In Vitro Functional Comparisons of HTLV-1 HBZ and HTLV-2 APH-2: Implications for Viral Persistence and Pathobiology

DISSEPTION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2014

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Abstract

Human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus found to cause diseases including neurological and inflammatory disorders as well as the aggressive T-cell malignancy adult T-cell leukemia/lymphoma (ATL). A second retrovirus, HTLV-2, is related to HTLV-1 but appears to be much less pathogenic in vivo. Chapter 1 is a literature review on the comparative biology and pathogenesis of HTLV-1 and -2. In subsequent chapters, we investigate mechanistic differences in the pathobiology between these two related viruses, mainly focusing on the roles of antisense transcribed gene products, HTLV-1 bZIP factor (HBZ) and the antisense protein of HTLV-2 (APH-2).

In Chapter 2, we examined how APH-2 affects pathways known to be modulated by HBZ and are hypothesized to be important for HTLV-1 pathogenesis. We found that APH-2 inhibited transforming growth factor β (TGF-β) signaling while HBZ enhanced it. However, HBZ and APH-2 repressed p65- and IRF-1-mediated transcription. Comparison of the stability of APH-2 to HBZ protein as well as their mRNA transcript levels in infected cells indicated a more
limited functional role for APH-2. Our findings reveal the importance of these pathways and their potential implications in HTLV-1 pathobiology.

Chapter 3 focuses on post-translational modifications (PTMs) of HBZ. Many cellular and viral proteins are regulated by PTMs. Therefore, we hypothesized that HBZ also might be regulated by PTMs. Mass spectrometry analysis revealed seven modifications to HBZ including phosphorylation, acetylation, and methylation. Using mutational analysis, we examined the role of the phosphorylated and acetylated residues in regulating protein expression/steady-state levels, as well as the ability of HBZ to repress Tax, c-Jun, p65, and IRF-1. We found that the newly discovered phosphorylated and acetylated residues do not play a role in regulating any of these pathways but we posit that these PTMs may be important in the regulation of other newly uncovered or yet to be discovered functions of HBZ.

Studies in Chapter 4 investigate the ability of HTLV-1 and HTLV-2 to modulate the type I interferon (IFN) response. Previous studies showed that type I IFN represses HTLV expression; however, HTLV-1 inhibits antiviral IFN expression through the induction of suppressor of cytokine signaling 1 (SOCS1). Although HTLV-2-immortalized T-cells (PBL/HTLV-2) and HTLV-1-immortalized T-cells (PBL/HTLV-1) express similar amounts of SOCS1, PBL/HTLV-2 cells expressed and secreted higher amounts of IFN-β than PBL/HTLV-1 cells. Furthermore, IFN-β expression led to an increase in antiviral IFN stimulated gene (ISG) expression in bystander cells incubated in PBL/HTLV-2 conditioned
medium compared to PBL/HTLV-1 conditioned medium. To elucidate the mechanism of IFN-β expression in PBL/HTLV-2, we examined how HTLV-2 proteins modulate the transcriptional activity of IFN regulatory protein 3 (IRF-3). Reporter assays demonstrated that the HTLV-2 proteins Tax-2 and APH-2 enhanced the induction of IFN-β through IRF-3. These studies collectively provide new insights into the differences in HTLV-1 and HTLV-2 that may explain their distinct pathobiological outcomes.
Dedication

To my family and close friends
Acknowledgements

When I came to OSU, I was lucky enough to join the Green lab. Dr. Green encouraged me to develop my own hypotheses and then trusted in me and allowed me to design my own experiments. His mentorship has allowed me to reach a new height in my scientific thinking process and career. I also thank every member of the lab, both past and present, but especially Dr. Amanda Panfil, who provided scientific insight and experimental data used in this thesis. Every project I worked on could not have been done without collaborate efforts. Noteworthy contributions came from Dr. Suresh De Silva of Dr. Wu’s lab and Dr. Nikoloz Shkriabai of Dr. Kvaratskhelia’s lab. Thank you for your help. My sincerest gratitude also to my undergraduate lab mentor and members for teaching me and allowing me to first discover my passion for research.

All my time, of course, was not spent in the lab. To all my friends, a huge thank you for keeping me sane and providing me with great experiences. Your encouragement played no small part in my completion of graduate school.

Lastly, I would like to thank Mr. G. Lucas and Mr. G. Roddenberry for inspiring me and getting me interested in the sciences at a young age. This passion was then supported and encouraged by my loving family, especially my
parents, Glenn and Margie Dissinger. Without them, I would certainly not have been able to get where I am today.
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Chapter 1

Literature Review

1.1 HTLV Discovery and Epidemiology

The first human retrovirus was discovered in 1980, after isolation from two cell lines and fresh peripheral blood mononuclear cells derived from a patient suffering from a cutaneous T-cell lymphoma [1]. In southwestern Japan, a retrovirus also was isolated from adult T-cell leukemia/lymphoma (ATL) patients[2,3]. Using antisera from ATL patients, these two viruses were shown to be identical and are now known as human T-cell leukemia virus type-1 (HTLV-1) [4]. The global spread of HTLV-1 was demonstrated when leukemia patients from the Caribbean were shown to be infected by this retrovirus [5]. Overall, it is estimated that there are currently 15-25 million people worldwide infected by HTLV-1 [6,7].

In 1982, a related human retrovirus was isolated from a patient with a T-cell variant of hairy cell leukemia [8]. This virus, named HTLV-2, is prevalent among isolated populations of Amerindians [9-11] in addition there is a growing endemic of HTLV-2 infections among intravenous drug users in the US and in Europe [1,12-14].
More recently, two other viruses related to HTLV-1 have been discovered. These viruses, named HTLV-3 and HTLV-4, were isolated from bush meat hunters in central Africa in 2005 [2,3,15,16]. The number of infections and global spread of these two viruses appear to be low, but research groups are studying and comparing them to other viruses within the HTLV family.

Phylogenetic studies of nonhuman primate retroviruses and HTLV-1 demonstrated a relationship, suggesting that HTLV-1 started as a zoonotic infection [4,17]. Further studies have shown a relationship between HTLV-2 and a simian T-cell leukemia virus counterpart (STLV-2) [5,18] as well as links between HTLV-3 and HTLV-4 with STLV-3 and STLV-4, respectively [6,7,16,19,20]. Together, the HTLV and STLV viruses make up the family of primate T-cell leukemia viruses (PTLVs).

1.2 HTLV Pathogenesis and Disease Association

HTLV-1 was the first human retrovirus to be linked to a disease [1,8]. However, of the millions of people infected with HTLV-1 globally, only 2-8% of individuals develop an associated disease [9-11,21,22]. HTLV-1 is the casual link to several diseases, including ATL [2,3,5], HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), and other inflammatory diseases. In all of these disease cases, it has been observed that the HTLV-1 proviral load is significantly higher than in asymptomatic carriers [23-29].
1.2.1 Adult T-cell Leukemia/Lymphoma (ATL)

ATL is a CD4$^+$ T-cell malignancy that develops after a latency period of approximately 40 years [21,22]. There are four subtypes of ATL that have slightly different presentations and outcomes [22,30]. These clinical subtypes, in increasing order of severity, are smoldering, chronic, lymphoma, and acute. ATL cells are characterized by their multi-lobulated nuclei, giving them the nickname of “flower cells” [21,22,30]. Sequencing of the proviral DNA in ATL cells has demonstrated a preferential selection for integrated proviruses that lack a 5’ long terminal repeat (LTR) (to be discussed in more detail in a later section).

Patients with ATL suffer from several abnormalities, including lymphadenopathy, hepatosplenomegaly, hypercalcemia associated with lytic bone lesions, elevated lactate dehydrogenase (LDH), as well as the presence of a soluble form of interleukin-2 (IL-2) receptor in the patient serum [30]. It is also common for ATL patients to suffer from massive cellular infiltration of organs such as the skin, liver, spleen, the gastrointestinal tract, and lungs [21,22,30].

Currently, there is no effective therapy for ATL; many cases of ATL are chemotherapy resistant and must rely on antivirals. The 5-year survival rate for patients receiving first-line antiviral treatment is only 46%, whereas survival for patients receiving first-line chemotherapy and first-line chemotherapy followed by antivirals are 20% and 12%, respectively [22]. The effectiveness of each treatment depends heavily on the subtype of ATL and other factors, such as the presence of functional p53. Unfortunately, the overall median survival of ATL patients is between 6 and 18 months [21,22].
1.2.2 HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

HAM/TSP is a neurological disease caused by HTLV-1. Most cases present in infected individuals in their fourth decade of life, but development can be rapid. Cases have been reported in children and in transfusion patients who received contaminated blood [7,31]. Development of HAM/TSP occurs in up to 4% of HTLV-1-infected individuals, and occurs more often in women than in men [7,31].

HAM/TSP is a slowly progressive spastic paraparesis pathologically characterized by severe white matter degeneration and fibrosis in the thoracic spinal cord [7,31]. Physically, patients suffer from weakness in their legs, stiffness, and an increased frequency and urgency to urinate [31]. It is currently hypothesized that the immune response to HTLV-1 is the cause of HAM/TSP: patients have an increase in circulating inflammatory cytokines and activated CD8+ T-cells [21,31,32]. It also has been reported that antibodies against the HTLV-1 Tax protein cross-react with the neuronal protein hnRNP A1, indicating that molecular mimicry may contribute to HAM/TSP development [33,34]. Other studies have reported an association between certain HLA types with higher proviral loads and HAM/TSP development [35], which would suggest a link between genetics and disease susceptibility.

1.2.3 HTLV-2 Associated Disease

Although HTLV-2 was first isolated from a cancer patient, there has been no causal link found between HTLV-2 and development of malignancy [8]. However, HTLV-2-infected individuals typically exhibit lymphocytosis [36]. There have been
a few reported cases of HAM/TSP-like disease in HTLV-2 infected individuals; however, the link between HTLV-2 and neurological disease is not definitive because other confounding factors are also usually present.

1.2.4 HTLV and HIV Co-infections

HTLV-1, HTLV-2, and HIV-1 share similar routes of infection and it is known that co-infections do occur, mainly in large metropolitan areas and endemic regions [37]. The effect of HTLV-1 and HIV-1 co-infections on disease progression is somewhat controversial. Some reports have indicated that HIV-1/HTLV-1 co-infection leads to a more rapid progression to AIDS and an increase in HAM/TSP incidence [37]. In contrast, other reports have suggested outcomes ranging from no effect to a slight protective effect against AIDS and HAM/TSP progression/development [37].

In contrast to what has been observed for HIV-1/HTLV-1 co-infections, it generally is accepted that HIV-1/HTLV-2 co-infection has a protective affect against AIDS progression. Individuals who are infected by both HTLV-2 and HIV-1, especially those with high HTLV-2 proviral loads, show a long-term non-progressor phenotype [38]. One mechanism for this protective effect is that Tax-2 induces CC-chemokines that block the CCR5 receptor for HIV-1, inhibiting its entry [39,40].
1.3 HTLV Transmission and Viral Life Cycle

1.3.1 HTLV Epidemiological Transmission

From an epidemiological standpoint, virus transmission occurs through several routes. Mother-to-child transmission of HTLV has been shown through breast feeding [41], which has been associated with an increased risk of developing ATL [42]. Sexual transmission is possible, and is more commonly seen as transmission from male-to-female rather than female-to-male [43-45]. Exposure to infected blood, either by whole cell blood transfusion or shared needles among intravenous drug users, is yet another mode of HTLV transmission [46].

1.3.2 HTLV Molecular Transmission

From a molecular perspective, cell-free HTLV transmission to T-cells is very inefficient [47]; instead, the virus requires cell-cell contact [47,48]. Infected cells form a virological synapse with uninfected cells, bringing budding virus in close contact with new cell targets, allowing for efficient infection [49]. Dendritic cells, however, are able to be infected by cell-free virus [50], allowing for transmission of virus from dendritic cells to T-cells both in cis and in trans.

1.3.3 HTLV Viral Life Cycle

The HTLV life cycle, like all retroviral life cycles starts with a mature virion expressing the surface unit (SU) of the viral protein envelope (Env) and binding to a specific cellular receptor (Figure 1.1). HTLV-1 and HTLV-2 use the cellular receptors glucose transporter-1 (GLUT-1) and neuropilin-1 (NRP-1). HTLV-1 also
utilizes heparan sulfate proteoglycan (HSPG) to facilitate binding, whereas HTLV-2 does not. After receptor binding, the transmembrane unit (TM) of Env mediates the fusion of the viral and cellular membranes. The uncoated viral core then is released into the cytoplasm and the packaged components of reverse transcriptase (RT) and tRNA\textsuperscript{Pro} bind to the genomic RNA subsequently reverse transcribing it into a double-stranded DNA intermediate. The double-stranded viral DNA along with viral and cellular proteins make up the pre-integration complex (PIC). The PIC is transported to the nucleus and the viral protein integrase mediates its integration into the host genome. This integrated form of viral DNA is known as the provirus. Utilizing cellular machinery, viral genes are transcribed to RNA and are exported to the cytoplasm or spliced and exported to the cytoplasm where they are processed and translated into viral proteins. Immature structural proteins assemble at the cellular membrane where a new virion forms. Enzymatic proteins and two copies of the full length RNA genome are packaged, and the new virion buds from the cell. Packaged viral proteases cleave the structural proteins, resulting in a fully mature and infectious virion.

### 1.4 HTLV Genomic Organization

The HTLV virion contains two copies of the single-stranded (+) sense RNA genome. Each RNA genome contains a cap at the 5’ end and a polyA tail at the 3’ end. At the 5’ end of the RNA genome, the coding sequence is flanked by a repeat (R) region and a unique 5’ (U5) region. Flanking the 3’ end of the coding
sequence is a unique 3’ (U3) region and a duplicated R region. Once the RNA genome is reverse transcribed to create a double-stranded DNA intermediate, the coding sequence is flanked by long terminal repeats (LTRs) consisting of the U3-R-U5 sequences. The U3 region of the 5’ LTR contains the promoter for all sense strand-encoded genes of the integrated provirus. The 5’ end of the provirus encodes the structural and enzymatic genes that are encoded by all retroviruses (Figure 1.2). Several of these genes are transcribed from the same RNA; therefore, ribosomal frame shifts are required to generate all of the viral protein products. The 3’ end of the provirus contains a unique region, termed the pX region, which encodes regulatory and accessory genes. It is because of this region that HTLVs are classified as complex retroviruses. HTLVs also contain another gene encoded by the antisense strand of the provirus whose promoter is contained in the 3’ LTR.

**1.5 HTLV-1 and HTLV-2 Viral Proteins**

As mentioned above, the HTLV provirus encodes for enzymatic and structural genes towards its 5’ end, which are common to all retroviruses. The pX region encodes for regulatory and accessory genes. The proteins of HTLV-1 and HTLV-2 share similar amino acid sequences and similar functions. Below, these proteins will be discussed and important differences between the HTLV-1 and HTLV-2 counterparts will be highlighted.
1.5.1 Structural and Enzymatic Proteins

The group-specific antigen (Gag) protein is a 55kD polypeptide precursor transcribed from the genomic RNA. It is subsequently cleaved into three proteins, the matrix (p19, MA), capsid (p24, CA) and nucleocapsid (p15, NC), which are essential for the proper assembly of mature infectious virions. During viral replication, infected cells release p19, which can be detected using an enzyme-linked immunosorbent assay (ELISA). This assay has come to be widely used as a correlative and reproducible measure of virus production.

Protease (Pro) is encoded by the same genomic RNA as Gag but is produced by ribosomal frameshifting. Autocatalysis cleaves the Pro polyprotein into its mature, active form. Subsequently, Pro cleaves the Gag and polymerase polypeptides into their cognate proteins.

The polymerase gene (pol) is the third gene encoded by the genomic RNA. A second unique ribosomal frameshift is needed to generate the polypeptide precursor. Pro cleaves the polypeptide into polymerase and integrase. Pol is an RNA-dependent DNA polymerase also known as reverse transcriptase (RT). The N-terminus of the protein contains the RT activity, whereas the C-terminus contains the RNase H activity. RNase H is needed to degrade RNA from the DNA:RNA hybrid after reverse transcription. Following complete reverse transcription, the double-stranded DNA intermediate is imported to the nucleus. Here, the viral integrase facilitates the proper integration of the DNA intermediate into the host genome, resulting in the provirus.
The envelope (env) gene is encoded by a singly spliced mRNA. The Env protein is a polypeptide that is cleaved into a surface unit (SU) and a transmembrane unit (TM). Env interacts with the receptors on target cells and mediates viral entry. It has been demonstrated that Env is the major determinant for HTLV CD4\(^+\) or CD8\(^+\) T-cell transformation tropism [51]. This tropism is, at least in part, determined by the SU subunit of Env and occurs post entry [52].

### 1.5.2 Regulatory Proteins

HTLVs encode two regulatory proteins, Tax and Rex that are encoded by the same doubly-spliced mRNA in two separate but overlapping reading frames.

#### 1.5.2.1 Tax

HTLV-1 Tax (Tax-1) and HTLV-2 Tax (Tax-2) are highly similar proteins (~78% amino acid identity, ~84% similarity) that act as transcriptional regulators for HTLV-1 and HTLV-2, respectively. Tax-1 is found predominantly within the nucleus while Tax-2 is found predominantly in the cytoplasm [53]. Tax-2, however, can also co-localize to nuclear bodies in a punctate manner, similar to Tax-1 nuclear bodies [54].

Tax associates with CREB transcription co-factors at 21-bp repeat sites within the LTR [55]. The Tax/CREB complex provides a docking site for CBP/p300 and acts as a tether to the DNA, which allows for acetylation of chromatin by the histone acetyl-transferase domain of CBP/p300 and activation of transcription [55,56].
Tax not only drives transcription of the provirus, but also can transactivate cellular pathways. Some of these pathways include PI3K/AKT, AP-1, CREB/ATF, MAPK, and NF-κB [53,57]. NF-κB is divided into the classical and alternative pathways. Activation of NF-κB by Tax is important for PBMC immortalization for both HTLV-1 [58] and HTLV-2 [59]. Both Tax-1 and Tax-2 activate the classical pathway to similar levels by increasing the kinase activity of IKKγ. Activation of IKKγ leads to phosphorylation and degradation of the inhibitor of NF-κB, thereby releasing the NF-κB dimer p65(RelA)/p50 to translocate to the nucleus to activate cellular genes [53,60,61]. Tax-1 also activates the alternative pathway by inducing the cleavage of p100 to p52 and nuclear translocation of the p52/RelB dimer [53,61]. The activation of this alternative pathway is important for the ability of Tax-1 to transform the mouse T-cell line, CTLL-2 [61]. Tax-2 is unable to efficiently activate the alternative NF-κB pathway, and therefore is unable to transform CTLL-2 cells by itself. However, when Tax-2 was expressed along with a constitutively active form of NF-κB inducing kinase (NIK), which induces processing of p100 into p52, transformation of CTLL-2 cells was partially rescued [61].

Tax modulates cell-cycle progression and DNA-damage repair pathways. During the G1 stage of the cell cycle, cyclins D and E increase in concentration and form complexes with cyclin-dependent kinases (Cdk) 4/6 and Cdk2, respectively. These cyclin/Cdk complexes target the hypophosphorylated tumor suppressor gene retinoblastoma (Rb), causing the release of transcription factor E2F. E2F subsequently induces the expression of genes necessary for the
transition into S phase for DNA replication. Formation of cyclin/Cdk complexes can be inhibited however, by Cdk inhibitors such as p16\textsuperscript{INK4a}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}. Tax accelerates the cell through G1 by inducing the expression of cyclin D, Cdk2 and Cdk4 [62,63] and strengthens the interaction between cyclin D and Cdk4 [64]. Tax also represses the expression of p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d} [65] and binds to p16\textsuperscript{INK4a} so it can no longer sequester Cdks [66]. A report has demonstrated that Tax can induce the degradation of hypophosphorylated Rb, committing the cell to progress to S phase [67].

ATL typically takes several decades to develop after infection by HTLV-1, indicating that several mutations must occur before development of cancer. Accumulating DNA damage has been observed in HTLV-infected cells both in vitro and in patients [68]. The ability of Tax to deregulate the DNA-damage repair response presents one possible mechanism of DNA damage. Tax is able to repress the nucleotide excision repair (NER) response [69] by inducing the expression of the gene proliferation cell nuclear antigen (PCNA) [70-72], which is the processivity component of DNA polymerase δ. Excess PCNA leads to read-through of DNA lesions and incorporation of the damaged sites into the DNA [73]. Tax also inhibits the base excision repair (BER) response by repressing the transcription of BER-linked DNA polymerase β.

1.5.2.2 Rex

The Rex protein is a post-transcriptional regulatory protein. The cell normally maintains mRNAs that include introns in the nucleus for further splicing or for eventual degradation. Following this programming, the cell tries to keep the
intron-containing full length HTLV genomic RNA, gag/pol mRNA, and the singly spliced env mRNA from being exported to the cytoplasm. To ensure protection from degradation and proper translation of the structural and enzymatic proteins for virion assembly, Rex binds to these mRNAs via their Rex responsive element (RxRE) and shuttles them out of the nucleus [74,75]. Studies have shown that while Rex is dispensable for T-cell transformation in vitro, it is necessary for replication and ultimately viral persistence in vivo [76].

1.5.3 HTLV Accessory Proteins

HTLV accessory genes play important roles for the virus in vivo. All of these genes have functions that contribute to the regulation of viral gene expression and/or prevent virus clearance by the host immune response.

1.5.3.1 HTLV-1 p12 and p8

p12 is a hydrophobic protein that is found predominantly in the ER-Golgi apparatus. It is capable of binding to several cellular proteins such as components of the IL-2 receptor, the MHC-I heavy chain, calreticulin, and vacuolar-ATPase. The binding of p12 to the MHC-I heavy chain inhibits the assembly of the full complex, limiting the amount of MHC-I on the cellular surface, which may help infected cells escape the immune response. Ablation of this gene in the context of a full-length HTLV-1 molecular clone reduces infectivity for human primary dendritic cells, human PBMCs, rabbits, and macaques [77,78].
p8 is encoded by the same mRNA as p12; in fact, it is produced by the cleavage of the p12 protein. Despite being processed from p12, p8 has a unique localization as it is found at the plasma membrane surface. Recently, p8 has been found to play a role in viral transmission [79], causing an increase in lymphocyte function-associated antigen-1 (LFA-1) clustering at the cell surface, creating a cellular conduit through which the virus can infect new targets.

1.5.3.2 HTLV-2 p10 and p11

HTLV-2 encodes p10 and p11 on the same mRNA by separate, but overlapping reading frames. Both proteins are functionally similar in that they both bind to the MHC-I heavy chain and reduce its expression on the cell surface [80], which promotes escape of infected cells from the immune response. Despite this similarity with HTLV-1 p12, p10 and p11 do not share the ability of p12 to interact with the IL-2 receptor or vacuolar-ATPases [80].

1.5.3.3 HTLV-1 p30 and p13

HTLV-1 encodes p30 on a doubly spliced mRNA. p30 is a nuclear protein that acts in a negative post-transcriptional manner. p30 binds to tax/rex mRNA and retains it in the nucleus, inhibiting viral replication [81]. p30 also binds to CBP/p300, keeping it from interacting with Tax [82]. In addition, this viral protein promotes survival of infected cells during genotoxic stress through its interactions with ataxia telangiectasia mutated (ATM) and Regγ [83].

p13 is encoded by the same open reading frame as p30 and consists of the C-terminal 87 amino acids of p30. p13 can be produced using the internal
methionine of p30 or from an alternatively spliced mRNA that lacks the p30 N-terminus coding region. Generally found to localize at the mitochondrial membrane, p13 can also be found in nuclear speckles where it interacts with Tax and inhibits viral replication [77,78,84].

1.5.3.4 HTLV-2 p28

HTLV-2 p28 is the counterpart of HTLV-1 p30 and it also acts as a negative post-transcriptional regulator, retaining tax/rex mRNA in the nucleus [81]. However, p28 does not further repress viral gene expression like p30 because it cannot interact with CBP/p300. p28 also does not interact with ATM or Regγ [85], and therefore does not support survival of infected cells under genotoxic stress.

1.5.3.5 HTLV-1 bZIP factor (HBZ)

HBZ is unique among the genes encoded by HTLV-1 in that it is encoded by an mRNA transcribed from the antisense strand of the proviral genome with its promoter in the 3’ LTR. An mRNA encoded by the antisense strand was first detected in 1989 [86], however the protein was not documented until 2002 [87]. HBZ contains three distinct domains: an activation domain at the N-terminus (which contains two LXXLL like motifs important for protein-protein interactions [88,89]), a central domain (which contains nuclear localization signals [90]), and a basic leucine zipper (bZIP) domain towards the C-terminus [87] (Figure 1.3). HBZ was first characterized by its ability to inhibit Tax transactivation using three mechanisms: 1) titrating away CREB proteins from Tax [87,91], 2) titrating away
p300 from Tax [88], and 3) inducing E3 ubiquitin ligase PDLIM2, which targets Tax for degradation [92-94].

There are two main isoforms of HBZ: a 209 amino acid protein encoded by an unspliced mRNA, and a 206 amino acid protein encoded by a singly spliced mRNA (SP1) [95]. These two isoforms differ only by seven amino acids within their N-termini. Another spliced variant of Hbz mRNA has been reported as well (SP2), but the SP1 variant that encodes the 206 amino acid protein is dominant [95].

Using a molecular clone with a stop codon introduced early in the HBZ coding sequence, Arnold et al. demonstrated that HBZ was dispensable for in vitro immortalization of PBMCs. However, HBZ was shown to be required for efficient infection, infected T-cell survival, and persistence of the virus in vivo [96]. Other studies have examined the role of HBZ in modulation of cellular pathways and observed that HBZ represses components of the AP-1 [97-100] and classical NF-κB signaling pathways [92,101]. HBZ also represses the tumor suppressor gene interferon regulatory factor-1 (IRF-1), which is down-regulated in many forms of leukemia [102].

Studies using HBZ transgenic mice have provided evidence that HBZ also alters the immune response and expression of cytokines. Sugata et al. observed that HBZ was capable of suppressing the cytokine production of Th1 cells [103]. It was further observed that HBZ was able to induce FoxP3 expression through the enhancement of TGF-β signaling, resulting in cells phenotypically resembling
induced T-regulatory cells (iTregs) [104,105]. However, these cells eventually lose FoxP3 expression and express IFN-γ, resulting in inflammation [106].

Although Tax activity is important during the early stages of ATL development, studies have shown the importance of the \( hbz \) gene as well. The majority of ATL cases no longer express Tax protein due to mutations within the \( tax \) gene [107], epigenetic silencing of the HTLV promoter [108,109], or deletion of the 5’ LTR promoter [110,111]. However, the \( hbz \) gene is expressed in the vast majority of HTLV-1 cell lines and ATL cases [112]. There is now ample evidence that HBZ promotes cell proliferation [112-115]. Furthermore, HBZ enhances JunD-mediated transcription, leading to an increase in human telomerase reverse transcriptase (hTERT) expression [89,116,117], which is a key late stage step in the development of cancer. The use of transgenic mice has shown that HBZ increases the incidence of lymphoma after a long latency period, similarly to what has been observed in the development of ATL [104].

1.5.3.6 Antisense Protein of HTLV-2 (APH-2)

APH-2, a 183 amino acid nuclear protein, was discovered in 2009 to be encoded by a singly-spliced mRNA transcribed from the antisense strand of the HTLV-2 provirus [118]. Like HTLV-1 HBZ, APH-2 represses Tax transactivation of the 5’ LTR promoter by titrating away CREB [118,119]. APH-2 binds to CREB despite the fact that it lacks a classical bZIP domain [118]. Instead, APH-2 binds to CREB via a C-terminal LXXLL-like motif [119] (Figure 1.3). HBZ also contains
LXXLL-like motifs but they are used to interact with p300 [88], which is a protein APH-2 is not able to bind [118].

After the discovery of APH-2, studies were conducted to compare it to HBZ in order to distinguish the essential roles of HBZ. Douceron et al. reported that unlike HBZ, APH-2 was unable to induce proliferation of cells [120]. Yin et al. used a rabbit model to demonstrate that APH-2 was not necessary for viral infection and persistence in vivo [119]. In fact, rabbits infected with HTLV-2 mutant viruses lacking APH-2 had increased antibody responses to HTLV-2 antigens and increased proviral loads. This finding showed that the virus was able to replicate significantly better when APH-2 was not present. It also was reported that APH-2 modulates JunB and c-Jun differently than HBZ, increasing the transcriptional activity of these AP-1 components instead of inhibiting them [121]. Further comparative studies between these two proteins will be discussed in Chapter 2.

1.6 Experimental Systems

1.6.1 Cell Culture

The study of HTLV, like other virus/model systems, is not without its complications. Cell-free infection is very inefficient and co-cultivation of T-cells with producer cells is needed for efficient propagation of the virus [47,48]. HTLV is able to infect a variety of cells, including T-cells, B-cells, monocytes, and endothelial cells, but only T-cells can be immortalized and transformed. T-cell
immortalization by HTLV is defined as continuous growth in the presence of IL-2, while transformation is defined as continuous growth without IL-2. Many informative over-expression assays have been performed in cell culture. These experiments have led to a better understanding of the multiple functions of HTLV genes. The use of HTLV molecular clones is also a very helpful tool, allowing for the study of genes in the context of the whole virus. Using mutagenesis, genes can be knocked out of the molecular clones; however, precautions must be taken in this approach as several HTLV open reading frames overlap.

1.6.2 HTLV Animal Models

There are many aspects of HTLV infection and pathogenesis that cannot be recapitulated using cell culture. To fully grasp the importance of gene expression and mechanisms of action, in vivo studies must be conducted. There are several useful animal models for HTLV, each with their own set of advantages and disadvantages.

Non-human primates are considered to be an excellent animal model for HTLV studies because they have several similarities to humans. Simian T-cell leukemia virus type-1 (STLV-1) is a useful tool as it can readily establish infections in monkeys and is highly related to HTLV-1. Miura et al. demonstrated that the Tax protein and bZIP factor protein encoded by STLV-1 have similar functions to HTLV-1 Tax and HBZ [122]. STLV-1-infected Japanese macaques were even used to test mogamulizumab, a humanized IgG1 monoclonal antibody against CCR4 that is used to treat relapsed ATL patients. Miura et al. showed
this antibody caused a significant decrease of infected cells and proviral load in these monkeys and suggested this model to be a suitable representative of HTLV-1 carriers [121]. However, the cost of housing and upkeep of monkeys is quite expensive. There is also no consistent induction of disease, so other animal models are frequently used.

Rabbit animal models have been used in HTLV research for thirty years to study transmission and persistent infections. Recently, rabbits have been used to demonstrate that HTLV immortalization tropism is a post-entry effect that results from clonal expansion [123]. It also has been demonstrated that Rex and HBZ are necessary for efficient infection and persistence using molecular clones in rabbits [76,96]. Although a useful model for persistence and transmission, rabbits do not develop HTLV disease.

Another small animal model used in HTLV research is the rat. Specific rat strains can be infected with HTLV and subsequently develop a HAM/TSP-like disease [124,125]. The histological presentation, however, is not a complete recapitulation of the disease in humans [126]. Rats also are capable of spreading the virus from infected mothers to newborn pups [127,128], allowing vertical transmission of HTLV to be studied.

The most common small animal model in HTLV research is the mouse. Severe combined immunodeficiency (SCID) mice have been used to successfully study the proliferation and tumorigenicity of HTLV-1-transformed cells [113,129]. SCID mice also develop complications, such as hypercalcemia, commonly
observed in ATL patients [130]. This model has been used to test potential ATL drugs [131-135].

The newest model being developed and used to study HTLV is the humanized mouse. Tezuka et al. used intra-bone marrow injection of CD133+ hematopoietic stem cells into NOD/Shi-scid/IL-2Rγc null (NOG) mice to develop a mouse with human circulating CD4+ T-cells [136]. Inoculation of these mice with irradiated HTLV-1-producing cells resulted in establishment of infection and development of leukemia with several ATL characteristics. These included the presence of the multi-lobulated nuclei flower cells, clonal proliferation of CD25+ T-cells, inflammatory hypercytokinemia, and an HTLV-1-specific adaptive immune response against Tax. While still in the early stages of investigation, this model shows promise for the study of HTLV infection and ATL pathogenesis.

1.7 Conclusion

HTLV-1 and HTLV-2 are human retroviruses that share a high percentage of sequence similarity, but have greatly divergent pathological outcomes. HTLV-1 is the etiological agent of the aggressive CD4+ T-cell malignancy ATL, as well as the neurodegenerative disease HAM/TSP. HTLV-2 infection, on the other hand, is considered to be asymptomatic, with only a few reported cases of HAM/TSP-like disease, and no causative links to cancer. The functional differences between HTLV-1 proteins and their HTLV-2 counterparts are hypothesized to play key roles in the different outcomes of viral infection. Many studies have focused on the Tax proteins, as Tax is known to transform T-cells in vitro. The
focus of my thesis is on comparisons between the antisense-transcribed proteins, HBZ and APH-2. Evidence that HBZ is critical for the development and maintenance of ATL is growing at a rapid rate. Comparative studies between HBZ and APH-2 will provide new insights into the leukemogenic process arising from HTLV-1 infection. Post-translational modifications of HBZ are also examined in my thesis, as we hypothesize that these modifications may play a role in the ability of HBZ to modulate viral and cellular gene expression.
Figure 1.1: Retroviral lifecycle. Schematic showing each step of the general retroviral lifecycle.
Figure 1.2: Genomic organization of HTLV-1. Proviral DNA measured in kilobases (Kb) containing the long terminal repeats (LTRs), mRNAs, and open reading frames (ORFs) are shown. ORFs are indicated by boxes. Spliced out RNA sequences are indicated by dotted lines.
Figure 1.3: HBZ and APH-2 domains. Schematic showing the key domains for HTLV-1’s major spliced variant HBZ (SP1) and HTLV-2’s APH-2. Numbers represent amino acids.
Chapter 2

Functional Comparison of HTLV-1 HBZ and HTLV-2 APH-2

2.1 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a complex oncogenic deltaretrovirus that infects an estimated 15-25 million people worldwide with endemic areas of infection in southwestern Japan, Africa, South America, and the Caribbean basin [7]. Approximately 2-5% of infected individuals develop disease related to HTLV-1 infection after a long clinical latency period upwards of four decades. HTLV-1 is the causative infectious agent of a highly aggressive CD4+ T-cell malignancy, adult T-cell leukemia/lymphoma (ATL) [2,3], and a neurodegenerative disease, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [137,138]. ATL is refractory to current chemotherapy, thus, there is currently no effective therapy. Even when treated aggressively, ATL patients can expect a median survival range of 8-10 months [21,22,30].

Human T-cell leukemia virus type 2 (HTLV-2) is a related retrovirus, sharing a similar genomic structure to HTLV-1. Both viruses encode the retroviral structural and enzymatic genes (gag, pol, and env), regulatory genes (tax and rex), and accessory genes important for viral infection and persistence [21].
HTLV-1 and HTLV-2 transmit most efficiently through cell-cell contact [47,48] and both viruses transform T-cells \textit{in vitro} [47,51,58,59,76]. Despite the strong genomic similarities, HTLV-2 is not closely associated with disease and has only been linked to a handful of cases with neurological disorders [31,139,140].

To better understand how HTLV-1 causes malignancy, comparative studies between HTLV-1 and HTLV-2-related gene products have been performed. Both viruses encode the regulatory gene, Tax. Tax-1 (HTLV-1) and Tax-2 (HTLV-2) have \~78\% identity at the amino acid level [53,141]. The Tax proteins have oncogenic properties and are sufficient to immortalize cells in culture [58,59,142]. Both Tax-1 and Tax-2 are transactivators of viral transcription from the 5' promoter (LTR) located in the positive sense strand of the proviral genome [141]. Tax-2 is able to modulate many of the same cellular pathways as Tax-1, including CREB/ATF and the classical NF-\kappa B pathway. However, there are some differences hypothesized to play a role in the different pathogenic outcomes, such as the ability of Tax-1 to activate the alternative NF-\kappa B pathway, which was shown to be important for the transformation of a mouse T-cell line. [53,141,143]. HTLV-1 and HTLV-2 also encode a post-transcriptional regulator of viral replication, p30 and p28, respectively [81,144]. Both p30 and p28 are required for \textit{in vivo} infection and function to retain the doubly spliced \textit{tax/rex} mRNA in the nucleus, thereby down-regulating viral gene expression [145,146].

Our lab has examined cellular host factor interactions with these proteins and discovered several similarities as well as differences in the types of cellular processes these proteins affect [85]. These differences may translate into
differences in disease development between HTLV-1 and HTLV-2.

Although Tax-1 is critical for efficient viral replication and cellular transformation, Tax-1 and gene expression from the sense strand of the HTLV-1 provirus are lost in ~70% of ATL cases [147,148]. This is caused by deletions of the viral 5’ LTR, non-sense mutations within Tax, or hyper-methylation and subsequent silencing of the viral 5’ LTR promoter [109]. The antisense strand of HTLV-1 encodes a nuclear protein, HTLV-1 bZIP factor (HBZ) that represses Tax transactivation by binding the cellular co-factors CREB and p300, preventing them from interacting with Tax [87,88,91]. HBZ is expressed in all ATL cell lines and in HTLV-1-infected individuals [112,113]. Our lab has shown that HBZ protein is required for efficient HTLV-1 infection and persistence in a rabbit model [96]. Several studies have fueled the hypothesis that HBZ is a secondary oncogene that plays a key role in cell proliferation [112,113,115,149] and cell survival [102,114].

More recently it was discovered that the antisense strand of HTLV-2 also encodes a protein, APH-2 [118]. APH-2 is also a nuclear protein that represses Tax transactivation through its interaction with CREB [118,119]. Using a rabbit model, our lab examined the role of APH-2 in viral infection and persistence, and found that APH-2 was dispensable. However, it was also clear that APH-2 does have an effect in vivo, because the APH-2 knockout virus was able to replicate better [119]. Based on these data, we hypothesized that comparative studies between HBZ and APH-2 may lead to a greater understanding of the role of HBZ in viral persistence and potentially ATL development. Several studies (Table 2.1)
have now compared how APH-2 affects known HBZ-modulated pathways. Herein, we expanded these studies to include APH-2 and HBZ modulation of TGF-β signaling, p65-mediated transactivation, and IRF-1-mediated transactivation, thereby demonstrating the involvement of both proteins in modulation of the immune response, cell growth, and survival of infected cells. Finally, our detailed comparisons between HBZ and APH-2 protein stability and RNA transcript abundance have provided us with a clearer picture of the viral landscape during natural in vivo infection.

2.2 Materials and Methods

**Cell lines and culture.** HEK293T and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher-Life Technologies, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL). Peripheral blood lymphocyte (PBL) lines were maintained in RPMI supplemented with 20% FBS, 20 U/ml recombinant human interleukin-2 (rhIL-2; Roche Applied Biosciences, Mannheim, Germany), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. All cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and air.

**Plasmids.** Flag-HBZ and Flag-APH-2 cDNA were cloned downstream of the CMV promoter and Flag-6xHis epitope in a modified expression vector. The TGF-β responsive 9xCAGA luciferase reporter plasmid was kindly provided by Dr. Masao Matsuoka, Kyoto University and described previously [105]. The IRF-1
expression plasmid and IRF-1 luciferase reporter plasmid were graciously provided by Dr. John Yim, Beckman Research Institute and described previously [150]. The p65 expression plasmid and κB luciferase reporter plasmid were a generous gift from Dr. Dean Ballard, Vanderbilt University and described previously [151]. The pME-APH-2 cDNA expression plasmid was generated and described previously [119]. The pME-HBZ cDNA expression plasmid was generated and described previously [96]. The transfection efficiency control plasmid TK-Renilla was described previously [81].

**Transient transfections.** HEK293T and HepG2 cells were transfected using either Lipofectamine Transfection Reagent (Thermo) or Lipofectamine 2000 Transfection Reagent (Thermo) respectively, according to the manufacturer's instructions. Each transfection experiment was performed at least three separate times with similar results.

**Reporter gene assays.** HEK293T cells were transfected using Lipofectamine Transfection Reagent (Thermo) in a 6-well dish with 20 ng TK-Renilla, 100 ng κB–luciferase construct and 50 ng of p65 expression plasmid for the p65 assays or 100 ng IRF-1 responsive luciferase construct and 25 ng IRF-1 expression plasmid for the IRF-1 studies, and 400 ng Flag-HBZ, 400 ng Flag-APH-2, or 1600 ng Flag-APH-2 expression plasmid. HepG2 cells were transfected using Lipofectamine 2000 Transfection Reagent (Thermo) in a 6-well dish with 50 ng TK-Renilla, 500 ng 9xCAGA luciferase construct, and 400 ng Flag-HBZ, 400 ng Flag-APH-2, or 1600 ng Flag-APH-2 expression plasmid. Exogenous TGF-β (Bio-Techne, Minneapolis, MN) was added to the HepG2 cell culture medium at
10 ng/ml 24 hours post-transfection. HEK293T and HepG2 cells were harvested 24 and 48 hours post-transfection, respectively, in Passive Lysis Buffer (Promega, Madison, WI), then analyzed using the Dual-Luciferase ® Reporter Assay System (Promega) according to the manufacturer's instructions. Levels of firefly luciferase and Renilla luciferase were measured using a Packard LumiCount luminometer (Packard Bioscience Company, Meriden, CT). Each condition was performed in triplicate.

**Immunoblotting.** Cell lysates were harvested in Passive Lysis Buffer (Promega) containing protease inhibitor cocktail (Roche) and quantitated using an ND-1000 Nanodrop spectrophotometer (Thermo). Equivalent amounts of protein (40 µg) were separated in Mini-PROTEAN ® TGX ™ Precast 4-20% Gels (Bio-Rad Laboratories Inc., Hercules, CA) and transferred to nitrocellulose membranes. Membranes were blocked in PBS containing 5% milk and 0.1% Tween-20 solution and incubated with primary antibody. The following antibodies were used: anti-HBZ ([96]; 1:1000), anti-APH-2 ([119]; 1:1000), anti-Flag clone M2 (Sigma Aldrich, St. Louis, MO; 1:5000), and anti-β-actin (Sigma; 1:5000). The secondary antibodies used were HRP goat-anti-rabbit and goat-anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000). Blots were developed using Immunocruz luminol reagent (Santa Cruz). Images were taken using the Fuji LAS 4000 Imaging System (GE Healthcare Life Sciences Piscataway, NJ).

**Quantitative RT-PCR.** Total RNA was isolated from 10^6 cells per condition using the Qiagen RNeasy Mini Kit (Qiagen Inc.-USA, Valencia, CA) according to the manufacturer’s instructions. Isolated RNA was quantitated and DNase treated.
Reverse transcription was performed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Thermo) according to the manufacturer’s instructions. The instrumentation and general principles of the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) are described in detail in the operator’s manual. PCR amplification was carried out in 96-well plates with optical caps. The final reaction volume was 20 μL consisting of 10μL iQ™ SYBR® Green Supermix (Bio-Rad), 300 nM of each specific primer, and 2 μL of cDNA template. For each run, standard cDNA, sample cDNA and a no-template control were all assayed in triplicate. The reaction conditions were 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. Primer pairs to specifically detect viral mRNA species (gag/pol, tax/rex, hbz, aph-2) and hGAPDH were described previously [123,152]. Data is presented in histogram form with standard deviations from triplicate experiments. Total copy number for each viral gene was determined using plasmid DNA standards and normalized to 10^6 copies of hGAPDH mRNA.

**Cycloheximide pulse-chase.** HEK293T cells were transiently transfected with either pME-HBZ or pME-APH-2. Twenty-four hours later, cells were treated with 100 μg/mL cycloheximide (Sigma) and then harvested at different time points (0, 3, 6, 9, 12, 15, and 24 hrs for HBZ; 0, 30, 60, 90, 120, 150, and 180 min for APH-2). Cell lysates were harvested in NP-40 lysis buffer containing protease inhibitor cocktail (Roche) and quantitated using the Pierce BCA Protein Assay Kit (Thermo). Extracted proteins were separated by SDS-PAGE and blotted with the appropriate antibodies as previously described in the Immunoblotting section.
Densitometric data was calculated with the Fuji LAS 4000 Imaging System (GE Healthcare Life Sciences)

Statistical analysis. Statistics were performed using the T-test function in Microsoft Excel.

2.3 Results
APH-2 protein is less stable than HBZ protein.

In order to conduct functional comparison studies between APH-2 and HBZ, we first determined measurable and comparable protein expression by western blot analysis. Both APH-2 and HBZ were cloned into a CMV-driven expression vector with the Flag epitope at the protein N-terminus. HEK293T cells were transfected with the Flag-APH-2 and Flag-HBZ vectors and western blot analysis was performed. Using equal amounts of transfected Flag-APH-2 and Flag-HBZ vectors, we detected a roughly 6-fold difference between the amount of Flag-HBZ and Flag-APH-2 protein (Figure 2.1A). We next titrated Flag-APH-2 vector DNA (400 ng, 800 ng, 1200 ng, and 1600 ng) against a constant amount of Flag-HBZ vector DNA (400 ng) in order to obtain similar levels of protein expression. We observed that transfection of 400 ng Flag-HBZ vector and 1600 ng Flag-APH-2 vector produced the most equivalent levels of HBZ and APH-2 proteins (Figure 2.1A). To determine if differences in HBZ and APH-2 transient expression levels were due to differences in protein stability, we performed a cycloheximide chase study to determine protein half-life of both HBZ and APH-2. In a previous study by Yoshida et al., the calculated HBZ half-life was
approximated at less than 12 hours [153]; however, no study to date has examined the half-life of APH-2. HEK293T cells were transfected with untagged APH-2 and HBZ expression constructs and then treated with the translation elongation inhibitor cycloheximide. Cell lysates were collected at defined time intervals and the expression levels of both HBZ and APH-2 were examined by western blot analysis. The calculated half-life of HBZ was approximately 6.5 hours (Figure 2.1B), whereas the calculated half-life of APH-2 was approximately 30 minutes (Figure 2.1C). Similar results were obtained using N-terminal tagged HBZ and APH-2 constructs (data not shown). Due to the large discrepancy in protein stability, we used two concentrations of Flag-APH-2 vector (compared to Flag-HBZ vector) in our studies: a matched DNA concentration (400 ng Flag-APH-2) and a protein-adjusted DNA concentration (1600 ng Flag-APH-2).

**HBZ enhances, while APH-2 represses, TGF-β signaling.**

HBZ was reported to enhance the activity of TGF-β by strengthening the interaction between p300 and the Smad complex [105], which in turn, drove the transcription of FoxP3, the master regulator of Treg cells [104,105]. We previously reported APH-2 was able to repress TGF-β signaling. However, these experiments were performed with comparable HBZ and APH-2 protein levels [154]. We reexamined this pathway using matched amounts of transfected HBZ and APH-2 DNA (Fig 2.2). Similar to our previous results, APH-2 significantly repressed TGF-β by approximately 50% (p<0.001), while HBZ enhanced TGF-β signaling approximately 2.5 fold (p=0.003).
HBZ and APH-2 repress p65 transactivation.

HTLV primarily replicates through mitosis of infected cells as opposed to spreading infection to new cells. HTLV also induces the classical NF-κB pathway through expression of the regulatory proteins Tax-1 and Tax-2 [61]. Although this pathway induces cellular growth, hyper-activation of the NF-κB pathway by Tax can lead to a phenomenon known as Tax-induced senescence (TIS) [101,155]. HBZ inhibits both Tax-1 and the classical NF-κB pathway component, p65, thereby preventing TIS [92,101]. This balance between HBZ and Tax-1 allows for continuous cell growth and evasion of cell senescence. Given the importance of the HBZ effects on p65 in HTLV-1 biology, we examined if APH-2 would also have similar effects on p65 by using a κB-luciferase reporter vector and a p65 expression vector. HEK293T cells were transfected with the κB-luciferase reporter, p65, Flag-HBZ or Flag-APH-2, and TK-Renilla for transfection efficiency (Figure 2.3). When equal amounts of Flag-APH-2 and Flag-HBZ DNA (400 ng) were used, HBZ significantly repressed (~60%, p<0.001), while APH-2 failed to repress, p65-mediated κB activation. When Flag-APH-2 DNA was increased 4-fold to produce equivalent protein levels with Flag-HBZ, we observed significant repression (~60%, p<0.001) of p65-mediated κB activation. Therefore, both APH-2 and HBZ were able to repress p65 transactivation.

HBZ and APH-2 repress IRF-1 transactivation.

IRF-1 is a component of the innate immune response and a tumor suppressor protein [150,156] that is required for the induction of apoptosis in response to DNA damage [157]. Interestingly, loss of IRF-1 expression has been
observed in several leukemias [158,159]. In 2011, Mukai and Ohshima found that HBZ interacted with IRF-1, inhibited its DNA binding ability, and induced its degradation, thereby significantly reducing the number of cells undergoing apoptosis [102].

To examine the effects of APH-2 on IRF-1, we utilized an IRF-1-responsive luciferase reporter vector and an IRF-1 expression plasmid. HEK293T cells were transfected with the IRF-1-luciferase reporter, IRF-1, Flag-HBZ or Flag-APH-2, and TK-Renilla for transfection efficiency (Figure 2.4). When equal amounts of Flag-APH-2 and Flag-HBZ DNA were used (400 ng each), both proteins repressed IRF-1-mediated transactivation, though to a greater degree by HBZ (11% repression vs 37% repression). When Flag-APH-2 DNA was increased 4-fold to compensate for reduced protein levels, we observed an even greater repression (48%) of IRF-1-mediated transactivation. Therefore, both HBZ and APH-2 repressed IRF-1-mediated transactivation.

*aph*-2 transcript level is reduced compared to *hbz* transcript level.

Given the difference in protein stability between APH-2 and HBZ (Figure 2.1), we used quantitative RT-PCR (qRT-PCR) to measure the relative RNA transcript levels of *aph*-2 and *hbz* (Figure 2.5). Total RNAs were collected and isolated from six separate clones of the immortalized primary human T-lymphocyte cell lines PBL/HTLV-1 and PBL/HTLV-2. RNA (500 ng) was reverse transcribed and qRT-PCR analysis was performed to determine copy numbers of *gag/pol*, *tax/rex*, *hbz*, and *aph*-2 transcripts relative to 10⁶ copies of *gapdh*. In each immortalized PBL/HTLV-2 cell line, *aph*-2 mRNA levels were considerably
lower than \textit{hbz} levels in the PBL/HTLV-2 cell lines. Therefore, HTLV-2 did not appear to compensate for the instability of APH-2 protein with an increased amount of \textit{aph}-2 transcripts.

\subsection*{2.4 Discussion}

HTLV-1 and HTLV-2 share similar genomic structure and the capability to transform T-lymphocytes \textit{in vitro}. However, HTLV-1 infection can lead to ATL development while HTLV-2 is not closely associated with disease. Comparative studies between HTLV-1 and HTLV-2 viral gene products allow for a better understanding of disease development in HTLV-1 infection. For example, studies comparing Tax-1 and Tax-2 demonstrated the importance of the alternative NF-κB pathway in HTLV-induced T-cell transformation \cite{61}. We hypothesized that comparative studies between the HTLV-1 antisense protein, HBZ, and its HTLV-2 counterpart, APH-2, would clarify the importance of these viral genes for cell proliferation and disease development.

Our study is the first to examine APH-2 protein stability, and we found that HBZ has a longer half-life of approximately 6.5 hours compared to the relatively short half-life of APH-2 of 30 minutes (Figure 2.1). Upon further investigation, we determined that \textit{aph}*-2 mRNA levels were decreased relative to \textit{hbz} mRNA levels, which indicated that HTLV-2 does not transcribe more APH-2 to increase protein levels (Figure 2.5) and the resulting amount of APH-2 molecules should be significantly less than HBZ molecules during infection of the respective viruses. These results suggest that between the two concentrations of
transfected Flag-APH-2, the matched concentration to Flag-HBZ would be the better functional comparison.

Activation of TGF-β signaling results in the induction of FoxP3, the master regulator of Treg cells. HBZ was reported to enhance TGF-β signaling and subsequent FoxP3 expression [105]. We found that while HBZ enhanced the activity of TGF-β, in contrast, APH-2 actually inhibited TGF-β activity (Figure 2.2). In a recent publication, HBZ-induced FoxP3 expression was reported to be labile; however, the experiments were performed in cells only expressing HBZ [106]. Other studies showed that Foxp3 expression in Treg cells was stabilized by the expression of the cellular protein SOCS1 [160]. SOCS1 protein is up-regulated by HTLV-1 infection, specifically by Tax [161,162]. Previous observations suggested that ATL cells which act phenotypically like Treg cells have high expression of Foxp3 and maintain Tax expression [163]. Taken together, our data suggests that HBZ enhances TGF-β signaling thereby inducing a Treg cell phenotype, whereas APH-2 inhibits the TGF-β signaling pathway and therefore does not induce the Treg cell phenotype.

Regulation of the classical NF-κB pathway by both Tax and HBZ during HTLV-1 infection is highly relevant for viral growth [101]. Regarding the classical NF-κB pathway, we found that APH-2 repressed p65 transactivation only at high levels of protein expression (Figure 2.3). However, at the more relevant comparative levels of APH-2, inhibition was not detected. It is possible that at a biologically relevant level where APH-2 stability is low, it is unable to sufficiently regulate NF-κB in a manner similar to HBZ. Therefore, differences seen in
pathological outcomes between HTLV-1 and HTLV-2 may be influenced by the differences in viral regulation of the NF-κB pathway.

IRF-1, a known tumor suppressor [150,156] that is down-regulated in several leukemias [158,159], was recently shown to be negatively regulated by HBZ [102]. Similar to our results for p65 transactivation, we found that APH-2 repressed IRF-1 transactivation to a degree similar to HBZ when equivalent levels of protein were used (Figure 4). When equivalent levels of DNA for both Flag-HBZ and Flag-APH-2 were used, inhibition of IRF-1 by APH-2 was less than the inhibition by HBZ. Once again, this finding suggests that with a short half-life at biological levels, APH-2 may not be able to inhibit IRF-1 enough to prevent apoptosis of infected, tumorigenic cells.

Our comparative studies have expanded our understanding of the role of HBZ during HTLV-1 infection (summarized in Figure 2.6). APH-2, unlike HBZ, has a repressive effect on TGF-β signaling, and therefore may steer infected CD4⁺ cells away from Treg differentiation. When similar levels of transfected DNA were used, APH-2 was unable to strongly repress either the classical NF-κB or IRF-1 signaling pathways, compared to HBZ. These effects showed that during HTLV-1 infection, HBZ may help infected cells evade an immune response, and promote growth and survival. Conversely, APH-2 would not permit HTLV-2-infected cells to escape the immune response or promote growth and survival. Even though APH-2 shares some functions with HBZ, its instability strongly limits its functional role. Taken together, these conflicting functions of HBZ and APH-2 help explain the pathological differences between HTLV-1 and
HTLV-2.
Table 2.1. Summary of compared functions of HBZ and APH-2.

<table>
<thead>
<tr>
<th>Function</th>
<th>HBZ</th>
<th>APH-2</th>
</tr>
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<tbody>
<tr>
<td>Required for <em>in vitro</em> immortalization</td>
<td>-[96]</td>
<td>-[119]</td>
</tr>
<tr>
<td>Required for efficient <em>in vivo</em> infection and persistence</td>
<td>+[96]</td>
<td>-[119]</td>
</tr>
<tr>
<td>Repression of Tax transactivation</td>
<td>++[87,88,91,96,119]</td>
<td>+[118,119]</td>
</tr>
<tr>
<td>Promotes T-cell proliferation</td>
<td>+[112,113,115,149]</td>
<td>-[120]</td>
</tr>
<tr>
<td>Modulation of AP-1</td>
<td>Represses c-Jun[97,98,100] and JunB[97,99], Enhances JunD[89]</td>
<td>Enhances c-Jun, JunB, and JunD[121]</td>
</tr>
</tbody>
</table>
**Figure 2.1: APH-2 protein is less stable than HBZ protein.** (A) HEK293T cells were transfected with Flag-HBZ (400 ng) and a titration of Flag-APH-2 (400 ng, 800 ng, 1200 ng, and 1600 ng) to obtain similar protein levels as detected by immunoblot. HEK293T cells were transfected with 6 µg (B) HBZ or (C) APH-2 expression plasmid in 10-cm dishes. Cells were trypsinized and re-plated 48 hrs after transfection into 6-well dishes to approximately 80-90% confluency. Cells were then treated with 100 µg/mL cycloheximide for the indicated times. Cells were collected by centrifugation, washed in PBS, and lysed using NP-40 lysis buffer. Western blot analysis was performed to detect HBZ, APH-2, and β-actin expression levels.
Figure 2.2: HBZ enhances, while APH-2 represses, TGF-β signaling. HepG2 cells were transfected with 500 ng TGF-β responsive luciferase reporter and 400 ng Flag-HBZ, 400 ng Flag-APH-2, or 1600 ng Flag-APH-2. TK-Renilla was also transfected to determine transfection efficiency. Twenty-four hours post-transfection, cells were treated with 10 ng/ml exogenous TGF-β and allowed to incubate for 24 hours. Cell lysates were collected and luciferase levels measured. Relative luciferase activity is shown as mean fold-change with the TGF-β treatment alone set at 1.
Figure 2.3: Both HBZ and APH-2 repress p65. HEK293T cells were transfected with 100 ng κB-luciferase reporter, 50 ng p65 expression plasmid, and 400 ng Flag-HBZ, 400 ng Flag-APH-2 or 1600 ng Flag-APH2. TK-Renilla was also transfected to determine transfection efficiency. Twenty-four hours post-transfection, cell lysates were collected and luciferase levels measured. Relative luciferase activity for the p65 alone condition was set at 1.
Figure 2.4: Both HBZ and APH-2 repress IRF-1. HEK293T cells were transfected with 100 ng IRF-1 responsive luciferase reporter, 25 ng IRF-1 expression plasmid, and 400 ng Flag-HBZ, 400 ng Flag-APH-2, or 1600 ng Flag-APH-2. TK-Renilla was also transfected to determine transfection efficiency. Twenty-four hours post-transfection, cell lysates were collected and luciferase levels measured. Bars represent the mean fold-change in luciferase levels. Relative luciferase activity for the IRF-1 alone condition was set at 1.
Figure 2.5: *aph-2* transcript level is reduced compared to *hbz* transcript level. Real-time RT-PCR was performed on mRNA isolated from A) HTLV-1 and B) HTLV-2 infected PBL cell lines as described in the Materials and Methods. Primer pairs and probes to specifically detect viral mRNA species were described previously [123,152]. Data is presented in histogram form with standard deviations from triplicate experiments. Total copy number below was determined using plasmid DNA standards and normalized to $10^6$ copies of GAPDH mRNA.
Our proposed model demonstrating the importance of HBZ modulation of cellular pathways that contrasts with APH-2. HBZ enhances TGF-β, which drives the differentiation of CD4⁺ T-cells to become iTregs that can suppress the immune response. APH-2 inhibits TGF-β signaling, reducing the amount of infected CD4⁺ that become iTregs. HBZ strongly inhibits Tax and p65 expression, keeping the level of NF-κB signaling low enough to avoid Tax-induced senescence, but high enough for continuous proliferation. APH-2 also represses Tax and p65; however, with low transcript levels and instability of the protein, these functions are highly reduced and could potentially allow for high enough levels of NF-κB signaling to induce senescence. Lastly, HBZ represses the tumor suppressor gene IRF-1, allowing cells to escape apoptosis and survive. APH-2 also is capable of inhibiting IRF-1 activity, but due to transcript levels and protein instability, would not overcome a strong apoptotic signal through IRF-1.
Chapter 3
Identification and Characterization of HTLV-1 HBZ Post-Translational Modifications

3.1 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus discovered to be associated with diseases [1,3,164] including the aggressive CD4+ T-cell malignancy, adult T-cell leukemia (ATL) [88,165,166], as well as the neurodegenerative disease HTLV-1-associated myelopathy/tropical spastic paraparesis, and other inflammatory diseases [167]. HTLV-1 encodes the structural and enzymatic proteins, Gag, Pol, and Env, as well as the regulatory proteins, Tax and Rex. The virus also encodes accessory proteins that are required for efficient infection and persistence in vivo, but are dispensable for T-cell immortalization in vitro [77]. The accessory protein HBZ is unique in that it is the only viral protein encoded by the minus strand of the proviral genome while the rest of the viral proteins are encoded by the plus strand [86,87,95]. HBZ is expressed in all HTLV-1 cell lines and cases of ATL; in fact, hbz is typically the only viral gene to be expressed in approximately 60% of ATL cases [9]. This finding is attributed to deletion or hyper-methylation-silencing of the promoter in
the 5’ LTR or a non-functional mutation in the Tax transactivator, which significantly disrupts plus-strand transcription [111,147].

When HBZ was discovered, it was first shown to repress Tax transactivation of the viral promoter [87,88,91]. Since then, other functions have been reported such as modulation of the AP-1 [89,97-100] and the classical NF-κB signaling pathways [92,101]. More recent studies have shown that HBZ may regulate the cell-mediated immune response to the virus infection [102,103]. There also is growing evidence that HBZ is important in the oncogenic process since it plays a role in driving infected cell proliferation [113-115], increasing hTERT transcription [116,117], and inhibiting apoptosis [102,168].

Post-translational modifications (PTMs) are chemical modifications added to proteins that can alter many aspects of a protein, including conformation, localization, and activity. This common mechanism of cellular regulation is utilized by several pathogens, including HTLV-1, to alter the expression of their own proteins. Tax contains several PTMs, for example, phosphorylation of Tax both stabilizes the protein [169] and inhibits its activity [170]. In addition, a phosphorylation site is required for the addition of an acetyl group that activates Tax to enhance NF-κB and induce transformation [171,172]. Furthermore, our lab has shown phosphorylation to be vital for the regulation of Rex function [164].

There currently are no published data about whether HBZ is post-translationally modified; however, it is known that HBZ interacts with acetyltransferases [88,166]. Therefore, we hypothesized that HBZ, like Tax and Rex, would contain PTMs that regulate important functions. In this study, we purified
an affinity-tagged-HBZ protein and analyzed this protein by LC-MS/MS. A high percentage of the protein, including the majority of the key leucine-zipper domain at the C-terminus, was covered in this analysis. This approach identified 7 modifications, which were further characterized by mutational analysis to determine if they regulated known HBZ functions.

3.2 Material and Methods

**Cells.** HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Thermo Fisher-Life Technologies, Waltham, MA) at 37°C in a humidified atmosphere of 5% CO₂ and air. Medium was supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

**Plasmids.** To generate the Flag-6xHis-HBZ construct, the HBZ cDNA was inserted downstream of an N-terminal Flag-6xHis affinity tag and expression was driven by a CMV promoter. Amino acid exchanges were made using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequencing and expression was verified by transfection and Western blot analysis. The pCMV-c-Jun and pLG4-10-6xAP-1-Luc plasmids [173] were graciously provided by Dr. John C. McDermott of York University. The p65 expression plasmid and κB-Luc plasmid [151] were a kind gift from Dr. Dean Ballard of Vanderbilt University. The IRF-1 expression plasmid and IRF-1 luciferase reporter plasmid [150] were graciously provided by Dr. John Yim of the Beckman Research Institute.
**Protein purification.** HEK293T cells were plated in six 100 mm dishes, three per condition, and each plate was transfected with 10 µg of empty vector or Flag-6xHis-HBZ plasmid using Lipofectamine (Thermo). Twenty-four hours post-transfection, cells were collected, combined, washed in cold 1x PBS, and lysed following the FLAG fusion protein immunoprecipitation and SDS-PAGE buffer elution protocols of the FLAG® M Purification Kit (Sigma Aldrich, St. Louis, MO). Samples were loaded on a large 12% SDS-PA gel and electrophoresed for 3 hours at 55 mA. The gel was washed with Millipore water and stained using GelCode Blue Stain (Thermo). The HBZ band was excised from the gel for further proteomic analysis.

**Mass spectrometry and proteomic analysis.** LC-MS/MS analysis was performed as described previously [1,3,174] with following modifications. HBZ excised gel slices were cut into small pieces (2-3 mm cubes) and incubated on a shaker overnight in 50% acetonitrile to distain gel pieces from Coomassie dye. Samples were reduced with 7.5 mM DTT in 75 mM ammonium bicarbonate solution at 50°C for 30 min, after which DTT was removed and the protein was alkylated with 40 mM iodoacetamide in 75mM ammonium bicarbonate solution for 20 min at room temperature in dark. The gel pieces were washed with acetonitrile, desiccated in a speed-vac. Aliquots were, subjected to in-gel proteolysis using the following endoproteinases (5 ng/µl): i) sequencing grade modified trypsin (Promega, Madison, WI); ii) sequencing grade chymotrypsin (Roche Applied Sciences, Mannheim, Germany); iii) sequencing grade endoproteinase Asp-N, (Roche) and iv) Trypsin/Asp-N combination. The resulting
peptides were extracted in 100 µl of acetonitrile by vortexing for 10 min. The solution was transferred to new small microcentrifuge tubes and desiccated in a speed-vac. Dryed samples were resuspended in 6 µl buffer A (2% acetonitrile, 0.2% formic acid,) and 5 µl were separated on a 15 cm x 0.075 mm fused silica capillary column packed with reversed phase 3 µm ReproSil-Pur C_{18AQ} resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a nano EASY HPLC. Peptides were eluted over 50 min by applying a 0-30% linear gradient of buffer B (80% acetonitrile, 19.8% water and 0.2% formic acid) at a flow rate of 350 nL/min. The Orbitrap (Thermo Fischer Scientific, San Jose, CA) was run in data dependent mode with 10 data-dependent scan events for each full MS scan. Normalized collision energy was set at 35, activation Q was 0.250. AGC target for MS was $1 \times 10^6$ and AGC target for MS/MS was $5 \times 10^4$. Dynamic exclusion was set to 60s and early expiration was disabled. Sequence analysis was performed with MASCOT (Matrix Sciences, London GB) software using an indexed human subset database of Swissprot, supplemented with HTLV, 263 contaminants and 114960 decoy sequences.

**Reporter assays.** Each functional reporter assay had its own set of conditions for plasmid concentrations. In brief, HEK293T cells were seeded in 6-well plates at $2 \times 10^5$ cells per well. Twenty-four hours post-plating, cells were transfected with 10 or 20 ng of TK-Renilla, a luciferase reporter, an expression plasmid of a specific transcription factor, and an HBZ expression plasmid at one of two concentrations (1:5 or 1:10 ratio). Empty vector was added to make the total DNA concentration equal among all transfections. Transfections were performed
using Lipofectamine (Thermo). Twenty-four hours post-transfection, cells were collected and analyzed using the Dual-Luciferase ® Reporter Assay System (Promega). Levels of firefly luciferase and Renilla luciferase were measured using a Packard LumiCount luminometer (Packard Bioscience Company, Meriden, CT). Each experiment was performed three independent times in duplicates.

**Western blot analysis.** Transfected cells were lysed in 1x Passive Lysis Buffer (Promega) with protease inhibitor cocktail (Roche). Protein concentrations were measured using a Nanodrop spectrophotometer (Thermo). SDS dye (6x solution) was added to the lysates and samples were boiled for 10 min. Twenty micrograms of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with a rabbit polyclonal anti-HBZ antiserum (1:1000), a mouse anti-Flag M2 antibody (1:5000) (Sigma Aldrich, St. Louis, MO), or mouse anti-Actin (1:10000) according to standard procedures. Secondary antibodies used included goat anti-rabbit and goat anti-mouse conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:2000. Blots were developed using Immunocruz luminol reagent (Santa Cruz) and imaged using the Fuji LAS 4000 imaging system (GE Healthcare Life Sciences, Piscataway, NJ). Densitometry was measured using Multi Gauge version 3.0 software (Fujifilm, Tokyo, Japan).
3.3 Results

Prediction of PTMs.

There are currently no reports on whether HBZ is post-translationally modified, but it is well known that PTMs can play a major role in the properties and functions of proteins. Two major types of modification are phosphorylation and acetylation. These modifications are reversible and are used to modify the activity of many transcription factors [165,175]. Using the online phosphorylation prediction tool, NetPhos 2.0 Server [167,176], we found 6 potential phosphorylation sites on HBZ (Table 3.1). We also used the online tool PAIL [77,177] for acetylation prediction (Table 3.2) that predicted 16 acetylated lysines. These data suggested that HBZ was likely modified by the cell. Instead of mutating all predicted modified residues, we first set out to identify modified residues by performing mass spectrometry (MS). This approach would allow us to identify both phosphorylation and acetylation added to HBZ within a eukaryotic cell in a single assay. This analysis was dependent on the production and purification of substantial quantities of HBZ protein.

Function and purification of Flag-6xHis-HBZ.

Varying amounts of the Flag-6xHis-HBZ construct were transfected into HEK293T cells along with a Tax expression plasmid and the HTLV-1-LTR-Luciferase reporter plasmid (Figure 3.1A). As expected, Flag-6xHis-HBZ was able to repress Tax transactivation in a dose-dependent manner similarly to untagged, wild-type HBZ. We next verified that we would be able to adequately purify HBZ for mass spectrometry analysis. Using components of Sigma’s FLAG
M Purification kit, lysates from HBZ-transfected HEK293T cells were collected and HBZ was purified using agarose beads conjugated with mouse anti-Flag antibody. SDS-PAGE and GelCode Blue visualization revealed a specific band correlating to tagged HBZ that could be processed for mass spectrometry (Figure 3.1B).

Identification of PTMs.

Multiple runs of LC-MS/MS were performed with protein digestion schemes described in the Materials and Methods section. Overall, we were able to obtain 68% coverage of the amino acid sequence, including the majority of the key leucine-zipper functional domain and identified several PTMs (Figure 3.2). We detected phosphorylation on S49, acetylation on K66 and K155, and methylation on K35, K37, K181 and K186 (Figure 3.2 and Tables 3.1-3.3). The majority of these modifications occur in the important protein-protein interaction domains of HBZ. Of the predicted phosphorylation sites, we covered 5 of the 6 predicted sites (Table 3.1), and 5 of the 16 predicted acetylation sites (Table 3.2). We compared MS spectrum counts for modified peptides with their unmodified counterparts, which allowed semiquantitative analysis for the frequency of modifications (Tables 3.1-3.3). Our data suggest that the phosphorylation of HBZ is an infrequent occurrence since S49 showed limited phosphorylation. The addition of an acetyl group to K155 also seems to be a rare event, being detected approximately 3% of the time. Of the discovered methylations, our data indicate that only K35 is methylated with some consistency. Furthermore, we provide evidence that K66 is constitutively acetylated, neutralizing the positive
charge of this amino acid. All these identified modifications are novel and, we hypothesized, could regulate the properties and/or functions of HBZ.

**Identified PTMs do not affect protein steady-state levels.**

In the current study, we decided to examine the roles of phosphorylation and acetylation individually by mutating modified amino acids to mimetic (S→D and K→Q, respectively) and inhibitory (S→A and K→R, respectively) residues for each PTM. We also created a phospho-, acetyl-mimetic mutant (PhAc-mim: S49D-K66Q-K155Q), and a phospho-, acetyl-inhibitory mutant (PhAc-inh: S49A-K66R-K155R) for the discovered modified sites to investigate if they act in concert. The approach of having mimetic and inhibitory mutants allowed us to compare each mutant to the wild-type protein and the paired residue mutation. It also is important to examine both the mimetic and inhibitory mutations as both phosphorylation and acetylation can positively or negatively regulate the functions of proteins. We decided not to focus on the discovered methylation sites currently since all methylated states were found in less than half the cases of detected peptides and we cannot create a methylated lysine mimetic mutation. If future studies identify methyltransferases that interact with HBZ, it would be interesting to see if over-expression of these enzymes modifies these residues of HBZ and are important for HBZ function.

After the creation of the mutant forms of HBZ in the Flag-6xHis vector, Western blot analysis was performed to examine if any of the modifications affected the stability of the protein, as indicated by steady-state levels. We hypothesized that acetylation of K66 would be important for protein stability as it
was found to be constitutively modified. However, probing for the affinity-tag showed that none of the modifications affected the steady-state level of the protein (Figure 3.3).

**Identified PTMs do not affect inhibition of Tax transactivation.**

HBZ inhibits Tax transactivation of the LTR promoter by binding to the co-activators CREB and p300 [86-88,91,95] and by up-regulating the ubiquitin E3 ligase PDLIM2, which targets Tax for degradation [14,111,147]. To examine if the phosphorylation and acetylation of HBZ were important for this function, HEK293T cells were transfected with an HTLV-1 LTR-luciferase reporter along with Tax and titrating amounts of wild-type HBZ, the PTM mutants, and a ΔLZ mutant (previously shown to repress Tax transactivation to a lesser extent than wild-type) [87,88,91] (Figure 3.4). As expected, wild-type HBZ and, to a lesser degree, HBZΔLZ, were able to repress Tax trans-activation of the HTLV-1 LTR promoter. We observed that all PTM mutants tested were able to inhibit Tax activity to a similar degree as wild-type HBZ and found no significant difference between paired mutants. It should be noted that this result for the S49 mutants was not unexpected because these mutations were reported previously to bind to p300, inhibiting it from interacting with Tax [88,89,97-100]. Since the PTM mutants all functioned at levels similar to WT HBZ, these data suggest that the tested PTMs do not affect the ability of HBZ to modulate Tax activity.

**Identified PTMs of HBZ do not affect c-Jun or p65 transcriptional activity.**

We next examined how the PTMs affect the ability of HBZ to repress c-Jun transcriptional activity because HBZ and APH-2, the HBZ counterpart in non-
pathogenic HTLV-2, differentially regulate this cellular pathway [92,97,101,121]. HEK293T cells were transfected with a 6xAP-1-luciferase construct along with pCMV-c-Jun and titrating amounts of HBZ. Because HBZ interacts with c-Jun through its leucine zipper domain [97,102,103], we also included the HBZΔLZ mutant (Figure 3.5). Our results show WT HBZ was able to repress c-Jun-mediated transcription and the HBZΔLZ mutant was unable to repress c-Jun. All PTM mutants acted in a manner similar to WT HBZ, indicating none of the PTMs affected the interaction of HBZ with c-Jun.

It previously was reported that HBZ represses the classical NF-κB pathway by inhibiting the DNA-binding of p65 and inducing p65 degradation [92,113-115]. This finding was important because this binding stopped cells from entering Tax-induced senescence [101]. We used the luciferase assay to examine if the discovered PTMs affected the ability of HBZ to repress p65 (Figure 3.6). These data demonstrated that the PTMs of HBZ, individually and in combination with each other, did not affect HBZ’s ability to repress p65 transcriptional activity. The data regarding c-Jun and p65 suggest that the phosphorylation and acetylation state at these residues are not important for the ability of HBZ to modulate the AP-1 and classical NF-κB pathways.

**Repression of IRF-1 is not dependent on identified PTMs.**

After testing whether PTMs regulate the ability of HBZ to repress viral expression and growth pathways, we next turned our attention to a component of the innate immune system. Interferon (IFN) regulatory factors (IRFs) are key components of the immune system as they control interferon production and
development of immune cells, but they also play a role in regulating oncogenesis [178]. IRF-1 induces the expression of type-I IFN and acts as a tumor suppressor by inducing apoptosis [150,156]. Clinical data have shown that IRF-1 expression is lost in many cases of leukemia [159]. Since HBZ is typically the only HTLV-1 protein expressed in cases of ATL, Mukai et al. investigated if HBZ and IRF-1 interacted and discovered that the N-terminus of HBZ was important for binding IRF-1 and repressing its activity. We performed a reporter assay to assess whether the PTMs of HBZ regulated the repression of IRF-1 transcriptional activity (Figure 3.7). All PTM mutants were able to repress IRF-1 in a dose-dependent manner and there were no significant differences between paired mutations. These data suggest that tested PTMs are not involved in the regulation of IRF-1 activity by HBZ.

3.4 Discussion.

Our present research is the first to report PTMs of HBZ and to define the potential role of these PTMs in the known functions of HBZ. Using online prediction tools, we found 6 potential sites of phosphorylation and 16 sites of acetylation. Our MS data covered 68% of the amino acids of HBZ, including 5 of the 6 potential phosphorylation sites and 5 of the 16 acetylation sites. In total, 7 modifications were identified: 1 phosphorylation, 2 acetylations, and 4 methylations. Three of these modifications occur in the N-terminal activation domain, one in between the activation domain and central domain, and the final three occur in the leucine zipper domain. Only acetylation of K66 occurred at a
high frequency, with the 6 other modifications occurring at low frequency. The negative predicted sites that were covered cannot be fully ruled out as being modified, but we are confident that the modifications do not occur at a high frequency. Of the uncovered predicted sites, it would seem likely that acetylation would occur more frequently than phosphorylation because there are more sites available and HBZ is known to interact with acetyltransferases.

We first examined the effect that these PTMs had on the steady-state levels of the protein, but found no difference between samples and controls. We next tested how PTMs affect the ability of HBZ to repress Tax transactivation. Because none of the mutants acted differently than the wild-type at a low or high concentration, we are able to infer two aspects of the identified phosphorylation and acetylation: 1) they do not affect the interaction of HBZ with p300, and 2) they do not affect the interaction of HBZ with CREB. The cellular signaling pathways AP-1 and NF-κB, along with IRF-1-mediated transcription were examined. Although the modifications found were in domains that modulate the activity of the tested transcription factors, they did not play any role in the ability of HBZ to repress these selected pathways.

HBZ is known to interact with several proteins and affect various cellular pathways. While we could not identify any role for PTMs in the pathways examined, it remains possible that these PTMs have a function. Although the enzymes that add PTMs to their cognate proteins within the cell are not 100% specific for functionality, their promiscuity is still expected to be limited due to the importance of strict regulation and localization. The possibility that a combination
of identified and unidentified PTMs may be necessary cannot be ruled out at this point. Furthermore, it is important to note that there could be unknown functions of HBZ that are regulated by these three PTMs. Future studies should focus on modifications that cannot be readily detected by MS such as SUMOylation [179,180] as these have also been shown to be important for regulating protein functions.
## Table 3.1: Predicted and identified phosphorylation sites of HBZ

<table>
<thead>
<tr>
<th>Predicted Phosphorylation Site</th>
<th>Score</th>
<th>Threshold</th>
<th>Times Modified/Times Detected</th>
<th>% Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>S29</td>
<td>0.974</td>
<td>0.5</td>
<td>0/5</td>
<td>0.00%</td>
</tr>
<tr>
<td>S49</td>
<td>0.975</td>
<td>0.5</td>
<td>1/27</td>
<td>3.70%</td>
</tr>
<tr>
<td>S54</td>
<td>0.965</td>
<td>0.5</td>
<td>0/15</td>
<td>0.00%</td>
</tr>
<tr>
<td>T73</td>
<td>0.991</td>
<td>0.5</td>
<td>0/5</td>
<td>0.00%</td>
</tr>
<tr>
<td>S150</td>
<td>0.993</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>S174</td>
<td>0.929</td>
<td>0.5</td>
<td>0/87</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

N.D. means Not Detected. N/A means Not Applicable
### Table 3.2: Predicted and identified acetylation sites of HBZ

<table>
<thead>
<tr>
<th>Predicted Acetylation Site</th>
<th>Score</th>
<th>Threshold</th>
<th>Times Modified/Times Detected</th>
<th>% Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>K66</td>
<td>2.09</td>
<td>0.5</td>
<td>39/39</td>
<td>100.00%</td>
</tr>
<tr>
<td>K84</td>
<td>2.64</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K86</td>
<td>1.65</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K88</td>
<td>2.42</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K89</td>
<td>1.96</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K93</td>
<td>1.47</td>
<td>0.5</td>
<td>0/2</td>
<td>0.00%</td>
</tr>
<tr>
<td>K106</td>
<td>0.79</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K110</td>
<td>1.37</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K119</td>
<td>1.51</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K120</td>
<td>2.43</td>
<td>0.5</td>
<td>0/1</td>
<td>0.00%</td>
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<tr>
<td>K128</td>
<td>0.75</td>
<td>0.5</td>
<td>N.D.</td>
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</tr>
<tr>
<td>K145</td>
<td>1.6</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K147</td>
<td>1.89</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K153</td>
<td>1.26</td>
<td>0.5</td>
<td>N.D.</td>
<td>N/A</td>
</tr>
<tr>
<td>K155</td>
<td>1.13</td>
<td>0.5</td>
<td>2/71</td>
<td>2.82%</td>
</tr>
<tr>
<td>K181</td>
<td>0.67</td>
<td>0.5</td>
<td>0/73</td>
<td>0.00%</td>
</tr>
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</table>

N.D. means Not Detected. N/A means Not Applicable
Table 3.3: Detected methylation sites of HBZ

<table>
<thead>
<tr>
<th>Methylated Residue</th>
<th>Times Modified/Times Detected</th>
<th>% Modified</th>
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</thead>
<tbody>
<tr>
<td>K35</td>
<td>3/10</td>
<td>30.00%</td>
</tr>
<tr>
<td>K37</td>
<td>1/6</td>
<td>16.67%</td>
</tr>
<tr>
<td>K181</td>
<td>3/73</td>
<td>4.11%</td>
</tr>
<tr>
<td>K186</td>
<td>1/80</td>
<td>1.25%</td>
</tr>
</tbody>
</table>
Figure 3.1: Flag-6xHis-tagged HBZ represses Tax transactivation and can be purified. (A) The functional activity of Flag-6xHis-HBZ was determined by measuring its ability to repress Tax trans-activation of a LTR1-luc reporter in HEK293T cells. Cells were transfected with S-tag Tax (200 ng), LTR-1 luciferase reporter (100 ng) TK-Renilla (20 ng) and a titration of HBZ constructs (100 and 500 ng.) Values represent mean measurements relative to Tax expression alone. Error bars represent standard deviation. Western blots were performed on 20 ug total cell lysate and probed with rabbit HBZ-specific antisera. Actin was used as a loading control. (B) Flag-6xHis-HBZ was purified from lysates of transfected HEK293T cells using agarose beads conjugated with anti-Flag antibody. Bands were resolved by SDS-PAGE and the gel was stained with GelCode Blue.
Figure 3.2: MS based analysis of PTMs in HBZ. (A) MS/MS fragmentation of HBZ peptide DGLLSLEEPK*, where Lys is methylated. (B) MS/MS Fragmentation of HBZ peptide GPPGEK*APPR, where Lys is acetylated. (C) MS/MS Fragmentation of HBZ peptide DGLLS*LEEESR, where Ser is phosphorylated. (D) Summary of MS results. The HBZ sequence is provided, with covered sequences underlined. PTMs found are highlighted in the sequence and marked relative to the three domains of HBZ in a cartoon.
Figure 3.3: Steady-state level of HBZ protein is not altered by identified PTMs. Lysates were collected from transfected HEK293T cells and were resolved by SDS-PAGE. The amount of lysate loaded was normalized to measured TK-Renilla values. The blot was probed with mouse anti-Flag M2 (1:5000). Densitometries were measured and the ratios are reported under the blot.
Figure 3.4: Phosphorylation and acetylation do not alter the ability of HBZ to repress Tax transactivation of the LTR promoter. HEK293T cells were transfected with LTR-1-luc reporter (100 ng), TK-Renilla (20 ng), S-tagged Tax (200 ng) and a titration of HBZ mutants (100 ng and 500 ng). DNA amounts were normalized with empty Flag-6xHis plasmid. Twenty-four hours post transfection, lysates were collected and luciferase levels were measured. Error bars represent standard deviations.
Figure 3.5: Phosphorylation and acetylation do not affect the ability of HBZ to inhibit c-Jun-mediated transcription. HEK293T cells were transfected with a 6xAP-1-luc reporter (50 ng), TK-Renilla (10 ng), pCMV-c-Jun (25 ng) and a titration of HBZ constructs (10 ng and 100 ng). DNA amounts were normalized with empty Flag-6xHis plasmid. Lysates were collected 24 hours post-transfection and luciferase levels were measured. Error bars represent standard deviations.
Figure 3.6: Phosphorylation and acetylation do not affect the ability of HBZ to repress p65-mediated transcription. HEK293T cells were transfected with κB-luc reporter (100 ng), TK-Renilla (20 ng), p65 expression plasmid (50 ng) and a titration of HBZ constructs (100 ng and 500 ng). DNA amounts were normalized with empty Flag-6xHis plasmid. Lysates were collected 24 hours post-transfection and luciferase levels were measured. Error bars represent standard deviations.
Figure 3.7: The ability of HBZ to repress IRF-1 activity is not affected by the identified PTMs. HEK293T cells were transfected with pIRF-1-luc (50 ng), TK-Renilla (20 ng), IRF-1 expression plasmid (100 ng) and a titration of HBZ constructs (50 ng and 500 ng). DNA amounts were normalized with empty Flag-6xHis plasmid. Lysates were collected 24 hours post-transfection and luciferase levels were measured. Error bars represent standard deviations.
Chapter 4
Differential Type I IFN Expression in HTLV-1 and HTLV-2

Immortalized T-cells: Implications for Pathogenesis

4.1 Introduction

Human T-cell leukemia virus type-1 (HTLV-1) and HTLV-2 are related complex deltaretroviruses with 70% nucleotide identity [21]. Both viruses infect T-cells, though HTLV-1 preferentially transforms CD4+ T-cells and HTLV-2 preferentially transforms CD8+ T-cells [123]. HTLV-1 and HTLV-2 also encode similar genes, including Tax and Rex regulatory genes, and genes on the antisense strand of the proviruses (HBZ and APH-2, respectively) [141,181]. Despite these similarities, HTLV-1 and HTLV-2 have distinct pathobiologies. HTLV-1 causes adult T-cell leukemia/lymphoma (ATL) [2,3,5], the neurological disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [138], and uvetis [182]. In contrast, while HTLV-2 has only been linked to a few sporadic cases of neurological disease [31] and is capable of causing lymphocytosis in infected people [36], virus infection has not been associated with any malignancies.
It is known that the innate immune system is able to detect viral pathogen-associated molecular patterns (PAMPs) through cellular sensors known as pathogen recognition receptors (PRRs) [183-186]. Recognition of a viral PAMP initiates a signaling cascade to activate the interferon (IFN) system by inducing the activation of interferon regulatory factors (IRFs) via phosphorylation. Phosphorylated IRFs then translocate to the nucleus and induce the transcription of IFNs [187]. IFNs are secreted from leukocytes, including lymphocytes, macrophages, and dendritic cells, where it binds to its cognate receptor. Binding starts a signaling cascade that ultimately induces the expression of IFN-stimulated genes (ISGs) [184,188,189], of which many, such as MxB, APOBEC, and TRIM proteins, have antiviral activities. Out of three types of IFN, type I IFNs (mainly IFN-α and IFN-β) have the greatest antiviral effects.

There have been numerous studies investigating HTLV-1 and type I IFNs. It has been reported that uninfected PBMCs treated with IFN prior to or during co-culture with a lethally irradiated HTLV-1 producer cell line were protected from HTLV-1 infection [190-192]. HTLV-1 expression is also repressed in infected cells when they are continuously exposed to type I IFN both in vitro and in vivo [193,194]. These data support the idea that type I IFNs might control HTLV infection and help in the management of disease. Currently, some of the best treatments for acute ATL involve the use of type I IFNs in combination with other drugs [22], and there is a case report of an ATL patient entering complete remission for at least ten months when treated with IFN-β [195]. Other studies
demonstrated that patients with HAM/TSP who were treated with IFN-β1a had reduced proviral loads and improved urinary and motor symptoms [196-198].

The induction of type I IFNs in response to HTLV-2 infection has not been examined. We hypothesized that HTLV-2 could modulate the IFN induction pathway differently than HTLV-1, and therefore might influence the differential pathological outcomes of the two related viruses. Upon examination of PBMCs immortalized by HTLV-1 and HTLV-2, we observed a difference in IFN-β expression. We then pursued this difference and examined the role of HTLV-2 proteins in regulating the expression of this potent antiviral cytokine.

4.2 Materials and Methods

**Cells.** PBL/HTLV-1 and PBL/HTLV-2 are immortalized PBL lines that were originally created via co-cultivation of human PBMCs with lethally irradiated virus-producing cells, then maintained in culture for over 10 weeks [123]. The cultures were maintained in RPMI medium (Thermo Fisher-Life Technologies, Waltham, MA) supplemented with 20% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 20 U/ml recombinant human interleukin-2 (rhIL-2; Roche Applied Biosciences, Mannheim, Germany). Jurkat cells were maintained in RPMI medium supplemented with 10% FBS, 2mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Thermo) at 37°C in a humidified atmosphere of 5%
CO₂ and air. Medium was supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

**Real-time RT-PCR.** RNA was collected from cells using the RNEasy and Qiashredder kits (Qiagen Inc.-USA, Valencia, CA). Isolated RNA was then treated with DNase (Genetech, San Francisco, CA). Total RNA (2 μg) was reverse transcribed with random hexamer primers using the SuperScript II Reverse Transcription kit (Thermo) according to the manufacturer’s instructions. The cDNA was diluted to 50 μl. Real-time PCR reactions were set up using 3 μl diluted cDNA, 5 μl iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA), 0.3 μl of both the 10 μM forward primer and reverse primer solution, and 1.7 μl of DEPC-treated water. Real-time PCR was performed using a Bio-Rad CFx96 machine. The primers used in this study are presented in Table 4.1. The PCR protocol for GAPDH, IFN-α, MxB, OAS1, and Viperin was a hot start at 95°C for 3 min followed by 40 cycles of 95°C for 20 s, 50°C for 20 s, and 72°C for 20 s. The protocol for IFN-β was a hot start at 95°C for 3 min followed by 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The SOCS1 protocol was a hot start at 95°C for 3 min followed by 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. A melting curve from 65°C-95°C was performed at the end of each run for all samples to ensure proper products were being made. Fold changes were measured using the ΔΔCt method with GAPDH being used as an internal control.

**IFN ELISAs.** Cells were seeded at 5x10⁵ cells/ml in a 12-well plate and cultured for 48 hours. The VeriKine Human Interferon Alpha Multi-Subtype ELISA Kit
(PBL Assay Science, Piscataway, NJ) and the Human Interferon-β ELISA Kit (Fujibebio Inc., Tokyo, Japan) were used to measure IFN levels in cell culture medium (after centrifuging cells at 20 x g for 10 minutes). Kit procedures were followed. Secreted units were calculated using the Prism5 program (GraphPad Software Inc., La Jolla, CA).

**Paracrine activation assay.** PBL/HTLV-1, PBL/HTLV-2, and Jurkat cells were plated in 6-well dishes at a density of 5x10^5 cells/ml and cultured for 24 hours. The cells were centrifuged at 20 x g for 10 minutes and the medium was added to fresh target Jurkat cells at a cell density of 5x10^5 cells/ml cells for 24 hours. The target Jurkat cells were then collected and the RNA was isolated and processed for real-time PCR.

**Reporter assays.** HEK293T cells were seeded in 6-well plates at 2 x 10^5 cells per well. Twenty-four hours post-plating, cells were transfected with 100 ng of TK-Renilla, 50 ng IFN-β-luc, 50 ng of pMe-eGFP, and 50 ng of a constitutively active form of IRF-3 (Flag-IRF-3(5D)). Plasmids expressing untagged Tax-2 and APH-2 were titrated (50 ng and 100 ng for Tax-2, 100 ng and 2000 ng for APH-2). Transfections were performed using Lipofectamine (Thermo) according to the manufacturer’s instructions. Twenty-four hours post-transfection, cells were collected and analyzed using a dual luciferase assay kit (Promega, Madison, WI). Levels of firefly luciferase and Renilla luciferase were measured using a Packard LumiCount luminometer (Packard Biosciences, Meriden, CT). The Flag-IRF-3(5D) expression plasmid was a kind gift from Dr. John Hiscott (McGill
University) [199]. The IFN-β-luc plasmid was generously provided by Dr. Xiaoyong Bao (University of Texas Medical Branch-Galveston) [200].

**Western blots.** Transfected cells were lysed in 1x Passive Lysis Buffer (Promega) with protease inhibitor cocktail (Roche). Protein concentrations were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Standard Western blot loading buffer (6x solution) was added to the lysates and samples were boiled for 10 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. GFP was detected using a rabbit antibody (Abcam, San Francisco, CA) at a dilution of 1:10,000. Flag-IRF-3(5D) was detected using a mouse α-Flag M2 antibody (1:5000) (Sigma Aldrich, St. Louis, MO) for the APH-2 assay system and mouse α-IRF-3 (1:2000, Abcam) for the Tax-2 assay system. Rabbit α-APH-2 polyanisera (1:1000) was used to detect APH-2. Secondary antibodies included goat α-rabbit and goat α-mouse conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:5000. Blots were developed using Immunocruz luminol reagent (Santa Cruz) and imaged using Hyperfilm ECL film (GE Healthcare Limited’s Amersham, Buckinghamshire, UK) and a Konica SRX-101A tabletop processor (Konica Minolta Medical Imaging USA, Inc., Wayne, NJ).

**Statistical Analysis.** Statistics were performed using the T-test function in Microsoft Excel.
4.3 Results

HTLV-2-infected cells express higher amounts of IFN-β than HTLV-1-infected cells.

To compare the type I IFN expression levels between HTLV-1 and HTLV-2 infected cells, we measured the relative expression of mRNA collected from PBMCs that were immortalized by HTLV-1 (PBL/HTLV-1) and HTLV-2 (PBL/HTLV-2) and used Jurkat cells as a negative control. We were unable to detect IFN-α mRNA expression from any cell line (data not shown); however, we detected a statistically significant increase in IFN-β mRNA expression from the PBL/HTLV-2 line compared PBL/HTLV-1 (~10 fold increase, p=0.002) (Figure 4.1A).

To verify the mRNA results and examine if detectable amounts of IFN proteins were being secreted, we next performed ELISAs on culture supernatants. IFN-α was undetectable in culture supernatants from any of the cell lines, consistent with our RT-PCR data. The IFN-β ELISA results also were consistent with our RT-PCR data, showing a significantly greater amount of IFN-β secretion from PBL/HTLV-2 cells compared to PBL/HTLV-1 (~4 fold increase, p<0.001) (Figure 4.1B). We next investigated whether the IFN-β was biologically active by performing a paracrine activation assay. Briefly, we cultured target Jurkat cells in conditioned medium from PBL/HTLV-1, PBL/HTLV-2, and Jurkat (control) cells. After culturing the target Jurkat cells for 24 hours in the conditioned medium, mRNA levels for three different ISGs (MxB, OAS1, and viperin) were examined (Figure 4.2). All three ISGs were significantly up-
regulated in the Jurkat cells cultured in the PBL/HTLV-2 conditioned medium compared to the PBL/HTLV-1 conditioned medium (MxB: ~4 fold, OAS1: ~8 fold, viperin: ~4 fold). This result demonstrated that the T-cell line immortalized by HTLV-2 expressed and secreted significantly higher amounts of biologically active IFN-β compared to cells immortalized by HTLV-1.

**HTLV-1 and HTLV-2 induce similar expression levels of SOCS1.**

It was previously reported that HTLV-1 inhibits type I IFN expression by the induction of the suppressor of cytokine signaling 1 (SOCS1) gene [161,162]. Moreover, knockdown of SOCS1 in an HTLV-1 infected cell line restored IFN-β expression [161]. Based on these results, we investigated the expression levels of SOCS1 to determine if HTLV-2-infected cells had lower expression than HTLV-1-infected cells. To our surprise, there was no significant difference in the level of SOCS1 mRNA expression between PBL/HTLV-1 and PBL/HTLV-2 cells (Figure 4.3). Therefore, we concluded that the increased type I IFN from HTLV-2-infected cells was not due to any difference in SOCS1 expression.

**HTLV-2 Tax-2 and APH-2 proteins enhance IRF-3 induction of IFN-β.**

Since PBL/HTLV-1 and PBL/HTLV-2 cells showed no significant difference in SOCS1 expression, we hypothesized that the induction of IFN-β by HTLV-2 infection could be caused by the ability of Tax-2 and/or APH-2 to augment the activity of IRF-3, the main inducer of IFN-β. We focused on Tax and APH-2 because the expression of these viral genes are positively correlated with HTLV-2 proviral load [120]. Using a luciferase assay, we observed that Tax-2 was capable of enhancing activated IRF-3 induction of IFN-β (Figure 4.4A).
Lastly, we examined the effect of APH-2 and observed it too augmented the activity of activated IRF-3 (Figure 4.4B) in a dose dependent manner. These results demonstrate that both Tax-2 and APH-2 strengthened the signal induced by phosphorylated IRF-3 and suggest a potential mechanism of IFN-β induction in HTLV-2-infected cells that is independent of SOCS1 levels.

4.4 Discussion

HTLV-1 and HTLV-2 share many similar properties at the gene level, however they have distinct pathological outcomes upon infection. Previous studies have shown the capability of the innate immune response, specifically the type I IFN system, to limit HTLV-1 infection [190-194,201]. Indeed, some of the current best treatments for ATL include an IFN-α component [22]. HTLV-1, however, has evolved a mechanism to inhibit the IFN response to permit continuous growth and eventual progression to disease [161,162].

To our knowledge, no previous studies have examined how HTLV-2 affects the type I IFN system. We hypothesized that HTLV-1 and HTLV-2 could affect this antiviral system differently, which might explain the differences in the pathogenicity of these two related viruses. Our results showed that neither HTLV-1 nor HTLV-2 immortalized cells expressed detectable amounts of IFN-α, however we observed a significantly higher amount of IFN-β expression and production from HTLV-2-infected cells. In addition, conditioned medium from PBL/HTLV-2 (which contained biologically active IFN-β) induced a significant increase in expression of ISGs in bystander cells compared to conditioned medium from PBL/HTLV-1.
To determine a mechanism for the greater amount of IFN-β generated in PBL/HTLV-2 cells, we investigated SOCS1 levels in both PBL/HTLV-1 and PBL/HTLV-2 lines, but observed no significant difference. We then tested the ability of Tax-2 and APH-2 to enhance the induction of IFN-β via activated IRF-3. Our results showed that both Tax-2 and APH-2 significantly enhanced IRF-3 activity. This result suggests that the effect of IFN-β under these experimental conditions was independent of SOCS1 expression.

Our findings have important biological implications. As mentioned above, many current ATL treatments include IFN-α as part of the therapeutic regimen. However, there have been case studies that have shown that IFN-β treatment can significantly ameliorate HTLV-1-associated disease [195-197]. Our study showed that a T-cell line immortalized by HTLV-2, which is considered to be non-pathogenic, expresses higher amounts of IFN-β than an HTLV-1 immortalized T-cell line and induced expression of ISGs in bystander cells. Furthermore, high level IFN- expression in PBL/HTLV-2 cells was independent of SOCS1 expression, which led us to investigate a different pathway for IFN induction mediated by IRF-3. Given that IFN-β has shown some utility in treatment of HTLV-1-infected individuals, our data provide a mechanistic rationale for further research into IFN-β as a treatment for HTLV-1-associated disease.

In support of our in vitro findings are reports of dual infections of people with HIV-1/HTLV-1 and HIV-1/HTLV-2, where there also are distinct disease outcomes. HTLV-1 enhances the replication of HIV-1, resulting in faster clinical progression and shorter survival time [154,202]. In contrast, studies have shown
that HTLV-2 has a protective effect against HIV-1 disease progression. For example, HIV-1/HTLV-2 co-infection results in lower plasma levels of HIV-1 mRNA compared to HIV-1-monoinfected patients [38,154,203,204]. In addition, there are a greater number of HIV-1/HTLV-2 individuals who become long-term non-progressors as compared to those infected with HIV-1 alone [38,154]. A study that examined long-term HIV-1 non-progressors found that CD8\(^+\) T-cells, which are the predominant cell type immortalized by HTLV-2 [123], expressed higher amounts of type I IFN [205]. Our data suggests that the higher amounts of IFN-β expressed in HTLV-2 infected cells could be a mechanism by which HTLV-2 exerts a protective effect against HIV-1 in co-infected patients.

In conclusion, this is the first study to compare type I IFN expression from HTLV-1 and HTLV-2 infected cells. We found that HTLV-2-immortalized T-cells expressed and secreted significantly higher amounts of IFN-β compared to PBL/HTLV-1 cells. This IFN-β was biologically active and able to induce antiviral ISGs in bystander cells. These findings suggest a mechanism that could help explain the different pathological outcomes between HTLV-1 and HTLV-2 infections. Furthermore, the results support the need to explore IFN-β as a treatment for HTLV-1 related disease.
### Table 4.1 Real-Time PCR Primers

<table>
<thead>
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<th>Gene Target</th>
<th>Primers</th>
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| hGAPDH[152] | Forward: 5'-CATCAATGACCCCTTCATTGAC-3'  
Reverse: 5'-CGCCCCACTTGGATTTTGGGA-3' |
| IFN-α[206]  | Forward: 5'-GACTCCATCTTTGGCTGTGA-3'  
Reverse: 5'-TGATTTTCTGCTCTGACAACCT-3' |
| IFN-β[207]  | Forward: 5'-AAACTCATGAGCAGTCTGCA-3'  
Reverse: 5'-AGGAGATCTCTAGTTTCGGAGG-3' |
| SOCS1[208]  | Forward: 5'-TTTTTCGGCCCTTAGCGTGA-3'  
Reverse: 5'-AGCAGCTCAGAAGGACAGTC-3' |
| MXB         | Forward: 5'-GATGATTTCATCCATCTTGAGTGA-3'  
Reverse: 5'-ACATGTGCTGTCTCCCTGTC-3' |
| OAS1[209]   | Forward: 5'-CCAGGAAATAGAGACAGGC-3'  
Reverse: 5'-GAGCGAACTCAGTACGAAGC-3' |
| Viperin[210]| Forward: 5'-CACAAAGAAGTGCTTGTCAGTGGT-3'  
Reverse: 5'AAGCGCATATATTTTACATCCAGATAAAG-3' |
Figure 4.1: HTLV-2 immortalized T-cells express higher amounts of IFN-β compared to HTLV-1 immortalized T-cells. (A) Real-time RT-PCR was performed on RNA collected from Jurkat, PBL/HTLV-1 and PBL/HTLV-2 cells. The mean fold change in IFN-β mRNA normalized to GAPDH is shown with standard deviation error bars. **p<0.01. (B) Secreted IFN-β was detected in supernatant from Jurkat, PBL/HTLV-1 and PBL/HTLV-2 cells. The means are reported as IU/ml with error bars representing standard deviation. ***p<0.001.
Figure 4.2: PBL/HTLV-2 cells induce a greater antiviral state in bystander cells than PBL/HTLV-1 cells. Mean fold changes in expression of three antiviral ISGs are presented from Jurkat cells grown in PBL/HTLV-1 and PBL/HTLV-2 conditioned medium. ISG mRNA levels were normalized to GAPDH. Error bars represent standard deviation. *p<0.05, **p<0.01, ***p<0.001.
Figure 4.3: PBL/HTLV-1 and PBL/HTLV-2 cells have similar levels of SOCS1 mRNA. Real-time RT-PCR was performed on RNA collected from Jurkat, PBL/HTLV-1 and PBL/HTLV-2 cells. Mean SOCS1 mRNA levels, normalized to GAPDH, are presented. Error bars represent standard deviation.
Figure 4.4: HTLV-2 proteins enhance IRF-3 induction of IFN-β. Luciferase assays were performed in HEK293T cells that had been transfected with IFN-β-luc and an activated IRF-3 construct. HTLV-2 proteins Tax-2 (A) and APH-2 (B) were titrated. Resulting mean fold changes are shown with standard deviations represented as error bars. *p<0.05, **p<0.01, ***p<0.001
Chapter 5
Synopsis and Future Directions

5.1 Synopsis of Thesis

HTLV-1 and HTLV-2 are highly related complex retroviruses that are the source of endemic infections around the world. These two viruses share properties such as genetic organization, transmission routes, and cellular tropism [21,141] and can transform primary human T-lymphocytes in vitro [47,51,58,59,76]. Despite these similarities, only HTLV-1 is clearly linked to diseases including the aggressive CD4+ T-cell malignancy ATL [2,3] as well as the neurodegenerative disorder HAM/TSP [137,138]. Although the regulatory protein Tax is the key protein that drives HTLV-1 pathogenesis, growing evidence points to the importance of HBZ as well. Our laboratory previously showed that HBZ is required for efficient viral infection and persistence in a rabbit animal model [96] and supports the growth of transformed cells [113]. More recently, we showed that APH-2, the HTLV-2 counterpart of HBZ, was dispensable for viral infection and persistence in vivo [119]. Since both HBZ and APH-2 repress Tax-mediated viral transcription, we hypothesized that HBZ and APH-2 modulate cellular pathways differently.
In Chapter 2, we performed direct comparisons of the effects of HBZ and APH-2 on three separate cellular pathways involved in the virus-host interaction. We confirmed previous results that HBZ enhanced TGF-β signaling while APH-2 inhibited this pathway. We also found that both HBZ and APH-2 repress p65 (NF-κB) and IRF-1-mediated transcription. Further evidence, however, showed that APH-2 was considerably less stable than HBZ, and that Aph-2 RNA transcript levels were significantly lower than Hbz levels post-infection. Thus, we concluded that the enhancement of TGF-β and inhibition of p65 and IRF-1 are distinct for HBZ compared to APH-2 during infection and contribute to the requirement of HBZ for viral persistence and eventual progression to disease.

In Chapter 3, we characterized the PTMs of HBZ and investigated their functional role in the Tax, c-Jun, p65 and IRF-1 pathways. The discovery of phosphorylation, acetylation, and methylation of HBZ are the first reported PTMs for this viral protein. However, using HBZ PTM mutants in functional assays, we found no significant difference in the repression of Tax, c-Jun, p65 and IRF-1. In addition, these PTMs do not appear to regulate the steady-state levels or function of HBZ.

Chapter 4 examined type I IFN expression in HTLV-1 and HTLV-2 immortalized cells. Previous reports found that HTLV-1 inhibits type I IFN through induction of SOCS1 [161,162]. We observed that despite expressing equal amounts of SOCS1 mRNA, HTLV-2 immortalized cells expressed and secreted significantly higher amounts of IFN-β compared to HTLV-1 immortalized cells. To explore a potential mechanism, we examined how HTLV-2 Tax-2 and APH-2
proteins affected IRF-3 activity. We found that both Tax-2 and APH-2 enhanced the ability of IRF-3 to induce IFN-β expression. These findings reveal another difference between HTLV-1 and HTLV-2 that may explain the pathological differences between the two viruses.

5.2 Short-Term Future Directions

The functional assays performed in these studies were conducted in HEK293T cells. This cell system allows for easy over-expression and optimization for reporter luciferase assays and provides important information on general mechanisms. However, HEK293T cells are not T-cells, the main target of HTLV infection and transformation. The next step for these studies will be to conduct these experiments in T-cells. Primary T-cells are difficult to transfect efficiently; therefore, we have begun to repeat the PTM functional studies in the Jurkat T-cell line (Figure 5.1 and 5.2). Our preliminary data gave similar results as observed in the HEK293T cells. From this we conclude that these PTMs do not modulate HBZ’s repression of Tax or p65 in T-cells.

Previous cohort studies showed that HIV-1/HTLV-1 co-infection leads to enhanced HIV-1 replication and shorter survival time [154,202], while co-infection with HTLV-2 has a protective effect against progression to AIDS [38,154,203,204]. Our data suggest that the protective effect of HTLV-2 may be due, in part, to its induction of IFN-beta expression. We plan to test this by growing target cells in PBL/HTLV-2 conditioned medium or in a trans-well system with PBL/HTLV-2 cells, and then challenging the target cells with GFP-expressing HIV-1. We will not be able to use Jurkat cells, the target cell used in
Chapter 4, because other studies have shown that IFN treatment does not have a protective effect in transformed T-cell lines [211,212]. THP-1, a monocyte cell line, and naïve T-cells will be used, as these can be protected from HIV-1 by IFN treatment [211,212]. We have already performed a pilot experiment confirming that IFN-β pretreatment of THP-1 cells inhibits HIV infection (Figure 5.3), indicating this is a viable approach to test our hypothesis.

5.3 Long-Term Future Directions

Determinants of the divergent disease outcomes between HTLV-1 and HTLV-2 are still not completely understood. HBZ, the protein encoded by the antisense strand of HTLV-1, is hypothesized to play a key role in ATL development and maintenance. HBZ is required for efficient infection and persistence of HTLV-1 in vivo [96] and promotes proliferation and survival of infected cells [102,112-115,213-215]. It is also the only viral gene found to be expressed in all HTLV-1 transformed cell lines and ATL patient cells [112,113]. APH-2 is the HTLV-2 counterpart of HBZ. Unlike HBZ, APH-2 is not required for persistence in vivo [119] or promote proliferation [120].

In this thesis, we used reporter assays to determine the effects of APH-2 on three known HBZ modulated pathways. Further comparative studies should be conducted to determine the effects of HBZ that are important for disease. HBZ causes anchorage-independent growth in vitro using a soft agar assay[149] and mice transgenic for HBZ have a higher incidence of lymphoma development compared to non-transgenic mice [104,215]. These studies should be conducted
with APH-2 to verify a difference in oncogenic potential. APH-2 transgenic mice would also allow for microarray comparisons to be performed. This comparison would show what genes would be up- or down-regulated differently between HBZ and APH-2 in vivo, which could further indicate important functions of HBZ in the development of ATL.

Overall, this thesis provides new insights into the differences in pathobiology between HTLV-1 and HTLV-2 and the roles of the antisense encoded proteins in development of disease (Figure 5.4). These results have the potential to reveal new targets and inform strategies for therapeutics against HTLV-1-related disease.
Figure 5.1: Phosphorylation and acetylation do not alter the ability of HBZ to repress Tax transactivation of the 5’ LTR promoter in Jurkat cells. 3.5x10^5 Jurkat cells were plated in RPMI medium (10% FBS, 2mM glutamine, no antibiotics). 24 hours after plating, cells were transfected with 300 ng LTR-1-luc construct, 100 ng TK-Renilla, 600 ng Tax expression plasmid, and 1500 ng Flag-HBZ. Transfection was done with Transfectin reagent (Bio-Rad) according to manufactures guidelines. Cells were harvested 40 hours after transfection and luciferase levels measured. Histograms represent the mean normalized luciferase levels. Error bars represent standard deviation.
Figure 5.2: Phosphorylation and acetylation do not alter the ability of HBZ to repress p65 mediated transcription in Jurkat cells. 3.5x10^5 Jurkat cells were plated in RPMI medium (10% FBS, 2mM glutamine, no antibiotics). 24 hours post plating, cells were transfected with 200 ng κB-luc plasmid, 100 ng TK-Renilla, 100 ng p65 expression plasmid, and 1000 ng Flag-HBZ. Transfections were performed using Transfectin reagent (Bio-Rad) according to manufacturers guidelines. Cells were harvested 40 hours post-transfection and luciferase levels were measured. Histograms represent mean normalized luciferase levels. Error bars represent standard deviation.
Figure 5.3: THP-1 cells can be protected from HIV infection by pretreatment with IFN-β. THP-1 cells were plated at $5 \times 10^5$ cells/ml in RPMI (10% FBS, 2mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml)). Cells were then treated with recombinant human IFN-beta (Thermo) at three concentrations (0 IU/ml, 10 IU/ml, 500 IU/ml) for 24 hours. Cells were then washed, and $2.5 \times 10^5$ cells were infected with inoculum containing single-round VSV-G pseudotyped HIV-1 virus encoding GFP (NL4-3ΔEnv+GFP) at 3 amounts (0 μl, 25 μl, 100 μl) for 2 hours. Cells were then washed and incubated in fresh medium for 2 days before GFP was detected using flow cytometry.
Figure 5.4: Model of HBZ and APH-2 interaction with cellular pathways. HBZ protein levels during infection should be higher than APH-2 due to its increase in protein stability and increase in transcript levels. Though APH-2 is capable of repressing p65 and IRF-1, its functional role on these pathways would be highly limited, making repression of both pathways distinct for HBZ. The affect of HBZ and APH-2 on TGF-β signaling are divergent, with HBZ enhancing the signal and APH-2 inhibiting it. These three functions of HBZ would contribute to viral evasion, continuous growth, and survival of an HTLV-1 infected cell and help explain why HBZ is required for viral persistence. We also observed that HTLV-2 immortalized T-cells secrete higher amounts of IFN-β than HTLV-1 immortalized T-cells and induced antiviral states in bystander cells, emphasizing a difference in HTLV-1 and HTLV-2 biology. Tax-2 (at lower levels) and APH-2 enhanced the activity of IRF-3 and could contribute to the increased levels of IFN-β. This could contribute to why the deletion of APH-2 results into the increase of HTLV-2 proviral load in vivo.
References


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