COMBATING BIOTERRORISM

Student Author

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Abstract

Outbreaks of the Venezuelan Equine Encephalitis Virus (VEEV) and the Chikungunya Virus (CHIKV) continue to emerge in Central and South America, Africa, and Asia, but there are currently no vaccines or anti-virals for these viruses. Given their ease of transmission, debilitating symptoms, and genetic alterability, VEEV and CHIKV have great potential for development into biological weapons. Therefore, there is an urgent need to determine possible methods of treatment or prevention. One possibility of prevention lies in determining the structure and biological function of the E3 protein that plays a key role in the infectivity of the viruses. In this research, heavy nitrogen (N^{15}) labeling and nickel-affinity purification were used to purify E3 from VEEV. The labeled E3 was used in nuclear magnetic resonance (NMR) to determine secondary structure, but protein aggregation resulted in poor signal. Although various purification techniques, including cobalt-affinity purification, were used to obtain small amounts of E3 protein from CHIKV, further work is needed in order to obtain enough protein to be used for NMR analysis.

Faculty Mentor

Dr. David Sanders is an associate professor of Biological Sciences at Purdue University. He received his Bachelor of Science degree from Yale College in Molecular Biophysics and Biochemistry. He conducted his Ph.D. research in Biochemistry with Dr. Daniel E. Koshland, Jr., who was then editor of the journal *Science*, at the University of California at Berkeley. Dr. Sanders joined the Markey Center for Structural Biology at Purdue University in 1995, where he is the leader of the Molecular Virology program and also a member of the Cancer Center. He was the discoverer of a biochemical reaction that leads to the entry of cancer-causing retroviruses into cells. His work on the Ebola virus led to his participation in the U.S. Defense Threat Reduction Agency's Biological Weapons Proliferation Prevention Program, a product of the Nunn-Lugar legislation. Dr. Sanders is a recipient of the National Science Foundation CAREER Award and an American Cancer Society Research Scholar.

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COMBATING BIOTERRORISM:

Purification and Structural Analysis of the E3 Proteins of the Venezuelan Equine Encephalitis Virus and the Chikungunya Virus

Tyshia Gwin, Cell, Molecular, and Developmental Biology

Image 1. Surface of the Venezuelan Equine Encephalitis Virus. The spikes seen on the surface (yellow) are responsible for the virus binding to the host cell. A matrix of proteins (blue) surrounds the virus core, which contains the genome needed for virus production inside the host cell (Paredes, Alwell-Warda, Weaver, Chiu, & Watowich, 2001, p. 9534). Reproduced with permission from American Society of Microbiology.

INTRODUCTION

Origin and history

The Venezuelan Equine Encephalitis Virus (VEEV) infects both equines and humans and was first reported in donkeys, mules, and horses in the 1930s in South America. Human cases were first documented in the 1950s when a small outbreak occurred in Colombia (Sanmartin-Barberi, Groot, & Osorno-Mesa, 1954, p. 283). Viral outbreaks were localized to the regions of northern South America until the 1990s when the virus began to spread into Central America, and outbreaks have since been reported as far north as Mexico (Weaver, Ferro, Barrera, Boshell, & Navarro, 2004, p. 146).

The first case of the Chikungunya Virus (CHIKV) was reported in humans in 1953 in Tanzania (Powers, Brault, Tesh, & Weaver, 2000, p. 471). Outbreaks occurred throughout the 1960s and 1980s in several regions of Africa, and it is suggested that the virus spread to Asia as several outbreaks arose concurrently (Powers, et al., 2000, p. 471). In the 2000s, cases have been reported in various other regions including Europe, North America, and Australia (Powers & Logue, 2007, p. 2365). Recently, large epidemics in India have caused concern about the growing rate of incidence (Pialoux, Gauzere, Jauréguiberry, & Strobel, 2007, p. 319).

Although their mortality rates are low, each of these viruses causes health problems including rash, fever, arthritis, nausea, diarrhea, weakness, and fatigue, which often last for weeks (Weaver et al., 2004, p. 151). Because of their ability to incapacitate the infected, as well as

the ease with which they can be genetically altered and transmitted, VEEV and CHIKV could easily be used as biological weapons. In 1959, Soviet scientists developed VEEV as a bioweapon and demonstrated the efficiency of aerosol transmission of the virus (Croddy, 2001, p. 211).

Existing treatments of the infection focus on alleviating symptoms, but additional research could determine possible methods of preventing infectivity. In order for the viruses to infect cells, the E3 protein of the viruses must be separated from the precursor protein known as pE2. The E3 protein acts as a natural inhibitor to virus penetration into cells; however, the biological function and three-dimensional structure of the E3 protein in VEEV and CHIKV is not known. Through determining the structure of the E3 protein, information on function could be obtained, or similar inhibitory molecules could be synthesized to prevent infection.

Infection

Both VEEV and CHIKV are transmitted by mosquitoes, and symptoms begin to appear soon after the onset of infection. Most of the symptoms subside after a few days, but weakness can last for several weeks, and infection with VEEV can progress to encephalitis (Weaver et al., 2004, p. 151).

Alphaviruses

VEEV and CHIKV are alphaviruses, which are members of the *Togaviridae* family of viruses (Jose, Snyder, & Kuhn, 2009, p. 837). The alphaviruses are categorized as Old World or New World viruses. Old World viruses

include Semliki Forest, Sindbis, and Chikungunya, which cause symptoms such as rash, fever, arthritis, and weakness. New World viruses include Western Equine Encephalitis, Eastern Equine Encephalitis, and Venezuelan Equine Encephalitis. These share the same symptoms as the Old World viruses, but can cause encephalitis in the host (Jose, Snyder, & Kuhn, 2009, p. 837).

The viruses in the alphavirus family share many structural similarities. Each alphavirus has an RNA genome that encodes four nonstructural proteins and the capsid, E1, E2, E3, and 6K proteins. The virus core, called the nucleocapsid, contains the genome surrounded by a lattice of capsid proteins (Jose, Snyder, & Kuhn, 2009, p. 841). A lipid bilayer taken from the host cell during virus release surrounds the nucleocapsid (Jose, Snyder, & Kuhn, 2009, p. 838). Inserted into the bilayer are the viral glycoproteins E1 and E2, which form 80 spikes on the virus surface (Paredes et al., 1993, p. 9096).

When the virus infects a cell, a cellular structure called the endosome contains the nucleocapsid. After the virus has entered the host cell, the nonstructural proteins are formed using the information in the viral genome. The nonstructural proteins replicate the genome and aid in the production of the capsid, E1, E2, E3, and 6K proteins. Initially, the E3 and E2 proteins are linked and are called pE2. pE2 and E1 pair up to form heterodimers or pE2/E1 pairs (Jose, Snyder, & Kuhn, 2009, p. 839). The pE2/E1 heterodimers are then moved through the cell to be displayed on the plasma membrane, or cell surface, of the host. At some point during movement, pE2 is separated into the E3 and E2 proteins (Strauss & Strauss, 1994, p. 504). At the plasma membrane, the E1/E2 heterodimers combine in groups of three that protrude from the virus and resemble spikes (Paredes et al., 1998, p. 1538). Then the newly formed nucleocapsid buds from the host cell, taking part of the host's lipid bilayer (plasma membrane) containing the E1/E2 glycoproteins (Jose, Snyder, & Kuhn, 2009, p. 838). The mature virus can then infect another cell. Part of the E1 protein, called the fusion peptide, is responsible for nucleocapsid release from the endosome and occurs as a result of E2 dissociating from E1 in the acidic environment of the endosome. This process releases the nucleocapsid into the cell, and viral replication and protein production begins again.

The E3 protein

Although it is not well studied, the E3 protein appears to act as a natural inhibitor to viral entry and infectivity. The E3 protein, after separation from E2, is a small protein roughly 7 kilodaltons in size (which is equal to approximately 100 quintillionth of a gram). The function of E3 has not been entirely determined, but it is likely that it is needed for stabilization of the E1/E2 heterodimers during movement to the cell surface (Jose, Snyder, & Kuhn,

2009, p. 839). If E3 is removed from E2 prematurely, then the E1/E2 heterodimers will dissociate and the progeny viruses will lack the E1/E2 spikes, preventing them from infecting other cells (Lobigs & Garoff, 1990, p. 1233). The conformation of the pE2/E1 heterodimers protects the fusion peptide of E1 from premature exposure; however, when E3 is separated from E2 the conformation of E2 changes and causes exposure of the fusion peptide (Voss et al., 2010, p. 711). These findings indicate that separation of E3 from E2 is necessary for exposure of the fusion peptide. If pE2 is not separated into E2 and E3, it can still be incorporated into the spikes displayed on the virus surface (Strauss & Strauss, 1994, p. 504). Because the fusion peptide of E1 is not exposed, the infectivity is very low (Strauss & Strauss, 1994, p. 504), resulting in no progeny virus production.

The reemergence of VEEV and CHIKV and the growing rate of incidence have caused great concern. Due to the ease of aerosol transmission, disease duration, and lack of prevention or treatment, the viruses pose a threat for use as biological weapons. If large numbers of a population were infected, the severity of the symptoms could have a dramatic impact on the health and economy of a country. For this reason, the study of the virus' methods of infectivity, particularly in regards to the significance of E3 in infection, is crucial.

The aim of this research is to determine the structure of the E3 protein from VEEV and CHIKV. Knowledge of the E3 structure would also provide insight into the related viruses of the alphavirus genus and could potentially be used to design a structurally similar drug that could prevent infection of the viruses in the same manner as E3. Additionally, the function and fate of E3 after cleavage from E2 is unclear, but structure can sometimes provide valuable information regarding the function of a protein. In order to learn about the structure and function of the E3 protein, protein purification techniques including nickel-affinity purification, cobalt-affinity purification, and anion-exchange purification were used to isolate large amounts of purified E3 to be used in the structural analysis via nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Expression of the E3 protein

In order to obtain the E3 proteins from VEEV and CHIKV to do structural studies, the first task was to use a system to produce large amounts of the protein. Because of the hazards and difficulties in obtaining the protein from the viruses, a system was designed to produce the protein in *E.coli*. Because of the internal environment of the *E.coli* cells, the E3 protein could not be produced

without a protein that would add stabilization. To accomplish this, the DNA sequence that encodes the E3 protein as well as the sequence that encodes thioredoxin, a stabilizing protein, were linked and put into *E.coli* cells. Contained within the thioredoxin (Trx) sequence is a sequence for a his-tag (6XHis), which aids in the purification of proteins from cells. The DNA sequence was put into *E.coli* cells, and the cells were forced to produce, or express, the protein. Large amounts of the recombinant protein (see Figure 1), which also contains a recognition sequence for TEV protease, were expressed in *E.coli*.

Purification of the E3 protein

Because *E.coli* normally expresses many different proteins, the recombinant protein needed to be separated from the other proteins (see Figure 2). To accomplish this, the recombinant protein was isolated from *E.coli* by passing it through a nickel column. This process is called nickel-affinity purification. After the *E.coli* cells expressed large amounts of the recombinant protein, they were broken open, or lysed. The contents, which contained normal *E.coli* proteins, the recombinant protein, and DNA and RNA, were applied to a column containing nickel. In order to separate the thioredoxin protein (Trx) from the E3 protein, TEV protease was added to the protein, which cuts the linker region between Trx and E3 protein. After cleavage with TEV, the proteins were added onto the nickel column again, but because the E3 protein then lacked the 6XHis tag, it was able to pass through the column and be collected.

RESULTS AND DISCUSSION

Expression and purification of the recombinant protein from VEEV

To produce large amounts of the recombinant protein containing E3 from VEEV, referred to as VEEV_{Trx-6XHis-E3}, the DNA sequence for VEEV_{Trx-6XHis-E3} was put into *E.coli* cells, and the cells were forced to express VEEV_{Trx-6XHis-E3} for two hours at 37°C. After expression, the cells were lysed, and the contents, called the lysate, were applied to a nickel column. After removal from the column, the purified VEEV_{Trx-6XHis-E3} was incubated with TEV protease for 16 hours at room temperature. The mixture of proteins was then applied to a nickel column, and the E3 protein (referred to as VEEV_{E3}) was collected and subsequently purified on an anion-exchange column (see Figure 3).

Expression, purification, and structural analysis of heavy nitrogen labeled VEEV_{E3}

Since large amounts of pure VEEV_{E3} were obtained, structural work could be started. In order to use NMR to determine the structure, the VEEV_{E3} had to be heavy

Recombinant Protein

Figure 1. Diagram of the recombinant protein. The recombinant protein contains thioredoxin protein (Trx) for stabilization of E3 in *E.coli*, a 6XHis tag for purification, a TEV cut site, or cleavage site, for separation from Trx and 6XHis, and the E3 protein.

Figure 2. Diagram of nickel-affinity purification.

Left: The contents of the *E.coli* cells, called the lysate, which include DNA, RNA, normal *E.coli* proteins, and the recombinant protein, are added to a column that contains nickel. The 6XHis of the recombinant protein binds tightly to the nickel and remains stuck inside the column while the other cell components lack 6XHis and can pass through and are discarded. The recombinant protein is then removed from the column.

Middle: The E3 protein needs to be separated from the thioredoxin protein (Trx) and 6XHis in order to do structural analysis. TEV protease is added to the recombinant protein, which cuts, or cleaves, the protein into Trx-6XHis and E3.

Right: The reaction mixture of TEV protease, Trx-6XHis, and E3 is added to a nickel column. TEV protease also contains 6XHis and will stick to the column along with Trx-6XHis, while the E3 protein does not and will pass through the column and can be collected. The now purified E3 can be used for structural analysis.

nitrogen (N^{15}) labeled. In order to label the protein, it was expressed in a minimal media supplemented with N^{15} , so that all of the amino acids of the recombinant protein would be labeled. The labeled recombinant protein, referred to as $VEEV_{Trx-6XHis-E3-N^{15}}$, was expressed in minimal media supplemented with N^{15} for two hours at $37^{\circ}C$, then for an additional 20 hours at $22^{\circ}C$. The $VEEV_{Trx-6XHis-E3-N^{15}}$ was then purified through nickel-affinity purification. The purified $VEEV_{E3-N^{15}}$ was used to perform a HSQC, which determines secondary protein structure. The measurements were made at $4^{\circ}C$ (see Figure 4).

In the figure, the signal is tightly clumped. This indicates that $VEEV_{E3-N^{15}}$ has aggregated, and as a result no information about the secondary structure could be determined. Investigation into different solution conditions (buffers) and additives is needed to prevent aggregation, so that a better HSQC can be obtained.

Expression and nickel-affinity purification of the recombinant protein from CHIKV

After the success with the expression and purification of $VEEV_{E3}$, the same techniques were applied to the purification of $CHIKV_{E3}$. $CHIKV_{Trx-6XHis-E3}$ was expressed at

$37^{\circ}C$ for four hours and was purified using nickel-affinity purification. $CHIKV_{Trx-6XHis-E3}$ was cleaved with TEV for 16 hours at room temperature and applied to a nickel column and $CHIKV_{E3}$ was collected (see Figure 5).

The expression of $CHIKV_{Trx-6XHis-E3}$ yielded similar amounts of protein as the $VEEV_{Trx-6XHis-E3}$ expression, and the nickel-affinity purification produced large amounts of relatively pure $CHIKV_{Trx-6XHis-E3}$. Unfortunately, after TEV cleavage and subsequent purification, the yield of $CHIKV_{E3}$ was very low.

There are several possibilities as to why low yields of $CHIKV_{E3}$ were obtained. The possibilities of low concentration and purity of TEV and $CHIKV_{Trx-6XHis-E3}$, as well as buffer conditions for the reaction, were tested and resulted in little improvement in the efficiency of the reaction. This led to the conclusion that that $CHIKV_{E3}$ could be sticking to the sides of the tube used in the cleavage reaction. To test this idea, $CHIKV_{Trx-6XHis-E3}$ was purified through cobalt-affinity purification, which is similar to nickel-affinity purification, except cobalt is used in the column instead of nickel. This improved the purity of $CHIKV_{Trx-6XHis-E3}$, and the TEV cleavage reaction was set up using fresh TEV. After the reactions were completed, the mixture was transferred into fresh tubes, and samples of the material from the sides of the old tubes were made (see Figure 6).

Interestingly, the material from the sides of the tube contain either $CHIKV_{Trx-6XHis-E3}$, TEV, or both, and possibly $CHIKV_{E3}$. This could explain why there has been poor cleavage efficiency and lack of $CHIKV_{E3}$. If the TEV, $CHIKV_{Trx-6XHis-E3}$, or both are sticking to the sides, they have less opportunity to contact each other for cleavage. The $CHIKV_{E3}$ would not be recovered because only the liquid mixture is applied to the nickel column. It is likely

Figure 3. Purification of $VEEV_{E3}$. The expression of $VEEV_{Trx-6XHis-E3}$ at zero hours (lane 1) and two hours (lane 2) at $37^{\circ}C$. The cell lysate containing $VEEV_{Trx-6XHis-E3}$ (lane 3) was purified through nickel-affinity purification (lane 4), cleaved with TEV protease, and $VEEV_{E3}$ was then purified by nickel-affinity purification and anion-exchange purification (lane 5).

Figure 4. HSQC of $VEEV_{E3-N^{15}}$ at $4^{\circ}C$.

Figure 5. Purification of $CHIKV_{E3}$. The expression of $CHIKV_{Trx-6XHis-E3}$ at zero hours (lane 1) and four hours (lane 2) at $37^{\circ}C$. The cell lysate containing $CHIKV_{Trx-6XHis-E3}$ (lane 3) was purified through nickel-affinity purification (lane 4), cleaved with TEV protease, and $CHIKV_{E3}$ was then purified by nickel-affinity purification (lane 5).

Figure 6. TEV cleavage of CHIKV_{Trx-6XHis-E3} after cobalt purification. CHIKV_{Trx-6XHis-E3} was cleaved using TEV (lane 1). The reaction at four hours (lane 2), at eight hours (lane 3), after 22 hours (lane 4), and material stuck to the sides of the tube (lanes 5 and 6) are shown.

that after cleavage CHIKV_{E3} becomes insoluble and sticks to the sides of the tube. Investigation into different buffers and different reaction tubes is needed to avoid this problem.

Conclusion

The optimal levels of VEEV_{Trx-6XHis-E3} expression at 37°C were reached within two hours, and large amounts of VEEV_{E3} were obtained from nickel-affinity purification and anion-exchange purification. Expression in minimal media of N¹⁵ labeled VEEV_{Trx-6XHis-E3} was successful, but HSQC to determine the secondary structure was not as a result of protein aggregation. More work is needed to identify buffer conditions that will prevent aggregation.

The optimal levels of CHIKV_{Trx-6XHis-E3} expression at 37°C were reached within four hours. However, CHIKV_{E3} appears to be sticking to the sides of the reaction tube, so more research is needed to fix the problem and to ensure that pure CHIKV_{E3} can be obtained and used for structural analysis.

With further research, the structure of E3 from the Venezuelan Equine Encephalitis Virus and the Chikungunya Virus can be determined. This information could be used by pharmaceutical companies to design drugs with similar structures that could prevent infection in much the same manner as E3. With a potential treatment, concern about the development of the viruses as biological warfare agents would be diminished, and the quality of health of individuals in geographic areas where the viruses persist would be greatly improved.

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