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Mutagenesis of Influenza Virus H5-Type Hemagglutinin to Identify Domains Important for Infection

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MUTAGENESIS OF INFLUENZA VIRUS H5-TYPE HEMAGGLUTININ TO IDENTIFY DOMAINS IMPORTANT FOR INFECTION

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ABSTRACT

Influenza virus hemagglutinin (HA) envelope protein aids in the recognition and attachment of the virion to host cell receptors. HA residues important for infection of the H5N1 type virus, an especially virulent type, are different than those previously characterized for other HA types. This project used mutagenesis to help identify H5 HA domains responsible for function. Of the 70 mutants tested, 25 showed infectivity levels similar to the WT, 24 showed no infectivity, 18 showed decreased infectivity, and 3 showed increased infectivity.

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ACKNOWLEDGEMENTS

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BACKGROUND

Introduction

Influenza, commonly referred to as the flu, is an acute respiratory disease that results from infection by influenza viruses. Cases of influenza can range from mild to severe, and can potentially lead to death. Influenza viruses are typically transmitted from person to person through air droplets generated by coughing, sneezing, or simply talking. According to the Center for Disease Control and Prevention, 5-20% of the United States population is infected with a seasonal influenza strain annually, causing 3,000 to 49,000 fatalities. Influenza strongly affects small children, elderly people, and those with weakened immune systems more severely than others (CDC.gov, 2010b). Only types A and B cause the seasonal epidemics in humans, while type C typically causes mild respiratory illness (Skehel, 2009). The incubation period for influenza is approximately 1-7 days, and people can be contagious one day prior to experiencing symptoms until about seven days after symptoms appear. Symptoms may include fever, cough, sore throat, runny nose, muscle aches, headaches, fatigue, vomiting, and diarrhea. Although most people recover from influenza in about two weeks, some develop life-threatening conditions such a pneumonia, bronchitis, and sinus and ear infections (CDC.gov, 2011b).

Influenza undergoes antigenic drift and shift to cause epidemics and pandemics. Antigenic drift refers to small changes in the virus that occur continually over time. Antigenic shift is a major change in the virus and typically, results in a worldwide pandemic (CDC.gov, 2011b). This is because it generates a new strain to which the population has no immunity. Several influenza pandemics have been documented throughout human history; however, the 1918 Spanish Flu resulted in the most deaths. In 1918, type A H1N1 influenza infected approximately one third of the world's population, and killed at least 50 million people

(Taubenberger and Morens, 2006). Other pandemics have occurred since the 1918 Spanish Flu, including the 1957 Asian Influenza (H2N2), the 1968 Hong Kong Influenza (H3N2), the 1977 Russian Influenza (H1N1), and most recently the 2009 "swine flu" (H1N1) (Horimoto and Kawaoka, 2005; Tang et al., 2011).

Influenza Virus

Influenza belongs to the Orthomyxovirus family and are negative-sense, single-stranded RNA viruses (Couch, 1996). The virus particle consists of an outer lipid envelope containing hemagglutinin (HA) and neuraminidase (NA) (**Figure 1**), which are knob-shaped glycoproteins between 80 and 120 nm in diameter and 200-300 nm in length. The surface glycoproteins, HA and NA, and the M2 ion-channel protein are all embedded in the viral envelope. The genome of influenza viruses is linear and segmented, and type A consists of 8 segments (**Figure 1**) (Horimoto and Kawaoka, 2005; Kawaoka et al., 2006).

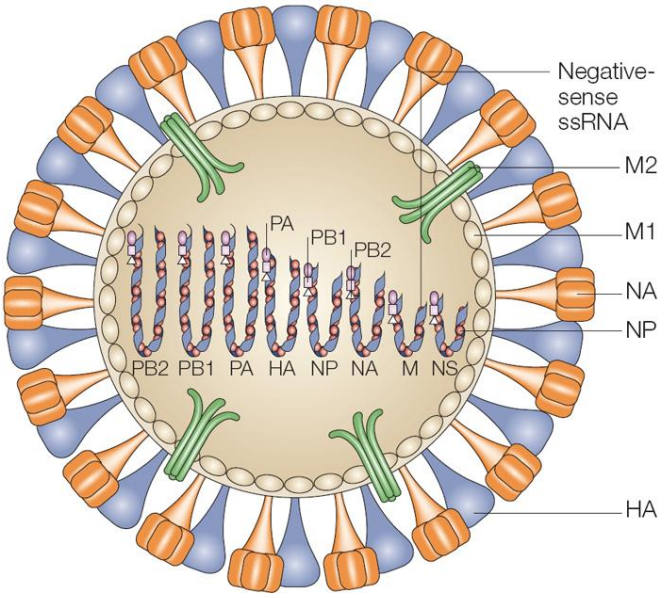

Figure-1: Diagram of Influenza-A Virion General Structure. The RNA virus is enveloped, and contains a segmented genome. Two surface glycoproteins, HA and NA, and the M2 ion-channel protein are embedded in the viral envelope, which is derived from the host plasma membrane (Horimoto and Kawaoka, 2005)

Nomenclature

Antigenic differences in their nucleoprotein (NP) and matrix protein (M) allow influenza viruses to be classified as types A, B, and C. Influenza A is the most virulent type and is further divided into different subtypes based on the antigenicity of the HA and NA surface glycoprotein. Currently 16 HA (H1-H16) and 9 NA (N1-N9) have been identified. The HA subtypes are differentiated by their reactivity with subtype-specific hyper-immune sera, while the NA subtypes differ in structure and sensitivity to antiviral drugs (Skehel, 2009). Currently, influenza A subtypes H1N1 and H3N2 are found to infect humans. Influenza type B viruses are not divided into subtypes, however, they can still be divided into different strains. Influenza A virus is clinically the most important of the influenza viruses because it is responsible for all the recent pandemics. In addition, since influenza A and B cause epidemics in humans, they are both included in annual vaccines (CDC.gov, 2011b).

Morphology of the Genome and Replication

Influenza virus infects the upper respiratory tract of the host, and mainly targets the columnar epithelial cells. The virus binds to the receptors through hemagglutinin (HA), and enters the cell by receptor-mediated endocytosis (**Figure 2**). The acidic pH of the endocytic vesicles induces the viral HA to undergo a conformation change from the nonfusogenic to the fusogenic form of the protein (Behrens and Stoll, n.d.). Following fusion of the viral envelope with the endosome, the viral genome is released into the nucleus. Viral RNP complex dissociates from the M1 protein and is imported to the host nucleus for transcription and translation. The progeny viruses are released from the host cell by budding. Budding is a process by which enveloped viruses detach from a cell by exocytosis in a pinching-off manner (Chazal and Gerlier,

2003). The neuraminidase (NA) activity of NA then cleaves the HA bound to sialic acid residues on the cellular surface that would otherwise retain the new virus at the cell surface. Localized infections typically occur because most influenza strains possess HAs that are only cleaved by proteases from the upper respiratory tract. However, systemic infections occur when viral strains have more universal cleavage sites compared to seasonal or less pathogenic viruses (Garten and Klenk, 1999).

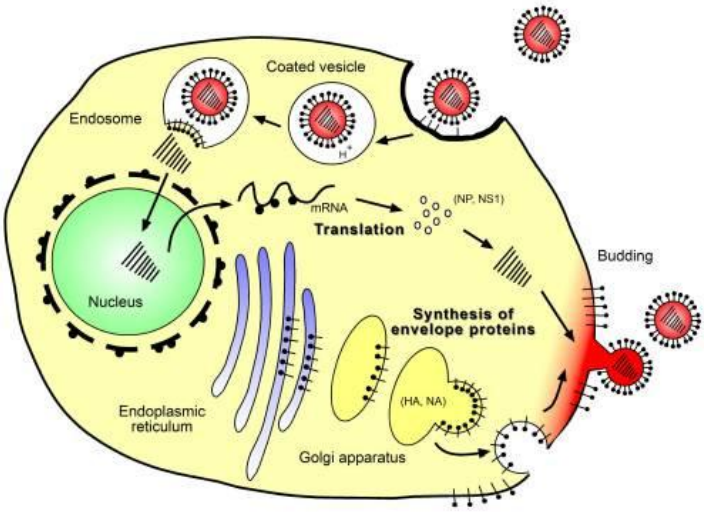

Figure-2: Diagram of Influenza-A Viral Replication. The virion attaches to the surface of cells via the binding of HA to sialic acid residues in surface proteins, and enters endocytic vesicles (upper diagram). Acidification of the vesicle results in viral RNA release into the nucleus (diagram center left) for transcription and translation. Mature virions exit the cell via exocytosis. (Behrens and Stoll, n.d.)

Current Treatments

Vaccines

Since the influenza virus is constantly undergoing antigenic drift, the annual seasonal influenza vaccine is continually modified. Three different types of influenza shots are available, however the most common influenza vaccine consists of inactive heat killed virus administered through a needle into the upper arm. According to the Center for Disease Control and Prevention, this form of vaccination is approved for people 6 months or older. This seasonal vaccine consists of three different influenza viral strains that scientists determine to be the

greatest risk in that year. For the current 2011-2012 season it includes two type A and one type B strains. The vaccine provides antigens to the body which stimulate the formation of protective antibodies against the virus (CDC.gov, 2011a).

A nasal-spray influenza vaccine (FluMist®) has also been approved for individuals between the ages of 2 and 49 who are not pregnant. This vaccine is made of attenuated, live influenza virus that is administered as a nasal spray. The virus is "cold-adapted" which means that it will only cause infection in the cooler temperatures found within the nose. The virus will not infect the lungs or other parts of the body where warmer temperatures exist (CDC.gov, 2011a).

Antiviral Treatments

Since the nature of pandemic influenza strains cannot be predicted, a vaccine against them cannot be developed. Antivirals are the first line of defense against a new influenza pandemic strain. They are also required for immune-compromised people who are non-responders or weak responders to the vaccine. The FDA-approved antivirals target either NA or M proteins. The FDA approved antiviral, Oseltamivir (commonly referred to as Tamiflu) is a neuraminidase inhibitor used to treat and prevent influenza A and B viruses in patients one year of age and older (Treanor et al., 2000; CDC.gov, 2009). Oseltamivir also decreases the risk of secondary complications including otitis media, bronchitis, pneumonia, and sinusitis (Gillissen and Hoffken, 2002). It is used both prophylactically and therapeutically. The antiviral is usually given orally to individuals who have experienced symptoms for less than two days as it is only effective during the viral replication phase when the neuraminidase is used to release viral

particles (Gillissen and Hoffken, 2002; NIH.gov, 2011). More recently, the majority of circulating influenza strains are resistant to Oseltamivir treatment.

Zanamivir is another FDA approved antiviral used to treat and prevent influenza A and B infections. This antiviral is a sialic acid analogue and a neuraminidase inhibitor (Hayden et al., 1997). Zanamivir is approved to *treat* infections in people seven years of age and older, and is approved to *prevent* infections in people five years of age and older (CDC.gov, 2009). This antiviral is administered orally or through inhalation (Treanor et al., 2000).

Amantadine and rimantadine are antivirals used to treat and prevent influenza A infections only. Amantadine is used in patients older than one year, and rimantadine is used to treat people older than thirteen years, and to *prevent* influenza in people older than one year (CDC.gov, 2009). These antivirals have no activity against influenza B strains, and can cause undesirable central nervous system and gastrointestinal side effects. Amantadine and rimantadine are both associated with the appearance of resistant influenza virus strains in treated patients (Treanor et al., 2000). Currently they are not used since most influenza strains are resistant to the two antivirals.

Pseudotype Viruses

Pseudotype virus has the backbone of one virus and the envelope proteins of another virus. They are widely used as surrogate models to study viral entry for high pathogenic viruses that required a BSL-3/4 facility, or for viruses that are difficult to generate. By using pseudotype viruses, the viral entry of these viruses can be studied in a BSL-2 facility. During this MQP project, the human immunodeficiency virus backbone was used to generate influenza pseudotype virus. Other viruses such as vesicular stomatitis virus and murine leukemia virus are also widely

used to generate pseudotype viruses. Since viral entry is solely determined by the interactions of its envelope glycoproteins and cellular receptors, the pseudotype virus behaves like influenza; however, it does not contain the envelope protein genes. Since the genes are not present, the pseudotype virus is only able to go through one cycle of replication and cannot produce progeny HIV virus (**Figure 3**). This system allows easy genetic manipulation, with the safety concerns alleviated.

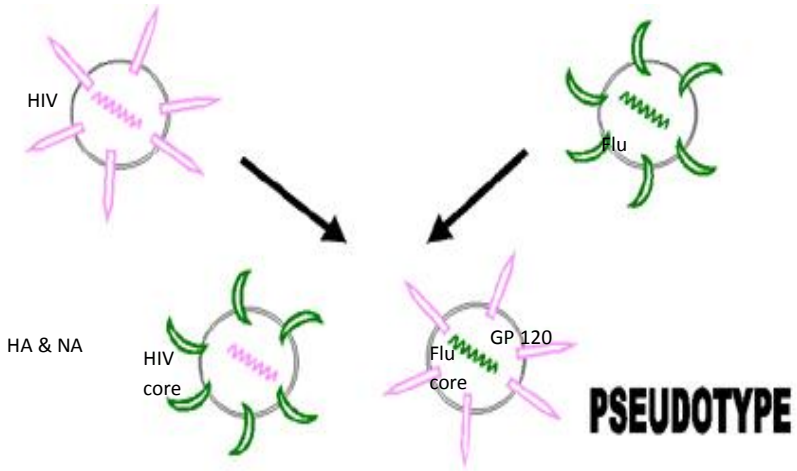

Figure-3: Diagram of Phenotypic Mixing to Produce Pseudotype Virus (Murray et al., 2010).

Hemagglutinin Structure

Hemagglutinin (HA) is a glycoprotein that plays an essential role in viral infection of host cells. The envelope HA spikes are transmembrane glycoproteins that are associated with the M1 protein at the inner surface of the membrane. Cleavage of HA by cellular proteases is required to expose the HA fusion peptide that is responsible for the fusion between the viral envelope and the endosomal membrane (Gamblin and Skehel, 2010).

Influenza virus hemagglutinin (HA) is responsible for binding the virus to host cell receptors, enabling infection. Due to this key role in infection, HA has been used as a target for

drug and vaccine development. Each monomer is synthesized as a single polypeptide chain (HA_0). Following the binding of the virion to the cell surface, the cleavage of HA_0 into HA1 and HA_2 is essential for HA activity. HA_1 mediates viral attachment to sialic acid on the host cell, while HA_2 mediates endosomal membrane fusion. HA is composed of a globular head containing the receptor-binding domain and alpha-helical stalks containing the HA_1/HA_2 cleavage site. All HA types have conserved receptor-binding domains, and the binding site is made up of three structural elements, essentially an alpha-helix and two loops. **Figure 4** shows the crystalline structure of Influenza strain Viet04 H5N1 HA (Stevens et al., 2006).

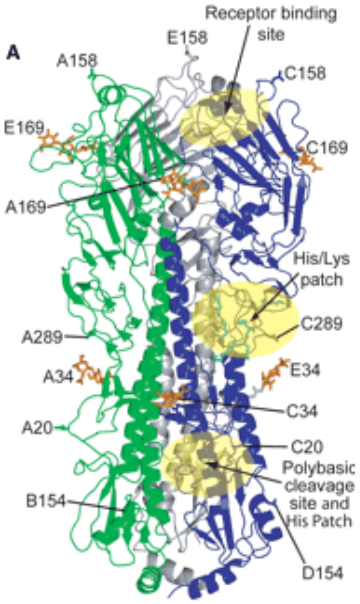

Figure-4: Crystalline Structure of Viet04 HA. Each monomer is shown in a different color, and carbohydrates are shown in orange (Stevens et al., 2006)

Mutational Studies of Influenza HA

Multiple mutational studies of this influenza HA protein have previously been performed for the purpose of identifying key amino acid residues important in viral function. Most of these studies, however, are focused mutations or are performed in HA from non H5 subtypes. In one of these studies, point mutations were made within the H5N1 hemagglutinin protein to determine

the role of individual amino acids in controlling the fusion acidity (Reed et al., 2009). The locations of eight key residues involved in pH control were identified by mutagenizing H5N1 HA2 coiled-coil residues that altered the pH in H3 and H7 HA proteins. Eleven other residues were also identified by sequence alignment of HA proteins from different subtypes. Seven of the mutations created were found to alter the pH, six of which were located in the fusion peptide pocket. Based on this Reed et al. (2009) study, it seems that fusion peptide pocket residues may commonly regulate HA acid stability across all HA subtypes (Reed et al., 2009).

Another HA mutagenesis study was performed by Su et al. (2008), and used pseudo-typed HIV-HA-luc viruses encoding mutated HA's to study the effects of HA mutations on infection (Su et al., 2008). The entry of HA mutants into live mammalian cells was measured by the amount of luciferase expression and compared with the sialic acid receptors on the cells. Typically, human influenza strains prefer to bind to terminal sialic acids with an α2,6 linkage, while avian strains prefer an α2,3 linkage. It is currently hypothesized that a switch in receptor specificity from avian to human preference is a key reason why influenza A viruses have crossed between species. Mutating only one amino acid in the HA sequence was shown to be adequate to make this switch. Mutations such as E190D, Q226L, and G228S were previously shown to induce a switch in receptor preference from avian to human in H1, H2, and H3 strains, but not in H5. In this 2008 study, six amino acid mutations located outside the receptor-binding domain of HA were tested, including, K35R, D45N, D94N, K35R/D45N, K35R/D45N/D94N, A267T. The results showed that none of the mutations influenced the receptor binding, except D94N. Mutation D94N improved receptor binding of H5 wild type HA to human cells, and decreased receptor binding to avian cells. The mutant viral titers produced by human cells were higher than the wild type viral titers (Su et al., 2008).

Other studies found that other amino acid residues were also important. Mutations S220T, D239G/N, A156T, D185N, Q226L, S227N, G228S, and K58I were found to hinder viral infection (Ayora-Talavera et al., 2009; Morlighem et al., 2011; Krenn et al., 2011). In this MQP project, mutations were performed in both HA1 and HA2 to identify important residues in both.

PROJECT PURPOSE

Influenza virus infects 5-20% of the United States population annually, and is responsible for 3,000-49,000 fatalities. Influenza strain H5N1 is a highly pathogenic avian influenza strain that currently rarely transmits to humans, but when it does, the mortality rate is usually high. The types of residues important for H5-type HA function are likely different than HA domains previously characterized for other strains (Su et al., 2008). The specific purpose of this MQP project was to investigate the effects of mutations in the H5 HA gene. The infectivity of the mutants was compared to that of the viruses with wild type HA to help map regions of the HA that are important for its function.

METHODS

Cell Maintenance

Two cell lines permissive for influenza infection were used throughout this project. A human embryonic kidney cell line (293FT) was used to generate pseudotype influenza virus (HIV/HA(H5)), and a human lung cancer cell line (A549) was used to determine the infectivity of the pseudotype viruses. Both cell lines were obtained from ATCC. Each cell line was grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Inc.) and supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco). The DMEM used to maintain the 293FT cells was also supplemented with 1% L-glutamine (200 mM solution) and 1% MEM Nonessential Amino Acids (100X solution) (Mediatech, Inc.).

Cells were split every 2-3 days when they reached approximately 90% confluence. Splitting was performed by removing the medium, washing the flask with 10 mL of 1X phosphate buffer saline (PBS) (Mediatech, Inc.) and adding 1 mL of 0.25% trypsin (Mediatech, Inc.). Once the cells became detached from the flask, they were passaged into a new T-75 flask at a ratio of about 1:5 cells to fresh media. Cells were not used for experiments until they reached passage 3. Cells were grown in a 37ºC incubator with 5% carbon dioxide.

Mutagenized HA Plasmids

A small mass of each plasmid encoding wild-type or mutagenized H5-type HA protein constructs, and pNL4.3 encoding the HIV backbone and luciferase reporter gene, were obtained from Dr. Lijun Rong (University of Illinois, Chicago) and Debra Mills, a technician at Microbiotix. These plasmids were transformed into E. coli to produce sufficient quantities for

use throughout this project. 1 µL of plasmid DNA solution was added to a 50 µL vial of competent DH5α *E. coli* (Invitrogen Inc.) on ice. The vial was incubated on ice for 30 minutes to allow the DNA to coat the surface of the cells. After the 30 minute incubation, the vial was transferred to a 42ºC water bath for 30 seconds to heat-shock them and allow the DNA to be taken into the cells, and then placed immediately back on the ice for 2 minutes. After the 2 minute incubation on ice, 500 µL of room temperature S.O.C. Medium (Invitrogen Inc.) was added to the vial, then the vial was vortexed and taped horizontally to a shaker platform at 37ºC. The vial was left to shake at ~300 rpm for 1 hour. Once the 1 hour of shaking was complete, the vial was transferred to a sterile 250 mL flask containing 100 mL of LB Miller Broth and 100 µg/mL of Ampicillin. The flask was placed in a shaker at 37ºC overnight.

Following the overnight incubation, the flask was removed, and plasmid DNA was isolated from the cells using the PureLink HiPure Plasmid Filter Midiprep Kit (Invitrogen Inc.). The cell suspension left overnight was poured into a 50mL conical tube and spun down at 4,000 x g for 10 minutes to pellet the cells. The supernatant was poured off, and the cells were resuspended in 10 mL of lysis buffer until homogenous. Next, 10 mL of precipitation buffer was added to the lysate and inverted to gently mix. This mixture was poured into the HiPure Filter Midi Column and left to filter through the column by gravity flow. Once all the liquid had passed through the column, 20 mL of wash buffer was added to the column to help remove any unwanted particles. The wash buffer was also left to filter through the column by gravity flow. Upon completion, a sterile 15mL centrifuge tube was placed under the filter column and 5 mL of elution buffer was added to the column to elute the plasmid through the column and into the tube. Once the solution had drained into the tube, 3.5 mL of isopropanol was added and the tube was centrifuged to pellet the plasmids at 15,000 x g for 30 minutes at 4°C. After 30 minutes, the

supernatant was removed, and 3 mL of ethanol was added to the tube. The tube was centrifuged again at 15,000 x g for 15 minutes. After the 15 minute centrifugation, the supernatant was discarded, and the pellet was left to air dry for about 10 minutes. After drying, the pellet was resuspended in 100 µL of 10 mM Tris-Cl, pH 8.5, and the optical density was taken using a spectrophotometer.

Upon completion of the plasmid prep, the plasmids were digested using KpnI HF and BamHI-HF (New England Biolabs) and run on an agarose electrophoresis gel to confirm the presence of the HA gene. When run on a gel, the vector (pcDNA3.1) showed a DNA band at 5 kb, and the HA insert showed a DNA band at 1.7 kb.

To verify the WT or mutant HA genotype, the plasmids were sent for sequencing at Sequegen, Inc. in Worcester, MA. Once verified, the plasmids were used to produce pseudotype virus.

Generation of Pseudotype Viruses

Pseudotype virus was generated by co-transfecting 293FT cells with plasmids encoding WT or mutant H5-type HA proteins and plasmid pNL4.3 R-E Luc+. pNL4.3 R-E Luc+ encodes for the HIV backbone and contains the luciferase reporter gene. 293FT cells were plated in 10-cm petri dishes which were coated with 10 mL of a solution of 1X PBS containing 0.001% solution Poly L-Lysine (Sigma-Aldrich) to help cells adhere to the plates. The plates were incubated at room temperature for about 30 minutes to allow Lysine attachment. T-75 flasks containing confluent 293FT cells were trypsinized, and the cells were suspended in 12 mL of media. 1.5 mL of the cell suspension was added to each 10-cm petri dish along with 12 mL of media 24 hours prior to transfection.

After 24 hours of cell growth, the 293FT cells were co-transfected with pNL4.3 Luc+R-E and either the wild type H5 HA construct or the mutant H5 HA construct. The transfection was as follows: For each 10-cm petri dish, 1.5 mL of Opti-MEM I (Reduced Serum Media 1X) (Invitrogen, Inc.) was added to a sterile 15 mL conical tube. 90 µL of Mirus transfection reagent (Mirusbio, Inc.) was added to the tube and gently mixed. After mixing, 7.5 µg of pNL4.3 Luc+R-E- and 7.5 µg of the H5 HA construct (wild type or mutant) were added to the tube and mixed gently. The mixture was incubated at room temperature for about 30 minutes. During the 30 minute incubation period, the 10-cm plate, containing the 293FT cells, was removed from the incubator and the medium was removed from it. 10 mL of 1x PBS was used to wash the plate, then it was removed. 15.5 mL of DMEM (containing Glutamine, NEAA, and 3% FBS) was added to the dish. After the 30 minute incubation of the conical tubes, the 1.5 mL of transfected cell suspension was added to the 10-cm dish, and the dish was returned to the incubator for 24 hours. After 24 hours of incubation, the medium was removed from the 10-cm petri dish and replaced with 15 mL of fresh DMEM (containing 2% Glutamine, 1% NEAA, 3% FBS, 10mM Hepes (Thermo Scientific), 10 mM $CaCl_2$, and 35 uL (5 units) of Neuraminidase (Sigma-Aldrich). Neuraminidase is added externally to the cells because it is not already provided in the pseudotype system and is essential for infection. After another 24 hours of incubation, the virus was harvested by centrifugation to pellet any cells, and the supernatant containing virus was poured into sterile conical tubes. The virus was stored at 4ºC until use.

Test of Viral Infection

After the wild type and mutant H5 pseudotype viruses were prepared, they were tested for infectivity in A549 cells by doing a pseudotype virus infection assay. 24 hours prior to infections, A549 cells were plated in 96-well CulturPlates (Perkin-Elmer) at a concentration of

$4x10^4$ cells/mL in complete medium. The 96-well plates were then incubated at 37ºC in 5% CO_2. After 24 hours of incubation, a separate 96-well plate was used to do a 1:5 dilution of virus in media (containing no FBS) from rows A to H. Each virus dilution was performed in duplicate. The 1:5 dilution was used to ensure that the undiluted reading of the plate was not above the linear range of the luminometer when assaying for Luciferase activity. The medium was removed from columns 2-11 of the 96-well plate containing cells. Medium was not removed from columns 1 and 12, and virus was never added to these columns. Columns 1 and 12 were used as background for the assay and represented the negative control "no infection". 100 µL of the dilutions made in the other 96-well plate were transferred to the plate containing A549 cells. After the virus was added to the 96-well plate, the plate was returned to the incubator for 5 hours. After 5 hours, the medium was removed from the plate and replaced with 100 µL/well of fresh DMEM (without phenol red, containing 2% Glutamine, 1% NEAA, 10% FBS) (Mediatech, Inc.). The plate was then returned to the incubator and left for 72 hours. After 72 hours, the plates were removed from the incubator, and 100 µL of BriteLite Plus (ultra-high sensitivity luminescence reporter gene assay system) containing substrate solution (Perkin-Elmer) was added to each well. Plates were sealed and left to shake at room temperature for about 5 minutes. A reading of luminescence was done by putting plates in an EnVision plate reader (2102 Multilabel Reader) (Perkin Elmer).

RESULTS

Influenza virus infects 5-20% of the United States population annually and causes thousands of fatalities. The influenza strain H5N1 is a highly pathogenic avian strain that is currently not transmitted to humans. The fear is that if this strain is able to transmit to humans, it will result in a worldwide pandemic. An essential structure of the virus involved in infection of the host cells is the envelop glycoprotein, hemagglutinin (HA). Although previous experiments (discussed in Background) have characterized several HA domains important for function in a variety of influenza strains, little is known about H5N1 type HA. During this project, a series of point mutations in the H5 HA gene were tested for their infectivity compared to the wild type H5 HA to identify domains important for HA function.

Table 1 shows a list of all the mutants tested in this project in numerical order. The first column shows the mutant tested, the second column shows the region of the HA that the mutant is found, and the third column describes the mutant infectivity relative to WT. As shown, 65 of the 70 mutants tested reside in the H5 HA_1 region, while 5 reside in the HA_2 region. Of the 70 mutants tested, 25 showed infectivity levels similar to the WT, 24 showed no infectivity, 18 showed decreased infectivity, and 3 showed increased infectivity. **Figure 5** shows the crystalline, monomeric structure of H5 HA denoting the mapped mutants of interest. All of the mutants in **Table 1** that showed higher, lower, or no infectivity compared to the wild type are mapped on this figure. Not shown are the mutants that had no effect on infectivity. The mutant D199A is shown in green as if it had higher infectivity than the wild type. This mutant did show slightly higher infectivity than the wild type, however, not significantly higher. As shown in the figure, the majority of the mutants showing no infectivity are located within the group specific

pocket and the stem region on the HA monomer. The mutants that showed decreased infectivity are located in the stem as well as in the beta-sheets of the head. The mutants showing increased infectivity are located in the head of the HA, as well as close to the group specific pocket. The mutant K64A is actually located within the group specific pocket.

Table 1: Complete List of Mutants Tested

Mutant	Region of HA	% Infectivity Compared to Wild Type
E30A	HA_1	Similar
M36A	HA_1	no infectivity
E37A	HA1	Lower
K38A	HA1	Lower
H44A	HA1	Similar
D47A	HA1	Lower
I48A	HA1	no infectivity
L49A	HA1	no infectivity
E50A	HA1	Similar
K51A	HA1	Lower
K52A	HA1	Similar
H53A	HA1	Similar
N54A	HA1	no infectivity
G55A	HA1	no infectivity
K56A	HA1	Lower
L57A	HA1	Lower
C58A	HA1	no infectivity
D59A	HA1	Similar
D61A	HA1	Similar
G62A	HA1	Lower
V63A	HA1	Lower
K64A	HA1	Higher
P65A	HA1	no infectivity
L66A	HA1	Lower
I67A	HA1	Lower
L68A	HA1	no infectivity
D84A	HA1	Similar
E85A	HA1	Similar
E115A	HA1	Similar

E115S	HA1	no infectivity
R123A	HA1	no infectivity
R123E	HA1	no infectivity
H126A	HA1	no infectivity
H126R	HA1	Lower
E128A	HA1	Lower
K129A	HA1	Similar
K135A	HA1	Lower
W138A	HA1	Lower
E142A	HA1	Similar
K156A	HA1	Similar
F159A	HA1	Lower
F160A	HA1	Lower
R161A	HA1	Lower
K168A	HA1	Similar
K169A	HA1	Similar
Y173A	HA1	Similar
N182A	HA1	no infectivity
E186A	HA1	Lower
D187A	HA1	no infectivity
D199A	HA1	Similar
E202D	HA1	no infectivity
K205A	HA1	Similar
Q223E	HA1	Similar
Q223L	HA1	Similar
K234A	HA1	Higher
K250A	HA1	Similar
D253A	HA1	no infectivity
D253E	HA1	Similar
D253K	HA1	no infectivity
E284A	HA1	Similar
E286A	HA1	Similar
K293A	HA1	no infectivity
E317A	HA1	no infectivity
K320A	HA1	no infectivity
R326A	HA1	Similar
R421A	HA2	Higher
R421G	HA2	No infectivity
L426A	HA2	no infectivity
F434K	HA2	no infectivity
F456A	HA2	no infectivity

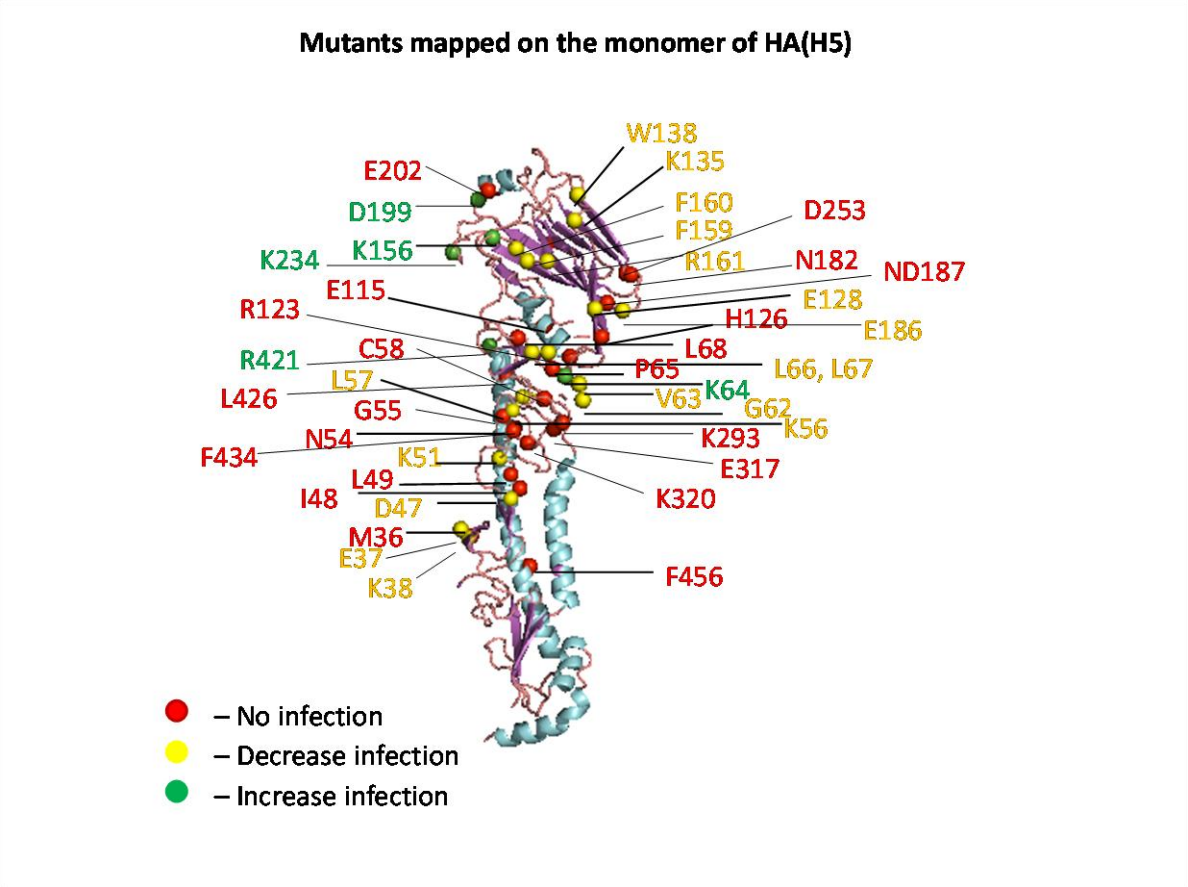

Figure 5: Structure of the H5 HA Monomer Showing the Positions of Mapped Mutants of Interest.

Mutants with Higher Infectivity

Figure 6 shows a summary of all three mutants displaying higher infectivity than WT. Mutants K64A and K234A all reside in the HA1 region, while R421A resides in the HA2 region, near the group specific pocket. The mutant K64A is located in the group specific pocket.

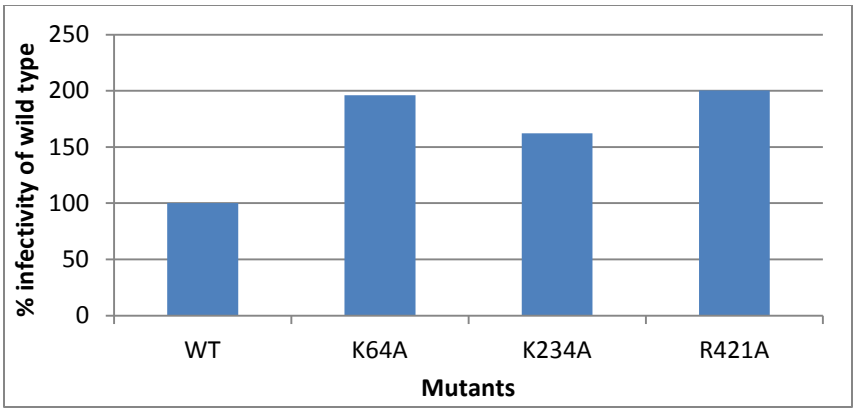

Figure 6: HA Mutations Showing Higher Infectivity Compared to Wild Type.

Mutants with Decreased Infectivity

Figure 7 shows a graph of all the mutants tested that showed infectivity that was less than the wild type.

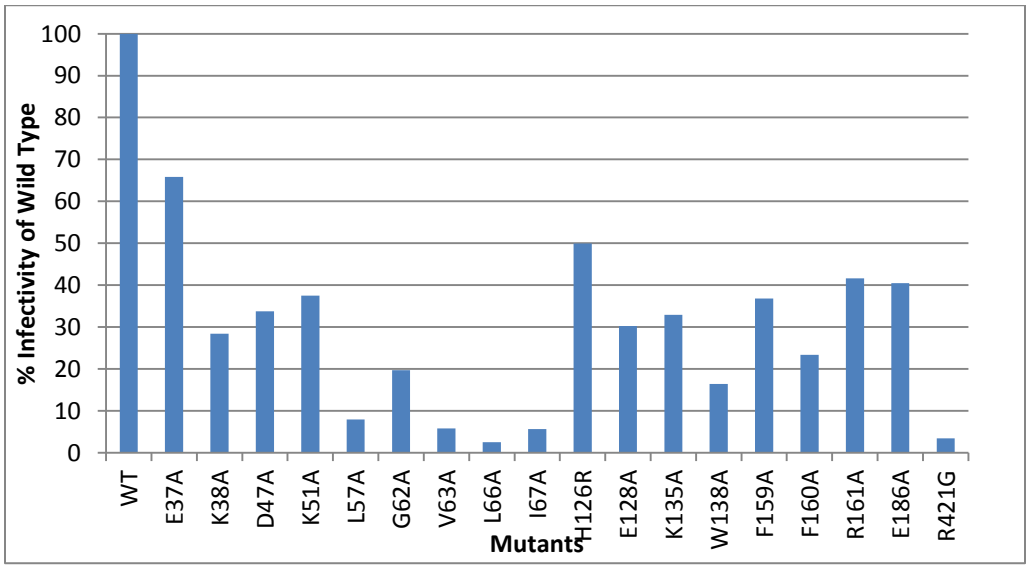

Figure 7: HA Mutations Showing Less Infectivity Compared to Wild Type.

Mutants with no Infectivity

Table 2 shows a chart of the mutations showing no detectable infectivity compared to the wild type. In these cases, the single amino acid mutation shown resulted in pseudotype virus that was no longer infectious to permissive cells.

Table 2: Mutants Showing No Infectivity Relative to Wild Type.

Mutant	% Infectivity of Wild Type
WT	100
M36A	0.00
I48A	0.09
L49A	0.00
N54A	0.03
G55A	0.01
C58A	0.00
P65A	0.68
L68A	0.00
E115S	0.47
R123A	0.00
R123E	0.16
H126A	0.04
N182A	0.00
D187A	0.01
E202D	0.21
D253A	0.01
D253K	0.05
K293A	0.16
E317A	0.02
K320A	0.05
R421G	0.10
L426A	0.00
F434K	0.08
F456A	0.00

Group Specific Pocket Region

Figure 8 shows the infectivity for a subset of the mutants tested in the group specific pocket region of HA at amino acids 50-70. This domain is important because it differs depending

on the influenza type, and divides HA into two separate groups. Of the 17 mutants tested in this domain, 6 showed decreased infectivity, 9 showed no infectivity, 1 showed infectivity equal to WT, and 1 (K64A) showed higher infectivity.

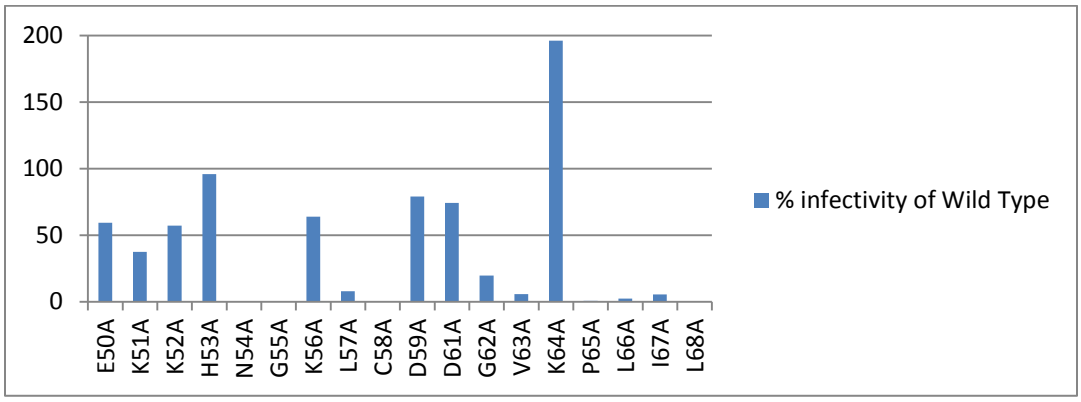

Figure 8: Summary of Infectivity for H5 HA Domain 50-70.

DISCUSSION

One of the main reasons strain H5N1 was studied throughout this project is because it is a highly pathogenic avian influenza strain. Although this influenza strain is rarely transmitted to humans, it is feared that, if this occurs, a worldwide pandemic will result. This study was performed to identify amino acids that are important for HA (H5) function. HA is an envelope glycoprotein present on the surface of the influenza virus. HA plays an essential role in infection of target host cells by recognition and binding of the virus to the host cell receptors. The pseudotype influenza strain H5N1 was used throughout this project. Little is known about H5N1 HA except that the key residues are likely different than previously characterized strains (Su et al., 2008). In this project, a series of 70 point mutations in the H5 HA protein were tested in pseudotype viruses against permissive A549 cells, a human lung cancer cell line. The infectivity of these mutants was compared to the infectivity of the wild type H5N1 pseudotype virus in order to identify domains important for H5 HA function. An important region in the HA gene resides between amino acids 50-70 and is called a group specific pocket. This domain differs between influenza types and divides HA into two separate groups (group 1 includes H1 and H5, while group 2 includes H3 and H7).

The results show that of the 70 HA mutants tested, 25 showed infectivity levels similar to the WT, 24 showed no infectivity, 18 showed decreased infectivity, and 3 showed increased infectivity. The fact that a large number of mutants showed either no infectivity, lower infectivity, or higher infectivity implies those residues are important for HA function and viral infection. The crystal structure of monomeric H5 HA displays a map of all the mutants that resulted in higher, lower, and no infectivity compared to the wild type (**Figure 5)**. As shown in the figure, most of the mutants that resulted in no infectivity are located in the stem region of the

structure. The mutants showing decreased infectivity are also located mostly in the stem region, as well as in the beta-sheets of the head region. Since mutants in these regions result in no infectivity or a decrease in infectivity, they are essential for HA function and viral infection. Mutants that resulted in increased infectivity are mostly located in the head region of HA (D199A, K156A, and K234A). Although the mutant D199A does not show a significant increase in infection compared to the wild type, it is still located in the head region. The other mutants that showed increased infectivity are located near (R421A) and within (K64A) the group specific pocket. These results indicate that mutants in the head and group specific pocket regions are both important for infectivity and are potential areas for mutations that could result in pandemics, since they enhance infectivity in human cells.

The group specific pocket differs in the region 50-70. Based on the 3D crystal structure of HA, it appears to be extremely important because the majority of mutants tested from this region resulted in no infectivity or decreased infectivity. The only mutant in this domain that resulted in higher infectivity compared to the wild type is K64A. This amino acid is usually a polar lysine, however, it was mutated to be a non-polar alanine. Since the switch from a lysine to an alanine resulted in an increase in infectivity, this shows that the non-polar amino acid actually aids in infection. Only five of the mutants tested in this region (E50A, K52A, H53A, D59A, and D61A) had no significant impact on infectivity. From these results, it is suggested that antivirals made to target the group specific pocket would be successful because it is an important region for infection.

Jean-Etienne Morlighem and her team previously tested mutation A156T in the H1N1 influenza strain, collected in Japan during the 2009 pandemic and resulted in severe cases of infection. Her results showed that this mutation is located in the Ca antigenic site, rather than the

receptor-binding site (Morlighem et al., 2011). In this MQP study, the mutation K156A in the H5N1 influenza strain was tested. Unlike Morlighem's results, this mutant showed infectivity that was about equal to that of the wild type. Morlighem's results showed increased infectivity. The discrepancy in results could be due to the fact that the influenza strains are different.

Overall, this research project went smoothly. One small problem was encountered during mutant sequencing. Some of the mutants did not sequence properly and could not be read, while other mutants contained the expected mutation but also contained an additional mutation. These mutants were not used in infection experiments. Amino acids 50-70 were expected to be important in HA function based on HA analyses in other strains, and we hoped to be able to test all of these mutants, but three of the mutant clones within this region were not able to be made due to errors with the PCR primers; so unfortunately mutants were not tested for amino acids 60, 69, or 70.

In the future, amino acids 60, 69, and 70 should also be tested to create a complete picture of this region and to make more definitive conclusions. Due to time constraints, not all amino acids in the HA protein were mutated. The remaining mutants should be made and tested for their infectivity compared to the wild type H5 HA and to other HA strains. Most of the mutants that were created in this project were mutated to an alanine, which is a hydrophilic, neutral amino acid. It would be interesting to also make mutations that are hydrophobic or charged to see how their infectivity compares.

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