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ROLE OF ACCESSORY PROTEINS OF HTLV-1 IN VIRAL REPLICATION, T CELL ACTIVATION, AND CELLULAR GENE EXPRESSION

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TABLE OF CONTENTS

1. ABSTRACT

Human T-cell lymphotropic virus type 1 (HTLV-1), causes adult T cell leukemia/lymphoma (ATLL), and initiates a variety of immune mediated disorders. The viral genome encodes common structural and enzymatic proteins characteristic of all retroviruses and utilizes alternative splicing and alternate codon usage to make several regulatory and accessory proteins encoded in the pX region (pX ORF I to IV). Recent studies indicate that the accessory proteins $p12^{I}$, $p27^{I}$, $p13^{II}$, and $p30^{II}$, encoded by pX ORF I and II, contribute to viral replication and the ability of the virus to maintain typical *in vivo* expression levels. Proviral clones that are mutated in either pX ORF I or II, while fully competent in cell culture, are severely limited in their replicative capacity in a rabbit model. These HTLV-1 accessory proteins are critical for establishment of viral infectivity, enhance T-lymphocyte activation and potentially alter gene transcription and mitochondrial function. HTLV-1 pX ORF I expression is critical to the viral infectivity in resting primary lymphocytes suggesting a role for the calcineurin-binding protein $p12^{I}$ in lymphocyte activation. The endoplasmic reticulum and cis-Golgi localizing $p12^{I}$ activates NFAT, a key T cell transcription factor, through calcium-mediated signaling pathways and may lower the threshold of lymphocyte activation via the JAK/STAT pathway. In contrast $p30^{II}$ localizes to the nucleus and represses viral promoter activity, but may regulate cellular gene expression through p300/CBP or related co-activators of transcription. The mitochondrial localizing $p13^{II}$ induces morphologic changes in the organelle and may influence energy metabolism infected cells. Future studies of the molecular details HTLV-1 "accessory" proteins interactions will provide important new directions for investigations of HTLV-1 and related viruses associated with lymphoproliferative diseases. Thus, the accessory proteins of HTLV-1, once thought to be dispensable for viral replication, have proven to be directly involved in viral spread *in vivo* and represent potential targets for therapeutic intervention against HTLV-1 infection and disease.

2. INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1), the first described human retrovirus, causes adult T cell leukemia/lymphoma (ATLL), an aggressive CD4-T cell malignancy and initiates a variety of immune mediated disorders including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a chornic neurodegenerative

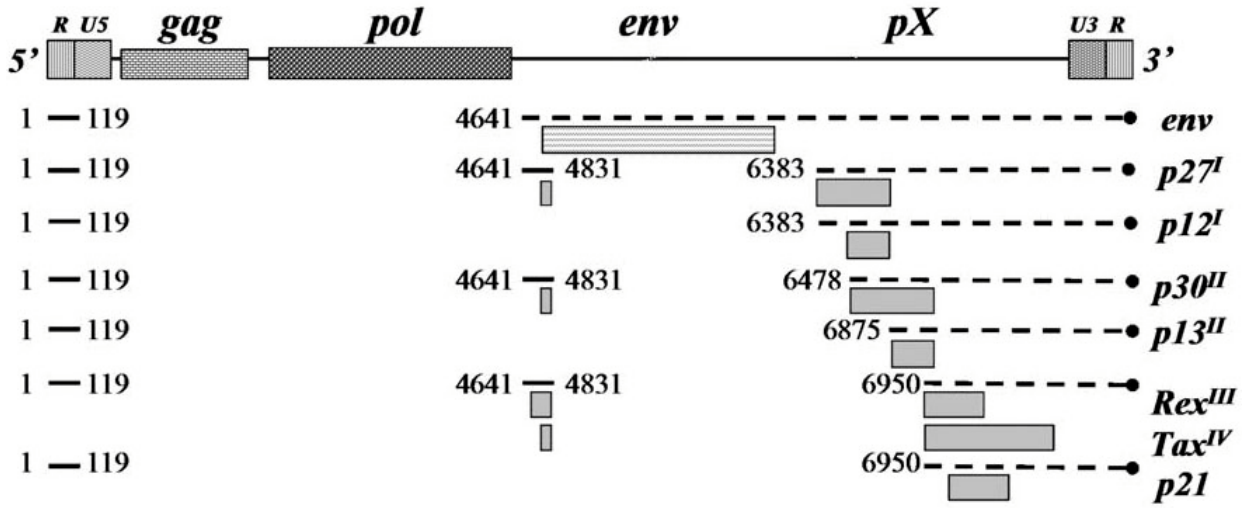

Figure 1. Diagrammatic illustration of HTLV-1 genome with nucleotide location (#) shown for spliced sites. Proteins produced or predicted from RNA shown on right with ORF origin shown as superscript.

disease (1-3). This virus infects approximately 20 million people worldwide and is endemic to the Caribbean, Japan, Africa, and South America (4-7). It is also a serious problem among at-risk groups in the United States (7). The details of how HTLV-1 promotes the development of these diseases is not clear, but is likely related to the ability of the virus to evoke lymphocyte activation (8-12). The epidemiology and diseases associated with HTLV-1 are well known, however the molecular mechanisms used by the virus to establish persistent infection and subsequently facilitate lymphocyte proliferation while circumventing immune elimination, remains less well defined.

The genome of HTLV-1 encodes structural (e.g., group specific core antigens, Gag) and enzymatic proteins (e.g., reverse transcriptase, RT) characteristic of all retroviruses. Additionally, HTLV-1, a complex retrovirus, utilizes alternative splicing mechanism and internal initiator codons, to make several regulatory and accessory proteins encoded by four open reading frames (ORFs) of the pX region (pX ORF I to IV) between the *env* gene and the 3' long terminal repeat (1, 13). ORF IV of HTLV-1 encodes the well-characterized Tax transactivating protein, while the ORF III encodes Rex, a key regulator of viral RNA transport. Tax is a 40 kD nuclear-localizing phosphoprotein, which interacts with cellular transcription factors to activate transcription from the viral promoter (Tax responsive element, TRE), as well as the enhancer elements of various cellular genes associated with host cell proliferation1 (4-18). Rex is a 27 kD, nucleolar-localizing phosphoprotein that functions to enhance nuclear export of unspliced or singly spliced viral RNA thus contributing to virus propagation (19-23). Both Tax and Rex have been the subject of recent reviews (15-17, 19, 24-27). In this review we focus on the important role that accessory proteins have in HTLV-1 replication and pathogenesis. Base on recent studies it has become clear that these proteins are, in fact, essential for the virus life cycle and may determine disease outcome associated with HTLV-1 infection.

3. HTLV-1 ACCESSORY PROTEINS ENCODED BY THE pX REGION

The HTLV-1 pX genome region includes ORF I and II that produce alternatively spliced forms of mRNA, which encode four accessory proteins, $p12^{I}$, $p27^{I}$, $p13^{II}$, and $p30^{II}$ (Figure 1) (28-31). pX ORF I mRNA is produced by alternative splicing events that combine the second exon of Tax with additional downstream sequences and encodes $p27^{I}$ (152 amino acids long) and $p12^{I}$ (99 amino acid long). $p12^{I}$ can be translated from a singly spliced message produced by direct splicing of nucleotide 119 to the splice acceptor at position 6383 or by initiation at an internal methionine codon in the $p27^{I}$ ORF I (32). $p12^{I}$ is thought to be preferentially expressed from the $p27^{I}$ mRNA since transfection of expression plasmids containing HA1-tagged versions of either the full-length $p27^{I}$ cDNA or the cDNA for the singly spliced $p12^{I}$ produced only the smaller $p12^{I}$ protein (30). However, using *in vitro* transcription-translation systems, Ciminale *et al*. (32) produced $p27^{I}$ from the doubly spliced mRNA. Therefore, removal of the internal $p12^{I}$ AUG start codon could yield detectable levels of $p27^{I}$. Interestingly, Pique *et al*. (33) demonstrated production of cytotoxic T cells in HTLV-1-infected subjects that were reactive against peptides representing all putative pX accessory proteins, including $p27^{I}$. The accessory proteins encoded by pX ORF II of HTLV-1 are produced from two alternatively spliced mRNAs. The larger protein, $p30^{II}$, is encoded by a doubly spliced message including the first and second exon of Tax spliced to the splice acceptor site at position 6478 (31). $p13^{II}$, the smaller protein contains the C-terminal 87 amino acids of $p30^{II}$ and is produced from a singly spliced message by splicing of the first Tax exon directly to the splice acceptor at position 6875 or translated from an internal methionine codon within $p30^{II}$ (30, 31). HTLV-1 accessory proteins were originally thought to be dispensable for viral

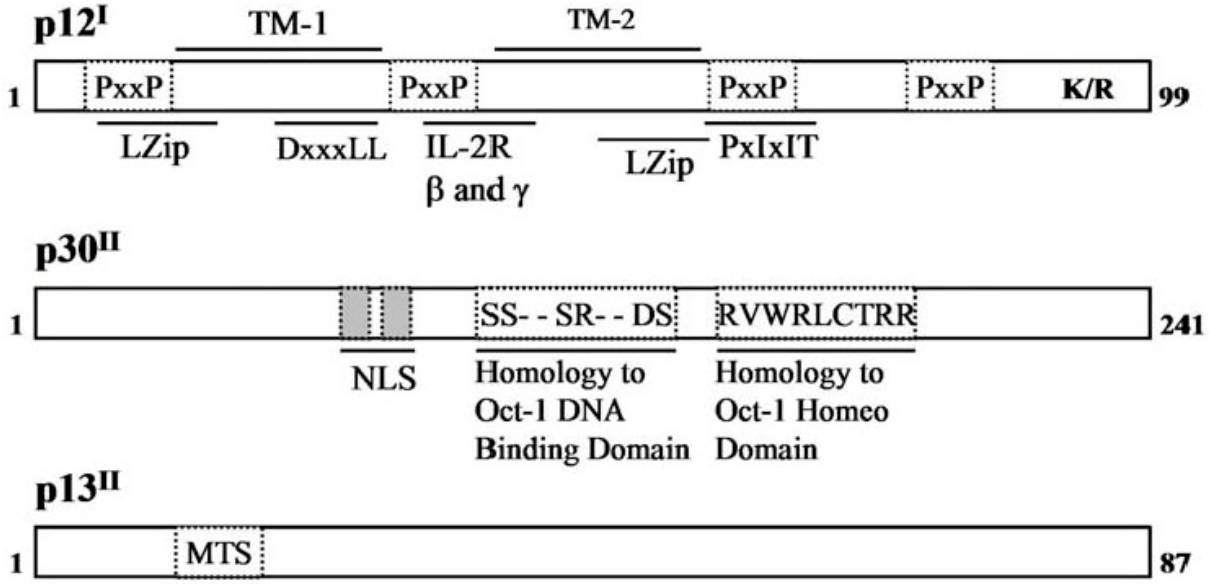

Figure 2. Diagram of key HTLV-1 accessory proteins with known motifs. PxxP = proline motifs predicted to be SH3 binding domains, LZip = leucine zipper like motif, DxxLL = putative AP-1 adaptor binding motif, IL-2R = region of IL-2 receptor binding, PxIxIT = calcineurin binding motif, NLS = nuclear localization motif, MTS = mitochondrial targeting signal.

replication (34). However, recent investigations performed by our laboratory and others have shed light into the role of the HTLV-1 accessory proteins in viral infectivity, maintenance of high viral loads, host cell activation, and regulation of gene transcription (35-48).There is evidence that pX ORFs I and II mRNAs and proteins are expressed both *in vitro* and *in vivo*. Studies employing reverse transcription-PCR (RT-PCR) assays have detected the presence of these mRNAs in infected cell lines and freshly isolated cells from HTLV-1-infected subjects (30) while semi quantitative RNase protection assays have identified these mRNAs in ATL and HAM/TSP patients (49). In HTLV-1 infected patients, and asymptomatic carriers, studies have determined the presence of antibodies (45, 50) and cytotoxic T cells (33) against recombinant proteins or peptides of the pX ORF I and II proteins. However, to date, there are no conclusive reports on the temporal expression of Tax, Rex and the four accessory proteins messages in terms of their specific quantities or relative levels. Chronically infected cell lines were found to have pX-tax/rex mRNA at 500-fold to 2500-fold higher levels than pX tax-ORF II mRNA and 1000-fold higher levels than pX-rex-ORF I mRNA (D. Derse personal communication).

Interestingly, analogous gene regions encoding the accessory proteins, especially the pX ORF I-encoded $p12^I$, are highly conserved in the closely related virus HTLV-2 and the nonhuman primate counterpart, simian T-cell lymphotropic virus type 1 (STLV-1) (13). Further illustration of the conserved nature of these gene regions comes from studies of another member of the deltaretroviruses, bovine leukemia virus (BLV). BLV, like HTLV-1, contains an X region between the *env* sequences and the 3' long terminal repeat. Two proteins are expressed from this region of BLV: the protein R3, which shares a common nuclear localization signal (NLS) with the Rex protein of HTLV-1, and G4, an arginine rich protein that may exist as two isoforms following protease processing (51, 52). Similar to HTLV-1, deletion of homologous sequences from BLV infectious molecular clones encoding these accessory proteins, R4 and G3, results in decreased viral loads in the experimental sheep model (51, 53, 54). Collectively these studies illustrate that these retroviruses, which are all associated with lymphoproliferative disorders, during the course of their evolution have retained conserved gene regions that apparently serve analogous functional roles.

4. pX ORF I $p12^I$

4.1. Biochemical Characteristics of $p12^I$: Features of a Signaling Molecule

HTLV-1 $p12^I$ is a highly hydrophobic protein, which contains a significant percentage of leucine (32%) and proline (17%) residues 30. Hydropathy and immunogenicity plots demonstrate a minimal number of soluble regions and two putative transmembrane domains extending from amino acid 12 to 30 and amino acid 48 to 67 (48, 55), which overlap with two predicted leucine zipper motifs that form alpha-helices. These distinct structural features could contribute to membrane localization or homo-oligomerization of the protein (Figure 2). Immunoprecipitation and immunoblot analysis have demonstrated that $p12^I$ indeed forms at least dimers, if not oligomers (48). However, helix-breaking proline residues within the predicted leucine zippers suggest that these may not be functional leucine zippers. Additionally, $p12^I$ contains four predicted SH3-binding motifs (Figure 2). Typically, SH3-binding motifs in cellular signaling proteins are proline rich with a minimal core of PXXP and are often preceded by an arginine residue at +2 (56). Interestingly, amino acids 8 to 11 and 70 to 74, encoding the first and third PXXP motifs of $p12^I$ are highly conserved among viral strains, suggesting a possible role for these domains in the function of $p12^I$. We have described another conserved

sequence (PSLP(I/L)T) extending from amino acid 48 to 99 in $p12^I$, highly homologous to the P*XIX*IT calcineurin-binding motif of NFAT and found to be critical for the interaction between $p12^I$ and calcineurin (35). In addition, $p12^I$ contains a dileucine motif (DXXXLL) at amino acid positions 26 to 31, with no functional role ascertained yet (Figure 2). However, dileucine motifs have been described in viral proteins such as HIV Nef and are commonly involved in directing protein trafficking through endosomal compartments by mediating association of the protein with adapter protein 1 (AP-1) to AP-3 (57). In addition to the motifs described above, sequence analysis of $p12^I$ suggests possible post-translational modifications of the protein. There is a ubiquitynation motif surrounding the lysine at position 88 of $p12^I$. Although the functional significance of this motif remains unclear, arginine substitution at this position, commonly found among natural HTLV-1 strains, significantly enhances the half-life of the protein (48). In addition to the ubiquitylation site, potential phosphorylation sites and glycosylation sites are also present in $p12^I$. Sequence analysis reveals a putative phosphorylation site of protein kinase C (LTMR) at amino acid 75, a potential phosphorylation site of casin kinase 2 (SPGD) at amino acid 23, a potential N-linked glycosylation site at amino acid 51 (Asn) and multiple potential O-linked glycosylation sites (Ser and Thr) in HTLV-1 $p12^I$. However, a deglycosylation study demonstrated that $p12^I$ is not a glycoprotein (55) while $p12^I$ does not appear to be a phosphoprotein (S. Kim, personal communication).

4.2. Subcellular Localization of $p12^I$ and Interactions with Cellular Proteins

HTLV-1 $p12^I$ was originally reported to localize in cellular endomembranes, which was suggested by a spider-like staining of cells expressing the viral protein (29). We characterized the subcellular localization of $p12^I$ in transfected 293T cells and Hela-Tat cells by multiple methods including immunofluorescent confocal microscopy, electron microscopy and subcelluar fractionation. Our findings indicated that $p12^I$ accumulates in the endoplasmic reticulum (ER) and cis-Golgi apparatus and remained unchanged following both cycloheximide (blocking *de novo* protein synthesis) and brefeldin A (disrupting ER-to-Golgi protein transport) treatments, indicating the protein is retained in the ER and cis-Golgi. Using coimmunoprecipitation assays, we identify the direct binding of $p12^I$ with both calreticulin and calnexin, resident ER proteins, which regulate calcium storage. These results indicated that $p12^I$ directly binds key regulatory proteins involved in calcium-mediated cell signaling and suggested a role of the viral protein in the establishment of HTLV-1 infection by activation of host cells (55).

HTLV-1 $p12^I$ shares sequence homology with the bovine papilloma virus (BPV) E5 protein and Epstein-Barr virus (EBV) LMP-1 protein (58). The region of highest homology starts after the first transmembrane domain and extends into the second. Like E5, HTLV-1 $p12^I$ also binds to the 16 kD subunit of the vacuolar H^+-ATPase (16K). Although $p12^I$ itself cannot induce the transformation of mouse C127 fibroblasts, it cooperates with E5 and potentiates the transforming ability of E5 (58). Further study to map the motif in $p12^I$ associated with 16K protein revealed that a central proline-rich region between aa 36-48 is required for the interaction, but this region alone is not sufficient for binding (59). Although this association appears to be required for the E5-mediated transformation of epithelial cells, there is no clear correlation with the weak transforming ability of $p12^I$, the $p12^I$-16K interaction and cooperative transformation with BPV E5. Thus, the functional significance of the $p12^I$-16K interaction remains to be determined. Interestingly, Nef, a key accessory protein of simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV), binds the catalytic subunit NBP-1 of the ATPase (60). NBP-1 association of Nef mediated by the Nef C-terminal flexible loop is critical for Nef-dependent internalization of CD4 and viral infectivity (61).

Analogous to E5, $p12^I$ also associates with immature form of IL-2 receptor β and γ chain when transiently co-expressed, resulting in reduced surface expression of the receptor chains (62). The IL-2R binding region of $p12^I$ mapped to the central proline-rich region (amino acids 37 to 47), which lie directly in front of the C-terminal proposed transmembrane domain of the protein. The $p12^I$-binding site on the IL-2R chain overlaps with the binding site for JAK kinases 1 and 3 and the adapter protein Shc. $p12^I$ does not influence JAK3 kinase activity directly, however it is considered to be accountable for the modest increase in STAT5 DNA binding activity in 293T cells co-transfected components of the IL-2 receptor signaling complex and in primary human lymphocytes transduced with a lentiviral $p12^I$-expressing vector (63). As a consequence, $p12^I$-expressing cells displayed a decreased requirement for IL-2 to induce proliferation during suboptimal stimulation with anti-CD3 and anti-CD28 antibodies (63). However, peripheral blood derived lymphocyte cell lines immortalized by a HTLV-1 proviral clone ablated for pX ORF I expression, ACH.$p12^I$, have intact IL-2 receptor signaling pathways (64). A possible explanation for these conflicting observations is that $p12^I$ may modestly activate IL-2 receptor pathways during the early stages of HTLV-1 infection before immortalization. Nevertheless, $p12^I$ does not appear to be necessary for the activation of the IL-2R-associated Janus kinases, JAK1 and JAK3, or their downstream effectors STAT3 and STAT5, after immortalization. Collectively, these studies indicate that $p12^I$ may induce STAT activity to confer a growth advantage to infected cells during the early stages of infection, before immortalization. Future studies are necessary to elucidate the JAK3-independent pathway $p12^I$ uses to induce STAT5 activation.

HTLV-1 $p12^I$ was reported to associate with immature forms of the major histocompatibility complex class I (MHC I), interfere with the interaction of MHC I with β_2-microglobulin, decrease the surface expression of transfected MHC-I and direct its degradation in the proteasome while co-expressed in Hela-Tat cells (65, 65). Additionally, the surface expression of endogenous MHC-I is decreased in Jurkat cells transduced with a $p12^I$ encoding

lentiviral vector. These results suggest that $p12^I$ might help the virus escape immune surveillance by down regulating MHC-I surface expression. However, levels of MHC-I and II were similar between T-lymphocytes immortalized with the wild type and $p12^I$-mutant HTLV-1 molecular clones (ACH and ACH.p12 respectively), indicating that $p12^I$-mediated modulation of MHC-I surface expression likely occurs only during the early stages of infection (64). Intriguingly, the accessory proteins $p10^I$ and $p11^V$ of HTLV-2 also associate with MHC-I, however these do not bind to either 16K or IL-2Rβ or γ (66). Additionally, HIV-1 Nef also binds to and down regulates the cell surface expression of MHC-I and is believed to contribute to immune evasion by HIV-1 (67). However, down regulation of MHC-I of virus-infected cells does not explain the early loss of infectivity of a molecular clone of HTLV-1 that lacks ORF I expression, as virus infection is blocked as early as 1 week post-inoculation, before a possible active immune response occurs (43). Future studies of early virus replication immediately after inoculation of virus infected cells in animal models might provide evidence to whether $p12^I$ indeed down regulates MHC-I expression on infected PBMC *in vivo* and actively contributes to viral spread or persistence.

In addition, $p12^I$ associates with two ER-resident calcium-binding proteins, calreticulin and calnexin (55) and the calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin (35). Calreticulin, a highly conserved and ubiquitous protein, serves as one of the major calcium-binding proteins in the ER, participates in calcium signaling, and has been linked to activation of the transcription factor nuclear factor of activated T cells (NFAT) (68). Through protein-protein interactions, calcineurin regulates transcriptional activation of NFAT by triggering the dephosphorylation and subsequent nuclear translocation of NFAT, which results in transactivation of NFAT-inducible cytokine genes including interleukin-2 (69-71). It would be advantageous for a virus to target such conserved proteins to dysregulate calcium signaling pathways to activate and modulate NFAT in infected T lymphocytes.

4.3. Role of 12^I in Regulation of Viral Infectivity *in vitro* and *in vivo*

Earlier reports showed that deletion of pX ORF I from HTLV-1 infectious molecular clones did not affect the viral infectivity and primary lymphocyte transformation mediated by HTLV-1 infection *in vitro* (34, 72). In contrast, our research group demonstrated that selective elimination of pX ORF I from the molecular clone ACH resulted in dramatically reduced viral infectivity *in vivo* (43). Rabbits inoculated with ACH.p12, harboring selective mutations that abolish the expression of pX ORF I mRNA, failed to establish persistent infection as indicated by reduced anti-HTLV-1 antibody responses, failure to demonstrate viral p19 antigen production in PBMC cultures, and only transient detection of provirus by PCR (43). A major difference between these *in vitro* and *in vivo* studies is that standard *in vitro* co-culture techniques used to transmit virus to naive PBMC utilize target cells stimulated by IL-2 and mitogen. However, *in vivo* the majority of circulating and tissue-associated lymphocytes are nondividing.

In support of these findings, using co-culture assays that would allow transmission of the virus to resting primary lymphocytes, our group demonstrated that pX ORF I mRNA is critical for viral infectivity in non-activated/quiescent PBMCs *in vitro* (42). In this study, HTLV-1 was transmitted by co-culturing naive PBMCs with three different virus producing cells, including HTLV-1 immortalized cell lines, ACH transfected 293 T cells and newly infected PBMCs, in the absence of exogenous stimuli to more accurately reflect the virus-cell interactions during the natural infection. Under these conditions, a significant decrease in viral infectivity of ACH.p12 producer cells was detected in primary lymphocytes. More importantly, viral infectivity was restored upon addition of IL-2 and mitogen to the co-cultured PBMCs (42). These data provided the first evidence that HTLV-1 $p12^I$ is required for optimal viral infectivity in nondividing primary lymphocytes and suggested a role of $p12^I$ in T-lymphocyte activation and in the early stage of viral infection. Analogously, studies of HIV-1 Nef indicate that the accessory protein is dispensable for transmission of the virus to activated target cells *in vitro* but is required for viral infectivity in nondividing lymphocytes (73-75).

4.4. Role of 12^I in Calcium-Mediated T Cell Activation

Reports from our laboratory illustrated that $p12^I$ expression in Jurkat cells results in ~ 20-fold activation of NFAT dependent gene expression, while AP-1 or NF-κB-mediated transcription remained unchanged (36). $p12^I$ specifically activates NFAT in synergy with Ras/MAP kinase activation stimulated by the phorbol ester, PMA. By inhibition of proximal signaling molecules, such as phospholipase C- γ (PLC-γ) and LAT, as well as distal signals, including calcineurin and NFAT, the function of $p12^I$ was mapped to be between PLC-γ and calcineurin (36). $p12^I$ mediated NFAT activation was dependent on cytosolic calcium since this function was abolished by BAPTA-AM treatment. Importantly, $p12^I$ functionally substituted for thapsigargin, which specifically depletes the ER calcium store by blocking the ER calcium ATPase. Therefore, HTLV-1 $p12^I$ was found to activate NFAT-mediated transcription in lymphoid cells in a calcium-dependent manner. Indeed, $p12^I$ expression increases the base-line cytoplasmic calcium concentration in Jurkat T cells. This basal elevated calcium is likely secondary to reduced ER stores calcium content and subsequently higher extracellular calcium entry (37). Both the calcium channel on ER membranes, IP_3R, and the calcium channels on plasma membranes, calcium release activated calcium channels (CRAC), contribute to the $p12^I$ mediated NFAT activation, strongly indicating the modulation role of $p12^I$ on calcium homeostasis.

The localization of $p12^I$ to the ER appears to be essential for NFAT activation, since diffusely localizing $p12^I$ truncation mutants were unable to activate NFAT, and partially restored the ability to activate NFAT when redirected to ER by addition of ER localization signal (76). $p12^I$ colocalizes with the ER-resident, calcium-binding

proteins calreticulin and calnexin, which are involved in multiple cellular functions including calcium homeostasis, protein folding, as well as integrin mediated signaling pathways (55). The direct binding between calreticulin and $p12^I$ does not correlate with the NFAT activation since expression of calreticulin dramatically reduced $p12^I$-induced NFAT activation independent of direct calreticulin-$p12^I$ interaction (37). Future studies are necessary to identify the biological significance of this protein-protein interaction in HTLV-1 infection and pathogenesis.

Interestingly, expression of $p12^I$ increased cytosolic calcium, indicating that HTLV-1 $p12^I$ induces release of calcium from the ER to activate NFAT (37), which would be an advantage for the virus during the early stages of HTLV-1 infection. Interestingly, this is similar to the function of a cellular protein CAML (Ca^{2+}-modulating cyclophilin ligand), which also contain two putative transmembrane domains like $p12^I$, colocalizes with calreticulin in the ER, induces calcium release from the ER and leads to NFAT activation (77). In addition, $p12^I$ mediated increase in NFAT activity could cause complete activation of cellular stimuli that would normally induce only partial activation of T cells (e.g., through AP-1). These stimuli could be triggered by cytokines or chemokines released from infected neighboring cells or by direct contact between viral envelope proteins and certain cell surface receptors on newly targeted lymphocytes prior to viral entry (78).

Expression of NFAT induces a highly permissive state to overcome the blockade at reverse transcription and permitted HIV replication in primary $CD4^+$ T cells, therefore it is possible that $p12^I$ causes T cells to be hypersensitive to T cell receptor and CD28 stimulation and thus highly permissive for subsequent viral infection. Interestingly, susceptibility of these cells to HIV infection could be restored by mitogen treatment, likely due to the phytohemagglutinin-induced upregulation of NFAT activity. This is similar to earlier reports from our laboratory that addition of mitogens can rescue the infectivity of a $p12^I$ mutant viral clone in resting PBMC (42), likely by overriding the requirement for $p12^I$-induced activation of NFAT. It will therefore be critical to examine the effect of cyclosporine in HTLV-1 replication in primary lymphocytes and compare the drug's capacity to affect replication of wild type and $p12^I$ mutant clones. Interestingly, cyclosporine reduces the infectivity of HIV and is strongly dependent on the presence of a functional *nef* gene (79).

Other retroviruses also encode proteins regulating Ca^{2+}- related signals by analogous or different mechanisms in T lymphocytes or other cell types. HIV accessory protein, Nef, also activates NFAT in synergy with Ras/MAPK pathway in a calcium dependent fashion (73, 80, 81). Similar to $p12^I$, Nef is dispensable for viral infection of activated target cells *in vitro*, but is required for viral infectivity in quiescent lymphocytes (75, 82-84). Nef contains SH3 domain binding motif responsible for the interaction of this protein with multiple cellular proteins and causes NFAT activation, which is dependent on its interaction with IP_3R (73).

Importantly $p12^I$ appears to enhance the production of a downstream gene of NFAT activation, interleukin-2 (IL-2) in Jurkat T cells and primary lymphocytes, which could be abrogated by calcium chelator BAPTA-AM and calcineurin inhibitor, cyclosporin A, suggesting the effect is calcium pathway-dependent (76). This increase in IL-2 could account for the decrease in requirement for the cytokine in proliferation of human primary lymphocytes in the presence of p12 (I63). Overall, $p12^I$ expression promotes T cell activation and likely facilitates the viral replication and productive infection, which correlates with our previous finding that $p12^I$ is necessary for viral infectivity in a rabbit model of infection (43).

Recent reports from our laboratory further characterized the $p12^I$-mediated NFAT activation by identifying the two positive (aa 33-47, aa 87-99) and two negative (aa 1-14, aa 70-86) regions in $p12^I$ that regulate the NFAT activation, using truncation mutants (76). Interestingly, these two positive and two negative regions contain individual SH3 binding domains (PXXP motif). SH3 binding domain has been demonstrated to be important for protein-protein interaction and the PXXP motif in HIV Nef was found to be necessary for Nef-mediated NFAT activation and viral infection (85-87). In a recent report from our laboratory, proline residues in these motifs were mutated into alanine residues to test the role of PXXP motifs in $p12^I$-mediated NFAT activation. Interestingly, the third SH3 binding domain (aa 70-73) was responsible for the negative effect of region aa 70-86 on NFAT activation (35, 76). Besides, mutations in the first two PXXP motifs (aa 8-11 and 35-38) caused only minimal changes in NFAT activation, while mutations in the last PXXP motif (aa 90-93) in the second positive region (aa 86-99), enhanced the NFAT activation. Future studies are necessary to elucidate the functional significance of specific residues within the PXXP motifs of $p12^I$ in NFAT activation.

We have demonstrated the role of the highly conserved calcineurin binding motif PSLP(I/L)T of $p12^I$ in its binding to calcineurin (35). Interestingly, $p12^I$ competes with NFAT for calcineurin binding as evidenced by the calmodulin bead pull-down experiments. More strikingly, alanine substitution mutations in PSLP(I/L) caused more NFAT nuclear translocation and increased NFAT transcriptional activity (~2-fold) than wild type $p12^I$ in a reporter gene assay (35). Interestingly, PSLP (I/L)T calcineurin binding site is within the aa 70–86 negative region and third P*XX*P motif which was responsible for the negative effect of region aa 70-86 on NFAT activation. Additionally, NFAT-inhibitory function of the PSLP(I/L)T motif in $p12^I$ was verified to be not from the inhibition of calcineurin phosphatase activity. PSLP(I/L)T is homologous to the conserved functionally critical calcineurin-binding motif (P*XIXI*T) in the N-terminal regulatory domain of NFAT, which binds both inactivated and activated calcineurin (88). Many calcineurin-binding

proteins such as the anti-apoptotic protein Bcl-2, calcineurin B homologous protein, a kinase anchoring protein AKAP79, and myocyte-enriched calcineurin-interacting protein 1 inhibit either calcineurin phosphatase activity or its substrate NFAT transcriptional activity (89-91). However, P*XLX*IT motif mediated binding itself does not inhibit the catalytic activity of calcineurin, because NFAT activation requires enzymatic activity of calcineurin as well as binding via this motif. Not surprisingly, $p12^I$ binding to calcineurin via a motif similar to P*XLX*IT did not inhibit calcineurin catalytic activity but instead influenced NFAT and calcineurin interaction by competing for binding with NFAT similar to artificial peptides representing this motif (88).

Due to the existence of a calcineurin-binding motif in $p12^I$, $p12^I$ may have at least two regulatory actions to modulate NFAT activation: (1) positive modulation by increasing cytosolic calcium concentration from ER stores and (2) negative modulation by calcineurin binding. It is unclear why $p12^I$ has two regulatory functions for NFAT transcriptional activity. It is notable that Bcl-2 has these similar properties like $p12^I$, however the functional relationship between calcium release from the ER and calcineurin binding of Bcl-2 is also still unresolved. Bcl-2 maintains calcium homeostasis and prevents apoptosis by localizing not only at the mitochondrial membrane but also in the ER membranes (92, 93). $p12^I$ may function like Bcl-2 at the ER membrane, acting like an ion channel protein to increase ER calcium permeability. Like Bcl-2, $p12^I$ may affect apoptosis in HTLV-1-infected T cells. Another ER membrane protein, CAML that activates NFAT by increasing calcium flux binds with calcineurin indirectly, through its association with cyclophilin (77). Overall, $p12^I$ interacts with calcineurin, an important regulator of NFAT signaling, via a highly conserved PSLP(I/L)T motif, to further T cell activation, an important antecedent to effective viral infection, via a calcium/calcineurin/NFAT pathway.

4.5. Putative Role of $p12^I$ Variants in Disease

Factors determining the progression from asymptomatic state to HAM/TSP and the contribution of viral factors are not fully understood. Recent studies aimed to identify sequence variation/ viral strains that are neuropathogenic suggest a possible role for $p12^I$ in the pathogenesis of HAM/TSP. The proteasome destabilization of viral proteins is thought to be an intracellular defense mechanism against viral infection (94, 95). Lysine is a known target for covalent binding of ubiquitin (94-96) and the metabolic instability $p12^I$ is mediated in part by ubiquitylation at a single lysine residue at position 88 and subsequent proteasomal degradation, as well as by destabilizing residues at its amino terminus (48). Interestingly, earlier analysis of $p12^I$ ORF in 21 HTLV-1 strains from different geographical areas (97) had demonstrated that $p12^I$ amino acid sequence is highly conserved and that, arginine residue is found more frequently at position 88 while the less frequent lysine carrying allele was found only in some TSP-HAM cases. Trovato *et al.* (48) extended this information by studying an additional 32 *ex vivo* samples from healthy carriers, TSP-HAM patients, ATLL patients and families in which both diseases occur, and confirmed that the lysine residue is found only in patients with TSP/HAM, irrespective of geographical locations, suggesting that a selective pressure over $p12^I$ might occur in the host. However, lysine residue at position 88 in $p12^I$ was not found in all HAM/TSP patients. It is hypothesized that the reduced stability of $p12^I$ in HAM/TSP patients due to this sequence variation may facilitate generation of a viral-specific CTL response, since degradation of $p12^I$ would alleviate the reduction of MHC class I molecules at the cell surface (98).

Martins *et al.* extended these observations further to verify whether the presence of lysine at position 88 could be used as a marker of progression to HAM/TSP, by analyzing 37 HAM/TSP patients and 40 asymptomatic carriers at different stages of infection (99). Interestingly, in this study, lysine residue at position 88 of $p12^I$ did not appear to be a universal diagnostic marker for HTLV-1-associated neurological disease, since this phenotype was found not only in HAM/TSP patient (1 out of 37), but also in asymptomatic HTLV-1 carrier (1 out of 40) who did not develop neurologic signs for 5 years (99). Even though this study analyzed a larger number of samples, all individuals in this study were born in the same geographic region, and thus it might simply represent a particular HTLV-1 carrier population in which the selective pressure on the $p12^I$ sequence would not be occurring. Overall, the significance of natural $p12^I$ alleles is unclear, and it is possible that the lysine at position 88 of $p12^I$ might have a significant effect on the biological effects of the protein in the host, including giving a possible selective advantage in individuals with a certain MHC I. Future studies including screening of HTLV-1-infected individuals in other populations may elucidate this further.

5. pX ORF II $p30^{II}$

Earlier studies suggested that ORF II was dispensable for expression of HTLV-1 proteins Tax, Rex, or Env, viral replication and immortalization of primary lymphocytes *in vitro* (34, 72). In addition, the isolation of a viral clone containing a premature stop codon in pX ORF II, from leukemic cells led to the conclusion that pX ORF II was not necessary for the outgrowth of leukemic clones *in vivo* (100). However, possible functional role of pX ORF II during early infection was not be ruled out by these initial studies. To specifically test the functional role of pX ORF II in viral replication *in vivo*, we inoculated rabbits with lethally irradiated cell lines expressing the wild-type molecular clone of HTLV-1 (ACH) and a clone containing selected mutations in pX ORF II (ACH.p30/13) (39). While all ACH-inoculated rabbits became infected as early as 2 weeks postinoculation, ACH.p30/13-inoculated animals failed to become infected or maintained low proviral copy numbers in their blood leukocytes. These animals also had weak and transient *ex vivo* p19 antigen production from their PBMC cultures and anti-HTLV-1 antibody titers declined towards the end of the study. Most strikingly, using quantitative competitive PCR, we demonstrated a dramatically reduced (up to 100-fold) viral load in the ACH.p30/13-infected animals (39). Taken together, these

data suggested that pX ORF II is in fact necessary for maintenance of high viral loads *in vivo*.

5.1. Biochemical Characteristics of p30^{II}: Features of a Transcription modulator

Several lines of evidence indicate that p30^{II} acts as a transcription factor. Importantly, the protein localizes to the nucleus, specifically the nucleolus of cells transiently transfected with a p30^{II} expression vector (29, 41). p30^{II} contains a highly conserved bipartite nuclear localization signal (NLS) between amino acids 71 to 98, which can be functionally substituted for the NLS of Rex (101). In addition, p30^{II} contains serine and threonine-rich regions that share distant homology to the activation domain of cellular transcription factors, such as Oct-1/2, Pit-1, and POU-1 (32) (Figure 2). Taken together, these characteristics suggest that p30^{II} has a role in transcription of viral and cellular gene expression.

5.2. Role of p30^{II} in Modulating HTLV-1 LTR-Mediated Transcription

We have reported that, when provided in limiting concentrations, p30^{II} expression stimulates HTLV-1 LTR-driven reporter gene activity, even in the presence of Tax, whereas higher concentrations represses LTR and CRE-driven reporter gene activity (41). These activities are analogous to herpes simplex virus type 1 (HSV-1) regulation of its immediate-early (IE) gene promoter. VP16, a potent transcription factor from HSV-1, binds the host cell protein HCF, which facilitates the stable complex formation of the viral protein with Oct-1 (102, 103). The IE gene promoter contains an Oct-1-like motif (TAATGARAT) that is important for IE gene expression, with both positive and negative effects, depending on the context of these cellular transcription factors and VP16 (102).

We have also demonstrated that p30^{II} co-localizes with p300 in the nucleus and physically interacts with CREB binding protein (CBP)/p300, at the highly conserved KIX domain, the domain HTLV-1 Tax also interacts with. In addition, p30^{II} is able to disrupt CREB-Tax-CBP/p300 complexes bound to the viral 21-bp Tax Responsive Elements (TRE) repeats (38). To recruit CBP/p300, Tax acts as a high-affinity binding site within this complex (104-106). Once associated with the viral promoter, CBP/p300 is believed to remodel chromatin and/or make communication with the basal transcription machinery possible.

HTLV-1 Tax, a transactivator of LTR mediated transcription, is a key player in the activation of the HTLV-1 viral genes through its interaction with the p300 and CBP coactivators (104, 105, 107, 108). Tax is able to regulate LTR mediated transcription by the recruitment of p300/CBP and P/CAF to these specific sites in the HTLV-LTR promoter (104, 108). Since p30^{II} and Tax interacts with CBP/p300 through the same KIX domain, it is possible that the competitive CBP/p300 binding between p30^{II} and Tax might be the mechanism by which p30^{II} attenuate the formation of these multiprotein complexes and thereby repress transcription on CREB-responsive promoters. Similarly, Tax expression has been demonstrated to interfere with the transcriptional activity of c-Myb and the binding of Tax and c-Myb to KIX domain of CBP was found to be mutually exclusive (109-112).

The coactivators CBP and p300 mediate transcriptional control of various cellular and viral DNA binding transcription factors. Although these coactivators have divergent functions, they are similar in nucleotide sequence, are evolutionarily conserved, and are commonly referred to as CBP/p300 (113, 114). CBP and p300 are highly related and share many functional properties, however there is evidence that these factors are not interchangeable. Several cellular and viral proteins that interact with either CBP or p300 have been identified, including steroid and retinoid hormone receptors, CREB, c-Jun, c-Myb, Sap-1a, c-Fos, MyoD, p53, Stat-1/2, NF-κB, pp90rsk, TATA-binding protein, TFIIB, HTLV-1 Tax, adenovirus E1A, Kaposi's sarcoma-associated herpes virus viral interferon regulatory factor protein, and simian virus 40 large T antigen (113-115). CBP/p300 protein is available only at limiting concentrations within the cell nucleus, causing an environment for competition between coactivators and transcription factors, thus providing an additional layer of regulated gene expression (104, 116, 117). There is evidence of a functional antagonistic relationship between transcription factors, as a result of competition for binding to common regions of CBP/p300 (104, 113, 118). Under such a condition of tight competition, relative concentrations of Tax/ p30^{II} at various stages of disease might be a critical factor in determining the levels of viral transcription. We hypothesize that, at higher concentrations, p30^{II} may support viral persistence by reducing viral expression, and thus reducing immune elimination of HTLV-1 infected cells. Additionally, p30^{II} might also repress cellular genes necessary to maintain viral persistence. However, at low concentrations, p30^{II} enhances TRE/viral over CRE/cellular mediated transcription. p30^{II} has the potential to play a role in promoting viral transcription, cell proliferation, competitively repressing CBP/p300-dependent cellular gene transcription (e.g., p53-dependent p21WAF1/CIP1 gene activity), and for promoting the spread of the virus *in vivo*. This is also consistent with our previous finding that an infectious HTLV-1 molecular clone failed to maintain viral loads *in vivo* when p30^{II} and p13^{II} expression was abolished (39).

Recently, it was reported that p30^{II} modulates LTR mediated transcription, in the context of the entire provirus, by a post-transcriptional mechanism (V. Franchini personal communication). However, our recent evidence indicates that, the role of CBP/p300 cannot be ruled out in the modulation of LTR mediated transcription by p30^{II}, in the context of the provirus (Michael *et al.*, manuscript in preparation). In the presence of increasing concentrations of p300, we were able to rescue the p30^{II}-mediated repression on LTR driven gene transcription, in a dose-dependent manner, irrespective of the presence or absence of the provirus (Michael *et al*., manuscript in preparation). CBP and p300 bridge transcription factors to relevant promoters, has intrinsic histone acetyltransferase (HAT)

activity, and form complexes with proteins such as CBP/p300 binding protein-associated factor, which also exhibits HAT activity (113). Recently, there is increasing knowledge of the mechanism and functional significance of the interactions between many viral proteins and CBP/p300. In the case of adenovirus oncoprotein E1A, interaction with CBP/p300 is critical for regulation of transcription, suppression of differentiation, and immortalization of cells in culture (113, 114, 119). The T antigen of SV40 regulates the expression of a group of cellular genes by modifying the HAT activity of CBP/p300 or by bridging the gap between DNA binding transcription factors and components of the general transcription machinery (113, 114). Identifying the molecular mechanism and functional significance of the interaction between $p30^{II}$ and p300 is very crucial in understanding of the role of $p30^{II}$ in the pathogenesis and replication of this important human pathogen. Therefore to further understand the molecular mechanism and functional significance of the interaction between $p30^{II}$ and p300, using N terminal and C terminal deletion mutants of $p30^{II}$, we have identified the motifs within $p30^{II}$ that are critical in binding CBP/p300 and in regulating LTR mediated transcription, in the presence/ absence of the provirus. Our recent study confirmed the role of $p30^{II}$ as a regulator viral gene transcription, in association with p300. In addition, we identified the amino acid sequence 100-179 of $p30^{II}$ to be the domain critical for its function as a repressor of LTR-mediated transcription, irrespective of the presence or absence of the HTLV-1 provirus (Michael *et al*., manuscript in preparation). This region contains various important features like DNA binding domain and serine and threonine rich residues with homology to Oct-1 and POU family of transcription factors. Interestingly, there are five lysine residues within this domain, all preceded by at least one serine residue (SK motif), the consensus acetylation sequence, thus representing potential acetylation sites for CBP/p300. Intriguingly, the motif found to be critical for binding p300 is amino acid sequence 1-132 (Michael *et al*., manuscript in preparation).

It will be important for future studies to define relevant $p30^{II}$ target genes and perhaps yet-unidentified direct $p30^{II}$-responsive DNA elements. These may include promoters of genes critical for T-cell function, such as the IL-2 promoter, which contains Oct-1-responsive elements (120-123). Further structure-function analyses of $p30^{II}$ will help define the roles of five lysine residues within the domain of $p30^{II}$ critical for repressing LTR mediated transcription. As CBP/p300-mediated acetylation has become a common theme for regulation of protein function, it will be interesting to test whether the intrinsic histone acetyltransferase activity of CBP/p300 can in fact function to acetylate and potentially regulate HTLV-1 $p30^{II}$.

As of yet, there is little information regarding the relative levels of various HTLV-1 proteins during various stages of the infection. It is possible that differences in expression levels of various viral proteins leading to differences in transcriptional regulation, might be the mechanism by which HTLV-1 infection/ disease progresses though various stages. This is likely to be in synergy with differential regulation of cellular gene regulation by Tax and/or $p30^{II}$ and thus changing the immune responses in accordance with different stages of progression of HTLV-1 infection and disease.

5.3. Role of $p30^{II}$ in Modulating Cellular Gene Transcription

Recently, using recombinant lentivirus expressing $p30^{II}$ in Jurkat T lymphocytes and Affymterix U133A human gene chip representing ~33000 genes, we demonstrated the role of $p30^{II}$ as a regulator of cellular gene expression. In addition, we identified several potential new functional roles for $p30^{II}$, in T cell activation or cell signaling and in regulation of apoptosis and cell cycle. More importantly, we illustrated the role of $p30^{II}$ as an activator of many key transcription factors involved in T cell signaling/ activation, such as Nuclear Factor of Activated T cells (NFAT), Nuclear Factor-Kappa B (NF-κB) and Activator Protein-1 (AP-1). Consistent with our previous hypothesis that $p30^{II}$ would modulate immune response, recently we found altered expression of cellular genes involved in immune modulation such as CD46, CD43, CD58, IFNγand CD72 when $p30^{II}$ was expressed (Michael *et al*., manuscript in preparation).

Our recent findings showed that expression of $p30^{II}$ was associated with altered expression of multiple genes associated with transcription and translation, including transcriptional control genes like TATA-binding protein associated factor 4 (TAF4), homeo box genes, T-box genes, proteins containing helix-loop- helix domain, zinc finger proteins, coiled coil proteins, histone deacetylase-6, nuclear receptor coactivator 3, GAS 7 and translation initiation / elongation factors (Michael *et al*., manuscript in preparation). Among these, histone deacetylase-6 (124, 125) and nuclear receptor coactivator 3 (CBP interacting protein) (126-128) with histone acetyltransferase and pCAF/CBP recruiting abilities are particularly interesting, since $p30^{II}$ contains multiple highly conserved lysines, which could play a role in acetylation. Another interesting candidate deregulated by $p30^{II}$ was GAS 7, which has sequence homology to Oct and POU family of transcription factors (129). Based on this, $p30^{II}$ appears to regulate transcription through various mechanisms and different levels, some of which could be attributed to the interaction between $p30^{II}$ and CBP/p300.

5.4. Potential Role $p30^{II}$ in T Cell Activation

Our recent findings showed that $p30^{II}$ expression enhanced NFAT, AP-1 and NF-κB mediated transcriptional activity. In addition, expression of $p30^{II}$ was associated with altered expression of multiple genes involved in various stages of T cell activation, including CD28, Vav-2, CD72, CD46, Lck tyrosine kinase, CHP (an endogenous calcineurin inhibitor), c-Jun and c-Fos, protein kinase D, epidermal growth factor, ETS domain transcription factor Elk-1, IKKγ, HPK-1, Ras GRP2 and NFAT (Michael *et al*., manuscript in preparation).

Expression from the IL-2 promoter requires binding of several transcription factors, including NFAT, AP-1 and NF-κB. NFAT is vital to proliferation of

peripheral lymphocytes for HTLV-1 infection (13) while AP-1 is linked to the dysregulated phenotypes of HTLV-1 infected T cells (130) and malignant transformation (131). Activation of AP-1 occurs through Tax-dependent and independent mechanisms in HTLV-1-infected T cells *in vitro* and in leukemia cells *in vivo* (130). NF-κB is highly activated in many hematopoietic malignancies, HTLV-1 infected T cell lines and in primary ATL cells, even when Tax expression levels are low (131) and due to its anti-apoptotic activity, it is considered to be a key survival factor for several types of cancer. HTLV-1 $p30^{II}$ is so far the only retroviral accessory protein to have broad modulating activities on the transcriptional activity of NF-κB, NFAT and AP-1.

It will be important for future studies to elucidate the mechanisms employed by $p30^{II}$ to enhance NFAT, AP-1 and NF-κB mediated transcription. Interestingly, HTLV-1 $p12^{I}$ stimulates NFAT mediated transcription, when stimulated with PMA, indicating that $p12^{I}$ acts synergistically with Ras/ MAPK pathway to promote NFAT activation and thus may facilitate host cell activation and establishment of persistent HTLV-1 infection (36). Since $p30^{II}$ enhances NFAT driven transcription significantly when stimulated with ionomycin or CD3 (Michael *et al.*, manuscript submitted), it likely employs a different mechanism than $p12^{I}$. It is possible that these two accessory proteins act synergistically to modulate NFAT driven transcription and subsequent T cell activation / signaling. AP-1 is able to interact with transcriptional coactivator CBP/ p300, as well as viral CREs and mediate HTLV-I gene expression. NF-κB and NFAT are also known to interact with transcriptional coactivator CBP/p300 (115). Therefore it is possible that $p30^{II}$ regulates the transcriptional activity of NFAT, NF-κB and AP-1, at least in part, by its interaction with CBP/p300.

5.5. Genes Modulated $p30^{II}$ in Apoptosis, Cell Cycle and Cell Adhesion

HTLV-1 mediated interference with normal T-cell apoptosis is thought to be a mechanism of tumorigenicity, but specific mechanisms by which HTLV-1 infection or any particular HTLV-1 gene products influence on T-cell survival are not fully understood. Similar to the effect of HTLV-1 Tax on apoptosis related genes (24, 132, 133), we have recently used gene array to demonstrate that $p30^{II}$ also deregulates multiple genes resulting in pro-apoptotic and anti-apoptotic effects including Bcl-2 related/interacting genes, the Fas mediated apoptosis pathway genes, caspases and genes associated with the DNA fragmentation pathway (Michael *et al*., manuscript in preparation). Since apoptosis is a well-known mechanism of cellular defense against viral infection, a possible role of $p30^{II}$ in lymphocyte apoptosis might correlate with the requirement of $p30^{II}$ in maintaining proviral loads *in vivo* (39).

Previous studies indicate that several members of the cell cycle machinery have altered expression in HTLV-1 infected cells (134-137). Several studies examined the aberrations in cell cycle caused by HTLV-1 Tax (134); however, not much is known about the role of other HTLV-1 proteins in causing abnormalities in cell cycle. Our data indicated that $p30^{II}$ expression altered the expression of multiple genes involved in regulation of different stages of cell cycle, including checkpoint suppressor 1, histone deacetylase 6, cyclin B1, WEE1 kinase, CDC14A, Lck, JAK2, GAS7, JUN and MDM2 (Michael *et al*., manuscript in preparation). Some of these genes are particularly interesting, for example, MDM2 is overexpressed in certain leukemias (138) and capable of enhancing the tumorigenic potential of cells by inhibiting p300 / PCAF mediated p53 acetylation (139). We also found that $p30^{II}$ expression was associated with altered expression of several genes involved in cell adhesion, including integrins, immunoglobulin (MADCAM1), cadherin, protocadherin, liprin, KIT ligand, CD84 / Ly-9, CD58, CD43 / sialophorin and glycosyl-phosphatidyl- inositol phospholipase D1 (Michael *et al*., manuscript in preparation).

6. pX ORF II $p13^{II}$

6.1. Biochemical Characteristics of $p13^{II}$: Mitochondrial Targeting

Less is known about the function of the smaller protein, $p13^{II}$, encoded by the 87 carboxy-terminal amino acids of the 241-residue Tof or $p30^{II}$ protein. Initial studies demonstrated $p13^{II}$ localization to the nucleus (30), but more-recent reports show mitochondrial localization of the protein (47, 140). Recently, by extraction of mitochondria-enriched fractions, D'Agostino *et al.* demonstrated that full-length $p13^{II}$ is mostly membrane-associated and partly soluble (140). Using electron microscopy, the sub-mitochondrial localization of $p13^{II}$ was further verified to be in the inner mitochondrial membrane and cristae (140). This localization is mediated by an atypical mitochondrial targeting sequence (MTS) in the N terminus of $p13^{II}$ between amino acids 22-31 (Figure 2). The 10-amino-acid MTS also targets green fluorescent protein to mitochondria when fused to the N terminus of green fluorescent protein (47). Importantly, a fusion protein of the $p13^{II}$ MTS with HIV Rev can localize to mitochondria, indicating that the $p13^{II}$ MTS is, at least in part, able to override the potent NLS of Rev (101). While the $p13^{II}$ MTS is also present in $p30^{II}$, $p30^{II}$ is not localized to the mitochondria, suggesting that the signal has to be near the amino-terminus to direct mitochondrial targeting (141). Analysis of the amino acid sequence of $p13^{II}$ reveals no characteristic DNA-binding motifs and previous data show neither DNA binding nor transcriptional activity (142). Since $p30^{II}$ and $p13^{II}$ are expressed from two different mRNAs and accumulate in separate cellular compartments, it is believed that the two proteins play distinct roles in the viral life cycle, possibly at the level of RNA processing ($p30^{II}$) and mitochondrial function ($p13^{II}$) (141).

6.2. $p13^{II}$ Alteration of Mitochondrial Morphology and Inner Membrane Potential

Functionally, expression of $p13^{II}$ disrupts the mitochondrial inner membrane potential and alters mitochondrial morphology and architecture, leading to apparent mitochondrial swelling and fragmentation of their normal interconnected string-like network, suggesting a role for $p13^{II}$ in induction of apoptosis (47). Additionally, it

is thought that $p13^{II}$ might possibly cause changes in mitochondrial permeability and/or alter processes like calcium signaling that relies on the mitochondrial network and the endoplasmic reticulum. Intriguingly, proteins that localize to mitochondria have been described for other human viruses including Vpr and Tat of HIV, vMIA of human cytomegalovirus, X of hepatitis B virus and BHRF-1 of Epstein-Barr virus (143-145). The retroviral proteins Vpr and Tat of HIV have been shown to disrupt mitochondrial inner membrane potential, resulting in rapid swelling of mitochondria and release of cytochrome c (145). Lately, mitochondrial swelling and altered permeability due to opening of the permeability transition pore (PTP) have been reported to play a critical role in triggering apoptosis, as demonstrated in the case of HIV-1 Vpr and X of hepatitis B virus (143, 144, 146). However, a study using the $p13^{II}$ amino acid region 9–41 did not show any involvement of the PTP in driven cation fluxes (140). Unlike protein X and Vpr, $p13^{II}$ does not cause causes substantial cytochrome *c* release (47). The biological significance of $p13^{II}$ mitochondrial localization and disruption of membrane potentials remains unclear.

In cells expressing $p13^{II}$-GFP fusion protein, only some cells showed apparent mitochondrial swelling and maintained their inner membrane potential, while others had a complete loss of inner membrane potential and marked perinuclear clustering, a characteristic of early apoptosis (47). Based on this, D'Agostino *et al.* (46) consider that $p13^{II}$ might initially induce selective permeability changes causing swelling and subsequent de-energization and irreversible swelling, and suggested that $p13^{II}$ triggers apoptosis. Despite these observations, thus far, increased levels of apoptosis have never been demonstrated in $p13^{II}$-expressing cells, leaving open the possibility for other mitochondrion-based functions of this viral protein. Such functions could simply include an increased respiratory activity of mitochondria, which is often accompanied by swelling or Ca^{2+} signaling and Ca^{2+} dependent regulation of the oxidative phosphorylation machinery. Thus, $p13^{II}$ may facilitate later stages of HTLV-1 infection such as assembly and release.

A computer prediction program indicated that the amino-terminal portion of $p13^{II}$ was predicted to contain a short hydrophobic leader (amino acids 1–5) followed by an α-helix (amino acids 21–30) that includes the MTS (47). The α-helix includes 4 arginine residues at positions 22, 25, 29, and 30, which are predicted to form a positively charged patch within the putative α-helix, thereby imparting amphipathic properties to this region. D'Agostino *et al.* (140) demonstrated that amino acids 9–41 of $p13^{II}$ which includes the MTS, folds into an α helix in the context of a membrane-like environment and has specific effects on the permeability of isolated mitochondria to small cations. Furthermore, the presence of the four arginines in the MTS is essential for the increase in mitochondrial ion conductance and *in situ* effects on mitochondrial morphology but not for mitochondrial targeting. In addition, circular dichroism analysis illustrated that efficient α-helical folding of amino acids 9–41 of $p13^{II}$ requires the presence of detergent or phospholipid micelles to mimic the membrane environment. This suggests that interaction with or embedding into membranes might be necessary for the correct folding of full-length $p13^{II}$ (140). However, the observation that the $p13^{II}$ MTS is not cleaved upon import into the mitochondria and that it does not require positively charged residues distinguishes it from most MTS, and suggests that the protein might utilize a mitochondrial import pathway distinct from that described for most MTS, which involves binding to a series of acidic receptors followed by cleavage of the signal.

6.3. $p13^{II}$ Cellular Protein Interactions

While screening a cDNA library from an HTLV-1-infected rabbit cell line by *Saccharomyces cerevisiae* two-hybrid assay, Hou *et al.* (147) discovered the association of $p13^{II}$ with two novel cellular proteins designated C44 and C254. C254 appears to be rabbit actin binding protein 280 (ABP280) present in the cytoskeleton of many different cell types and functions in the modulation of cell shape and polarity and is essential for migration in melanocytes and other cultured cells. More importantly, ABP-280 is important in the insertion of adhesion molecules into the cell membrane. While the region of ABP-280 that interacts with $p13^{II}$ is also involved in interactions with integrin B1, tissue factor, and presenilin, other regions of ABP-280 binds to glycoprotein and the cytoplasmic domain of furin (147). C44 shares homology with archeal adenylate kinases, the eukaryotic homologues of which localize to mitochondria and are involved in energy metabolism. Interestingly, the human homologue of C44 is expressed in the Jurkat T-cell line and proliferating, but not resting, PBMC (147). These studies were performed using two molecular clones of HTLV-I, K30p and K34p, which have amino acid differences in *rex,* $p13^{II}$, and $p30^{II}$. Interestingly, only $p13^{II}$ K34, but not $p13^{II}$ K30 allow the interaction with C44. The amino acid sequence of the $p13^{II}$ variant used by Ciminale *et al.* (47) was most similar to $p13^{II}$ K30, as it had the 25- amino-acid carboxyl tail and that it would not bind C44. This might provide clues to the role of specific amino acids within $p13^{II}$, which are critical in binding C44 and in causing apoptosis. Additionally, $p13^{II}$ binds to farnesyl pyrophosphate synthetase (FPPS), an enzyme involved in the mevalonate/squalene pathway, and in the synthesis of farnesyl pyrophosphate, a substrate required for prenylation of Ras (53). Interestingly, G4, a mitochondrial targeting accessory protein of BLV also binds to FPPS. Future studies on the functional significance of the interaction between $p13^{II}$ and these cellular proteins will possibly elucidate the role of $p13^{II}$ in altering mitochondrial physiology and thus in HTLV-1 replication and pathogenesis.

Furthermore, Mahana *et al*. (148), reported an increase in Vav phosphorylation in rabbit cells transfected with an HTLV-1 molecular clone that contains two mutations in pX ORF II, resulting in expression of truncated $p13^{II}$ and $p30^{II}$. Vav is a hematopoietically restricted guanine nucleotide exchange factor for the Rac/Rho family of GTPases and is necessary for T-cell activation (149). These findings suggest that $p13^{II}$ may play

a role in controlling the activation state of Vav, which may relate to viral infectivity and leukemogenesis.

7. ACCESSORY GENE PRODUCTS OF RELATED DELTARETROVIRUSES

Deltaretroviruses like HTLV-1 HTLV-2, STLV, and BLV have similar genomic structure, viral replication, conserved organizational structure and pathology (13, 150, 151). HTLV-1, HTLV-2 and BLV are complex pathogenic retroviruses of the oncovirinae family that cause lymphoproliferative diseases and encode conserved regulatory and accessory genes from pX region ORFs at the 3' portion of the viral genome (13). There is increasing knowledge about the role of the homologous gene products in the pathogenesis of HTLV-2 and BLV, after the advent of infectious molecular clones.

HTLV-1 and HTLV-2 are distinct but genetically related viruses that share 60% amino acid identity, yet they differ in their pathogenicity *in vivo*. HTLV-1 is found mainly in $CD4^+$ T cells, whereas HTLV-2 is mainly in $CD8^+$ T cells *in vivo* (152). However, both viruses are capable of infecting and transforming T cells *in vitro*. More importantly, HTLV-2 is also associated with leukemia and neurologic disease, although less frequently than HTLV-1 (7, 153). Analyzing the viral determinants that contribute to the pathogenesis and identifying the similarities and differences between HTLV-1 and -2 may provide a better understanding of HTLV pathogenesis. In addition, HTLV-2 is thought to be an important model to study HTLV pathogenesis, due to the similarities in genome structures and *in vitro* biological properties. As with HTLV-1, rabbits can be successfully infected with molecular clone of HTLV-2 (154) and although proteins encoded by the pX region between *env* and the last exon of *tax*/*rex* of HTLV-2 appear to be dispensable for viral replication and cellular transformation *in vitro* (155), they are important for viral replication in the rabbit model (156). Thus, like HTLV-1, proteins encoded in the pX region of HTLV-2 are likely to play an important role in viral life cycle during the natural infection. Further studies will be required to determine the role of these accessory genes in the disease syndromes associated with HTLV-2 infections. Based on initial studies, HTLV-2 ORF I protein $p10^I$ and ORF V protein $p11^V$ which overlaps the ORF I region appear to be analogous to HTLV-1 $p12^I$ in binding MHC-I molecules and perhaps able to down regulate this important surface protein on infected cells (66). Interestingly, $p10^I$ localizes within the nucleus (28). However, unlike $p12^I$, $p10^I$ and $p11^V$ could not bind 16-kDa vacuolar H^+ ATPase or the α, β or γ_c chains of the IL-2R, suggesting that the basic mechanisms of host-virus interaction may be different between HTLV-1 and –2 (66). More strikingly, C-terminal portion of HTLV-1 $p30^{II}$ shares 77.5% sequence homology with the N-terminal 49 amino acids of HTLV-2 ORFII protein $p28^I$ (I28). There are recent findings indicating that $p30^{II}$ and $p28^{II}$ can inhibit Tax and Rex function by retaining tax/rex RNA in the nucleus, thus decreasing the doubly spliced RNA pool in the cytoplasm, leading to decrease in Tax and Rex production (V. Franchini and P. Green personal communications).

Bovine leukemia virus infection of sheep offers a reliable model of disease associated with deltaretrovirus infections. The BLV accessory proteins R3 and G4 share varying degrees of homology with the HTLV-1 accessory proteins $p12^I$, $p13^{II}$ and $p30^{II}$ (157). The mRNAs coding for both R3 and G4 were originally identified by RT-PCR in PBMCs from BLV-infected cattle. G4 message is expressed at the beginning of the lymphoproliferative stage of BLV-induced disease while the R3 message is produced during the period surrounding seroconversion (54). Similar to initial reports of HTLV-1 deletion mutants, BLV molecular clones that disrupted the expression of pX ORF genes, encoding the G4 and R3 accessory proteins, failed to influence virus replication and infectivity in cell culture systems but reduced the virus transmission and more importantly the provirus load, leading to a drastic decrease in virus propagation in sheep (54). Importantly, BLV is the closest model system to study apoptotic process since leukemia occurs *in vivo* in experimental sheep models. Role of HTLV-1 in apoptosis is not clear, since reports are contradictory depending on experimental conditions and an appropriate animal system to elucidate the role of HTLV-1 in the apoptosis is deficient. Interestingly, attenuated mutant proviruses that harbor deletions in the G4 and/or R3 genes decrease the susceptibility of the PBMC to apoptosis and prevent uninfected cells from undergoing programmed cell death at similar levels as that of the wild-type virus, indicating that R3 and G4 genes are not required to maintain both direct and indirect protection against apoptosis *in vivo* (158). In addition, a live attenuated BLV vaccine with deletions in R3 and G4 genes was found protect 8 out of 9 sheep against a challenge by either wildtype BLV or BLV from blood of infected animals (159).

The BLV G4 protein shares structural features and cellular distribution patterns with HTLV-1 $p13^{II}$, while BLV R3 appears to be functionally related more closely to HTLV-1 $p12^I$. G4 has 105 amino acids, including an N-terminal stretch of hydrophobic residues (aa 1-24) followed by putative proteolytic cleavage sites and an arginine-rich region (aa 58-72) in the middle while R3 has 44 amino acids, including an N-terminal hydrophilic region followed by hydrophobic sequences. The hydrophilic portion of R3 corresponds to the first 17 amino acids of Rex, which include the domain responsible for accumulation of the protein in the nucleus and in binding to its RNA target, the Rex-responsive element (157). Like $p12^I$, R3 accumulates in the cellular endomembranes. However, since it also accumulates in the nucleus consistent with the presence of the Rex NLS at the N-terminal, it is speculated to regulate post-transcriptional activity of Rex (52). On the other hand, like $p13^{II}$, G4 also contain a mitochondrial targeting signal and amphipathic α helix. G4 contain 3 helical segments; a long helix lying in the hydrophobic region (aa 8 to 22), another long helix at the second lying near the C- terminus (aa 76 to 89) and an amphipathic short helix in the arginine rich region (aa 63 to 69), among which the first and the last are required for mitochondrial targeting. Importantly, like $p13^{II}$, G4 also localize to the nucleus and mitochondria (53). To test the functional properties of the viral proteins

Table 1. Summary of the functional role of HTLV-1 accessory proteins

Protein	pX ORF	Subcellular localization	*In vitro* functional activity	*In vivo* effects
$p12^{I}$	I	ER and cis-Golgi	Induces release of calcium from ER, interact with calcineurin as well as calreticulin and cause calcium-mediated NFAT activation; decreases IL-2 requirement for T-cell activation; enhances the production of IL-2; decreases MHC-1 surface expression	Abolished infectivity in rabbit model; reduced infectivity in non-dividing primary human T-cells
$p27^{I}$	I	Unknown	Unknown	Unkown, However, recognized by CTLs of infected subjects,
$p30^{II}$	II	Nucleus	Binds p300/CBP; differentially modulates HTLV-1 LTR mediated transcription; enhances transcription NFAT, NF-κB and AP-1 mediated transcription.	Reduced viral load in rabbit model[1]
$p13^{II}$	II	Mitochondria and nucleus	Mitochondrial swelling and disruption of $\Delta\psi$	Reduced viral load in rabbit model[1]

[1] Based on the reduced viral load from ACH.30/13 double-knockout proviral clone in rabbit model of infection (39, Bartoe *et al.* 2000)

further, Kerkhofs *et al.* (51) tested the oncogenic potential of R3 and G4, by determining their ability to transform primary rat embryo fibroblasts. In this system, G4 (analogous to HTLV-1 $p13^{II}$), but not R3 (analogous to HTLV-1 $p12^{I}$) cooperated with the Ha-*ras* oncogene to induce tumors in nude mice. Using yeast two-hybrid system and confocal microscopy, G4 was demonstrated to interact with farnesyl pyrophosphate (FPP) synthetase, an enzyme in the mevalonate/squalene pathway that is critical for synthesis of FPP, a substrate required for prenylation of Ras (52). Interestingly, the arginine rich domain of G4 was found to be important in its oncogenic potential and in interacting with FPPS. Analogously, HTLV-1 $p13^{II}$ was also found to specifically interact with FPP synthetase and to colocalize with G4 in mitochondria. Whether these observations explain the function of G4 and $p13^{II}$ is yet to be determined, however these findings provide new directions for research in the role of these accessory proteins in signal transduction pathways, leading to cell transformation and potential therapeutic approaches to eliminate virus replication. Interestingly, infectious molecular clones of BLV with mutations in gene regions encoding G4 and R3 were limited in their ability to maintain proviral loads in infected sheep (51). More importantly, while wild-type BLV typically produces leukemias and/or lymphosarcomas in the majority of infected sheep during the course of the infection, none out of 13 sheep infected with viruses with mutations in G4 or in R3 and G4 developed disease, indicating that G4 is required for pathogenesis *in vivo* (51). Whether this diminished pathogenic ability is specifically related to these gene products or a generalized attenuation of replication capacity by the virus has not been resolved. Despite this, the BLV model provides an important system to test the role of the regulatory and accessory genes in the pathogenesis of the deltaretroviruses and in exploring possible vaccine trials.

8. PERSPECTIVE

The mechanism of HTLV-1 mediated T call activation and transformation has been extensively investigated. Most of these studies are focused on Tax, which is known to be a critical transcriptional activator and a key protein in cell transformation. The oncogenic potential of Tax has been demonstrated in animal models, as well as *in vitro* transformation assays (15, 17, 25). Therefore, Tax apparently may be responsible for many of the required events necessary for HTLV-1-mediated lymphocyte immortalization. Nonetheless, it is uncertain whether Tax aids the virus in establishing persistent infection, a prerequisite for basal transcription of Tax itself. Recent studies demonstrate that expression of the accessory proteins encoded by pX ORFs I and II is critical for efficient HTLV-1 infection *in vivo*, although potentially dispensable for viral replication under activation conditions *in vitro*.

Based on recent findings from our own laboratory and others, we propose molecular functions for pX ORF I-encoded $p12^{I}$ and ORF II encoded $p30^{II}$ in HTLV-1-induced T-cell activation (Table 1). While the mechanism of $p30^{II}$ function awaits additional research, $p12^{I}$ function has been established to be calcium-dependent, independent of Tax and possibly occurs before Tax is expressed during a natural infection. However highly activated T cells mediated by expression of $p12^{I}$ and/or $p30^{II}$ likely allow HTLV-1 provirus to integrate into host cell genome and permit the early viral infection. Since Tax is also able to cause T cell activation, it appears to be redundant for HTLV-1 to use multiple proteins to activate T lymphocytes. It is possible that these proteins act coordinately or synergistically. We propose that, by modulating the expression of various HTLV-1 proteins, the virus employs selective use of different viral proteins during different stages of the infection. However, since information on the expression profile of HTLV-1 proteins during different stages of the infection is limited, additional studies designed to test the temporal expression patterns of HTLV-1 regulatory and accessory proteins during viral infection are required to explore this possibility. Additionally, it will be crucial to determine if $p12^{I}$ is present in HTLV-1 viral particles or selectively expressed before viral integration. If $p12^{I}$ is expressed after viral integration and not early in viral infection, it is likely to influence late events, such as viral replication, particle

assembly and release. Functional and biological significance of the interaction of $p12^{I}$ with calcineurin and the regulatory functions on NFAT transcriptional activity also await further investigation. Additionally, $p12^{I}$ may play a role in regulation of cell cycle and apoptosis, since it is able to increase the intracellular calcium like the cellular protein Bcl-2 and modulate NFAT activity. However, $p12^{I}$ activates NFAT while Bcl-2 blocks NFAT-induced FasL transcription and retards the G0 to S phase transition (160). Therefore, the mechanism employed by $p12^{I}$ and Bcl-2 might be distinct and future studies are necessary to test the possible role of $p12^{I}$ in cell cycle progression and apoptosis in T lymphocytes. Understanding the role of $p12^{I}$ in HAM/TSP also awaits further investigation.

There is increasing understanding of the functional significance of $p30^{II}$ $p13^{II}$. Emerging evidence suggests that these proteins may act during later stages of infection to promote viral persistence and potentially aid in virus assembly. Currently, we are evaluating the effect of single $p13^{II}$ and $p30^{II}$ knockout mutations on the infectivity of HTLV-1 viral clones *in vivo*. Importantly, recent findings based on microarrays from our laboratory shed light on the possible mechanisms by which $p30^{II}$ functions in HTLV-1 pathogenesis and in leukemogenesis. However, based on the clues available from this study, future studies are essential not only to verify the findings, but also to test their functional and biological significance. Additional research is also crucial in elucidating the mechanisms employed by $p30^{II}$ to enhance NFAT, AP-1 and NF-κB mediated transcription. Such studies may possibly define additional $p30^{II}$ target genes and $p30^{II}$-responsive DNA elements. Interestingly, one of the lysine residues within $p30^{II}$ appears to be critical for its ability to inhibit the HTLV-1 LTR mediated transcription, irrespective of the presence or the absence of the provirus (B. Michael, unpublished data). On the light of this finding, our current research focus on the intrinsic histone acetyltransferase activity of CBP/p300, whether CBP/p300 acetylates and potentially regulates HTLV-1 $p30^{II}$ via acetylation. In addition, findings from the deletion and site directed mutational analysis are valuable in designing experiments to reintroduce these mutations into infectious molecular clones to test their functional significance *in vivo*.

Little is known about the function of $p13^{II.}$ Future studies on the $p13^{II}$ protein are essential to determine if the mitochondrial swelling induced by $p13^{II}$ is significant in apoptosis or viral assembly. Functional significance of the interaction between $p13^{II}$ and cellular proteins like FPPS also await additional research. Biological significance of $p13^{II}$ mitochondrial localization and disruption of membrane potentials is also uncertain. *In vivo* experiments using infectious molecular clones with mutations in the motifs critical for the functions *in vitro* are necessary to determine the biological significance of these findings. Such detailed mutational analyses with each of the accessory proteins will be central in understanding the effect of specific mutations on protein function in the context of the whole virus *in vitro* or *in vivo*. In addition to the HTLV-1 rabbit animal model, BLV sheep model can be used to to test specific mutations of analogous gene regions in a disease model.

In conclusion, emerging evidence indicates that the accessory proteins of HTLV-1 and other deltaretroviruses associated with lymphoproliferative diseases, though once thought to be dispensable for viral replication, are in fact multifunctional proteins, critically involved in viral transmission and propagation and may be of potential use in designing therapeutic measures and vaccines.

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