  IGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDCI  418
            IGRTKS+ SR+GFEM+WD NGW  TD + +  QDI+  N WSGYSGSF    E TG +C
H8N4  360  IGRTKSLESRSGFEMVWDANGWVSTDKDSNGVQDIIDNNNWSGYSGSFSIRWETTGRNCT  419

H1N1  419  RPCFWVELIRGRPKENTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDK  469
             PCFWVE+IRG+PKE TIWTSGSSI+FCGVNSDT GWSWPDGA LPF IDK
H8N4  420  VPCFWVEMIRGQPKEKTIWTSGSSIAFCGVNSDTTGWSWPDGALLPFDIDK  470
```

(d)

```
H1N1  1    MNPNQKIITIGSVCMTIGMANLILQIGNIISIWISHSIQLGNQNQIETCNQSVITYENNT  60
           MNPNQKIITIGS  + + + N++L + +I    IS    +   N++  CN + + Y
H6N5  1    MNPNQKIITIGSASLGLVIFNILLHVASITLGIIS----VTKDNKVHICNTTEV-YNETV  55

H1N1  61   WVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGDVFVIRKP  120
            V    + ++NT +   +      L     LC VSG+AI SKDN +RIGS+G +FVIR+P
H6N5  56   RVETVVIPVNNTIYLNHEPEF---LNNTEPLCDVSGFAIVSKDNGIRIGSRGHIFVIREP  112

H1N1  121  FISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVAWS  180
           F++C P ECRTFFLTQGALLNDKHSN T+KDRSPYR LMS P+G  P+ Y ++FESV WS
H6N5  113  FVACGPSECRTFFLTQGALLNDKHSNNTVKDRSPYRALMSVPLGSSPNAYQAKFESVGWS  172

H1N1  181  ASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSCFT  240
           A+ACHDG  W+ IG+SG D+ A AV+ Y G+ TD I+SWR  ILRTQES C C+ G C+
H6N5  173  ATACHDGKKWMAIGVSGADDDAYAVIHYGGVPTDVIRSWRKQILRTQESSCVCIKGECYW  232

H1N1  241  VMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNWHGSN  300
           VMTDGP+N QASYKIF+ +KG +V   E++    H EECSCYP+  ++ CVCRDNW+G N
H6N5  233  VMTDGPANNQASYKIFKSQKGMVVDEKEISFQGGHIEECSCYPNMGKVECVCRDNWNGMN  292

H1N1  301  RPWVSFNQNLEYQIGYICSGIFGDNPRPNDK--TGSC-GPVSSNGAN--GVKGFSFKYGN  355
           RP + F++ LEY++GY+C+GI  D PR  D   TGSC   V  +G N  GVKGF F+ GN
H6N5  293  RPILIFDEKLEYEVGYLCAGIPTDTPRVQDSSFTGSCTNAVGGSGTNNYGVKGFGFRQGN  352

H1N1  356  GVWIGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGL  415
            VW GRT S+SSR+GFE++   +GW       S K +++    WSGYSG+F     +T
H6N5  353  SVWAGRTISVSSRSGFEVLLIEDGWIRPSKTISKKVEVLNNKNWSGYSGAFTIPTAMTSK  412

H1N1  416  DCIRPCFWVELIRGRPKENTIWTSGSSIS-FCGVNSDTVGWSWPDGAELPFTIDK  469
           +CI PCFW+E+IRG+P+E T   + SS + FCGV+S+  GWSW DGA LPF IDK
H6N5  413  NCIVPCFWLEMIRGKPEERTSIWTSSSSTVFCGVSSEVPGWSWDDGAILPFDIDK  467
```

(e)

```
H1N1  1    MNPNQKIITIGSVCMT-------IGMANLILQIGNIISIWISHSIQLGNQNQIETCNQSV  53
           MNPNQKII I +  MT       IG+ANL L IG    +  +  + + N N+  +    +
H4N6  1    MNPNQKIICISATGMTLSVVSLLIGIANLGLNIGLHYKVGDTPDVNIPNVNRTNSTTTII  60

H1N1  54   ITYENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGD  113
                N + N T + I N N           L     LC V+ W I SKDN++RIG
H4N6  61   NNNTQNNFTNITNI-IQNKNEE------RTFLNLTKPLCEVNSWHILSKDNAIRIGEDAH  113

H1N1  114  VFVIRKPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSR  173
           + V R+P++SC P  CR F L+QG  L  +H+NGTI DRSP+R L+S  +G+ PSPYN++
H4N6  114  ILVTREPYLSCDPQGCRMFALSQGTTLRGRHANGTIHDRSPFRALVSWEMGQAPSPYNAK  173

H1N1  174  FESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECAC  233
            E + WS+++CHDGI+ ++I +SGP+N A AV+ Y G     I SW  NILRTQESEC C
H4N6  174  VECIGWSSTSCHDGISRMSICMSGPNNNASAVVWYGGRPVTEIPSWAGNILRTQESECVC  233

H1N1  234  VNGSCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCR  293
             G C  VMTDGP+N +A+ KI   ++GKI K  E+     H EECSCY     I C+CR
H4N6  234  HKGVCPVVMTDGPANNRAATKIIYFKEGKIQKIEELTGKAQHIEECSCYGAGGVIKCICR  293

H1N1  294  DNWHGSNRPWVSFN-QNLEYQIGYICSGIFGDNPRPNDKT-GSC-GPVSSNGAN-GVKGF  349
           DNW G+NRP ++ + + + +   Y+CS +  D  RPND T G+C  P++    + GVKGF
H4N6  294  DNWKGANRPVITIDPEIMTHTSKYLCSKVLTDTSRPNDPTNGNCDAPITGGSPDPGVKGF  353

H1N1  350  SFKYGNGVWIGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQH  409
           +F  G   W+GRT S  SR+G+EM+  PN  T T +     Q IV    WSGYSG+F+ +
H4N6  354  AFLDGENSWLGRTISKDSRSGYEMLKVPNAETDTQSGPISHQMIVNNQNWSGYSGAFIDY  413

H1N1  410  PELTGLDCIRPCFWVELIRGRPKENTI-WTSGSSISFCGVNSDTVGWSWPDGAELPF  465
                 +C  PCF+VELI GRPKE+++ WTS S ++ CG       WSW DGAE+ +
H4N6  414  --WANKECFNPCFYVELITGRPKESSVLWTSNSIVALCGSRERLGSWSWHDGAEIIY  468
```

(f)

```
H1N1 1    MNPNQKIITIGSVCMTIGMANLILQIGNI---ISIW------ISHSIQLGNQNQIETCNQ  51
          MNPNQK+     + + +G+ NL++ I N+   IS++       S ++   N NQ  T
H7N7 1    MNPNQKLFASSGIAIALGIINLLIGISNMSLNISLYSKGENHKSDNLTCTNINQNNT--T  58

H1N1 52   SVITYENNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSK  111
           V TY NNT +      + N  +          L  N SLC V GW + +KDN++R G
H7N7 59   MVNTYINNTTIIDKNTKMENPGY----------LLLNKSLCNVEGWVVIAKDNAIRFGES  108

H1N1 112  GDVFVIRKPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYN  171
            + V R+P++SC PL C+ + L QG  + +KHSNGT  DR+ +R L+S P+G  P+  N
H7N7 109  EQIIVTREPYVSCDPLSCKMYALHQGTTIRNKHSNGTTHDRTAFRGLISTPLGNPPTVSN  168

H1N1 172  SRFESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESEC  231
          S F  V WS+++CHDG++ +TI + G +  A A + YN  +T TIK+W  NILRTQESEC
H7N7 169  SEFICVGWSSTSCHDGVSRMTICVQGNNENATATVYYNKRLTTTIKTWAKNILRTQESEC  228

H1N1 232  ACVNGSCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCV  291
           C N +C  VMTDGP+N QA  K+    KG I+K   +     H EECSCY  +  +TCV
H7N7 229  VCHNSTCVVVMTDGPANNQAFTKVIYFHKGTIIKEEPLKGSAKHIEECSCYGHNQRVTCV  288

H1N1 292  CRDNWHGSNRPWVSFNQ-NLEYQIGYICSGIFGDNPRPNDKT-GSC-GPVS-SNGANGVK  347
          CRDNW G+NRP +  +  NLE+   YIC+G+  D  RP DK  G C  P++ S GA G+K
H7N7 289  CRDNWQGANRPVIEIDMNNLEHTSRYICTGVLTDTSRPKDKAIGECFNPITGSPGAPGIK  348

H1N1 348  GFSFKYGNGVWIGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFV  407
          GF F   N  W+GRT S   R+GFEM+  PN  T  D+    +Q+IVG + WSGYSGSF+
H7N7 349  GFGFLNENNTWLGRTISPKLRSGFEMLKIPNAGTDPDSKIKERQEIVGNDNWSGYSGSFI  408

H1N1 408  QHPELTGLDCIRPCFWVELIRGRPKENTI--WTSGSSISFCGVNSDTVGWSWPDGAELPF  465
           +      +C  PCF+VELIRGRP+E     WTS S I+ CG        S+PDGA++ +
H7N7 409  DYWN-DNSECYNPCFYVELIRGRPEEAKYVEWTSNSLIALCGSPIPVGSGSFPDGAQIKY  467
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(g)

```
H1N1   1    MNPNQKIITIGSVCMTIGMANLILQIGNIISIWISHSIQLGNQNQIETCNQSVITYENNT  60
            MNPN+KIITIGS+ + + + N++L I +II       + LG   +  +CN++V+   N T
H10N8  1    MNPNKKIITIGSISLGLVVFNVLLHIVSIIVT----VLVLGKGEKNGSCNETVVREYNET  56

H1N1   61   WVNQTYVNISNTNFAAGQSV--VSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKGDVFVIR  118
               +  +   NT+             +    ++C V G+A +SKDN +RIGS+G VFVIR
H10N8  57   VKVEKVIQWHNTSVIEHIPYWNGGTYMNNTEAICDVKGFAPFSKDNGIRIGSRGHVFVIR  116

H1N1   119  KPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVA  178
            +PF+SCSP ECRTFFLTQG+LLNDKHSNGT+KDRSP+RTLMS  +G+ P+ Y +RFE+VA
H10N8  117  EPFVSCSPKECRTFFLTQGSLLNDKHSNGTVKDRSPFRTLMSVEVGQSPNVYQARFEAVA  176

H1N1   179  WSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGSC  238
            WSA+ACHDG  W+TIG++GPD+ AVAV+ Y G+ TD I SW  +ILRTQESEC C+ G C
H10N8  177  WSATACHDGKKWMTIGVTGPDSKAVAVIHYGGVPTDVINSWAGDILRTQESECTCIQGDC  236

H1N1   239  FTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNWHG  298
            + VMTDGP+N QA Y+I++  +G+I+  ++++    H EECSCYP+  ++ CVCRDNW G
H10N8  237  YWVMTDGPANRQAQYRIYKANQGRIIGQIDVSFNGGHIEECSCYPNDGKVECVCRDNWTG  296

H1N1   299  SNRPWVSFNQNLEYQIGYICSGIFGDNPRPNDK--TGSCGPVSSNGANGVKGFSFKYGNG  356
            +NRP +  + +L Y++GY+C+G+  D PR  D   TGSC     N   GVKGF F+ G+
H10N8  297  TNRPILVISPDLSYRVGYLCAGLPSDTPRGVDAQFTGSCTSPMGNQGYGVKGFGFRQGSD  356

H1N1   357  VWIGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLD  416
            VW+GRT S +SR+GFE++   NGWT T      +Q +V    WSGYSGSF    E++G D
H10N8  357  VWMGRTISRTSRSGFEILRIKNGWTQTSKEQVGRQVVVDNLNWSGYSGSFTLPVEMSGRD  416

H1N1   417  CIRPCFWVELIRGRPKENTIWTSGSSISFCGVNSDTVGWSWPDGAELPFTIDK  469
            C+ PCFWVE+IRG+P+E TIWTS SSI  CGV+     W+W DGA LPF IDK
H10N8  417  CLVPCFWVEMIRGKPEEKTIWTSSSSIVMCGVDYKVADWTWHDGAILPFDIDK  469
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(h)

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H1N1  1    MNPNQKIITIGSVC-------MTIGMANLILQIGNIISIWISHSIQLGNQNQIETCNQSV  53
           MNPNQKI+   +         + IG+ANL L IG  +    + S Q   Q +    +Q++
H5N9  1    MNPNQKILCTSATAIAIGAIAVLIGIANLGLNIGLHLKPSCNCSHQ---QPEATNASQTI  57

H1N1  54   ITYENNTWVNQTYV-NISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSVRIGSKG  112
           I    N + N+T +  ISNTN    +           LC ++ W I+ KDN+VRIG
H5N9  58   I----NNYYNETNITQISNTNIQVEEKANRDFNNLTRGLCTINSWHIFGKDNAVRIGEDS  113

H1N1  113  DVFVIRKPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNS  172
           DV V R+P++SC P ECR + L+QG  +  KHSNGTI DRS YR L+S P+   P+ Y+S
H5N9  114  DVLVTREPYVSCDPDECRFYALSQGTTIRGKHSNGTIHDRSQYRALISWPLSSPPTVYSS  173

H1N1  173  RFESVAWSASACHDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECA  232
           R E + WS+++CHDG   +++ ISGP+N A AV+ YN      I +W  NILRTQESEC
H5N9  174  RVECIGWSSTSCHDGKARMSVCISGPNNNASAVVWYNRRPVTEINTWARNILRTQESECV  233

H1N1  233  CVNGSCFTVMTDGPSNGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVC  292
           C NG C  V TDG + G A  +++  ++GK +K   +     H EECSCY + + ITC C
H5N9  234  CHNGVCPVVFTDGSATGPAETRVYYFKEGKTLKWEPLAGTAKHIEECSCYGERAGITCTC  293

H1N1  293  RDNWHGSNRPWVSFNQ-NLEYQIGYICSGIFGDNPRPNDKT-GSCG-PVSSNGANGVKGF  349
           RDNW GSNRP +  +   + +   YICS +  DNPRPND T G C  P   N  NGVKGF
H5N9  294  RDNWQGSNRPVIRIDPVAMTHTSQYICSPVLTDNPRPNDPTVGKCNDPYPGNNNNGVKGF  353

H1N1  350  SFKYGNGVWIGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQH  409
           S+  G   W+GRT S +SR+G+EM+  PN  T   +  +  Q IV   +WSGYSGSF+ +
H5N9  354  SYLDGGNTWLGRTISTASRSGYEMLKVPNALTDDRSKPTQGQTIVLDTDWSGYSGSFMDY  413

H1N1  410  PELTGLDCIRPCFWVELIRGRPKENTI-WTSGSSISFCGVNSDTVGWSWPDGAELPFTI  467
                +C R CF+VELIRGRPKE+ + WTS S +S C        W+WPDGA++ + +
H5N9 414   --WAEGECYRACFYVELIRGRPKEDKVWWTSNSIVSMCSSTEFLGQWNWPDGAKIEYFL  470
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