Mechanisms employed by HIV and SIV to overcome the antiviral activity of BST-2/tetherin

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Abstract: English

BST-2 (tetherin/CD317/HM1.24) is an interferon-inducible cellular factor that is able to retain fully formed viral particles at the cell surface. This effectively prevents the release of a wide range of viruses including HIV-1 and SIV. Viruses in turn have evolved mechanisms to overcome this restriction, for example, HIV-1 uses Vpu to promote viral release and SIV uses Nef and/or Env. These evasion mechanisms are not fully understood and elucidating these mechanisms may lead to the development of therapies to combat AIDS and other viral infections. Our study focuses on two aspects of BST-2 antagonism: 1) mechanism employed by SIV to overcome BST-2 and 2) mapping the interaction site between BST-2 and Vpu. In part one, we demonstrate that SIV infection of COS-7 cells can downregulate simian BST-2 at the mRNA level in a species-specific manner. Further experiments indicate that this may be part of a broadly neutralizing mechanism to silence not only BST-2, but also other interferon-induced antiviral factors. In the second part of our study we use a bioluminescence energy transfer (BRET) technique to identify essential residues that contribute to the interaction between Vpu and BST-2, and viral release. Overall, this study provides important insight into mechanisms employed by viruses to evade BST-2 restriction.

Abstract: Français

BST-2 (tetherin/CD317/HM1.24) est un facteur cellulaire dont l'expression peut être induite par l'interféron et qui a la propriété de retenir les particules virales à la surface de la cellule. BST-2 est capable d'empêcher la relâche de particules virales matures et infectieuses d'un large groupe de virus incluant le VIH-1 et le VIS. Par conséquent, les virus se sont adaptés en développant leur propre mécanisme pour combattre cette restriction. Par exemple, le VIH-1 utilise la protéine virale u (Vpu). Une meilleure connaissance de ces mécanismes d'évasion est indispensable afin de permettre le développement de nouveaux médicaments pour combattre le SIDA et les autres infections virales. Cette étude est centrée sur deux aspects de l'antagonisme de BST-2: 1) les mécanismes utilisés par le VIS pour combattre BST-2 et 2) l'interaction entre BST-2 et Vpu. Dans la première partie, on démontre que l'infection des cellules COS-7 par le VIS réduit l'expression de BST-2 au niveau de l'ARNm. De plus, ce mécanisme ne semble être qu'une partie d'un mécanisme général ciblant les gènes stimulés par l'interféron (ISGs). Dans la deuxième partie de l'étude, on utilise une technique de transfert d'énergie bioluminescent (BRET) pour déterminer les résidus responsables de l'interaction entre BST-2 et Vpu. Dans son ensemble, cette étude apporte à une meilleure compréhension des mécanismes utilisés par les virus pour échapper à la restriction par BST-2.

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Publications

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Abbreviations

Ad5	Adenovirus serotype 5
agm	African green monkey
AIDS	Acquired immune deficiency syndrome
AP2	Adaptor protein 2
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
BiFC	Biomolecular fluorescence complementation
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
BST-2	Bone marrow stromal cell antigen 2
CA	Capsid
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
cdk9	Cyclin-dependent kinase 9
CMV	Cytomegalovirus
cpz	Chimpanzee
Creb	cAMP response element-binding
CXCR4	CXC chemokine receptor 4
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
Env	Envelop
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
EYFP	Enhanced yellow fluorescent protein
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde phosphate dehydrogenase
gor	Gorilla
gp	Glycoprotein
GPI	Glycosylphosphatidylinositol
HAD	HIV associated dementia
hBST-2	Human BST-2
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HTLV-1	Human T cell leukemia virus type 1
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IN	Integrase
IRF	Interferon regulatory factor

ISG	Interferon stimulated genes
ISRE	Interferon stimulated response element
kb	Kilobase
KSHV	Kaposi's sarcoma-associated herpesvirus
LAV	Lymphadenopathy associated virus
LEDGF	Lens epithelium-derived growth factor
LTNPs	Long term non-progressors
LTR	Long terminal repeat
MA	Matrix
mac	Macaques
MAP	Mitogen activated protein
MCMD	Minor cognitive motor disorder
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MPMV	Mason-Pfizer monkey virus
mRNA	Messenger RNA
MSM	Men who have sex with men
MTOC	Microtubule-organizing center
NC	Nucleocapsid
Nef	Negative factor
NES	Nuclear export signal
NLS	Nuclear localization signal
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
P-TEFb	Positive transcription-elongation factor b
PBS	Phosphate-buffered saline
PBS	Primer binding site
pDC	Plasmacytoid dendritic cells
PIC	Pre-integration complex
PPT	Polypurine tract
PR	Protease
PVDF	Polyvinylidene Fluoride
Rev	Regulator of viral gene expression
rh	Rhesus macaque
RLU	Relative light unit
Rluc	Renilla luciferase
RNA	Ribonucleic acid
RRE	Rev response element
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RTC	Reverse transcription complex
SIV	Simian immunodeficiency virus
smm	Sooty mangabey

Stat	Signal transducers and activators of transcription
SU	Surface
tan	Tantalus
TAR	Transactivation response
Tat	Transcriptional activator
TCA	Trichloroacetic acid
TGN	trans-Golgi network
TM	Transmembrane
Trim5α	Tripartite motif 5 alpha
Vif	Viral infectivity factor
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus glycoprotein

Chapter 1: Introduction

1.1 Discovery

Incidents of acquired immune deficiency syndrome (AIDS) were first reported in 1981 among homosexual men that were infected with *Pneumocystis carinii* pneumonia and had an overall weakness in their immune system [1, 2]. After this first report, there was a surge in the number of cases of rare opportunistic infections in previously healthy homosexual men [3]. The cause for this outbreak was unknown and researchers quickly went to work to identify the etiological agent of AIDS. In 1983, human immunodeficiency virus (HIV), then known as lymphadenopathy associated virus (LAV), was first proposed to be linked to AIDS [4]. This was later confirmed and we now know that HIV is the etiological agent of AIDS and can be spread through sexual contact, blood (transfusions and exchange of dirty needles) and from mother-to-child [5-7].

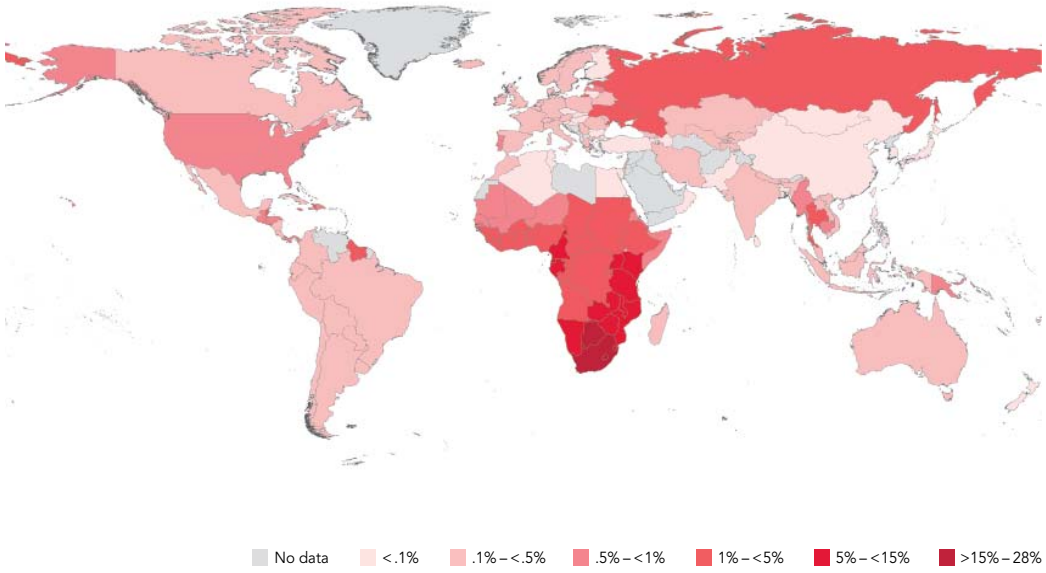
1.2 Epidemiology

HIV remains the most elusive virus in history due to its ability to establish latency early in infection and its high mutation rate allowing it to develop drug resistance. Researchers have worked tirelessly since the first identification of HIV to find a solution to the spread of the virus, but have yet to find a cure or vaccine. Once considered fatal, the medical advancements made over the last few decades have transformed AIDS into a chronic disease [8]. Nevertheless, in less developed countries where antivirals are not easily

accessible, HIV/AIDS remains a considerable health problem. As of 2010 the World Health Organization estimated 33.3 million people were living with HIV with ~2.6 million new infections in 2009 [9]. This represents a 19% decrease of newly infected individuals since the peak of the epidemic in 1999 [9]. However, there is still much work to be done as only ~35% of HIV-infected individuals are receiving treatment [9]. Sub-Saharan Africa still continues to have the highest incidence of HIV infection in the world with an increasing proportion of individuals living with HIV despite a lack of increase and, in some countries a decrease, in the number of new infections (Figure 1). In this region, most cases of infections are due to unprotected heterosexual intercourse [9]. It is estimated that sexual transmission makes up to 80% of total HIV infections with a transmission rate of about 1:1000 sexual encounters [10]. In certain countries in Eastern Europe and Central Asia the incidence of HIV is on the rise primarily among intravenous drug users, sex workers and men who have sex with men (MSM) [9]. Of note, there has been some evidence of an increase in the number of HIV infections in many high-income countries within the community of MSM [9]. This demonstrates that HIV/AIDS is a problem that is continually affecting the world and prevention programs and research towards therapies are still in need. Prevention programs are an essential element to the fight against AIDS and include education on safe sex practices, needle exchange programs, programs to increase the availability of condoms, and programs to encourage and facilitate HIV testing.

A **Global prevalence of HIV, 2009**

Source: UNAIDS.



B **Changes in the incidence rate of HIV infection, 2001 to 2009, selected countries**

Source: UNAIDS.

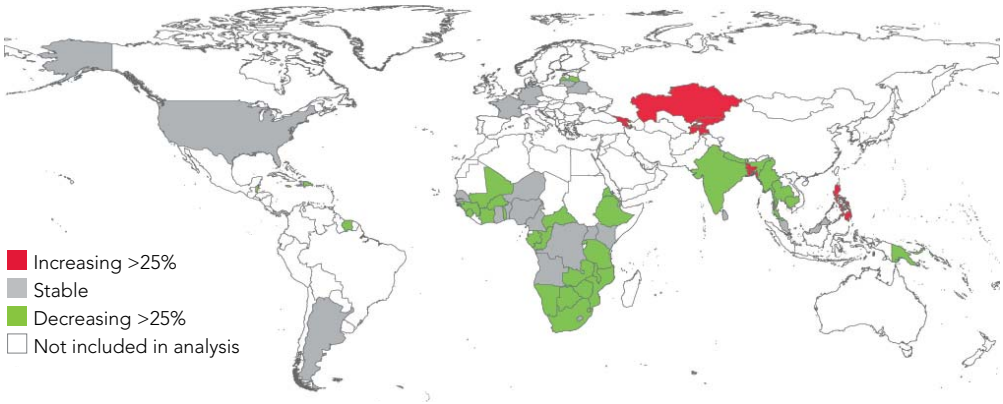


Figure 1 World map (A) Global prevalence of HIV and (B) Change in incidence rate of HIV infection [9].

1.3 Classification, evolution and origins of HIV

HIV is classified under the family of *Retroviridae* and the genus of lentiviruses due to its ability to cause disease after long incubation periods [11]. Lentiviruses may be further divided into five groups according to the target host, which are ovines-caprines, bovines, felines, equines and primates [11]. Since the first identification of HIV-1, a second virus, HIV-2 was isolated and also identified to cause AIDS in rare instances [12, 13].

The origins of HIV-1 have been traced back to several independent transmission events from SIV in chimpanzees (SIVcpz) to humans that eventually gave rise to HIV-1 pandemic group M (main), rare group N (non-M, non-O) and non-pandemic group O (outlier) [14]. A recently identified HIV-1 group P likely arose from transmission from SIV in gorillas (SIVgor) to humans [15]. HIV-1 can further be divided into nine clades/subtypes (A-D; F-H; J-K) of which subtype C is the most globally prevalent [16]. HIV-2 originated from cross species transmission of SIV in sooty mangabeys (SIVsmm) to humans [17, 18]. Zoonotic transmission of SIVsmm to Asian macaques also gave rise to pathogenic infections in macaques [18] (Figure 2).

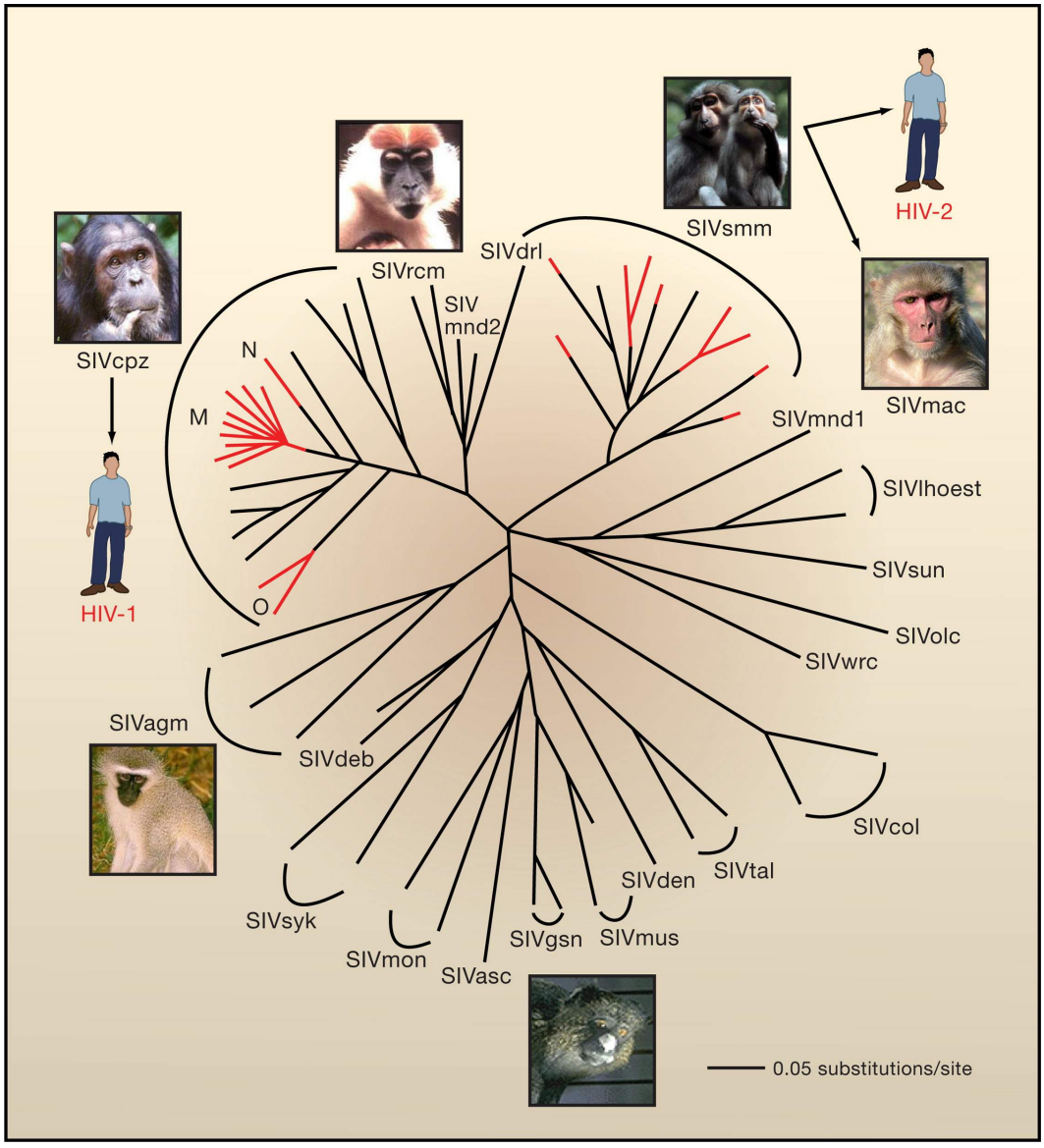


Figure 2 Phylogenic relationship of SIV and HIV species [18].

1.4 Pathogenesis

1.4.1 Pathogenesis of HIV infection

The two major cellular targets of HIV-1 are T lymphocytes (T cells) and macrophages. Infection of T cells usually results in cytopathic effects while infected macrophages may continue to survive for months *in vivo* and *in vitro* [19]. Once HIV-1 infects a host there is a rapid surge of virus replication that occurs in the first few weeks following infection. This is the acute phase of infection and infected individuals may only display flu-like symptoms. As T cells are activated and begin to clear the viruses, viremia decreases and eventually reaches a steady-state termed the "set point", which may vary from one individual to another. An individual's set point may be a predictor of progression to AIDS [18, 20]. This is the chronic phase of infection and usually occurs weeks after the acute phase [16]. During this time the infected individual is asymptomatic and HIV-1 replication is taking place a low levels [16]. This chronic phase may last up to 10 years or more before the infected individual displays signs of AIDS including decreased $CD4^+$ T cell counts (<350cells/μl), loss of immune activity and destruction of lymphoid tissue [16]. This leaves the individual susceptible to opportunistic infections and viral co-infections including *Mycobacterium tuberculosis*, cryptococcal infection, hepatitis C virus (HCV), cytomegalovirus (CMV), etc [21]. If left untreated this can become fatal. In addition to causing AIDS, HIV-1 is able to cross the blood brain barrier soon after initial infection resulting in

the development of motor and cognitive deficiencies referred to as HIV-associated dementia (HAD) and minor cognitive motor disorder (MCMD) [22-25]. HIV-2 is also capable of causing AIDS, but only in a minority of infected individuals [26].

1.4.1 Nonprogressive vs progressive viral infections

SIV infection of its natural host does not result in progression to AIDS despite long term infection and the presence of high viral loads (termed nonprogressive infection) [27]. These include SIV infections of African green monkeys (SIVagm) and Sooty mangabeys (SIVsmm). On the contrary, SIV infection of Asian macaques (a non-natural host) and HIV-1 infection of humans results in a chronic infection and usually results in AIDS (progressive infection) [27]. Unlike SIV infection of natural hosts, progressive SIV and HIV infection induces high levels of immune activation during the chronic phase of infection [27]. Interestingly, SIV infection of natural hosts results in strong innate immune responses in the acute phase of infection including production of high levels of interferon and upregulation of interferon-stimulated genes (ISGs), however this level of immune activation is rapidly resolved [28, 29]. Additionally, SIVagm and SIVsmm are able to induce disease in a non-natural host indicating that these viruses are indeed capable of pathogenicity in the absence of blocks present in their natural hosts. It is likely that the evolution of the virus and the host has resulted in a symbiotic relationship. An understanding of the SIV infection in its natural host will

hopefully give insight into the mechanism of disease progression in HIV-1-infected humans.

Many studies have attempted to elucidate the determinants of the different immune responses in progressive and nonprogressive infection but none have provided a clear explanation. One hypothesis is that there is an increase in microbial translocation in progressive infection that leads to chronic immune activation that is not observed in nonprogressive infections and depletion of Th17 cells from the gastrointestinal tracts in progressive infections may be a contributing factor [27]. Another theory suggests that natural hosts have a lower percentage of $CCR5^{+}CD4^{+}$ T cells (the target of the virus) and therefore depletion of these cells would be of little consequence to the host [27]. In this case, immunological function would be performed by another subset of T cells that are resistant to SIV infection.

Interestingly, a small population of HIV-infected individuals (~2-5%) known as "long term non-progressors" (LTNP) may never develop AIDS despite being treatment naïve [30]. These individuals are able to control the virus early in infection maintaining low viral loads (<10 000 copies/ml) and high $CD4^{+}$ T cell counts throughout the chronic phase of infection [30]. A subset of LTNPs known as "elite controllers" sustain levels of viral RNA below the limits of detection (<50 copies/ml) for months to years [30]. One explanation for this phenomenon is that LTNPs are infected with an attenuated virus that have deletions or alterations in the sequence that

causes it to be less fit. Another explanation proposes that LTNPs have polymorphisms in the target receptor that impairs virus entry, however more research is required to understand this phenomenon [30].

1.5 Treatment and vaccine development

1.5.1 Anti-retroviral therapy

The introduction of highly active antiretroviral therapy (HAART) has provided an effective therapy to substantially reduce viremia and have changed AIDS from being a fatal disease to a chronic disease in most developed countries. However, much of these new therapies are costly and unattainable in developing countries where HIV-1 infection is the most prevalent.

There are approximately 30 antiviral products available to treat HIV-infected patients [8]. The first antiviral drugs consisted of dideoxynucleosides called nucleoside reverse transcriptase inhibitors (NRTIs). This class of drugs binds to the catalytic site of reverse transcriptase (RT) and competitively inhibits reverse transcription resulting in chain termination [8]. The NRTI 3'-azido-2',3'-dideotythymidine (AZT) was the first therapy available for HIV-infected patients [31]. Following the NRTIs, nonnucleoside reverse transcriptase inhibitors (NNRTIs) were discovered, which bind to a distinct site on RT separate from the catalytic site. Subsequently, inhibitors that targeted other steps in the viral life cycle were produced including steps in entry, maturation and integration [8].

One major downfall to HAART is the ability of the virus to develop resistance as a result of the low fidelity of RT. Mathematical calculations predicted that using mono- or dual- therapy, the number of mutations that arose would quickly produce resistant viruses, however the likelihood of sufficient mutations to simultaneously arise to overcome three or more therapies was highly unlikely [18, 32]. This became the basis for HAART that now uses a combination of three or more drug therapies. Even still, drug resistance continues to be a problem and there is a continual need for new and more effective drugs.

1.5.2 Vaccine development

Researchers have been working towards a vaccine against HIV-1 since its discovery in the early 1980s but have failed to produce an effective HIV-1 vaccine. Challenges impeding the development of an HIV-1 vaccine include the diversity and adaptability of the virus, lack of broadly reactive neutralizing antibodies, and ability of the virus to evade immune responses [33].

Over 30 potential HIV-1 vaccines have gone into clinical trials since 1987 [34]. Vaccine strategies that have been pursued include HIV-1 peptides to induce neutralizing antibodies, recombinant viral vectors that express HIV-1 antigens, and HIV-1 DNA plasmids [34, 35]. Two phase 2b/3 trials, the STEP study sponsored by Merck and the Phambili trial sponsored by the NIH, used a replication-incompetent recombinant adenovirus serotype 5 (Ad5)

vector expressing HIV-1 Gag, Pol and Nef [36, 37]. Unfortunately both trials failed to elicit protective immunity and even increased the number of HIV-1 infections in individuals with pre-existing Ad5 neutralizing antibodies [33]. More recently a phase 2b/3 trial in Thailand involving over 16,000 individuals and sponsored by the US Army Medical Research and Material Command and the US National Institute of Allergy and Infectious Diseases, reduced the number of HIV-1 infections by 31.2% [38]. This vaccine was based on a primer recombinant canarypox vector vaccine (ALVAC-HIV [vCP1521]) that was boosted with a recombinant gp120 vaccine (AIDSVAX B/E) [38]. The vaccine, however, did not influence the viral load or $CD4^+$ T cell counts in HIV-1 infected subjects. Although the results are promising, they are modest and there continues to be ongoing efforts to create a more fully protective HIV-1 vaccine.

1.6 Structure and genome of HIV

The HIV-1 genome is approximately 9kb in length and contains nine genes that encode 15 proteins (Figure 3) [39]. These include Gag, Pol and Env and six accessory proteins named Nef, Vif, Vpr, Vpu, Tat and Rev. Gag and Env encode the structural elements of the of the virus, Pol encodes the enzymatic proteins of the virus, which include RT, protease (PR) and integrase (IN). The six accessory proteins encode regulatory elements required for effective viral production, and proteins required to evade host defense mechanisms *in vivo*. In addition to these nine genes, the HIV genome contains a 5' and 3' long terminal repeat (LTR). The LTR is further divided into U5, R and U3 regions

whose sequences are important for transcriptional initiation and regulation as well as integration of the viral genome into the host DNA [40].

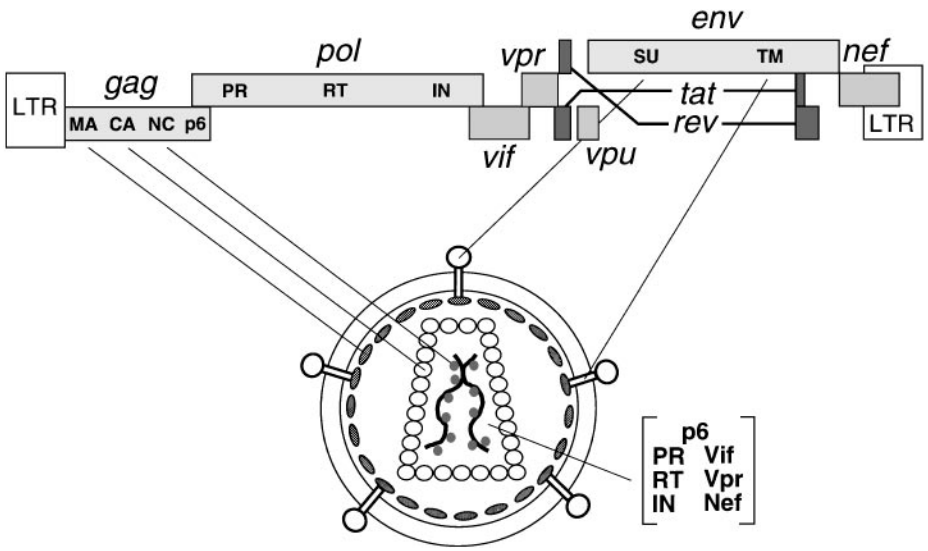


Figure 3 Organization of the HIV-1 genome and virion [39].

The Gag protein is transcribed to create the $Pr55^{gag}$ polyprotein that is later cleaved by protease to form matrix (MA/p17), capsid (CA/p24), nucleocapsid (NC/p7) and p6 [39, 41]. The MA protein covers the inner surface of the virion membrane (Figure 3). CA makes up the core of the virus, which consists of an asymmetric cone containing two single-stranded RNA (ssRNA) genomes that form a dimer, and other viral and cellular proteins. NC binds to the RNA genome within the core formed by CA. The Env protein is produced as the gp160 polypeptide precursor and is cleaved in the ER to form the gp120 (SU) and gp41 (TM) subunits [39, 41]. The two subunits interact with each other noncovalently and are located on the viral membrane as trimers. The gp41 subunit spans the membrane and gp120 sits

on the surface of the membrane. They are required for recognition of the host receptor and co-receptor to initiate entry into a target cell.

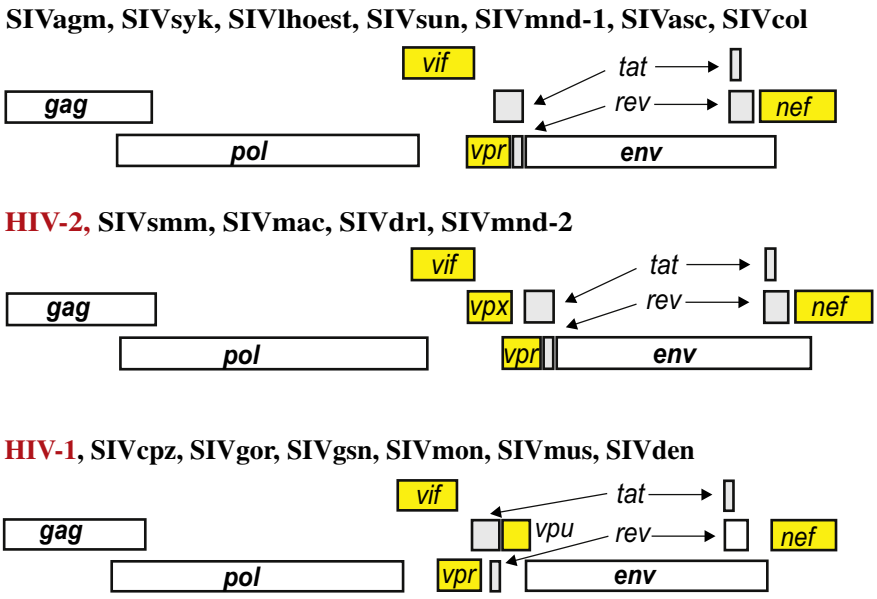


Figure 4 Genomic organization of HIV and SIV [11]

The genomes of various SIV strains and HIV-2 are structured similarly to HIV-1 but do differ in certain areas (Figure 4). For example, HIV-2, SIV from SIVsmm and SIVmac contain a *vpx* gene that is thought to have originated from a duplication of *vpr* or from acquisition of a heterologous *vpr* gene through recombination events [42, 43]. The *vpu* gene is thought to have been acquired by an ancestor of the current SIV found in *Cercopithecus* monkeys. This SIV is thought to have recombined in a chimpanzee host with another SIV from red-capped mangabeys to form the current vpu-containing SIVcpz [11]. This SIVcpz subsequently spread to gorillas and humans creating SIVgor and HIV-1, respectively.

1.7 HIV-1 Life Cycle

1.7.1 Entry

Infection is initiated by the recognition of the host CD4 receptor by gp120 where it then undergoes a conformational change allowing it to recognize a second receptor referred to as a co-receptor [44]. The association with a co-receptor is required for membrane fusion to take place. Although many different chemokine receptors may be used as co-receptors by HIV-1, the CCR5 and CXCR4 co-receptors are used most frequently [45]. Viruses that use the CCR5 co-receptor (R5 viruses) are macrophage-tropic and those that use the CXCR4 co-receptor are T cell-tropic viruses (X4 viruses). X4 viruses tend to emerge late in infection and are associated with rapid progression to AIDS while R5 viruses initiate infection either through the mucosa or intravenously [16]. Following the recognition of the co-receptor, gp41 becomes exposed and inserts itself into the target cell thereby bringing the two membranes together. Although direct fusion of HIV-1 to the target cell has long been considered the principle mechanism of entry, recent studies have provided evidence that the virus may also use endocytosis to gain entry into the cell [46, 47].

1.7.2 Uncoating and reverse transcription

Following viral entry, the viral core is released into the cell and uncoating of the viral capsid occurs. Whether uncoating occurs soon after entry near the plasma membrane or closer towards the nucleus and if it

occurs before, during or after reverse transcription is unclear [48]. One model proposes that the viral capsid remains intact until reaching the nuclear pore and after reverse transcription has been completed. In this model the capsid serves as a means to keep the concentration of RT high around the template and prevent diffusion of the enzyme through the cytoplasm thereby increasing the efficiency of reverse transcription [48]. At the same time the capsid would still allow the diffusion of important small molecules into the capsid such as nucleotide triphosphates [48].

Whether reverse transcription occurs before, during or after uncoating it is nevertheless a crucial step of the viral life cycle and takes place in the cytoplasm within the reverse transcription complex (RTC). The RTC contains MA, Vpr, RT, IN and the two ssRNA viral genomes [49, 50]. Reverse transcription allows the virus to convert its ssRNA genome to double-stranded DNA (dsDNA) and this is critical for the viral genes to be transcribed and translated by the host cell. The star of this process is the RT enzyme, which actually contains two enzymatic domains. RT is able to act as a DNA polymerase that can use both RNA and DNA as the template and it also has RNase H activity that degrades RNA in RNA-DNA complexes. However, special regions called polypurine tracts (PPTs) contain a unique sequence and structure that prevents it from being degraded by the RT's RNase H activity [51]. Before reverse transcription can begin, cellular $tRNA^{Lys3}$ must bind to the primer-binding site (PBS) located at the 5' end of the RNA template (Figure 5). RT then is able to initiate the synthesis of the minus-

strand DNA from the $tRNA^{Lys3}$ up until the 5' cap of the RNA. This forms the minus-strand strong-stop DNA [(-)sssDNA]. The RNase H activity of RT degrades the RNA in the RNA:DNA duplex and this is followed by the first strand transfer where the (–)sssDNA binds to the complementary R sequence in the 3' LTR of the genomic RNA. From here the minus-strand DNA is elongated and the RNA template is degraded with the exception of the PPT. The PPT serves as the primer for the synthesis of the positive-strand DNA and forms the positive-strand strong stop DNA [(+)sssDNA]. The PPT RNA is degraded and the second strand transfer now occurs where the (+)sssDNA aligns its PBS with the PBS of the 3' end of the minus-strand DNA. The rest of the sequences are synthesized and the result is a dsDNA genome. Upon the completion of reverse transcription the pre-integration complex (PIC) is formed which includes the dsDNA, IN, MA, Vpr, RT and other cellular proteins [41, 52, 53].

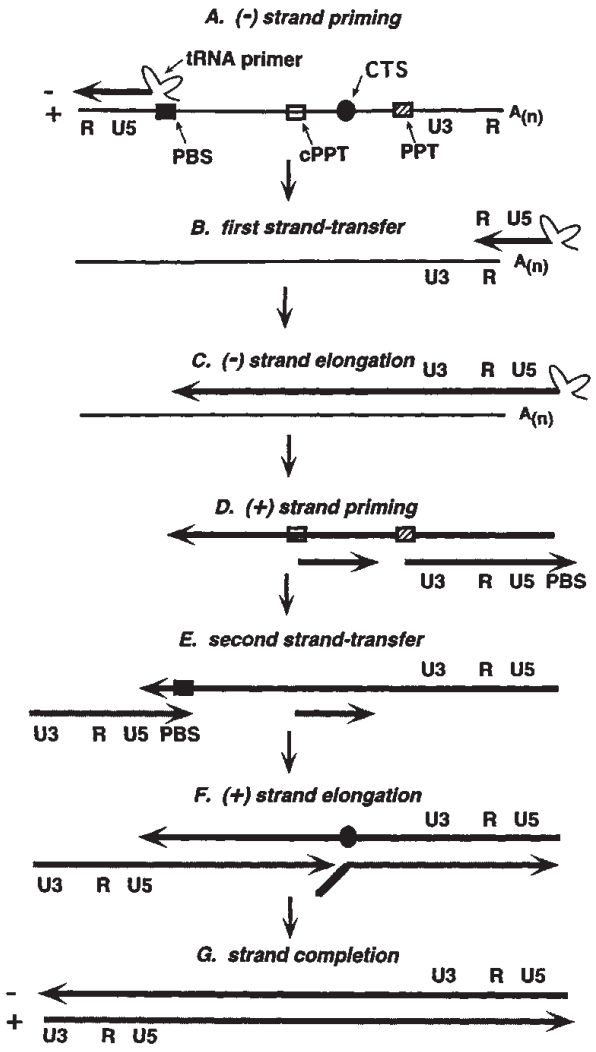


Figure 5 HIV-1 Reverse Transcription [54].

1.7.3 Nuclear import and integration

HIV-1 is able to infect both dividing and non-dividing cells including terminally differentiated macrophages which means HIV-1 has a mechanism to enter the nucleus of non-dividing cells [55]. The PIC is larger than a nuclear pore and therefore cannot simply enter the nucleus by diffusion [50]. This necessitates a mechanism of nuclear import that would allow the virus

to introduce its genomic material to the host transcriptional machinery. Although the mechanism of nuclear import is not well understood it has been proposed to require help from MA [56, 57], vpr [58, 59], IN [60] as well as many cellular factors [50]. There is evidence, which indicates that PICs traffic to the nucleus by using microtubule networks and have been reported to accumulate at the microtubule-organizing center (MTOC) located near the nucleus [61].

Integration is initiated when IN binds to the LTR at both ends of the viral DNA and catalyzes the cleavage of a dinucleotide from each end [62, 63]. This process is known as 3'-processing and the end result is a two-nucleotide (CA) overhang at either ends of the viral DNA. DNA strand transfer then takes place where IN catalyzes two transesterification reactions between the exposed 3'OH groups of the viral DNA and the phosphodiester bond in the target host DNA and joins the 3' ends of the viral DNA to the host DNA [64]. Host cellular machinery then fills in the remaining gaps between the 5' viral DNA and host DNA and integration is complete. Several cellular proteins have been demonstrated to be involved in the integration process including the integrase-interactor lens epithelium-derived growth factor (LEDGF)/p75 [65].

1.7.4 Transcription and RNA export

Once integrated into the host genome the virus may remain unexpressed (latent infection) or transcription of its genome may begin

(productive infection). The triggers that determine whether the integration results in a latent or productive infection are not well understood but may have a relation with the chromosome environment surrounding the site of integration [66]. The 5' LTR also contains many regulatory sequences that control transcription of the viral genome including the TATA box and promoter and enhancer sequences. The HIV-1 LTR is normally a poor promoter and only a small number of transcripts are produce at the beginning. The Tat protein that is produced early in the viral life cycle enhances transcription. This occurs when Tat along with cyclin T1 binds to the transactivation response (TAR) element located in the 5'LTR [67, 68]. This then recruits cellular proteins including cyclin-dependent kinase 9 (cdk9) to form the positive transcription-elongation factor b (P-TEFb) complex and results in the phosphorylation of RNA polymerase II, which then enters into the elongation step [67, 68].

To regulate its gene expression the virus employs alternative splicing and produces three forms of mRNA transcripts: unspliced (9kb), singly spliced (4-5kb) and multiply spliced (2kb) [69]. The multiply spliced mRNA is produced first and is transported to the cytoplasm giving rise to Nef, Tat and Rev (the early genes) [70]. The singly spliced and unspliced transcripts are retained in the nucleus and are only exported to the cytoplasm later in the viral life cycle [70]. The full-length transcript produces both Gag and Gag-Pol proteins and the singly spliced transcripts give rise to Env, Vpu, Vpr and Vif.

Multiply spliced RNA is exported by cellular machinery, but Rev is critical for the export of singly spliced and unspliced mRNA [71]. As such, the export of singly spliced and unspliced RNA does not occur until sufficient Rev has accumulated from the multiply spliced mRNA. Rev contains both a nuclear localization signal (NLS) and a nuclear export signal (NES), which allows it to shuttle in and out of the nucleus [72]. Once inside the nucleus it will bind to an RNA stem loop called the Rev response element (RRE) that is located within the *env* gene. This promotes the export of singly spliced and unspliced transcripts into the cytoplasm via the CRM1/exortin-1 pathway [41]. The balance of multiply spiced, singly spliced and unspliced transcripts is essential to a productive infection.

1.7.5 Virus assembly, release and maturation.

Assembly of the virus occurs at the plasma membrane in T cells and is thought to occur in intracellular compartments in macrophages [19]. The Gag polyprotein drives viral assembly on the plasma membrane and is specifically targeted to lipid rafts due to a myristylated N terminus and basic residues in MA [73]. This is followed by multimerization of the Gag polyproteins through interactions of CA forming spheres at the plasma membrane [74]. NC binds to a packaging signal (ψ) on the viral genomic RNA allowing the incorporation of the viral genome into the viral particle [74, 75]. The p6 protein then recruits endosomal sorting complexes required for transport (ESCRT) to the site of assembly which is necessary for the virus to bud off from the host membrane [75]. Maturation requires PR that cleaves

the Gag polyprotein to form the individual MA, CA, NC, p6 and results in significant morphological changes to the virus [74, 75]. This process may occur during or after budding and is required to create infectious particles [74]. Once budding and maturation is complete the virus is free to infect other cells and may do so by cell-free spread, but more commonly proceeds using cell-cell spread through virological synapses [76].

1.8 Host restriction factors

Restriction factors are cellular proteins that are able to interfere with the successful replication of the virus. There are several restriction factors that have been discovered to impede retroviral replication and three that have made a great impact on the field of virology. They are APOBEC3G [77], Trim5α [78] and recently identified BST-2 (tetherin/CD317/HM1.24) [79, 80]. Their expression is induced by interferon and they are therefore called interferon-stimulated genes (ISGs). Although these proteins represent potent blocks of distinct steps in the virus life cycle, the virus in turn has evolved mechanisms to overcome this restriction. For example, APOBEC3G is a cytidine deaminase that converts deoxycytidine to deoxyuridine and results in the introduction of lethal mutation during reverse transcription [11]. However, the presence of the HIV-1 protein Vif overcomes this restriction by inducing the proteosomal degradation of APOBEC3G [81]. Trim5α from monkey cells is able to interfere with the uncapping of the incoming HIV-1 particle thereby imparting resistance to HIV-1 infection, but human Trim5α is unable to target HIV-1 [78]. Finally, BST-2 has been shown to interfere

with virus release and as it is the focus of this study, will be reviewed in detail.

1.9 Bone marrow stromal cell antigen 2

1.9.1 Discovery

BST-2 was first identified as a marker of terminally differentiated normal and neoplastic B cells and was proposed to be used as a target for treatment of multiple myeloma [82, 83]. Over a decade after its first discovery, a proteonomics screen identified BST-2 as a target of the K5 protein from Karposi's sarcoma-associated herpesvirus (KSHV), which triggered the downregulation of BST-2 from the cell surface [84]. This was the first clue that indicated BST-2 may function as an antiviral protein. Vpu has long been known to be required in certain restrictive cell types (such as HeLa) but not others (such as COS-7) for efficient virus release, which pointed to the presence of an inhibitor of virus release in these restrictive cell types [85-87]. An explanation for this phenotype was revealed in 2008 when two studies reported the ability of BST-2 to restrict HIV-1(Vpu$^-$) release that could be rescued by Vpu [79, 80].

1.9.2 Structure and function

BST-2 is constitutively expressed in mature B cells, bone marrow stromal cells, macrophages and plasmacytoid dendritic cells (pDCs) [83, 88-90]. The promoter of BST-2 contains an interferon-stimulated response element (ISRE) and STAT3 binding sites allowing its expression to be

induced by interferon [83, 88]. BST-2 can be found at the trans-Golgi network (TGN), at the plasma membrane in lipid rafts and in recycling endosomes, and is thought to traffic between these compartments [91, 92]. Lipid rafts are also the main assembly and budding sites of HIV-1 [93].

BST-2 has been shown to "tether" fully formed viral particles to the cell surface as well as to link viral particles to each other [79, 80]. This antiviral activity of BST-2 targets a wide range of viruses including HIV, SIV, alpharetroviruses (RSV), betaretroviruses (MPMV), gammaretroviruses (HTLV-1), filoviruses (Marburg and Ebola), arenaviruses (Lassa) and herpesviruses (KSHV) [79, 80, 94-97]. BST-2 is a type II membrane protein with an N-terminal cytosolic domain, followed by a transmembrane domain, an extracellular coiled-coil domain and a C-terminal glycosylphosphatidyl-inositol (GPI) anchor [91] (Figure 6A). The N-terminal cytosolic domain contains a dual tyrosine motif (Y_6Y_8) that is required for the AP1/AP2-dependent clathrin-mediated endocytosis [98]. The coiled-coil domain contains two N-glycosylation ($N_{65}N_{92}$) sites and three cysteines ($C_{53}C_{63}C_{91}$). These cysteines may form disulfide bonds to stabilize the homodimerization of BST-2 and formation of at least one disulfide bond is essential to restrict viral release [99-101]. The presence of both a transmembrane domain and a GPI anchor makes this protein atypical, as the prion protein is the only other mammalian protein known to exhibit this topology [91].

There are two predominant models to explain how BST-2 is able to

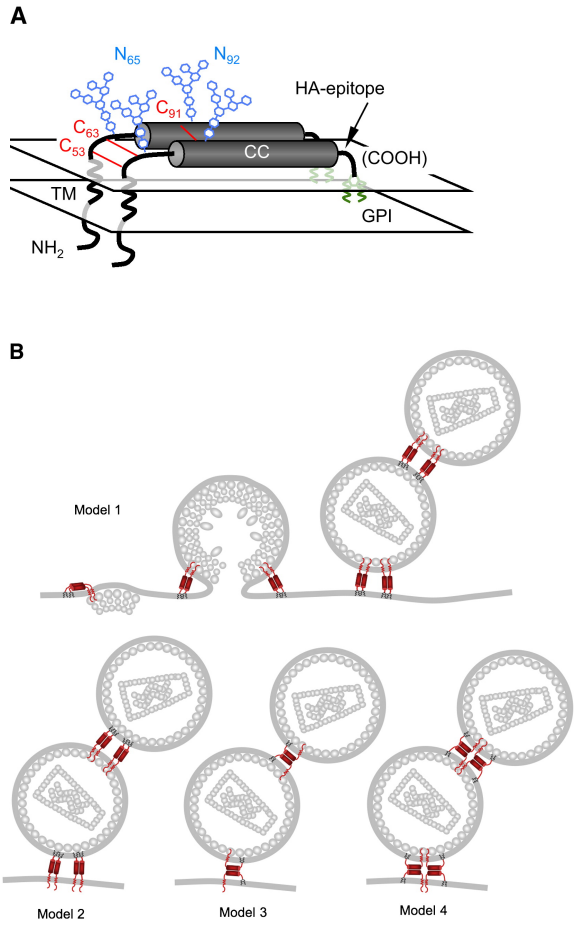


Figure 6 Structure and function of BST-2. (A) Diagram depicting the structure and dimerization of BST-2. (B) Model 1 and 2 depicting BST-2 acting as a physical bridge by inserting one end of the protein into the cell membrane and the other end into the viral membrane. Model 3 and 4 showing the physical bridge forming primarily through interactions between the extracellular domains BST-2 proteins. Figure adapted from [100].

tether viral particles to the cell surface and to each other (Figure 6B). Both models suggest that BST-2 acts as a physical tether of the viral particles. The first model proposes that a BST-2 homodimer inserts one end of the protein

into the viral particle while the other end remains attached to the host cell surface thereby linking the viral particle to the cell surface [79, 100]. A recent X-ray crystallography structure of the extracellular domain of BST-2 demonstrates that the dimers form in parallel [102]. In an alternative model BST-2 on the cell surface and BST-2 incorporated into the viral membrane would interact through their extracellular domains and become stabilized through the formation of disulfide bonds [99-101, 103]. Immuno-electron microscopy data have shown that BST-2 is incorporated into viral particles and is present at viral budding sites on the plasma membrane [92, 103, 104]. BST-2 has also been detected between viral particles as well as between the host plasma membrane and the viral particle, supporting the role of BST-2 as a physical tether of viral particles [103, 104]. The result is a marked accumulation of HIV-1(Vpu$^-$) viral particles on the surface of BST-2 expressing cells as visualized by electron microscopy [79, 100, 103]. Interestingly, an "artificial" BST-2 comprised of the transmembrane domain of the transferrin receptor, the coiled coil domain from the dystrophia myotonica protein kinase and the GPI anchor from urokinase plasminogen activator receptor was able to effectively prevent viral release despite the lack of sequence identity to BST-2 [100]. This supports the mechanism of a physical tether and suggests that a cofactor is not involved.

1.9.3 Evasion of BST-2 restriction

Viruses have evolved different strategies to evade BST-2 restriction and many viruses encode BST-2 antagonists including: Vpu from HIV-1,

SIVcpz and SIVgor; Env from HIV-2, SIVtan and Ebola; Nef from SIV; and K5 from KSHV (summarized in Table 1). The evasion mechanisms used by HIV-1 and SIV will be highlighted below.

Vpu is an 81 amino acid membrane protein that is required for effective virus release in a cell type dependent manner. It also induces the proteasome degradation of CD4 in the ER and therefore prevents CD4 expression on the cell surface [105]. Most recently, HIV-1 Vpu was shown to overcome BST-2 restriction and was observed to downregulate BST-2 from the cell surface as well as deplete levels of intracellular BST-2 [79, 80]. To promote viral release Vpu may induce the degradation of BST-2 via the proteosomal and/or the endolysosomal pathway or Vpu may sequester BST-2 at the TGN thereby preventing BST-2 from reaching its sites of action (Figure 7) [89, 92, 106-112]. Importantly, Vpu acts in a species-specific manner and can only overcome the restriction of human BST-2. Extensive domain switching experiments have shown that this activity of Vpu requires its transmembrane domain, and the transmembrane domain of BST-2 is required for susceptibility of BST-2 to Vpu [108, 113-115]. Although some SIV strains express Vpu (including SIVcpz and SIVgor), only Vpu proteins from SIVgsn/mus/mon are able to antagonize BST-2 [116]. Therefore, the ability of SIV to overcome BST-2 restriction is likely provided by another viral protein. Indeed, it has been shown that several SIV strains including SIVmac, SIVsmm, SIVblu and SIVagm use Nef to antagonize BST-2 [117, 118].

Tetherin antagonists	Tetherin surface downregulation	Tetherin intracellular depletion	Target domain in tetherin	Intracellular relocalization of tetherin	Species specificity	References
HIV-1 Vpu	Yes	Yes	TM domain	TGN	Yes	Neil et al. (2008), Van Damme et al. (2008), Mitchell et al. (2009), Goffinet et al. (2009), Douglas et al. (2009), Mangeat et al. (2009), Iwabu et al. (2009), Dubé et al. (2010)
SIVcpz/gor Vpu	?	?	?	?	Yes	Sauter et al. (2009), Yang et al. (2010b)
SIVgsn/mus/mon Nef	?	?	Cytoplasmic tail (G/DDIWK)	?	Yes	Sauter et al. (2009), Yang et al. (2010b)
SIVmac/smm Nef	Yes	?	Cytoplasmic tail (GDDIWK)	?	Yes	Jia et al. (2009), Zhang et al. (2009)
SIVagmSab/Tan Nef	?	?	Cytoplasmic tail (DDICK)	?	Yes	Zhang et al. (2009)
HIV-2 Env	Yes	No	Ectodomain	TGN	No	Le Tortorec and Neil (2009), Jia et al. (2009), Hauser et al. (2010), Abada et al. (2005)
Ebola GP	No	No	?	?	No	Kaletsky et al. (2009), Lopez et al. (2010)
SIVtan Env	Yes	No	Ectodomain	Perinuclear region	No	Gupta et al. (2009b)
KSHV K5	Yes	Yes	Cytoplasmic tail(K18)	Late endosomes	Yes	Mansouri et al. (2009), Pardieu et al. (2010)

Table 1 Summary of viral antagonists towards BST-2 (tetherin) [119].

Nef (negative factor) is a membrane-associated protein that is critical to the pathogenesis of the virus [120]. It is known to downregulate several cellular proteins from the cell surface including CD4 and MHC class I [120]. Recently, Nef from SIV was shown to deplete cell surface expression of BST-2 in a species-specific manner [117, 118]. The sensitivity of simian BST-2 to SIV Nef has been mapped to a region in the cytoplasmic domain ($_{14}$G/DDIWK$_{18}$) of simian BST-2 that has been lost in human BST-2 [117, 118]. This may explain why HIV-1 has evolved the use of Vpu to antagonize BST-2 and it is interesting to note that Nef proteins from HIV-1 and HIV-2 are able to counteract BST-2 from Old World monkeys (Rhesus macaque and Sooty mangabey), which contain the $_{14}$G/DDIWK$_{18}$ target sequence for Nef [117].

The mechanism used by Nef to overcome BST-2 restriction is not well characterized but mutations in the myristoylation site and cholesterol recognition motif in Nef impairs its ability to antagonize BST-2 [117, 118].

In addition to Nef, the Env protein from SIV is able to overcome BST-2 restriction however it does not seem as widely used as the Nef protein among SIV strains. Only Env from SIVtan has been demonstrated to have BST-2 antagonizing properties [121]. SIVtan Env is able to downregulate cell surface BST-2 and does not affect intracellular levels of BST-2. Additionally, a point mutation (A100D) in the ectodomain of BST-2 is able to confer resistance to SIVtan Env suggesting the extracellular domain as the target of SIVtan Env [121].

It is clear that BST-2 is a potent antiviral factor, however both HIV-1 and SIV have developed efficient mechanisms to overcome this barrier. There still remains much that is unknown about these evasion mechanisms and further research in this area may allow us to exploit the antiviral activity of BST-2 for future therapies

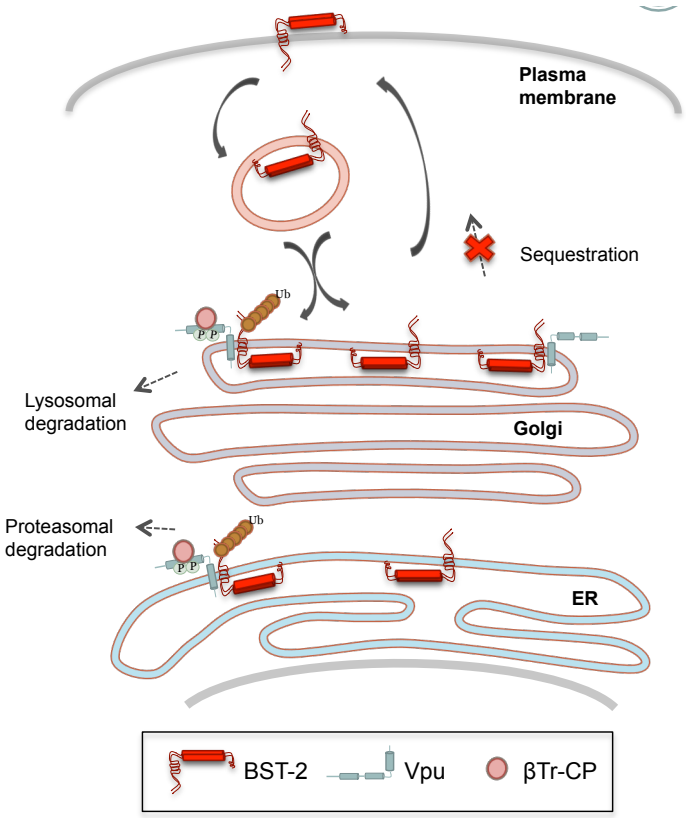


Figure 7 Proposed mechanism of Vpu antagonism of BST-2. BST-2 traffics to the plasma membrane and is internalized through recycling endosomes. Vpu interrupts this cycle by sequestering BST-2 at the TGN or by inducing the degradation of BST-2.

1.10 Project objectives

The research conducted herein revolves around the notion of virus evasion mechanisms of host restriction factors. Specifically, our objective was to gain further insight into the mechanisms used by both HIV and SIV to overcome restriction by BST-2.

The research was divided into two parts. In part one, we examined the mechanism used by SIVagm to overcome BST-2 restriction. Although recently it has been shown that SIVagm may employ both Nef and Env to antagonize BST-2 [118, 121], a separate study proposed that SIVagm is unable to counteract BST-2 altogether [122]. Further research is needed to reconcile these studies and here we uncovered an alternative mechanism that may be used by SIVagm to overcome BST-2 restriction. In part two, we examined the interaction of BST-2 and Vpu through their respective transmembrane domains. Although it was well known that this interaction occurred, the exact residues involved in the interaction were unknown. Additionally, it was not well known if the interaction was absolutely necessary for Vpu to counteract BST-2. We have developed an assay to measure Vpu-BST-2 interaction and through mutational analysis have identified several critical residues that are required to mediate interaction of the two proteins.

Chapter 2: Materials and Methods

2.1 Cell culture conditions and transfections

HeLa, COS-7 and HEK293T cells were all cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) containing 10% FBS. Cells were cultured at 37°C in an environment containing 5% CO_2.

For transfections, HEK293T cells were plated at 0.4 x 10^6, and COS-7 and HeLa cells were plated at 0.2 x 10^6 in 6-well plates. For other formats, the number of cells used was adjusted accordingly. The following day transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Opti-Modified Eagle Medium (Opti-MEM) (Invitrogen) was used as the diluting media. Cell culture media was changed 4-6h post-transfection and cells were collected 48h post-transfection unless otherwise indicated.

2.2. Reverse transcriptase (RT) assay

The reaction mixture to measure viral RT activity consisted of 40µl of reverse transcriptase reaction cocktail made up of 2M Tris-HCl (pH 7.9), 0.2M $MgCl_2$, 2M KCl, 20mM EGTA, 2% Triton X-100, 80% ethylene glycol, 0.2M DTT, 12mM GSH, poly(rA)-oligo(dT) (10U/ml) (Midland Certified Reagent Company), and [^{3}H]dTTP (2.5µCi/µl) (Perkin Elmer), and 10µl of cultured supernatant in 96-well format. The reaction was incubated for 3h at 37°C and then 150µl of cold 10% TCA was added for 30min at 4°C to precipitate the reaction. The reaction mixture was then passed through

MultiScreen glass fiber filter plates (Millipore) on the MultiScreen$_{HTS}$ Vacuum Manifold (Millipore). The filters were washed two times with cold 10% TCA and once with cold 95% ethanol. EcoLite™ scintillation cocktail (MP Biomedicals) was added to the filters in scintillation vials. A liquid scintillation counter (Wallac 1410; Perkin Elmer) was used to measure emission.

2.3 Viral infectivity assay

Tzm-bl cells were seeded into 24-well plates at 0.04 x 10^6 cells/well. The next day 25µl of cultured supernatant was added to each well. The cells were lysed in 100µl 1X passive lysis buffer (Promega) 48h later and incubated for 10min at room temperature. Luciferase substrate (Promega) was added to each well and luminescence of firefly luciferase was detected using the GLOMAX20/20 luminometer (Promega).

2.4 Viral stocks and infections

To create viral stocks, HEK293T cells were first seeded in 10-cm plates (~3.0 x 10^6 cells/plate). The next day 0.2µg of VSV-G and 4µg of either BH10, BH10(Vpu-) or SIVagm plasmids were transfected into HEK293T cells. The media containing VSV-G pseudotyped viruses was collected 48h post-transfection and centrifuged at 3000rpm for 15 min at 4°C. The viral stock was then passed through a 0.2µm sterile syringe filter, divided into aliquots and stored at -80°C until required.

COS-7 and HeLa cells seeded one day before were infected at an MOI=2 using VSV-G pseudotyped viruses. The next morning (18h post-infection), the culture media was changed and the cells were treated with IFNα2b (500IU/ml). The cells were collected 48h post-infection for Northern blot and Western blot and the culture supernatant was used for viral infectivity and viral RT assays.

2.5 Western blot

Cells were lysed using CytoBuster™ protein extraction reagent (Novagen) and were run on 10% or 12% SDS-polyacrylamide gels. Transfer was done either overnight or for 2h at 4°C on PVDF membranes (Roche) and membranes were then blocked in PBST (PBS containing 0.05% tween-20) with 5% non-fat milk. Primary antibodies were incubated with the membrane for 2h at room temperature or overnight at 4°C. The membrane was then washed with PBST (4 x 5 min) and the secondary antibody was added and incubated for 45min at room temperature. The membrane was washed again with PBST (4 x 5min) and enhanced chemiluminescence (ECL) substrate (PerkinElmer) was applied to the membrane according to the manufacturer's instructions. The membrane was then exposed on HyBlotCL® autoradiography film (Denville Scientific). As a loading control, the membrane was stripped in a solution containing 100mM β-mercaptoethanol, 2% SDS and 62.5mM Tris-Cl pH 6.8 at 50°C for ~15min and probed for β-tubulin.

The primary antibodies used were anti-HIV-1 p24 (CA) mouse monoclonal antibody (1:5000), mouse monoclonal anti-Flag (1:1000) (Sigma-Aldrich) for detection of Flag-tagged human BST-2, mouse monoclonal anti-β-tubulin (Santa Cruz Biotechnology) (1:5000), rabbit polyclonal anti-IFITM3 (Proteintech Group) (1:1000), anti-Stat1, anti-Stat2 and anti-ISG56. All primary antibodies were diluted in PBST containing 5% BSA, 0.05% NaN_3.

The secondary antibodies used were horseradish peroxidase-linked donkey anti-rabbit IgG (GE Healthcare) (1:5000) and horseradish peroxidase-linked sheep anti-mouse IgG (GE Healthcare) (1:5000). All secondary antibodies were diluted in PBST containing 5% non-fat milk.

2.6 Northern blot

Cells were collected in TRIzol® reagent (Invitrogen) and RNA was extracted according to the manufacturer's instructions. RNA was separated on an agarose-formaldehyde gel (1% agarose, 2.2M formaldehyde, 1X MOPS) in 1X MOPS-2.2M formaldehyde running buffer. The gel was then treated with 0.1M NaOH for 20min at room temperature followed by 0.3M NaOAC (pH 5.2) for 20min at room temperature and included rinsing with autoclaved water in between treatments. Transfer occurred overnight in 10X SSC using the Amersham Hybond™-N (GE Healthcare) membrane. The membrane was incubated in 6X SSC for 5 min at room temperature then the RNA was UV cross-linked to the membrane. The membrane was then incubated in 10ml of ULTRAhyb® Ultrasensitive Hybridization Buffer

(Ambion) for 1h at 42°C with rotation. The human BST-2 and agmBST-2 [^{32}P]-labeled probes were created by using the Nick Translation Kit (Roche) according to the manufacturer's instructions. Following pre-hybridization the probe diluted in hybridization buffer was incubated with the membrane at 42°C overnight. The next day the membrane was washed in 2X SSC, 0.1% SDS for 2 X 10 min or until the background radioactivity signal diminished. The membrane was then exposed on HyBlot CL® autoradiography film (Denville Scientific). As a loading control, the membrane was stripped in 1X SSC buffer containing 0.5% SDS at 60°C for 1h and then probed for GAPDH.

2.7 Promoter activity assay

To sequence the agmBST-2 promoter COS-7 cells were sent for chromosome walk analysis at Bio S&T Inc. (Montreal). DNA was isolated from COS-7 and HEK293T cells using DNeasy Blood and Tissue Kit (Qiagen) and served as the template to clone the human and agmBST-2 promoters. For the agmBST-2 promoter the forward primer used was 5'-TTTCTCGAGAGGGGCACTGGATGAAGCCC - 3' and the reverse primer used was 5' - TTTAAGCTTCCAGATCTCCCCTCTAGCTG -3'. For the human BST-2 promoter, the forward primer used was 5' – TTTCTCGAGAGAGGC ACTGGATGAAGCCC - 3' and the reverse primer used was 5' - TTTAAGCTTCCAGATCTCCCCTTTAGCTG - 3'. This covered ~500bp upstream of the transcription start site. Both promoters were cloned into the pGL3-Basic vector (Promega) using the Xhol and HindIII restriction sites creating pGL3-agmBST2 and pGL3-hBST2. The reporter vector (20ng of either

constructs) was transfected into COS-7 cells in 24-well plates and infected with VSV-G pseudotyped BH10 or SIVagm 6h post-transfection. Viruses were removed 18h post-infection and IFNα2b (500IU/ml) was added either 2h before infection or 18h post-infection. The cells were collected 48h post-infection in 1X passive lysis buffer (Promega) and the culture supernatant was used to assay viral infectivity.

2.8 Bioluminescence resonance energy transfer (BRET) assay

Human BST-2 was cloned into the pRluc-C3 vector (BioSignal Packard) using the forward primer 5'-AAAGGTACCTCATGGCATCTACTTCGT ATGAC-3' and reverse primer 5'- TTTGGATCCTCACTGCAGCAGAGCGCTGAG-3' in the KpnI and BamHI restriction sites. Vpu was cloned into the pEYFP-N1 vector (Clontech) using the forward primer 5' AAAGGTAC CATGGTGCCCATTATTGTCGCC 3' and reverse primer 5' TTTGGATCCC CCAGGTCGTCAATGTCCCA 3' in the KpnI and BamHI restriction sites. The constructs were transfected into HEK293T cells in 6-well plates and collected 48h posted transfection. Cells were detached using PBS containing 5mM EDTA. The cells were centrifuged at 1500rpm at 4°C for 5min and washed 2 times in PBS. White opaque 96-well plates were used for the measurement of the Rluc and EYFP and black opaque 96-well plates were used to measure EYFP signal following laser stimulation. Approximately 100,000 – 500, 000 cells were used per well. To measure fluorescence of Rluc coelenterazine H was added to a final concentration of 5μM in a total volume of 100μl of PBS. For measurement of EYFP only, cells were resuspended in PBS. The

measurements were carried out using a Synergy™ 4 Multi-Mode Microplate Reader (Bioteck).

Point mutations within the BST-2 and Vpu transmembrane domains were created using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions.

Chapter 3: Results

3.1 Mechanism of SIV to overcome BST-2

3.1.1 SIVagm is able to replicate effectively in HeLa cells

We sought to understand the ability of SIV from African green monkey (SIVagm) to overcome BST-2 restriction of viral release. There are two previous reports that Env and Nef from SIVagm is able to counteract BST-2, but the mechanism is currently unknown [118, 121]. We first infected HeLa cells with three VSV-G pseudotyped viruses consisting of wild type HIV-1 (BH10), Vpu-deleted HIV-1 (BH10(Vpu^-)) [115], or wild type SIVagm. HeLa cells are a cervical cancer cell line and endogenously express low levels of BST-2. The cells were treated with IFNα2b 18 hours post infection to further induce the expression of BST-2.

The results show that the endogenous human BST-2 inhibited the production of infectious BH10(Vpu^-) by approximately 100-fold as compared to the level of BH10 (Figure 8A). As anticipated, BH10 (which expresses Vpu) was able to effectively overcome BST-2 even in the IFNα treated cells (Figure 8A). Interestingly, high levels of infectious SIVagm were detected in the culture supernatant (Figure 8A), which was at least partially attributed to downregulated endogenous human BST-2 of cells lacking IFN treatment at the protein level similar to what was seen with BH10 infection (Figure 8B). Neither BH10 infection nor SIVagm infection influenced human BST-2 mRNA levels (Figure 8C). These data suggest that SIVagm, similar to BH10, has a

strategy to evade the restriction by human BST-2 that may occur through downmodulation of human BST-2 protein.

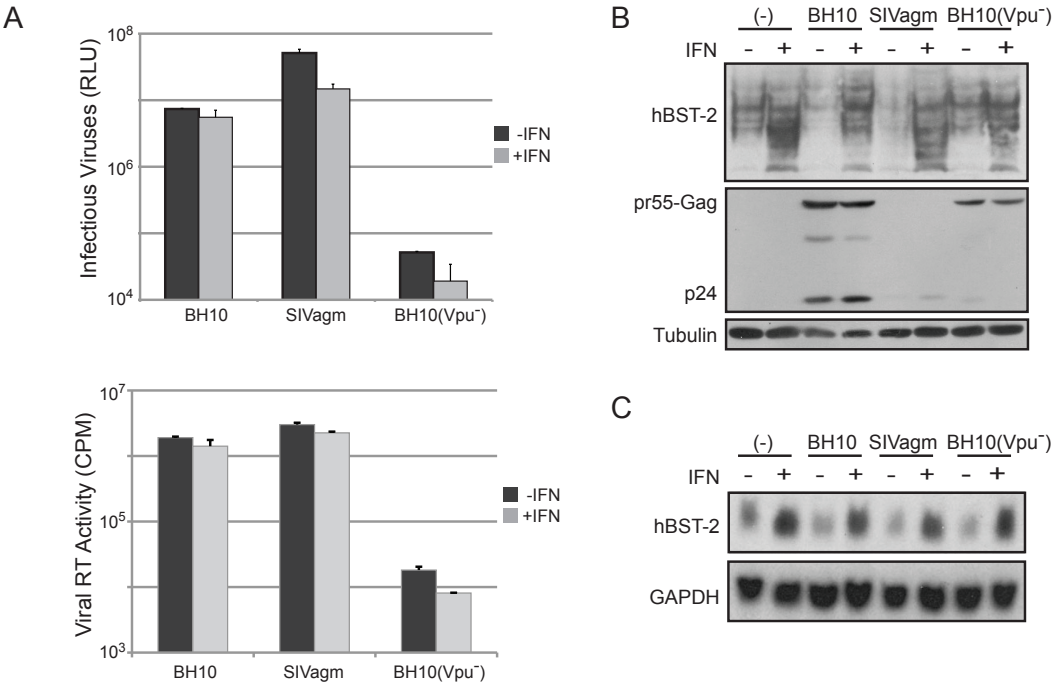


Figure 8 SIVagm is highly produced in HeLa cells. HeLa cells were infected with VSV-G pseudotyped BH10, SIVagm or BH10(Vpu$^-$) and treated with IFNα2b post infection. (A) The level of infectious viruses in the culture supernatant (top). The viral RT activity measured from the culture supernatant (bottom). Results shown represent three independent experiments. (B) Western blot to detect expression of human BST-2 in HeLa cells and amount of cellular p24. Tubulin was detected as an internal control. (C) Northern blot to detect human BST-2 mRNA. Detection of GAPDH mRNA was used as a control. Negative control represents uninfected HeLa cells.

3.1.2 Endogenous agmBST-2 mRNA level is reduced upon SIV infection of COS-7 cells

We next tested whether SIVagm is also able to overcome the restriction by BST-2 from African green monkey (agmBST-2). To this end, we infected the African green monkey kidney cell line, COS-7, with VSV-G pseudotyped BH10 or SIVagm viruses. COS-7 cells do not endogenously express BST-2. The cells were treated with IFNα2b to induce agmBST-2 expression. In line with previous reports showing that HIV-1 Vpu does not counteract agmBST-2 [19, 21, 22], production of infectious BH10 was diminished by more than 10 fold upon interferon treatment (Figure 9A). Interestingly, SIVagm production was not markedly affected by the addition of interferon and indicates SIVagm may have a mechanism to overcome agmBST-2 (Figure 9A).

Considering there is no commercially available antibody to detect agmBST-2, we were unable to assess the effect of SIVagm infection on interferon-induced agmBST-2 protein. Instead, we performed a Northern blot to determine whether SIVagm infection exerted any effect on interferon-induced agmBST-2 mRNA expression. Interferon produced a robust induction of agmBST-2 mRNA in uninfected cells and in BH10 infected cells (Figure 9B). Surprisingly, SIVagm infection virtually abolished this induction while BH10 infection exerted no effect (Figure 9B). Similar observations were made when different doses of SIVagm were used for infection (data not shown). These data suggests that SIVagm has evolved a means to counteract restriction by agmBST-2 and that this mechanism acts on the level of mRNA.

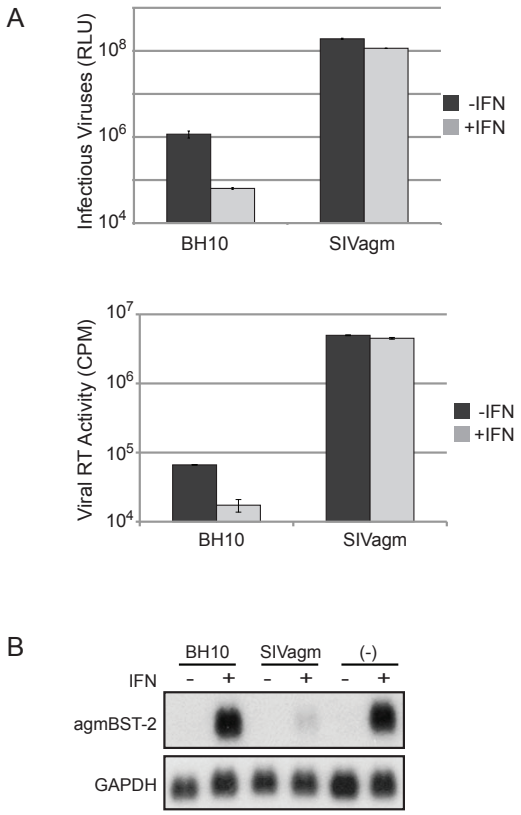


Figure 9 SIVagm infection of COS-7 cells decreases agmBST-2 mRNA levels. COS-7 cells were first infected with VSV-G pseudotyped viruses BH10 or SIVagm, then treated with IFNα2b 18h post infection. (A) The level of infectious viruses (top) and the viral RT activity (bottom) (B) Northern blot to detect agmBST-2 mRNA. GAPDH mRNA was detected as control. Negative control represents uninfected COS-7 cells.

3.1.3 Reduced agmBST-2 expression is not due to lack of promoter activity

The promoter of the *bst-2* gene bears an IFN stimulated response element (ISRE) that allows for an increase in *bst-2* expression upon interferon treatment. We hypothesized that SIVagm infection may hinder interferon-stimulated transcription from the *agmbst-2* promoter. To test this, we first determined the promoter sequence of *agmbst-2* by chromosome walk analysis using the known coding sequence of *agmbst-2* as a starting point. The *agmbst-2* promoter DNA sequence was then aligned with a published human *bst-2* promoter sequence (Figure 10A) [91]. Although there was a high homology between the two promoter sequences, the *agmbst-2* promoter lacked four short DNA segments at positions -391, -369, -285 and -206. We next inserted the ~500bp promoter sequences of human *bst-2* or *agmbst-2* genes upstream of a *firefly luciferase* gene in the context of the pGL3 basic reporter construct. These DNA constructs were then transfected into COS-7 cells, infected with either BH10 or SIVagm and treated with IFNα2b. The results of luciferase assays showed that both human *bst-2* and *agmbst-2* promoters responded to interferon treatment as demonstrated by the increased luminescence (Figure 10B). However, neither BH10 nor SIVagm infection exerted a significant effect on interferon-stimulated luciferase luminescence (Figure 10B). These results suggest that BH10 or SIVagm infection does not affect the activity of human *bst-2* or *agmbst-2* promoters. It is possible that DNA sequences further upstream our cloned

DNA region may contain regulatory elements that control the expression of BST-2 mRNA but were not included in the reporter DNA constructs that we created. BST-2 mRNA transcription is also subject to regulation that occurs at the chromatin structure level, which may not be reflected in a transient transfection using a reporter DNA construct. The mechanism behind the decrease in agmBST-2 mRNA expression following SIVagm infection of COS-7 cells awaits further investigation.

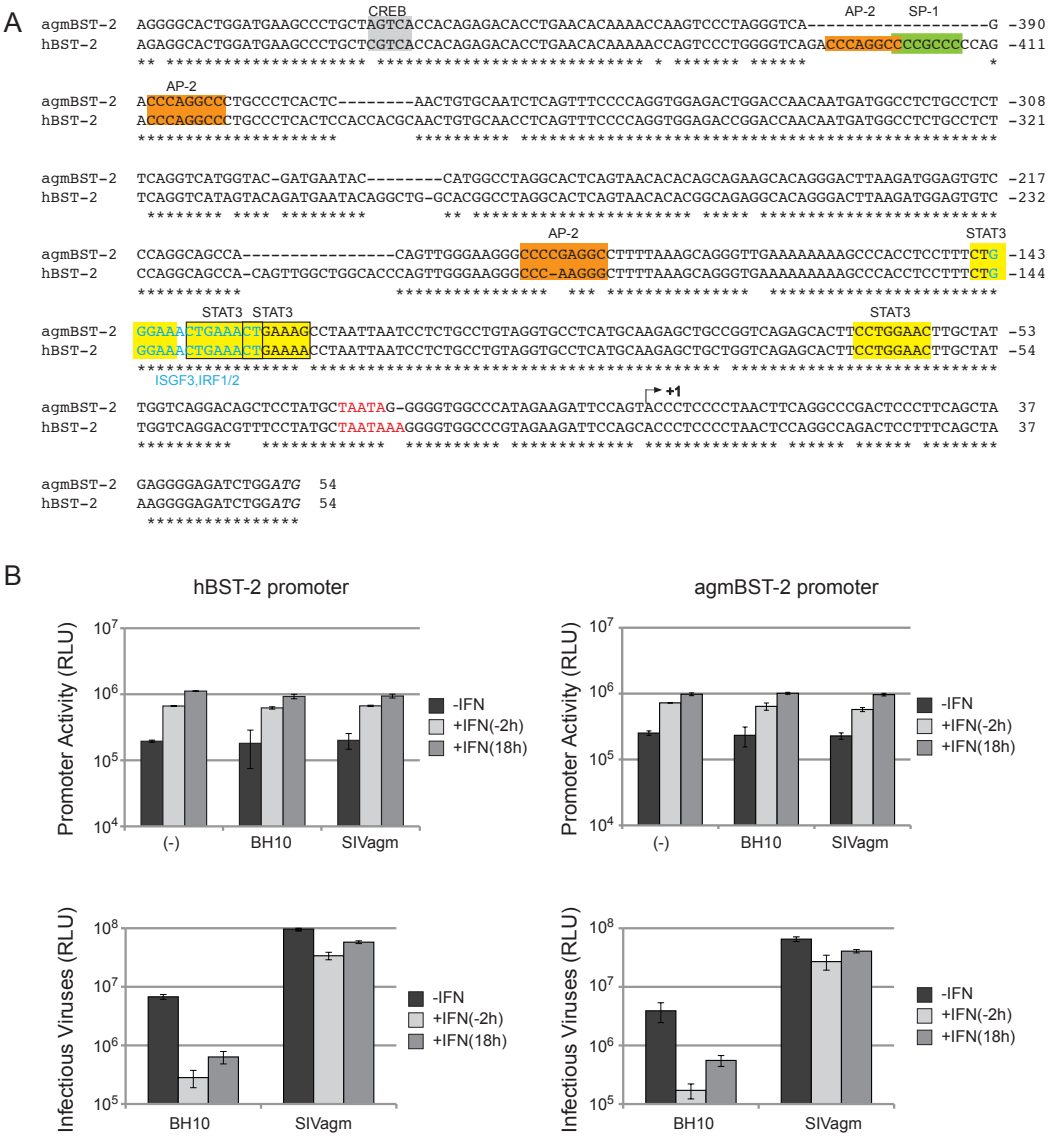


Figure 10 Promoter activity of the *bst-2* gene is unaffected by viral infection. (A) Sequence alignment of the promoter of *agmbst-2* and human *bst-2*. Putative Stat 3, AP2, CREB and SP-1 binding sties are highlighted in yellow, orange, grey and green, respectively [24]. The ISGF3 and IRF1/2 sites are indicated in blue, a TATA box is indicated in red and the translation starting codon ATG is italicized. (B) The luminescence measured from the firefly luciferase in the promoter-luciferase fusion constructs was a measure of the promoter activity. The culture supernatant was also used to measure the level of infectious viruses.

3.1.4 SIVagm infection of COS-7 diminishes the expression of other ISGs

Given BST-2 is an ISG we asked whether SIVagm infection also affects the expression of other ISGs. To address this question, we infected COS-7 cells with either VSV-G pseudotyped BH10 or SIVagm viruses and then treated the cells with IFNα2b. The cells were collected for Western blot analysis using antibodies against various ISGs including ISG56, Stat1, Stat2 and interferon induced transmembrane protein 3 (IFITM3). The expression of ISG56 and IFITM3 was decreased upon SIVagm infection but not with HIV-1 infection (Figure 11). Stat2 showed a slight decrease with SIVagm infection, but the level of Stat1 remained comparable to the control (Figure 11). These data suggest that SIVagm targets and reduces the expression of select ISGs.

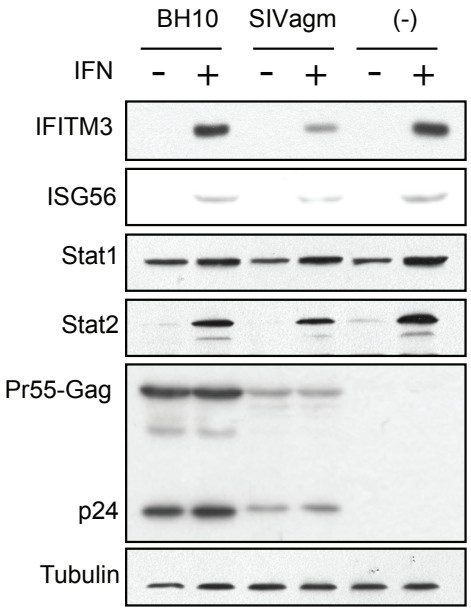


Figure 11 SIVagm infection of COS-7 diminishes IFITM3 protein expression. Western blot to detect the ISGs IFITM3, ISG56, Stat1 and Stat2 following infection with either VSV-G pseudotyped BH10 or SIVagm. An antibody towards HIV-1 p24(CA) was used to detect viral Gag expression in cells. Tubulin was used as a loading control.

3.2 Monitoring Vpu-BST-2 interaction using BRET

3.2.1 Development of an assay to monitor Vpu and BST-2 interaction

It is well known that Vpu and BST-2 are able to interact with each other through their respective transmembrane domains [113-115]. However, the exact nature of this interaction and the residues involved were not well characterized. In order to gain insight into the interaction between HIV-1 Vpu and human BST-2 we developed an assay to monitor the interaction using the principles of bioluminescence resonance energy transfer (BRET). We generated fusion proteins of BST-2 fused with Renilla luciferase (Rluc-BST2) at its N-terminal and Vpu fused with EYFP at its N-terminal (EYFP-C1-Vpu) or C-terminal (EYFP-N1-Vpu).

Rluc-BST2 was cotransfected with either EYFP-C1-Vpu or EYFP-N1-Vpu into HEK293T cells. Coelenterazine h was then added and became oxidized by Rluc resulting in an emission of energy. If Rluc and EYFP were within 100Å of each other, the energy emitted by Rluc may be transferred to EYFP resulting in an emission of energy by EYFP (Figure 12A). The BRET ratio = $\frac{emission\ of\ EYFP}{emission\ of\ Rluc}$ was used to asses the proximity of the two proteins. The negative control used was an empty EYFP-N1 vector cotransfected with Rluc-BST2 and the positive control was a vector with EYFP fused to Rluc (EYFP-Rluc). The results show that cotransfection of EYFP-N1-Vpu and Rluc-BST2 exhibited a BRET ratio that is greater than the positive control (EYFP-Rluc) suggesting that EYFP-N1-Vpu and Rluc-BST2 interact strongly with each

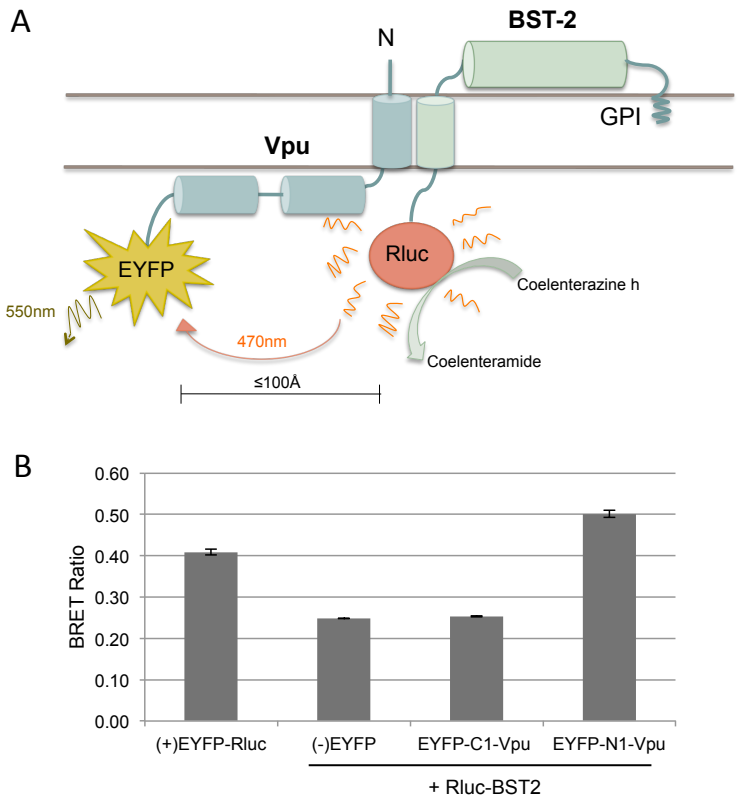


Figure 12 Verification of BRET assay. (A) Diagram depicting the principles of the BRET assay. (B) 293T cells were transfected with either the positive control EYFP-Rluc, the negative control EYFP and Rluc-BST2, EYFP-C1-Vpu and Rluc-BST2, or EYFP-N1-Vpu and Rluc-BST2. The BRET ratio was used to determine the level of interaction of BST-2 and Vpu.

other (Figure 12B). Interestingly, EYFP-C1-Vpu and Rluc-BST2 have a BRET ratio that is comparable to the BRET ratio measured in the negative control. This is likely due to the placement of EYFP at the N-terminal of Vpu that would position it at the opposite face of the plasma membrane to Rluc. This placement would interfere with any energy transfer from Rluc to EYFP even if BST-2 and Vpu did interact. Based on these results we continued further experiments using EYFP-N1-Vpu (from now on referred to as EYFP-Vpu).

3.2.2 Mutations in specific residues of the Vpu and BST-2 affect their ability to interact with each other

To determine the essential residues within BST-2 and Vpu involved in mediating the interaction between the two proteins, we selectively mutated the non-alanine residues to alanine in single, double or triple mutations of Vpu in the EYFP-Vpu construct (Figure 13). Additionally, we mutated residues within the transmembrane domain of BST-2 in the Rluc-BST2 construct to the corresponding residues in agmBST-2 or rhBST-2 because the simian BST-2s are known to be resistant to HIV-1 Vpu antagonism (Figure 14). We also included mutations that have been shown in the literature to be resistant to Vpu antagonism (ΔLL22/23) [115] and additional triple alanine mutations to cover the remaining BST-2 transmembrane domain. These EYFP-Vpu mutants were cotransfected with wild type Rluc-BST2, and Rluc-BST2 mutants were cotransfected with wild type EYFP-Vpu into HEK293T cells and their interaction was assessed using BRET.

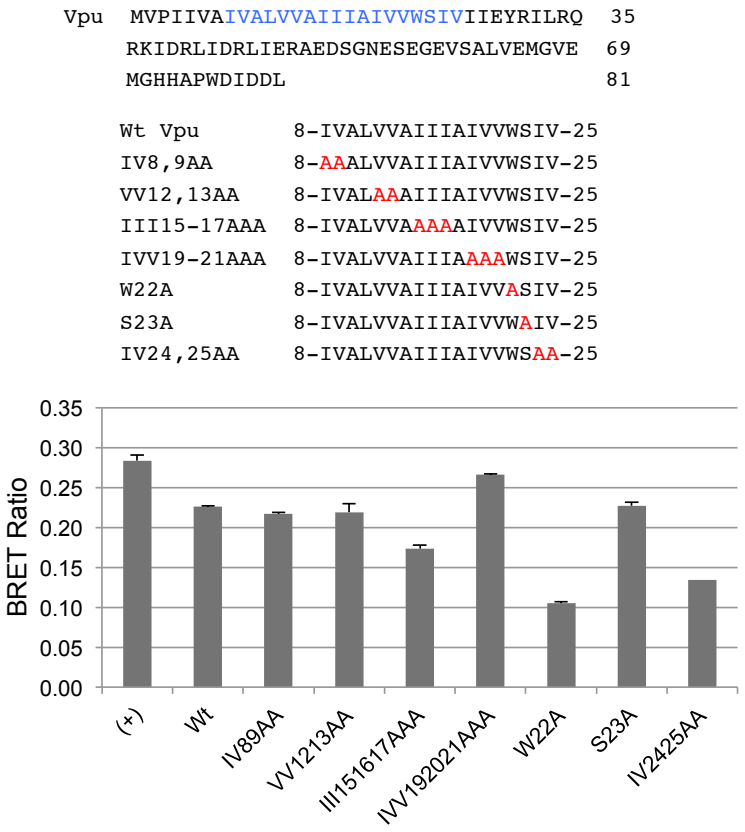


Figure 13 Vpu transmembrane mutants. The Vpu sequence is shown with the transmembrane domain indicated in blue. The names of the Vpu mutants are listed and the mutated residues of each mutant are indicated in red. The graph shows the BRET ratio of the mutant EYFP-Vpu cotransfected with Rluc-BST-2 in HEK293T cells. The positive control is transfection of EYFP-Rluc alone. The BRET ratios have been adjusted by subtracting the value of the negative EYFP control.

Results of the BRET assay show a reduced BRET ratio of Vpu mutants W22A and IV24-25AA and to a lesser extent mutant III15-17AAA (Figure 13). These results suggest that the residues W22, I24, V25 and possibly I15, I16 and I17 are involved in interacting with BST-2. The residues that indicate the

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agmBST-2      37-LAV--GILGLLVIVLLGVPLIFFI-48
rhBST-2       37-LVV--GILGLLVIVLLGVLLIFFI-48
hBST-2        22-LLLGIGILVLLIIVILGVPLIIFT-45
                 * :  *** **:**:*** **:*

Del22/23LL    22--LGIGILVLLIIVILGVPLIIFT-45
G25A          22-LLLAIGILVLLIIVILGVPLIIFT-45
Del25/26GI    22-LLL--GILVLLIIVILGVPLIIFT-45
V30G          22-LLLGIGILGLLIIVILGVPLIIFT-45
I33V          22-LLLGIGILVLLVIVILGVPLIIFT-45
IVI34-36AAA   22-LLLGIGILVLLIAAALGVPLIIFT-45
I36L          22-LLLGIGILVLLIIVLLGVPLIIFT-45
LGV37-39AAA   22-LLLGIGILVLLIIVIAAAPLIIFT-45
PLI40-42AAA   22-LLLGIGILVLLIIVILGVAAAIFT-45
I43F          22-LLLGIGILVLLIIVILGVPLIFFT-45
T45I          22-LLLGIGILVLLIIVILGVPLIIFI-45
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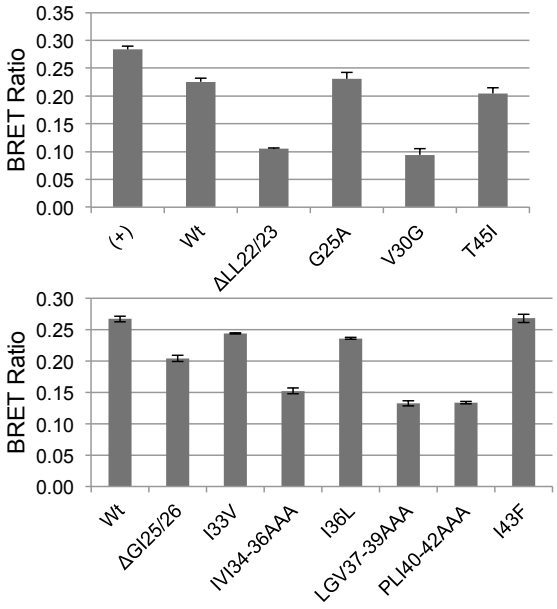

Figure 14 BST-2 transmembrane mutants. A sequence alignment of the BST-2 transmembrane domain from African green monkey (agmBST-2), rhesus macaque (rhBST-2) and human (hBST-2) is shown. The human BST-2 mutants that were created are listed and the mutated amino acids are indicated in red. The graphs show the BRET ratio of the mutant Rluc-BST2 cotransfected with wild type EYFP-Vpu in HEK293T cells. The positive control is HEK293T cells transfected with EYFP-Rluc. The Bret ratios have been adjusted by subtracting the value of the negative EYFP control.

strongest interaction with BST-2 cluster at the C-terminal end of Vpu. It is possible that the C-terminal end of the protein may form the predominant link to BST-2 and other residues may be involved in stabilizing the interaction.

The BST-2 mutants ΔLL22/23 and V30G were significantly impaired in their ability to interact with Vpu and the triple mutants, IVI34-36AAA, LGV37-39AAA and PLI40-42AAA all demonstrate a reduced BRET ratio (Figure 14). Considering, all the triple mutations of BST-2 resulted in a reduced BRET signal there is a possibility that the mutations changed the structure of the transmembrane domain too dramatically and thereby affected the ability of BST-2 to interact with Vpu. The residues L22, L23 and V30 are all located proximal to the N-terminal region of BST-2 and show the greatest decrease in binding to Vpu as assessed by the BRET assay. Interestingly, this region corresponds to the C-terminal side of Vpu where the residues seemed to have had the greatest influence on binding to BST-2. This indicates that specific and strong binding may occur in this region.

3.2.3 Residues in the C-terminal end of Vpu essential to promote viral release

We next sought to determine whether a defect in the ability of Vpu to interact with BST-2 would affect its ability to rescue the release of HIV-1 in the presence of BST-2. To ensure that EYFP does not interfere with the protein function of Vpu we generated the same Vpu mutants in the absence

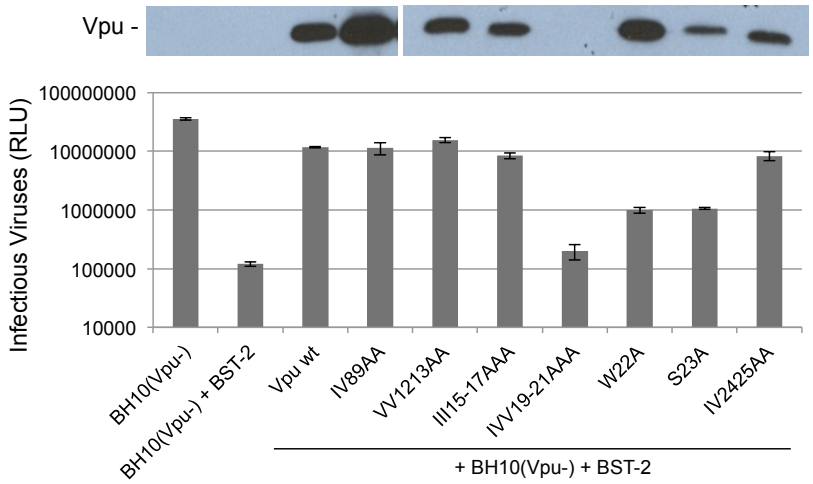

Figure 15 Vpu W22A and S23A unable to rescue HIV-1 viral release. Vpu mutants were transfected into HEK293T cells along with BH10(Vpu$^-$) and wild type BST-2. The protein expression of Vpu was measured in a Western blot. The ability of each Vpu mutant to rescue BH10(Vpu$^-$) is shown in the amount of infectious viruses produced.

of the EYFP tag. The wild type Vpu or Vpu mutants were then cotransfected with BH10(Vpu$^-$) and wild type BST-2 into HEK293T cells. The results of viral infectivity show the W22A mutant was highly impaired in its ability to overcome BST-2 restriction of viral release (Figure 15). There was a 10-fold decrease in viral infectivity in the presence of W22A as compared to the wild type Vpu. This is in line with the inability of the W22A mutant to interact with BST-2 as shown using the BRET assay. However, the S23A mutant, which did not show an inability to interact with BST-2 was also impaired in its ability to increase viral infectivity (Figure 15). The IVV19-21AAA mutant was the most severely impaired in rescuing viral release, however this

mutant was not expressed. Surprisingly, III15-17AAA and IV24-25AA mutant was able to effectively promote virus despite its inability to interact with BST-2 as was shown in the BRET assay (Figure 15). This suggests that the ability of Vpu to overcome BST-2 restriction is more complex than simply being required to bind and interact with BST-2. Furthermore, the mutated residues that influence the ability of Vpu to bind to BST-2 or have an effect on the ability of Vpu to overcome BST-2 restriction all lie in the C-terminus of the Vpu protein. This further points to the C-terminal end of Vpu as a critical determinant of its ability to overcome BST-2 restriction.

Chapter 4: Discussion

4.1 SIVagm prevents the interferon-induced expression of BST-2 mRNA

Previous studies have shown that SIVagm Nef and Env have the ability to overcome BST-2 [118, 121]. Here we have demonstrated that SIVagm has an additional mechanism to overcome BST-2 restriction of viral release. This mechanism is not shared by HIV-1, is species specific and acts at the level of mRNA. There have been no studies thus far that have examined the expression of BST-2 at the mRNA level in face of viral infection and we believe that this decrease of agmBST-2 mRNA by SIVagm infection may be indicative of a broader viral mechanism to overcome host defenses.

The reduction of agmBST-2 mRNA may be a result of several factors such as an increase turnover rate of agmBST-2 mRNA or a decrease in the level of transcription. We have attempted to address the mRNA stability of agmBST-2 however the results were inconclusive (data not shown). To examine the transcriptional activity of BST-2 we generated reporter constructs with the BST-2 promoter upstream of a *firefly luciferase* gene, however the results indicated that the BST-2 promoter activity was unaffected with SIVagm infection (Figure 10). It is important to note that the reporter construct does not necessarily recapitulate gene expression as it occurs naturally. It is possible that there are certain regions of the chromosome that contain promoter or enhancer elements essential for the expression of BST-2 mRNA that is not present in the reporter construct. In

the natural environment these regions may be targeted by SIVagm infection and hinder mRNA expression. It is also possible that chromosome modifications are involved in suppressing mRNA expression following SIVagm infection that would not be observable in the context of a reporter construct. The mechanism behind the decrease in agmBST-2 observed following SIVagm infection of COS-7 cells awaits further investigation.

The observation that SIVagm is also able to reduce the expression of other ISGs lead us to consider the possibility that SIVagm may have a mechanism to target a subset of ISGs to escape the antiviral activity of these genes. This is supported by the fact that IFITM3 is also downregulated by SIVagm and has recently been shown to be a potent antiviral factor [123-127]. It has been previously reported that SIV infection of its natural host results in a strong induction of a type I IFN response and production of ISGs during acute infection that is resolved once it reaches the chronic phase of infection [29, 128, 129]. This was not observed in pathogenic SIV infections of non-natural hosts and is one of the distinguishing features between non-pathogenic and pathogenic infection [29, 128, 129]. The mechanism behind the resolution of the IFN response in non-pathogenic SIV infection is unclear and may be a consequence of evolution and adaptation of the host and the virus to one another. Additionally, it is unknown whether it is the host that controls the IFN response or whether SIV is able to shut down the IFN pathway. Through our observations we propose that SIVagm may have a mechanism to downregulate the expression of a subset of ISGs including BST-

2 and IFITM3 (Figure 9, 11). Not only would this prevent chronic immune activation, but it would also thwart the expression of antiviral ISGs. The group of ISGs tested represents a small subset of ISGs and more data is required to determine whether or not SIVagm is indeed able to downregulate a broad spectrum of ISGs and whether this mechanism is able to contribute to the non-pathogenesis of SIV infection. Furthermore, how and at which step of IFN induction this inhibition occurs remains unknown. It is possible that SIVagm infection interferes with the IFN pathway or with a transcription factor that is essential to the expression of a large group of ISGs. A key experiment to be done in the future would be a microarray analysis of the whole genome expression profile of African green monkey cells to test whether or not there is a global inhibition of ISG mRNA production following SIVagm infection. Although there is no array currently available for African green monkey, the possibility of using Rheusus macaque arrays to detect African green monkey genes exists.

It is interesting to note that SIVagm has also developed the use of Nef and Env as a method to overcome BST-2. The fact that two viral proteins from SIVagm have acquired the ability to overcome BST-2 points to the importance of overcoming this antiviral factor. Here, we have shown that BST-2 is downregulated at the mRNA level by SIVagm infection. It may be possible that this observed phenotype maybe be a result of either the action of Nef or Env to overcome BST-2. There is not much known about the mechanisms used by Nef or Env to counteract BST-2. Further studies are

required to understand the role and mechanism of BST-2 mRNA downregulation.

4.2 Insights into Vpu and BST-2 interaction and viral release

The mechanism whereby Vpu antagonizes BST-2 is presently unclear. Previous studies have suggested more than one mechanism may be at place where Vpu can overcome BST-2 restriction by either sequestering BST-2 at the TGN or causing degradation of BST-2 through the proteosomal and/or endolysosomal pathway [89, 92, 107, 108, 110, 130]. Sequestration at the TGN would prevent BST-2 from reaching the cell surface and degradation of BST-2 would reduce intracellular levels of BST-2 as well as the amount of BST-2 that would recycle to the cell surface. However, the study by Miyagi *et al.* demonstrated that neither cell surface nor intracellular depletion of BST-2 is required for Vpu sensitivity in certain cell types (such as H9) [90]. This observation would suggest that the mechanism used by Vpu is cell type dependent and may require certain cellular factors that are not ubiquitously expressed. Whether the dominant mechanism is sequestration or degradation, if they are dependent on each other and whether the mechanism requires specific cellular factors are unknown. In this study we have identified several residues within the Vpu and BST-2 transmembrane domain that when mutated abrogate their ability to interact with each other. These results are discussed in detail in the following sections.

4.2.1 Vpu transmembrane domain

The Vpu mutants W22A, IV2425AA and to a smaller extent III15-17AAA have a decreased ability to interact with BST-2 as measured using the BRET assay. W22A also lost its ability to promote viral release and is consistent with the view that without interaction with BST-2, Vpu would be unable to overcome BST-2 restriction. However, both III15-17AAA and IV2425AA were able to promote viral release despite being unable to interact with BST-2. This result is puzzling as it would seem logical that interaction with BST-2 would be required for Vpu to antagonize BST-2 regardless of the mechanism used to overcome BST-2; be it sequestration or degradation. Further experimentation would be required to understand this observation, however this may indicate that Vpu has a third mechanism to overcome BST-2 that is indirect. For example, III15-17AAA and IV2425AA may be residues that are critical to a Vpu function to modify or activate another cellular protein, which in turn would act on BST-2 to prevent it from restricting viral release. Additionally, it is possible that the BRET assay may give false positives. For example, if both BST-2 and Vpu are expressed in high amounts in the same compartment, they may give off a positive BRET signal even though there is no specific interaction. Verification of the BRET results using immunoprecipitation may help clarify these results.

Interestingly, the mutant S23A was impaired in its ability to promote viral release yet it was still able to interact with BST-2. This would suggest that interaction with BST-2 is not sufficient for Vpu to overcome BST-2. In

line with the degradation model of BST-2 antagonism, S23 may be involved in recruiting cellular factors that are involved in the degradation of BST-2 and mutations in S23 may alter the structure or sequence of Vpu and abrogate this ability. The reduced ability of S23A to promote viral release may also be due to the lower expression level of this mutant compared to the wild type Vpu.

A recently published study by Vigan *et al*. identified three residues in Vpu that when mutated lost the ability to co-immunoprecipitate with BST-2, downregulate BST-2 from the cell surface and to promote viral release in the presence of BST-2 [131]. These residues are A14, W22 and to a lesser extent A18. These residues are located on the same side of the alpha-helical transmembrane domain and the authors propose that in the absence of interaction with BST-2, the ability of Vpu to antagonize BST-2 is impaired. This study only tested if Vpu mutants that were impaired in promoting viral release were also unable to co-immunoprecipitate with BST-2. They did not test the effect of mutations of other residues on the ability of Vpu to co-immunoprecipitate with BST-2. Therefore, it is unclear if promoting viral release always necessitates binding to BST-2.

Our BRET data demonstrated that W22A showed the greatest decrease in BRET ratio suggesting it to have the strongest influence on the interaction of Vpu with BST-2. W22 is the only residue within the Vpu transmembrane domain that contains a bulky side chain that would allow it to protrude into

the groove of an interacting protein. The other residues with the exception of S23 have small hydrophobic side chains that would make it difficult to mediate interaction with another protein in the hydrophobic lipid bilayer. However, other residues such as A14, A18, I24 and V25 may be involved in stabilizing the interaction. A14 and A18 have the smallest side chains and may form pockets where residues from BST-2 can enter to stabilize the interaction.

Altogether our results indicate that the residues on the C-terminal end of Vpu form the strongest interaction with BST-2. Mutations in this area also greatly affect the ability of Vpu to rescue viral release. Although the role of each residue is unclear, it is apparent that simply interacting with BST-2 is not sufficient for Vpu to promote viral release (as in the case of S23A). Further investigation of the Vpu transmembrane mutants will hopefully shed light on the mechanism Vpu employs to overcome BST-2.

4.2.2 BST-2 transmembrane domain

The BST-2 mutants that showed diminished binding to Vpu are ΔLL22/23, V30G, IVI34-36AAA, LGV37-39AAA, and PLI40-42AAA. Previously, our group demonstrated the ΔLL22/23 mutant had a reduced ability to co-immunoprecipitate with Vpu and was resistant to Vpu antagonism [115]. Our BRET data here confirms this previous observation and provides additional validation of the BRET assay. Additionally, V30G has been previously shown to reduce sensitivity to Vpu and our BRET results demonstrate it is unable to

interact with Vpu [113-115]. These data suggest a link between Vpu-BST-2 interaction and the ability for Vpu to overcome BST-2.

A recently published study using a biomolecular fluorescence complementation (BiFC) assay confirmed many of our results [132]. This BiFC assay used complementary fragments of the KG fluorescent protein fused to either BST-2 or Vpu to monitor their interaction [132]. If the two fragments were brought in close proximity to each other (<15nm) by the interaction of BST-2 and Vpu then the fluorescence created could be visualized and measured. Similar to the BRET assay, both techniques allow the detection of the interaction of two proteins in live cells and rely on fluorescence as a read out. Unlike BiFC, BRET requires an energy transfer to occur and basal emission from EYFP may contribute to background signals. However, BRET requires the two proteins to be within 10nm of each other and may therefore be slightly more sensitive than BiFC. Additionally, these techniques do not distinguish between a direct and indirect interaction and it is therefore possible that a cellular factor may help mediate interaction between the two proteins.

Using the BiFC technique, Kobayashi *et al.* identified the same triple mutations in BST-2 that abrogated interaction with Vpu (IVI34-36AAA, LGV37-39AAA, and PLI40-42AAA) [132]. They conducted further mutagenesis studies within these triple mutations and identified three critical residues that mediated interaction with Vpu, which are I34, L37 and

L41 [132]. Alanine substitutions of these residues resulted in resistance to Vpu antagonism and prevented interaction with Vpu. Computational modeling indicated that these three residues lie on the same side of the BST-2 helical transmembrane domain [132]. This would be consistent with the study by Vigan *et al.* where the residues identified to influence Vpu interaction with BST-2 also were located on one face of the transmembrane domain. The three residues identified in BST-2 are conserved among simian BST-2, including those that are not susceptible to Vpu antagonism. The authors propose that it is therefore not the primary sequence but rather the secondary structure that is the determinant of BST-2 sensitivity to Vpu. Intriguingly, in their study the mutant P40A was resistant to Vpu antagonism yet was still able to interact with Vpu [132]. Similarly, the BST-2 mutant T45I was shown to be resistant to Vpu antagonism [113-115, 132], however T45I did not abrogate interaction with Vpu (Figure 13) [132]. Altogether these data indicate that in the absence of interaction between BST-2 and Vpu, Vpu is unable to antagonize BST-2. However, interaction of the two proteins is not sufficient to render BST-2 Vpu-sensitive.

The studies so far indicate there are two mechanisms at work: sequestration and degradation. The fact that mutations in residues P40 and T45 do not affect binding of BST-2 to Vpu but does increase resistance to Vpu antagonism seems to suggest that these residues are involved in triggering degradation of BST-2. They may be targets for cellular factors that are recruited by Vpu to degrade BST-2. The hypothesis that there are two

mechanisms at play is supported by the fact that the mutation T45I substantially reduces the ability of Vpu to antagonize BST-2, but does not entirely abrogate this ability [113, 114]. Additionally, intracellular levels of T45I are not degraded [121]. This suggests that Vpu is no longer able to trigger the degradation of BST-2, but it may still be able to sequester BST-2 and may help explain why there is not a complete insensitivity to Vpu.

Altogether these results indicate that Vpu may employ both mechanisms in overcoming BST-2 restriction and that interaction between the two proteins is not sufficient for Vpu to promote viral release. This interaction may allow Vpu to sequester BST-2, however the second mechanism of degradation appears to be necessary for full antagonism of BST-2 restriction. We propose that certain residues within BST-2 (L22, L23, V30) and Vpu (W22) mediate interaction of the two proteins and other residues (such as T45 and P40 in BST-2 and S23 in Vpu) are involved in triggering the degradation of BST-2. Additionally, the C-terminal of Vpu is essential to both interaction with BST-2 and antagonism of BST-2. Further investigation is required to understand the extent to which both mechanisms are used and to understand the interplay between these two mechanisms.

4.3 Concluding remarks

This study furthers our comprehension of mechanisms used by SIV and HIV to overcome BST-2 restriction and gives insight into the determinants of pathogenic infections. Although further investigation is required, a

knowledge of the elements differing between pathogenic and non-pathogenic infections will have significant implications in the development of therapies against AIDS and may one day lead to a cure and/or effective prevention of this disease. Additionally, a detailed understanding of the residues in Vpu and BST-2 that are essential to promote/prevent viral release may in the future be used in the design of drugs therapies that may exploit BST-2 as an antiviral factor. Such therapies may include competitive inhibitors that interfere with the interaction of Vpu and BST-2. As resistance mutations to existing drugs continually emerge, there is no doubt that there is a persistent need for novel drugs and drug targets.

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