A NOVEL TECHNIQUE FOR THE EXPRESSION AND

PURIFICATION OF HIV-1 *VIF* CO-FACTOR APOBEC3G

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ABSTRACT

HIV-1 has evolved to protect itself against the body's innate viral defense mechanisms. One such example of this phenomenon is illustrated by the viral protein *vif,* which is expressed in the later stages of infection. Vif binds the host cell protein Apobec3G, a cytidine deaminase which is ectopically expressed in non-permissive cell lines to allow for reverse transcription of the HIV-1 viral genome. Apobec3G has the ability to prevent HIV-1 replication by mutating the cDNA, so the goal of this research was to develop a novel technique for the expression and purification of Apobec3G. An expression and purification technique was developed, and the purified Apobec3G protein was shown to have the capacity for binding viral Vif *in vitro*.

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ACKNOWLEDGEMENTS

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BACKGROUND

Human Immunodeficiency Virus type 1 (HIV-1)

General Background and Discovery

The Human Immunodeficiency Virus Type 1 (HIV-1) is a lentivirus of the family *Retroviridae*. Its infection in humans is characterized by a dramatic reduction in number of CD4 cells, and the developments of certain opportunistic infections, such as Kaposi's sarcoma, and other malignant cancers in its hosts (Levy, 1998). The disease is part of a group of diseases caused by immunosuppressive viruses including HIV-2 and SIV (SIV is speculated to be the virus from which HIV-1 evolved from in non-human primates) (Knipe and Howley, 2001). The HIV-1 family of viruses, is also divided into three different groups dubbed M (major), O (outlier) and N (non-M or O); and those groups are also organized into a number of different sub-clades of HIV-1 which are thought to have evolved from recombination between subtypes and mutations resulting in the formation of unique viral strains (*see* Figure 1) (Knipe and Howley, 2001).
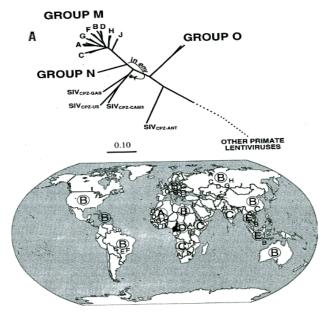

Figure 1: Genetic Subtypes of HIV-1 and Their Worldwide Distribution. (A) Phylogenic relationships of each HIV subgroup. Sequence homology was determined by comparing full-length *pol* sequences (Knipe and Howley, 2001). (B) World distribution of each subtype of HIV-1 (Knipe and Howley, 2001).

The first documented evidence of HIV infection in humans can be traced to an African serum sample collected in 1959, but the first acknowledged human infection with HIV/AIDS was documented in 1983 at the Pasteur Institute (Barre-Sinoussi *et al*, 1983). Subsequent studies recognized the fact that patients with Acquired Immunodeficiency Syndrome (AIDS) had similar preceding symptoms (Levy, 1998), leading scientists to believe that there was an etiologic agent required for the progression of AIDS. Human T-cell leukemia virus (HTLV) and lymphadenopathy-associated virus (LAV) were initially speculated to be this agent, but further investigation concluded it was a new lentivirus (Gallo and Montagnier, 2003) that had so many new characteristics, the International Committee on Taxonomy of Viruses gave it a separate name – Human Immunodeficiency Virus (HIV) (Coffin *et al*, 1986).

HIV-1 Virion

The HIV-1 virion has a cone shaped *core* that contains a genome composed of two identical RNA strands, molecules of viral dependent DNA polymerase (Reverse Transcriptase -- RT) and the nucleocapsid proteins (NC, p9) (Levy, 1998). The core/nucleus is surrounded by capsid protein (CA, p24) and the accessory viral protein R (vpf, p15) (Gelderblom *et al*, 1989). It is this core, or "nucleoid", that is injected into host cells to propagate infection (Knipe and Howley, 2001). The inner core is surrounded by the matrix protein (MA, p17), which provides both the structural and vital integrity of the virion (Gelderblom *et al*, 1989). Other proteins which are proposed to be contained within the viral core include the viral protein vif, on the order of 1 molecule per every 20-30 molecules of p24 (Liu *et al*, 1995), and the accessory protein nef, which is thought to

be present on the order of ~10% of RT incorporation (Welker *et al*, 1996). Their presence in the core suggests that they play an important role in early infection. The core and matrix envelope are surrounded by a lipid membrane studded with the surface glycoprotein gp120 (SU) and the transmembrane glycoprotein gp41 (TM), which act as binding sites for receptors on host cells so as to initiate infection. Figure 2 illustrates the structure the HIV-1 virion.
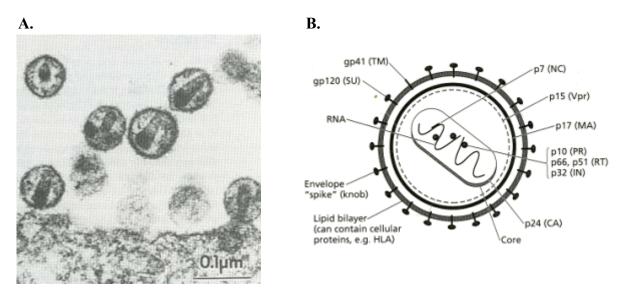
A.

B.

Figure 2: HIV-1virion structure. (A) Scanning micrograph of budding HIV-1 particles on the surface of a T-lymphocyte. Notice the cone shaped core of each virion (Levy, 1998). (B) Schematic of the HIV-1 virion structure. Each component of the infectious virion is listed (Levy, 1998).

HIV-1 Infection

HIV is transmitted either by exposure to the oral, rectal or vaginal mucosa during sex or breast feeding, by intravascular inoculation with infected blood products, or by maternal transmission from mother to child (Jaffe *et al*, 1983). Three primary factors influence established infection of HIV-1 including the characteristics of the infectious agent (virulence and infectiousness), host related factors (susceptibility, contagiousness and immune response) and environmental factors (social, cultural and political) (Levy,

1998). These factors contribute to the uniqueness of each virus between hosts. They also contribute to the classifications HIV based on the extent of infection.

Classification of Infection

HIV infection can be classified on the basis of three distinct characteristics. The replication rate of the virus has been delineated to be either that of the "slow/low" type, which refers to the fact that the virus replicates *slowly* and produces a *low* amount of infectious progeny, or "fast/high" which confers to the exact opposite scenario (Fenyo et *al*, 1988). Slow/low progression can often lead to an infection classified as a long-term non-progressor (LTNP), in which the viral loads of the host seem characteristic of HIV, but the number of viable CD4 T-cells remains fairly constant (Learmont *et al*, 1992). Slow/low infection is also often associated with a Vif deficient virus replicating in non-permissive cells (which will be explained in further detail later in the paper, and relates directly to this MQP).

HIV infection can also be classified based on the formation of syncytium in host cells (Koot *et al*, 1992). Syncytium is the formation of multinucleated giant cells induced by cell:cell fusion (*see* Figure 3) (Levy, 1998). Syncytium inducing viruses (SI) are usually associated with advanced infection, while non-syncytium inducing viruses (NSI) are associated with "slow/low" replicating viruses.
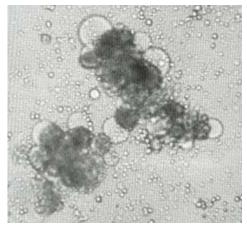
Figure 3: Syncytia formation caused by cell:cell fusion during acute infection of PBMC by HIV-1 (Levy, 1998).

Lastly, the virus can be classified by its stages of infection. Tropism refers to the cell lines in which the virus establishes infection. M-tropic strains of HIV-1, which are mainly present in early infection, are associated with a slow/low rate of replication. They reproduce primarily in monocyte-derived macrophages. T-tropic viruses replicate primarily in CD4 t-cells, and are associated with fast/high replication (Collman *et al*, 1989). T-tropic infection is usually established after long-term incubation of the virus and is associated with the later stages of infection. It accounts for the characteristic reduction in CD4 counts among HIV infected patients.

Viral Entry

Infection by an HIV-1 virion begins with the binding of the surface glycoprotein gp120 to the CD4 receptor of the host cell (Dalgleish *et al*, 1984). Binding of soluble CD4 to gp120 results in the rapid dissociation of gp120 from its complex with the transmembrane glycoprotein gp41 (Moore *et al*, 1990). CD4, however, is not the only cell surface receptor needed for viral entry. Research proved that the β-chemokine

receptor CC-CKR-5 (CCR-5) was the principle co-factor for the entry of m-tropic viruses into cells (Deng *et al*, 1996). Conversely, complementary DNA cloning proved that Fusin (now dubbed CXCR-4) was the co-receptor for t-tropic HIV-1 infection (Reng *et al*, 1996). The dissociation of gp120 allows it to bind a co-receptor, such as CCR-5, which brings the virion closer to the cell surface. Exposure of gp41 allows it to bind a fusion receptor, which permits the fusion of the virion to the cells (Sattentau and Moore, 1991). After the virion has bound to the hosts' receptors, it fuses with cell in a pH independent manner (McClure *et al*, 1988) and injects the core nucleoid into the cytoplasm of the host cell (*see* Figure 4).
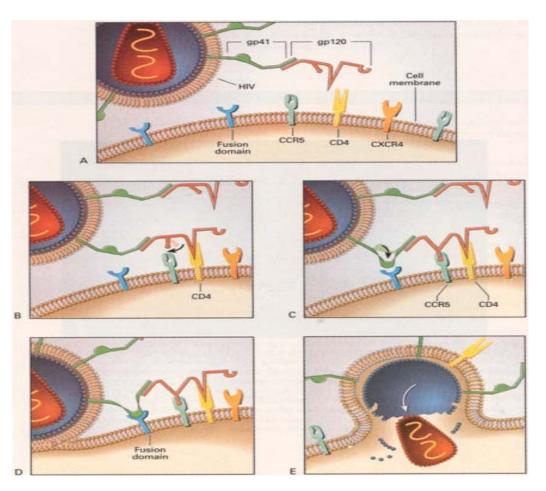

Figure 4: Interactions between the HIV-1 virion and the host cell surface. (A) The binding site on HIV-1 gp120 (red) interacts with the host cells' CD4 receptor (yellow). (B) Dissociation of gp120

allows it to bind a co-receptor, such as CCR-5 (turquoise), and that dissociation results in (C) the exposure of gp41 (green). (D) Exposure of gp41 allows it to bind a fusion receptor (blue) on the cell surface which allows the virion (E) to enter the host cell and propagate infection. (Levy, 1998).

HIV-1 Genome

Once the viral core has been injected into the host cell, the propagation of the virus can begin. The 9.8 kb HIV genome contains open reading frames (ORF's) encoding several viral proteins (*see* Figure 5) (Levy, 1998).
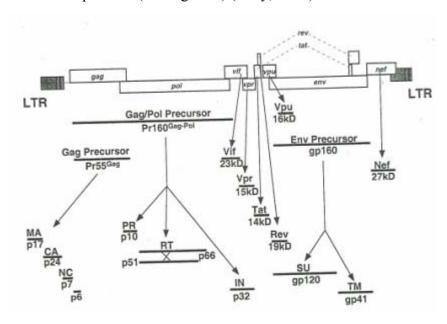

Figure 5: HIV-1 Open Reading Frames. The location of each gene, the relative size of the primary translation products, and the processed mature viral proteins are shown (Knipe, 2001).

Reverse transcription of the viral genome occurs within subviral particles in the cytoplasm of the host cell (Knipe, 2001). The double stranded DNA product is then transported to the nucleus by a nucleoprotein-preintegration complex (PIC) where integration into the chromosomal DNA, mediated by the virus-encoded integrase protein, takes place (*see* Figure 6) (Brown *et al*, 1987). Once the viral DNA has been

incorporated into the host cells DNA, production of viral mRNA begins, followed by the translation of proteins needed to construct infectious progeny. Initially, the full length viral mRNA is translated into the gag and pol precursors, which are proteolytically cleaved into the matrix (MA, p17), capsid (CA, p24) and nucleocapsid proteins (NC, p6 & p9) (from gag precursor), protease (P, p10), reverse transcriptase (RT, p66, p51), and integrase (IN, p32) (from gag-pol polyprotein) (*see* Figure 5) (Gelderblom *et al*, 1989). The envelope precursor protein (gp16) is then cleaved into the surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41) (McCune *et al*, 1988). Other viral regulatory and accessory proteins such as Tat (p14), Tev (p26), Rev (p19), Nef (p27), vif (p23), vpr (p15) and vpu (p16) are not processed until later in infection (Levy, 1998).
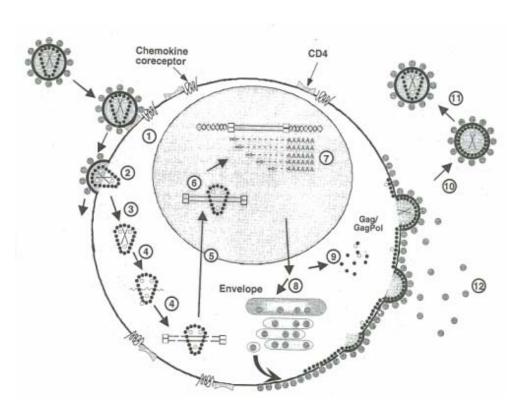

Figure 6: Viral life cycle. (1) Virion binds CD4 and respective fusion receptor and (2) the virion fuses to the host cell in a pH independent manner, releasing its encapsidated genome into the cytoplasm. (3) Partial uncoating of the virions is followed by the (4) reverse transcription of its double stranded

RNA genome in a subviral particle in the cytoplasm. (5) The resulting double stranded DNA is then transported to the nucleus where integration into chromosomal DNA takes place. (6) Viral mRNA's are then transcribed from the chromosomal DNA and (7) translated into the viral proteins. (8) The translated Env, Gag and Pol proteins assemble and (9) are transported to an area near the surface of the cell. (10) Viral progeny particles begin budding off the surface of the host cell. (11) Subsequent proteolysis by the viral protease allows for the release of mature virions. (12) Non-virion associated gp120 is also released from the cells (Knipe and Howley, 2001).

After the production of the viral proteins, newly synthesized genomic RNA is incorporated into the capsid protein, which had been previously translated in the cytoplasm, and the gag-pol proteins are processed near the cell surface (Knipe, 2001). This gives rise to the to production of a new infectious virion which then buds through the host cell membrane and is released to subsequently infect other cells in the same manner (Levy, 1998).

HIV-1 Virion Infectivity Factor (vif)

The HIV-1 accessory gene *vif* encodes a basic protein of 192 amino acids with a molecular weight of approximately 23 kDa (Kan *et al*, 1986). The translated protein is predominantly localized to the cytoplasm, but some presence of the protein has been linked to the nucleus as well, indicating that Vif is involved in some kind of transcriptional mechanism.

The translated Vif protein has the ability to form multimers (Yang *et al*, 2001). The portion of the genome responsible for this action falls between amino acids 154-164, and a deletion in this region dramatically reduced both the ability of the protein to form these multimeric factors and the efficiency of the virus to produce infectious progeny (Yang *et al* 2001). Since this mutation affected the viruses ability to infect host cells, it suggests that Vif's ability to form multimers is essential for its function. This same domain

(ser144), which is adjacent to the proposed oligomerization domain, is phosphorylated, and a deletion mutation of that particular residue also severly reduces Vif's funciton (Yang *et al*, 1996). This suggests that the phosphorylation of Vif is also essential to its function.

Early research showed that the amino acid sequence of Vif was highly conserved among many different HIV-1 isolates, as well as in HIV-2 isolates, and in other lentiviruses (Strebel *et al*, 1987). This sequence conservation lead scientists to believe that Vif played a vital role in the in the viral life cycle of HIV-1. Viruses with a mutated *vif* gene, in most cases, showed rates of infectivity that were ~1000X less than that of the wild type virus. However it was also shown that in some cell lines, the presence of vif is not required for infectivity (Strebel *et al*, 1987).

A. MENRWQVMIVWQVDRMRIRTWKSLVKHHMYVSGKARGWFYRHHYESPHPRISSEVHIPLGDAR
LVITTYWGLHTGERDWHLGQGVSIEWRKKRYSTQVDPELADQLIHLYYFDCFSDSAIRKALLGHI
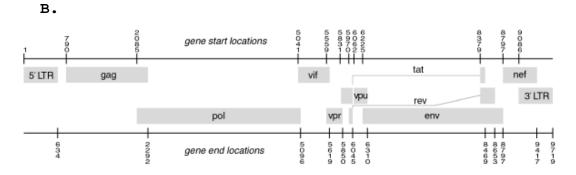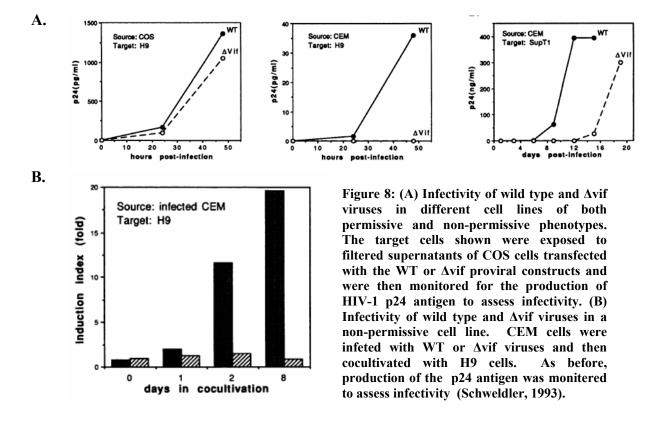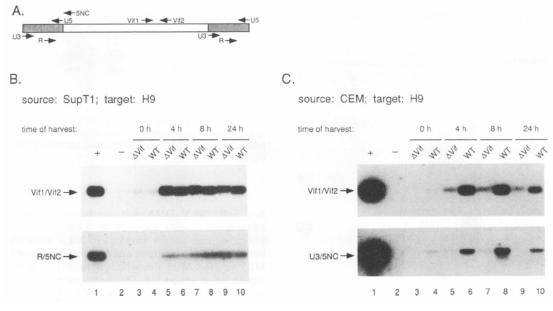VSPRCEYQAGHNKVGSLQYLALAALITPKKIKPPLPSVTKLTEDRWNKPQKTKGHRGSHTMNGH

B.

Figure 7: (A) Amino acid sequence of the HIV-1 accessory protein vif. (B) Organization of open reading frames in HIV-1's genome. (HIV Sequence Database--http://www.hiv.lanl.gov/content/hiv-db/ALIGN_CURRENT/ALIGN-INDEX.html).

With the idea that the expression of Vif is necessary for the infection of host cells, the function of the viral protein was investigated further. PCR analysis using primers specific for viral RNA intermediates and DNA products of reverse transcription proved that Vif is required for the synthesis of proviral DNA (Schweldler *et al*, 1993). Transfection experiments using both WT and Δvif viruses showed that Vif is also required for the production of infectious virions in certain cell lines (Schweldler *et al*, 1993). The latter lead to the discovery of the two primary phenotypes of host cells for HIV-1. Cell lines, such as macrophages, H9 T-lymphatic cells and CEM cells, in which the presence of Vif was crucial to produce infectious progeny were labelled as "non-permissive"; and those cell lines, such as Sup-T1 and Jurkat, which produced infectious virions even in the absence of vif were deemed "permissive" (Schweldler *et al*, 1993). This finding proved that the ΔVif phenotype was cell specific. Heterokaryon experiments also proved that the non-permissive phenoype is dominant over the permissive phenotype; meaning that Vif deficient virions produced in non permissive cells who infect permissive cells create non infectious progeny (Madani and Kabat, 1998). This finding suggests either the presence of a host cell factor in non-permissive cells that counteracts the effects of Vif, or a cell factor in permissive cell lines that functions similarly to Vif in its absence.

Experiments performed by Schweldler *et al* (1993) (*see* Figure 8), proved that when vif was mutated in HIV-1 strains and transfected into non-permissive, human PBL's or macrophages, which are primary cell resivoirs for HIV-1 in vivo, the virions produced were nearly completely defective.

A.

B.

Figure 8: (A) Infectivity of wild type and Δvif viruses in different cell lines of both permissive and non-permissive phenotypes. The target cells shown were exposed to filtered supernatants of COS cells transfected with the WT or Δvif proviral constructs and were then monitored for the production of HIV-1 p24 antigen to assess infectivity. (B) Infectivity of wild type and Δvif viruses in a non-permissive cell line. CEM cells were infeted with WT or Δvif viruses and then cocultivated with H9 cells. As before, production of the p24 antigen was monitered to assess infectivity (Schweldler, 1993).

The mechanism by which this phenomenom was carried out was shown to be linked with the reverse transcription of genomic RNA into proviral DNA. As stated previously, PCR analysis proved that proviral DNA synthesis was impaired in Δvif virions (Schweldler *et al*, 1993). Further investigation (*see* Figure 9) showed that the amounts of reverse transcriptase and genomic RNA were relatively the same for both wild type and Δvif viruses, but the production of proviral DNA from HIV-1 genomic RNA, in non-permissive cells, was significantly reduced in Δvif viruses (Schweldler *et al*, 1993). This proposed the question, is the viral DNA being degraded during or after synthesis, or is it even being synthesized at all?
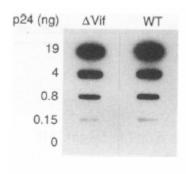
Figure 9: PCR analysis of proviral DNA synthesis and analysis of RNA content. (A) HIV-1 specific PCR primers used to amplify proviral DNA. (B) Time course analysis of viral DNA synthesis in non-permissive H9 cells after exposure to WT and Δvif viruses grown in permissive SupT1 cells. (C) Time course analysis of viral DNA synthesis in non-permissive H9 cells after exposure to WT and Δvif viruses grown in non-permissive CEM cells. (D) RNA content of WT and Δvif virions. RNA was extracted fom both WT and Δvif virions and then normalized for p24 antigen content. (Schweldler *et al*, 1993)

Next, the mechanism by which Vif might possibly synthesize or protect nascent proviral DNA was investigated. Research showed that a defective virus can enter cells and begin reverse transcription during primary infection, but the resulting viral DNA is prematurely degraded, redering it unable to cause further rounds of infection. This defectiveness of the vif mutant must, therefore, be dependent on the cellular source of the virus (Madani and Kabat, 1998). With that in mind, that same group investigated the permissive and non-permissive cell phenotypes. The fact that vif deficient viruses

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produced in permissive cells still had the ability to produce infectious virions, while those produced in non-permissive cell lines did not, suggested that there was a cellular co-factor expressed in non-permissive cells that was overcome by vif in non-permissive cells. This cellular cofactor was discovered via subtractive cloning techniques to be CEM15 (now known as apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G or APOBEC3G) (Sheehy *et al*, 2002). The presence of this protein in non-permissive cells, but its absence in permissive lines, proved that there was a cellular inhibitor which is overcome by vif in non-permissive cell lines (Sheehy *et al*, 2002).

In non-permissive cells expressing Apobec3G, WT viruses (expressing vif) replicate normally (*see* Figure 10) while vif deficient viruses show impaired replication. Conversely, in permissive cells transgenically expressing Apobec3G, both Δvif and wild type viruses are able to replicate normally (Sheehy *et al*, 2002).

A.   **B.**

Figure 10: Expression of CEM15 (APOBEC3G) in permissive and non permissive cell lines. (A) Replication of HIV-1 in permissive and non-permissive cells. CEM (non-permissive) and CEM-SS (permissive) cells were infected with WT or Δviruses, and p24 antigen accumulation was monitored to assess viral replication. (B) Northern blot analysis of the expression of CEM15 in permissive and non-permissive cell lines. RNA's from the listed cells were extracted and resolved by electrophoresis. (Sheehy *et al*, 2002)

Ectopic expression of genes from non-permissive cells in permissive cell lines, an event that occurs when a virion produced in a non-permissive cell infects a permissive cell, converts them to a nonpermissive phenotype, further supporting the fact that the non-permissive phenotype is dominant; and Apobec3G can be incorporated into virions, which may account for the ability of the protein to change the phenotype of the cell (Sheehy *et al*, 2002).

Sheehy *et al* also noticed that the nucleotide sequence of Apobec3G was similar to that of Apobec1, a cytidine deaminase. This observation proved to be relevant when later research showed that HIV-1 DNA is a target substrate for Apobec3G (Zhang *et al*, 2003). The confirmation that Apobec3G was, in fact, a cytidine deaminase that induced G to A hyper-mutation in the minus strand of proviral DNA, gave insight into the protein's native function. This DNA mutator may play an active role in the viral defense mechanism in host cells that can induce lethal hyper-mutation of the incoming nascent viral reverse transcripts. It is thought that this hyper-mutation accounts for the Δvif phenotype (Zhang *et al*, 2003).

Sheehy *et al* states, "Because DNA deamination takes place after virus entry into target cells, Apobec3G function is dependent on its association with the viral nucleoprotein complexes that synthesize cDNA and must therefore be incorporated into virions as they assemble in infected cells." This group also discovered that packaging of 3G was almost eliminated in WT virions, and that levels of 3G in infected cells was also reduced (but not to the same degree as in the virions) (*see* Figure 11). Protease inhibitor experiments proved that the direct interaction of Vif with Apobec3G in host cells, resulted in the induction of proteosome-mediated degredation.
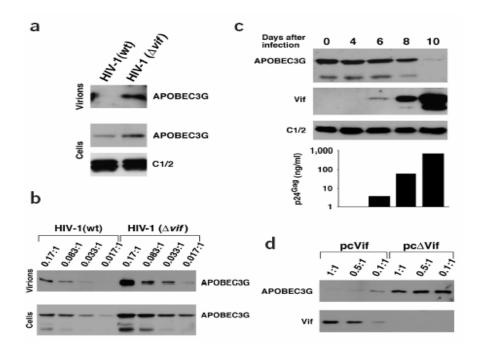
Figure 11: Vif inhibits the packaging of Apobec3G into virions while also effectively binding it in host cells. (a & b) Levels of Apobec3G in host cell lysates as well as virions. 293 T-cells were transfected with varying amounts of plasmids containing Apobec3g and either WT or Δvif viruses. Occlusion of Apobec3G was monitored. (c) Permissive cells' production of vif vs. Apobec3G. CEM-SS (permissive cell line) cells expressing Apobec3G were challenged with WT HIV-1 and the intracellular levels of Apobec3G were monitored. (d) Immunoblot analysis of 293 T-cells transfected with vectors containing WT or Δvif viruses and a constant amount of Apobec3G. The intracellular levels of both vif and Apobec3G were monitored. (Sheehy et al, 2003).

The current theory for the function of vif is that it has the ability to both partially exclude Apobec3G from being packaged into virions and also signal for it's proteosomal degradation through a direct interaction (Sheehy et al, 2003). The binding of Apobec3G by vif in the host cell enables the production of proviral DNA and subsequent infection of other cells. It is for this reason that my research has centered around this topic. The primary reservoir for HIV-1, as stated before, is in non-permissive cells, and by disabling Vif's function, you can almost completely block infection of HIV-1.

PROJECT PURPOSE

It has been speculated that the viral protein *vif* evolved in an effort to protect HIV-1 from the human body's natural viral defense systems. Subsequent research validated this fact and gave insights into the mechanisms by which this feat was accomplished. Now with the knowledge that Vif binds the host cell cytidine deaminase, Apobec3G, in an effort to protect the minus strand of the reverse transcripts from being prematurely degraded, this Apobec3G-induced viral destruction mechanism was selected as a possible target for anti-retroviral therapies. The main goal of my research was to identify a novel technique for the expression and purification of Apobec3G, so as to use it for structural or biochemical studies which could give insights into the mechanisms of HIV-1 and the human body's innate anti-viral defense. Targeting Vif for these experimental therapies could prove to be useful knowing that Vif deficient viruses in non-permissive cells (which are the primary reservoir for HIV-1 in vivo) are unable to produce infectious progeny. Many of the processes in which Vif participates in are still somewhat obscure, and the knowledge gained from this research could prove to be valuable in the identification of targets for experimental therapies.

MATERIALS AND METHODS

Expression of Apobec3G

Apobec3G-encoding plasmid constructs were created with KpnI forward and reverse primers. The cDNA constructs, which were amplified using the polymerase chain reaction (PCR), and the pDuet vectors were then digested with KpnI. The Novagen® pDuet vectors were digested in a total volume of 25 µL as follows: 10 µl pDuet Vector, 2.5 µL Buffer I (NEB buffer), 2.5 µL KpnI, 10 µL sterile water. The PCR products were digested in a total volume of 50 µL as follows: 35 µL 3G PCR product, 5 µL Buffer I, 5 µL KpnI, 5 µL sterile water. Each reaction was incubated in a 37°C water bath for 1 hour and the products were purified using the Qiagen® PCR purification kit. The PCR products were ligated into the pDuet vectors as follows: 13 µL purified Apobec3G cDNA insert, 3 µL digested pDuet vector, 2 µL T4 Buffer, 2 µL T4 Ligase (total volume of 20 µL). The ligation reaction was incubated at room temperature for ~1.5 hours. To transform the ligations into cells for expression, 7 µL of the ligation reaction was added to 100 µL of competent BL-21 (DE3) cells and incubated on ice for 30 minutes (tapping the tube occasionally). The reaction was then heat shocked for 90 seconds at 42°C to "open" the cell walls, and then an incubation at room temperature for ~5 minutes will "close" the cell walls and ensure transformation. 1 mL of LB media was then added to the cells, and the entire reaction was plated on LB plates containing Chloroamphenicol (since the pDuet vector confers chloroamphenicol resistance) and were incubated a 37°C overnight. Once colonies had grown, "over-night" cultures were prepared as follows, 50 mL Terrific Broth (TB) medium, 50 µL of Chloroamphenicol, 1 colony from previously plated cells. Those cultures (10 were made) were then incubated on a shaker overnight at

37°C. After 24 hours, the 50 mL overnight cultures were each added to a liter of TB media with 1 mL of chloroamphenicol. Those 1-liter cultures were incubated at 37°C until the cells were in log phase. To determine when the cells were growing in log phase, the absorbance of each culture was taken, and an OD_{600} reading between 0.6-0.8 indicated log phase growth. Once log phase had been reached, the temperature was re-set to 28°C (which was found to be the optimal temperature) and the cultures were allowed to equilibrate for 30 minutes. Expression of Apobec3G was induced using 300 µM IPTG (which was found to be the ideal concentration of IPTG). After induction, the cultures were allowed to grow for another 3 hours (which was discovered to be the optimal allowed time for growth), and then harvested by spinning at 5000 rpm for 30 minutes. The pellets were then resuspended in 50 mM NaP, 300 mM NaCl, 10 mM Imidazole and transferred to a 50 mL conical tube where they were spun again at 5000 rpm for 20 minutes. The supernatant was then decanted, and the pelleted cells, now expressing Apobec3G, were stored at -80°C.

Lysing of Apobec3G Pellets

 Bacterial cell pellets were thawed and then resuspended in 25 mL of 50 mM NaP, 300 mM NaCl, 10 mM Imidazole, 1 mM DTT, 1 mM PMSF. Cells were then lysed by submitting them to one pass through the cell disruptor, which lyses the cells via intense pressure.

Purification of Apobec3G Using HIS-Select Nickel Affinity Gel

Lysed Apobec3G pellets were incubated with HIS-Select Nickel resin, and rocked on a nutator in the cold, for 45 minutes to 1 hour. With the HIS-tagged proteins now bound

to the nickel beads, the suspension was poured onto a gravity column and the "flowthrough" was collected. The resin was then washed with 10 bed volumes (50 mL) of 50 mM NaP, 300 mM NaCl, 10 mM Imidazole, 1 mM DTT, 1 mM PMSF, and the "wash" sample was also collected. The fusion protein was eluted off of the beads by washing the bed with 50 mM NaP, 300 mM NaCl, 250 mM Imidazole, and this "elution" sample was collected in ~0.5 mL fractions. The beads were then washed again with 50 mM NaP, 300 mM NaCl, 10 mM Imidazole to ensure the viability of the resin.

Purification of Apobec3G Using High Performance Q Sepharose Beads

The purified protein was first diluted with Tris-HCl pH 8.0 in a 1:3 ratio, filtered into a 150 mL "superloop" and then loaded onto a mq10 ion exchange column. The column was first equilibrated with 5 column volumes of 100% 20 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM DTT and then with the same buffer containing 5% as much NaCl salt as originally (all other components of the buffer are exactly the same). The FPLC was then set using the following parameters: flow rate--4.0 mL/min, column pressure limit--4 Mpa, wavelength--280nm, eluate fraction size--2 mL, and with the gradient using a starting concentration of 15% 20 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM DTT and a target concentration of 85% 20 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM DTT. Using the elution profile created by the FPLC, fractions were analyzed via an SDS-PAGE gel to detect the presence of the fusion protein. Those fractions containing the Apobec3G fusion were then pooled and further purified.

Purification of Apobec3G Using S-Protein Agarose

The protein fraction from the FPLC which conferred the greatest presence of the fusion protein on the SDS-PAGE gels was incubated with 0.5 mL of S-Agarose on the nutator in the cold for 45 minutes. The reaction was then spun at 5000 rpm for 10 minutes, and the supernatant was saved as the "flowthrough" fraction. The beads were then resuspended in 2 bed volumes (1 mL) of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, and spun again at 5000 rpm for 10 minutes. The "wash" step was repeated two more times. To elute the protein, the beads were resuspended in 200 mM NaCitrate pH 2 (made in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100), spun for 10 minutes at 5000 rpm, and the supernatant was saved as the eluted protein sample.

Purification of Apobec3G Using a PD-10 Sizing Column

The column is first equilibrated with 25 mL of 20 mM HCl, pH 8.0, 500 mM NaCl. The protein sample (which had already been purified by the previously described methods) was then passed through the resin and bound to the beads. The column was then eluted with 3.5 mL of the same buffer. The PD-10 column also serves to change the buffer of a protein sample in solution.

Co-Immunoprecipitation of Vif and Apobec3G

The initial part of this experiment deals with the binding of Vif and Apobec3G. The two proteins were incubated in the cold on a nutator overnight in a 1:3 ratio (25 μL of purified Vif fusion protein and 50 μL of purified Apobec3G fusion protein --> this was

done twice). Each reaction was then incubated with either anit-Vif or anti-3G antibodies overnight in the same manner. To bind the binding reaction to the protein A agarose, the resin was first equilibrated with 50 mM NaP, 300 mM NaCl, 10 mM Imidazole, and was then incubated overnight in the same manner with the bound fusion proteins containing the given antibodies. To assay the reaction via SDS-PAGE gel and by western blotting, the gel was washed three times with 50 mM NaP, 300 mM NaCl, 10 mM Imidazole, and 50 μL of the same buffer was added to the reaction again. 25 μL of this was run on an SDS-PAGE gel without dye, for western blotting, and 25 μL of loading dye (without reducing agent) was added to the remainder of the reaction to be assayed by SDS-PAGE. The SDS gel and Western blot film were then observed for the presence of the bound Vif-3G fusion protein.

RESULTS

Expression of Apobec3G

The main purpose of this MQP was to identify a novel technique for the expression and purification of soluble Apobec3G for use in structural and biophysical studies. A protocol for the expression and purification of Apobec3G was devised using a NusA-Apobec3G fusion protein. In addition, a potential protocol for the cleavage of the fusion protein was devised, which would leave free purified Apobec3G.

To begin, I first had to identify an ideal environment in which to express the protein. The human Apobec3G cDNA was amplified from another stock plasmid using PCR, and cloned into the Novagen® pDuet plasmid expression vector using KpnI restriction sites. The successful cloning was confirmed via sequencing – data not shown.

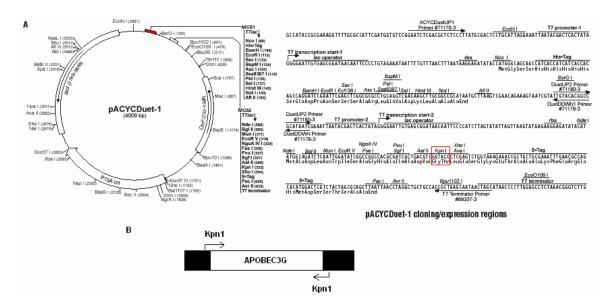**Figure 12: Expression Vector and Primers Used for Expression. (A) Novagen® pDuet vector used for expression. The human Apobec3G cDNA sequence was cloned into the MCS-1 (multiple cloning site 1 – highlighted in red) and then transformed into competent BL-21 cells. (B) Primers used to amplify Apobec3G DNA. Primers were constructed with Kpn1 forward and reverse primers, and Apobec3G DNA was amplified from stock plasmids using PCR.**

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The expression plasmid encoding Apobec3G was then transformed into competent BL-21(DE3) CodonPlus cells as described in the methods section of this report. Chloramphenicol-resistant colonies were then chosen based on their isolated positions on the agar plate, and 12 different isolates were cultured in 250 mL of TB (terrific broth) media. I then selected a range of temperatures (18°C, 28°C & 37°C) in which to grow the cultures, and varying concentrations of isopropyl thiogalactoside (IPTG) (50 µM, 300 µM, 600 µM & 1 mM) in which to induce expression of the protein (Table 1). 5 mL time points were taken before induction, every hour for 3 hours post-induction and after overnight growth.

18°C				28°C			
250 mL culture	250 mL culture	250 mL culture	250 mL culture	250 mL culture	250 mL culture	250 mL culture	250 mL culture
50 µM IPTG	300 µM IPTG	600 µM IPTG	1 mM IPTG	50 µM IPTG	300 µM IPTG	600 µM IPTG	1 mM IPTG
--							
		37°C					
	250 mL culture	250 mL culture	250 mL culture	250 mL culture			
	50 µM IPTG	300 µM IPTG	600 µM IPTG	1 mM IPTG			

Table 1: Illustration of Expression Experiment. Cultures were grown at varying temperatures, and expression was induced with differing molarities of IPTG to find the ideal environment in which to express Apobec3G.

The time point samples were then pelleted and resuspended in 100 µL of Tris – EDTA (TE), pH 7.4 and transferred to 1.5 mL eppendorf tubes. The cells were then lysed via a sonicator, which ruptures cells by utilizing high frequency vibrations. After sonication, the samples were spun down to remove cell debris, and the supernatant (soluble fraction) was analyzed further. Due to a low protein concentration as determined

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by a Bradford assay, the protein samples were then precipitated using TCA to make sure that all proteins present in the solution would be visible on a gel. Loading dye was added to each sample, and they were analyzed via SDS-PAGE gel (*see* Figure 13).

18°C										
MARKER	50 μM Pre	50 μM 1hr	50 μM 2hr	50 μM 3hr	50 μM o/n	300 μM Pre	300 μM 1hr	300 μM 2hr	300 μM 3hr	300 μM o/n

28°C										
50 μM Pre	50 μM 1hr	50 μM 2hr	50 μM 3hr	50 μM o/n	300 μM Pre	300 μM 1hr	300 μM 2hr	300 μM 3hr	300 μM o/n	MARKER

18°C										
600 μM Pre	600 μM 1hr	600 μM 2hr	600 μM 3hr	600 μM o/n	MARKER	1 mM Pre	1 mM 1hr	1 mM 2hr	1 mM 3hr	300 μM o/n

28°C										
600 μM Pre	600 μM 1hr	600 μM 2hr	600 μM 3hr	600 μM o/n	MARKER	1 mM Pre	1 mM 1hr	1 mM 2hr	1 mM 3hr	300 μM o/n

37°C										
MARKER	50 μM Pre	50 μM 1hr	50 μM 2hr	50 μM 3hr	50 μM o/n	300 μM Pre	300 μM 1hr	300 μM 2hr	300 μM 3hr	300 μM o/n

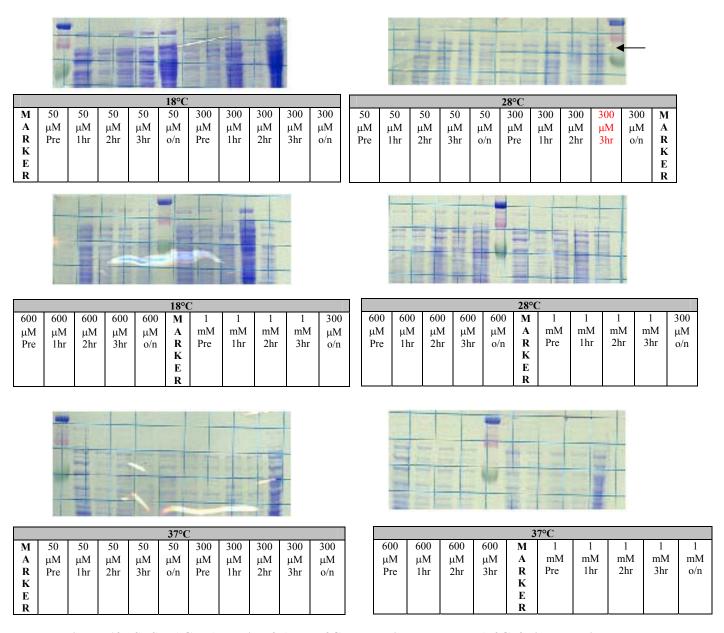| 37°C									
600 μM Pre	600 μM 1hr	600 μM 2hr	600 μM 3hr	MARKER	1 mM Pre	1 mM 1hr	1 mM 2hr	1 mM 3hr	1 mM o/n

Figure 13: SDS PAGE Analysis of Apobec3G Expression. The NusA-3G fusion protein has a molecular weight of 120 kDa (black arrow on the right). The green band on the gel represents a MW of ~80 kDa and the magenta band represents a MW of ~165 kDa. The samples in each lane are indicated in the tables below the gels, as well as the temperature at which the cultures were grown. In the last gel (37°C, 600 μM & 1 mM) the 600 μM overnight samplewas discarded due to a spill. This accounts for the missing lane.

After analyzing the expression, I determined that cultures grown at 28°C for 3

hours and induced with 300 μM IPTG proved to express Apobec3G the most efficiently

relative to other bacterial proteins.   The presence of the fusion protein in the uninduced

lanes are most likely due to a leaky promoter.

To confirm Apobec3G expression, a 1 L culture was expressed using the above

criteria. The cells were then purified over the HIS-Select Ni2+ resin, which binds to the

6XHis tag expressed in the NusA-3G fusion protein. The eluted fractions were then

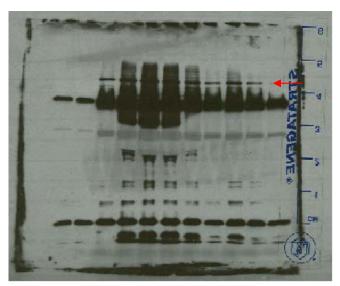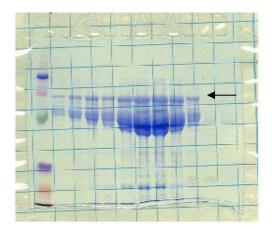assayed via an SDS-PAGE and western blot (*see* Figure 14).

Figure 14: Purification of His-Tagged Protein. Western blot using anti-apobec3g antibodies of expression trial at 28°C inducing with 300 μM IPTG after being purified over the HIS-Select Ni2+ resin. The presence of the NusA-3G fusion protein (delineated by the red arrow) is clearly seen. Each lane is loaded with a fraction eluted off of the HIS-Select Ni2+ column.

As seen in the western blot film, the red arrow delineates the Apobec3G-Fusion

protein. The large band beneath that is the free NusA in the solution. It is illuminated by

the 2° Ab which the membrane was incubated with. Protein purified from non-induced

cultures are not shown.

After confirmation that Apobec3G was being expressed, I could concentrate on finding an ideal method in which to purify and cleave the NusA-3G fusion protein in order to provide free Apobec3G.

Nickel Column Purification of Apobec3G

The purification of Apobec3G required the utilization of different affinity, sizing and ion exchange columns. To begin, I first lysed one pellet from the 10 L culture and purified it over the HIS-Select Nickel resin. The eluted fractions were then collected and analyzed via SDS-PAGE gel (*see* Figure 15).
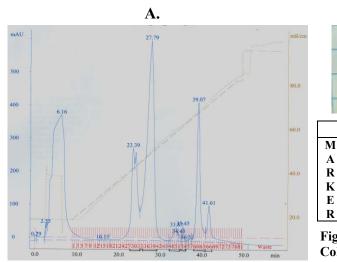

Organization of Lanes											
MARKER	FRAC. 1	FRAC. 3	FRAC. 5	FRAC. 7	FRAC. 9	FRAC. 11	FRAC. 13	FRAC. 15	FRAC. 17	FRAC. 19	FRAC. 21

Figure 15: Analysis of Nickel Column Fractions by SDS-PAGE. The fusion band is delineated by the black arrow. The table displays the organizion of the lanes in the gel.

The band marked by the black arrow represents the Apobec3G-NusA fusion protein. As seen previously, the large band beneath the fusion is free NusA. Protein from non-induced culture is not shown.

Mono Q Column Purification of Apobec3G

After purifying the fusion protein over the nickel resin, I pooled Nickel column fractions numbers 9 through 19, which contained the greatest amount of protein, and passed that sample over the High Performance Q-Sepharose beads using the Fast Performance Liquid Chromatography (FPLC). These beads are anion exchangers, so they bind to proteins with a net negative charge (PI of Apobec3G ~ 5.4). The peak fractions were assayed via SDS-PAGE electrophoresis (*see* Figure 16).

A.	B.

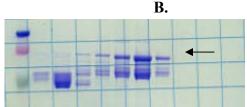| Organization of Lanes											
MARKER	FRAC. 28	FRAC. 30	FRAC. 32	FRAC. 34	FRAC. 36	FRAC. 38	FRAC. 40	FRAC. 50	FRAC. 59	FRAC. 61	FRAC. 66

Figure 16: (A) Elution Profile From the Mono-Q Column. Absorbance of each eluted fraction was taken at 280 nm, and the peaks correspond to the absorbance units in each fraction (B) Analysis of various column fractions by SDS-PAGE. The fusion band is delineated by the black arrow.

S-Tag Purification of Apobec3G

Fractions 32-40 from the mono Q colunm were then pooled and passed over the S-protein Agarose which is specific to the S-Tag that is expressed as part of the NusA-Apobec3G fusion protein. The protein was eluted in one fraction, and 100 µL of that fraction was TCA precipitated and analyzed via SDS-PAGE electrophoresis (*see* Figure 17). This purification left us with a protein that was ~85% pure, so I then focused on cleavage trials to separate Apobec3G from its NusA-Tag.
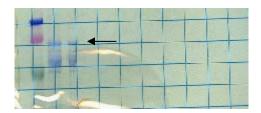
Figure 17: Analytical SDS-PAGE Gel of the Protein Eluted From the S-Protein Agarose Column. The farthest lane to the left is the marker followed by the TCA precipitated eluate, then the non-precipitated fraction. The fusion protein is marked by the black arrow.

Cleavage of Apobec3G

NusA-Apobec3G, which had now been purified over HIS-Select nickel resin, High Performance Q-Sepharose beads and S-Protein Agarose, was digested with the protease Thrombin at 1:25, 1:50 and 1:100 dilutions for 1 hr and 3 hr, respectively. The reactions were stopped using 1 mM PMSF, and the samples were analyzed via SDS-PAGE electrophoresis (*see* Figure 18). The presence of free Apobec3G was confirmed by western blot. This experiment, however has yet to be optimized further. As one can see, from both the SDS-PAGE gel and the western blot, there is still some free NusA as well as some other contaminants that have to be eliminated before the protein can be used for structural or biochemical studies. In addition, the experiment has yet to be optimized for protein yield.
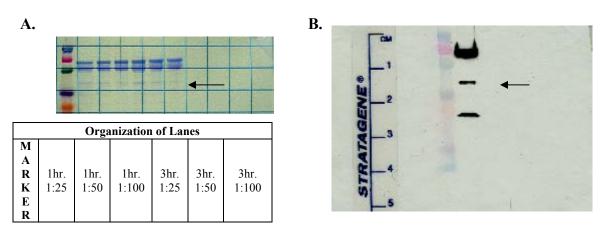
A.

B.

Organization of Lanes						
M A R K E R	1hr. 1:25	1hr. 1:50	1hr. 1:100	3hr. 1:25	3hr. 1:50	3hr. 1:100

Figure 18: (A) SDS-PAGE gel of thrombin digests of purified NusA-Apobec3G fusion protein. Free Apobec3G has an approximate MW of 48 kDa and is delineated by the black arrow on the gel. **(B)** Western blot of best cleavage reaction, which proved to be 1:25 digest for 1 hour. The free protein is shown by the black arrow.

The western blot confirms the presence of free Apobec3G, but, as can be seen from the gel, most of the protein remains in uncleaved form, so the protocol has still yet to be optimized further. I speculate that by purifying the sample once more using a sizing column, most of the unwanted proteins still present in the solution could be eliminated (*see* Table 2). Another pass through the S-Protein Agarose could also eliminate most of the free NusA.
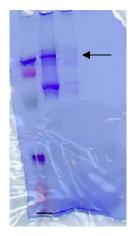
Purification and Cleavage Protocol						
Step 1	*Step 2*	*Step 3*	*Step 4*	*Step 5*	*Step 6*	*Step 7*
Lyse pellet	Purify over HIS-Select Ni2+ Resin	Purify over MonoQ Column using FPLC	Purify over S-Protein Agarose	Cleave using 1:25 Thrombin for 3 hrs.	*Purify over PD-10 sizing column*	*Purify over S-protein agarose*

Table 2: Organization of Protocol for the Purification and Cleavage of the NusA-3G Fusion Protein. Steps in red are yet to be optimized. The research only utilized up to step 5, but steps 6 & 7 are the proposed final procedures.

Co-Immunoprecipitation of Vif and Apobec3G

Lastly, a co-immunoprecipitation experiment was done to prove that the Apobec3G being expressed is capable of binding HIV-1 vif. To prove that Vif binds Apobec3G, and that this expression and purification produce functional Apobec3G, I co-immunoprecipitated the two proteins and analyzed them via SDS-PAGE electrophoresis and western blot (*see* Figure 19). This co-immunoprecipitation experiment suggests that Vif binds the bacterially expressed Apobec3G, indicating that Apobec3G at least retains its ability to bind vif.
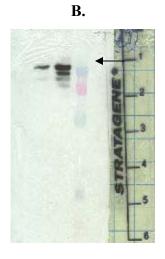
A.   **B.**

Figure 19: (A) SDS-PAGE analysis of Co-Immunoprecipitation of Vif and Apobec 3G. The Vif-Apobec3G dimer has a MW of ~215 kDa. The top blue band of the gel represents a MW of ~206 kDa. The Co-immunoprecipitated protein is delineated by the black arrow. (B) Western blot of co-immunoprecipitation experiment. As before, the black arrow arrow illustrates the Vif-3G dimer.

Organization of Lanes (A)		
M A R K E R	Anti-Vif 1° Ab	Anti-3G 1° Ab

Organization of Lanes (B)		
Anti-3G 1° Ab	Anti-Vif 1° Ab	M A R K E R

DISCUSSION

The HIV-1 viral protein *vif* is a 23-kDa protein transcribed in the later stages of infection (Levy, 1998). It was recently discovered that, once transcribed, Vif binds to the cytidine deaminase Apobec3G, which is readily translated in non-permissive cells (the primary reservoir for HIV-1 in vivo), and allows for the reverse transcription of the viral genome (Sheehy *et al*, 2003). The reason this protein has been selected as a possible target for the creation of experimental therapies is because *vif*-deficient viruses, replicating in non-permissive cells, produce non-infectious viral progeny (Schweldler *et al*, 1993). Therefore, a patient infected with a ΔVif virus will show little or no presence of infectious virions in their body, and the number of viable CD4 T-cells will remain normal. The body's immune system will then not be affected, due to the fact that infectious viral progeny are not being produced.

The purpose of this MQP was to target the Vif-Apobec3G reaction in order to create experimental therapies for HIV-1. From this research, I've proposed a novel technique for the expression and purification of Apobec3G. Ideal conditions for the expression of the protein were determined to use the following criteria: expression was induced using 300 μM IPTG, and the cultures were grown for 3 hours at 28°C. With that protocol, I then concentrated on the purification of Apobec3G. The protein sample was purified using various affinity and ion exchange columns. The developed process was determined to be as follows: lysed pellet passed over the HIS-select Ni_{2+} column; eluted fractions pooled and passed through high performance Q-Sepharose beads; peak fractions pooled and passed over the S-Protein agarose; eluted protein cleaved using 1:25 thrombin for 3 hours at room temperature. This protocol still has to be optimized further. I

speculate that the cleaved protein can be purified further using the S-Protein agarose, which will bind all of the free NusA still present in the solution, and then using a sizing column which should eliminate the rest of the undesirable contaminants. Once a sample of desirable concentration has been purified, the protein can be used for chemical and biophysical studies. I propose to begin crystal trials and binding assays. With these assays, insights into the structure and function of Apobec3G and Vif can be assessed. Investigating binding constant and rates can reveal the binding and active sites, which may serve as targets for possible experimental therapies. This is the goal that this research inevitably strives to reach, and work will continue until definitive results have been achieved.

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