INVESTIGATION OF THE EFFECTS OF SIV_{AGM} TAT PROTEIN ON SLPI GENE EXPRESSION IN PRIMATE CELLS

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF SIV_{AGM} TAT PROTEIN ON SLPI GENE EXPRESSION IN PRIMATE CELLS

Old World monkeys one of which is African Green monkey (AGM) have resistance against to HIV-1. They can be infected by species-specific simian immunodeficiency viruses (SIV), however they do not develop AIDS-like syndrome. There are several restriction factors already known that prevent HIV-1 replication protecting these organisms from infection. However, although Old World monkey cells can be successfully infected with HIV-1 by using high viral loads, the virus eventually become undetectable, indicating the presence of yet unidentified anti HIV-1 factor(s) that confer resistance to these cells. Old world monkeys may have evolved resistance mechanisms by switching specific genes on in response to the synthesis of viral proteins. Since HIV-1 Tat protein is the first viral protein synthesized after viral entry and its presence is required for the synthesis of other HIV-1 proteins, it may also play an important role on the activation of such resistance mechanism. In the previous studies conducted in our laboratory, Secretory Leukocyte Protease Inhibitor (SLPI) which is a 11,7 kDa protein with antiprotease, antimicrobial and antiviral activities was identified as a candidate restriction factor. It was observed to be overexpressed in AGM cell lines in the presence of HIV tat. Although, HIV Tat and SIV Tat have similar functions in the infected cells, they are highly dissimilar in their aminoacid composition ($\approx$40% similarity). Therefore the effects of HIV-1 Tat and SIV Tat on SLPI gene expression might be different. The aim of this study was to investigate the effects of SIV agm Tat on SLPI expression in AGM cells. For this purpose, CV-1 cell lines stably expressing SIV tat gene were established and the expression of SLPI was determined by Q-PCR and Western blotting. A slight decrease in SLPI expression in stable monkey cells that express SIV Tat was observed while HIV Tat expressing stable cells showed SLPI overexpression.

ÖZET

SIV_{AGM} TAT PROTEİNİNİN PRİMAT HÜCRELERİNDEKİ SALGISAL LÖKOSİT PROTEAZ İNHİBİTÖRÜ GENİ İFADELENMESİ ÜZERİNDEKİ ETKİSİNİN İNCELENMESİ

Afrika Yeşil maymunu (AYM) gibi Eski Dünya maymunları HIV-1'e karşı dirençlidirler. Türlerine spesifik simian bağışıklık yetmezlik virüsü (SIV) ile enfekte olabilmelerine rağmen AIDS benzeri bir sendrom geliştirmezler. Bazı restriksiyon faktörlerinin, HIV-1 replikasyonunu engelleyerek bu organizmaları enfeksiyona karşı koruduğu bilinmektedir. Eski Dünya maymunları hücreleri çok yüksek viral yük kullanıldığında, başarılı bir şekilde enfekte edilebilmelerine ragmen, bu hücrelerde virüs sayısının zamanla saptanamayacak seviyelere düşmesi, bu hücrelere dirençlilik sağlayan henüz tanımlanmamış restriksiyon faktörlerinin varlığını göstermektedir. Eski Dünya maymunları viral proteinlerin üretimine karşılık olarak bazı genlerinin ekspresyonlarını değiştirerek bir direnç mekanizması oluşturmuş olabilirler. HIV-1 Tat, virüsün hücre içine girişinden sonra üretilen ilk viral protein olduğundan ve diğer HIV-1 proteinlerinin sentezlenmesi için varlığı gerekli olduğundan, bu maymunların geliştirdiği direnç mekanizmasında önemli bir rol oynaması mümkündür. Laboratuarımızda yapılan ön çalışmalarda, antiproteaz, antimikrobiyel ve antiviral aktivitesi olan Salgısal Lökosit Proteaz İnhibitor (SLPI) proteini olası bir restriksiyon faktörü olarak belirlenmiştir ve AYM hücrelerinde HIV tat varlığında bu proteinin aşırı ifadelendiği gözlemlenmiştir HIV ve SIV tat genlerinin işlevleri benzer olduğu halde, aminoasit dizilimleri çok farklıdır ($\approx\%40$ benzerlik). Bu nedenle HIV-1 Tat ve SIV-Tat proteinlerinin SLPI geni ifadelenmesine etkileri farklı olabilir. Bu çalışma AYM hücrelerinde SIV AYM Tat proteininin SLPI üretimine etkisini incelemeyi amaçlamaktadır. Bu amaçla, SIV tat genini kalıcı olarak ifadeleyen AYM hücrelerinde SLPI ifadelenmesi Q-PCR ve Western blotlama yöntemleriyle incelenmiştir. SIV Tat varlığında SLPI ifadelenmesi belirgin olmayan bir şekilde azalırken, HIV Tat varlığında anlamlı bir artış gözlemlenmiştir.

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CHAPTER 1

INTRODUCTION

1.1. Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV)

Human and Simian immunodeficiency viruses are representatives of the genus lentiviruses in retroviridae family.

Mainly HIV-1 and rarely HIV-2 damage the host immune system and cause AIDS which is characterized by progressive loss of CD4+ T cells leading to immunodeficiency, opportunistic infections such as tuberculosis, fungal infections and Kaposi's sarcoma, neurological infections and problems (Hutchinson, 2001).

CD4+ cells (T-helper cells) signal other immune cells to function, so loss of these cells resulting from destruction with HIV makes the immune system defective leaving the host vulnerable to the opportunistic infections (Klimas et al., 2008).

While HIV causes AIDS in humans, SIV is generally asymptomatic in its natural African host despite showing significant CD4+ T cell loss. It was observed that mucosal CD4+ T cells decrease rapidly and critically in both SIVsmm-infected sooty mangabeys and SIVagm-infected African green monkeys, however after some time cell loss stops or is recovered in sooty mangabeys and agms respectively (Paiardini et al., 2009). HIV-1 and HIV-2 are very similar in genetic structure but they share just 60% of nucleotide homology (Requejo, 2006).

Earliest step of the viral life cycle involves the binding of virus to the host cell through receptors. Several kinds of molecules can be used as receptors and some viruses use one receptor for both binding and fusion, while others need one receptor to bind and one co-receptor to fuse. Being infected by the virus depends on the existence of receptors in the species and cells (Goff, 2004a).

HIV-1 generally needs to interact with CD4 and one of the chemokine co-receptors (CXRC4, CCR5) to enter the target cell (Klimas et al., 2008). While virus mostly interacts

with CCR5 co-receptor, some variants evolve through AIDS progression to use CXCR4, sometimes together with CCR5 generally in late stages (Requejo, 2006), (Vicenzi and Poli, 2013).

1.1.1. Transmission

There are several ways of transmission of AIDS mainly by sexual contact, from mother to child, via interaction with contaminated blood. The rate of transmission from mother to infant by breast-feeding, intrauterinal or parturition is about 1% in HIV-2, whereas 42% in HIV-1. Breast-feeding doubles the risk of transmission of disease to offspring (Grant and De Cock, 2001).

1.1.2. Structure

HIV is spheroid in shape and approximately 0.1 μm crosswise. A lipid bilayer envelope carries numerous spikes as shown in Figure 1.1, each containing four gp120 and gp41 molecules that are inserted in the envelope. Under the envelope, matrix proteins enclose the capsid, which is shaped like trimmed cone and composed of p24 proteins that hold RNA of the virus. RNA, viral enzymes, p6 and p7 proteins are located inside the capsid (Hutchinson, 2001), (Pantophlet and Burton, 2006).

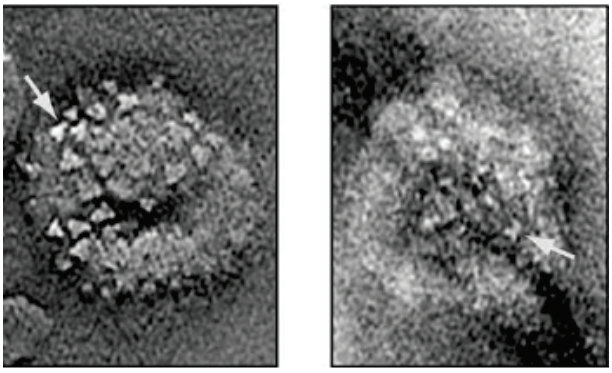

Figure 1.1. 3D topograms of SIV and HIV. White arrows indicate envelope spikes (Source: Pantophlet and Burton, 2006

1.1.3. Evolutionary History of HIV

It has been widely accepted that the natural hosts of HIV-1 and HIV-2 are not humans, indeed zoonotic or cross-species transmission lead to infection of humans with these viruses (Gao et al., 1999).

Based on molecular phylogenetic studies, it was determined that HIV-1 and HIV-2 have evolved from different SIVs; HIV-1 is most closely related to SIVcpz from chimpanzees (Pan triglodytes triglodytes) populating in regions of Africa where HIV-1 groups M (for major), N(for non-M/non-O) and O(for outliner) are seen in a great genetic diversity and have transmitted on at least three different occasions explaining the emergence of three major groups of HIV-1 (Rambaut et al., 2004). HIV-2 evolved from SIVsm from sooty mangabeys (Cercocebus atys) on multiple transmission events yielding HIV-2 subtypes A through H. In some regions of Africa, where HIV-2 is frequently seen, sooty mangabeys have been hunted for food or kept as pets (Hutchinson, 2001), (Locatelli and Peeters, 2012).

It is widely accepted that current HIV-1 strain causing AIDS is 50 to 100 years old (Requejo, 2006). Also a study, which interpreted the date of most recent common ancestor of HIV- 1 group M was predicted to 1930's by using linear regression of genetic divergence against the time of isolation of the viruses, intensifies the previous estimation of age of HIV-1 (Rambaut et al., 2004).

Figure 1.2. Human exposure to monkeys in Central Africa with the photographs (Source: Locatelli and Peeters, 2012)

1.1.4. Genome Structure

All retroviruses have similar genomic structures having the genes, Gag, Pol and Env encode for the enzymes and proteins used in the replication cycle. HIV and SIV are more complex having more genes named as accessory genes ordered as 5'-LTR-gag- pol-vif-vpr-tat-rev-vpu(vpx in the cases of HIV-2 and SIV)-env-nef-LTR-3' (Nielsen et al., 2005). 9749 bp-long HIV-1 genome contains 9 ORFs encoding 15 proteins (Hutchinson, 2001). The accessory genes are vif, vpr, vpu and nef for HIV-1 and vif, vpr, vpx and nef for HIV-2 and SIV (Miller et al., 2000).

Gag and Pol proteins; Vif, Vpr, Vpu, Env and truncated form of Tat proteins; and Rev, Nef and full form of Tat proteins are expressed from unspliced, full-length RNA; partially spliced mRNAs of ≈4 kb; and fully spliced mRNAs of ≈2 kb in size, respectively (Cullen and Malim, 1991).

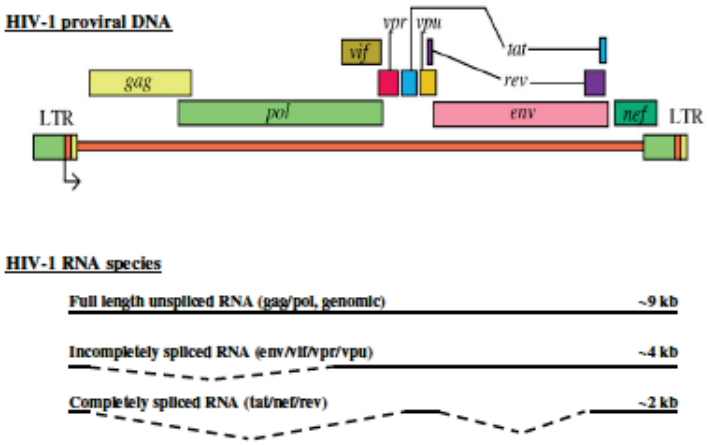

Figure 1.3. HIV-1 open reading frame
(Source: Nielsen et al., 2005)

The virus has 2 identical copies of RNA molecules each having a 5' cap and a 3' poly(A) tail (Goff, 2004a).

Products of Gag cleavage such as nucleocapsid (NC), capsid (CA) and matrix (MA); make a protein core, which is enclosed by lipid bilayer formed by envelope glycoproteins, surrounding the RNAs. Also in this core, viral enzymes, integrase (IN), protease (PR) and reverse transcriptase (RT) produced by proteolysis of Pol are packaged (Miller et al., 2000).

There are 400000 to 2.5 million copies of viral RNA in an infected cell and the virus can use as much as 40% of cellular protein synthesis machinery to produce gag protein and protein synthesis of a virus can reach to a point that it can inhibit or compete with host's (Hutchinson, 2001).

HIV has the highest mutation rate because of either exchange of RNA parts accumulated in the cell cytoplasm or high mutation rate of RT. The high genetic diversity of HIV caused from this high mutation rate gives scientists a hard time to develop treatment. While mutation rate is high in env, gag and pol genes, they are not remarkably changeable. The most conserved protein is CA among viruses (Hutchinson, 2001).

1.1.4.1. LTR

LTR, which flanks provirus, has 4 functional regions as for control of HIV-1 transcription: TAR element, core promoter, enhancer region, and modulatory region. While 5' LTR stimulates transcription by means of promoter regulatory elements located within its U3 region, 3' LTR is necessary to be added polyA tails to viral transcripts. TATAA box and specificity protein (Sp) binding sites are involved within the core region of LTR and crucial for transcription initiation and elongation (Li et al., 2012). The enhancer region has 2 copies of NFκB binding sites and is important for the both HIV activation and proviral latency (Burnett et al., 2009). The modulatory region is composed of binding sites for several proteins depending on cell phenotype, and a number of other factors (Li et al., 2012). Most importantly interaction with Sp1 and TFIID, host transcription factors, is crucial for promoter to function properly (Cullen, 1992).

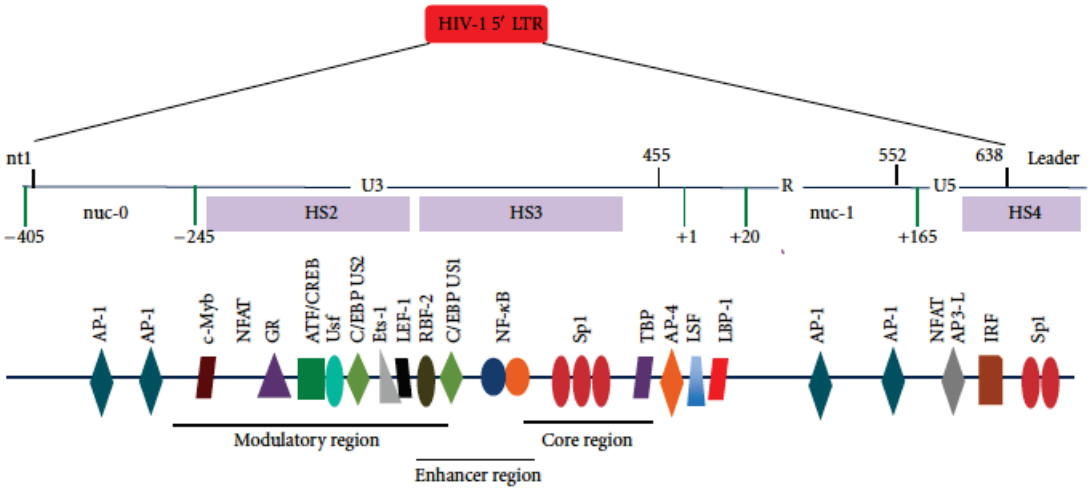

Figure 1.4. HIV-1 LTR
(Source: Li et al., 2012)

1.1.4.2. GAG

It is responsible for the synthesis of core proteins, nucleocapsid (NC), capsid (CA), matrix (MA) and p6. Its presence is crucial for virus to move out of the cell (Hutchinson, 2001).

MA assists the localization of the Gag and Gag-Pol to the plasma membrane prior to viral assemblage. CA constitutes the core of the virion having 2000 copies in one and operates in assembly, especially in viral uncoating. NC coats the viral RNA preventing its cleavage and transfers full-length RNA into the virus. p6 binds to Vpr to integrate it in the time of assembly and aids providing effective virion release (Frankel and Young, 1998).

1.1.4.3. POL

Pol encodes for the viral enzymes, proteases (PR), reverse transcriptases (RT), integrases (IN) and ribonucleases (Hutchinson, 2001). Protease cuts Gag to CA, NC, MA and p6; and Pol to IN, PR and RT to produce mature, infectious virions from immature particles (Frankel and Young, 1998). Reverse transcriptase is a heterodimer contains two subunits; p66 and p51 that originate from Pol, both having a polymerase domain and p66

has a RNase H domain. RT generates the duplex DNA by catalyzing both RNA-dependent and DNA- dependent DNA polymerization prior to integration (Katz and Skalka, 1994). Integrase integrates viral double stranded DNA into the host genome (Frankel and Young, 1998).

1.1.4.4. ENV

Env codes for the envelope proteins, which are surface protein (SU) or gp120 and transmembrane protein (TM) or gp41 (Hutchinson, 2001). SU is placed on the viral membrane and important for the viral entry, because it binds to distinct receptors via its variable V3 loop interacting with the host cell. Its high affinity interaction with CD4 creates a conformational transition in Env easing coreceptors binding and following viral entry. After the SU and receptors interact, TM initiates the fusion of viral and cellular membranes, and it conducts the fusion and anchors Env in the viral membrane (Frankel and Young, 1998).

1.1.4.5. VIF

Vif, virion infectivity factor, is expressed late in the infection (Trkola, 2004). In the absence of Vif, proviruses are not able to integrate into host genome, so it is concluded that Vif affects the late stages of assembly (Miller et al., 2000). Also from the finding that this effect is just seen in some cell types such as primary CD4+ T cells, it is resolved that Vif cancels out the antiviral activity of a negative factor named as APOBEC3G, function of which is discussed later, is expressed by primary cells. Also it may ensure the presence of Gag-derived proteins for the association with other viral proteins by preventing immature cleavage of Gag by proteases (Trkola, 2004).

1.1.4.6. VPR

After uncoating is completed, Vpr transports Reverse Transcription Complex from cytoplasm to nucleus through its nuclear localization signal, which directs Vpr to the interior of the nucleus rather than nuclear complex because of having a different structure than

the common canonical karyophilic NLS that uses importin-dependent pathway (Frankel and Young, 1998).

1.1.4.7. VPU

Vpu maintains transportation of Env glycoproteins, which are sometimes kept in endoplasmic reticulum by interacting with CD4, to the cell surface by destroying CD4 molecules that are in complex with these glycoproteins to help virus budding. Also Vpu stimulates the down regulation of MHC class I proteins thereby protecting infected cell from immune surveillance. Furthermore, it also promotes virus release (Frankel and Young, 1998).

1.1.4.8. NEF

Nef, negative factor for virus replication, is seen around 70 copies per virion and promotes the down regulation of CD4 and MHC class proteins by adjusting endosome trafficking pathway of the cell providing protection from potential immune vigilance, like Vpu (Trkola, 2004). In more detail, it assists the guiding of CD4 molecules to lysosome for degradation (Frankel and Young, 1998).

1.1.4.9. VPX

Vpx is found on HIV-2 and SIV strains. It increases viral replication by neutralizing the activity of host factor SAMHD-1 that typically halters DNA synthesis by driving dNTPs to hydrolysis (Vicenzi and Poli, 2013).

1.1.5. Early and Late Phases of Viral Gene Expression

For simple retroviruses, sequence-specific interactions with host transcription factors are enough to induce generation of viral mRNAs. However these interactions can only activate a basal, low degree of transcription. Tat, that is one of these initial gene products,

enhances its own transcription through its trans-acting activity. As a result of increased transcription, a second regulatory protein, named as Rev, is accumulated leading to activation of unspliced or partially spliced mRNA's expression. So viral gene expression is divided in to two phases named as an early, regulatory phase and a late, structural phase by the production of Tat and Rev (Cullen, 1992).

1.1.5.1. TAT

The ORF of Tat consists of 2 exons code for proteins; 101 amino acids for HIV-1 and 130 amino acids for HIV-2. Tat is expressed in the early stage of infection and activates gene expression from the promoter within the LTR (Miller et al., 2000). When unspliced RNAs are transported to the nucleus by the help of Rev protein, truncated form of Tat, which is encoded by the first exon bordered by a conserved translation stop signal at its 3' end, is produced and able to transactivate the promoter (Hetzer et al., 2005), (Cullen, 1992).

In some lentiviruses, Tat binds to a 3-nucleotide bulge region of a 59-nucleotide RNA stem-loop structure, located soon after downstream of the transcription start site, called TAR (trans-activating region) (Frankel and Young, 1998).

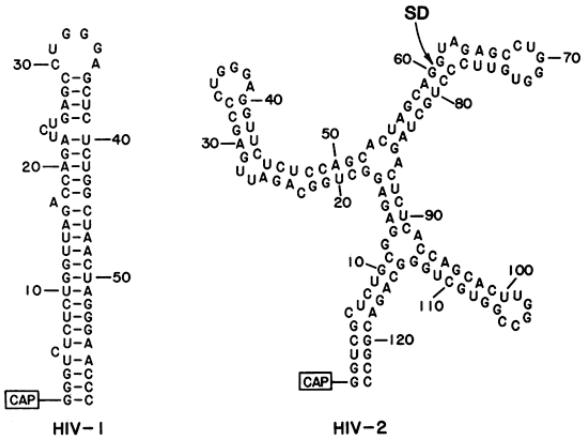

Figure 1.5. Predicted secondary structures of HIV-1 and HIV-2 TARs
(Source: Cullen, 1992)

Tat increases the rate of transcription by the help of two different cofactors. One of them is cyclin-T1 encoded by cell; it enhances the affinity and specificity of the Tat-TAR interaction. The other one is the Tat-associated kinase Cdk9 produced by host; it phosphorylates RNAPII resulting in increased transcription elongation. How hyperphosphorylation of RNAPII stimulates transcription elongation is enlightened recently and demonstrated in the Figure 1.6. DSIF (DRB-sensitivity-inducing factor) and NELF (negative elongation factor) bind to the RNAPII, soon after transcription is initiated, by haltering elongation. However phosphorylation of carboxy terminal domain of the RNAPII by Tat-recruited kinases detaches these negative factors counteracting their inhibitory effects (Nakatani, 2002).

Moreover, Tat induces transcription independently from TAR, as long as enhancer region of LTR, where NFκ B family protein binds, is complete. Normally these proteins are held in cytoplasm bounded with inhibitory IκB proteins. Tat translocates NFκB to nucleus degrading IκB (Marcello et al., 2001).

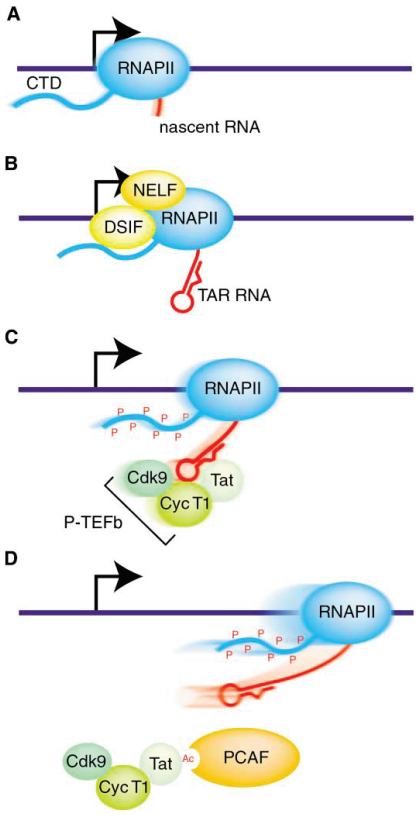

Figure 1.6. Transcriptional elongation by HIV-1 Tat
(Source: Nakatani, 2002)

1.1.5.2. REV

Rev, which is a 116 amino acid protein, is located in nucleus and nucleolus. In its primary structure, the most noteworthy sequences are a highly basic strand involves 10 arginine residues mediates binding of Rev to RRE and Rev multimerization, and cause nuclear accumulation of Rev functioning as nuclear localization signal; and a leucine-rich sequence that serves as an activation domain and nuclear export signal (Cullen and Malim, 1991), (Vicenzi and Poli, 2013). By having nuclear localization signal in N terminal and nuclear export signal in C terminal, Rev is able to shuttle between nucleus and cytoplasm. RRE is a 234 nucleotide RNA stem-loop structure located within the HIV-1 Env coding sequence. Unspliced and partially spliced RNAs have RRE, while fully spliced RNAs do not have (Groom et al., 2009).

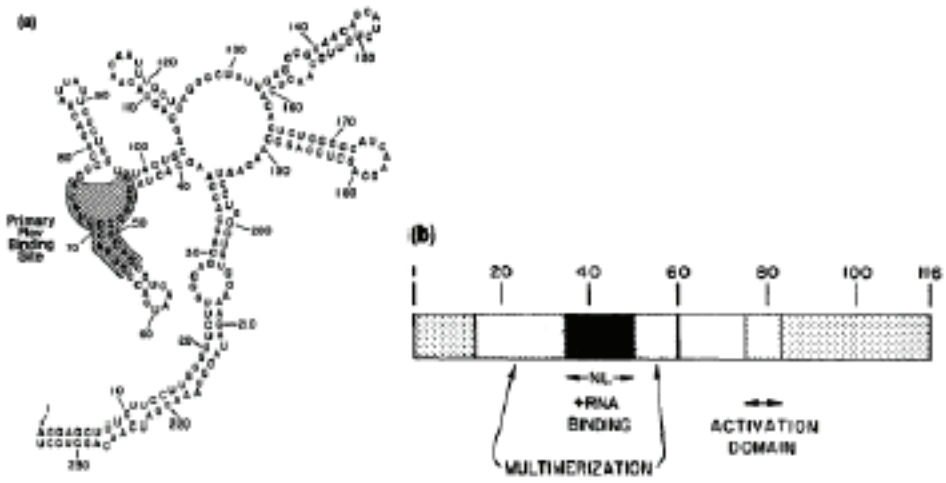

Figure 1.7. Structural Organization of RRE and Rev
(Source: Cullen and Malim, 1991)

Rev is expressed in the early stage of infection and stimulates the transportation of unspliced or partly spliced mRNAs from nucleus to cytoplasm. In eukaryotes, firstly introns have to be spliced in the cell nucleus and then gene products are exported to the nucleus for translation. Intron-containing mRNAs are held in the nucleus, until their splicing is completed. As Tat, Rev and Nef are products of spliced RNA, they are exported after splicing. However export of products of partially spliced or unspliced mRNAs which are Gag, Pol, Vif, Vpr, Vpu and Env should be overcome in some way, so that virus can be

replicated successfully. After Rev enters the nucleus via its nuclear localization signal, it maintains unspliced RNAs transport by binding to RRE (Rev-response element) found on RNAs, the binding targets RNAs for the export with the nucleus export signal sequence located on the carboxy domain of Rev and transport is managed by the host's export proteins mainly by CRM-1 (Exportin 1). Full-length transcript is used to translate Gag, Pol and Env proteins and is organized as genome in new virions (Miller et al., 2000), (Cullen, 2003).

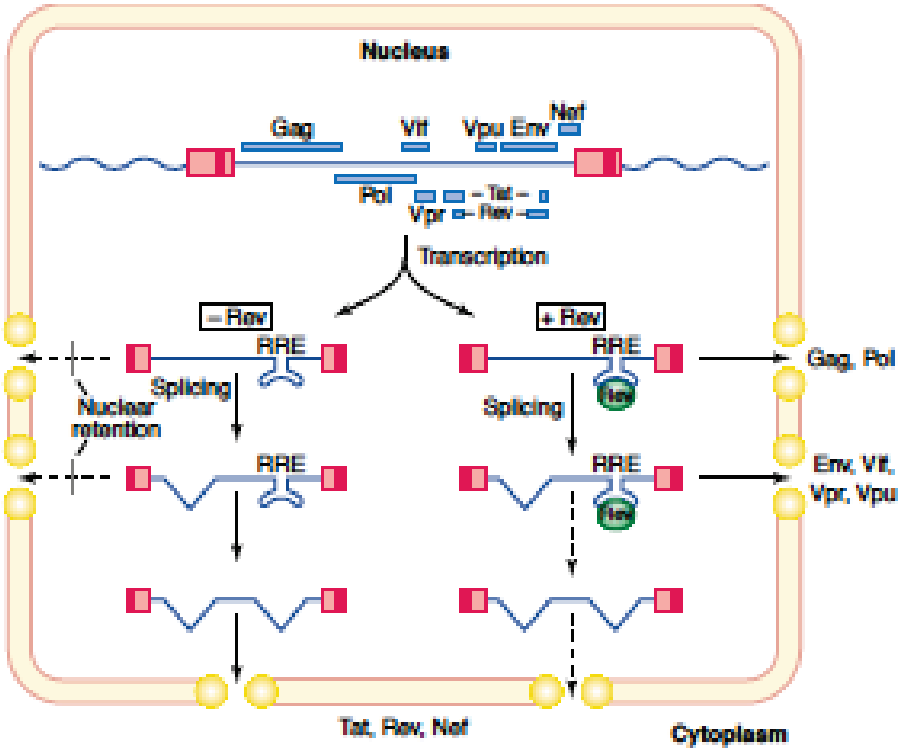

Figure 1.8. Rev-dependent transport
(Source: Cullen, 2003)

1.1.6. Life Cycle

HIV infects the cells through the interaction between the viral envelope protein gp120 and CD4 and chemokine or closely related seven-transmembrane coreceptors. Also gp120 can interact with DC-SIGN (dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin) and glycoproteins like syndecan-1, which leads to capturing the

virus, so that they can engage with their appropriate receptors (Trkola, 2004).

Once virus engages with host cell, membrane fusion is stimulated through the conformational changes in the transmembrane proteins and virus discharges its content to host cytoplasm. Several proteins like p17 matrix, Nef and Vif along with host factors assist the processes of fusion and disgorgement (Klimas et al., 2008), (Frankel and Young, 1998). When the uncoating is completed, reverse transcription complex (RTC), consisting of viral RNA, reverse transcriptase, integrase, tRNAlys, p17 matrix protein, nucleocapsid protein p7, Vpr and a series of host proteins, is formed (Trkola, 2004). Reverse transcription begins in RTC, while the complex is still in cytoplasm. In this step, reverse transcriptase synthesizes minus-strand DNA from plus-strand viral RNA and its RNase activity cleaves the viral RNA and then plus-strand DNA is made by using minus-strand DNA as a template. DNA stays in the pre-integration complex (PIC) formed simultaneously with DNA synthesis and the termini of the DNA are altered by cutting near the 3' ends of each strand by viral integrase creating free 3' OH termini, soon after a highly conserved CA dinucleotide pair (Goff, 2004a). PIC transports genetic information from cytoplasm to nucleus by interacting with proteins importin α and β. DNA, found in nucleus as either circular containing one or two LTRs or linear, which are the only form capable to integrate is inserted the host DNA by joining altered 3' ends covalently with 5' ends of the host genome. Then host enzymes cut unjoined 5' ends, fill in the 5 bp gaps and ligate viral DNA generating integrated provirus. Consequently, provirus is a lasting inhabitant of the host.

Even though how integration is activated is still unknown, it doesn't seem like an accidental mechanism, because HIV integrates favorably at transcriptionally active parts of genome by benefitting from chromosomal environment (Vicenzi and Poli, 2013).

If the infected cell is activated, it proceeds to produce viral proteins by converting viral DNA back to RNAs, called mRNA, via RNA polymerase II and transports transcribed mRNAs from nucleus to cytoplasm. When mRNAs move to cytoplasm, they are used as template to initiate viral protein synthesis by cellular machinery. Products translated from viral mRNAs composing the envelope and core proteins are then spliced to smaller functional units by viral proteases (Klimas et al., 2008). Once spliced, Gag and Gag-Pol proteins are processed to core proteins; MA, CA, NC, p6, and viral enzymes; IN, PR, RT gather within the cell membrane with viral RNA. Then virus is coated with SU and TM proteins, buds from and pinches off the cell by forming a mature virus that is able to infect another cell (Frankel and Young, 1998).

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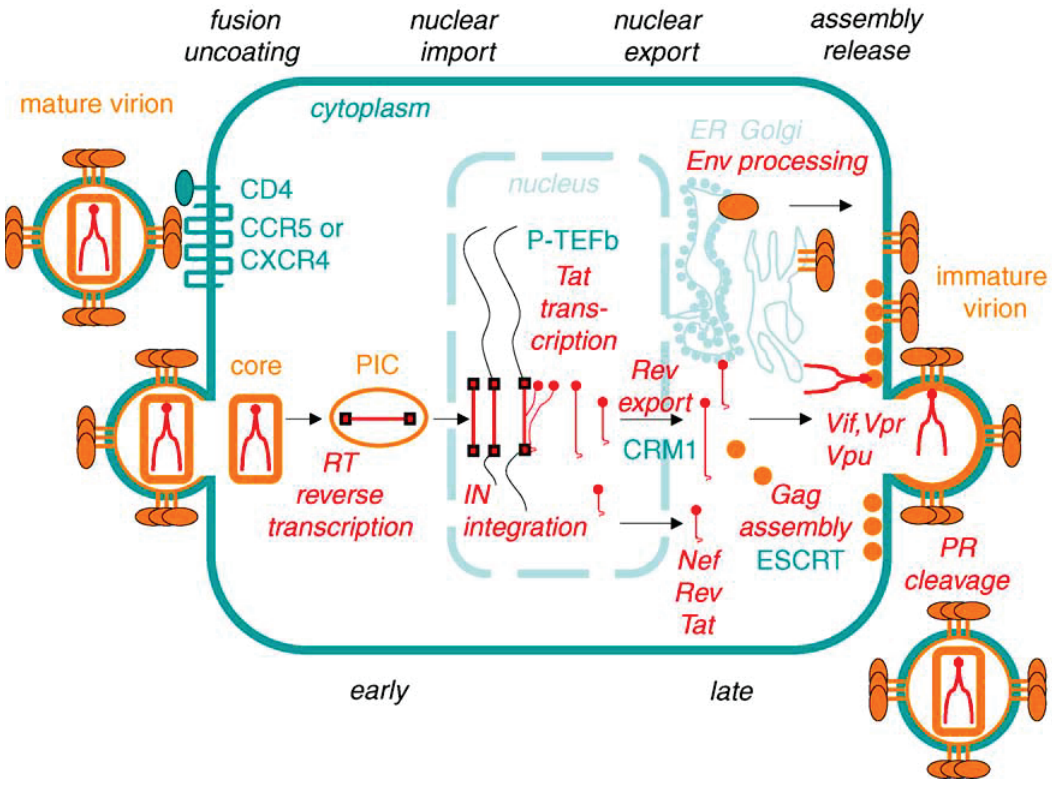

Figure 1.9. HIV life cycle
(Source:Zheng et al., 2005)

1.2. Host Restriction Factors

Infection with HIV influences people differently; some show rapid progression to AIDS, some survive more than ten years, and even some stay uninfected. The difference results from the difference in host factors' activity; for example 32 bp deletion in CCR5 co-receptor confers resistance to R5-tropic HIV-1 by blocking the expression of receptor on the cell surface. Researches proposed that a pathogen also used this receptor led to an epidemic by resulting in the selection of mutated allele among ancestral Caucasians by looking at the geographic distribution of CCR5Δ32 (Sverdlov, 2000).

Retroviruses have generally an antagonistic relationship with the host. As a result of coevolution of viruses with hosts, virus proteins have evolved to have specific binding

sites for host proteins to utilize host mechanisms for stimulating uncoating, nuclear localization and formation of new viruses. In turn, host cells have evolved strong mechanisms to restrict virus replication (Goff, 2004a), (Wolf and Goff, 2008).

After the presence of restriction systems was discovered in some primates including AGM, intensive researches have focused on identification of the mechanisms to facilitate establishment of an animal model for HIV treatment (Bieniasz, 2003).

1.2.1. HIV-1 Counteracts General Restriction Factors

1.2.1.1. APOBEC3G

HIV replication depends on Vif expression in nonpermissive cells such as primary CD4 T cells, PBMCs, macrophages, while permissive cells like 293T, HeLa and SupT1 cell lines do not require Vif. The discovery of APOBEC3G was derived from the observation that there should be an antiviral factor expressed in nonpermissive cells to block Vif-deficient HIV replication. This antiviral factor, APOBEC3G, is coded on chromosome 22 of human and a member of cytosine deaminase protein family (Hache et al., 2006), (Chiu and Greene, 2009).

APOBEC3G can incorporate into virion via specific association with the nucleocapsid region of Gag protein. Its mode of action is that it deaminates cytidines hydrolyzing them to uridines in cDNA of the retrovirus, after transcription occurs, it results in G to A transition in the sense strand DNA (Hache et al., 2006). As a result of APOBEC3G activity, 1-2% of all C residues in DNA are altered to U residues. These mutations can lead to introduction of stop codons into ORFs damaging replication of virus (Goff, 2003). Mutation rate is highest at the 5' end of the nef gene and lowest at the 5' UTR, increasing 5' to 3' direction. Some of the extremely mutated viral DNA is targeted for degradation before it is reverse-transcribed (Aguiar and Peterlin, 2008).

Conticello and colleagues found that when Vif was coexpressed, APOBEC3G production was suppressed. So Vif likely blocks the association of APOBEC3G with virion by triggering its degradation (Hache et al., 2006), (Goff, 2004b). APOBEC3F also restricts HIV replication as APOBEC3G does (Huthoff and Towers, 2008).

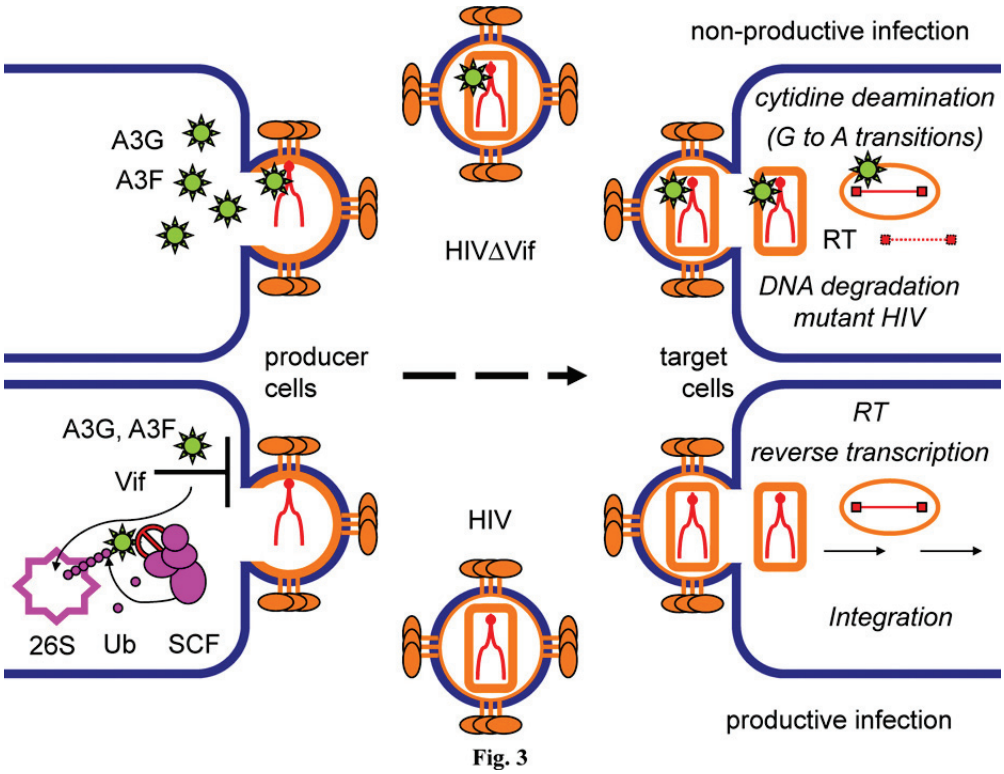

Figure 1.10. Proposed model of APOBEC proteins' restriction
(Source: Aguiar and Peterlin, 2008)

1.2.2. Species-Specific Restriction Factor: TRIM5α

One of the most important restriction factor has been identified so far is Trim5α, since it restricts replication immediately after viral entry. Most primate species including rhesus macaque, African green monkey and owl monkey, are resistant to HIV-1. Researches demonstrated that this resistance defeated when the cells were infected with high titer of virus. In order to evidence this effect, these resistant cells must have one or more restriction factors that show saturable effect (Goff, 2004a). Also several studies proposed that this inhibition appears at a post-entry step most likely by hindering the initiation of reverse transcription. (38) Stremlau and colleagues determined that the factor provides resistance to HIV-1 is Trim5α in rhesus macaques (Stremlau et al., 2004).

TRIM5α from different species can restrict a wide range of viruses in different variations (Nakayama and Shioda, 2015). For example TRIM5α of rhesus macaques

and cynomolgus monkeys inhibit HIV-1, but not SIVmac (Williams and Burdo, 2009). Correspondingly, TRIM5α found in human cannot block HIV-1 capsid while sharing 87% resemblance with its rhesus homolog, but strongly restricts murine leukemia virus (Zheng et al., 2005). TRIM5α of AGM can block HIV-1 and SIVmac, while cannot SIVagm. The observation that TRIM5α block the SHIV chimeric viruses, SIV virus with HIV-1 capsid region, proves that it interferes with HIV-1 CA (Nakayama and Shioda, 2015).

TRIM5α is a member of TRIM (tripartitate motif) family proteins. Proteins of this family localize to distinct positions forming specific bodies there and drive other proteins to these structures (Goff, 2004a).

Four regions of 493 residues TRIM5α has been described are; a RING domain, B box 2, a coiled-coil domain, and a PRYSPRY domain (Zheng et al., 2005).

It has been suggested that RING domain degrades the core of virus having E3 ubiquitin ligase activity (Nakayama and Shioda, 2015). Ubiquitination normally targets proteins for proteasomal degradation, but also it functions in immunity. E3 ligase activity of TRIM5α regulates immune responses by activating MAPK and NFκB signaling pathways through the activation of TAK1 (transforming growth factor beta-activated kinase 1) kinase complex (De Silva and Wu, 2011). B box 2 domain plays in multimerization together with coiled-coil domain (Nakayama and Shioda, 2015). PRYSPRY domain contributes the targeting of capsid proteins (James et al., 2007). Inhibition of HIV by OWM, but not by human relies on a single amino acid change in this domain; from arginine to proline at position 332. Substitution of arginine to proline in human TRIM5α restores its ability of HIV restriction in vitro (Williams and Burdo, 2009).

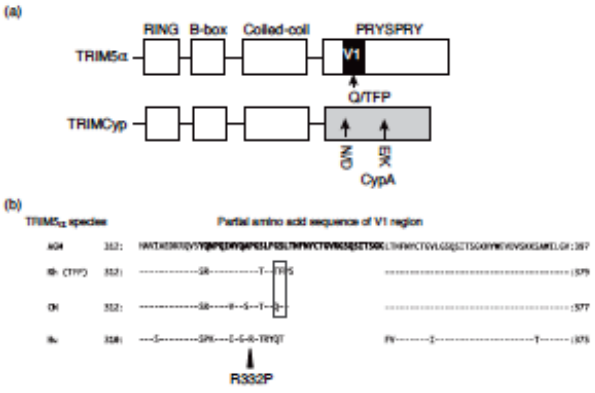

Figure 1.11. Organization of Trim genes and proteins
(Source: Nakayama and Shioda, 2015)

When virus enters the cytoplasm, TRIM5α attacks and develops a sophisticated hexagonal frame on top of the capsid expediting viral uncoating which prevents infection (De Silva and Wu, 2011). In human, a protein referred to as Cylophilin A (CypA) attaches to the HIV-1 capsid, increases infectivity possibly by shelding viral capsid from activity of a restriction factor. Intriguingly owl monkey, exclusive member of New World primate, expresses a TRIM5-CypA fusion protein resulting from insertion of CypA in the place of PRYSPRY domain (Sokolskaja et al., 2006) and the factor inhibits incoming viruses consisting of HIV-1, FIV and several SIVs by binding capsid proteins via its CypA domain rather than PRYSPRY domain as in Trim5α (Huthoff and Towers, 2008).

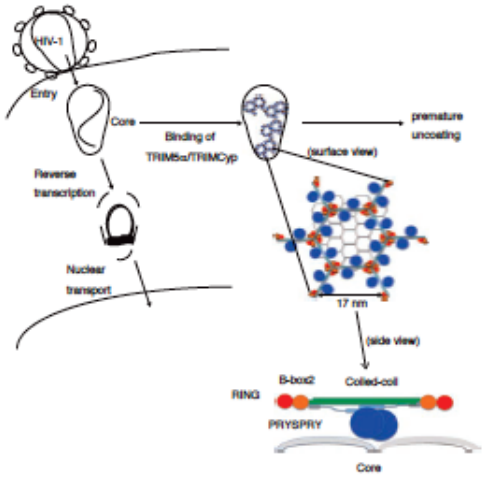

Figure 1.12. Proposed model of Trim proteins' restriction
(Source: Nakayama and Shioda, 2015)

1.2.3. Other Restriction Factors

As mentioned earlier, Vpu maintains detachment of virus from cell surface in some cells. However overexpression of several host factors weakens the release of virion.

BST-2, also known as Tetherin, one of these factors identified so far, aroused interest, because its overexpression is observed mostly in Vpu-dependent cells (Strebel et al., 2009). BST-2, which is a approximately 30 aa transmembrane protein, has a N terminus resided at the cytoplasm, a transmembrane-spanning region and a C terminus with a GPI anchor (Saito and Akari, 2013). So it is proposed that it tethers virus particles

to the membrane through N terminal transmembrane region and GPI anchor.

In human cells, Vpu interacts with transmembrane domain of BST-2 and decreases its expression both endogenously and exogenously (Strebel et al., 2009). Also human BST-2, like TRIM5α, regulates the generation of cytokines by inducing TAK1 dependent NFκB pathway (Saito and Akari, 2013).

Furthermore HIV-2 Env and SIV Nef counteracts with BST-2 by targeting N terminal and C terminal, respectively, resulting in a reduction in the level of BST-2 in cell surface. However, it may be untrue to refer it as a restriction factor, because absence of Vpu doesn't affect viral spread despite of reduced viral release by Tetherin (Strebel et al., 2009).

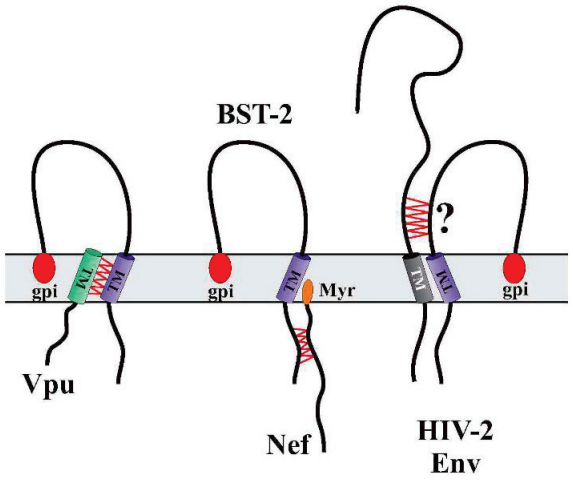

Figure 1.13. Interaction of Tetherin with HIV Proteins
(Source: Strebel et al., 2009)

Myeloid cells, like dendritic cells and macrophages, can restrict the HIV-1 virus and it has been observed that this restriction can be overcome by the expression of Vpx of HIV-2 or SIV by degrading this restriction factor, named as SAMHD1. SAMHD1 is thought to block reverse transcription step by decreasing dNTP concentration induced by dGTP binding (Saito and Akari, 2013).

1.3. Preliminary Studies

In previous studies conducted in our lab, CV-1 cells derived from African green monkey (*Chlorocebus aethiops*) kidney tissue were transiently transfected with pBudCE4.1 plasmid containing HIV-1 tat. Also CV-1 cell lines was transfected with empty parental vector were used as a control. To identify different proteins expressed by these two cells, 2D-gel electrophoresis was performed and obtained protein spots were analyzed with MALDI TOF/TOF. When the overexpressed proteins were identified with mass spectrometry, SLPI was found to be as overexpressed and its overexpression in the presence of tat was confirmed via Q-PCR, western blotting and viral studies. These results are exciting for us because when we checked the literature, we realized that SLPI has already known as having extracellular anti-HIV activity. Also Annexin was found to be one of the proteins overexpressed in tat-expressing cells as a result of 2D- PAGE.

1.4. Secretory Leucocyte Protease Inhibitor (SLPI)

Human secretory leukocyte protease inhibitor (SLPI) is a small, nonglycosylated, 107 aa, cysteine-rich, 11.7 kDa, cationic protein with antiprotease, antifungal, antibacterial and anti-HIV activities (Turpin et al., 1996), (Challacombe and Sweet, 2002). It is expressed mostly in saliva, breast milk, semen, and other mucous tissues in which probability of encountering with a pathogen is high (Hocini et al., 2000).

SLPI looks like a boomerang in its tertiary structure, of each end holding one domain. Disulfide bridges are established between cysteine residues in each domain and interactions between two domains are the main determinant for the conformation and efficiency of the protein. The gene expressed SLPI is located on chromosome 20 and has three introns and four exons extending $\approx$2.6 kb in length (Doumas et al., 2005).

The discovery that no oral transmission of HIV had been observed and saliva contained antiviral activity, led to the discovery of SLPI as an anti-HIV-1 factor (McNeely et al., 1997), (Doumas et al., 2005).

The physiological concentration of SLPI in saliva (1-10 μg/ml) has been found to be capable of inhibiting HIV-1 and 61 its level increases with infection with HIV-1; furthermore its depletion causes impaired antiviral functioning (McNeely et al., 1995). Transmission from infected mother to infant by nursing can be explained by the inappre-

ciable level of SLPI and outrageous level of viral load found in breast milk (Challacombe and Sweet, 2002).

There is no evidence that SLPI interacts directly with viral proteins, and it appears to exert its activity just after binding of virus to the host receptor blocking the viral entry. Also this antiviral activity had been determined to be independent of its antiprotease characteristics (Tomee et al., 1998). Hence its mechanism of action may include interaction with another molecule/molecules crucial for viral entry or a novel mechanism to inhibit infection (McNeely et al., 1997).

It has been discovered that monotropic strains of HIV-1 is more susceptible to restriction by SLPI than dualtropic strains probably due to having several routes of entry by using two coreceptors (Skott et al., 2002). Moreover there is no other known activity of SLPI against other lentiviruses. SLPI blocks proteases providing protection from cleavage by proteolytic enzymes produced as a consequence of inflammation via its C terminal domain and the active core. Its N terminal domain may stabilize the interaction of inhibitor with proteases and increase its antiprotease activity via a conformational change induced by heparin (Doumas et al., 2005).

SLPI exerts its antibacterial activity on both Gram-negative and -positive bacteria in a dose-dependent manner via its N terminal domain and its binding to mRNA and DNA of bacteria is likely involved in this inhibition, like as proposed for its antifungal activity. Another proposed mechanism is that SLPI inhibits NFκB activation by protecting IκB from proteolytic cleavage so that increased expression of pro-inflammatory genes by NFκB is prevented (Hiemstra, 2002), (Tomee et al., 1998). It was found that SLPI also has antifungal activity against several pathogenic fungi such as Candida albicans. So decreased SLPI level in saliva associated with aging may be the cause of the higher tendency of aged toward oral fungal infections than young individuals (Doumas et al., 2005).

1.5. Annexin-2

Annexin 2 is a calcium-dependent membrane binding protein with several functions including calcium-mediated exocytosis, activation of DNA replication, actin-based motility control, fibrinolysis, trafficking of intermediates to late endosomes. Besides, it has been found to play a role in cytomegalovirus binding and fusion and influenza virus

replication (Rai et al., 2010).

Recently, its possible role in HIV-1 infection has been proposed from the observation that binding of SLPI to macrophage cells involves Annexin 2.

The evidences that phosphatidyl-inositol (PI) and phosphatidyl-serine (PS) on the membrane that incorporate into viral envelope during maturation act as a cofactor for infection of macrophages, assembly of the HIV-1 mediated by Gag is preferred at lipid rafts which contain PI and PS, interaction of Annexin 2 with Gag is crucial for infectivity in macrophages and SLPI protects macrophages from infection with HIV-1, led researchers to the proposal that SLPI may prevent the infection by disrupting the interaction between Anx2 and PS (Woodham et al., 2016), (Herbeuval et al., 2005), (Harrist et al., 2009).

1.6. Aim of the Study

The aim of the thesis was to establish CV-1 cell lines expressing HIV-1 tat and SIV tat proteins stably in order to investigate possible resistance mechanism in African green monkeys provided by SLPI in the light of previous findings. As HIV tat was found to cause overexpression of SLPI in agm cells and HIV tat and SIV tat are similar in function despite of differences in their sequences, we aimed to investigate whether SIV tat affect the SLPI expression the same way as HIV tat.

Moreover, Annexin2 was observed as overexpressed in the presence of HIV-1 tat in agm cells, it was also aimed to investigate its expression in the presence of HIV-1 tat and SIV tat in agm cells.

In order to shed light on SLPI mechanism, it was aimed to observe the effects of HIV- 1 tat and SIV tat on HIV LTR.

Also, 293T cell lines expressing HIV-1 tat and SIV tat genes stably were established for further studies.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Frequently used kits, plasmids and primers in this study were listed below.

2.1.1. Kits

Table 2.1. Commercially available kits used in this study

KITS	**SUPPLIER**
PCR Master Mix	Thermo Fisher Scientific
GeneJET RNA Isolation Kit	Thermo Fisher Scientific
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific
DNase I	Thermo Fisher Scientific
RealQ Plus Master Mix Green without ROX	Thermo Fisher Scientific
TurboFect Transfection Reagent	Thermo Fisher Scientific
QIAquick Gel Extraction Kit	Qiagen
T4 Ligase	Thermo Fisher Scientific
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific
NucleoBond® Xtra Midi Plasmid Isolation Kit	Macherey-Nagel
Nano-Glo® Luciferase Assay System	Promega
ProteoJET Cell Lysis Reagent	Thermo Fisher Scientific
Min Elute Reaction Clean up Kit	Qiagen

2.1.2. Plasmids

The plasmids were used in this study were listed below in Figures 2.1, 2.2, 2.3, 2.4, 2.5, 2.6 and 2.7.

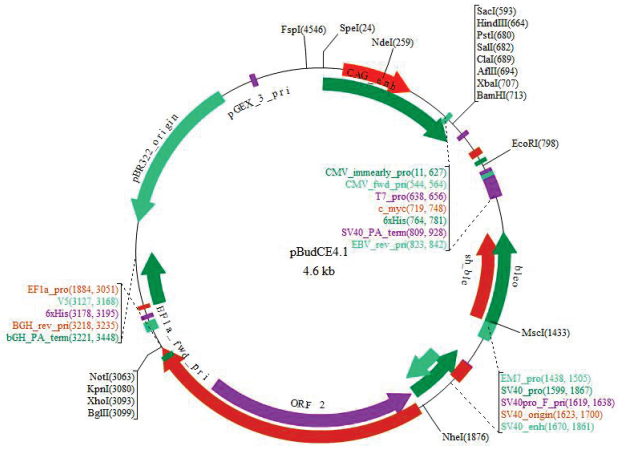

Figure 2.1. pBud CE4.1 Mammalian Expression Vector (Invitrogen)

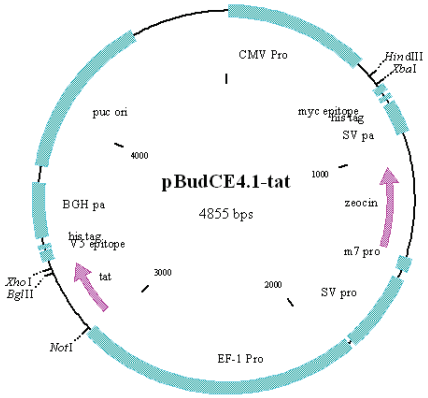

Figure 2.2. pBudCE4.1-tat Mammalian Expression Vector
(Source: Burcu Sengez Thesis, 2011)

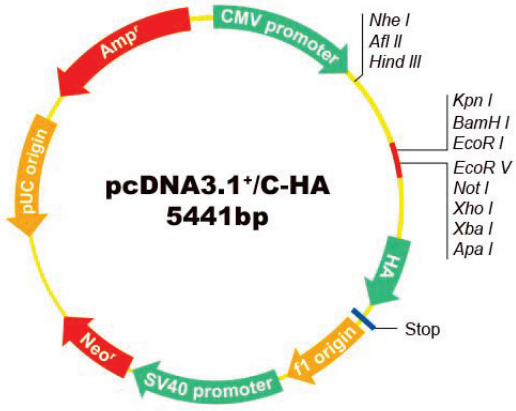

Figure 2.3. pcDNA+C-HA Mammalian Expression Vector (Genscript)

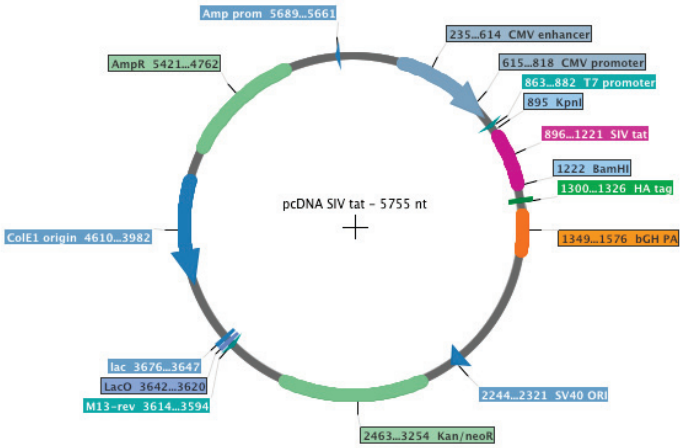

Figure 2.4. pcDNA-SIV-tat Mammalian Expression Vector (Genscript)

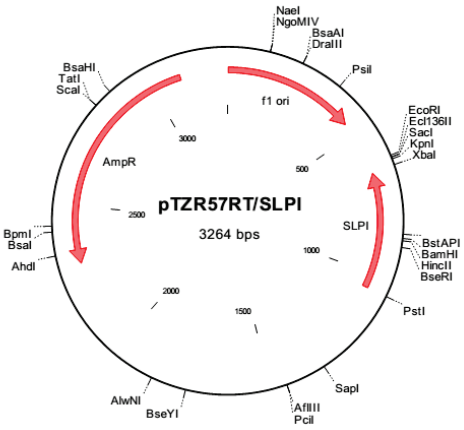

Figure 2.5. pTZ57R/T-SLPIagm

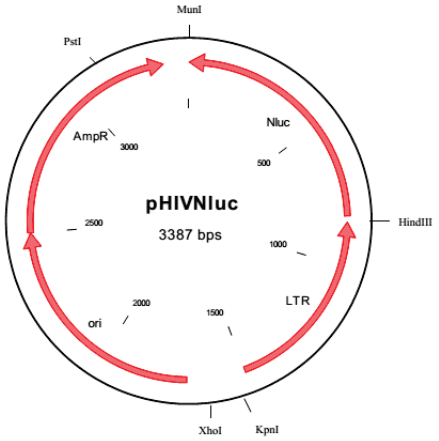

Figure 2.6. pHIVLuc plasmid
(Source: Selcuk OZDEMIR Thesis, 2015)

2.1.3. Primers

Primers were designed according to the region to be amplified and ordered from Sentromer DNA Technology. Primer names, sequences and the expected size of amplified regions for this study were shown in Table 2.2.

Table 2.2. Primer sequences used in this study

Primer Name	Sequence	Expected Size
q-Zeocin F	5' TGATGAACAGGGTCACGTCGTC 3'	
q-Zeocin R	5' AAGTTGACCAGTGCCGTTCCG 3'	145 bp
Htat NotI	5' GCGGCCGCACCATGGAGCCAGTA 3'	
Htat BglI	5' AGATCTATTCCTTCGGGCC 3'	276 bp
pcDNA F	5' ACGACTCACTATAGGGAGACCC 3'	
pcDNA R	5' TAGAAGGCACAGTCGAGGCT 3'	183 bp
Stat F	5' GCTATCATTGTCCGCTTTGCTTC 3'	
Stat R	5' CCAGGTGTATGATCTGCTGTCG 3'	184 bp
GAPDHq F	5' AGAAGGCTGGGGCTCATTTG 3'	
GAPDHq R	5' AGGGGCCATCCACAGTCTTC 3'	258 bp
SLPI2q F	5' CTAGGGAAGAAGAGATGTTG 3'	
SLPI2q R	5' CCTCCAAATCACAGGAATC 3'	245 bp
SLPI-PstI	5' CTGCAGCACCATGAAGTCCAG... 3'	
SLPI-XbaI	5' TCTAGATCAAGCTTTCACAGG... 3'	415 bp
AnnexinA2-Q-F	5' GCAATGCACAGAGACAGGATA 3'	
AnnexinA2-Q-R	5' CTGAGCAGGTGTCTTCAATAGG 3'	134 bp

Expected sizes given in Table 2.2 are for commonly used samples in this study. So they might have shown differences when used to amplify cDNA or genomic DNA, depending on whether amplified region contains intron.

2.2. Methods

2.2.1. Bacterial Techniques

2.2.1.1. Preparation of Chemically Competent Cells and Transformation

A spot of bacteria from E.coli DH5α stock was taken via a loop and inoculated onto LB agar plate. Plate was incubated for overnight at 37°C. A single colony was taken and inoculated into LB medium. 4 hours after inoculation, starter culture was added into LB medium with large volume and allowed to grow over night. When the OD_{600} reached

to 0.55, cells were harvested with centrifugation at 2500x g, for 10 minutes, at 4°C. Supernatant was discarded and pellet was dissolved in filter sterilized Inoue transformation buffer (55 mM $MnCl_2.4H_2O$ (Applichem), 15 mM $CaCl_2.2H_2O$ (Applichem), 250 mM KCl (Applichem), 10 mM PIPES(Applichem) pH 6.7 and H_2O). Cells were harvested again by centrifuging at 2500x g, for 10 minutes, at 4°C. Supernatant was discarded and pellet was dissolved in filter sterilized Inoue transformation buffer and DMSO (Santa Cruz) was added. Aliquots of suspension were dispensed by snap-freezing and aliquots were stored at -80°C.

In order to calculate transformation efficiency, competent cells were transformed with 10 pg PUC19 plasmid (Invitrogen) by heat shock and inoculated onto LB agar plate without antibiotics. After plate was incubated at 37°C overnight, colonies were counted and efficiency was calculated according to the formula; TE=Colonies/DNA concentration in μg/Dilution, where TE stands for transfection efficiency, and dilution refers to the total dilution of the DNA before plating.

2.2.1.2. Plasmid Amplification and Isolation

Desired plasmid was transformed into competent DH5α cells with heat shock method and cell was inoculated into LB agar plate containing antibiotic whose resistance gene is found in the transformed plasmid. After plate was incubated at 37°C, over night, single colony from plate was selected and inoculated into LB with antibiotic and medium was incubated at 37 °C with shaking, over night. Cells were harvested using centrifuge and plasmid isolation was performed by using GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the instructions described in the protocol. Before isolated plasmid was verified with restriction, its concentration was measured via Nanodrop. Plasmid was cut with appropriate restriction enzyme(s) and amplified by PCR for double-check. Then verified plasmids were isolated with NucleoBond® Xtra Midi plasmid isolation kit (Macherey-Nagel) and stored at -20 °C for further usage.

2.2.1.3. Molecular Cloning

Target gene and target plasmid that includes gene was cloned into were double digested with appropriate enzymes and they were loaded into agarose gel, bands with desired length after digestion were sliced and extracted from gel by using QIAquick Gel Extraction Kit (Qiagen) in concordance with the protocol. Thereafter concentrations of isolated bands were measured with Nanodrop and double-checked with agarose gel electrophoresis. The gene and plasmid were ligated by using T4 DNA ligase (Thermo Scientific) in a vector to insert ratio of 1:3 according to the protocol. Ligation mixture was transformed to the competent cells and transformed cells were inoculated. Several colonies were selected and presence of insert was checked with colony PCR by using appropriate primers. For colony PCR, 25μl PCR reaction was prepared as listed in Table 2.3 and a small amount of single colonies was picked up with a pipette tip and tip was dipped into reaction mixture. One of the colonies containing insert was selected and grown in the medium. Thereafter, plasmid isolation was done as described previously and plasmid was cut by restriction enzyme(s) to ensure the target gene is inserted.

Table 2.3. Colony PCR Components

Component	**Volume**
2x Master Mix (Thermo Scientific)	12.5 μl
Primer F (10 μM)	1 μl
Primer R (10 μM)	1 μl
Water	Up to 25 μl

pBudCE4.1 and pTZ57R/T+SLPIhuman plasmids was double digested with KpnI and PstI restriction enzymes (Thermo) and cut plasmids were loaded into agarose gel. After gel was run, band with expected sizes were sliced and extracted by using QIAquick Gel Extraction Kit (Qiagen), plasmid concentrations were measured via Nanodrop. Bands that were isolated from gel were verified with agarose gel electrophoresis. Ligation reaction was set up according to the protocol by considering 1:3 vector to insert ratio. After transformation and inoculation, some colonies were selected and controlled with colony PCR by using SLPI-PstI and SLPI-XbaI primers. Two of the colonies containing transformed plasmid were grown and plasmid isolation was performed with GeneJET Plasmid Miniprep Kit (Thermo Scientific). After isolated plasmids were double-checked with PCR and sequencing, plasmid isolation from higher volume of inoculum was performed

with NucleoBond® Xtra Midi plasmid isolation kit (Macherey-Nagel). For sequencing, SLPIhuman gene cloned into pBudCE4.1 vector was amplified with PCR and PCR mixture was purified by using Min Elute Reaction Clean Up Kit (Qiagen).

2.2.1.4. Construction of SIVtat Plasmid

The complete coding sequence of Tat gene of SIV from agm.Sab92018 was accessed from NCBI database and sequences recognized by KpnI and BamHI were added to the 5' and 3' ends, respectively to be used for cloning if needed.

SIVagm.Sab92018-tat gene with KpnI and BamHI sequences in pcDNA3 cloning vector and pCDNA3.1+C-HA (empty parental plasmid) were purchased from GenScript. Lyophilized plasmids were dissolved in 40 μl ddH2O (100 ng/μl) as indicated in the protocol. Plasmids were transformed into the DH5α competent cells. Then cells were plated onto LB-ampicillin plates. Plates were incubated at 37°C over night. One colony for each plasmid was chosen from LB-amp plates and inoculated into LB broth containing ampicillin. It was incubated at 37°C over night at 200 rpm. Plasmids were isolated with Nucleobond Xtra midi-plus kit (Macherey-Nagel) and their concentrations were measured via Nanodrop. In order to check plasmids, pcDNA3.1-SIVtat plasmid and pcDNA3.1+C-HA plasmids were double digested with KpnI and BamHI; and SspI and BamHI, (Thermo) respectively and confirmed with agarose gel electrophoresis.

2.2.2. Cell Culture Techniques

In this study, CV-1 and 293T cell lines were used. CV-1 cells are derived from epithelial kidney cells of African green monkeys (*Chlorocebus aethiops*) and 293T cells are originated from human embryonic kidney cells. Both cells are adherent and immortal.

2.2.2.1. Cell Passaging

First of all, cell culture media were prepared by adding 10% FBS (Biochrom) and 1% penicillin-streptomycin (Sigma) into either DMEM- high glucose (4500 mg/L

glucose, L-glutamine) (Sigma) or IMDM (with 25 mM Hepes, without L-glutamine) (Lonza). After cells reach to 80-90% confluency, medium was removed, cells were washed with 1X PBS, sufficient amount of trypsin was added to detach cells from surface by cleaving proteins via its serine protease activity. Cells were incubated with trypsin (Sigma) in a 37°C incubator with 95% air-5% CO_2 for 2 minutes and harvested into a falcon by resuspended in DMEM and later they were centrifuged at 1200x rpm for 2 minutes. Supernatant was aspirated, pellet was dissolved in medium and desired concentration of cells was added onto medium-containing plate. After plates were wigwagged, they were kept in the incubator. For maintenance, cells were subcultured in a ratio of 1:6 in every 3-4 days, for transfection, optimum dilution of cells was determined and used.

2.2.2.2. Cell Freezing and Thawing

In order to stock the cell lines, cells were waited to reach to 80-90% confluency in 10 cm plate. Medium is removed, cells are washed with 1X PBS and harvested with trypsin treatment, added into a falcon and cells centrifuged at 1200x rpm for 2 minutes. After supernatant is removed, cell pellet is dissolved in a freezing solution that contain 70% DMEM, 20% FBS and 10% DMSO and the cell mixture is put into a 2 ml cryovial. To make sure temperature will decrease steadily, vial is wrapped by paper towel and put into -80°C for 1 day in a container before kept at liquid nitrogen (between -196°C and -210°C) for long duration of storage.

In case of need, immediately after cryopreserved cells were removed from liquid nitrogen tank, they were put into 37°C water bath and swirled for less than one minute until a small bit of ice cube was left in the vial. Then the vial was placed into hood after its outside was cleaned with 70% ethanol. Stocked cells were transferred into a plate with desired size to obtain high density and pre-warmed medium was added drop-wise. After cells were attached, medium was changed to remove DMSO, because it is toxic for cells.

2.2.2.3. Transfection & Establishment of Stable Cell Lines

Lipofectamine 2000 (Thermo Scientific), Fugene (Roche), ExGen500 (Thermo Scientific), X-tremeGENE Q2 (Roche) and Turbofect (Thermo Scientific) reagents were

initially tested by transfecting eGFP-C3 plasmid to the cells and 48 h after transfection, GFP expression was observed under fluorescent microscope. Turbofect was determined to be the most effective one, so further experiments were conducted by using Turbofect.

Plasmids were linearized with suitable restriction enzymes; for pBudCE4.1 and pBudCE4.1-tat plasmids, NheI and for pcDNA3.1 and pcDNA3.1-SIVtat plasmids, BglII were used and efficiency of linearization was checked with agarose gel electrophoresis.

The day before transfection, 293T and CV-1 cells were split into 24-well plate with 1:4 dilution. Next day, cells with 70-90% confluency were transfected with linearized plasmids by using Turbofect as recommended. For the control of transfection efficiency, eGFP plasmid was transfected to cells. 48 h after transfection, cells transfected with GFP were observed under the fluorescent microscope to observe the ratio of transfected cells over nontransfected cells.

Cells transfected with linearized plasmids were passed to 6-well plates without any dilution. IMDM with a final concentration of 200 μg/ml of Zeocin (Invivogen) was added to the cells transfected with pBud and pBud-tat. DMEM with a final concentration of 1500 μg/μl of G418 (Sigma) was added to the cells transfected with pcDNA and pcDNA SIV tat. As pBudCE4.1 plasmid contains Zeocin resistance gene and pcDNA3.1 has Geneticin (G418) resistance gene, these antibiotics were used to select cells into which plasmids were inserted.

In every 3-4 days, media supplemented with appropriate antibiotic concentrations was replaced. After colony formation was seen, single colony from the 6 well plates was transferred to 24 well plates by using cloning disc according to the manufacturer's recommendation. Colony was allowed to grow. When the cells reached to confluency, they were transferred to 60 mm plate and passaged regularly.

To check whether desired genes are expressed regularly in RNA level and in protein level; total RNA from cells were isolated, reverse transcribed to cDNA and controlled with PCR, and total protein from stable cell lines were extracted and controlled with Western blotting as described below.

2.2.3. RNA Isolation & cDNA Synthesis

Confluent stable cells were harvested with Trypsin treatment and were precipitated with centrifugation at 1200x rpm for 2 minutes. RNA isolation was performed by

using GeneJET RNA Purification Kit (Thermo Scientific) according to the instructions described by the manufacturer. Concentrations of RNA samples were measured with Nanodrop and 1 μg of RNA was treated with DNase I (Thermo Scientific) to eliminate any DNA contamination. 1.2% agarose gel was poured, RNA samples were loaded into gel and gel was run at 60 V for 1,5 hour to confirm RNA integrity. Before RNA agarose gel electrophoresis, tanks were washed with distilled water and TAE buffer was replaced to minimize RNase level. RNAs of cells were checked with PCR to verify the efficacy of Dnase treatment.

Then cDNA was synthesized from RNA samples with RevertAid First Strand Synthesis Kit by using oligo(dT)18 primer.

Establishment of stable cell lines were verified with PCR by amplifying cDNAs of pBud, pBud-HIVtat, pcDNA, and pcDNA-SIVtat expressing cells with Zeo, Tat, pcDNA and Stat primers, respectively. 2X PCR master mix (Thermo Scientific) was used for this experiment and PCR conditions concordance with the manual are listed in Table 2 .4.

Table 2.4. PCR Conditions

Number	**Cycle**	**Temperature(°C)**	**Time**
1	Initial denaturation	95	3 minutes
30	Denaturation	95	30 seconds
	Annealing	60	30 seconds
	Extension	72	30 seconds
1	Final extension	72	10 minutes

2.2.4. Analysis of Proteins

2.2.4.1. Total Protein Extraction

Confluent stable cells in 100 mm plates were harvested by trpsinization and precipitated by centrifugation, as described. 20 volumes of ProteoJET Cell Lysis Reagent was added to 1 volume of packed cells and cells were resuspended by vortexing. After 10 minutes of incubation at room temperature with agitation, cell lysate was clarified by

centrifuging at 20000 x g for 15 minutes and supernatant was transferred to a new tube and stored at -80°C for future usage.

2.2.4.2. Bradford Assay

In order to identify protein concentration, Bradford assay was performed. First of all, Bradford reagent was prepared as described by He (He, 2011). Then BSA standard (Sigma) was dissolved in UP water such that the final concentration is 400 μg/ml. After that BSA standard solution was diluted 4 fold with UP water to obtain 100 μg/ml BSA solution. Standards contain 1 μg, 2 μg, 4 μg, 6 μg, 8 μg and 10 μg BSA in 100 μl were prepared and 900 μl Bradford reagent was added. At the same time extracted proteins were diluted to 1:10, 1:100 and 1:1000 with total volume of 100 μl and 900 μl Bradford reagent was added. 200 μl of each samples was put into 96-well plate (Corning) in triplicate and absorbance values were measured at 595 nm with a spectrophotometer.

A standard curve as shown in Figure 2.7 was plotted by using the absorbance values of BSA standards and protein concentrations of cells were calculated according to the standard curve.

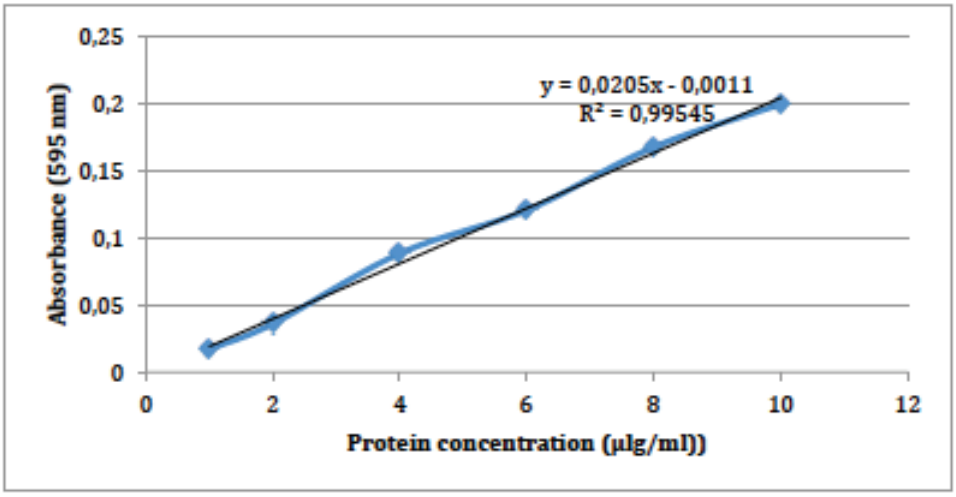

Figure 2.7. BSA Standard Curve

2.2.4.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

As GAPDH, SLPI and TAT proteins which are small proteins with molecular weights of 37 kDa, 11.7 kDa and 9 kDa, respectively were checked; it was appropriate to use 15% separating gel and 5% stacking gel. GAPDH was used as an internal control.

Gels were prepared by using 40% acrylamide-polyacrylamide (Applichem), Tris (Applichem), APS (Applichem), SDS (Applichem), TEMED (Applichem), and water. 30 μg of each protein sample extracted from stable cell lines was mixed with 6X protein loading dye and denatured by boiling at 95-100 °C for 5 minutes. Mixtures were vortexed, before and after heating and loaded into SDS gel and gel was run for 1 hour at 90 V and for 1,5 hour at 120 V in Mini-PROTEAN® Tetra Vertical Electrophoresis Cell system (Biorad), by adding 1X running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). After running the gel was completed, transfer of proteins to the PVDF membrane (Thermo Scientific) was acomplished using Pierce G2 Fast Blotter (Thermo Scientific) according to the protocol proteins with low molecular weight. Membrane was activated by wetting it with methanol (Sigma-Aldrich), before it was equilibrated in 1-Step Transfer Buffer (Pierce) together with filter paper. After transfer had been completed, membrane was blocked in blocking buffer (5% (w/v) BSA (Sigma Aldrich) in 1X TBST (Tris-buffered saline, 0.1% (v/v) Tween 20 (Applichem))) by incubating for 1 hour at room temperature with agitation. Membrane was rinsed for 30 seconds in TBS-T with shaking, and it was incubated with diluted primary antibody in TBS with 5% BSA to a concentration predetermined up to overnight, at 4°C, with agitation. Dilutions were determined as 1:1000 for GAPDH Rabbit mAb (Cell Signaling), 1:1000 for rabbit SLPI polyclonal antibody (MyBioSource) and 1:500 for rabbit HIV tat polyclonal antibody (MyBioSource). After incubation with primary antibody, membrane was washed 5 times in TBS-T while agitating, for 5 minutes per wash and it was incubated with Goat anti-Rabbit IgG F(ab')2 Secondary Antibody, HRP (Pierce) diluted 1:3000 for 1 hour, at room temperature, with shaking. Before visualization, membrane was washed 5 times in TBS-T while agitating, for 5 minutes per wash, was incubated with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) for 5 minutes and visualized by using Fusion FX (Vilber). After membrane was checked for desired protein, either SLPI or TAT, it was stripped with Restore^{TM} PLUS Western Blot Stripping Buffer (Thermo Scientific) to investigate

the presence of GAPDH as an internal control. For this reason, all steps were applied to the membrane again to detect GAPDH.

2.2.5. Real Time PCR

SLPI and Annexin expression levels of stable cell lines were measured with Q-PCR by using GAPDH as a standard. For this reason, RNA was isolated from cell lines and was converted to cDNA after DNase treatment. Synthesized cDNAs were used as template and expression levels were described by using RealQ Plus Master Mix Green without ROX (Ampliqon) with Q-PCR whose parameters are described in Table 2.5 and Table 2.6.

Table 2.5. Q-PCR Components

Component	**Volume**
2x Master Mix	5 μl
Primer F (10 μM)	0.25 μl
Primer R (10 μM)	0.25 μl
cDNA	1 μl
Water	3.5 μl

Table 2.6. Q-PCR Conditions

Number	**Cycle**	**Temperature(°C)**	**Time**
1	Initial denaturation	95	10 minutes
40	Denaturation	95	30 seconds
	Annealing	60	30 seconds
	Extension	72	60 seconds

2.2.6. Luciferase Assay

Luciferase assay was performed to confirm SIV-tat functionality and to investigate the effect of SLPI(human) on HIV-LTR by utilizing Nano-Glo® Luciferase Assay System (Promega).

In the first experiment 293T cells with equal confluency were transfected with pBudCE4.1, pBudCE4.1-HIVtat, pcDNA3.1-SIVtat and pcDNA3.1+C-HA plasmids separately. Also all cells were co-transfected with pHIVluc plasmid (Selcuk OZDEMIR Thesis, 2015). 48 hour after transfection, luciferase reagent was prepared as defined in the protocol and added to transfected cells medium. In order to make sure that measurement is in the dynamic range of the equipment, cell culture media of the samples were diluted 100 fold and their luminescence values were also measured.

Before measurement was done, cells with reagents were incubated for 5 minutes and luminescence was measured for 10.000 mc with Varioskan Flash (Thermo Scientific) in Biotechnology and Bioengineering Research and Application Center, Izmir Institute of Technology. Luminescence values were normalized considering cells only transfected with pHIVluc plasmid as a blank and increase in luciferase activity in cells transfected with plasmids contains HIVtat and SIVtat were evaluated according to the activity in cells transfected with empty parental vectors.

In the second experiment, CV-1 cells with equal confluency were co-transfected with pBudCE4.1-SLPI(human) and pHIVluc plasmids. Luciferase assay was performed as outlined above and luciferase activity was interpreted according to the activity in cells co-transfected with pBudCE4.1 and pHIVluc. As a positive control, cells co- transfected with pHIVluc and pBudCE4.1-SLPI(agm) were used.

2.3. Statistical Analysis

Statistical analysis was performed by using one-way ANOVA and $P<0.05$ was considered statistically significant. Data shown are representative of 3 independent experiments.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Construction of Plasmids

SLPIhuman from pTZ57R/T+SLPIhuman plasmid and pBudCE4.1 plasmids were restricted with PstI and XbaI enzymes by creating sticky ends that allow the foreign gene and vector to anneal. As a result of agarose gel electrophoresis of double digestion reactions, a 415 bp band for SLPI gene and a 4.6 kb band for pBudCE4.1 vector were observed as expected as seen in Figure 3.1. The bands with expected lengths were sliced and extracted from gel and then ligated as described before. As a result of colony PCR (Figure 3.2), colony 4 and colony 9 were picked and inoculated. Plasmids isolated from these colonies were controlled with PCR by using SLPI-PstI and SLPI- XbaI primers as seen in Figure 3.3. Also isolated plasmids were sequenced with 16- capillary 3130xl Genetic Analyzer (Applied Biosystems) in Biotechnology and Bioengineering Application and Research Center, Izmir Institute of Technology as a double-checking. As a result of sequencing, no base change was observed.

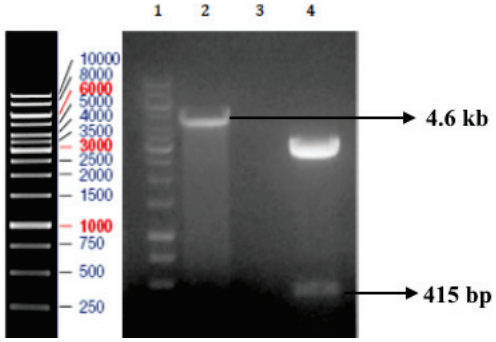

Figure 3.1. GeneRuler 1 kb DNA ladder and Agarose gel electrophoresis of double digestion of pBudCE4.1 and pTZ57R/T+SLPIhuman plasmids.
Lane 1: GeneRuler 1kb DNA ladder Lane 2: Cut pBudCE4.1 plasmid Lane 4: Cut pTZ57R/T SLPI human plasmid

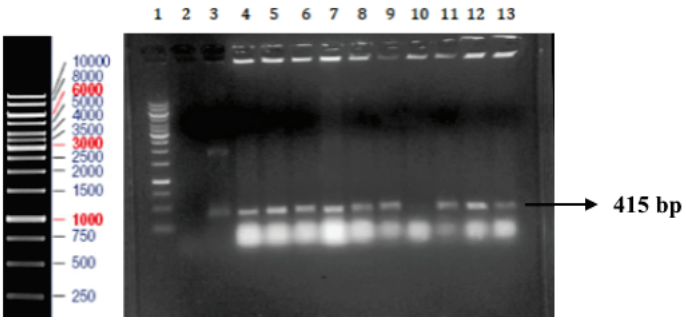

Figure 3.2. Agarose gel electrophoresis of colony PCR.
Lane 1: GeneRuler 1 kb DNA ladder, Lane 2: Nontemplate control Lane 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13: colonies 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 respectively.

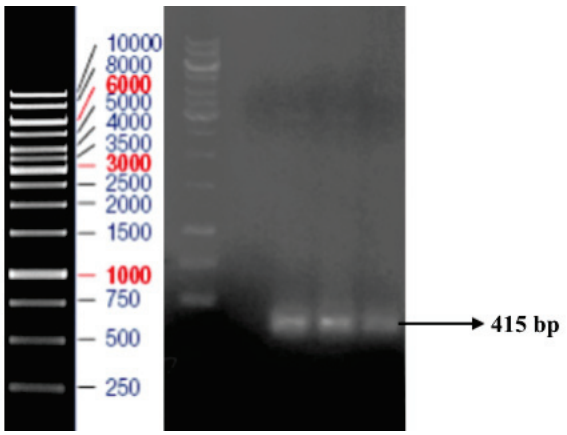

Figure 3.3. Agarose gel electrophoresis of PCRs of isolated plasmids from colony 4 and 9.
Lane 1: GeneRuler 1 kb DNA ladder, Lane 2: PCR of nontemplate control, Lane 3: PCR of isolated plasmid from colony 4 and Lane 4: PCR of isolated plasmid from colony 9, Lane 5: positive control.

pCDNA3.1 and pcDNA3.1-SIVagm.Sab92018-tat plasmids purchased from GenScript were amplified and verified with double digestion. As a result of agarose gel electrophoresis of cut plasmids, bands with expected sizes were observed as in Figure 3.4. Restriction of pcDNA3.1 with BamHI and SspI generated two sequences 1046 bp and 4395 bp in length and restriction of pcDNA3.1-SIVtat with BamHI and KpnI engendered two sequences 324 bp and 5431 bp in length, as expected. Also isolated plasmids were sequenced with 16-capillary 3130xl Genetic Analyzer (Applied Biosystems) in Biotechnology and Bioengineering Application and Research Center, Izmir Institute of Technology as a double-checking. As a result of sequencing, no base change was observed.

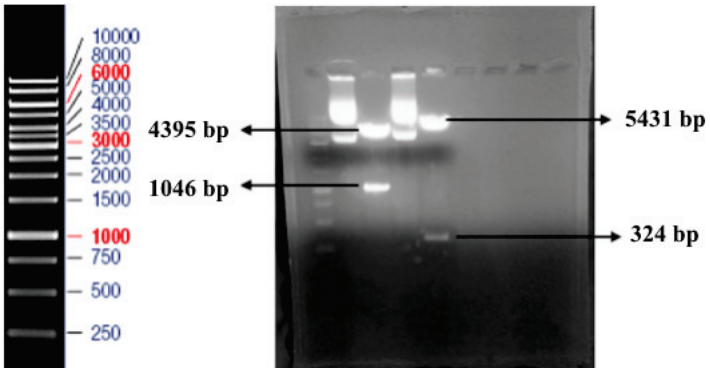

Figure 3.4. The result of gel electrophoresis of double digested pcDNA3.1+C-HA and pcDNA3.1-SIVtat plasmids.

Lane 1: GeneRuler 100 bp DNA ladder (Thermo Scientific), Lane 2: uncut pcDNA3.1+C-HA, Lane 3: cut pcDNA3.1+C-HA, Lane 4: uncut pcDNA3.1-SIVtat and Lane 5: cut pcDNA3.1-SIVtat.

3.2. Determination of the Most Efficient Transfection Reagent

In order to test the efficiencies of different transfection reagents, CV-1 cells were transfected with eGFP plasmid by using Exgen 500, FuGene, Lipofectamine 2000 and Turbofect reagents. All transfection experiments were performed on the CV-1 cells with same confluency and according to the protocol of reagent itself. 48 hour transfection, expressed GFP was observed under fluorescence light and Turbofect was determined to be most efficient as represented in Figures 3.5, 3.6, 3.7 and 3.8 below.

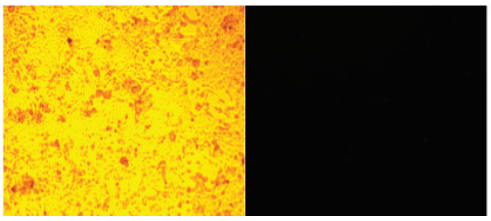

Figure 3.5. Exgen 500 transfection control after 48 h

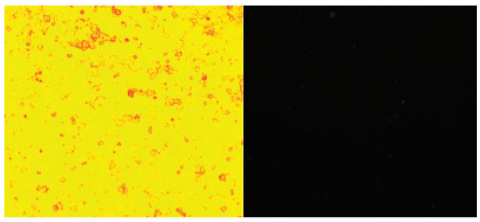

Figure 3.6. FuGene transfection control after 48 h

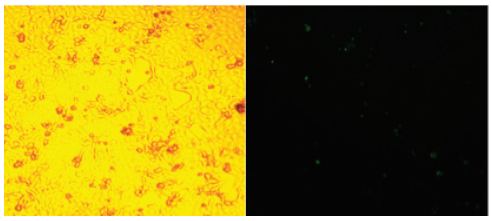

Figure 3.7. Lipofectamine 2000 transfection control after 48 h

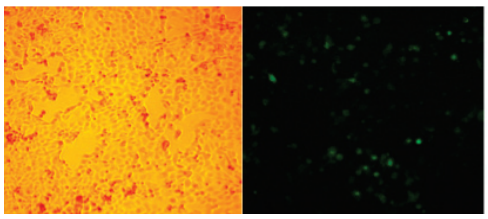

Figure 3.8. Turbofect transfection control after 48 h

All of the reagents that were used are cationic lipid- and/or polymer-based reagents, however as seen in figures below, each has different efficiencies on CV-1 cells. These differences can be resulted from several factors, such as different culture conditions, transfection formulations, quality of reagent, quality of cells, presence of serum etc. As Turbofect is most efficient reagent and requires no additional optimization, it was further used for establishment of stable cell lines.

3.3. Confirmation of SIV tat Functionality with Luciferase Assay

As it is known that Tat is a transactivator of transcription from LTR by acting on TAR region and HIV-1 and SIV tat are similar in function so that they can transactivate one another's LTRs (Viglianti and Mullins, 1988), the functionality of synthesized SIV tat gene was confirmed by investigating its activity on HIV-LTR. For this reason, 293T cells were co-transfected with pcDNA3.1-SIVtat plasmid and pHIVluc plasmid that contains HIV-1-LTR and luciferase gene, thus the activity of luciferase represented the activation of HIV-1-LTR by SIV-tat. Also some other conditions were considered; cells were transfected with pHIVluc to observe its activity only, cells were co-transfected with pcDNA3.1+C-HA and pHIVluc to examine the affect of empty parental vector. Moreover, pBudCE4.1-HIVtat plasmid were transfected into 293T cells along with pHIVluc vector for verification of transactivation of HIV-LTR by HIV tat by normalizing its activity with reference to the activity of luciferase in cells co-transfected with pBudCE4.1 and pHIVluc. In consequence of Luc assay, it was observed that SIV tat transactivates HIV-1 LTR but not to the full extent as shown in Figure 3.9. SIV tat can transactivate HIV-1 LTR only up to 55,3% of HIV-1 tat. This result was consisted with those reported in different studies. SIV MAC tat was found to transactivate HIV-1 LTR up to 20% of HIV-1 tat (Viglianti and Mullins, 1988), and tat of SIV AGM , SIV MND and HIV-2 can transactivate HIV-1 LTR less than 70% relative to HIV-tat (Sakuragi et al., 1991).

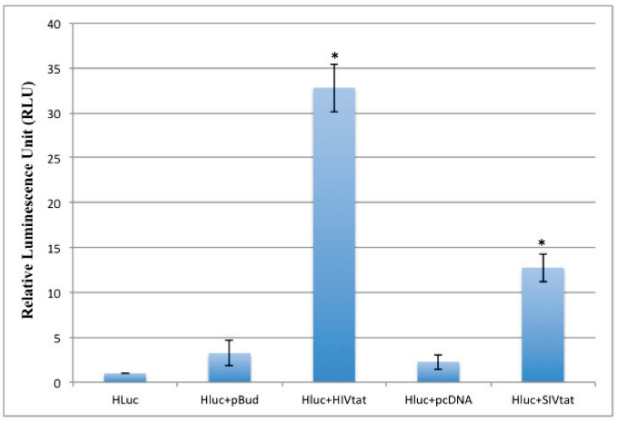

Figure 3.9. Measured luminescence values of transfected cells' relatively to 293T cells transfected with pHIVLuc only.

pBudCE4.1, pBudCE4.1-HIVtat, pcDNA3.1 and pcDNA-SIVtat along with pHIVLuc transfected cells were used and measurements were done 48 h after transfection. (*:$p<0.05$)

3.4. Confirmation of Stable Cell Lines with Reverse Transcriptase PCR

The minimum concentrations of Zeocin and G418 that kill all cells in one week were found to be 200 μg/ml and 1500 μg/ml, respectively. In order to establish CV-1 and 293T cell lines express HIV-tat and SIV-tat stably, pre-determined concentrations of antibiotics were added to the media. For Zeocin selection, IMDM (Iscove's Modified Dulbecco's Medium) that is a hypotonic medium relatively to DMEM was preferred, as activity of Zeocin is reduced in the presence of high salt. Under stress conditions, cells might exhibit some morphological changes such as increase in size, abnormal shapes and appearance of protrusions, thus the occurrence of cells with abnormal shapes in the period that antibiotic selection was applied was considered to be reasonable and cells with abnormal shapes were shown in Figure 3.10. After 3-4 weeks of period of antibiotic treatment, several loci of cells that express desired proteins were seen as in Figure 3.11.

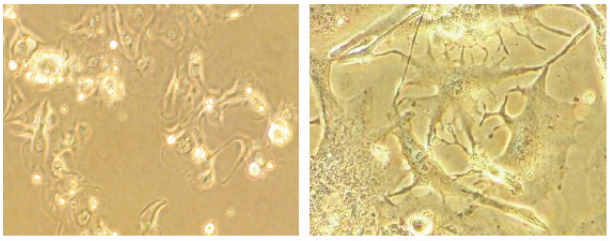

Figure 3.10. Cells with abnormal shapes under antibiotic treatment.

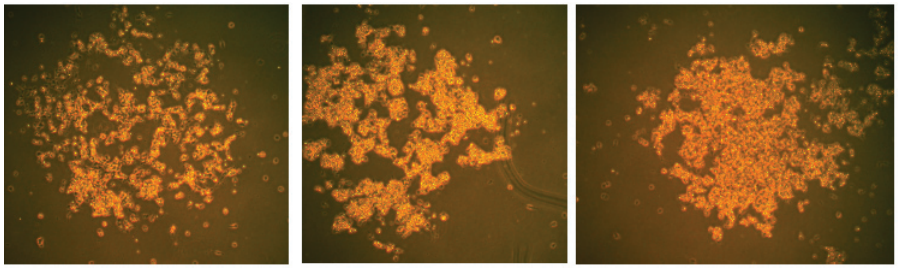

Figure 3.11. CV-1 colonies stably express desired proteins.

Because level of expression can differ cell to cell and the insertion of desired gene into a silence region of DNA might occur, it is important to isolate and transfer several colonies. At the end, CV-1 and 293T cells expressing HIV-tat along with CV-1 and 293T cells expressing pBudCE4.1 mock vector to be used as control, CV-1 and 293T cells expressing SIV-tat along with CV-1 and 293T cells expressing pcDNA3.1+C-HA mock vector to be used as control were established.

As described in section 2.2.3 , total RNA from stable cell lines was extracted, treated with DNase I, and checked with agarose gel electrophoresis for integrity. RNAs were observed for integrity in agarose gel as seen in Figure 3.12. After RNA was treated with DNase I, DNA contamination was invsetigated with PCR by using RNAs as template. As expected, no bands was observed when PCR reactions loaded into agarose gel as in Figures 3.13 and 3.14. After RNA integrity and eliminaton of DNA contamination were testified, RNAs were reverse transcribed to cDNA. Achievement of establishing stable cell lines express desired genes was checked with PCR and the result was shown in Figures 3.15 and 3.16.

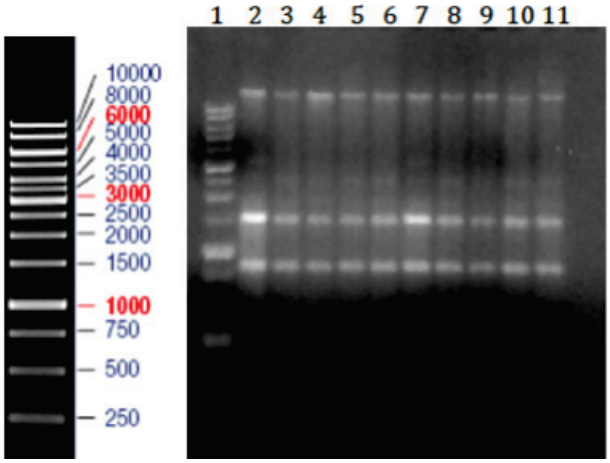

Figure 3.12. Total RNAs of stable cell lines.

Lane 1: GeneRuler 100 bp DNA ladder (Thermo Scientific), Lane 2: non-transfected 293T RNA, Lane 3: pBud-293T RNA, Lane 4: HIVtat-293T RNA, Lane 5: pcDNA- 293T RNA, Lane 6: SIVtat-293T RNA, Lane 7: non-transfected CV-1 RNA, Lane 8: pBud-CV-1 RNA, Lane 9: HIVtat-CV-1 RNA, Lane 10: pcDNA-CV-1 RNA, Lane 11: SIVtat-CV-1 RNA.

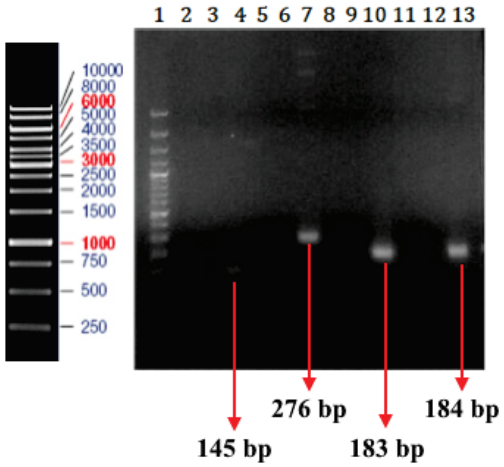

Figure 3.13. Control PCR of DNA contamination with 293T RNAs

Lane 1: GeneRuler 100 bp DNA ladder (Thermo Scientific), Lanes 2,3,4: NTC, pBud-293T, PC with Zeo primers, respectively, Lanes 5,6,7: NTC, HIVtat-293T, PC with Htat primers, respectively, Lanes 8,9,10: NTC, pcDNA-293T, PC with pcDNA primers, respectively, and Lanes 11,12,13: NTC, SIVtat-293T, PC with Stat primers, respectively where NTC stands for non-template control and PC stands for positive control.

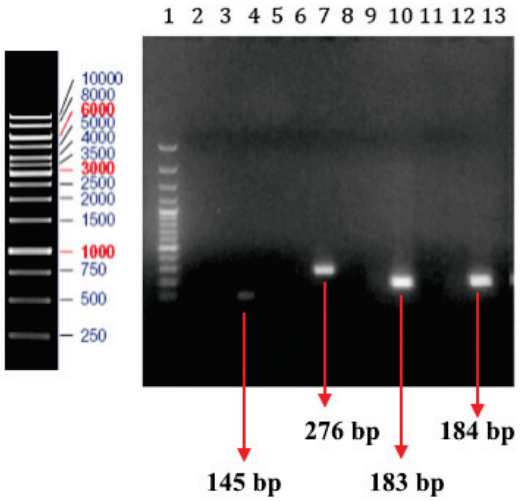

Figure 3.14. Control PCR of DNA contamination with CV-1 RNAs

Lane 1: GeneRuler 100 bp DNA ladder (Thermo Scientific), Lanes 2,3,4: NTC, pBud-CV-1, PC with Zeo primers, respectively, Lanes 5,6,7: NTC, HIVtat-CV-1, PC with Htat primers, respectively, Lanes 8,9,10: NTC, pcDNA-CV-1, PC with pcDNA primers, respectively, and Lanes 11,12,13: NTC, SIVtat-CV-1, PC with Stat primers, respectively where NTC stands for non-template control and PC stands for positive control

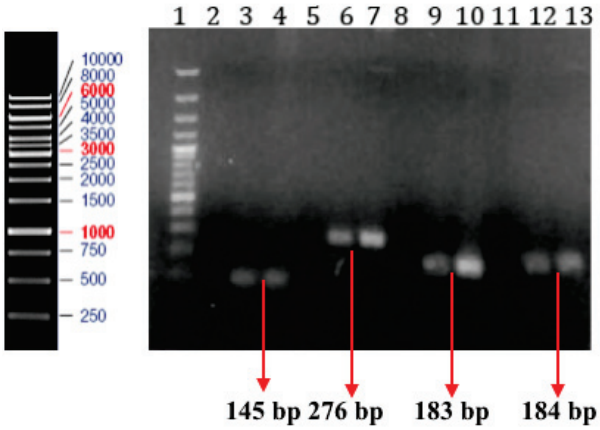

Figure 3.15. Control PCR of cDNAs of 293T stable cells.

Lane 1: GeneRuler 100 bp DNA ladder (Thermo Scientific), Lanes 2,3,4: NTC, pBud-293T, PC with Zeo primers, respectively, Lanes 5,6,7: NTC, HIVtat-293T, PC with Htat primers, respectively, Lanes 8,9,10: NTC, pcDNA-293T, PC with pcDNA primers, respectively, and Lanes 11,12,13: NTC, SIVtat-293T, PC with Stat primers, respectively where NTC stands for non-template control and PC stands for positive control

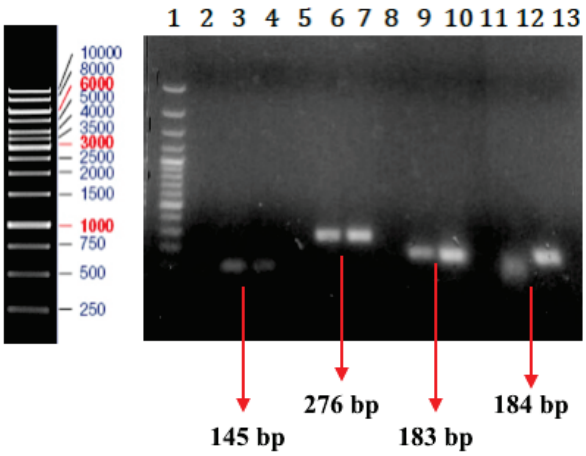

Figure 3.16. Control PCR of cDNAs of CV-1 stable cells.

Lane 1: GeneRuler 100 bp DNA ladder (Thermo Scientific), Lanes 2,3,4: NTC, pBud-CV-1, PC with Zeo primers, respectively, Lanes 5,6,7: NTC, HIVtat-CV-1, PC with Htat primers, respectively, Lanes 8,9,10: NTC, pcDNA-CV-1, PC with pcDNA primers, respectively, and Lanes 11,12,13: NTC, SIVtat-CV-1, PC with Stat primers, respectively where NTC stands for non-template control and PC stands for positive control

3.5. Analysis of SLPI and Annexin Gene Expression with Q-PCR

From the cDNAs extracted from stable CV-1 cell lines, SLPI and Annexin expression levels were investigated by performing Q-PCR. In all experiments, GAPDH expression was used as an internal control and changes in SLPI and Annexin expression were calculated relatively to GAPDH expression. SLPI overexpression was observed in HIV tat expressing CV-1 cells relatively to the mock transfected cells. Moreover, no increase in SLPI expression was observed in CV-1 cells express SIV tat compared to the cells express empty parental vector. Indeed SLPI expression in CV-1-Htat cells was 24.2 times of mock transfected cells,whereas its expression in CV-1-Stat cells was 0.54 times of mock transfected cells. The result shows HIV-1 tat induces SLPI overexpression; however there is no significant change in the presence of SIV tat. As sequences of HIV-1 tat and SIV tat are significantly different than each other in spite of their similar function in viral cell cycle, it is not surprising that both have different effects on SLPI expression.

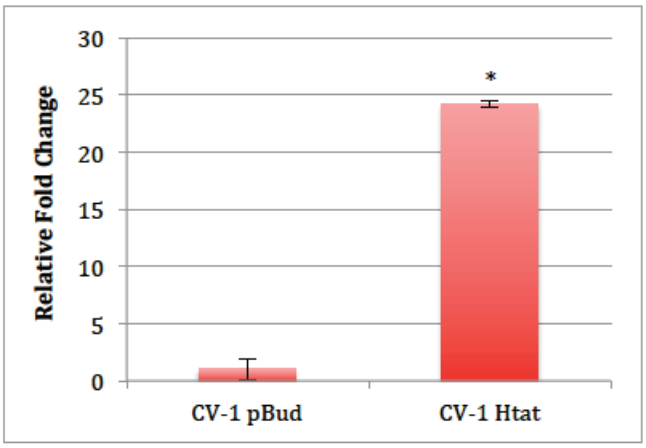

Figure 3.17. Comparison of SLPI expression in CV-1 pBud and HIV tat cell lines (*:$p<0.05$)

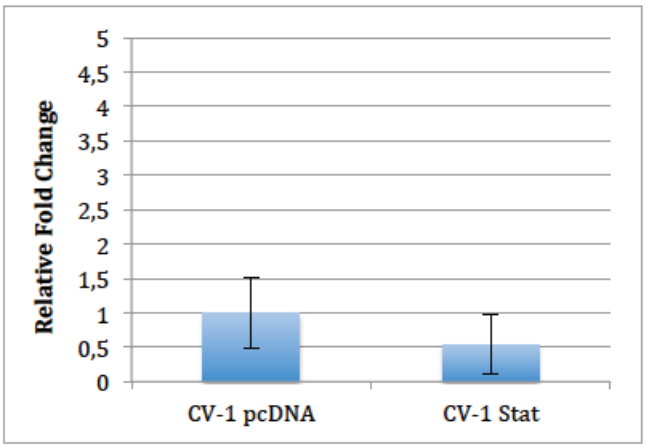

Figure 3.18. Comparison of SLPI expression in CV-1 pcDNA and SIVtat cell lines

If Annexin was concerned, neither HIV tat nor SIV tat have a significant effect in its expression. Annexin expression in CV-1-Htat and CV-1-Stat cells were 2.77 times and 0.52 times of mock transfected cells, respectively.

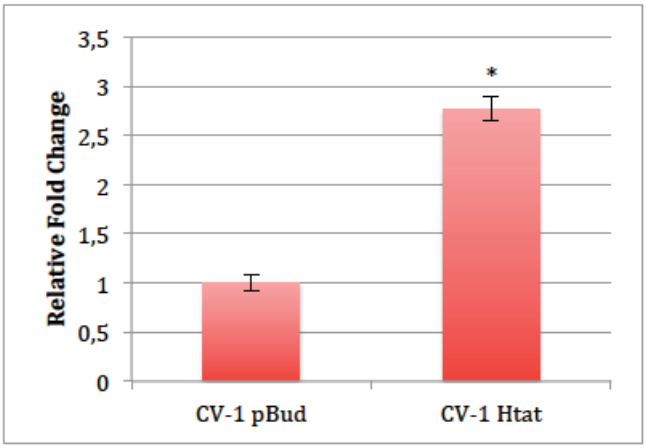

Figure 3.19. Comparison of Annexin-2 expression in CV-1 pBud and HIV tat cell lines (*:$p<0.05$)

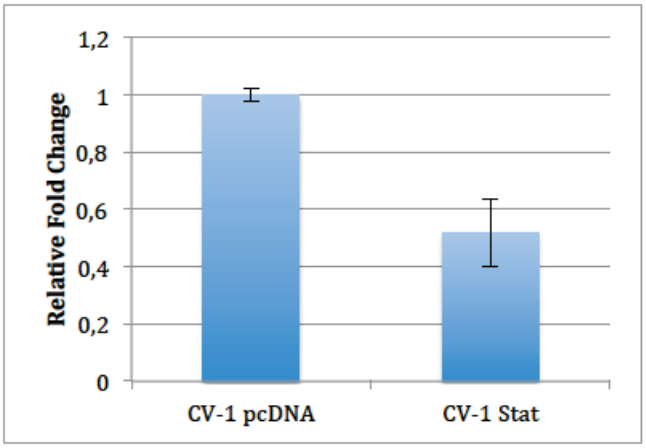

Figure 3.20. Comparison of Annexin-2 expression in CV-1 pcDNA and SIV tat cell lines

Also Q-PCR samples were loaded on agarose gel to check whether they are at correct size. As a result of agarose gel electrophoresis, bands of PCRs with GAPDH gave 258 bp, with SLPI gave 245 bp and with Anx2 gave 134 bp bands as in Figure 3.21.

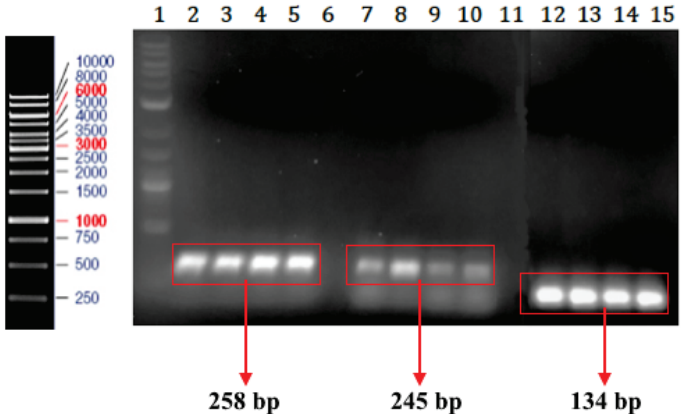

Figure 3.21. Agarose gel electrophoresis of Q-PCR samples.

Lane 1: GeneRuler 100 bp DNA ladder (Thermo Scientific), Lanes 2,3,4:5: pBud-, Htat-, pcDNA- and Stat-CV-1 with GAPDH primers, respectively, Lanes 7,8,9,10:, pBud-, Htat-, pcDNA- and Stat-CV-1 with SLPI primers, respectively,and Lanes 12,13,14,15: pBud-, Htat-, pcDNA- and Stat-CV-1 with Anx2 primers, respectively.

3.6. Analysis of SLPI Protein Expression with Western Blotting

After total protein isolation was done and protein concentrations were determined via Braford assay, 40 μg protein from CV-1 cells transfected with pBudCE4.1 plasmid as a negative control and CV-1 cells stably expressing HIV-1 Tat protein were loaded on a SDS gel with 15% separating and 5% stacking gels. SDS gel was run and proteins were transferred onto PDVF membrane as described before. After blocking, washing, and incubation with primary and secondary antibody were performed, membrane was visualized. As a result of visualization, Tat protein was observed in lysate of cells stably transfected with pBudCE4.1-Htat plasmid but not in lysate of cells stably transfected with mock plasmid. GAPDH was used as an internal control and observed, too. The results of Western blotting for GAPDH and Tat proteins is given in the Figure 3.22, below.

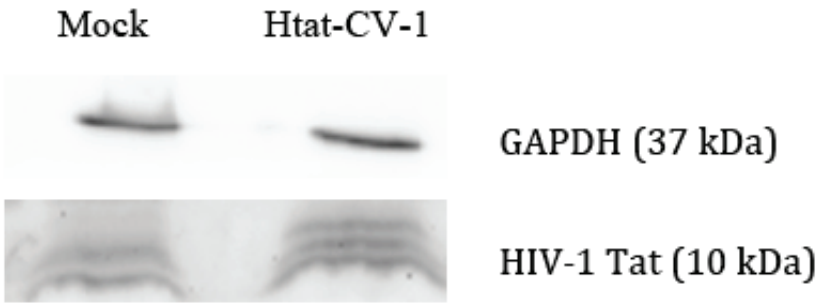

Figure 3.22. Western blot analysis of pBud-CV-1 and Htat-CV-1 cell lysates.

In order to investigate SLPI expression in protein level, pBud-CV-1, Htat-CV-1, pcDNA-CV-1 and Stat-CV-1 cells were lysed and cell lysates were analyzed with SDS-PAGE and Western blotting as described below. SLPI expression increased in the presence of HIV -1 tat, but slightly decreased in the presence of SIV tat, in accordance with Q-PCR results.

Figure 3.23. Western blot analysis of pBud-CV-1 and Htat-CV-1 cell lysates

Figure 3.24. Western blot analysis of pBud-CV-1 and Htat-CV-1 cell lysates

When Western blotting results were analyzed via ImageJ, SLPI expression increased to 5.5 fold in the presence of HIV-1 Tat and to 0.78 fold in the presence of SIV Tat in concordance with Q-PCR results.

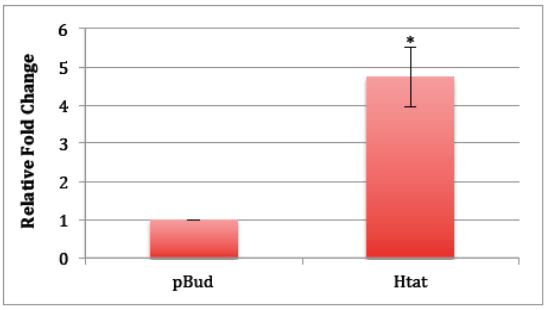

Figure 3.25. Analysis of Western blots for pBud- and Htat-CV-1 cells via ImageJ (*:$p<0.05$)

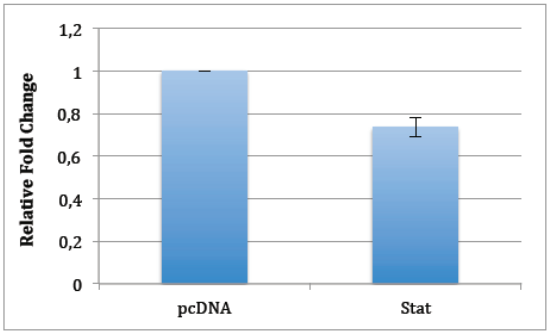

Figure 3.26. Analysis of Western blots for pcDNA- and Stat-CV-1 cells via ImageJ

3.7. Determination of the Effect of SLPIhuman Protein on HIV-LTR Promoter with Luciferase Assay

Because it is known that SLPI has an inhibitory effect on NFκB activation (Hiemstra, 2002), (Tomee et al., 1998) and HIV-1 LTR has NFκB binding sites (Li et al., 2012); it was proposed that SLPI may affect transcription from HIV-1 LTR. The effect of SLPIagm on HIV-1 LTR was reported in previous studies conducted in our laboratory, thus it was aimed to investigate the effect of SLPIhuman on HIV-1 LTR via luciferase asssay, as both protein have similar function, structure and sequence. For this reason, CV-1 cells were co-transfected with pbud-SLPIhuman and pHIV-Luc plasmids and luciferase activity was monitored 48 hour after transfection. As a mock control, cells were co- transfected with empty parental vector (pBudCE4.1) and pHIVLuc plasmids. As a positive control, luciferase activity of cells were co-transfected with pBud-SLPIagm and pHIV-Luc plasmids were also monitored. As a result, it was seen that SLPIagm decreased the luciferase activity about 165 fold; however SLPIhuman decreased about 17.5 fold. The result represented in Figure 3.27 shows that both SLPIhuman and SLPIagm have negative effects on LTR activation as expected because, HIV-LTR has 2 NFκB sites which are important for LTR activation and SLPI has a inhibitory effect on NFκB. Thus SLPI from both agm and human are able to impair HIV-1 LTR activation indirectly by acting on NFkB activation by binding to NFκB binding sites on LTR.

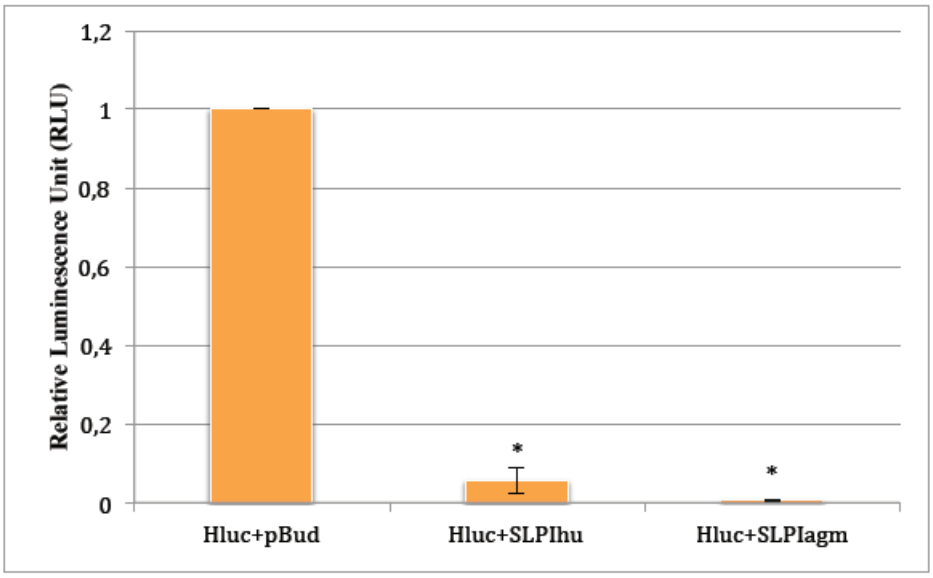

Figure 3.27. Measured luminescence values of transfected cells' relatively to CV-1 cells co-transfected with pHIVLuc and pBudCE4.1.

pBudCE4.1-SLPIhuman and pBudCE4.1-SLPIagm along with pHIVLuc transfected cells were used and measurements were done 48 h after transfection.(*:$p<0.05$)

CHAPTER 4

CONCLUSION

Recent studies indicate that Old World Monkeys including African green monkeys are resistant to HIV-1, and they do not develop AIDS-like syndrome even they can be infected by using high viral titer. Among the restriction factors determined up to now, no one acts after viral entry. Because of the fact that these primates can prevail over infection, there should be other restriction factor(s) unidentified yet.

Since Tat is one of the firstly synthesized proteins and plays an important role in transcription activation and also there are reports that have shown the effect of HIV-1 tat on host cell gene expression, it might trigger the ambiguous resistance mechanism. On this basis, Secretory leukocyte protease inhibitor (SLPI) has been nominated as a candidate for the restriction with previous studies conducted in our laboratory, because it is over expressed in the presence of HIV-1 tat.

In order to investigate SLPI expression, stable CV-1 cell lines that express HIV-1 tat and SIVagm tat proteins were established and SLPI levels were compared by using RT-PCR. Establishment of stable cell lines is important for reproducibility of experiments, so these stable cell lines were used for Q-PCR and Western blotting.

As a conclusion SLPI expression significantly increased in the presence of HIV-1 tat and slightly decreased in the presence of SIV tat. Since SIVs and their hosts have coevolved for hundreds of years, AGMs may have developed a completely different mechanism to suppress SIV than to repress HIV.

Annexin-2 is crucial for HIV infectivity and SLPI may inhibit infection by disrupting Annexin2-Gag interaction. HIV tat slightly increased Annexin2 expression, while SIV tat decreased. Thus HIV might have developed a defense mechanism by increasing Annexin-2 expression.

Also it was seen that both SLPI human and SLPI agm decreased HIV-1 LTR activation. In other words, SLPI decreases viral gene expression by downregulating LTR. So AGMs may have developed a resistance mechanism by increasing SLPI concentration to minimize viral gene expression. For the future, Annexin-2 expression should be investigated in human cells to enlighten its role in infection. Also SLPI mechanism should be

further studied in order to understand resistance mechanisms of AGMs.

Furthermore in vivo studies can help us to determine whether SLPI expression will be affected the same when cells are infected with virus, rather than transfected with Tat only.

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