IDENTIFICATION AND CHARACTERIZATION OF THE POST-TRANSLATIONAL MODIFICATIONS OF THE HTLV TYPES 1 AND 2 REGULATORY PROTEIN REX

DISSERTATION

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ABSTRACT

Human T-cell Leukemia Virus (HTLV) types 1-4 are classified as complex retroviruses and are members of the genus Deltaretrovirus. HTLV-1 and HTLV-2 are the most prevalent worldwide with approximately 10-20 million people infected, whereas HTLV-3 and HTLV-4 were discovered recently in a very limited number of individuals in Africa. Of the HTLV isolates, only HTLV-1 has clearly been linked to the development of adult T-cell leukemia/lymphoma (ATL/ATLL), an aggressive CD4+ T-lymphocyte malignancy and the neurodegenerative disease, HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Disease association with HTLV-2 is less clear, with only a few cases of leukemia and neurological diseases reported. The difference in pathology between the two related viruses has yet to be elucidated, but likely results from the activities of the regulatory and accessory proteins.

HTLV Rex is a trans-acting regulator of viral replication. Rex acts post-transcriptionally by binding and exporting unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm. We previously showed that Rex-2 is phosphorylated primarily on serine residues and that site-specific serine phosphorylations are critical for Rex-2 function. It had been proposed that Rex-1 function also may be
regulated through phosphorylation. Although these results suggest an important role for
phosphorylation in Rex function, a complete mapping of the phosphorylation sites within
Rex has yet to be reported. Identification and characterization of these distinct
phosphorylation sites would facilitate comparative biological studies between Rex-1 and
Rex-2, leading to the functional importance of phosphorylation in regulating Rex
function and ultimately understanding the pathogenic differences between HTLV-1 and
HTLV-2.

In this dissertation, we report molecular studies regarding the role
phosphorylation plays in Rex function. In Chapter 2, we provide further characterization
of the Rex-2 carboxy terminal phosphorylation domain (CTPD). Our studies identified
the ability of the CTPD to regulate Rex-2 function and its importance for HTLV-2-
mediated cellular proliferation and immortalization \textit{in vitro} and its contributions to virus
infection and persistence in a rabbit model of infection. Introduction of a
phosphomimetic amino acid (negative charge) into the Rex-2 c-terminus or deletion of
certain c-terminal amino acids can maintain Rex-2 in a highly functional state. Studies
also found that this domain plays an important role in Rex-2 protein stability. Thus, our
data provides evidence that the Rex-2 c-terminus contains a functional inhibitory domain
that is regulated via phosphorylation. Cells harboring HTLV Rex-2 CPTD mutant
proviruses display enhanced infection \textit{in vitro} and increased virion production.
Furthermore, these mutants promoted HTLV-2- induced proliferation of human primary
T-cells and displayed increased replication in inoculated rabbits as measured by
significantly stronger antibody responses as compared to wtHTLV-2 infected animals.
Overall, our study demonstrated the importance of Rex-2 post-translational modifications and their role in regulating protein stability and function.

Chapters 3 and 4 focus on identifying phosphorylation sites within Rex-1 and Rex-2 \textit{in vivo}. We conducted a phosphoryl mapping of mammalian-expressed Rex-1 and Rex-2 proteins using a combination of affinity purification, liquid chromatography tandem mass spectrometry, and site-directed substitution mutational analysis. We achieved 100\% physical coverage of both Rex-1 and Rex-2 sequences. In Chapter 3, we report the identification of six novel phosphorylation events in Rex-2 at Thr-19, Ser-117, Ser-125, Ser-151, Ser-153, and Thr-164. We evaluated the functional significance of these novel phosphorylation sites and found that only phosphorylation within the CPTD at Ser-151, Ser-153, and Thr-164 are critical for Rex-2 function \textit{in vivo}. Indirect immunofluorescence revealed that Ser-151 is the only site important for proper subcellular localization of Rex-2.

In Chapter 4, we report the identification of five novel phosphorylation sites within Rex-1 at Thr-22, Ser-36, Thr-37, Ser-97, and Ser-106. We also confirmed two previously identified residues Ser-70 and Thr-174. The functional significance of these phosphorylation sites was evaluated. We determined that phosphorylation of Ser-97 and Thr-174 are critical for Rex-1 function, but in contrast to Rex-2, phosphorylation does not play a role in subcellular localization of the Rex-1 protein.

Overall, this work is the first to identify novel phosphorylation sites \textit{in vivo} that regulate Rex function and will provide important insight not only into unraveling the mechanism(s) by which Rex function is regulated, but also lend a better understanding of how Rex controls HTLV replication and pathogenesis.
Dedicated to:
Justin M. Marrie
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CHAPTER 1

INTRODUCTION

LITERATURE REVIEW

1.1 Human T-cell Leukemia Virus History and Epidemiology

The first pathogenic human retrovirus isolated and later associated with a human neoplastic malignancy was human T-cell leukemia virus type-1 (HTLV-1) (116, 265). T-cell lines established from patients with cutaneous T-cell lymphoma and leukemia were shown to produce retroviral particles as well as antigens that were reactive against sera from adult T-cell leukemia/lymphoma (ATLL) patients (116, 265). Numerous epidemiological and molecular studies have proven that HTLV-1 is the etiological agent of ATLL, an aggressive CD4+ T-lymphocyte malignancy, and a slowly progressive neurological disorder termed HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (77).

Human T-cell leukemia virus type 2 (HTLV-2) was discovered and characterized a few years after the isolation of HTLV-1. HTLV-2 was isolated from a patient with a rare variant of hairy cell leukemia (147) and in another patient with a CD8+ T-cell
leukemia and coexisting hairy B-cell leukemia (278). Reports suggest that HTLV-2 appears to be involved with rare T-cell lymphoproliferative (200, 349) and neurological disorders (111, 119, 134), but a definitive causal link between HTLV-2 viral infection and disease association has yet to be established.

Although HTLV-1 and HTLV-2 are highly homologous at the nucleotide sequence level and in genomic organization, it is hypothesized that the two viruses originated and evolved independently from distinct lineages of simian T-lymphotropic viruses (STLVs) type 1 and 2 (317). Recently, two more HTLV isolates (HTLV-3 and HTLV-4) have been identified and isolated in a very limited number of bushmeat hunters in Africa (330). Phylogenetically, HTLV-3 is related to STLV-3 (39, 330), whereas HTLV-4 belongs to a phylogenetic lineage that is unrelated and distinct from all known HTLVs and STLVs (330). To date, there have been no reports linking HTLV-3 or HTLV-4 infections to disease.

HTLV-1 and HTLV-2 are the most prevalent worldwide, but have two very distinct geographical distributions. Approximately 10-20 million people are estimated to be infected with HTLV-1 (297), where it is endemic in Japan, South America, Africa, Melanesia, the Caribbean basin, and southeastern United States (28, 29, 53, 66, 220, 246, 329, 336). It is predicted that after a long latency period spanning 20 or more years, only 2-5% of infected individuals will develop ATLL (69, 214, 230), and 0.25-3% will develop HAM/TSP (93, 133, 252). Contrary to HTLV-1, the geographic origin of HTLV-2 is less defined and has been found to be endemic in Europe, southeast Asia, and regions in the Americas (85, 87, 176, 189). Due to the low percentages of infected individuals
that develop an HTLV-associated disease, we hypothesize that HTLV induces a chronic, asymptomatic, lifelong infection.

Contrary to human immunodeficiency virus (HIV-1) and other animal retroviruses, HTLV is a highly cell-associated virus and efficient routes of transmission require that a virally infected cell passes into an uninfected individual. This allows the infected cell to dock with the target cell to assure cell-cell contact facilitating the formation of a polarized cell-cell junction and the formation of the virological synapse (124). HTLV infection is spread horizontally via sexual transmission (229) and exposure to contaminated blood products (209). Although sexual transmission could be bidirectional, there is greater risk of male-to-female transmission. This scenario also is affected by other factors, such as genital ulcers, high viral load, and anti-HTLV antibodies. Although HTLV primarily is spread horizonally, vertical transmission from mother-to-child has been reported. Viral transmission is dependent on ingestion of infected milk-borne lymphocytes, duration of breast feeding (180, 328), and maternal antibodies against HTLV. The most efficient route of HTLV transmission leading to rapid disease development is via transfusion of whole blood.

1.2 Experimental Systems to Study HTLV

The study of HTLV has been facilitated via many experimental systems. Over the last 30 years, researchers have struggled with the poor replication kinetics of the virus in vitro, lack of consistent animal models, and frequency and time course of disease. In cell culture, HTLV has the capacity to infect a wide range of human and nonhuman cell types
including B-cells, T-cells, endothelial cells, glial cells and monocytes (4, 120, 122, 174). To date, only primary T-lymphocytes have been found to be susceptible to HTLV immortalization/transformation making this an accepted experimental system for exploring the early events associated with malignancy. HTLV is a highly cell-associated virus, and cell-free infection is very inefficient. To achieve efficient HTLV infection, PBMC target cells require co-cultivation with lethally irradiated HTLV-producer cells.

Initial HTLV studies were limited to examination of infected patient material, over-expression of viral genes using reporter assays \textit{in vitro}, or characterization of infected cell lines using viral isolates obtained directly from patients. These studies continue to be valuable tools for understanding HTLV biology. Over the years, advances in isolation and manipulation of proviral clones capable of generating infectious virus, and the development of methodologies for characterizing these clones in primary human T-lymphocytes using relevant animal models has vastly increased our understanding of these complex retroviruses (43, 67, 168).

A variety of animal models to study HTLV infection and transformation have provided important insight into viral-host interactions and determinants for HTLV pathogenesis/oncogenesis. As with all animal models, each has its unique advantages and disadvantages, and animal models for HTLV infection are no exception (183). First, tumor transplant models utilizing genetically engineered severe combined immunodeficiency (SCID) mice have yielded important information on the tumorigenic potential of ATL cells and tumor outgrowth in these animals has allowed evaluation of potential therapeutic drugs (79, 126). Furthermore, the mouse models have provided insight into Tax-mediated dysregulation of cellular processes involved in lymphocyte
transformation and leukemogenesis (24). In addition to the mouse, Rats have been used to study viral infection and neurological disease association along with testing the role of cell-mediated immunity to viral infection (154, 248, 342). Researchers have successfully infected squirrel monkeys with HTLV, which offers an attractive nonhuman primate model for vaccine studies (161). Lastly, the rabbit has been the most widely used animal model for HTLV due to the ease and consistency of viral transmission. Early studies utilizing the rabbit model provided important information regarding bodily fluids likely to contain the virus (blood, semen, breast milk) and effective methods to prevent viral transmission (6, 160, 173, 183, 314). The rabbit model also has been used for the evaluation of the host immune response against viral infection and in attempts to generate a vaccine (309). More recently, the rabbit model has been successfully used to evaluate the pathogenesis of infectious molecular clones of both HTLV-1 and HTLV-2 (55, 57). Briefly, HTLV established viral cell lines are used as virus producer cells. These cells are lethally irradiated and inoculated into rabbits allowing viral replication, immune response, and persistence to be monitored over time. Studies using these models have provided a better understanding of the importance of viral accessory proteins, although dispensable in vitro, we have learned that they play a critical role in vivo. Ultimately, these model systems can be used to understand viral pathogenesis and develop potential therapeutic strategies against HTLVs in the future.
1.3 HTLV Pathogenesis and Disease Association

HTLV-1 and HTLV-2 are transforming retroviruses that have the capacity to infect and promote T-lymphocyte activation and proliferation in vitro and in vivo (105, 237, 272, 280). One of the hallmark features of HTLV is its ability to infect and immortalize/transform primary human T-lymphocytes in vitro. Immortalization is defined as continuous growth of T-lymphocytes in the presence of exogenous interleukin 2 (IL-2), and typically evident in culture microscopically as refractile cell clusters within 7-10 weeks of co-cultivation with lethally irradiated HTLV-producing cells. Transformation is defined as continuous growth in the absence of exogenous IL-2; the establishment of hardy IL-2-independent transformed T-cell lines typically requires months in culture. Although the molecular basis for cellular transformation by HTLV is not completely understood, data generated from multiple experimental systems clearly identified the viral protein Tax as the critical determinant. This makes HTLV different from animal transforming retroviruses such as Rous sarcoma virus or Abelson murine leukemia virus in that HTLV does not encode viral homologues of cellular proto-oncogenes (175, 290). Interestingly, despite a wide variety of human and nonhuman cells that can be infected by HTLV in vitro (52, 175), HTLV-1 preferentially transforms CD4+ T-cells, whereas HTLV-2 preferentially transforms CD8+ T-cells, and to some extent CD4+ T-cells (184, 270, 323, 339). Both HTLV-1 and HTLV-2 transform T-cells in vitro and in vivo. Since only a fraction of HTLV-1 infected individuals actually develop ATLL or HAM/TSP, and an even smaller fraction develops an HTLV-2 associated malignancy, this suggests that the disease association with HTLV is a multistep process that requires the accumulation of genetic mutations over an extended period of time. However, there is
sufficient evidence to suggest that HTLVs play a critical role in these processes. Below are detailed examples of HTLV-associated diseases.

1.4 HTLV-1 Pathogenesis

To date, HTLV-1 infection has clearly been linked to the development of ATLL. Throughout its aggressive clinical course, infected cells infiltrate the skin, liver, gastrointestinal tract, and the lungs. Furthermore, HTLV-1 infection also is associated with various lymphocyte-mediated inflammatory diseases including HAM/TSP, uveitis, infectious dermatitis, and arthropathy (18, 77, 89, 181, 224, 225, 245, 255, 341).

1.4.1 Adult T-cell Leukemia/Lymphoma (ATLL)

As early as 1977, physicians identified an unusual clustering of ATLL patients in concentrated areas of Japan. These events led them to believe that a transmissible agent was involved in the disease occurrence (313). Later it was elucidated that HTLV-1 was, in fact, the causative agent of ATLL. This aggressive lymphoproliferative disease is characterized clinically into five different stages: asymptomatic, pre-leukemic, chronic/smoldering, lymphoma, and acute. Despite the fact that the majority of infected individuals are asymptomatic, these individuals are still able to transmit the virus. While approximately half of the pre-leukemic individuals undergo spontaneous regression, some will progress to the next phase of infection. The chronic/smoldering phase of ATLL is characterized by skin lesions and bone marrow involvement, along with elevated
numbers of circulating leukemic cells. These infected T-cells typically present with highly lobulated or “flower-shaped” nuclei with a phenotype of CD2+, CD3+, CD4+, CD8-, CD25+, and HLA-DR+ (313). Finally, a small fraction of patients will develop acute ATLL, which is accompanied and characterized by hypercalcemia, elevated levels of LDH, skin lesions, lymphadenopathy, lymphomatous meningitis, lytic bone lesions, spleen or liver involvement, and immunodeficiency (141). Even with intense chemotherapy, the median survival time for acute ATLL patients is 6-10 months (292).

1.4.2 HTLV-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

Similar to what is seen with ATLL, only a small fraction of infected individuals (0.2-5%) will develop HAM/TSP (153). It is a chronic and progressive demyelinating disease that predominantly affects the spinal cord and seems to be more prevalent in women than men (195, 252). Contrary to ATLL, the onset of HAM/TSP is much faster and can present as early as six months after transfusion (148, 320). HAM/TSP initially presents with clinical signs of weakness and stiffness of the lower limbs. As the disease progresses, more symptoms present including constipation, impotence, and hyperreflexia. Pathologic studies reported perivascular and parenchymal infiltration of mononuclear lymphoid cells which correlate with myelin and axonal degeneration, ultimately resulting in severe degeneration of white matter. Reports indicate that the virus-host immune system interaction plays a critical role in the development of HAM/TSP. Briefly, high proviral load accompanied with increased viral gene expression leads to the processing and presentation of HTLV-1-specific peptides, specifically those of Tax-1, which causes
activation and expansion of antigen-specific CD4+ and CD8+ T-cells. Infiltration and localization of HTLV-1-specific CD8+ cytotoxic T-lymphocytes (CTLs) to the central nervous system implicates those cells in disease development (77, 135).

1.5 HTLV-2 Pathogenesis

As mentioned above, the disease association for HTLV-2 is less clear and lacks epidemiological evidence. Interestingly, HTLV-2 was isolated from patients with a rare variant of hairy cell leukemia (147), CD8+ T-cell leukemia (278), mycosis fungoides (349), and large granular lymphocytic leukemia (200). These findings suggest an association between HTLV-2 infection and T-cell lymphoproliferative disorders. Consistent with the \textit{in vitro} tropism of HTLV-2, several of the HTLV-2 associated leukemia cases involve the CD8+ T-cell lineage. Furthermore, HTLV-2 infection has been reported in association with spastic ataxia and chronic neurodegenerative diseases (111, 119). Interestingly, HTLV-2 infection has been reported in a patient with a chronic progressive neurological disease that is clinically identical to HAM/TSP (134). Finally, HTLV-2 has been associated with increased incidence of pneumonia, bronchitis, and inflammatory conditions such as arthritis, and perhaps increased mortality (9, 282).

1.6 HTLV Classification, Genome Organization, and Life Cycle

HTLV types 1-4 are members of the \textit{Retroviridae} family with C-type morphology. Virions assemble at the plasma membrane and possess a central,
symmetrically placed spherical inner core that contains a homodimer of linear, positive sense, single stranded RNA genome of about ~9kb. HTLVs as well as the related simian and bovine T-cell leukemia viruses (STLV and BLV) have been placed in the genus Deltaretrovirus (95) and are classified as complex retroviruses. The distinguishing feature that separates simple and complex retroviruses is that in addition to the structural and enzymatic genes gag, pol, and env, complex retroviruses contain regulatory and accessory genes that encode for proteins that support viral replication, persistence, and ultimately pathogenesis (Figure 1.1). Complex retroviruses differ from other transforming animal retroviruses in that they do not harbor viral homologues of cellular proto-oncogenes. The ability of the virus to generate diverse mRNAs and proteins from a relatively small genome occurs, in part, via at least three mechanisms. First, the virus utilizes frame shifting and internal initiation codons in the generation of gag, pol, and pro encoded from a single mRNA transcript with three reading frames (232, 233). Second, the cleavage of certain large protein precursors such as Gag generates smaller peptides with discrete functions. Finally, HTLVs use alternative splicing resulting in multiple mRNA transcripts that encode distinct proteins.

The RNA genome organization as well as that of the DNA intermediate (provirus) is very similar for all HTLVs. The RNA genome is capped at the 5’ end and contains a ~200 nucleotide poly(A) tail at its 3’ end. The proviral genome contains a long terminal repeat (LTR) at both the 5’ and 3’ ends that encompass sequence blocks that are critical for replication and regulation of viral gene expression. The LTR is composed of a unique 3’ (U3) region present only at the 3’ end of the viral RNA genome, a repeated (R) region present at both ends of the viral RNA genome, and a unique 5’ (U5) region present only
at the 5’ end of the viral RNA genome. The remaining portion of the viral genome between the LTRs is the coding region for *gag*, *pol*, and *env*. In addition, there are four open reading frames (ORFs) in HTLV-1 and five ORFs in HTLV-2 that historically were referred to as the X region (Figure 1.1).

The replication life cycle of HTLVs is similar to that of other retroviruses. First, the viral glycoprotein surface unit (SU) component of Env recognizes and binds to a cell surface receptor(s) leading to the viral and target cell membrane fusion that is facilitated by the TM component of Env. Membrane fusion is followed by the release and uncoating of the viral core into the cytoplasm of the target cell. Next, due to a unique enzyme produced by all retroviruses (reverse transcriptase), the RNA genome is reverse transcribed to generate a double stranded linear DNA intermediate that is transported into the nucleus where it becomes stably integrated into the target cell’s genome. This integration event is catalyzed through the enzymatic activity of the viral protein, integrase. From this point, the integrated HTLV genome is referred to as a provirus and becomes a stable part of the target cell’s genome. Next, the cellular RNA polymerase II initiates transcription from the viral promoter located in the U3 region of the 5’ LTR. Viral transcription results in the production and accumulation of transcripts that are spliced (with the exception of the genomic mRNA), processed, exported, and translated into distinct viral proteins. Lastly, the assembly of the virion and the packaging of the genomic RNA follows. HTLVs utilize a sequence called the psi element ($\psi$) for encapsidation (233). Intriguingly, the $\psi$ element is spliced out in all of the other viral transcripts, ensuring that only the unspliced genomic RNA is packaged into the virion, which then buds from the cytoplasmic membrane. The newly released virions are
immature, and require the activity of protease to cleave the structural proteins for proper maturation of the virion (95, 182).

1.7 HTLV Structural and Enzymatic Genes

HTLVs encode for the structural and enzymatic genes: \textit{gag}, \textit{pol}, \textit{pro}, and \textit{env}. The structural proteins are encoded by the \textit{gag} and \textit{env} genes, whereas the \textit{pol} gene provides the enzymatic activity. Interestingly, HTLV-1 and HTLV-2 have structural and enzymatic proteins that are similar to other retroviruses, yet they do not appear to ensure proper and efficient cell-free transmission (186). The reason(s) for this low efficiency is still not clear.

1.7.1 Gag

The main function of Gag (internal group specific antigen) is to promote the assembly and release of virus particles. HTLV Gag is made as a polypeptide precursor, then later through proteolytic cleavage, generates the matrix (MA, p19), capsid (CA, p24), and nucleocapsid (NC, p15) proteins (250). The targeting of MA and its Gag precursor p55 to the inner surface of the plasma membrane is achieved via myristylation at the N-terminal end of the protein (112, 249). Similar to other retroviruses, HTLV MA has an N-terminal cluster of basic amino acids that have been shown to play numerous roles in virus replication including infectivity, particle release, precursor cleavage and ultimately cell-to-cell transmission (187). The two remaining Gag products (CA and NC)
of HTLV have not been studied in great detail, but their contribution to viral replication likely is similar to other retroviruses including Moloney MLV and HIV-1 and in their interactions with cellular restriction factors such as APOBEC3G (236).

1.7.2 Pro

A frameshift that occurs during the synthesis of Gag (233) results in the production of the viral protein protease (Pro) whose reading frame overlaps the 3’ end of \textit{gag} and the 5’ part of \textit{pol} (100). Pro is responsible for the processing of the precursor Gag and Pol polypeptides to produce smaller functional units. Thus, Pro is essential for proper maturation of the HTLV viral particle (233).

1.7.3 Pol

Another ribosomal frameshift during the translation of Gag results in the synthesis of Gag-Pol. The carboxy terminal region of Gag-Pol is cleaved by Pro to release integrase, the viral protein needed for the stable integration of the viral DNA provirus into the host genome. The remaining part of Pol contains two functional regions. It has reverse transcriptase activity at the N-terminus and RNase H activity at the C-terminus. These functions are critical after HTLV infection to generate the double-stranded DNA intermediate from the viral RNA genome (269).
1.7.4 Env

The Envelope (Env) protein of HTLV is encoded from a singly spliced mRNA. This protein is post-translationally modified, thus resulting in a 61-69 kDa glycoprotein (192, 289). The Env precursor is cleaved by a cellular protease to generate the surface unit (SU, gp46) and the transmembrane subunit (TM, gp21). Similar to other retroviral Env proteins that are organized as oligomers, HTLV Env is assembled at an early stage in the endoplasmic reticulum (ER) as a dimer (253). The SU glycoprotein is composed of three domains that harbor the receptor binding determinants. The TM, on the other hand, contains a fusion peptide at the N-terminus and is directly responsible for the initiation of fusion pores. Mutational analysis of TM demonstrated that its role in post-fusion events is required for infection (187).

1.8 Cellular Tropism

Despite the wide variety of human and nonhuman cells that can be infected by HTLV \textit{in vitro}, the \textit{in vivo} tropism or cellular targets are more selective. Investigations of the cellular tropism of HTLV in asymptomatic patients and those with neurological disease indicated that HTLV-1 has a preferential tropism for CD4+ T-cells with CD8+ T-cells being an additional viral reservoir in HAM/TSP patients. In contrast, the tropism of HTLV-2 \textit{in vivo} is less clear but seems to favor CD8+ T-cells. The difference in cellular tropism between HTLV-1 and HTLV-2 has led to the hypothesis that cellular tropism may be an important determinate for differences in leukemogenic capacity and disease associations (78, 205).
One study detected proviral sequences predominantly in CD8+ T-lymphocytes from HTLV-2 infected individuals, whereas others have detected HTLV-2 in both CD4+ and CD8+ T-cell subsets, with a greater viral burden in CD8+ T-cells. This distinct *in vivo* T-cell tropism has been recapitulated *in vitro* utilizing immortalization/transformation assays where lethally irradiated HTLV producer cells were co-cultured with freshly isolated human peripheral blood mononuclear cells (PBMCs). These studies showed that the majority of cells transformed by HTLV-1 *in vitro* were CD4+ T-lymphocytes (78), whereas HTLV-2 preferentially transformed CD8+ T-cells (333). Recent studies using recombinant viruses revealed that the *env* gene was the major viral determinant responsible for the distinct *in vitro* cellular transformation tropism of HTLV-1 and HTLV-2 (333).

Although the precise mechanism of cell-to-cell transmission remains to be elucidated, several studies have indicated that the HTLV receptor may be multi-component in nature (54, 92, 94, 143, 169, 299). Utilizing various assay systems, several cell surface molecules have been shown to be important for HTLV entry into cells. The first molecule that was associated with HTLV infection was the glucose transporter 1 (GLUT 1) (206-208). Recently, heparin sulfate proteoglycans (HSPGs), and neuropilin-1 (NRP1) were identified (94). Current studies demonstrated that NRP1, the receptor for semaphoring-3A and VEGF-A165 and a member of the immune synapse, is also a physical and functional partner of HTLV-1 Env protein and over-expression of NRP-1 increases HTLV-1 Env-dependent syncytium formation (307). Careful study of the cell surface of activated primary T-cells revealed that CD4+ T-cells expressed significantly higher levels of HSPGs than CD8+ T-cells, whereas CD8+ T-cells expressed GLUT-1 at
strikingly higher levels than CD4+ T-cells. Work from Jones et al. showed that HTLV-2 Env binding and viral entry were significantly higher on CD8+ T-cells while HTLV-1 Env binding and viral entry were higher on CD4+ T-cells. In addition, the authors reported that over-expression of GLUT-1 in CD4+ T-cells enhanced HTLV-2 entry, while expression of HSPGs on CD8+ T-cells increased HTLV-1 entry. Collectively, these studies demonstrated that HTLV-1 and HTLV-2 differ in their T-cell binding and entry requirements and together with the viral recombinant data suggest that the distinct difference in the in vitro cellular transformation tropism and in vivo pathobiology of these viruses may result from different and distinct interactions between their related Env proteins and cellular surface molecules on CD4+ and CD8+ T-cells.

Cell-free HTLV virions are poorly infectious in vitro. In a recent study, HTLV-1 was found to efficiently infect myeloid and plasmacytoid dendritic cells (DC) (145). In addition, DCs exposed to HTLV-1, both before and after being productively infected, can efficiently transfer virus to autologous primary CD4+ T-cells. The DC-mediated transfer of HTLV-1 involves HSPGs and NRP-1, which results in long-term productive infection of the CD4+ T-cells. This study along with observations of HTLV-1-infected DCs in the peripheral blood of infected patients, indicates that DCs likely have a central role in HTLV-1 transmission and persistence in vivo (145).

1.9 HTLV Accessory Proteins

HTLVs utilize alternative splicing and internal initiation codons to generate several regulatory and accessory proteins. Although initially coined “accessory” because
these gene products were believed to be dispensable for viral replication (68, 273), extensive research has proven that these proteins are critical for different aspects of HTLV replication including regulation of transcription, infectivity, maintenance of viral load, host cell activation, and modulation of immune response (8). To date, the expression of these accessory proteins has not been shown directly in vivo, although there is indirect evidence for expression from studies that detected serum antibodies and CTLs specific for HTLV-1 ORF-I and –II gene products in asymptomatic carriers as well as patients (45, 65, 261). These reports suggest that the accessory proteins are produced at some point during the course of HTLV infection at levels high enough to elicit a specific immune response. In HTLV-1, the accessory proteins p12 and p27 are produced from ORF-I, whereas the p30 and p13 gene products are produced from ORF-II (Figure 1.1). Below is a brief summary of what is known about the function of ORF-I and ORF-II proteins.

1.9.1 HTLV-1 ORF-I p12 and p27

p12 is a 99 amino acid (a.a.) hydrophobic protein that is generated from a singly spliced mRNA. Besides being rich in leucine and proline residues (172), p12 contains two putative transmembrane domains, four predicted SH3-binding motifs, a leucine zipper region, and a calcineurin-binding domain (8, 166, 172, 312). This protein appears to be a modulator of T-lymphocyte proliferation and immune function. It has been shown to localize to both the endoplasmic reticulum (ER) and Golgi, but also interacts with the IL-2 receptor α and γc chains and MHC class I heavy chains disrupting their surface
expression. p12 also interacts with the 16kDa subunit of the vacuolar ATPase, a complex important for the function of lysosomes and endosomes and implicated in transformation pathways. It also has been shown to interact with calreticulin and calnexin, important ER regulators of calcium storage and calcium-mediated cell signaling. Analysis of p12 in the context of the HTLV-1 provirus suggested that p12 does not play a role in the IL-2-dependent proliferation response or JAK/STAT activation of HTLV-1 immortalized cells (70, 227). It has been shown that p12 may activate quiescent T-cells as well as provide growth advantage for primary human PBMCs in the presence of suboptimal antigen stimulation. This has lead to the hypothesis that p12 possibly plays a role in early HTLV-1 infection (56, 243). Subsequently, studies revealed that p12 is essential in the establishment of persistent in vivo infection using the rabbit model (7, 58). Recently, a p12-deficient virus study demonstrated that p12 promoted cell-to-cell spread by inducing LFA-1 clustering on T-cells via calcium-dependent signaling (167). Collectively, these reports demonstrate that p12 is a multifunctional protein and its interaction with key cellular proteins involved in immune recognition and cell proliferation facilitates viral infection, host cell proliferation, and survival by providing growth advantages to infected cells during the early stages of HTLV-1 infection.

p27 is a 152 a.a. protein that is generated from a doubly-spliced mRNA and shares the first 20 a.a. with Rex and the last 99 a.a. with p12. The exact role of p27 in HTLV-1 replication and pathogenesis has yet to be elucidated; however, one study reported CTLs against the p27 protein are generated during HTLV-1 infection providing evidence for the chronic production of p27 in vivo (65).
1.9.2 HTLV-1 ORF-II p30 and p13

HTLV-1 p30 is a 241 a.a. nuclear/nucleolar protein produced from a doubly-spliced mRNA (26). It is a multi-functional protein with transcriptional and post-transcriptional roles in regulating viral gene expression. p30 has domains that share homology with the DNA binding domain and homeodomain of the transcription factor Oct-1 and a bipartite nuclear localization signal (61, 171). p30 also contains serine and threonine-rich regions that share homology with the activation domain of the POU family of transcription factors, such as Oct-1/2, Pit-1, and POU-1 (49). p30 has also been shown to interact directly with CREB binding protein (CBP)/p300, TIP60, and Rex (295, 346, 347).

Early studies by Lairmore and colleagues provided evidence that p30 is involved in modulating HTLV-1 as well as cellular gene expression (347). In vitro studies demonstrated that at low concentrations, p30 differentially regulated cellular and viral promoters through its interaction with the conserved KIX domain of the transcriptional coactivator CBP/p300. In contrast, at high concentrations p30 functions as a repressor of viral gene transcription by competing with the viral protein Tax for cellular factors and disrupting the CREB-Tax-CBP/p300 complexes that are bound to the HTLV-1 viral promoter. Similarly, p30 also may repress the activation of cellular gene CREB-responsive promoters by sequestering the cellular pool of CBP/p300. Utilizing microarrays, it was shown that p30 has the capacity to alter the expression of multiple cellular genes that are involved in transcription and translation, T-cell activation, apoptosis, cell cycle, and cell adhesion (221). Further reports demonstrated that cellular
genes are modulated by p30 in a TIP-60-dependent manner (221). TIP-60 has been shown to be a partner of the transcription activator Myc. These reports demonstrated that p30 enhances Myc-dependent cellular transcription and its ability to transform human fibroblasts, which might contribute significantly to ATLL progression (15). p30 has also been shown to repress viral replication at the post-transcriptional level by binding to and retaining tax/rex mRNA in the nucleus (242). The suppression of Tax protein expression abrogates HTLV-1 transcription and thus down-regulates replication. Recently, it was reported that p30 and the positive post-transcriptional regular, Rex, form ribonucleoprotein complexes specifically on tax/rex mRNA (295). Rex neutralizes the p30-mediated suppression of viral mRNA expression and restores cytoplasmic levels of tax/rex mRNA and Tax protein expression. This interaction is speculated to act as the switch between virus latency and replication.

A recent study showed that p30 expression results in activation of the G2-M cell cycle checkpoint in Jurkat T-cells (64), which suggests that p30 is involved in events that would promote early viral spread and T-cell survival. Although p30 has been shown to be dispensable for HTLV-1-mediated cellular transformation in vitro, it has been determined that p30 is essential early in infection to sustain high viral loads in rabbits and promote persistence (20). It is now becoming clear that p30 is a multi-functional protein that assists the virus at multiple steps throughout infection contributing to virus survival and pathogenesis.

Another protein expressed from ORF-II is p13. p13 initiates at an internal methionine codon present in the singly-spliced mRNA. Its open reading frame correlates to the last 87 a.a. of p30 (63). p13 targets to the mitochondria where it disrupts the
mitochondrial inner membrane potential and ion flux and alters mitochondrial morphology (51, 62). In the mitochondria, p13 interacts with farnesyl pyrophosphate synthase, an enzyme involved in post-translational farnesylation of Ras (193). In addition, p13 seems to sensitize cells to apoptosis induced by pro-apoptotic agents such as ceramide (117). Another report showed that p13 significantly reduced the incidence and growth rate of tumors arising from c-myc and Ha-ras-co-transfected rat embryo fibroblasts, as well as reduced proliferation at high density in vitro (293). Similar to p30, p13 has been found to be dispensable for HTLV-1 infection and immortalization of PBMCs in vitro, whereas rabbits inoculated with a p13 deficient virus failed to induce a significant immune response and establish a persistent infection (20, 294).

1.9.3 HTLV-1 Antisense Gene, HBZ

It has only been recently that natural retrovirus antisense transcripts have received significant attention. The HTLV-1 basic leucine zipper factor (HBZ) gene is encoded from the minus strand of the proviral genome; the mRNA is synthesized from a promoter located in the 3’LTR (219). In addition to HTLV-1, antisense viral transcripts have been identified in other retroviruses including HIV-1, feline immunodeficiency virus (FIV), simian immunodeficiency virus-1 (STLV-1), and recently in HTLV-3 (36, 185, 304, 318). Proteins from these transcripts have been postulated to play key roles in the infection cycle and/or pathophysiology of the virus. Interestingly, the non-pathogenic HTLV-2 does not contain a minus strand transcript. Although it remains unknown if HTLV-3 induces cancer, the current data allow one to speculate that HTLV types that
induce T-cell neoplasia encode genes that are transcribed from the negative strand that may play a role in oncogenesis. Moreover, unlike the 5’ proviral LTR that contains the promoter for all other HTLV-1 genes, the 3’ LTR remains functionally intact and unmethylated at all stages of ATL development, suggesting a potential role of Hbz in the maintenance of leukemia. In support of this, Hbz transcripts have been detected in all ATL cells studied to date, whereas tax mRNAs are present in only ~ 40% of leukemic cells (41).

Recent research in the field suggests that the Hbz gene may function at both the mRNA and protein levels. The HBZ protein contains an N-terminal transcriptional activation domain and a leucine zipper motif in its C-terminus (41, 91). Exogenously over-expressed HBZ protein interacts with CREB-2 and down-regulates Tax-mediated HTLV-1 transcription and interacts with and disrupts the DNA binding activity of JunB and c-Jun (AP-1 components). In addition, HBZ promotes c-Jun degradation through a proteasome-dependent degradation pathway. HBZ also has been shown to interact and activate JunD. The Jun family of transcription factors regulates gene expression of a number of cellular genes via interactions with the AP-1 sites present in the promoter region. HBZ increases the expression of human telomerase reverse transcriptase (hTert) due to the presence of five putative AP-1 binding sites within the hTert promoter (213, 215). Therefore, it has been hypothesized that HBZ may play an important role in HTLV-1 biology and the development leukemia by counteracting the effects of Tax-mediated transcription and/or attenuating or activating cellular gene expression.

Satou et al. recently reported that short interfering RNAs (siRNA) to Hbz significantly decreased proliferation of ATL cells (286). Furthermore, these authors
reported that \(Hbz\) mRNA rather than HBZ protein promoted proliferation of a human T-cell line (286). Mutational analysis suggested that the structure of the \(Hbz\) mRNA is important for its role as a negative regulator of proliferation. In support of this conclusion, DNA microarray analysis demonstrated that increased \(Hbz\) mRNA expression correlated with up-regulated expression of the cellular transcription factor E2F1 and subsequently many cellular E2F1-responsive genes. These results provide further evidence that \(Hbz\) could be critical for the regulation of cell proliferation and development of ATL. To evaluate the role that HBZ plays in HTLV-1 associated diseases, such as chronic inflammatory diseases that often are seen in individuals with ATL, \(Hbz\) transgenic mice were generated. Histological analyses of these mice revealed severe dermatitis with massive infiltration of lymphocytes to both the dermis and epidermis. In addition, lymphocyte infiltration also was observed in the alveolar septa and bronchi of the lungs. Interestingly, the spontaneous skin and lung lesions in the HBZ transgenic mice resembled those observed in HTLV-1 infected individuals. This, along with the findings that the HBZ transgene promoted CD4+ T-lymphocyte proliferation, are consistent with the conclusion that HBZ is involved not only in the progression of oncogenesis, but also plays a critical role in HTLV-1-associated chronic inflammatory diseases (219, 286).

Recent studies by Arnold et al. utilizing an infectious molecular clone of HTLV-1 indicated that the HBZ protein is dispensable for immortalization/transformation of primary T-lymphocytes in cell culture (10). Furthermore, rabbits infected with this HBZ protein knockout virus became persistently infected but displayed a decreased antibody response to viral gene products and reduced proviral load in PBMCs as compared to wild
type HTLV-1 infected animals. The data provide important evidence that HBZ is required for the establishment of chronic viral infections \textit{in vivo} (10).

Recent findings suggested that \textit{hbz} mRNA supports proliferation of ATL cells. Suppression of \textit{hbz} gene transcription by short interfering RNA (siRNA) significantly decreased proliferation of ATL cells. In contrast to \textit{tax} mRNA, \textit{hbz} mRNA is expressed in all fresh ATL cells. Lentiviral vectors that express \textit{hbz}-specific short hairpin RNA effectively decreased both \textit{hbz} mRNA and protein expression in transduced HTLV-1-transformed SLB-1 T-cells. \textit{Hbz} knockdown correlated with a significant decrease in T-cell proliferation in culture and tumor formation in animals challenged with SLB-1-\textit{hbz} knockdown cells (11). Recently, Li \textit{et al} utilized real-time RT-PCR to determine the kinetics of viral gene expression in cells transiently transfected with an HTLV-1 proviral plasmid, newly infected human PBMCs, and PBMCs from newly infected rabbits. The expression profiles in transiently transfected and infected cells were similar over time. Although \textit{hbz} levels were significantly lower than \textit{tax/rex}, they were detectable and increased over time. Interestingly, in rabbits, \textit{Hbz} mRNA was detectable at one week post-infection and increased and stabilized over the course of infection (Li \textit{et al} 2009 JV In Press).

Taken together, the data support the hypothesis that the HBZ protein suppresses Tax-mediated transcription from the 5’ LTR, and the \textit{Hbz} RNA promotes ATL cellular proliferation (Figure 1.2). However, further studies are needed to dissect the precise mechanisms by which HBZ protein and mRNA promote HTLV-1 pathogenesis.
1.10 HTLV-2 Accessory Proteins

In HTLV-2, the accessory protein p10 is produced from ORF-I, p28 from ORF-II, p22/20 from ORF-III, and p11 is produced from ORF-V. To date there is limited information on the function of these proteins and their roles in HTLV-2 biology. However, the fact that the pX region from which they are expressed is well conserved among retroviruses suggests that they provide essential functional contributions to the virus. One study showed that an HTLV-2 viral clone where the pX region was removed was able to replicate in vitro, but was attenuated in terms of infectivity and persistence in an in vivo rabbit model (55). Since the entire pX was deleted in this study, individual contributions of each gene could not be determined. Below is a brief description of the proteins generated from transient transfection of the cDNAs for ORF-I, ORF-II, ORF-III, and ORF-V.

1.10.1 HTLV-2 ORF-I p10

HTLV-2 p10 is an 83 a.a. protein that is expressed from a doubly spliced bicistronic mRNA that also encodes ORF-V p11. The protein is mostly hydrophobic and shows some homology to HTLV-1 p12. Similar to p12, p10 associates with MHC-I but does not bind to IL2-Rβ, γ, or 16K, suggesting that despite the homology between p12 and p10, the two proteins are functionally distinct (142). Consistent with this hypothesis, p12 localizes to the ER and the cis-Golgi, whereas p10 accumulates in the periphery of the nucleoli and in nuclear speckles. This distinct subcellular localization could be
attributed to the first 21 arginine-rich amino acids that are derived from the Rex-2 ORF and serve as a nuclear localization signal (48).

1.10.2 HTLV-2 ORF-II p28

The HTLV-2 accessory gene product p28 is a 216 a.a. protein that can be translated from two singly spliced, bicistronic mRNAs. Although p28 potentially could be translated from tax/rex mRNA, it has been shown that when the AUGs for Tax and Rex are functional, a minimal amount of p28 is produced from this mRNA (48). In comparison, the first 49 N-terminal amino acids of p28 contain 77.5% homology with the C-terminal region of HTLV-1 p30. p28 localizes predominantly to the nucleus, and acts as a negative regulator of both Tax and Rex by binding to and retaining tax/rex mRNA in the nucleus (344). It is hypothesized that the reduction of viral replication in a cell carrying the provirus may allow escape from the host’s immune system. Younis et al. found that p28 is recruited to the viral promoter in a Tax-dependent manner and travels with the transcription elongation machinery until its target mRNA is synthesized.

Experiments that artificially directed p28 to the promoter indicated that p28, unlike HTLV-1 p30, does not have transcriptional activity.

p28 has the potential to be expressed from two distinct singly-spliced mRNAs. These mRNAs use different splice acceptor sites and have the potential to encode both p28 and two truncated Rex-2 products (p22/p20Rex). The p22 and p20 truncated Rex products appear to localize to the cytoplasm, but their function has yet to be elucidated.
1.10.3 HTLV-2 ORF-V p11

Translation of the HTLV-2 accessory protein p11 is initiated from the AUG of Tax/Env that is linked to ORF-V sequences, although the protein appears to be larger than its predicted molecular weight of 8.4 kDa. A 10 a.a. stretch in p11 shows strong homology to part of the musculoaponeurotic fibrosarcoma (MAF) nuclear transforming protein, however, a functional correlation has yet to be determined (48). Similar to p10, p11 associates with MHC-I but does not bind to 16K, or IL2Rβ or γ (142). Finally, p11 localizes to the nucleus, and to a lesser extent, to the cytoplasm (48).

1.11 HTLV Regulatory Proteins

HTLV encodes two positive regulatory proteins, Tax and Rex, from a single bicistronic mRNA. Both proteins are essential for efficient viral infection and replication in vitro and in vivo. Tax increases the rate of transcription from the viral promoter located in the viral LTR and modulates the transcription or activities of numerous cellular genes involved in cell growth/survival and differentiation, cell cycle control, and DNA repair (97). Rex is a nucleolar-localizing and shuttling phosphoprotein that acts post-transcriptionally by preferentially binding, stabilizing, and selectively exporting the unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm, thus primarily controlling the expression of the structural and enzymatic proteins that are essential for production of viral progeny (345).
1.11.1 HTLV ORF-IV Tax-1 and Tax-2

HTLV Tax is a pleiotropic phosphoprotein that regulates viral replication as well as HTLV-mediated transformation and disease progression. The prototypical HTLV-1 Tax is a 353 a.a. protein that is predominately nuclear but has been shown to shuttle between the nucleus and the cytoplasm (37, 218). Whereas Tax-2, on the other hand, is located predominantly in the cytoplasm and is 331 a.a. in size (reported from the pH6neo HTLV-2A molecular clone) (218).

1.11.2 The Role of HTLV Tax in Cellular Transformation and Pathogenesis

One of the hallmark features of HTLV-1 is its ability to infect and immortalize/transform primary human T-lymphocytes in cell culture. As mentioned earlier, immortalization is defined as continuous growth of T-lymphocytes in the presence of exogenous interleukin 2 (IL-2), and typically evident in culture microscopically as refractile cell clusters within 7-10 weeks of co-cultivation. Transformation is defined as continuous growth in the absence of exogenous IL-2; the establishment of hearty IL-2-independent transformed T-cell lines typically requires months in culture. Although the molecular basis for cellular transformation is not completely understood, data generated from multiple experimental systems clearly identified the viral transactivator Tax as the critical determinant. The current understanding is that Tax is involved very early in the viral infection and sets the stage for cellular transformation and ultimately, disease progression. Initial experiments
revealed that Tax has oncogenic potential as demonstrated by induction of tumors in transgenic animals, transformation of rodent fibroblasts, and *Herpes samiri* vector immortalization of human T-lymphocytes (78). Interestingly, over-expression of Tax in transgenic mice resulted in formation of mesenchymal tumors, salivary and lacrimal gland exocrinopathy, lymphadenopathy or splenomegaly, and lymphoma or leukemia (99, 105, 237, 256). Studies using infectious molecular clones showed directly that Tax is the essential gene product required for HTLV-mediated cellular transformation of primary human T-cells in culture (68, 101, 274, 281, 338). The precise mechanism by which Tax contributes to the malignant process is unclear but is proposed to involve several points of cellular dysregulation culminating in the accumulation of genetic mutations and uncontrolled lymphocyte growth. Due to its established role as a critical component of the transforming capacity of HTLV, Tax has been categorized as functionally analogous to the regulatory proteins encoded by several DNA tumor viruses including adenovirus E1A and simian virus 40 (SV40) large T antigen (73). However, data is emerging that other viral genes have important roles in the biology of the virus by contributing to virus survival and ultimately its oncogenic properties. We discuss in more detail below specific activities of Tax that have been implicated in the transformation process.
1.11.3 HTLV Tax and Transcriptional Activation

Tax is one of the first proteins expressed early after viral infection and is a transactivator of viral gene expression. Tax transcriptionally activates the HTLV promoter via three 21 bp repeat sequences termed the Tax response element (TRE). The TRE contains DNA sequences identical to part of the cyclic adenosine monophosphate (cAMP)-responsive element (CRE). The CRE, which is contained in many cellular gene promoters, is responsive to cAMP and binds to members of the CRE binding protein/activating transcription factor (CREB/ATF-1) family of transcription factors in a Tax-dependent manner. In vitro, Tax contacts GC-rich DNA that flanks the TRE-1 or CRE sequence and recruits the cellular co-activator (CREB) to the transcription complex. The Tax/CREB heterodimer interacts with the CRE-like sequence of the viral promoter to activate viral transcription. Tax directly interacts with the CREB-binding protein (CBP) and p300 to form a Tax/CREB/p300/CBP complex. Interestingly, CREB recently has been designated as a proto-oncogene due to its role in promoting abnormal survival and proliferation of hematopoietic cells (157). Recruitment of another host cell factor, p300/CBP-associated factor (PCAF), which directly interacts with Tax, is essential for transcription initiation. Tax also modulates the activity of other cellular transcription factors including serum response factor (SRF) and AP-1, which activate a plethora of early response genes that regulate proliferation contributing to the survival of the infected cell. The role of Tax in this process has been tested directly using Tax mutants that have been identified that fail to activate the CREB/ATF pathway and are defective for transactivation of the viral promoter (211, 279). Over-expression of these and other Tax mutants in various assay systems has been invaluable for dissecting cell signaling
pathways and for determining the association between Tax and cellular transformation. Functional analysis of Tax in the context of an infectious virus has presented a unique challenge since a knockout of Tax, or more specifically, the lack of Tax to activate the CREB/ATF pathway, disrupts overall viral gene expression and replication. Ross et al. circumvented this problem by generating a unique HTLV provirus that replicates by a Tax-independent mechanism due to replacement of the TRE with the cytomegalovirus immediate-early promoter enhancer (279). Therefore, viral gene expression and replication are not disrupted significantly by mutations in Tax. Initial Tax knockout studies revealed that Tax was absolutely required for T-lymphocyte transformation providing the first direct evidence (in the context of a virus) that Tax was the critical viral transforming protein (279). Subsequent studies revealed that CREB/ATF activation by Tax is required to promote sustained cell growth of CD4+ T-cells and IL-2 independent cellular transformation (274, 280).

In addition to transactivating the viral promoter, Tax modulates the transcription or activity of numerous cellular genes involved in cell growth and survival, cell cycle control, and DNA damage/repair (97). The ability to modulate the expression or activity of a variety of viral and cellular gene products is proposed to be the key mechanism by which Tax induces cellular transformation. The sections below will focus on the ability of Tax to modulate gene expression and/or impinge on critical cellular activities and regulatory control pathways consistent with its transforming capability.
1.11.4 HTLV Tax and Cell Survival

It is a well established paradigm that cancer is a multi-step process in which several events are required for cellular transformation and ultimately, disease progression. The culmination of HTLV-1 viral infection is the development of ATL, a CD4+ T-cell malignancy. In order for this rare event or endpoint to occur, the virus must facilitate the conversion of a normal T-cell into a transformed leukemic cell. To accomplish this, the virus must modify or overcome a number of cellular defense barriers and checkpoints, most importantly, apoptosis and/or senescence (156, 178). Experimental evidence has revealed that HTLV-1 employs Tax to manipulate and exploit multiple pathways to facilitate cell survival. One of the major cell growth and survival pathways that Tax targets directly involves the activation of NFκB. NFκB consists of a family of inducible transcription factors that regulate multiple biological functions including the growth and survival of T-cells. Aberrantly activated NFκB has been associated with multiple human cancers (302); and while under tight regulation in normal T-cells, NFκB is constitutively active in HTLV-1 infected and Tax-expressing T-lymphocytes (115). NFκB normally is sequestered in the cytoplasm primarily by physical interaction with inhibitor proteins IκBα and IκBβ. Although Tax activates multiple members of the NFκB pathway, it is clear that Tax alone cannot directly activate NFκB via physical interactions. Evidence has implicated the cellular protein IκB kinase (IKK) in the Tax-mediated activation of NFκB. The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ (also referred to as NEMO). Tax targets and binds the IKKγ subunit creating a constitutively active IKKγ, which is a hallmark of both HTLV-1 infected and Tax transfected T-lymphocytes (97, 137). The aberrant IKKα-
IKKβ-IKKγ complex phosphorylates the cytoplasmic inhibitors IκBα and IκBβ leading to their proteasomal-mediated degradation, which allows the nuclear translocation of NFκB (RelA/p50). This event results in transcriptional activation of the NFκB-responsive genes IL-2, IL-2Rα, IL-3, GM-CSF, bcl-xL, and survivin, which are key regulators of proliferation and apoptosis (107). Dual expression of IL-2 and IL-2Rα within an infected T-cell leads to an autocrine stimulatory signal for T-cell proliferation via the Janus kinase/signal transducer activator of T-cells (JAK/STAT) pathway. Therefore, constitutive activation of this pathway has been proposed as one of the key steps required to reach the fully transformed state. Detailed mutational analysis of Tax has identified specific mutants and/or domains important for activation of NFκB signaling (157, 279).

Studies utilizing HTLV infectious molecular clones indicated that immortalization of T-lymphocytes in cell culture are dependent on Tax activation of NFκB (274, 280). The critical role for NFκB activation by Tax in the process of HTLV-1 associated malignancy also is supported by in vivo observations. In addition, NFκB and NFκB target genes are found to be activated in ATL, ATL transplanted NOD-SCID INFγ knockout mice, and tumors arising in Tax transgenic mice (183). Approaches to block NFκB using drugs or peptide inhibitors have resulted in tumor cell regression in various animal models, which is consistent with the importance of this transcription activation pathway in tumor cell survival (183). However, there remains a disconnect between Tax activation of NFκB and induction of ATL, since many leukemic cells no longer express Tax but show constitutive NFκB activation. Thus, it is clear that Tax activation of NFκB provides a critical proliferative or survival signal early in the cellular transformation process, but not for
maintenance of the leukemic state. As referred to above, HBZ may be the critical maintenance factor needed to sustain the leukemic state.

A second important pathway by which Tax represses apoptosis involves phosphatidylinositol 3-kinase (PI3K) and its downstream target Akt. This pathway normally is activated in response to cytokine and/or T-cell receptor signaling and is an important mediator of cell survival and proliferation (300). PI3K-Akt has been found to be activated in HTLV-1 transformed Rat-1 cells and is thought to be involved in cellular transformation. Tax, via binding to PI3K, promotes site-specific phosphorylation and activation of Akt. Akt is a serine/threonine kinase that influences many downstream signaling cascades culminating in the activation of numerous transcription factors including activator protein 1 (AP1), (258) a factor found to be highly expressed in invasive human cancers including ATL. The critical role for Tax-PI3K-Akt activation in the survival and proliferation of virus-infected Tax expressing cells is supported by PI3K inhibitor experiments that block Akt phosphorylation and induce cell death (125).

1.11.5 HTLV Tax and Cell Cycle Deregulation

A hallmark of all cancers is that the tumor cells display increased DNA replication and cellular proliferation. Oncoproteins must activate not only the progression of the cell cycle, they also must facilitate escape from cellular checkpoints and dismantle tumor suppressors that are in place to maintain and protect the integrity and efficiency of each mitotic cellular division. Tax has evolved various strategies to counteract at least
three distinct cellular tumor suppressors including retinoblastoma (Rb), p53, and human Drosophila large disc (hDLG).

Tax has the ability to override cell cycle control and stimulate G1-to-S-phase transition in HTLV-1 infected and Tax expressing cells (239, 288). Cell cycle progression is tightly controlled in mammalian cells through sequential activation and degradation of proteins called cyclins and cyclin-dependent kinases (Cdks). Together, cyclins and Cdks form complexes that phosphorylate target proteins within specific regulatory cascades. The specific phosphorylations result in the activation or inactivation of target proteins that collectively dictate passage through the cell cycle. Tax stimulates the G1-to-S-phase transition in three distinct ways: (i) transcriptional up-regulation of cyclin D2; (ii) direct binding and activation of the kinase holoenzyme; and (iii) repression of Cdk inhibitors. Tax, along with increased IL-2R signaling, increases the transcriptional expression of the G1 phase D cyclins (specifically cyclin D2) by directly activating their promoters (5, 131, 284). Tax also has been shown to modify the cell cycle by directly binding cyclin dependent kinases (CDK)-4 and (CDK)-6 and by repressing inhibitors such as the INK4A-D and KIP1 (97, 108, 215). The resulting activation of the cyclin D/CDK4/6 kinase holoenzyme results in the hyper-phosphorylation of the Rb tumor suppressor (162). Rb is the founding member of the “pocket” protein family and has been shown to be the major tumor suppressor that regulates G1-to-S phase transition. Rb functions by binding and inhibiting the transcription factor E2F1, which regulates genes involved in S phase progression and/or apoptosis (131, 194, 287). During normal cell cycle progression into S phase, Rb becomes phosphorylated by the cyclin D holoenzyme leading to its proteasomal degradation. In addition to activating the holoenzyme, Tax may bind Rb
directly inducing its degradation and subsequent release of the transcription factor E2F1, thereby promoting G1-to-S transition (90, 194).

In conjunction with Rb, p53 has been established as a DNA binding transcription factor that plays a pivotal role in protection against structurally damaged DNA, oncogene activation, and cellular transformation. This is accomplished by p53-mediated cell growth arrest or apoptosis through transcriptional activation of cell cycle regulatory proteins. This tumor suppressor was first discovered as a 53 kDa protein that co-immunoprecipitated with SV40 large T-antigen. Its function was later elucidated as the guardian of the G1-to-S-phase transitional checkpoint, and plays a central role in maintaining genomic stability after DNA damage has occurred. p53 is activated in response to double-stranded DNA breaks and functions to increase the transcription of Cdk inhibitor proteins as well as activate Bax, a pro-apoptotic protein (196). It is hypothesized that Tax disruption of the p53 pathway would be advantageous for progression of the cell cycle even in the presence of DNA damage. It has been reported that p53 is mutated in 50% of all human cancers. Inactivation of p53 seems to be a major target in a number of virally transformed cells. There are several viral oncogenes that have been found to interfere with p53 function including SV40 large T-antigen, hepatitis B X protein, adenovirus E4 ORF6, cytomegalovirus IE2, and the human papillomavirus E6 and E7 proteins (90). Interestingly, in HTLV-induced leukemia, p53 itself is not mutated yet the p53 regulated checkpoint that guards the transition between G1-to-S is defective (305). The mechanism by which Tax alters p53 activity is not only complex, but unique as well. Tax does not bind p53, repress its gene transcription, alter its subcellular localization, or disrupt its DNA binding activity (228, 262). To date, two
hypotheses have been proposed to explain how Tax inactivates p53. First, findings indicate that Tax abrogates p53 function by directly competing for binding to the ubiquitous transcriptional co-activator CBP/p300. The amino terminal transactivation domain of p53 interacts with multiple cellular transcription factors, including TFIID, TFIIH, and CBP/p300, which facilitates p53-mediated transcription of cell cycle control genes (90). The oncoprotein interference and sequestering of these critical cofactors ultimately decreases the ability of p53 to activate target gene expression. The second possibility is that Tax acts through an NFκB complex to inactivate p53 function (263). To date, neither the NFκB nor the CBP/p300 mechanisms fully explain the Tax-mediated loss of p53 (223), and additional studies will be required to fully elucidate the role of Tax in p53 inactivation and DNA damage in ATL.

It has become clear that many cancer causing viruses utilize similar strategies to promote cellular transformation. It first was demonstrated over a decade ago that the carboxyl terminus of HTLV-1 Tax and the E6 proteins of the highly oncogenic human papillomavirus (HPV) encode a PDZ binding motif (PBM) that targets cellular proteins with PDZ protein-protein interaction domains (118, 191). Interestingly, the PBM is absent from HPVs that are poorly oncogenic and also is absent in Tax from the rarely pathogenic HTLV-2 (75, 78, 118). The PDZ domain was named after the first PDZ-containing proteins identified, which were postsynaptic density protein (PSD-95), Drosophila discs large protein (DLG), and epithelial tight junction protein (Zonula Occludens-1). Other identified PDZ domain-containing proteins include the human homolog of the Drosophila discs large tumor suppressor protein (hDlg), the human homolog of the Drosophila scribble tumor suppressor protein (hScrib), the membrane-
associated guanylate kinase with inverted orientation (MAGI-1, -2, -3), and a multi-PDZ protein (MUPP1). PDZ domain-containing proteins also possess other binding motifs including SH3, pleckskin, and protein tyrosine phosphatase, which implicate the involvement of these proteins in numerous signaling processes (107) that regulate cell cycle/proliferation. To date, three PDZ containing proteins (pro-IL-16, MAGI-3, and hDlg) have been shown to interact directly with Tax via their respective PDZ domains (107, 283, 303). Pro-IL-16 is abundantly and constitutively expressed in human peripheral blood T-lymphocytes and promotes cell growth arrest (107). MAGI-3 plays a role in several cell survival signaling pathways and is a key mediator/regulator in cell polarity. hDlg is a scaffolding protein that contains three PDZ domains and has been shown to signal downstream of Wnt and frizzled. While all of the functions of hDlg have not yet been fully elucidated, it is clear that hDlg functions as a tumor suppressor (129).

In addition to Tax, oncoproteins of several DNA tumor viruses, including the E6 protein from the highly oncogenic HPVs and the adenovirus type 9 E4ORF1 oncoprotein, also bind and deregulate hDlg (190). In addition, hDlg binds the C-terminus of the adenomatous polyposis complex (APC) tumor suppressor, which regulates cellular proliferation and cell cycle phase transition (212). To overcome this cellular protection barrier, the HPV oncoprotein E6 binds to hDlg and promotes its proteasomal-mediated degradation, whereas E4ORF1 and Tax are thought to interfere with the binding of hDlg to APC via competition of the same PDZ domain of hDlg. Another report showed that Tax inactivates hDlg by inducing its hyper-phosphorylation and disrupting its subcellular localization (118). The involvement of the PBM of Tax in ameliorating cell cycle arrest is supported by the observation that the over-expression of hDlg in mouse fibroblasts results
in cell cycle arrest at G0/G1, which can be abrogated by the expression of Tax (129, 130, 303). Rat fibroblasts expressing a chimeric Tax-2 encoding the last 53 amino acids of Tax-1 (Tax221), which contains the PBM, demonstrated increased transforming potential (75). Furthermore, it was demonstrated that deletion of the PBM from Tax-1 abrogated hDGl binding, resulting in reduced micronuclei/DNA damage, and repression of transformation activity in rat fibroblasts and an IL-2-dependent mouse cell line (118, 130, 335). The contribution of the Tax-1 PBM to HTLV-induced proliferation and immortalization of primary T-cells in vitro and viral survival in an infectious rabbit animal model recently was investigated (335). Using both virus gene PBM knockout and knockin approaches, the Tax-1 PBM was found to significantly increase both HTLV-1- and HTLV-2-induced primary T-cell proliferation. Viral infection and persistence were severely attenuated in rabbits inoculated with an HTLV-1 provirus containing a deletion in the four amino acid PBM motif. Together, these studies support the conclusion that the PBM of Tax-1 and its interacting partners, the cellular PDZ domain containing proteins (e.g. hDGl), are important in cellular transformation.

1.11.6 HTLV Tax Promotes Aneuploidy and Genetic Instability

Chromosomal abnormalities (aneuploidy and/or polyploidy) and structurally damaged DNA are common features of cancer cells. Nearly 70% of all human cancers demonstrate aneuploidy and genetic instability, including both HTLV-1-infected and ATL cells (199, 211). Although investigators have proposed aneuploidy to be a cause of cellular transformation (267), it still remains unclear if it is in fact a cause or a
consequence. Aneuploidy can arise from multipolar mitosis, which typically occurs when centrosome replication mistakenly generates greater than two spindle poles in a single cell. This defect of centrosome replication is observed commonly in multiple human cancers including breast, lung, colon, and prostrate. Given the strong association between centrosome abnormalities and transformation, it is not surprising that human cancer viruses (HPV, HBV, and EBV) would use their oncoproteins to corrupt centrosomal replication (72, 81, 188). Several studies have shown that HTLV-1 Tax induces multipolar mitosis by targeting and disrupting the function of two key cellular proteins, TAX1BP2 and RANBP1. By interacting with TAX1BP2, which normally functions in blocking centriole replication, Tax increases abnormal duplication of centrioles (47). Furthermore, Tax interacts with RANBP1 during mitosis, which leads to fragmented spindle poles and enhanced multipolar segregation (257). Together, these Tax-mediated mechanisms clarify and support the observations of aneuploidy and multipolar spindles in HTLV-1-induced leukemic cells.

The high incidence of aneuploidy in ATL cells combined with the very low occurrence of genetic defects/mutations in mitotic checkpoint genes (155) has led researchers to search for the viral mechanism that can destabilize and sabotage the function of the mitotic spindle assembly checkpoint (MSC), which guards against chromosomal instability (231). The MSC ensures that the correct number of chromosomes align properly before the transition to anaphase. Studies have reported that perturbation of the MSC correlates with the development of aneuploidy (244). The MSC is regulated by multiple proteins including the family of mitotic arrest defective proteins (MAD)-1, -2, and -3, the budding uninhibited by benzimidazole (BUB) -1, -2, -3, and
monopolar spindle 1 (MPS1). The first hint that HTLV-1 is involved in MSC regulation came with the discovery that Tax binds and inactivates MAD1 (132, 140). Tax expressing cells displayed aberrant cytokinesis resulting in multinuclei formation. MAD1 is required to deliver MAD2 to the kinetochores, where these two MSC proteins function as a heterodimer to regulate proper microtubule attachment and correct chromosomal segregation during mitosis (231). Later studies revealed that in the presence of Tax, both MAD1 and MAD2 were improperly localized in the cytoplasm as opposed to the nucleus. Thus, the sequestering of MAD1 by Tax inhibits proper localization of MAD2 in the nucleus, which results in defective MSC and is consistent with the development of aneuploidy and ATL progression. Tax also can support the unscheduled degradation of securin and cyclin B1 by binding to and activating the anaphase promoting complex (APC) (197). Through this interaction, Tax is thought to induce early mitotic exit, which contributes to abnormal chromosome segregation and subsequent aneuploidy.

1.11.7 HTLV Tax Modulates DNA Damage Repair Pathways

Many cancer cells contain a large number of chromosomal abnormalities including deletions, translocations, rearrangements, duplications, and aneuploidy (38). Structurally damaged DNA frequently is found in both HTLV transformed T-lymphocytes isolated directly from patients and those immortalized in culture (210). To date, there is no evidence that directly links a specific karyotypic chromosomal damage/abnormality to the development of ATL. As discussed above, Tax has the ability to manipulate multiple cellular proteins, signaling pathways, and critical check points.
Although these activities of Tax can have catastrophic effects on chromosomal integrity (316), there is no evidence that Tax directly induces DNA damage. Instead, the current understanding is that Tax inhibits the ability of the cell to repair DNA damage introduced from exogenous sources (203, 222). DNA mistakes normally are identified and corrected by multiple cellular checkpoints and mechanisms. Tax has been shown not only to abrogate DNA damage-induced checkpoints that normally monitor chromosomal integrity, but also to repress the expression of several overlapping DNA repair pathways including the expression of DNA β-polymerase, which is used for nucleotide excision repair (NER), base excision repair (BER) (138, 260), mismatch repair (MMR), and recombination repair (226). It is not hard to imagine that the suppression of these pathways by Tax would destroy the integrity of the cell and create an environment that would allow the incidence of DNA damage to increase. The first DNA repair pathway shown to be independently suppressed by Tax was NER (152). The NER pathway is used by the cell to repair some of the most commonly found forms of DNA damage such as UV-induced cyclobutane pyrimidine dimers, photoproducts, and intrastrand crosslinks. It has been demonstrated that defects in the NER pathway function are associated with predisposition to develop numerous cancers including xeroderma pigmentosum and Cockayne’s syndrome (23). The NER pathway functions in two ways. The first is a more precise transcription-coupled NER, where NER components identify stalled RNA polymerase complexes that are blocked by DNA damage. The second is a global genomic NER, which examines the entire genome for helix distortions that are caused by DNA damage. To date, there is no direct evidence to support a role for the involvement of Tax in transcription-coupled NER; however, a Tax-mediated mechanism does affect global
NER (150). Upon DNA damage, expression of the p21\textsubscript{Waf/Cip1} cyclin-dependent kinase inhibitor is induced, which results in suppression of PCNA-dependent replication but not PCNA-dependent repair. Reports have shown that excess PCNA expression can overcome the block in replication and enable the progression of the DNA polymerase to proceed through the DNA template lesion before the cell has time to correct the error. Tax has been shown to activate the PCNA promoter, thus increasing the amount of PCNA available in the cell (151, 268). This mechanism explains how Tax expression can abrogate normal NER function and account for the accumulation of errors and genomic abnormalities frequently seen in ATL cells.

Soon after the discovery that Tax inhibits NER, Philpott and Buehring demonstrated that Tax also suppresses BER. Tax initially was shown to trans-repress the promoter of DNA $\beta$-polymerase, the enzyme required for single-nucleotide gap filling reactions and necessary for proper function in both the BER and MMR mechanisms (138). BER is responsible for the removal of several types of DNA lesions including spontaneous hydrolytic depurination of DNA, deamination of cytosine and 5-methylcytosine, products of reactions resulting in hydroxyl-free radical formation, and covalent DNA adducts (331). BER, like NER, can be divided into two separate pathways. First, the short-patch repair pathway (major pathway) is needed to remove individual damaged nucleotides. The second pathway is the long-patch repair pathway (minor pathway), which resolves DNA segments ranging from two to ten nucleotides in length. Through repression of DNA $\beta$-polymerase, Tax plays a role in the deregulation of the short-patch repair pathway of BER. It is unclear whether Tax affects the long-patch pathway. This may be due to the fact that unlike the short-patch pathway, the long-patch
pathway is mediated by a combination of enzymes including DNA β/δ/ε-polymerases together with PCNA. Although recent studies have proposed that p53 plays an indispensable role in BER through direct interactions with AP endonuclease and DNA β-polymerase, very little is known about the intricacies of Tax-mediated repression of BER (348). Still, we must entertain the possibility that Tax may affect BER at other stages of the repair pathway such as glycosylase-mediated recognition of template lesions, strand excision by the exonuclease or transactivation of other BER components.

To date, very few have studied the precise role that Tax plays in the dysregulation of the MMR pathway. However, similar to the other DNA repair pathways, inactivation of the DNA MMR results in increased accumulation of spontaneous mutations, leading to microsatellite instability. Furthermore, one study reported that 11 ATL patients showed a decrease or loss in expression of multiple MMR genes (226). This observation of microsatellite instability in primary ATL cells along with the suppression of DNA β-polymerase by Tax strongly suggests the possibility that Tax may disrupt MMR function.

Chromosome end-to-end fusion and shortened telomeres are observed commonly in many cancers and are present in ATL cells (22). Studies show that Tax plays a pivotal role in abrogating not only double strand break (DSB) repair by the suppression of Ku80, but also deregulates the expression of human telomerase reverse transcriptase (hTert) (88). DSBs induce chromosomal abnormalities including chromosomal breakage and translocations. These breaks are repaired by non-homologous end joining, which is a process that requires several cellular components. The first component is a DNA-dependent protein kinase complex (DNA-PK), which is composed of three subunits, Ku70, Ku80, and the DNA-PK catalytic subunit (203). Gene array studies showed a
correlation between the expression of Tax and significantly reduced levels of Ku80 mRNA, which is speculated to reduce the capacity of the cell to repair new DSBs (240). Studies also showed that early in the cellular transformation process, Tax suppresses the expression of hTert by inhibiting transcription from the hTert promoter (80). hTert is an enzyme that extends the ends of chromosomes with specific nucleotide repeats to form functional units called telomeres. The telomeres protect the DNA from end-to-end fusions that form dicentric chromosomes, which are prone to breaking and degradation by exonucleases. Although it is clear that Tax is involved in abrogating DNA repair, continued research on the effects of HTLV within this mechanism will provide insight on the interplay between defective DNA repair and cellular transformation (211).

1.11.8 Down-Regulation of Tax Provides Selective Advantage to ATL Cells

Interestingly, during the course of HTLV-1 infection, the levels of Tax within infected cells fluctuate. These documented changes have raised questions among researchers for years as to what advantages the virus would gain from silencing Tax expression. As discussed above, Tax is required for the virus to transform cells, however, studies demonstrate that Tax transcripts are detectable in only ~ 40% of all ATL cells (285). A current hypothesis as to why ATL cells would want to silence Tax, even though its expression is absolutely necessary to induce cellular transformation and leukemogenesis, is that Tax is required early during infection to initiate transformation, but at some point, likely the result of other genetic events, Tax becomes dispensable. One reason for silencing Tax is that it is the main target of the cytotoxic T-lymphocyte (CTL)
response. Therefore, ATL cells that down-regulate Tax expression would have an advantage in evading host immune-surveillance (216). HTLV-1 carriers with a high proviral load are more prone to develop ATL if the anti-Tax immune response is weak. This HTLV-1 model where Tax is not required for maintenance differs from other oncogenic virus systems, such as HPV, in which continuous viral oncoprotein expression is necessary to sustain the viral-induced cancer (73). Research into HTLV-1 biology and analysis of Tax transcript expression has revealed three mechanisms for the silencing of Tax expression. First, over time, Tax has been shown to accumulate nonsense mutations, insertions, and deletions that abrogate its expression/function (86, 306). Second, regions of the provirus are methylated, which results in transcriptional repression (170). Third, portions or all of the 5’LTR are deleted thereby eliminating or severely disrupting viral transcription (308). Genetic alterations within Tax are seen in roughly 10% of ATL cells, whereas DNA methylation is found in 15%, and 5’LTR deletions are present in 27% of the ATL cells analyzed. As mentioned in a previous section, we discussed the role of the viral HBZ protein in down-modulation of Tax-mediated viral gene expression that likely would contribute to infected cell survival and ultimately, development of leukemia (Figure 1.2).

1.12 HTLV ORF-III Rex-1 and Rex-2

HTLV Rex is a trans-acting regulator of viral replication. Rex is a nuclear-localizing and shuttling phosphoprotein that acts post-transcriptionally by binding and selectively exporting the unspliced and incompletely spliced viral mRNAs from the
nucleus to the cytoplasm, thus controlling the expression of the viral gene products that are essential for viral progeny production (114, 127, 179). The 189 a.a Rex-1 and 170 a.a Rex-2 proteins encoded by ORF-III of the pX region share 60% homology at the amino acid level. When analyzed by SDS-PAGE, Rex-1 has an apparent size of 27kDa, and Rex-2 is detected as two major bands of 24 and 26 kDa (100). The two isoforms of Rex-2, p24\textsuperscript{Rex} and p26\textsuperscript{Rex}, have the same amino backbone but differ by post-translational modifications; specifically, serine phosphorylations resulting in a charge-induced conformational change (100, 234, 235). Both HTLV-1 and HTLV-2 produce truncated forms of Rex from alternatively spliced mRNAs. These proteins, named p21\textsuperscript{Rex-1} and p22/20\textsuperscript{Rex-2}, lack the N-terminal sequences of Rex that are responsible for nuclear localization and RNA binding, and that interfere with Rex localization and function (50, 113, 177).

Mutational analysis has defined several functional domains within Rex. Both Rex-1 and Rex-2 have an RNA binding domain (RBD), nuclear localization signal (NLS), and a central core activation domain (AD) encompassing a nuclear export signal (NES). The AD is flanked by two multimerization domains (MD). The AD/NES interacts with chromosome region maintenance interacting protein 1 (CRM1)/Exportin 1 (3, 32, 82, 83, 325, 326). The RBD binds specifically to a complex RNA structure termed the Rex response element (RxRE) found in viral mRNAs (345). Recently, a unique C-terminal domain has been identified for Rex-2 that is a target for serine phosphorylation and has been found to be important in nucleo-cytoplasmic protein shuttling (235). These RNA-protein-protein interactions are all critical for proper Rex function. Data in Chapter
2 of this thesis will focus on characterizing and understanding the role that this newly identified domain plays in Rex-2 function.

Studies using transiently transfected or stably infected cells have demonstrated that HTLV-1 Rex increases the amount of unspliced viral RNA by reducing the rates of splicing and degradation of unspliced RNA in the nucleus (104). Rex also facilitates the nuclear-cytoplasmic transport of the unspliced and incompletely spliced viral mRNAs resulting in increased structural and enzymatic protein production. The accumulation of viral structural proteins is dependent on Rex, yet Rex itself is generated from completely spliced mRNA. The virus has a biphasic life cycle: an early Rex-independent phase and a late Rex-dependent phase. Early during infection, most viral mRNAs are doubly spliced, which is a default splicing mechanism by the host cellular machinery, which increases the pool of Tax and Rex proteins. Accumulation of sufficient levels of Rex results in the expression of incompletely spliced mRNA in the cytoplasm, thus leading to the production of structural and enzymatic gene products and assembly of infectious virus particles. Therefore, Rex may be the switch that determines whether the virus exists in a latent or productive state.

The current model for Rex function involves multiple steps and interactions with both cellular and viral proteins and mRNA. First, a pool of Rex accumulates in the cytoplasm and shuttles into the nucleus via its NLS. Rex then binds to the RxRE present in the viral mRNA, resulting in protection of this mRNA from splicing/degradation. Rex then multimerizes and forms a RNA/Rex/CRM1/Ran-GTP complex. This complex interacts with the nuclear pore complex present at the inner surface of the nuclear membrane and then is actively exported into the cytoplasm. Finally, hydrolysis of Ran-
GTP to Ran-GDP results in the dissociation of the complex and subsequent release of the viral mRNA cargo, which allows Rex to return to the nucleus to start another cycle (Figure 1.3) (163, 345).

The RxRE of HTLV-1 is a 205 nucleotide RNA stem loop structure that is found in the 3’ LTR of viral mRNAs (32, 98, 163, 315). Mutational analysis revealed that the actual Rex binding domains within the RxRE are relatively short. Only a stretch of ~ 43 nucleotides constitute the high affinity Rex-1 binding motif and is sufficient to mediate Rex function (32, 33). It has been shown that the ability of the RxRE to fold into the stem loop structure brings the polyadenylation signal (AAUAAA) into close spatial proximity to the GU rich polyadenylation site, giving the full RxRE a novel role in ensuring efficient polyadenylation of the viral mRNA (3, 19). Substitutions and mutational analysis have demonstrated that proper folding of the RxRE is required to provide a docking site for Rex, and hence, is essential for its function in vivo (338).

Cellular proteins also have been shown to bind the viral RxRE. Reports demonstrated that the heterogeneous ribonucleoprotein particle A1 (hnRNP A1), which competes with Rex for binding to the RxRE, results in impairment of Rex function in cells over-expressing hnRNPA1 (71, 109). Another study showed that Rex interacts with translational initiation factor 5A (eIF-5A) (158, 159). It is hypothesized that this interaction may facilitate translational efficiency of the Rex-bound mRNA, which would be consistent with HIV-1 Rev data (13, 60, 179).

To date, numerous studies have investigated the role phosphorylation plays in regulating the function of both Rex-1 and Rex-2 (1, 2, 102, 103). This thesis focuses on elucidating the unique phosphorylation sites within Rex and determining their functional
significance both *in vitro* and *in vivo*. Rex has been shown to be essential for HTLV gene expression; a full understanding of its regulation remains a critical objective in HTLV research and could yield insight into developing new therapeutic interventions.
Figure 1.1 Organization of the HTLV-1 and HTLV-2 genomes. A detailed representation of the HTLV-1 and HTLV-2 proviral genome in kilobases showing the long terminal repeats (LTR), mRNAs, and open reading frames (ORFs) is shown. ORFs are indicated by boxes: structural and enzymatic proteins (black); regulatory protein ORFs (grey), accessory protein ORFs (white); HBZ antisense ORF (shaded).
Figure 1.2 Known activities of HBZ. Current data suggest that Hbz mRNA increases expression of the transcription factor E2F1 thereby activating E2F1-responsive genes leading to increased cellular proliferation. Data also show that HBZ protein down-regulates Tax-mediated HTLV-1 (5’LTR promoter) transcription by binding to CREB-2. HBZ protein also interacts with JUNB and c-Jun, components of Activator Protein-1 (AP-1), and disrupts their DNA binding activity. In addition to AP-1, HBZ protein binds to JunD and activates JunD-responsive cellular genes, which are involved in growth, proliferation, and apoptosis.
Figure 1.3 Life cycle of HTLV Rex protein. 

**Early phase:** Initial viral transcription results in mRNAs that are completely spliced resulting in the production of *tax/rex* mRNA. 

**Late phase:** Pools of Rex protein accumulate in the cytoplasm and shuttle into
the nucleus via its NLS. Rex then binds to the RxRE present in the viral mRNA, resulting in protection from splicing/degradation. Rex then multimerizes and forms a RNA/Rex/CRM1/Ran-GTP complex. This complex is actively exported into the cytoplasm. Finally, hydrolysis of Ran-GTP to Ran-GDP results in the dissociation of the complex and subsequent release of the viral mRNA cargo, which facilitates translation of the viral mRNAs and allows Rex to return to the nucleus to start another cycle.
CHAPTER 2

HUMAN T-CELL LEUKEMIA VIRUS TYPE-2 REX CARBOXY TERMINAL IS
AN INHIBITORY/STABILITY DOMAIN THAT REGULATES REX
FUNCTIONAL ACTIVITY AND VIRAL REPLICATION

2.1 Abstract

Human T-cell leukemia virus (HTLV) regulatory protein, Rex, functions to increase
the expression of the viral structural and enzymatic gene products. The phosphorylation
of two serine residues (S151 and S153) at the c-terminus is important for the function of
HTLV-2 Rex (Rex-2). The Rex-2 phosphomimetic double mutant S151D, S153D is
locked in a functionally active conformation. Since rex and tax genes overlap, Rex
S151D and S153D mutants were found to alter the Tax oncoprotein coding sequence and
transactivation activities. Therefore, additional Rex-2 mutants including P152D, A157D,
S151Term, and S158Term were generated and characterized. All Rex-2 mutants and
wtRex-2 localized predominantly to the nucleus/nucleolus, but in contrast to the detection
of phosphorylated and unphosphorylated forms of wtRex-2 (p26 and p24), mutant
proteins were detected as a single phosphoprotein species. We found that P152D, A157D,
and S158Term are more functionally active than wtRex-2 and that the Rex-2 c-terminus and its specific phosphorylation state are required for stability and optimal expression. In the context of the provirus, the more active Rex mutants (A157D or S158Term) promoted increased viral protein production, increased viral infectious spread, and enhanced HTLV-2-mediated cellular proliferation. Moreover, these Rex mutant viruses replicated and persisted in inoculated rabbits despite higher antiviral antibody responses. Thus, we identified in Rex-2 a novel c-terminal inhibitory domain that regulates functional activity and is positively regulated through phosphorylation. The ability of this domain to modulate viral replication likely plays a key role in infectious spread and viral induced proliferation.

2.2 Introduction

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2) are related complex oncogenic retroviruses that transform primary human T-cells in culture and are associated with leukemia and neurological disorders in humans (341). In addition to the typical retrovirus structural and enzymatic genes \( \text{gag, pol, and env} \), HTLV encodes two \textit{trans}-regulatory gene products, Tax and Rex, in partially overlapping open reading frames, as well as accessory gene products (182). The viral oncoprotein Tax increases the rate of transcription from the viral long terminal repeat (LTR) (40, 76, 128). In addition, Tax can modulate the expression or activities of numerous cellular proteins involved in cell proliferation and differentiation, cell cycle regulation, and DNA repair processes (35,
The pleiotropic effects of Tax on cellular processes are required for its transforming and oncogenic capabilities, and play a critical role in HTLV-induced cellular transformation (90, 274, 280, 281).

Rex is a key regulator of viral replication. At the molecular level, the basic role of Rex is to regulate cytoplasmic levels of viral genomic unspliced mRNA \((gag/pol)\) and singly-spliced \((env)\) mRNA, thus controlling the expression of the structural and enzymatic gene products that are essential for production of viral progeny (114, 179). Rex functions by binding viral mRNAs via a \textit{cis}-acting RNA Rex-response element (RxRE) and facilitating the export of these mRNA species from the nucleus to the cytoplasm (17, 27, 32). Previous studies revealed that HTLV-1 Rex (Rex-1), HTLV-2 Rex (Rex-2) and their RxREs are structurally similar and functionally interchangeable (163, 339). Mutational analyses of Rex-1 and Rex-2 have defined several domains critical for their functional properties. These include the arginine-rich N-terminal sequences that serve both as an RNA binding domain and as a nuclear localization signal, the central leucine-rich activation domain encompassing the nuclear export signal (NES), and the multimerization domain composed of two regions flanking the NES (31, 32, 110, 123, 235, 254, 271, 296, 325, 326).

Both Rex-1 and Rex-2 are phosphoproteins, and phosphorylation has been shown to be critical for their function (1, 2, 103). In HTLV-2-infected cells, as well as in cell lines transfected with Rex-2 expression plasmids, two major species of Rex-2 (p24 and p26) have been detected. Both Rex-2 species have the same amino acid backbone and differ by a conformational change that is induced by serine phosphorylation (102, 182,
This is unique to Rex-2 as Rex-1 presents as a single 27 kDa protein. Rex-2 p24 is found primarily in the cytoplasm, whereas the p26 phosphorylated form localizes predominantly to the nucleus and nucleolus (50, 340). In addition, phosphorylation of Rex-2 correlates with its binding to RxRE-containing RNA and inhibition of mRNA splicing (16, 103). A mutational analysis of Rex-2 that targeted all serines and threonines revealed a novel c-terminal functional domain containing two critical phosphorylated residues at serine 151 and 153 (234, 235). Rex-2 mutants containing alanine substitutions at either of these two serines (S151A, S153A) displayed reduced phosphorylation, impaired RNA binding capacity, diffused cytoplasmic localization, and decreased functional activity. In contrast, replacement of both serine residues with phosphomimetic aspartic acids (S151D, S153D) resulted in detection of only the p26 species in cells, enhanced RNA binding capacity of Rex-2, and an intense speckled nucleolar localization (234, 235). Interestingly, this Rex-2 mutant was locked in a phosphorylated active conformation since it could not be altered by phosphatase treatment in vitro. These results suggest an important role of the Rex-2 c-terminus in its functional regulation.

We have proposed that the regulation of Rex-2 through phosphorylation provides a critical control in HTLV-2 replication cycle at the cellular level, which would allow the virus to better adjust to environmental stimuli (234, 345). Mutant Rex (S151D, S153D) is locked in an active form potentially removing at least one of its key regulatory controls, thus providing a unique reagent with which to evaluate the role Rex-2 in regulating viral replication and cellular transformation in vitro and viral persistence in vivo. One caveat is that the mutations (S151D, S153D) in Rex-2 affected the amino acid sequence and
disrupted the transactivation activities of the viral oncoprotein Tax. Therefore, to facilitate our studies several new Rex-2 mutants were generated that do not significantly affect Tax function. These mutants include two phosphomimetic or charged mutants, P152D and A157D, and two deletion mutants, S151Term and S158Term. We found that the introduction of aspartic acid into the c-terminus or deletion of the c-terminal sequences downstream of serine 158 resulted in a highly functional Rex protein; a phenotype consistent with the disruption or removal of a carboxy terminal inhibitory domain. Our data indicate that the c-terminus is indispensable for Rex-2 protein stability, whereas the phosphorylation status of the c-terminus dictates the function of the Rex-2 protein, but does not affect protein stability. In the context of full length infectious virus, the more functionally active Rex-2 mutants, A157D and S158Term, showed increased viral gene expression in infected primary T-cells, enhanced viral infectivity, and promoted HTLV-2-mediated cellular proliferation of primary T lymphocytes. Lastly, HTLV-2 mutant viruses containing the Rex-2 mutations at either A157D or S158Term successfully replicated and persisted in inoculated rabbits and resulted in stronger antibody responses to viral antigens compared to wild type HTLV-2. Thus, we identified a novel c-terminal inhibitory domain in Rex-2 that regulates functional activity and this domain itself is positively regulated through phosphorylation or charge-induced conformation alterations. The ability of this domain to modulate viral replication likely plays a key role in HTLV infectious spread and virus-mediated cellular proliferation and cell survival.
2.3 Materials and Methods

**Cells**

293T and Hela cells were maintained in Dulbecco's modified Eagle's medium and 729 human B-cell and Jurkat T-cell lines were maintained in Iscove's medium and RPMI 1640 medium. Medium was supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Human peripheral blood mononuclear cells (PBMCs) were isolated and cultured as described previously (335).

**Plasmids**

The Rex-2 expression vector BC20.2, containing the HTLV-2 tax/rex cDNA expressed from the cytomegalovirus (CMV) immediate-early gene promoter and the Rex-1 expression vector SE356, containing the HTLV-1 tax/rex cDNA expressed from the cytomegalovirus (CMV) immediate-early gene promoter, have been described previously (102, 339). The rex mutations were generated in either BC20.2 (Rex-2) or SE356 (Rex-1) using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Various Rex-2 mutants were transferred to the HTLV-2 proviral clone pH6neo (44). Mutations were confirmed by DNA sequencing. The human immunodeficiency virus type 1 (HIV-1) Tat expression vector, pcTat and the Rex-2 reporter plasmid (pCgagRxRE-II) were described previously (234). The LTR-2-luciferase Tax reporter plasmid, κB-Luc Tax reporter plasmid, CMV-luciferase (firefly) plasmid and Thymidine kinase-Renilla
luciferase plasmid were described previously (339). Wild type and mutant Rex-2-GFP constructs were generated by inserting Rex-2 sequences into the EGFP-N3 vector (Promega, Madison, WI) upstream of the GFP open reading frame. FLAG-tagged Rex-2 constructs were generated by insertion of the FLAG-tag sequence into vector BC20.2 upstream of the Rex-2 open reading frame using primers Sph1-flag (sense)
5’-GCATGCTCGATTACAAGGATGATGATGATAAGGGCGGCATGC-3’
and Sph1-flag (antisense)
5’-GCATGCCGCCCTTATCATCATCATCATCCTTGTAATCGAGCATGC-3’.

**Tax and Rex functional reporter assays**

The ability of Tax to activate CREB/ATF (viral LTR) or NF-κB was determined by dual luciferase assay as described (335). The Rex functional assay was performed as described previously with slight modification (234). Briefly, Rex cDNA expression plasmids were cotransfected into 293T cells with 0.05µg of CMV-luc, 0.25 µg of pcTat and 0.5 µg of Rex reporter plasmid pCgag-RxRE. Cell lysates were prepared at 48 h post transfection and luciferase activity was determined to control for transfection efficiency. HIV-1 p24 Gag level in the cell lysates was determined by ELISA (Beckman-Coulter, Fullerton, CA). All transfection experiments were performed in triplicate in three independent experiments.
p19 Gag ELISA and isolation of HTLV-2 stable producer cell lines

Virion production of HTLV proviral clones from transiently transfected 293T cells was measured by a commercially available p19 matrix antigen ELISA (ZeptoMetrix, Buffalo, NY). To generate stable transfectants, proviral plasmid clones containing the Neo gene were introduced into 729 B-cells by electroporation as described previously (101). Stable transfectants containing the desired proviral clones were isolated and characterized as previously described (333).

DNA preparation and PCR

Genomic DNA was isolated from permanently transfected cell clones or from immortalized PBMCs using the PUREGENE® DNA purification system (Gentra, Minneapolis, Minn.). Genomic DNA (1µg) was subjected to 30-cycle PCR. The forward primer 670 (179) and the reverse primer PG201 (5’-GCTGGTATAGGTATAGGCTA-3’) were used to amplify a specific 437-bp fragment from the HTLV-2 tax/rex region. The PCR-amplified product was separated on agarose gels and visualized by ethidium bromide staining. Mutations were confirmed by DNA sequencing. For infected rabbit PBMCs, 1µg DNA was subjected to 40-cycle PCR using primers 670 and 671 (179) to amplify a 159-bp fragment specific for the HTLV-1/2 tax/rex region. In addition, 40 cycles of real-time Taqman PCR were conducted to quantitate proviral copy number per cell as described (10). Rabbit PBMC DNA was subjected to PCR in duplicate using the HTLV-specific primer pair AAM.001 (5’-CGGATACCCAGTCTACGTGTTT-3’) and AAM.002 (5’-CTGAGCCGATAACCGTCTCCTTA-3’) and probe
(5’-FAM-ATCACCTGGGACCCCATCGATGGA-TAMARA-3’), and final values were averaged. The 25µl reactions contained 500ng DNA, 100ng (25ng/ml) of each primer and probe concentration of 100pmol/µl. Copy number was determined based on a standard curve generated from duplicate samples of dilutions of a plasmid containing the tax gene sequences. The copy number per cell value for a sample was generated based on the estimation that 1µg PBMC DNA is equivalent to 134,600 cells.

**Western blot, antibodies and pulse-chase immunoprecipitation assay**

Cells were lysed with modified RIPA buffer (0.05M Tris-Cl [pH 8.0], 0.15M NaCl, 1% Nonidet P-40, 0.5% desoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 20 µg/ml of aprotinin, 1mM Na₃VO₄ and 1mM NaF) on ice for 30 min. After centrifugation, the cell lysates were subjected to 12% SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell Biosciences, Keene, NH). Western blots were performed as recommended by the manufacturer. Proteins were visualized using the enhanced chemiluminescence (ECL) Western blot analysis system (Santa Cruz Biotechnology, Santa Cruz, CA). Rex-1, Rex-2 and Tax-2 were detected using protein-specific rabbit polyclonal antisera. α-EGFP antibody, α-FLAG M2 monoclonal antibody, α-actin monoclonal antibody, goat-α-rabbit IgG antibody, and goat-α-mouse IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

The half-life of Rex was determined by pulse-chase experiments. Briefly, 10⁶ 293 T cells were transfected with 10 µg of wild-type rex or various rex mutant expression
plasmids or negative control using lipofectamine®PLUS reagent (Invitrogen, Carlsbad, CA). Cells were metabolically labeled with [35S]methionine-cysteine (Trans-35S-label, 100 mCi/ml; Amersham) in methionine-cysteine-free RPMI 1640 supplemented with 20% dialyzed fetal calf serum for 6 h. Cells were chased with cold medium for the indicated time points, then harvested and lysed in RIPA buffer on ice for 30 min. Lysates were clarified by centrifugation at 17,000 × g for 30 min at 4°C. Subsequently, equal amounts of cell lysates from the different time points were immunoprecipitated using rabbit α-Rex antisera for 16 h at 4°C. The immune complexes were collected using Protein A-Sepharose CL-4B (Sigma) and subjected to 12% SDS-PAGE; 35S-labeled proteins were visualized and quantified by Typhoon analysis (Molecular Dynamics).

**Short-term coculture microtiter proliferation and long-term immortalization assays**

Short-term microtiter proliferation assays were performed as described previously with some modifications (259). Briefly, freshly isolated human PBMCs were prestimulated with 2µg/ml phytohemagglutinin (PHA) and 10U/ml IL-2 (Roche Diagnostic Corporation, Indianapolis, IN) for two days. One hundred 729 HTLV producer cells were irradiated (10,000 rads) and cocultured with 10⁴ prestimulated PBMCs in the presence of IL-2 in round bottom 96-well plates. Wells were enumerated for growth and split 1:4 at weekly intervals. At week seven, cell proliferation was confirmed by 3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2-H-tetrazolium, inner salts (MTS) assays using CellTiter 96® Aqueous One
Solution Reagent as recommended by the manufacturer (Promega, Madison, WI). The long-term immortalization assays were performed as described previously (335).

**Detection of HTLV-2–infected T-cells by flow cytometry analysis**

Irradiated 729 HTLV producer cells (2 x 10^5) were cocultured with 10^6 PBMCs in the presence of IL-2. Two days after plating, cells were washed with PAB (PBS, 0.1% NaN3, 1% BSA) and stained with phycoerythrin-Cy5–conjugated mouse α–human CD3 antibody. The samples were washed, fixed, and permeabilized with Fix&Perm reagents (Serotec, Raleigh, NC). For detection of intracellular viral protein, cells first were incubated with HTLV-2 Gag p19 detector antibody (Zeptometrix), washed, incubated with fluorescein isothiocyanate (FITC)–conjugated secondary antibody goat anti–mouse IgG2b, and analyzed for CD3/p19 double-positive cells using a BD Biosciences (San Jose, CA) fluorescence-activated cell scanner (FACScan).

**Rabbit inoculation procedures**

Twelve-week-old specific pathogen-free New Zealand White rabbits (Hazelton, Kalamazoo, MI) were inoculated via the lateral ear vein with approximately 1 x 10^7 gamma-irradiated (7500 rad) 729wtHTLV-2, 729HTLV-2/RexA157D, 729HTLV-2/RexS158Term or 729 uninfected control cells (six rabbits per group). Cell inocula were equilibrated based on their p19 Gag production per cell (a surrogate for virion production) prior to inoculation. At weeks 0, 1, 2, 4, 6, and 8 after inoculation, 10 mL of
blood was drawn from the central ear artery of each animal. Serum reactivity to specific viral antigenic determinants was detected using a commercial ELISA (BioMerieux, Inc., Durham, N.C.) (1:100 dilution) and HTLV Western blot assay (ZeptoMetrix) (1:200 dilution) adapted for rabbit plasma by use of avidin-conjugated goat anti-rabbit IgG (1:2000 dilution) (Sigma, St Louis, MO) (294). Serum showing reactivity to Gag [p24 capsid (CA) or p19 matrix (MA)] and Env [gp21 transmembrane (TM) or gp46 surface unit (SU)] antigens was classified as HTLV-2 sero-positive. Estimated proviral loads were determined and quantified using genomic DNA from PBMC samples by PCR and real-time Taqman PCR as described above.

2.4 Results

Generation of Rex-2 mutants

Rex-2 is detected in virus infected cells as two distinct protein species or conformations as detected by Western blot (p26 and p24). Both Rex-2 species have the same amino acid backbone and differ by serine phosphorylation (p26 is nuclear and the active species). In particular, serines 151 and 153 were identified as two phosphorylation sites that play a critical role in the functional regulation of Rex-2 (103, 234). Replacement of these two serines with alanines not only disrupted Rex-2 function, but shifted the Rex-2 steady state concentration to the p24 Rex species: the p26/24 steady state ratio for wild-type Rex is approximately 1, whereas S151,153A mutant the ratio was approximately 0.5. Moreover, phosphomimetic aspartic acids (S151D, S153D) locked
Rex-2 in a phosphorylated and active p26 conformation (234, 235). The primary objectives of this study were to understand the functional role of the carboxy terminal Rex-2 phosphorylation domain and to address whether the ability to regulate Rex function was important for HTLV-2-mediated cellular proliferation and immortalization in vitro and survival in a rabbit model of infection. Numerous studies have demonstrated the essential role of the viral oncoprotein Tax in HTLV-mediated cellular proliferation and transformation. Since the Tax and Rex proteins are encoded by separate, but partially overlapping reading frames, mutations in rex could alter the amino acid sequence of Tax and disrupt critical transcriptional activities required for viral replication and cellular transformation. Indeed, our previously described highly active Rex mutants (S151D and S153D) resulted in alteration of the Tax coding sequence and significantly impaired Tax CREB/ATF and NFκB transactivation activities (280) (Table 1).

It has been well documented that phosphorylation events can result in a charge-induced conformational change of a protein, which may alter its stability or function (30, 241, 298, 337). Therefore, we hypothesized that introducing a phosphomimetic amino acid into the Rex-2 c-terminus, but not necessarily at serine 151 or serine 153, might result in the same conformational alteration as reported for Rex mutant S151D, S153D (234). Based on this hypothesis, two Rex-2 mutants were generated in the 170 amino acid Rex-2 polypeptide, P152D and A157D. Mutant A157D did not have any alteration in the Tax amino acid sequence, whereas P152D resulted in a single amino acid change in Tax (A132G). Using LTR-Luc and κB-Luc reporter assays, we showed that the Tax A132G mutation resulted in a 50% reduction in CREB/ATF transactivation activity, but
induction of nearly wild-type levels of NFκB activity (Table 1). A157D did not show altered Tax expression or function. One hypothesis consistent with our previous Rex-2 phosphorylation and functional data was that the c-terminus of Rex-2, when non-phosphorylated, inhibits protein function but can be positively regulated or activated by phosphorylation (234, 235). To test this hypothesis, we generated two Rex c-terminal deletion mutants, S151Term and S158Term, in which the codon for serine 151 or serine 158, respectively, was mutated to a termination codon. These mutations maintained the Tax amino acid sequence and its transactivation function (Table 1).

**Protein expression and functional activity of Rex-2 mutants**

We initially evaluated protein expression of Rex-2 mutants in transfected 293T cells by Western blot using rabbit polyclonal α-Rex antisera. As expected, in the cells transfected with the wtRex-2 expression construct, both p24 and phosphorylated p26 protein species were detected (Fig 2.1A). Consistent with our previous report, expression of Rex S151D or S153D resulted in the detection of only the phosphorylated p26 (234)(Fig. 2.1A). Rex mutants that contained a phosphomimetic or charged residue within this region, P152D and A157D, also exhibited a single phosphorylated p26 species. The two c-terminal Rex deletion mutants, S151Term and S158Term, displayed a single protein form consistent with their predicted gel mobility (Fig 2.1A). However, it was notable that the amount of steady state protein for the two termination mutants was lower than the wtRex-2 or aspartic acid point mutants (Fig 2.1A).
We next tested the Rex-2 mutants for their ability to function in our quantitative reporter bioassay in which HIV-1 p24 Gag production was measured and used as a read-out of Rex functional activity (234). P152D, A157D, and S158Term were significantly more active than wtRex-2 at the lowest Rex-expression plasmid concentration tested. This difference gradually was minimized and disappeared at higher concentrations, which likely was due to a plateau effect (outside the linear range of the assay). These results were consistent with our hypothesis that the Rex-2 c-terminus inhibits its own function and this inhibition can be removed either through deletion of the c-terminal sequence or via introduction of a phosphomimetic amino acid (negative charge) within the c-terminus.

In contrast, the activity of S151Term was approximately 25 to 40 % that of wild type over the range of Rex expression plasmid concentrations tested (0.02 to 0.5 µg), suggesting that the c-terminal sequence from amino acid 151 to amino acid 157, which includes serines 151 and 153, was required for optimal Rex function.

It is also worthy to note that Rex functional activity of S158Term is higher than that of wtRex-2 despite its lower steady-state level of Rex protein, which suggests that the c-terminus inhibits Rex-2 activity. This inhibitory effect of the Rex c-terminus can act either in cis through induction of conformational change, or in trans through competition of cellular factors required for Rex-2 function. To distinguish between these two possibilities we performed a simple experiment in which a GFP-Rex c-terminal fusion construct (GFP-Rex151-170 or GFP-Rex154-170) was cotransfected into 293T cells with either wtRex-2 or Rex S158Term. We did not observe any significant inhibition to Rex-2 function (data not shown), consistent with our hypothesis that the Rex-2 c-terminus negatively regulates Rex-2 through conformational change or spatial effect.
Subcellular localization of Rex-2 carboxy terminus mutants

Previous studies indicated that the phosphorylated form of Rex-2, p26, is found primarily in the nucleus and nucleolus, whereas the p24 inactive form displayed diffuse cytoplasmic and nuclear localization, indicating that the proper subcellular localization of Rex-2 correlated with its function (16, 50, 103). We determined the subcellular localization of the newly generated Rex-2 mutants in transiently transfected Hela-Tat cells using Rex-EGFP fusion proteins. EGFP alone displayed bright, diffuse staining throughout the cytoplasm as well as in the nucleus. In contrast, all of the Rex-EGFP fusion proteins tested, including S151Term-EGFP, exhibited predominant nuclear localization with some weak cytoplasmic staining. This result indicated that the capacity of Rex-2 to localize to the nucleus was not significantly affected by the c-terminal mutations (Fig 2.2A). We confirmed the relatively equal stable protein expression of these Rex-EGFP fusion proteins by Western blot using either α-Rex or α-EGFP specific antisera. Furthermore, their functional activity was comparable to that of their untagged protein forms (compare Fig 2.1B 0.5 μg concentration versus Fig 2.2C). It is important to note that addition of the GFP tag at the carboxy terminus of S151Term and S158Term stabilized these deletion mutants in comparison to their untagged counterparts. We attribute this to the location and possibly the size of the tag since S158Term FLAG-tagged at the amino terminus resulted in a protein stability similar to the untagged protein (see Fig. 2.3C).
**Phosphorylation at Ser151 and/or Ser153 enhances Rex-2 functional activity**

Our results demonstrated that only S151Term, but not the other three c-terminal mutants including P152D, A157D and S158Term, exhibit significantly impaired activity as detected by reporter assay. One important difference between these mutants was that serines 151 and 153 were deleted and not available to be phosphorylated in S151Term. In order to determine whether it was the loss of phosphorylation at serines 151 and 153 that contributed to the impaired functional activity of S151Term, we introduced serine 151 and 153 alanine encoding mutations in combination with P152D, A157D, and S158Term; mutants were termed P152D-2A, S157D-2A, and S158Term-2A, respectively. The functional activity of these Rex mutants was examined by quantitative reporter assay (Fig 2.3A). Consistent with our previous report (234), the functional activity of Rex S151,153A was approximately 30% that of wtRex-2 (Fig 2.3A). Similarly, the functional activity of P152D-2A, A157D-2A, and Rex S158Term-2A was about 70% lower than their parental mutants, suggesting that phosphorylation at Ser 151 and/or 153 was required for a fully functional Rex-2 (Fig 2.3A). We next compared the expression profile and steady state levels of Rex from the parental mutants with the serine 151, 153 alanine mutants. Introduction of the additional S151/153A mutations into Rex P152D, A157D and S158Term did not alter the mobility of the Rex proteins on SDS-PAGE (Fig 2.3B). As noted above, we detected increased Rex protein expression of phosphomimetic point mutants but decreased protein expression of the c-terminal deletion mutants. Interestingly, additional mutation of the phospho-acceptor serine residues 151 and 153 by alanine substitution appeared to significantly decrease the steady state amounts of all Rex.
mutants. To eliminate the possibility that the decreased steady-state levels of protein seen on Western blot was not a reflection of altered epitopes and recognition by the α-Rex-2 antisera, we generated FLAG-tagged Rex constructs and examined Rex protein expression using α-FLAG M2. Consistently, Rex steady state protein level detected in mutants A157D-2A and S158Term-2A was significantly lower than A157D and S158Term, respectively (Fig 2.3C). To determine the half-life of the wild-type and Rex-2 mutant proteins, we performed pulse-chase experiments. As shown in Figure 2.3D, the half-life of the functionally active wild-type p26 was approximately twelve hours and more stable than the inactive p24 species (half-life is approximately nine hours). In mutant S151,153A the half life of both p26 and p24 is approximately twelve hours. Thus, replacement of these two serine residues to alanine alone does not result in an unstable p26 or p24 protein species. Indeed, the slight increase in the S151,153A p24 species is consistent with the overall steady state increase of p24 relative to p26 and that phosphorylation at one or both of these sites on p24 contributes to the conformational shift to p26. For mutants S151Term and S158Term, deletion of the c-terminal sequence not only decreased Rex steady-state level, but also substantially decreased Rex half-life to approximately two hours. Together, these results indicated that in addition to its role in regulation of Rex-2 functional activity, the c-terminus and phosphorylation status of Rex-2 played an important role in protein stability and optimal expression of steady state protein levels.

Establishment and characterization of stable virus producer cell lines
Next, we generated mutant HTLV-2 proviral clones including HTLV-2/RexA157D and HTLV-2/RexS158Term (termed H2A157D and H2S158Term, respectively) to assess how viral replication would be affected by the unique phenotype of these Rex mutants. Both H2A157D and H2S158Term are competent for viral protein production as determined by p19 Gag ELISA in transient transfected 293T cells: consistent with the more functionally active phenotype displayed by these Rex mutants, both show increased levels of p19 production relative to wild-type (data not shown). In order to determine the capacity of H2A157D and H2S158Term proviral clones to replicate and induce cellular immortalization/transformation in primary human T-cells, permanent 729 B-cell transfectants expressing the two mutant viruses were isolated and characterized. For each of the stable transfectants, the expected mutations were confirmed by diagnostic genomic DNA PCR and DNA sequencing (data not shown). To monitor the production of viral proteins in these stable transfectants, the concentration of p19 Gag in the culture supernatant of several cell clones was quantified by ELISA. As shown in Figure 2.4A, the amount of p19 Gag expression from each stable cell clone tested was variable. This likely is attributable to the chromosomal location of integrated proviral sequences and the overall proviral copy number in each cellular clone. All stable cell clones expressing the Rex mutant viruses produced slightly higher p19 Gag as compared to our well-characterized HTLV-2 producer cell line, 729pH6neo. This result is also consistent with proviral clone transfection data and the more functionally active phenotype displayed by these Rex mutants. We confirmed the expression of Rex and Tax by Western blot in the cell lines selected for subsequent coculture assays (Fig 2.4B). We consistently observed slightly lower Tax expression in the two mutant producer cell
lines relative to wildtype, again which correlates with a more functional Rex and redistribution of viral mRNA (greater gag/pol and env mRNA at the expense of tax/rex).

**Functionally more active Rex promoted HTLV-2 infectivity and virus-induced cellular proliferation**

We performed short-term microtiter proliferation assays and long-term immortalization assays to assess the capacity of the Rex-2 mutant viruses on infecting and immortalizing human PBMCs (101, 259, 335). These coculture assays use freshly isolated PBMCs and cell-associated virus transmission designed to mimic the *in vivo* infection. The *in vitro* long-term coculture immortalization assays were carried out initially in 24-well plates in order to monitor the immortalization process and the characteristic expansion of infected PBMCs. After seven weeks of coculture, infected PBMCs were transferred to 25cm² flasks to expand. The growth curve shown in Figure 2.5A indicated that both H2A157D and H2S158Term induce progressive growth patterns consistent with wtHTLV-2 immortalization. Viral protein production from the infected PBMCs was quantified by Gag p19 ELISA. As shown in Figure 2.5B, PBMCs infected with HTLV-2 Rex mutants continuously produced more p19 Gag than those infected with wtHTLV-2, indicating more robust viral replication and virion production due to the more active Rex. As previously reported, the immortalized PBMCs were shown to harbor and express HTLV-2 as determined by immunofluorescence for p19 (335) and the genetic mutations were confirmed by PCR amplification of *rex*-specific sequences from genomic DNA followed by DNA sequencing (data not shown). These results indicated
that although H2A157D and H2S158Term Rex mutant virus infected cells produce more virions, this phenotype did not translate into a significant increase in immortalization of PBMCs. It is important to note that HTLV long-term immortalization of T-lymphocytes is relatively inefficient, involves a selection for growth of a rare number infected cell clones, and is not quantitative.

In an effort to obtain a more quantitative measure of the ability of these viruses to infect and immortalize PBMCs, $10^4$ PBMCs were cultured with 100 virus-producer cells in 96-well plates. At weekly intervals, individual wells were assayed for proliferation as measured microscopically by increased cell number or by MTS assay. In addition, the cells in individual wells were split weekly at a 1:4 ratio. Therefore, slowly growing or non-dividing cells are eliminated very quickly and the percentage of surviving wells is an accurate measure of the immortalization efficiency of viruses. The Kaplan-Meir plot of HTLV-2-induced T-cell proliferation demonstrated that the percentage of wells containing proliferating lymphocytes in coculture with HTLV-2 mutants with the more active Rex was substantially higher than those in coculture with wtHTLV-2 (Fig. 2.5C). Furthermore, flow cytometry analysis revealed that the percentages of HTLV-2 Rex mutant-infected T-cells were significantly higher than wtHTLV-2-infected T-cells at two days post-plating (Fig 2.5D). These data indicate that maintenance of Rex-2 in the active state can enhance infectivity and increase proliferation of HTLV-2 infected cells.

**HTLV-2 mutants with more active Rex persisted in the inoculated rabbit model**
In order to evaluate the role of Rex functional regulation in HTLV-2 infection and replication *in vivo*, we inoculated rabbits with 729 control, 729wtHTLV-2, 729H2A157D or 729H2S158Term cells. Rabbits were inoculated with lethally irradiated cell lines and rabbit blood was sampled at weeks 0, 1, 2, 4, 6, and 8 post-inoculation. Serum antibody titers to viral antigens increased over the course of the study in the majority of the rabbits (Fig 2.6A). Statistical analysis confirmed a significantly higher antibody response to HTLV-2 antigens in the two groups of HTLV-2 Rex mutant-infected rabbits compared with the wild-type HTLV-2 group at eight weeks post-inoculation. In addition, proviral loads were examined by amplification of specific HTLV-2 genomic fragments from rabbit PBMCs. We detected proviral signals in all inoculated rabbits, which was consistent with the seroconversion data. However, quantitative real-time Taqman PCR over time revealed that proviral loads in rabbits infected with either Rex-2 mutant virus was not significantly different than the proviral loads of rabbits inoculated with wild-type HTLV-2 (Table 2). Interestingly, the enhanced antibody responses in Rex mutant virus-infected rabbits did not attenuate viral infection (represented as proviral load) over the time course of this study, indicating that HTLV-2 Rex mutant viruses could successfully infect and persist in inoculated rabbits. We did not observe any *in vivo* reversion to wild type HTLV-2 sequence in Rex mutant virus-infected rabbits (data not shown).
2.5 Discussion

The goal of this study was to better understand the functional role of the carboxy terminal Rex-2 phosphorylation domain (CTPD) and to address whether the ability to regulate Rex function is important for HTLV-2-mediated cellular proliferation and immortalization \textit{in vitro} and virus survival in a rabbit model of infection. We have shown that introducing a phosphomimetic amino acid (negative charge) into the Rex-2 c-terminus or deletion of certain c-terminal sequences could maintain Rex-2 in a highly functional state. Interestingly, we also found that the c-terminus played an important role in Rex-2 protein stability and/or protein expression. Thus, our data provided evidence that the Rex-2 c-terminus contains a functional inhibitory domain that also is regulated by phosphorylation status. Cells harboring HTLV-2 mutants with more active Rex showed enhanced infection \textit{in vitro} and increased virion production. Furthermore, these mutants promoted HTLV-2-induced proliferation of human primary T-cells and displayed increased replication in inoculated rabbits as measured by significantly stronger antibody responses as compared to wtHTLV-2-infected animals. However, proviral load levels over time and the ability of the mutant viruses to persist was similar to wtHTLV-2 inoculated rabbits. Thus, although HTLV requires Rex to efficiently replicate and persist in inoculated rabbits, the ability to modulate Rex function does not significantly alter the \textit{in vivo} course of infection, including the proviral load set point and the establishment of persistence.

We have shown that deletion of the c-terminus destabilizes Rex-2 protein by significantly decreasing its half-life. In addition, we observed that substitution of negatively charged amino acids (aspartic acid) within the Rex-2 carboxy terminus
increased steady state protein levels and functional activity, whereas specific mutation or substitution of serines 151 and 153 with alanine resulted in decreased protein steady state levels and impaired Rex-2 function. Presented in Figure 2.7 is a model consistent with our Rex-2 biochemical and functional data and the published literature. The initial translation product of Rex-2, p24, is located primarily in the cytoplasm. We propose that phosphorylation of this inactive p24 at an unidentified serine(s) removes the inhibitory effect of the c-terminus, results in a conformational change, giving rise to an active p26 intermediate that can translocate to the nucleus and interact with target RNA. This intermediate is likely unstable and a potential substrate for dephosphorylation (return to the p24 conformation) or subsequent phosphorylation at serine 151 and/or 153 resulting in a more stable p26 conformation (Fig 2.7A). Substitution of serine 151 and serine 153 with phosphomimetic aspartic acid residues (S151D, S153D) disrupts the c-terminal inhibitory domain and results only in the detection of a stable (phosphatase resistant) and highly active p26 (Fig 2.7B) (234). Substitution of negatively charged aspartic acid for other residues in the c-terminus of Rex-2 (P152D or A157D) can override the initial phosphorylation at an unidentified serine(s), remove the inhibitory effect of the c-terminus and quickly drive the equilibrium forward. As a result of this open conformation, serines 151 and/or 153 likely are more easily accessible and quickly phosphorylated, resulting in the detection of only the stable and active p26 (Fig 2.7C). In S158Term, the inhibitory c-terminal domain is removed by the deletion of sequences downstream of serine 158 and as a result of this open conformation, serines 151 and/or 153 are quickly phosphorylated, generating a single stable active protein (Fig 2.7D). Similarly, although S151Term is active in a single detectable protein conformation, its
functional activity is significantly attenuated likely due to the loss of the phospho-
acceptor serines located at position 151 and 153 (Fig 2.7E). This is consistent with the
phenotype of combination mutants containing additional serine 151 and 153 alanine
substitutions (P152D-2A, A157D-2A, and S158Term-2A), which display reduced
stability and functional activity. Studies currently ongoing in our laboratory to identify
other key phosphorylation sites and their respective cellular kinases may further facilitate
our understanding of the functional regulation of Rex-2.

The question arises as to whether this c-terminal regulatory domain is unique to
Rex-2 or if it also is contained in the highly related Rex-1. Rex-1 is a 189 amino acid
protein and although Rex-1 and Rex-2 have the capacity to functionally substitute for
each other (163, 339), the carboxy terminus of the two related proteins at the amino acid
level is quite divergent (234, 235). In cells, Rex-1 is detected as a single phosphoprotein
species of 27 kDa and one study using the H7 kinase inhibitor suggested that
phosphorylation is important for its function (1, 2). Our initial studies revealed that two
Rex-1 deletion mutants (P180Term and L170Term) have reduced steady state protein
levels as compared to wild-type Rex-1 yet their function remains similar (Fig 2.8A and
8B). Moreover, deletion of part of the c-terminus reduces the half-life of Rex-1 from 12
hours to approximately 7 hours (Fig 2.8C). Thus, this phenotype is nearly identical to
Rex-2 S158Term suggesting that Rex-1 also contains a carboxy terminal
inhibitory/stability domain. Additional studies will be required to understand what, if any,
role phosphorylation plays in the regulation of this domain.

In the context of the provirus, the functionally more active Rex-2 mutants, A157D
and S158Term, led to increased production of p19 Gag as measured in the culture
supernatant; ultimately increasing HTLV-2 infectious spread and HTLV-2-mediated cellular proliferation as determined in our short-term quantitative assays. Both HTLV-2 Rex mutants can successfully infect and persist in inoculated rabbits. Consistent with increased expression of structural proteins in vitro, rabbits inoculated with Rex-2 mutants A157D and S158Term exhibited a significantly higher antibody response to viral antigens as compared to wtHTLV-2 infected rabbits. The common expectation is that higher antibody titer will more efficiently limit viral replication, leading to lower proviral load and ultimate elimination of viral infection. However, we did not observe any significant difference in proviral loads between wild type and Rex-2 mutants. Our Western blot analysis indicated that the increased antibody response was directed primarily against the viral Env and Gag. Although antibody response is a strong indicator of viral replication, there is no evidence that these antibodies are neutralizing or significantly control the infection. Previous studies provided strong evidence that an efficient cytotoxic T lymphocyte (CTL) response is the major host immune surveillance against HTLV-1-infected cells and possibly pathogenesis (18). Tax protein has been identified as the dominant target of HTLV-1-specific CTLs (136, 149), which is expressed from double spliced viral mRNA and minimally affected by alterations in Rex functional activity (179). More importantly, various studies have suggested that host genetic polymorphisms play a critical role in determining the efficacy of an individual immune response to HTLV-1 or HTLV-2 (139, 321). Consistently, we detected highly variant serological responses in inoculated rabbits irrespective of inoculum.

Overall, our studies characterized the c-terminus of Rex-2 and emphasized its important role in regulation of Rex-2 protein function and expression, and in HTLV-2
replication and infectious spread, along with viral induction of cellular proliferation. This finding also provides some important information regarding HTLV-2 replication in the rabbit animal model and its crosstalk with the host immune system.

**Acknowledgments**

We thank Joshua Arnold and Romi Doueiri for their assistance with the rabbit experiment, Tim Vojt for preparation of the figures, and Kate Hayes-Ozello for editorial comments. This work was supported by a grant from the National Institutes of Health (CA100730) to PLG.
Figure 2.1 Expression and functional activity of Rex-2 mutants. (A) Western blot of Rex-2 protein expressed from 293T cells transiently transfected with Rex-2 cDNA plasmids. Proteins were detected using rabbit Rex-2-specific antisera. Wild-type p24 and p26 are indicated and arrows identify truncated Rex proteins. (B) Functional activity of Rex-2 cDNA mutants was determined using the modified HIV p24 Gag reporter assay. 293T cells were transfected with 0.25µg pcTat, 0.5µg pcGagRxRE-II, 0.05µg CMV-luc, and increasing concentrations of wtRex or mutant Rex plasmids as indicated (0.02 to 0.5µg). Forty-eight hours after transfection, cells were harvested and assayed for p24 Gag. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations.
Figure 2.2 Subcellular localization of Rex-2 mutants. HeLa-Tat cells were transfected with 1 µg of various Rex-2-EGFP plasmids or EGFP-N3 negative control as indicated using Lipofectamine® PLUS (Invitrogen, Carlsbad, CA). (A) For EGFP detection, cells were plated and visualized using a Zeiss LSM 510 microscope (GFP and the light field are shown. (B) Expression of Rex-2-EGFP fusion proteins was detected by Western blot using α-Rex-2 antisera or α-EGFP antibody. (C) The functional activity of Rex-2-EGFP fusion proteins was determined by HIV p24 Gag reporter assay. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations.
Figure 2.3 The c-terminus is crucial for Rex protein expression and stability. (A) Disruption of phosphorylation at serines 151 and 153 (by deletion or alanine substitution mutation) consistently impairs Rex functional activity. 293T cells were transfected with 0.25µg pcTat, 0.5µg pcGagRxRE-II, 0.05µg CMV-luc, and 0.1µg of wtRex or mutant Rex plasmids as indicated. Forty-eight hours after transfection, cells were harvested and assayed for p24 Gag. The values, which are normalized and shown relative to wtRex-2, represent relative p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations. (B) Rex-2 proteins expressed from transiently transfected 293T cells were detected by Western blot using α-Rex-2 specific antisera. Wild-type p24 and p26 are indicated and the arrow identifies the truncated Rex. Detection of cellular β-actin was used as a loading control. (C) The altered expression level of Rex-2 mutants is not attributable to the detection sensitivity of our α-Rex-2 antisera. Flag-tagged Rex protein expression (FLAG-wtRex, FLAG-RexA157D, FLAG-RexA157D-2A, FLAG-RexS158Term, and FLAG-RexS158Term-2A) were detected from transiently transfected 293T cells using α-FLAG monoclonal antibody M2. Wild-type p24 and p26 are indicated and the arrow identifies the truncated Rex. (D) The half-life of wtRex, S151D, A157D, S158Term, and S151Term were determined by pulse-chase experiments as described in Material and Methods. Quantification of protein at different time points using the Typhoon imaging system was utilized to determine the protein half-life.
Figure 2.4 Establishment of permanent producer cell lines of HTLV-2 mutants. (A) Viral protein expression in permanent transfectants. Three independently isolated 729 stable producer cell clones for H2A157D and H2S158Term were isolated as described in Materials and Methods. Cells (5 x 10^6) were plated in each well of 6-well plates and p19 Gag production was measured in 48 h culture supernatants by ELISA. The 729wtHTLV-2 cell line was used as the positive control. (B) Viral protein expression, including Rex and Tax, and β-Actin as a loading control, were detected by Western blot for selective stable producer cell lines (shown as black bar in panel A) to be used in coculture analysis. Wild-type p24 and p26 are indicated and the arrow identifies the truncated Rex.
Figure 2.5 HTLV-2 T-lymphocyte immortalization and proliferation assays. PBMCs (2x10^6) were cultured with irradiated donor cells (1x10^6) in each well of 24-well plates. (A) Representative growth curves for HTLV-2-infected cells are shown. Cell viability was determined weekly by trypan blue exclusion (0-11 wks post-cocultivation). The mean and standard deviation for each time point were determined from three independent wells. (B) HTLV-2 gene expression was confirmed by detection of p19 Gag protein in the culture supernatant using ELISA. (C) Representative Kaplan-Meir plots for T-lymphocyte proliferation in short-term microtiter assay. Prestimulated PBMCs (10^4) were cocultured with 100 irradiated 729 stable producer cells per well in 96 well plates. The Kaplan-Meir plot shows the percentage of proliferating wells as a function of time (wks). (D) Functionally more active Rex enhances viral infectivity in coculture assays. Irradiated 729 stable producer cells (2x10^5) were cocultured with 10^6 PBMCs in the presence of IL-2. The percentages of newly infected T-cells (CD3^+, p19^+) were enumerated two days post-plating by immunofluorescence analysis. The mean and standard deviation for each sample were determined from three independent experiments using PBMCs from three different healthy donors. The mean values are indicated by the horizontal lines. The percentages of H2A157D and H2S158Term infected T-cells are both significantly higher than that of wtHTLV-2-infected T cells (p<0.001) as determined by ANOVA followed by Tukey’s post-test.
Figure 2.6 Assessment of HTLV-2 infection in inoculated rabbits. Antibody responses against HTLV-2 from each rabbit were measured by anti-HTLV-2 ELISA assay, using both HTLV-2 Gag and envelope proteins as antigens. Each dot represents the absorbance value of a single inoculated rabbit at 0, 1, 2, 4, 6, and 8 weeks post inoculation within each group. The inocula as indicated below include 729.wtHTLV-2 (n=6), 729.H2A157D (n=6), 729.H2S158Term (n=6), or 729 (n=2). The horizontal line represents the average of the rabbit group at each weekly time point. Statistical analysis (ANOVA followed by Tukey’s) of titers at four and eight weeks after inoculation revealed significantly higher antibody responses to HTLV-2 antigens in the 729/H2A157D (P<0.01) and 729/H2S158Term (P<0.05) inoculated rabbits (denoted by *) compared with the wild-type control group. Week 6 displayed borderline significance (P=0.058).
Figure 2.7 Model for Rex-2 phosphorylation and functional regulation. (A) The primary Rex-2 translation product p24 is inactive. An initial phosphorylation on unidentified serine(s) induces a conformational alteration and results in an unstable but functionally active p26 intermediate. This intermediate can be further stabilized by subsequent phosphorylation on serine 151 and or 153, generating a fully functional, stable p26 form. “Pi” represents phosphorylation, triangles denote non-phosphorylated serines, and solid triangles denote phosphorylated serines. (B) Mutant S151,153D disrupts the equilibrium between inactive p24\textsuperscript{rex} and active p26\textsuperscript{rex} because the aspartic acids (depicted by filled diamonds) are not subjected to dephosphorylation. (C) In mutants P152D and A157D, introduction of a phosphomimetic aspartic acid (filled diamond) into the carboxy terminus functionally overrides the initial phosphorylation on unidentified serine(s), removes the inhibitory effect of the carboxy terminus, and results in an unstable p26\textsuperscript{rex} active form. Rex-2 is locked in the p26\textsuperscript{rex} form because the aspartic amino acid is not subjected to dephosphorylation. (D) Deletion of the sequence downstream of Ser158 permanently removes the inhibitory carboxy terminus and interrupts the equilibrium between p24\textsuperscript{rex} inactive form and p26\textsuperscript{rex} active form. The p26\textsuperscript{rex} intermediate can be stabilized by phosphorylation on serine 151 and or 153 (depicted by triangles), whereas Rex expressed from the S151Term mutant (E) is conformationally unstable because serines 151 and 153 are deleted.
Figure 2.8 Expression and functional activity of Rex-1 mutants. (A) Western blot of Rex-1 protein expressed from 293T cells transiently transfected with wtRex-1 and deletion mutants, P180Term L170Term, cDNA plasmids. Proteins were detected using rabbit Rex-1-specific antisera. Wild-type p27 Rex is indicated and the arrows identify the truncated Rex proteins (B) Functional activity of Rex-1 was determined using the modified HIV p24 Gag reporter assay. 293T cells were transfected with 0.25µg pcTat, 0.5µg pcGagRxRE-I, 0.05µg CMV-luc, and 0.1mg Rex-1 wild-type and mutants DNA. Forty-eight hours after transfection, cells were harvested and assayed for p24 Gag production. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations. (C) The half-lives of wtRex-1 and P180Term were determined by pulse-chase experiments as described in Material and Methods. Proteins were quantified at different time points using the Typhoon imaging system.
Table 2.1 Rex-2 mutants and their Tax transactivation activity. * The Rex-2 mutants generated were named based on the mutated amino acid (aa) and its position within the 170-aa HTLV-2 Rex. For example, S151D indicates that serine residue at aa 151 was substituted with aspartic acid. ** S, serine; D, aspartic acid; G, glycine; P, proline; A, alanine; L, leucine; F, phenylalanine, R, arginine; Term, termination codon. *** Tax transactivation activity on CREB/ATF- and NFκB-responsive luciferase reporter genes was examined using dual luciferase assay as described in the Materials and Methods. The data is based on three independent experiments and normalized to wtRex-2 (set as 1.0).
Table 2.2 Detection and quantification of HTLV-2 DNA in rabbit PBMCs. Genomic DNA isolated from rabbit PBMCs were subjected to PCR and to real-time Taqman PCR (weeks 0, 2, 4, 8 and 11) using HTLV-2-specific primers (670/671). Numbers in parentheses denote copy number x10^{-2} per cell in rabbit PBMCs. –, indicates no amplified PCR fragment; +, amplified PCR fragment. As determined by ANOVA, copy numbers in rabbits inoculated with the mutant proviruses were not significantly different from wild type. The P-values for wk 2, wk 4, wk 8, and wk 11 are 0.216, 0.517, 0.498, and 0.443 respectively.

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CHAPTER 3

SITE-SPECIFIC PHOSPHORYLATION REGULATES HUMAN T-CELL LEUKEMIA VIRUS TYPE-2 REX FUNCTION IN VIVO

3.1 Abstract

The human T-cell leukemia virus type-2 (HTLV-2) Rex is a trans-acting regulatory protein required for efficient cytoplasmic expression of the unspliced and incompletely spliced viral mRNA transcripts encoding the structural and enzymatic proteins. Previously, it was demonstrated that phosphorylation of Rex-2, predominantly on serine residues, correlated with an altered conformation as observed by a gel mobility shift and the detection of two related protein species (p24Rex and p26Rex). Rex-2 phosphorylation is required for specific binding to its viral mRNA target sequence, inhibition of mRNA splicing, and may be linked to subcellular compartmentalization. Thus, the phosphorylation-induced structural state of Rex in the infected cell may be a switch that determines whether HTLV exists in a latent or productive state. We have conducted a phosphoryl and functional mapping of both structural forms of mammalian cell-expressed Rex-2 using affinity purification, liquid chromatography tandem mass spectrometry, and site-directed substitutional mutational analysis. We identified two phosphorylation sites in p24Rex at Ser-117 and Thr-164. We also have identified six
phosphorylation sites in p26\textsuperscript{Rex} at Thr-19, Ser-117, Ser-125, Ser-151, Ser-153, and Thr-164. We evaluated the functional significance of these phosphorylation events and found that phosphorylation on Thr-164, Ser-151, and Ser-153 is critical for Rex-2 function \textit{in vivo}, and that phosphorylation of Ser-151 is correlated with nuclear/nucleolar subcellular localization. Overall, this work is the first to completely map the phosphorylation sites in Rex-2 and provides important insight into the phosphorylation continuum that tightly regulates Rex-2 structure, cellular localization, and function.

3.2 Introduction

Human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are closely related complex retroviruses that have the ability to transform primary human T-cells in culture and are associated with leukemia and a variety of human diseases (341). HTLV-1 is causally associated with adult T-cell leukemia, an aggressive CD4\textsuperscript{+} T-cell malignancy, and a chronic neurodegenerative disorder, HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (96, 324). Disease association with HTLV-2 is less clear, with only a few cases of leukemia and neurological diseases reported (119, 276, 278). The difference in pathogenesis between the two related viruses has yet to be elucidated, but likely results from the activities of the regulatory and accessory proteins as well as their distinct Env cell surface molecule interaction profiles (78, 143, 144, 333).

In addition to the archetypal structural and enzymatic retroviral genes gag, pol, and env, HTLV encodes two \textit{trans}-regulatory genes, \textit{tax} and \textit{rex}, which are essential for efficient viral replication/transformation, as well as several accessory genes important for
viral infection and persistence *in vivo* (182). The viral oncoprotein Tax acts to *trans*-activate transcription from the viral long terminal repeat (LTR) (40, 76) and modulates the transcription/function of numerous cellular genes involved in cell growth, cell cycle control, DNA repair, and cell differentiation (35, 228, 266, 268, 287). The pleiotropic affects of Tax on such numerous cellular processes are critical for its ability to induce cellular transformation and oncogenesis (78, 90, 274, 280).

HTLV Rex is a positive *trans*-acting regulator of viral replication. Rex is a nuclear-localizing and shuttling phosphoprotein that acts post-transcriptionally by binding and selectively exporting the unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm, thus controlling the expression of the viral gene products that are essential for viral progeny production (114, 179). Therefore, it has been proposed that Rex is critical for the transition from the early, latent phase to the late, productive phase of HTLV infection. Rex binds viral mRNAs via a *cis*-acting viral RNA structure termed the Rex-response element (RxRE) located in the R region of the viral LTR (14, 247, 311). Previous analysis of HTLV-1 Rex (Rex-1), HTLV-2 Rex (Rex-2) and their RxREs, revealed structural homologies and showed that they are functionally interchangeable (339, 340). Rex mutational analysis has defined several domains that are critical for its functions that include an arginine-rich N-terminal sequence that functions as an RNA binding domain (RBD), which also overlaps with a nuclear localization signal (NLS), a leucine-rich central core activation domain that contains a nuclear export signal (NES), and two flanking Rex-Rex multimerization domains (31, 32, 110, 123, 235, 254, 271, 296, 326). More recently, it has been reported that Rex-2 contains a novel C-terminal inhibitory/stability domain that regulates protein shuttling, subcellular
localization and functional activity and is positively regulated through phosphorylation (234, 235, 334).

Phosphorylation is a well-documented regulatory event with a pivotal role in controlling the activity/function of proteins (310). Earlier studies have demonstrated that both Rex-1 and Rex-2 are phosphoproteins, and that this modification is critical to their function (1, 2, 103). In HTLV-2-infected T-cells and cell lines transfected with Rex-2 expression plasmids, two species of Rex-2 (p24\textsuperscript{Rex} and p26\textsuperscript{Rex}) are detected. Both Rex-2 species have the same amino acid backbone and differ by a charge-induced conformational change due to a serine/threonine phosphorylation (100, 102, 234). This altered gel mobility is unique to Rex-2 since Rex-1 presents as a single 27 kDa phosphoprotein. We reported the identification of important Rex-2 phosphorylation sites using targeted serine or threonine to alanine substitution mutational analysis. This study identified at least one critical phosphorylation site at serine 151 (234). A serine-to-alanine substitution at this residue (S151A) resulted in reduced phosphorylation, impaired RNA binding capability, diffused cytoplasmic localization, and decreased functional activity. In contrast, replacement of the serine with a phosphomimetic aspartic acid (S151D) resulted in detection of only the p26\textsuperscript{Rex} species in cells. The S151D mutant was locked in a p26\textsuperscript{Rex} active conformation, since \textit{in vitro} phosphatase treatment failed to alter its mobility. This protein displayed enhanced RNA binding capacity and intense localization to the nucleus (234, 235).

Our earlier studies suggest a linear relationship between phosphorylation, conformational changes, and Rex-2 function implicating phosphorylation as a key regulatory event within the productive life cycle of the virus (234). To understand the
regulation of Rex-2 function by phosphorylation, we performed complete phosphorylation mapping of both p24\textsubscript{Rex} and p26\textsubscript{Rex}. In this study, we combined liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (201) of affinity-purified Rex-2 protein with substitution mutational analysis to identify and functionally characterize key phosphorylation sites. We achieved 100% coverage of both p24\textsubscript{Rex} and p26\textsubscript{Rex}. We have identified two phosphorylation sites in p24\textsubscript{Rex} and six total phosphorylation sites in p26\textsubscript{Rex}. We also determined three specific amino acid phosphorylation events found to be critical for Rex-2 function \textit{in vivo} (Ser-151, Ser-153, Thr-164). Phosphorylation at Ser-151 and Ser-153 is the major switch event for the conformational change between p24\textsubscript{Rex} and p26\textsubscript{Rex}. Phosphorylation of one of these sites (Ser-151) was critical for nuclear subcellular localization of the Rex-2 protein. Taken together, our data support a model in which a phosphorylation continuum regulates the biological properties of Rex controlling viral replication and ultimately, disease.

### 3.3 Materials and Methods

**Cell Culture**

293T and HeLa-Tat cells were maintained in Dulbecco’s modified Eagle’s medium at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} in air. Medium was supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, penicillin (100U/ml), and streptomycin (100\mu g/ml).
Mammalian Expression Plasmid

The Rex-2 expression vector BC20.2, which contains the HTLV-2 tax/rex cDNA expressed from the cytomegalovirus (CMV) immediate-early gene promoter, was described previously (102, 339). The S-tagged expression vector S-Rex-2 was constructed by inserting the HTLV-2 rex open reading frame from BC20.2 into pTriEx4-Neo (Novagen, Madison, WI) in-frame with the amino-terminal His-tag and S-tag via SmaI and BamHI. All generated rex expression vectors contained a previously described mutation in the overlapping tax reading frame (F4Term), which had no effect on the Rex amino acid sequence, but severely truncated Tax, completely knocking out expression and function (281). The various rex-2 targeted mutations were generated using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequence analysis and vector expression was verified by transfection and Western blot analysis. The human immunodeficiency virus type 1 (HIV-1) Tat expression vector, pcTat, Rex-2 reporter plasmid (pCgagRxRE-II) and CMV-luciferase (firefly) were described previously (234).

Rex Functional Reporter Assay

The Rex-2 functional assay was performed as described previously with slight modifications (234). Briefly, 0.1μg Rex-2 cDNA expression plasmids were cotransfected into 293T cells with 0.05μg of CMV-luc, 0.25μg of pcTat, and 0.5μg of Rex reporter
plasmid pCgag-RxRE-II using Lipofectimine Reagent (Invitrogen, Carlsbad, CA). Cell lysates were prepared at 24 h post-transfection in Passive Lysis Buffer (Promega, Madison, WI) with a protease inhibitor mixture (Roche Applied Science Indianapolis, IN) on ice for 30 min. Luciferase activity was determined and used as a transfection efficiency control. HIV-1 p24 Gag levels in the cellular lysates were determined by ELISA (ZeoMetrix, Buffalo, NY). All transfection experiments were performed in triplicate in three independent experiments.

**Immunoblot and Immunofluorescence Analysis**

Cell lysates were prepared at 24 h post-transfection in Passive Lysis Buffer (Promega, Madison, WI) with a protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) on ice for 30 min. After centrifugation, total protein concentrations were determined by Bradford protein assay (Bio-Rad). The cell lysates were subjected to 12% SDS-PAGE, and transferred to nitrocellulose (Schleicher & Schuell Biosciences, Keene, NH). Western blots were performed as recommended by the manufacturer and proteins were visualized using enhanced chemiluminescence (ECL) Western blot analysis system (Santa Cruz Biotechnology, Santa Cruz, CA). Rex-2 was detected using protein-specific rabbit polyclonal antisera. Subcellular localization of Rex-2 was performed as previously described (322) with slight modifications. HeLa-Tat cells were transfected with 2μg of vector control plasmid, wtRex-2, or various Rex-2 mutants. At 24 h post-transfection, cells were washed and fixed in PBS containing 2% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 and 0.5% fetal bovine serum for 15
min at 4°C. Cells were incubated in blocking buffer (0.5% fetal bovine serum and 2mg/ml human IgG) for 30 min at room temperature. Staining was conducted in blocking buffer with rabbit Rex-2 specific antisera followed by incubation with secondary antibody conjugated to FITC AlexaFluor 488 (Molecular Probes, Eugene, OR). Nuclear staining was performed using 4’6-diamidino-2-phenylindole (DAPI) Slow Fade Gold (Invitrogen, Carlsbad, CA). Fluorescence was visualized on an epifluorescence microscope (Olympus, Melville, NY), and digital images were taken using Optronics Imaging System (Goleta, CA).

**Purification of Rex-2 Protein**

Protein purification was performed as described previously with slight modifications (74). Briefly, cell lysate (1.5 ml volume) was incubated with a 75 μl bed volume of S-protein agarose (Novagen, Gibbstown, NJ) overnight at 4°C, washed twice with a high salt modified RIPA buffer (0.05M Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 1.0M NaCl, 0.01M EDTA) and twice with a low salt modified RIPA buffer (0.05M Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 150mM NaCl). S-protein agarose beads were resuspended in 100 μl SDS loading dye containing β-mercaptoethanol and subsequently boiled for 2 min. Samples were electrophoresed in a 12% SDS one-dimensional polyacrylamide gel and visualized by Coomassie Blue staining. Both the p24\textsuperscript{Rex} and p26\textsuperscript{Rex} bands were excised from the gel separately for further proteomic analysis.
**Mass spectrometry analysis**

LC-MS/MS analysis was performed as described previously with slight modifications (74). Briefly, p24\textsubscript{Rex} and p26\textsubscript{Rex} excised gel slices were cut into 1-2 mm cubes; washed three times with 500 µl ultra-pure water and incubated in 100% acetonitrile for 45 min. Samples were reduced with 50 mM DTT at 56°C for 45 min then alkylated with 55 mM iodoacetamide for 1 h at room temperature. The material was dried in a speed-vac, rehydrated in a 12.5 ng/µl modified sequencing grade trypsin solution (Promega, Madison, WI) and incubated in an ice bath for 40-45 min. The trypsin solution was removed and replaced with 40-50 µl of 50mM ammonium bicarbonate and 10% acetonitrile (pH 8.0), and the mixture was incubated overnight at 37°C. Elastase digests were performed as described for trypsin at an enzyme concentration of 15 ng/µl in the absence of acetonitrile in the reaction buffer. Peptides were extracted two times with 25 µl 50% acetonitrile, 5% formic acid and dried in a speed-vac. Digests were resuspended in 20 µl buffer A (5% acetonitrile, 0.1% formic acid, 0.005% heptafluorobutyric acid) and 3-6 µl were loaded onto a 12-cm x 0.075 mm fused silica capillary column packed with 5µM diameter C-18 beads (The Nest Group, Southboro, MA) using an N2 pressure vessel at 1100 psi. Peptides were eluted over 55 min by applying a 0-80% linear gradient of buffer B (95% acetonitrile, 0.1% formic acid, 0.005% HFBA) at a flow rate of 150µl/min with a pre-column flow splitter resulting in a final flow rate of ~200nl/min directly into the source. In some cases, the gradient was extended to 150 min to acquire more MS/MS spectra. An LTQ™ Linear Ion Trap
(ThermoFinnigan, San Jose, CA) was run in automated collection mode with an instrument method composed of a single segment and five data-dependent scan events with a full MS scan followed by four MS/MS scans of the highest intensity ions. Normalized collision energy was set at 35, activation Q was 0.250 with minimum full scan signal intensity at $1 \times 10^5$ with no minimum MS$^2$ intensity specified. Dynamic exclusion was turned on utilizing a three minute repeat count of 2 with the mass width set at 1.0 m/z. Sequence analysis was performed with TurboSEQUEST™ (ThermoFinnigan, San Jose, CA) or MASCOT (Matrix Sciences, London GB) using an indexed human subset database of the non-redundant protein database from National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov).

### 3.4 Results

**Functional Domains of HTLV-2 Rex**

Two species of Rex-2 ($p24^{Rex}$ and $p26^{Rex}$) are detected in viral infected cells. Both Rex-2 species have the same amino acid backbone and differ by a charge-induced conformational change due to a serine/threonine phosphorylation (100, 102, 234). The $p26^{Rex}$ species is localized primarily to the nucleus and its presence correlates with Rex-2-specific RxRE target RNA binding (103, 340). Mutational analysis permitted the assignment of functional properties to distinct domains of the Rex-2 protein (Fig. 3.1). In addition to the nuclear localization signal/RNA binding domain, central core activation domain/nuclear export signal, and multimerization domains also shared with the functionally homologous HTLV-1 Rex (235), we also reported that Rex-2 contained a
unique C-terminal inhibitory/stability domain found to be important for proper nucleo-
cytoplasmic shuttling, subcellular localization, and function (235, 334). This domain also
encompasses at least one phosphorylated serine residue (S151), identified by
substitutional mutational and peptide phosphoamino acid analysis, that is critical for Rex-
2 function in vivo (234, 235). Replacement of this serine with phosphomimetic aspartic
acid (S151D) resulted in detection of only the p26\textsuperscript{Rex} species in cells. The S151D mutant
was locked in a p26\textsuperscript{Rex} active conformation, since in vitro phosphatase treatment failed to
alter its mobility. Therefore, since phosphorylation of Rex-2 may be a key regulatory
event, a complete analysis of Rex-2 phosphorylation is well-justified.

Expression and Detection of Biologically Active Affinity S-tagged Rex-2 Protein

To determine the distinct phosphorylation events present in both p24\textsuperscript{Rex} and
p26\textsuperscript{Rex} that occur in vivo, we used a tandem affinity tagged Rex-2 construct that was
expressed in and purified from mammalian cells. The Rex-2 vector (S-Rex-2) expresses
full-length Rex-2 protein fused to amino-terminal His\textsubscript{6} and S-tag (Fig. 3.2A). Since the
HTLV regulatory proteins Tax and Rex are translated from the same mRNA in partially
overlapping reading frames, we utilized a cDNA mutant sequence containing an amino
terminal stop codon in the Tax reading frame leaving the Rex-2 reading frame unchanged
(280). This mutation abrogated Tax protein expression (data not shown). The S-Rex-2
and the wtRex-2 expression constructs were transiently transfected into 293T cells and
protein expression was determined by Western blot analysis using rabbit polyclonal α-
Rex antisera (Fig. 3.2B). As expected, the wtRex-2 transfected cells produced both
p24\textsuperscript{Rex} and p26\textsuperscript{Rex} species. S-Rex-2 transfected cells also produced two distinct species of increased molecular weight consistent with the addition of the tags. We performed further analyses to confirm that the S-tagged Rex-2 species had similar properties as wtRex-2 (p24\textsuperscript{Rex} and p26\textsuperscript{Rex}). Pro Q diamond staining as well as protein phosphatase treatment indicated that the upper band was a phosphorylated form of the lower band consistent with what had been reported previously for wtRex-2 (103) and (data not shown). We then tested S-Rex-2 for its ability to function in our quantitative reporter bioassay in which HIV-1 p24 Gag production was measured and used as a read-out of Rex functional activity (234). As shown in Figure 3.2C, S-Rex-2 displayed significant functional activity, although slightly lower than wtRex-2. We hypothesize that this reduced activity likely is due to the proximity of the amino terminal tag to the RNA binding domain. Taken together, these data demonstrate the proper and efficient expression of a functionally active S-tagged Rex-2 from mammalian cells.

**Affinity Purification of Rex-2 from Mammalian Cells**

We successfully purified S-tagged Rex-2 protein from transfected 293T cells using S-protein-agarose beads as described in the “Experimental Procedures.” This purification procedure is based on the strong affinity between the 15-amino acid S-tag and the S-protein that is immobilized on the agarose beads, both of which are derived from RNase S (164). The affinity purified Rex-2 protein was resolved by SDS-PAGE, where both species of S-tagged Rex-2, equivalent to wild-type p24\textsuperscript{Rex} and p26\textsuperscript{Rex}, were detected by Coomassie Blue staining (Fig. 3.2D). This purification process produced
adequate quantities of highly purified Rex-2 protein from mammalian cells and allowed for the subsequent post-translational modification analysis by LC-MS/MS.

**Phosphopeptide Mapping of Rex-2 Using LC-MS/MS**

Multiple strategies were employed to identify the phosphorylation sites within Rex-2. The two species of S-tagged Rex-2 were excised and treated separately. This ensured that we could determine the specific phosphorylation maps of each species independently in an effort to better understand how phosphorylation alters Rex-2 conformation and regulates its function. Initially, the proteins were subjected to trypsin enzymatic digestion. The tryptic peptides that were too large to detect were either further digested with elastase or independently digested with elastase. This combined analytical approach allowed us to obtain a detailed physical map covering 100% of the polypeptide for both species of S-tagged Rex-2 (Fig.3.3A). Our analysis identified two phosphorylation sites, Ser-117 and Thr-164, in the lower species (p24\textsuperscript{Rex} equivalent). In addition to Ser-117 and Thr-164, we also identified four additional residues (Thr-19, Ser-125, Ser-151, and Ser-153) that are phosphorylated in the upper S-tagged species (p26\textsuperscript{Rex} equivalent). Figure 3.3B shows a representative MS/MS spectrum of the tryptic phosphopeptide that identified phosphorylation at Ser-151. Furthermore, phosphorylation of Ser-151 previously was shown to be important for Rex-2 subcellular localization, RNA binding, and Rex-2 function in vivo (234).

**Substitution Mutational Analysis of the Identified Rex-2 Phosphorylation Sites**
To determine possible regulatory roles of the six identified phosphorylation sites, we generated single alanine amino acid substitutions and tested these Rex-2 mutants for their ability to function in our quantitative reporter bioassay. The Rex-2 mutants were transiently co-transfected into 293T cells with pcTat and pCgagRxRE-II along with a CMV-luciferase transfection efficiency control. We identified three mutants S151A, S153A, and T164A within the C-terminal domain of Rex-2 that displayed significantly reduced function (Fig. 3.4A). Further mutational analysis of these three residues by converting them to phosphomimetic aspartic acid (S151D, S153D, and T164D) restored functional activity to wild-type Rex-2 levels indicating that phosphorylation plays a positive functional role (Fig. 3.4A). Aspartic acid substitution of Thr-19, Ser-117, or Ser-125 had no effect on protein function consistent with the conclusion that phosphorylation of these three residues does not negatively regulate function, but is silent (data not shown). The steady state expression levels of the wild-type and mutant Rex-2 proteins were determined by Western blot analysis using rabbit polyclonal α-Rex antisera (Fig. 3.4B). All of the Rex-2 mutants were stably expressed. With the exception S151D and S153D detected only as p26\textsuperscript{Rex}, all of the mutants displayed both the p26\textsuperscript{Rex} and p24\textsuperscript{Rex} forms. We previously reported that the p26\textsuperscript{Rex} to p24\textsuperscript{Rex} ratio of wtRex in transfected cells is greater than or equal to one (234). One exception was mutant S151A, which showed a reduction in the amount of p26\textsuperscript{Rex} relative to p24\textsuperscript{Rex} implicating a role for phosphorylation of this residue in altering the conformation/mobility of the protein. It is notable that T164A displayed a similar reduction in p26\textsuperscript{Rex} indicating that phosphorylation of both Thr-164 and Ser-151 correlate with p26\textsuperscript{Rex} expression. Moreover, substituting threonine with the phosphomimetic aspartic acid (T164D) resulted
in an increase in $p26^{\text{Rex}}$ and the restoration of function to wild-type levels (Fig. 3.4A), which also is consistent with the conclusion that $p26^{\text{Rex}}$ is the active form of the protein. Taken together, our results help further define a critical functional domain that is regulated via phosphorylation of three key amino acids, Ser-151, Ser-153, and Thr-164. We hypothesize that the altered ratio of $p24^{\text{Rex}}$ to $p26^{\text{Rex}}$ found in the T164A mutant and previously reported for S151A is indicative of an important role for this residue in the Rex-2 phosphorylation continuum.

**Subcellular Localization of Rex-2 Carboxy Terminus Mutants**

We next wanted to determine if the phosphorylation of key Rex-2 residues identified in the carboxy terminus regulates subcellular localization of the protein. It has been documented that $p26^{\text{Rex}}$ is found primarily in the nucleus and nucleolus, whereas the inactive $p24^{\text{Rex}}$ is diffusely dispersed throughout the cytoplasm and nucleus (235, 340). We utilized indirect immunofluorescence to assess whether the three phosphor acceptor residues (Ser-151, Ser-153, and Thr-164) that were critical for function in our bio reporter assay, were important for nuclear localization. HeLa-Tat cells were transiently transfected with wtRex-2, and each of the alanine or aspartic acid substitutional mutants. Wild-type Rex, as well as all three phosphomimetic mutants (S151D, S153D, and T164D) localized predominantly to the nucleus with the detection of very minimal cytoplasmic staining (Fig. 3.5). Mutant S151A was widely diffused throughout the cell with significant cytoplasmic staining (Fig. 3.5). Mutants S153A and T164A showed significant nuclear staining similar to wild-type Rex, but also displayed some moderate
cytoplasmic staining. Together, we conclude that although phosphorylation of Ser-151, Ser-153, and Thr-164 are pivotal for Rex-2 function, only the substitution of the Ser-151 phospho acceptor residue for alanine resulted in a significant change in subcellular localization to the cytoplasm.

**Protein Expression and Functional Activity of Rex-2 Combination Mutants**

Combining the results from this study with previous published work suggests that phosphorylation of the carboxy terminus of Rex-2 triggers a protein conformation change that correlates with nuclear localization, protein stability, and function. Since individual mutations at the carboxy terminus still maintain partial function and the fact that p24\textsuperscript{Rex} shows Thr-164 phosphorylation, we hypothesized that phosphorylation occurs on a continuum and that phosphorylation of all three residues are required for optimal biologic activity. To test this hypothesis and determine if there is a functional relationship between Ser-151, Ser-153, and Thr-164, we generated and characterized a combination of Rex-2 alanine mutants for function and protein species expression. As shown in Figure 3.6, the two double mutants S151A,T164A and S153A,T164A display similar but significantly reduced functional activity as compared to wtRex-2. However, their functional activity was comparable to single alanine substitution mutants at either of those residues (compare Fig. 3.2A and Fig. 3.6). As seen with the S151A and T164A single mutants, Western blot analysis of the double mutants revealed a reduction of the p26\textsuperscript{Rex} relative to p24\textsuperscript{Rex} and the functional activity directly correlated with the levels of p26\textsuperscript{Rex} detected. The function of the triple mutant, S151A,S153A,T164A was knocked down even further,
but some activity over background levels was observed. Western blot analysis revealed a significant and nearly complete loss of the $p26^{\text{Rex}}$ species (Fig. 3.6): some $p26^{\text{Rex}}$ was detected on longer exposure, which correlated with the residual functional activity. Taken together, these data allow us to formulate a new more complex Rex-2 phosphorylation continuum model that initiates with phosphorylation of Thr-164 followed by subsequent phosphorylation of Ser-151 and Ser-153 thus tightly regulating Rex-2 function and ultimately, viral production.

### 3.5 Discussion

Phosphorylation is a well-known regulatory event that plays a key role in controlling the function of cellular and viral proteins (1, 146, 202, 301, 310, 327). The modulation of protein function can occur by multiple mechanisms including regulation of subcellular localization, protein-protein interactions, protein stability, and protein-nucleic acid binding. Previously, it was demonstrated that phosphorylation of HTLV-2 Rex, predominantly on serine residues, correlated with an altered conformation as observed by a gel mobility shift and the detection of $p24^{\text{Rex}}$ and $p26^{\text{Rex}}$ protein species (102, 234). Rex-2 phosphorylation and the presence of the $p26^{\text{Rex}}$ species correlated with functional activity including specific binding to its viral mRNA target sequence, cytoplasmic export, and translation of the target mRNA (234, 235). Moreover, we recently reported that the carboxy terminus of Rex-2 contains a stability/inhibitory domain that is positively regulated through phosphorylation (334). However, a complete map of site-specific phosphorylation of Rex-2 and the precise role it plays in protein function remains
unclear. The goal of this study was to use affinity purification and liquid chromatography tandem mass spectrometry to identify all phosphorylation sites in both p24\textsuperscript{Rex} and p26\textsuperscript{Rex} to provide insight into a possible phosphorylation continuum and the mechanism that controls Rex-2 function and ultimately the “on/off” switch that regulates productive viral replication. Consistent with previous reports, we confirmed that Rex-2 is phosphorylated predominantly on serine residues and some threonine residues, and although the p26\textsuperscript{Rex} species is heavily phosphorylated, p24\textsuperscript{Rex} does contain minimal phosphorylation. We report the phosphorylation of p24\textsuperscript{Rex} at two sites, Ser-117 and Thr-164. We also reconfirmed phosphorylation of Ser-151 in p26\textsuperscript{Rex} (234) and identified five additional phosphorylation sites including Thr-19, Ser-117, Ser-125, Ser-153, and Thr-164.

Using mutational analysis substituting the identified phospho acceptor residues in Rex-2 with alanine or phosphomimetic aspartic acid, we showed that: 1) the site-specific phosphorylation at Ser-151, Ser-153, and Thr-164 all positively contributed to Rex-2 function; 2) the phosphorylation of Ser-151, Thr-164, and to a lesser extent Ser-153 dictate p24\textsuperscript{Rex} to p26\textsuperscript{Rex} ratio and thus Rex conformational switching, and; 3) the phosphorylation of Ser-151 regulates the ability to efficiently localize to the nucleus. Both p24\textsuperscript{Rex} and p26\textsuperscript{Rex} are phosphorylated at Ser-117 and Thr-164, but only phosphorylation at Thr-164 is functionally significant. Thus, since we do not have evidence that Ser-151 or Ser-153 are phosphorylated without prior phosphorylation of Thr-164 a logical conclusion is that phosphorylation occurs on a continuum with phosphorylation of Thr-164 an earlier event with subsequent phosphorylation of Ser-151 and/or Ser-153. In Figure 3.7, we present a model consistent with the published literature and our data as to how phosphorylation regulates Rex-2 function. The initial translation
product of Rex-2, p24^{Rex}, is located primarily in the cytoplasm. It is an inactive species in a structural conformation in which the C-terminus is configured in such a way that it prevents efficient phosphorylation of Ser-151 and Ser 153 and generation of an active p26^{Rex} mRNA binding protein. We propose that the phosphorylation continuum starts with the phosphorylation of Thr-164, which begins the rotation of the C-terminal inhibitory domain exposing Ser-151 and/or Ser-153 making them available to be phosphorylated by the as yet unknown cellular kinase. However, since proteins are dynamic and not fixed in space, as long as one or more key phosphor acceptor sites are available within the C-terminus, a hierarchical phosphorylation pattern could be side-stepped at a low frequency in mutant Rex proteins. This is consistent with functional and Western blot results of our single (S151A or T164A) and double (S151A,T164A or S153A,T164A) alanine mutants that maintain some function, and the expression of the active p26^{Rex}. However, the triple alanine mutant (T164A, S151A, S153A), which contains none of the identified key phosphor acceptor residues, shows minimal functional activity and very little detection of the active p26^{Rex} species.

Typically, stable protein conformational changes require bond rotations. Interestingly, using proteomic protein identification (mudpit) analysis, we recently identified the association of the cellular protein Pin1 in the Rex-2 pull-down complex (data not shown). Pin1 is a peptidylproline cis-trans isomerase (PPIase) of Ser/Thr peptide bonds N-terminal to proline residues. Pin1 is known to induce conformational changes that can affect enzymatic activities, phosphorylation status, subcellular localization, protein stability, and protein/protein interactions (59, 84, 146, 202, 291, 301, 343). One hypothesis consistent with our data is that once Thr-164 is phosphorylated,
Pin1 could bind and cause a structural conformational change rotating the inhibitory C-terminal domain allowing access of the cellular kinase to phosphorylate Ser-151. The single mutants alone only hinder the ability of the kinase to reach its target consequence sequence. For example, S151A or S153A could be phosphorylated at Thr-164 facilitating subsequent phosphorylation of either Ser-153 or Ser-151, respectively, resulting in the formation of p26\textsuperscript{Rex} and modest function. In the case of the mutant T164A, the C-terminal domain is not efficiently dislocated, and since a biological protein is not static, a minimal amount of phosphorylation can occur on both Ser-151 and Ser-153. Whereas, in the double mutant we inhibit not only Thr-164 phosphorylation and thus the conformational change of the C-terminus, but also block any phosphorylation of Ser-151. With this mutant, we detected a greater increase in the amount of p24\textsuperscript{Rex}, which correlates with the reduced function of the mutant protein, with the only possible phosphorylation being Ser-153.

Taken together, the data presented in this study provide a wealth of knowledge in the cellular signaling controlling Rex-2 protein function, structure, and localization. Further studies will be aimed at identifying the cellular proteins including the kinase(s) involved in the regulation of Rex-2 function.

**ACKNOWLEDGMENTS**

We thank Tim Vojt for preparation of the figures and Kate Hayes-Ozello for editorial comments. This work was supported by a grant from the National Institutes of Health (CA100730) to PLG.
Figure 3.1 Functional domains of HTLV-2 Rex. The functional domains of the 170 aa Rex-2 protein are depicted in shaded boxes. The nuclear localization signal (NLS) and the RNA binding domain (RBD) are positioned within the first 19 amino acids of the N-terminus. The activation domain and the nuclear export signal (NES) are located between residues 81-94. This region is flanked by the two multimerization domains. The first lies between amino acids 57-71, whereas the second spans amino acids 124-132. The C-terminal domain that lies between amino acids 150-170, has been reported to be important for Rex-2 functions such as nucleo-cytoplasmic shuttling, and this domain also plays a functional role in protein localization and function.
Figure 3.2 Efficient expression and functional activity of Rex-2. A, illustration of the S-tagged Rex-2 (S-Rex-2) expression vector construct (not drawn to scale). B, Western blot of S-Rex-2 protein expressed from 293T cells transiently transfected with Rex-2 cDNA plasmids. Proteins were detected using rabbit Rex-2 specific antisera. Wild-type p24Rex and p26Rex are indicated and arrows identify S-tagged Rex-2 proteins. C, the functional activity of S-Rex-2 was determined using the modified HIV p24 Gag reporter assay. 293T cells were transfected with 0.25μg pcTat, 0.5μg pCgagRxRE-II, 0.05μg CMV-luc, and 0.1μg of wtRex-2 or S-Rex-2 plasmids. Twenty-four hours post-transfection, cells were harvested and assayed for p24 Gag. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations. D, affinity purification of S-tagged Rex-2 from mammalian cells. 293T cells were transfected with S-Rex-2, purified by S-protein agarose beads, eluted and resolved by SDS-PAGE analysis, and detected by Coomassie Blue staining.
Figure 3.3 Mapping Rex-2 p24 & p26 phosphorylation sites by mass spectrometry. 
A, a compilation of the results obtained with LC-MS/MS analysis of Rex-2. The 170 aa Rex-2 protein is depicted with phosphorylation sites identified (*). The table (inset) shows % total amino acid coverage from LC-MS/MS analysis for both p24\textsuperscript{Rex} and p26\textsuperscript{Rex}. 

<table>
<thead>
<tr>
<th>Rex-2 Species</th>
<th>% Residues mapped</th>
<th>Novel phosphorylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>p26</td>
<td>100</td>
<td>T19, S117, S125, S151, S153, T164</td>
</tr>
<tr>
<td>p24</td>
<td>100</td>
<td>S117, T164</td>
</tr>
</tbody>
</table>

B, a representative MS/MS spectrum of the tryptic phosphopeptide \textit{R.FHLPSFQCESTPPTEMDAWNQPSGISSPPSPSPNLASYPKT}, which identified phosphorylation at Ser-151 is shown. CID Mass spectrum of m/z 1506.8 (3+) revealed a 41 aa peptide of Mₗ 4517.3. B and Y ion designations marked with (+++) are doubly charged. The presence of the y11 ion (*) and the accompanying upstream y-series to S-158 mapped the phosphorylation to Ser-151. The MASCOT peptide score was 77 with an expected score of 0.00043.
Figure 3.4 Mutational analysis of Rex-2 phospho-specific mutants. A, the functional activity of either wtRex-2 or Rex-2 mutants as indicated, were determined using the modified HIV p24 Gag reporter assay. The specific amino acid substitution for each Rex-2 mutant is shown. 293T cells were transfected with 0.25μg pcTat, 0.5μg pCgagRxRE-II, 0.05μg CMV-luc, and 0.1μg of wtRex-2 or Rex-2 mutant plasmids. At 24 h post-transfection, cells were harvested and assayed for p24 Gag. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations. T, threonine; S, serine; A, alanine; D, aspartic acid. B, Western blot analysis of wild-type and Rex-2 mutants. Whole cell lysates normalized for transfection efficiency were subjected to Western blot using rabbit Rex-2-specific antisera. p24Rex and p26Rex are indicated. Densitometry was used to quantitate the amount of p26Rex and p24Rex and the numbers below the gel designate the p26/p24 ratio.
Figure 3.5 Subcellular localization of Rex-2 mutants. To determine the subcellular localization of the Rex-2 mutants, HeLa-Tat cells were transfected with 1μg of a control plasmid, wtRex-2, or various Rex-2 mutants. At 24 h post-transfection, cells were stained with rabbit Rex-2-specific antisera (Green). Nuclei were stained with DAPI (Blue).
Figure 3.6 Functional analysis of Rex-2 S151A, S153A, and T164A combination mutants. A, the functional activity of either wtRex-2 or a Rex-2 mutants as indicated were determined using the modified HIV p24 Gag reporter assay. 293T cells were transfected with 0.25μg pcTat, 0.5μg pCgagRxRE-II, 0.05μg CMV-luc, and 0.1μg of wtRex-2 or Rex-2 plasmids. Twenty-four hours post-transfection, cells were harvested and assayed for p24 Gag. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations.

B, Western blot analysis of wild-type and Rex-2 mutants. Whole cell lysates normalized for transfection efficiency were subjected to Western blot using rabbit Rex-2 specific antisera. p24\text{Rex} and p26\text{Rex} are indicated. Densitometry was used to quantitate the amount of p26\text{Rex} and p24\text{Rex} and the numbers below the gel designate the p26/p24 ratio.
Figure 3.7 Rex-2 phosphorylation continuum and function model. The newly Rex-2 translated product p24^{Rex} is inactive due to the structural and spatial inhibition of the carboxy terminal domain (black ribbon) that masks the RNA binding domain (RBD) and nuclear localization signal. Initial phosphorylation (Pi) of threonine 164 induces a conformational change that results in a p24^{Rex} intermediate. This intermediate can be stabilized further by continual phosphorylation (Pi) of serine 151 and 153 facilitating the dislocation of the inhibitory C terminal domain resulting in a detectable conformation change/gel mobility shift. The conformational change, which results in nuclear localization, allows the fully functional and stable p26^{Rex} protein, via its exposed RBD, to interact with the viral target mRNA (depicted with RxRE secondary structure).
CHAPTER 4

PHOSPHORYLATION REGULATES HUMAN T-CELL LEUKEMIA VIRUS TYPE-1 REX FUNCTION

4.1 Abstract

Human T-cell leukemia virus type 1 (HTLV-1) is a pathogenic complex deltaretrovirus, which is the causative agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis. In addition to the structural and enzymatic gene products, HTLV-1 encodes the positive regulatory proteins Tax and Rex along with viral accessory proteins. Tax and Rex proteins orchestrate the timely expression of viral genes important in viral replication and cellular transformation. Rex is a nucleolar-localizing shuttling protein that acts post-transcriptionally by binding and facilitating the export of the unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm. Rex-1 is a phosphoprotein and general protein kinase inhibition correlates with reduced function. Therefore, it has been proposed that Rex-1 function may be regulated through site-specific phosphorylation. We conducted a phosphoryl mapping of mammalian-expressed Rex-1 using a combination of affinity...
purification and liquid chromatography tandem mass spectrometry. We achieved 100% physical coverage of the Rex-1 polypeptide and identified five novel phosphorylation sites at Thr-22, Ser-36, Thr-37, Ser-97, and Ser-106. We also confirmed two previously identified residues, Ser-70 and Thr-174, but found no evidence of phosphorylation at Ser-177. The functional significance of these phosphorylation events was evaluated by site-directed substitutional mutational analysis. Our results indicate that phosphorylation at Ser-97 and Thr-174 is critical for Rex-1 function in vivo. We completely mapped the site-specific phosphorylation of Rex-1 identifying a total of seven residues; Thr-22, Ser-36, Thr-37, Ser-70, Ser-97, Ser-106, and Thr-174. Overall this work is the first to completely map the phosphorylation sites in Rex-1 and provides important insight into the regulation of Rex-1 function.

4.2 Introduction

Human T-cell leukemia virus types 1-4 are related complex retroviruses that are members of the genus *Deltaretrovirus* (182). HTLV-1 and HTLV-2 are the most prevalent worldwide, whereas HTLV-3 and HTLV-4 were discovered recently in a limited number of individuals in Africa (39, 330). Of the HTLV isolates, only HTLV-1 infection has been clearly linked to the development of adult T-cell leukemia/lymphoma (ATL), an aggressive CD4+ T-lymphocyte malignancy, and various lymphocyte-mediated inflammatory diseases such as HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (77, 251, 341). However, a few cases of atypical hairy cell leukemia or neurologic disease have been associated with HTLV-2 infection (25, 111,
Although the difference in pathology between HTLV-1 and HTLV-2 has yet to be elucidated, it likely results from differential activities of the regulatory and accessory proteins.

In addition to the typical structural and enzymatic retroviral genes gag, pol, and env, HTLV encodes two trans-regulatory genes, tax and rex, which are essential for efficient viral replication/transformation, as well as several accessory genes important for viral infection and persistence in vivo (182). The viral oncoprotein Tax increase the rate of transcription from the viral promoter located in the 5’ long terminal repeat (LTR) (40, 76, 128) and modulates the transcription and activity of numerous cellular genes involved in cell growth, cell cycle control, DNA repair, and cell differentiation (35, 228, 266, 268, 288). The pleiotropic effects of Tax make it essential for efficient viral replication as well as cellular transformation and oncogenesis (274, 280, 332).

HTLV-1 Rex (Rex-1) is a nuclear-localizing and shuttling phosphoprotein that acts post-transcriptionally by preferentially binding, stabilizing, and selectively exporting the unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm, thus controlling expression of the structural and enzymatic proteins that are essential for production of viral progeny (17, 179, 234). Therefore, it has been proposed that Rex-1 regulates the switch from the early, latent phase to the late, productive phase of HTLV infection. Rex-1 binds viral RNAs via a cis-acting RNA sequence termed the Rex-response element (RxRE), which is located in the R region of the viral LTR (345). Mutational analysis of Rex-1 has identified several domains including an arginine-rich N-terminal sequence that functions as an RNA binding domain (RBD) that overlaps with a nuclear localization signal (NLS), a leucine-rich central core activation domain that
contains a nuclear export signal (NES), two flanking Rex-Rex mutimerization domains, and a C-terminal stability domain (31, 32, 110, 123, 235, 254, 271, 296, 326, 334).

Phosphorylation is a well known reversible regulatory event that controls the activity/function of proteins in eukaryotic cells (310). It has been demonstrated that both Rex-1 and Rex-2 are phosphoproteins, and that this modification is critical for their function (1, 2, 102, 103, 234). One study investigating the possible relationship of Rex-1 function and phosphorylation showed that treatment of HTLV-1 infected cells with the protein kinase C inhibitor H-7 [1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine] specifically blocked cytoplasmic accumulation of Rex-dependent gag-pol mRNA (2). The same group reported that Rex-1 is phosphorylated on Ser-70, Ser-177, and Thr-174, with Ser-70 phosphorylation being 12-O-tetradecanoyl-phorbol-13-acetate (TPA) dependent (1). However, a complete phosphorylation map and the identification of the key residues required for function have yet to be elucidated.

In this study, we combined liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (74) of affinity-purified Rex-1 protein in combination with substitution mutational analysis to identify and functionally characterize key phosphorylation sites. The LC-MS/MS analysis achieved 100% coverage of the Rex-1 sequence and revealed five novel phosphorylation sites. We also identified two specific amino acid phosphorylation events found to be critical for Rex-1 function in vivo (Ser-97 and Thr-174). Overall, this work highlights the importance of phosphorylation and how it regulates the biological properties of Rex-1 ultimately controlling the distribution of viral gene expression and productive viral replication.
4.3 Materials and Methods

Cells

293T and HeLa-Tat cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air in Dulbecco’s modified Eagle medium. Medium was supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, penicillin (100 U/ml), and streptomycin (100μg/ml).

Mammalian Expression Plasmid

The Rex-1 expression vector SE356, which contains the HTLV-1 tax/rex cDNA expressed from the cytomegalovirus (CMV) immediate-early gene promoter, has been described previously (76, 339). The S-tagged Rex-1 expression vector S-Rex-1 was constructed by inserting the HTLV-1 tax/rex open reading frame from SE356 into pTriEx4-Neo (Novagen, Madison, WI) in-frame with the amino-terminal His-tag and S-tag via SmaI and BamHI. All generated rex expression vectors contained a previously described mutation in the overlapping tax reading frame (F4Term), which had no effect on the Rex-1 amino acid sequence, but severely truncated Tax-1, completely knocking out expression and function (281). The various rex-1 targeted mutations were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) to introduce targeted amino acid changes. All mutations were confirmed by DNA sequence analysis and vector expression was verified by transfection and Western blot analysis. The human immunodeficiency virus type 1 (HIV-1) Tat expression vector, pcTat, Rex-1
Rex-1 Functional Reporter Assay

The Rex-1 functional assay was performed as described previously with slight modifications (234). Briefly, 0.1 μg Rex-1 cDNA expression plasmid was cotransfected into 293T cells with 0.05 μg of CMV-luc, 0.25 μg of pcTat, and 0.5 μg of Rex-1 reporter plasmid pCgag-RxRE-I using Lipofectimine Reagent (Invitrogen, Carlsbad, CA). Cell lysates were prepared at 24 h post-transfection in Passive Lysis Buffer (Promega, Madison, WI) with a protease inhibitor mixture (Roche Applied Science Indianapolis, IN) on ice for 30 min. Luciferase activity was determined to control for transfection efficiency. HIV-1 p24 Gag levels in the cellular lysates were determined by ELISA (ZeptoMetrix, Buffalo, NY). All transfection experiments were performed in triplicate in three independent experiments and presented as an average with standard deviation.

Immunoblot and Immunofluorescence Analysis

Cell lysates were prepared 24 h post-transfection in Passive Lysis Buffer (Promega, Madison, WI) with a protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) on ice for 30 min. After centrifugation, total protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA). To detect Rex-1, 50
μg of total cell lysates from transfected cells was separated by SDS-PAGE (12%) and transferred to a nitrocellulose membrane (Schleicher & Schuell Biosciences, Keene, NH). Proteins were visualized using polyclonal rabbit α-Rex-1 specific antisera and the enhanced chemiluminescence (ECL) Western blot analysis system (Santa Cruz Biotechnology, Santa Cruz, CA). Subcellular localization of Rex-1 was performed as previously described (322) with slight modifications. HeLa-Tat cells were transfected with 2μg of a control plasmid or S-Rex-1. At 24 h post-transfection, cells were washed and fixed in PBS containing 2% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 and 0.5% FBS for 15 min at 4°C. Cells were incubated in blocking buffer (0.5% FBS and 2mg/ml human IgG) for 30 min at room temperature. Staining was conducted in blocking buffer with rabbit α-Rex-1 specific antisera followed by secondary antibody conjugated to FITC Alexa 488 (molecular Probes, Eugene, OR). Nuclear staining was performed using 4’6-diamidino-2-phenylindole (DAPI) Slow Fade Gold (Invitrogen, Carlsbad, CA). Fluorescence was visualized on an epifluorescence microscope (Olympus, Melville, NY), and digital images were taken using Optronics Imaging System (Goleta, CA).

**Purification of Rex-1 Protein**

Protein purification was performed as described previously with slight modifications (74). Briefly, cell lysate (1.5ml) was incubated with a 75 μl bed volume of S-protein agarose (Novagen) overnight at 4°C, washed twice with a high salt modified RIPA buffer (0.05 M Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 1.0 M NaCl, 0.01
M EDTA) and twice with a low salt modified RIPA buffer (0.05 M Tris-HCl, pH 8.0, 0.1% SDS, 1% Triton X-100, 150 mM NaCl). 100 μl SDS loading dye with β-mercaptoethanol was added to the washed beads followed by boiling for 2 min. Samples were electrophoresed in a 12% SDS one-dimensional polyacrylamide gel and visualized by Coomassie Blue staining. The S-tagged Rex-1 band was excised from the gel for further proteomic analysis.

**Mass spectrometry Analysis**

LC-MS/MS analysis was performed as described previously with slight modifications (74). Briefly, the S-tagged Rex-1 protein band was excised from 1-D polyacrylamide gel, cut into 1-2 mm cubes; washed three times with 500 μl ultra-pure water and incubated in 100% acetonitrile for 45 min. Samples were reduced with 50 mM DTT at 56°C for 45 min and then alkylated with 55 mM iodoacetamide for 1 h at room temperature. The material was dried in a speed-vac, rehydrated in a 12.5 ng/μl modified sequencing grade trypsin solution (Promega, Madison, WI) and incubated in an ice bath for 40-45 min. The excess trypsin solution was removed and replaced with 40-50 μl of 50 mM ammonium bicarbonate, 10% acetonitrile (pH 8.0), and the mixture was incubated overnight at 37°C. Elastase digests were performed as described for trypsin at an enzyme concentration of 15 ng/μl, but without acetonitrile in the reaction buffer. Peptides were extracted 2X with 25 μl 50% acetonitrile, 5% formic acid and dried in a speed-vac. Digests were resuspended in 20 μl Buffer A (5% acetonitrile, 0.1% formic
Acid, 0.005% heptafluorobutyric acid) and 3-6 µl were loaded onto a 12-cm x 0.075 mm fused silica capillary column packed with 5 µM diameter C-18 beads (The Nest Group, Southboro, MA) using a N2 pressure vessel at 1100 psi. Peptides were eluted over 55 min, by applying a 0-80% linear gradient of Buffer B (95% acetonitrile, 0.1% formic acid, 0.005% HFBA) at a flow rate of 150 µl/min with a pre-column flow splitter resulting in a final flow rate of ~200 nl/min directly into the source. In some cases, the gradient was extended to 150 min to acquire more MS/MS spectra. A LTQ™ Linear Ion Trap (ThermoFinnigan, San Jose, CA) was run in an automated collection mode with an instrument method composed of a single segment and 5 data-dependent scan events with a full MS scan followed by four MS/MS scans of the highest intensity ions. Normalized collision energy was set at 35, activation Q was 0.250 with minimum full scan signal intensity at 1 \times 10^5 with no minimum MS^2 intensity specified. Dynamic exclusion was turned on utilizing a three minute repeat count of 2 with the mass width set at 1.0 m/z. Sequence analysis was performed with TurboSEQUEST™ (ThermoFinnigan, San Jose, CA) or MASCOT (Matrix Sciences, London GB) using an indexed Human subset database of the non-redundant protein database from National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/).
4.4 Results

Functional Domains of HTLV-1 Rex

Mutational analyses permitted the assignment of functional properties to distinct domains of the Rex-1 protein (Fig. 4.1). In addition to the characterized nuclear localization signal/RNA binding domain, central core activation domain, two multimerization domains, and the newly identified C-terminal stability domain, three phosphorylation sites have been identified at Ser-70, Ser-177, and Thr-174 by the use of reverse-phase HPLC and sequential Edman degradation (1). However, this approach only provided limited mapping coverage of Rex-1 and the functional relevance of the identified sites were not addressed. To date, no further studies have examined the possibility of other phosphorylation events or the effect of these post-translational modifications on Rex-1 function.

Expression and Detection of Biological Active Affinity S-tagged Rex-1

To identify the amino acid residues of Rex-1 that are phosphorylated in vivo, we employed a tandem affinity purification approach of Rex-1 expressed in mammalian cells. The S-tagged Rex-1 vector (S-Rex-1) expressed full-length Rex-1 protein fused to amino-terminal His₆ and S-tags (Fig. 4.2A). Since the HTLV-1 regulatory proteins Tax and Rex are expressed from the same mRNA in partially overlapping reading frames, a point mutation was made in the nucleotide sequence which added a stop codon in the tax-I reading frame that left the Rex-1 amino acid sequence unchanged (279). This mutation completely abrogated Tax-1 protein expression and function (data not shown). The S-
Rex-1 expression construct was transiently transfected into 293T cells, and the appropriate nuclear subcellular localization of Rex-1 was confirmed by indirect immunoflorescent microscopy (Fig. 4.2B). Wild type Rex-1 was shown as a single 27 kDa band by Western blot analysis using rabbit polyclonal α-Rex-1 antisera (Fig. 4.2C). Next we determined if the S-tagged Rex-1 retained its ability to function in our quantitative reporter bioassay in which HIV-1 p24 Gag production is measured and used as a read-out of Rex-1 functional activity in vivo. As shown in Figure 4.3A, S-Rex-1 displayed significant functional activity, although slightly lower than wtRex-1. We hypothesize that this reduced activity likely is due to the proximity of the amino terminal tag to the RNA binding domain. Taken together, these data demonstrate the proper nuclear subcellular localization and efficient expression of a functionally active S-tagged Rex-1 from mammalian cells.

**Affinity Purification of Rex-1 from Mammalian Cells**

We successfully purified S-tagged Rex-1 protein from transfected 293T cells using S-protein-agarose beads as described in the “Methods”. This purification procedure is based on the strong affinity between the 15-amino acid S-tag and the S-protein that is immobilized on the agarose beads, both of which are derived from RNase S (165). The affinity purified S-tagged Rex-1 protein was resolved by SDS-PAGE and detected by staining with coomassie blue (Fig. 4.3B). This purification process produced adequate quantities of highly purified S-tagged Rex-1 from mammalian cells and allowed the subsequent post-translational modification analysis by LC-MS/MS.
Phosphopeptide Mapping of Rex-1 Using LC-MS/MS

Multiple strategies were employed to determine the phosphorylation sites within Rex-1. The affinity purified S-tagged Rex-1 band was excised and treated as follows. First, the protein was subjected to trypsin enzymatic digestion. The tryptic peptides that were too large to detect were either digested further with elastase or independently digested with elastase. This combined analytical approach allowed us to obtain a detailed physical map covering 100% of the Rex-1 sequence (Fig. 4.4A). Our analysis identified four serine phosphorylation sites at Ser-36, Ser-70, Ser-97, and Ser-106. We also identified three threonine phosphorylated residues at Thr-22, Thr-37, and Thr-174. We did not identify tyrosine site-specific phosphorylation, which is consistent with an earlier report (1).

Substitutional Mutational Analysis of the Identified Rex-1 phosphorylation Sites

To determine possible regulatory roles of the seven identified phosphorylation sites, we generated single alanine amino acid substitutions and tested these Rex-1 mutants to see if they retain their ability to function in our quantitative reporter bioassay. The Rex-1 mutants were transiently co-transfected into 293T cells with pcTat and pCgagRxRE-I, along with CMV-luciferase to account for transfection efficiency. We indentified two mutants S97A and T174A that displayed significantly reduced function (Fig. 4.5A). Further mutational analysis of these two residues by converting them to
phosphomimetic aspartic acid (S97D and T174D) restored functional activity to wtRex-1 levels indicating that phosphorylation plays a positive functional role (Fig. 4.5A). Aspartic acid substitution of Thr-22, Ser-36, Thr-37, Ser-70, or Ser-106 had no effect on protein function, which is consistent with the conclusion that phosphorylation of any of these five residues does not negatively regulate function, but is silent (data not shown). The steady state expression levels of the wild-type and mutant Rex-1 proteins were determined for each mutant by Western blot analysis and detected using rabbit polyclonal α-Rex-1 antisera (Fig 4.5B). All of the Rex-1 mutants were stably expressed. Taken together our data identified five novel phosphorylation sites within Rex-1 and have determined that phosphorylation of Ser-97 and Thr-174 is critical for Rex-1 function in vivo.

4.5 Discussion

Phosphorylation plays a key role in regulating the function of cellular and viral proteins (1, 146, 202, 310, 334). Previously, it was demonstrated that Rex-1 is a phosphoprotein and that phosphorylation may play a role in Rex-1 function (1, 2). It also has been shown that Rex-1 is essential for efficient viral replication and survival in vivo (338). Given the importance of this protein in HTLV biology, we sought to understand how Rex-1 function is regulated. Multiple studies have been directed at understanding the importance of phosphorylation in HTLV Rex-2 function (102, 103, 234, 235). These studies reported that phosphorylation at the carboxy terminus of Rex-2 is critical for protein stability, shuttling, and cellular localization, all of which are positively regulated.
through phosphorylation (235, 334). There have been some efforts aimed at determining
the role of phosphorylation in the regulation of HTLV Rex-1 (1, 2). The first studies,
using thin layer chromatography and tryptic peptide analysis, reported that the native
protein was phosphorylated mainly on serine and threonine. Subsequently, it was
reported that Rex-1 was phosphorylated on three residues; Ser-70, Ser-177, and Thr-174.
This group also speculated that protein kinase C may play a role in Rex-1
phosphorylation, which was supported by drug studies using the more global kinase
inhibitor H-7 (2). Neither study could conclusively identify all phosphorylation sites
within Rex-1 nor were any of the sites further tested for their biological relevance.

In this current study, we were able to not only identify phosphorylated Rex-1, but
also assign phosphorylation to site-specific residues by peptide sequencing using tandem
mass spectrometry. Consistent with previous reports, we confirm that Rex-1 is
phosphorylated predominantly on serine and threonine residues. We report the
identification of five novel phosphorylation sites, Thr-22, Ser-36, Thr-37, Ser-97, and
Ser-106 and also confirm the phosphorylation on Ser-70 and Thr-174. Furthermore, we
identified specific phosphorylation sites that are critical for Rex-1 function in vivo,
specifically Ser-97 and Thr-174. We previously reported that phosphorylation of a
specific residue of Rex-2 at the carboxy terminus (Ser-151) is important for proper
protein nuclear localization (235, 334). Evaluation of functionally disrupted substitution
mutants, S97A and T174A, for subcellular localization revealed no difference when
compared to wild-type (data not shown). It is important to note, that Ser-97 falls within
the previously characterized central core activation domain/nuclear export signal (121),
and that phosphorylation of this residue may be pivotal for proper Rex-1 function.
Previous studies of both HIV-1 Rev and HTLV-1 Rex showed that mutations within the NES interfere with the ability of these proteins to associate with CRM1, a cellular protein that belongs to the importin β family and functions as a nuclear export receptor for NES-containing proteins, and the Rev- and Rex-dependent viral mRNAs encoded by these complex retroviruses (31, 34, 106, 121, 204, 319).

Thr-174, which is located in the carboxy terminus of Rex-1, was identified as a critical phosphorylation site. It previously was shown that Ser-151 located in the carboxy terminus of Rex-2 is a key phosphorylation site, important for Rex-2 function in vivo (234, 235). We also demonstrated that Rex-1 and Rex-2 share a similar stability domain located within their carboxy terminus. (334). We hypothesized that phosphorylation of Thr-174 of Rex-1 (Fig. 4.4B) could play a similar role in regulating Rex-1 function similar to Rex-2 Ser-151. Further C-terminal comparison analysis is ongoing to elucidate further homology between these two related proteins.

One previous study identified phosphorylation on Ser-177 of Rex-1 (1). Throughout our studies, we were unable to confirm this finding, but did identify multiple new sites. One explanation for why these new phosphorylation sites were not identified in the earlier studies could be that the high performance liquid chromatography fraction procedure used may have resulted in a loss of other phosphopeptides within the protein. The selective loss of phosphopeptides can result from the addition of a phosphate group, thus reducing hydrophobicity, which may cause failure of the protein to be retained on the reverse-phase material used in purification (238). An additional consideration is that the previous study analyzed Rex-1 protein derived from Cos-7 cells (African Green Monkey kidney), which may produce alternative post-translational modification patterns
when compared to human cells. Although not without its own caveat and limitations, LC-MS/MS provides a more robust method for the comprehensive mapping of phosphorylation sites (12, 201, 217, 238).

In summary, our data indicate that phosphorylation of specific residues regulates Rex-1 function in vivo. Utilizing a combination of affinity purification, liquid chromatography tandem mass spectrometry, and site-directed mutational analysis we identified two phosphorylated residues, Ser-97 and Thr-174 that are critical for Rex-1 function. Ongoing research in our lab is focused on comparative studies to better characterize the homology of the carboxy terminus of Rex-1 and Rex-2. These studies are focused on uncovering Rex cellular binding partners and kinase(s) and their functional relationship in order to better understand how phosphorylation regulates Rex-1 function. These studies will enable us to determine the differences between the two related proteins and perhaps gain insight into the distinct pathology following HTLV-1 and HTLV-2 infection.
Figure 4.1 Functional domains of HTLV-1 Rex. The functional domains of the 189 aa Rex-1 are depicted in shaded boxes. The nuclear localization signal (NLS) and the RNA binding domain (RBD) are positioned within the first 19 amino acids of the protein. The activation domain and the nuclear export signal (NES) are located between residues 79-99. This region is flanked by the two multimerization domains, the first lies between amino acids 57-66, whereas the second spans amino acids 106-124. Recently, a C-terminal stability domain has been identified spanning amino acids 170-189 (334). Three previously identified phosphorylation sites are indicated: Ser-70, Thr-174, and Ser-177 (1).
Figure 4.2 Efficient expression and detection of affinity-tagged Rex-1. (A) Illustration of the S-tagged Rex-1 (S-Rex-1) expression vector construct (not drawn to scale). (B) To determine the subcellular localization of the S-tagged Rex-1, HeLa-Tat cells were transfected with 1μg of S-Rex-1 or wtRex-1 expression plasmids. At 24 h post-transfection, cells were stained with rabbit α-Rex-1 specific antisera (Green). Nuclei were stained with DAPI (Blue). (C) Western blot of Rex-1 proteins expressed in 293T cells transiently transfected with S-Rex-1 and wtRex-1 cDNA plasmids. Proteins as indicated were detected using rabbit α-Rex-1 specific antisera.
Figure 4.3 Functional activity and expression of S-tagged Rex-1. (A) The functional activity of S-tagged Rex-1 was determined using a HIV p24 Gag reporter assay. 293T cells were transfected with 0.25μg pcTat, 0.5μg pcGagRxRE-I, 0.05μg CMV-luc, and 0.1μg of wtRex-1 or S-Rex-1 expression plasmids. 24 h post-transfection, cells were harvested and assayed for p24 Gag. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations. (B) Affinity purification of S-tagged Rex-1 from mammalian cells. 293T cells were transfected with S-Rex-1 and S-tagged Rex-1 was purified by S-protein-agarose beads, eluted and resolved by SDS-PAGE analysis, and detected by Coomassie Blue staining.
Figure 4.4 Mapping Rex-1 phosphorylation sites by mass spectrometry. (A) A compilation of the results obtained with LC-MS/MS analysis of S-tagged Rex-1. The 189 aa Rex-1 protein is depicted with phosphorylation sites identified (*). The table (inset) shows % total amino acid coverage from LC-MS/MS analysis. (B) A representative MS/MS spectrum of the tryptic phosphopeptide, which identified phosphorylation at Thr-174 is shown. CID Mass spectrum of m/z 1020.42 (3+) revealed a 29 aa peptide of Mr 3058.23. B and Y ion designations marked with (0) indicate a loss of H2O. are doubly charged. The presence of the b10, b11 and b12 ions map the phosphorylation to Thr-174. The MASCOT peptide score was 82 with an expected score of 0.0028.
Figure 4.5 Mutational analysis of Rex-1 phospho-specific mutants. (A) The functional activity of either wtRex-1 or a Rex-1 mutants as indicated, were determined using the modified HIV p24 Gag reporter assay. The specific amino acid substitution for each Rex-1 mutant is shown. 293T cells were transfected with 0.25 μg pcTat, 0.5 μg pCgagRxRE-II, 0.05 μg CMV-luc, and 0.1 μg of wtRex-1 or Rex-1 mutant plasmids. At 24 h post-transfection, cells were harvested and assayed for p24 Gag. The values represent actual p24 Gag production from a representative experiment performed in triplicate. Error bars indicate standard deviations. T, threonine; S, serine; A, alanine; D, aspartic acid. (B) Western blot analysis of wild-type and Rex-1 mutants. Whole cell lysates normalized for transfection efficiency were subjected to Western blot using rabbit Rex-1-specific antisera. Rex-1 is indicated.
CHAPTER 5

SYNOPSIS AND FUTURE PERSPECTIVES

The human T-cell leukemia type 1 (HTLV-1) and type 2 (HTLV-2) are pathogenic human retroviruses that have the ability to transform primary human T-lymphocytes in cell culture and in vivo. Although a plethora of reports have identified the viral protein Tax as a major determinant in this process, the exact mechanism(s) by which HTLV induces cellular transformation has yet to be elucidated. Experiments utilizing the in vitro coculture assays identified that the viral protein Tax is a major determinant in HTLV-mediated transformation, but more studies are needed to unravel the specific mechanism(s) that drive this process. In Chapter 1 we provided a comprehensive overview of HTLV history and biology, dissecting the intricacies and mechanisms of how HTLV infects cells, persists in the host, and ultimately causes disease. Although the last 30 years of research has provided a wealth of knowledge, more studies are needed to fully understand this multifaceted virus. Incorporated within this Chapter, we provide a comprehensive review of the HTLV Tax protein summarizing years of reports on this pleitropic viral protein. In this dissertation, we sought to expand our knowledge of HTLV at both the molecular level, focusing on the post translational modifications of the regulatory protein Rex, and then in vivo to understand how regulating Rex affects HTLV
biology. It has long been thought that Rex may be critical for the transition from the early, latent phase of infection to the late productive phase. It would provide great insight into HTLV biology if we could determine the mechanism(s) involved in regulating Rex function to better understand viral persistence/progression which ultimately leads to disease. A better understanding of the intricate interplay between the virus and host will assist us in developing novel and effective therapies to combat the virus and limit disease.

Tax and Rex are the positive trans-regulatory proteins that are essential for HTLV replication (42, 100, 275). As described in Chapter 1, there have been extensive research efforts that have explored Tax, whereas our understanding of Rex has been more limited. Previous studies utilizing mutational analysis of both Rex-1 and Rex-2 defined several domains within the protein that are critical for function (234, 235). In Chapters 2 and 3 we set out to better understand and characterize the unique functional carboxy terminal domain that was previously identified in Rex-2 and dissect the role phosphorylation plays in Rex function. Using similar experimental approaches, Chapter 4 investigated the role that phosphorylation plays in Rex-1 function. The goal of this research is to underscore the functional similarities and differences between HTLV-1 Rex and HTLV-2 Rex and perhaps gain insight into the precise mechanism(s) that regulate these two proteins and ultimately understand the pathogenic differences between these two closely related viruses.

We conducted extensive in vitro molecular studies to determine the role that the carboxy terminus of Rex-2 plays in regulating function. We built off the identification of a unique phosphorylation mutant S151D/S153D which we previously characterized as being “locked” in an active conformation and functional state (234). This mutant was a
unique reagent, in that by substituting aspartic acids for the serine residues, we eliminated at least one key regulatory control of Rex-2. One caveat is that the mutations (S151D/S153D) in rex-2 also affected the overlapping tax-2 sequence disrupting the transactivation activities of the Tax oncoprotein. To circumvent this problem, we generated new aspartic acid mutants that did not significantly affect Tax-2 function. These mutants included two phosphomimetic charged mutants (P152D and A157D), and two deletion mutations (S151Term and S158Term). Our data indicated that the carboxy terminus of Rex-2 acts as an inhibitory and stability domain for the viral protein, and that this domain is controlled via phosphorylation. We also show that Rex-1 shares a similar stability domain in its carboxy terminus. We propose that the regulation of Rex-2 through phosphorylation provides critical control over the HTLV-2 replication cycle and plays a role in the virus’s ability to infect \textit{in vitro} and persist \textit{in vivo}. In the context of the provirus, the functionally more active Rex-2 mutants A157D and S158Term displayed increased viral protein production, increased HTLV-2 infectious spread, and HTLV-2-mediated cellular proliferation as determined by our short-term quantitative assay. Consistent with our \textit{in vitro} data, rabbits inoculated with the mutant viruses became infected and produced a higher antibody response. Despite the increased antibody response, the infection persisted and the mutant Rex-2 viruses did not alter the proviral load set point when compared to the wild type virus. Although this data expanded our current understanding of Rex-2, we are now confronted with questions regarding the phosphorylation of the protein that needed further investigation.

To determine all phosphorylation events that occur \textit{in vivo}, we conducted a phosphoryl and functional mapping of both structural forms (p24$^\text{Rex}$ and p26$^\text{Rex}$) of
mammalian cell-expressed Rex-2 using affinity purification, liquid chromatography and tandem mass spectrometry. We achieved 100% physical coverage and identified two novel phosphorylation sites in p24Rex at Ser-117 and Thr-164. We also identified six phosphorylation sites in p26Rex at Thr-19, Ser-117, Ser-125, Ser-151, Ser-153, and Thr-164. We then utilized site-directed substitutional mutational analysis by substituting the identified phosphor acceptor residues in Rex-2 with either alanine or aspartic acid. We showed that site-specific phosphorylation at Ser-151, Ser-153, and Thr-164 positively regulates Rex-2 function. We also demonstrate that phosphorylation of Ser-151 is critical for proper protein localization to the nucleus. Building on previous reports and the data presented in Chapter 3, we developed and presented a new Rex-2 phosphorylation continuum model. In Chapter 4 we again used LC-MS/MS to identify key phosphorylation sites within HTLV Rex-1. Using similar techniques, we identified five novel phosphorylation sites at Thr-22, Ser-36, Thr-37, S-97, and S-106. We also confirmed two previously identified residues at Ser-70 and Thr-174 (1). We also showed that Ser-97 and Thr-174 are critical for Rex-1 function.

The data presented here has set the stage for the next wave of experiments that will further divulge into the intricate regulation of Rex. Open questions still remain on the homology between Rex-1 and Rex-2. We have shown that Rex-1 contains a carboxy terminal stability domain similar to that of Rex-2. It is worthy to note that although Rex-1 does not contain heavy phosphorylation on its carboxy terminus, Thr-174 was critical for Rex-1 function. It would be advantageous to carry out further comparison analysis between the two viral proteins in the future. Although the majority of this work is focused on phosphorylation, we have not ruled out other post translational events that could be
participating in Rex functional control. We should take a step back and review other post translational modifications. It would be advantageous to review the Mascot Mass Spec data already retrieved, and look for other modifications such as acetylation, sumoylation, or ubiquination for they have been found to play regulating roles in many cellular and viral processes (46, 74, 198).

Recent experimental designs used to identify cellular binding partners for Tax-1 have been described (35, 74). This group has utilized affinity purification and liquid chromatography tandem mass spectrometry, to identify cellular binding partners for Tax and have compiled a growing list that they call the HTLV-1 Tax “interactome”. It would be valuable to use this method with Rex. Not only would it provide insight into cellular binding partners and possibly identify novel functional roles for Rex, but could potentially identify cellular kinases that are involved in Rex phosphorylation. In addition to the proteomic approaches, computational based bioinformatics, kinase drug inhibition studies, and the use of MEF (Mouse Embryonic Fibroblasts) kinase knockout cell lines could be used to identify cellular kinases and signaling pathways involved in Rex function.

Although, extensive mutational analysis has yielded a wealth of knowledge about Rex structure/function, we still do not have a 3-dimensional/spatial understanding of the viral protein. In order for us to move forward and answer the pressing questions regarding protein-protein interactions, protein-RNA interactions, and the hopes of developing small molecule drug inhibitors we need a crystal structure of Rex. Although extensive research for the last 30 years have given us vast information on the function of Rex, little has been achieved on structure, in fact, there is so little known that new
computational bioinformatic prediction programs are unable to develop a working model for Rex. It would assist us to advance our efforts into the chemistry of Rex via nuclear magnetic resonance (NMR) and protein structural crystallography. The data gained from these studies would greatly enhance our current knowledge on Rex-RxRE binding and also Rex-protein interaction and protein conformations. These techniques are not new to the field. The NMR of HIV-1 Rev and its RNA binding RRE were resolved more than a decade ago (21). Since that discovery, crystal structures have been elucidated. I propose to build on these developed techniques to unravel the mystery of Rex.

Current and continued investigations of these future directions have several implications. First, they will expand our knowledge of basic HTLV biology and retroviruses in general. Second, HTLV-associated malignancies continue to be a vital problem in many parts of the world, thus the need to develop a vaccine has never been greater. Understanding the mechanism of the regulatory proteins and how they regulate viral replication might prove to be critical for the development of vaccines or novel therapeutic strategies.


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