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Critical Role of Human T-Lymphotropic Virus Type 1 Accessory Proteins in Viral Replication and Pathogenesis

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INTRODUCTION

Human T-cell lymphotropic virus type 1 (HTLV-1) infection currently persists in 10 to 20 million people worldwide but is a particular problem in regions of endemicity in the Caribbean, Japan, Africa, and South America and among at-risk groups in the United States (10, 39, 85, 94). The virus is the etiologic agent of adult T-cell lymphoma or leukemia, an aggressive malignancy of $CD4^+$ T lymphocytes, and initiates the neurodegenerative disease tropical spastic paraparesis–HTLV-1-associated myelopathy (15, 55, 118). The virus infection is also associated with a variety of other immune-mediated disorders, most likely through its ability to induce T-lymphocyte activation (10, 56, 88). HTLV-1 is a highly cell-associated virus, and transmission occurs through routes that promote lymphocyte transfer (44, 119). While the epidemiology and diseases associated with HTLV-1 are well characterized, the molecular mechanisms used by the virus to establish persistent infection and subsequently promote lymphocyte proliferation while evading elimination by the host immune response remain poorly defined.

The genome of HTLV-1 encodes the common structural and enzymatic proteins typical of all retroviruses (i.e., Gag, Pol, and Env). In addition, as a complex retrovirus, HTLV-1 uses alternative splicing and internal initiation codons to produce several regulatory and accessory proteins encoded by four open reading frames (ORFs) predominantly located in the pX region (pX ORF I to IV) of the viral genome between *env* and the 3′ long terminal repeat (42). A doubly spliced, 2.1-kb mRNA containing elements of the 5′ long terminal repeat, *pol*, and the pX region encodes the regulatory proteins Tax (pX ORF IV) and Rex (pX ORF III) (49, 68, 77, 95). Through interaction with cellular transcription factors, Tax potently activates transcription not only from the viral promoter (Tax-responsive element) but also from the enhancer elements of many cellular genes involved in host cell proliferation (47, 97, 104, 105, 128). Rex is responsible for nuclear export of unspliced or singly spliced viral RNA (52). For a more detailed discussion on Tax and Rex function the reader is referred to a number of recent reviews (59, 77, 95, 105).

HTLV-1 ACCESSORY PROTEINS EXPRESSED FROM pX GENE REGION

In addition to Tax and Rex, pX ORFs I and II produce alternatively spliced forms of mRNA, which encode four accessory proteins, p12^{I}, p27^{I}, p13^{II}, and p30^{II} (Table 1) (13, 24, 45, 71). These alternative mRNA and protein products were initially believed to be dispensable for viral replication (37, 107). However, over the past several years a number of reports have been published demonstrating that the HTLV-1 accessory proteins are critical for viral infectivity and maintenance of high viral loads, host cell activation, and regulation of gene transcription (3, 12, 25, 29, 60, 75, 96, 129, 130). These studies illustrate the critical function of these HTLV-1 accessory proteins during viral replication, and as a result these proteins should more appropriately be considered essential proteins that regulate virus-host cell interactions during the natural infection.

In addition to the bicistronic mRNA encoding Tax and Rex, HTLV-1 expresses at least five other species of pX mRNA,

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TABLE 1. Summary of HTLV-1 accessory proteins

Protein[a]	pX ORF	Subcellular distribution	In vitro functional activity	In vivo effects of proviral clone gene mutation
$p12^{I}$	I	ER and *cis*-Golgi	Calcium-mediated NFAT activation; decreases IL-2 requirement for T-cell activation	Abolished infectivity in rabbit model; reduced infectivity in nondividing primary human T cells
$p13^{II}$	II	Mitochondria and nucleus	Mitochondrial swelling and disruption of $\Delta\psi$	Reduced viral load in rabbit model[b]
$p30^{II}$	II	Nucleus	Transcriptional regulator; binds p300/CBP	Reduced viral load in rabbit model[b]

[a] $p27^{I}$, predicted to be expressed from pX ORF I, is recognized by T cells derived from infected subjects (103), but limited functional studies have been reported.
[b] Viral load from ACH.30/13 double-knockout proviral clone in rabbit model of infection (12).

including those encoding the less well characterized $p21^{Rex}$ (Fig. 1) (13, 24, 71). mRNA species specific for four accessory proteins—$p12^{I}$, $p27^{I}$, $p13^{II}$, and $p30^{II}$—can be detected in cells transfected with HTLV-1 molecular clones, HTLV-1 immortalized or transformed cell lines, and freshly isolated leukocytes from virus-infected subjects with or without disease (13, 19, 24, 71). Similar to the circumstances of Tax and Rex, there exists limited detailed knowledge about the specific quantities and timing of expression for these four accessory proteins during the course of the natural infection. Indirect evidence of their expression in vivo has been revealed from studies of the immune response to the viral infection. Serum antibodies (20, 35) as well as cytotoxic T cells (103) directed against recombinant proteins or peptides representing these proteins have been detected in diseased patients, as well as from asymptomatic carriers. Thus, proteins encoded by pX ORF I and II are produced during the course of the viral infection at levels sufficient to elicit both specific antibody and cell-mediated immune responses in infected subjects.

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 of HTLV-1, simian T-cell lymphotropic virus type 1 (23, 110, 115). Further illustration of the conserved nature of these gene regions comes from studies of another member of the deltaretroviruses, bovine leukemia virus (BLV). This virus, 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 (62, 76, 122). 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 (62, 123). Collectively these studies illustrate that these retroviruses, which are all associated with lymphoproliferative disorders,

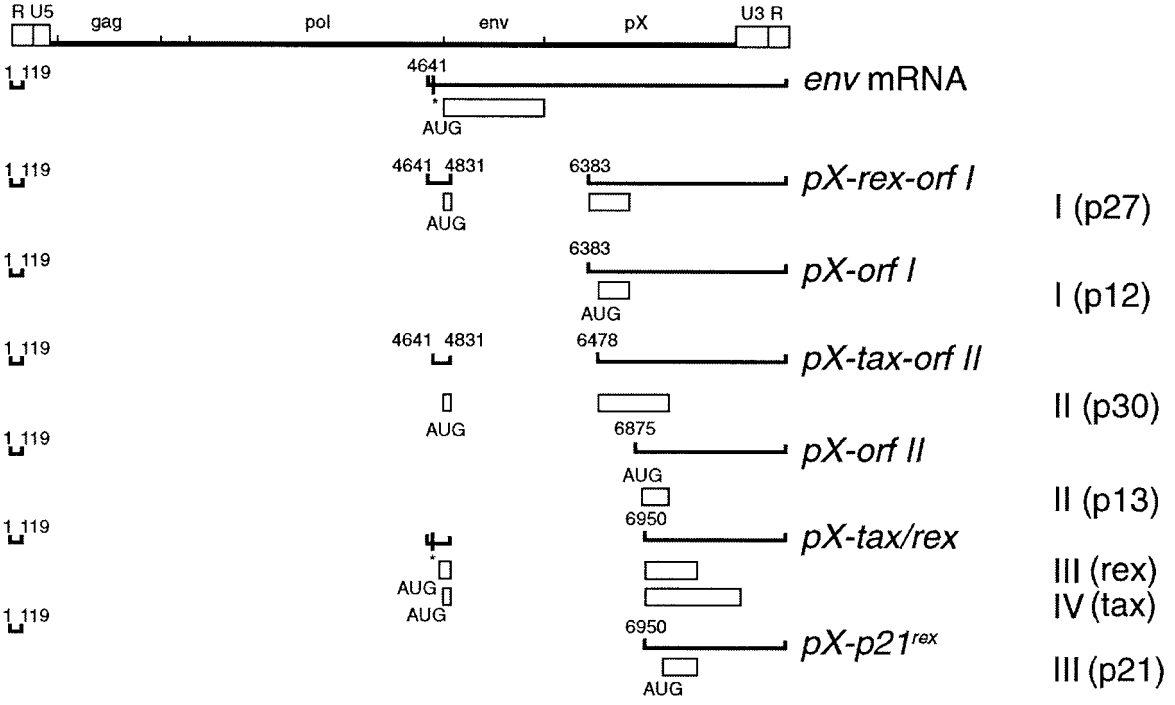

FIG. 1. Diagram of HTLV-1 genome and alternatively spliced mRNA and protein species from pX ORF. Lines below the proviral genome (top) represent spliced RNA, and boxes below each line represent protein products. The ORF and protein size in kilodaltons are represented to the right of each predicted protein.

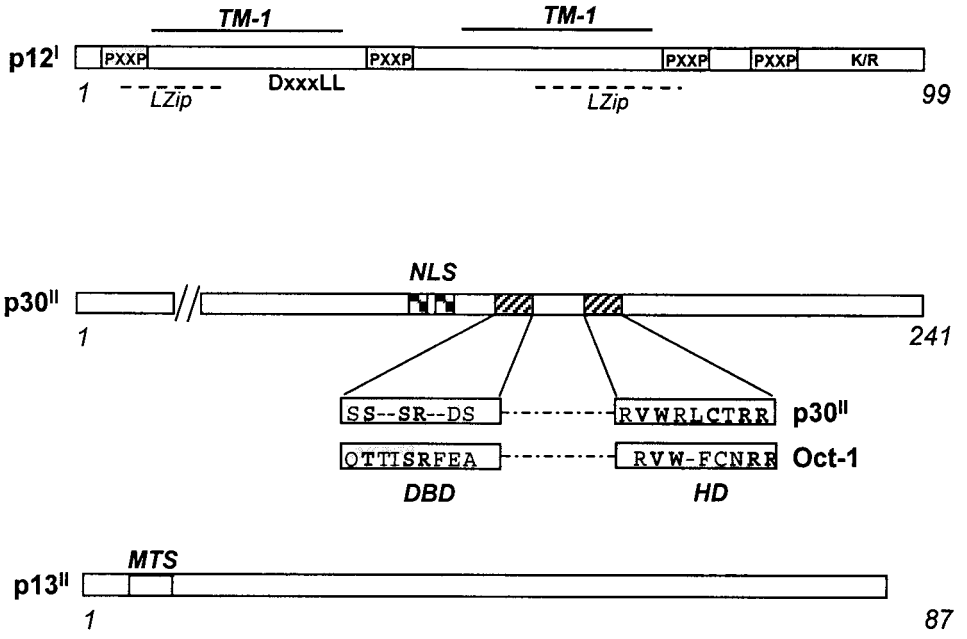

FIG. 2. Diagram of $p12^{I}$ (top), $p30^{II}$ (middle), and $p13^{II}$ (bottom) with predicted functional motifs in shaded boxes. Abbreviations: TM, transmembrane region; LZip, leucine zipper motif; DxxxLL, dileucine motif; PxxP, SH3 binding motif; K/R, lysine-to-arginine variant at position 88 (arginine at this position increases stability of protein). Boxes below $p30^{II}$ (middle) indicate regions sharing sequence homology with the DNA binding domain and homeodomain of Oct-1. Numbers below bars are amino acid position numbers.

during the course of their evolution have retained conserved gene regions that apparently serve analogous functional roles.

ROLE OF pX ORF I $P12^{I}$ IN VIRAL REPLICATION AND T-CELL ACTIVATION

Alternative splicing events that combine the second exon of Tax with additional downstream sequences produce pX ORF I mRNA. This message potentially encodes two accessory proteins, the 152-amino-acid $p27^{I}$ and the 99-amino-acid $p12^{I}$. Translation of the latter is initiated at an internal methionine codon in the $p27^{I}$ ORF. Alternatively, $p12^{I}$ can be translated from a singly spliced message produced by direct splicing of nucleotide 119 to the splice acceptor at position 6383 (24, 71). Interestingly, transfection of expression plasmids containing HA_1-tagged versions of either the full-length $p27^{I}$ cDNA or the cDNA for the singly spliced $p12^{I}$ yield only the smaller $p12^{I}$ (71). In contrast, using in vitro transcription-translation systems, Ciminale et al. (24) produced $p27^{I}$ from the doubly spliced mRNA. Thus, it is possible that $p12^{I}$ is preferentially produced from the $p27^{I}$ mRNA and that removal of the internal $p12^{I}$ AUG start codon could yield detectable levels of $p27^{I}$. Pique et al. (103) demonstrated production of cytotoxic T cells in HTLV-1-infected subjects that were reactive against peptides representing all putative pX accessory proteins, including $p27^{I}$. These results suggest that $p27^{I}$, along with $p12^{I}$, $p13^{II}$, and $p30^{II}$, is produced during the course of the natural infection in vivo.

Biochemical Features of a Signaling Molecule

Amino acid sequence analysis of $p12^{I}$ reveals a highly hydrophobic protein with 32% of its amino acids being leucine and 17% being proline (71). Hydropathy and immunogenicity plots alike demonstrate a minimal number of soluble regions and suggest two transmembrane domains extending from amino acid 12 to 30 and amino acid 48 to 67 (71, 117). These putative transmembrane domains roughly overlap with two predicted leucine zipper motifs, which form alpha-helices. These structural features could contribute to membrane localization or homo-oligomerization of the protein (Fig. 2). Using HA_1-and AU_1-tagged versions of $p12^{I}$ transiently overexpressed in HeLa-Tat cells, Trovato et al. (117) demonstrated by immunoprecipitation and immunoblot analysis that the protein indeed forms at least dimers, if not oligomers. However, the presence of several helix-breaking proline residues within the predicted leucine zippers warrants caution about whether functional leucine zippers or simply hydrophobic protein domains contribute to the oligomerization of the protein. Furthermore, $p12^{I}$ contains at least three predicted SH3-binding motifs (42, 117) (Fig. 2). Similar motifs in cellular signaling proteins are typically proline rich with a minimal core of PXXP and are often preceded by an arginine residue at +2 (18, 73). Interestingly, the sequences encoding the first and third PXXP motifs of $p12^{I}$ (amino acids 8 to 11 and 70 to 74) are highly conserved among viral strains, suggesting a role for these domains in the function of $p12^{I}$ (42). In addition, a dileucine motif (DXXXLL) is found at amino acid positions 26 to 31 (Fig. 2). As shown for HIV Nef, dileucine motifs 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 (30, 106). However, no functional role has yet been established for the proposed motif in HTLV-1 $p12^{I}$. A ubiquitylation motif surrounds the lysine at position 88 of HTLV-1 $p12^{I}$. While the functional significance

of this motif remains unclear, arginine substitution at this position, which is commonly found among natural HTLV-1 strains, significantly enhances the half-life of the protein (117).

Cytoplasmic Expression and Cellular Protein Interactions

When transiently expressed in fibroblasts HTLV-1 $p12^I$ localizes to the endoplasmic reticulum (ER) and *cis*-Golgi compartments (38, 61, 70, 72). Intriguingly, we recently demonstrated that $p12^I$ associates with two ER-resident calcium-binding proteins, calreticulin and calnexin (38). 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) (74, 90). Furthermore, calreticulin functions as a regulator of neoangiogenesis, and the N terminus of the protein, designated vasostatin, is used as a therapeutic angiogenesis inhibitor (102). It would be advantageous for a virus to target such a conserved protein in order to dysregulate calcium signaling pathways and activate NFAT in infected T lymphocytes.

Overall HTLV-$p12^I$ shares sequence homology with bovine papillomavirus (BPV) E5 and Epstein-Barr virus LMP-1 (43). The region of highest homology starts after the first and extends into the second transmembrane domain of $p12^I$. Interestingly, $p12^I$ functionally cooperates with BPV E5 in transformation of mouse C127 fibroblasts and, like E5, binds to the 16-kDa subunit of the vacuolar H^+ ATPase (16K) (43). Although this association appears to be required for the E5-mediated transformation of epithelial cells, no clear correlation was found between $p12^I$-16K interaction and cooperative transformation with BPV E5, leaving the functional significance of the $p12^I$-16K interaction to be determined. Attempts to further map the motif in $p12^I$ responsible for the association with 16K did not clearly identify a specific domain in the viral protein. Although the region between amino acids 36 and 48 of $p12^I$ is necessary for the interaction, it alone is not sufficient for binding (72). Interestingly, Nef, a key accessory protein of simian immunodeficiency virus and human immunodeficiency virus (HIV), binds the catalytic subunit NBP-1 of the ATPase (78). NBP-1 association of Nef mediated by the Nef C-terminal flexible loop is critical for Nef-dependent internalization of CD4 and viral infectivity (81).

Several reports have suggested an involvement of HTLV-1 $p12^I$ in the modulation of T-cell-specific signal transduction pathways. Using transient transfections in HeLa-Tat cells, Mulloy et al. (93), using transient-coexpression assays, reported that HTLV-1 $p12^I$ interacts with the immature forms of the interleukin-2 receptor β (IL-2Rβ) and γ chains, resulting in reduced surface expression of the receptor chains. The IL-2R binding region of $p12^I$ mapped to 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. Although $p12^I$ does not influence JAK3 kinase activity directly, Nicot et al. (96) recently demonstrated a modest increase in STAT5 activity in 293T cells transfected with $p12^I$ and all components of the IL-2R signaling complex and in primary human lymphocytes transduced with a $p12^I$-expressing lentiviral vector. 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 (96). Conversely, peripheral blood-derived lymphocyte cell lines immortalized by transfection with HTLV-1 infectious molecular clones with selected elimination of pX ORF I have intact IL-2R signaling pathways (28). Thus, following immortalization $p12^I$ 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. Taken together, these findings indicate that $p12^I$ may induce STAT activity to confer a growth advantage to infected cells during the early stages of infection that precede the immortalized T-cell state. It remains to be elucidated by which JAK3-independent pathway $p12^I$ induces STAT5 activation.

When coexpressed in HeLa-Tat cells, $p12^I$ binds immature forms of the major histocompatibility complex class I (MHC-I) and directs its degradation in the proteasome (60, 61). In this system $p12^I$ localizes to the ER and decreases the surface expression of transfected MHC-I in HeLa-Tat cells and endogenous MHC-I in Jurkat cells transduced with a $p12^I$ encoding lentiviral vector. These data suggest that $p12^I$-mediated down regulation of MHC-I surface expression might aid the virus in escaping immune surveillance. In contrast, T-lymphocytes immortalized with the wild type and $p12^I$-mutant clones ACH and ACH.p12 expressed equal levels of MHC-I and -II, indicating that if $p12^I$ modulates MHC-I surface expression it is likely to occur only during the early stages of infection (28). In a similar manner, the accessory proteins $p10^I$ and $p11^V$ of HTLV-2 also associate with MHC-I, but these do not bind to either 16K or IL-2Rβ or -γ (60). These results are intriguing, because HIV type 1 (HIV-1) Nef binds to and down regulates the cell surface expression of MHC-I and is believed to contribute to immune evasion by HIV-1 (100). Despite this evidence down regulation of MHC-I of virus-infected cells also does not appear to 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 postinoculation, prior to the time one would expect an active immune response (29). It remains to be shown whether HTLV-1 $p12^I$ down regulates MHC-I expression on infected peripheral blood mononuclear cells (PBMC) in vivo and actively contributes to viral spread or persistence. To address this question studies of early virus replication immediately after inoculation of virus-infected cells in appropriate animal models will be required.

Regulation of Viral Infectivity by HTLV-1 $p12^I$

Initial studies reported that deletion of pX ORF I from HTLV-1 infectious molecular clones had no adverse effects on the virus' ability to infect and transform primary lymphocytes in vitro (37, 107). 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 (29). Rabbits inoculated with ACH.p12, which is mutated and does not express 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 (29). The most striking difference

between these in vitro and in vivo studies is that standard in vitro coculture techniques used to transmit virus to naïve PBMC utilize target cells stimulated by IL-2 and mitogen. However, in vivo the majority of circulating and tissue-associated lymphocytes are nondividing. To test whether $p12^I$ is critical for optimal viral infectivity in nonactivated primary cells in vitro, we designed coculture assays that would allow transmission of the virus to resting primary lymphocytes (3). These assays were based on the coculture of a variety of HTLV-1 producing cells with naïve (nondividing) PBMC in the absence of exogenous stimuli to more accurately reflect the virus-cell interactions during the natural infection. Under these conditions, we demonstrated a dramatic reduction in the viral infectivity of the $p12^I$ mutant ACH.p12 in primary lymphocytes. Furthermore, upon addition of mitogen to the coculture, the mutant's ability to infect primary cells was restored (3). 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. 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 (22, 31, 79, 99, 101, 112, 127).

We have recently reported that $p12^I$ expression in Jurkat cells results in an approximately 20-fold activation of NFAT-dependent gene expression, while AP-1- or NF-κB-mediated transcription remained unchanged (5). HTLV-1 $p12^I$ specifically induced NFAT-mediated transcription in synergy with the Ras/mitogen-activated protein kinase (MAPK) pathway. Inhibition of calcium-dependent signals by cyclosporine, BAPTA-AM and a dominant negative mutant of NFAT2 abolished the $p12^I$-mediated activation of NFAT-dependent transcription. In contrast, inhibition of more proximal signaling, such as that through phospholipase C-gamma, did not affect $p12^I$-induced NFAT activity (5). Importantly, $p12^I$ functionally substituted for thapsigargin, which selectively depletes intracellular calcium stores. Thus, HTLV-1 $p12^I$ in a calcium-dependent manner appears to activate NFAT-mediated transcription in lymphoid cells. These recent studies collectively implicate a novel mechanism by which this HTLV-1 accessory protein may dysregulate common T-cell activation pathways critical for the virus to establish persistent infection.

Subcellular localization studies indicated that $p12^I$ colocalizes with the ER-resident, calcium-binding proteins calreticulin and calnexin (38). Most strikingly, expression of $p12^I$ results in increased cytosolic calcium, indicating that HTLV-1 $p12^I$ induces release of calcium from the ER to activate NFAT (W. Ding, J. Virol., in press). Thus, the viral protein appears to act in the ER to activate calcium-mediated signaling, which would be an obvious advantage for the virus by activating T cells during the early stages of HTLV-1 infection. Cellular stimuli that would normally induce only partial activation of T cells (e.g., through AP-1) could through the influence of $p12^I$ become fully activated due to enhanced NFAT activity. 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 (9, 114). Calreticulin and calnexin each have been demonstrated to modulate calcium storage and control protein folding, including several viral glycoproteins, in the ER (74, 91). Within the ER, $p12^I$ may serve to modulate calcium-mediated signals involved in cell activation. Alternatively, these proteins may serve as molecular chaperones to regulate the folding of $p12^I$. Further studies will be required to determine the possible role of $p12^I$ in calcium storage and release from the ER. Interestingly, Johnson et al. (60, 61) have reported that $p12^I$ binds to the heavy chain of MHC-I and prevents its association with β_2-microglobulin, impairing the traffic of the protein complex. Calreticulin also acts as a chaperone in the assembly and expression of MHC-I molecules in activated human T lymphocytes (8). One potential mechanism to explain the ability of $p12^I$ to interfere with MHC-I complex transport is by binding and retaining calreticulin–MHC-I complexes in the ER or *cis*-Golgi.

Together our data support the tenet that HTLV-1 $p12^I$ causes an increase in calcium release from the ER to activate NFAT. Interestingly, the cellular protein CAML (Ca^{2+}-modulating cyclophilin ligand) induces calcium release from the ER in a fashion proposed for HTLV-1 $p12^I$ (120). Like HTLV-1 $p12^I$, CAML contains two putative transmembrane domains, colocalizes with calreticulin in the ER, and leads to NFAT activation (53, 54). Thus, the accessory protein $p12^I$ of HTLV-1 appears to mimic the function of a host cell protein to increase cytosolic calcium and thus facilitate pathological T-cell activation and eventually viral infection and replication.

Role in T-Cell Activation

Calcium-dependent activation of NFAT facilitates productive infection of primary lymphocytes by HIV (69). Primary $CD4^+$ T cells stably expressing NFAT2 became highly susceptible to HIV infection, while cells transduced with empty vector did not. Susceptibility of these cells to HIV infection could be restored by phytohemagglutinin treatment, which is most likely due to the phytohemagglutinin-induced upregulation of NFAT activity. These data concur with our findings that addition of mitogens can rescue the infectivity of a $p12^I$ mutant viral clone in resting PBMC (3), most 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 (1). Manninen et al. have delineated the induction of NFAT activity in Nef-expressing Jurkat cells (83, 84). Similarly to HTLV-1 $p12^I$, this induction required calcium signaling and the synergistic action of the Ras/MAPK pathway. In sharp contrast to HTLV-1 $p12^I$, however, Nef-mediated NFAT activation is dependent on plasma membrane localization of the viral protein, as well as association with PAK2, suggesting an involvement of the PAK2/Vav/CASK pathway. This indicates that HIV Nef induces NFAT activity via a molecular mechanism that is clearly distinct from that used by HTLV-1 $p12^I$. Nevertheless, despite the apparent differences in the mechanisms of calcium regulation between HTLV-1 $p12^I$ and HIV Nef, the two accessory proteins apparently play a critical role in enhancing viral infectivity in primary lymphocytes by upregulation of NFAT.

pX ORF II P30^{II}: MODULATOR OF TRANSCRIPTION

The accessory proteins encoded by pX ORF II of HTLV-1 are produced from two alternatively spliced mRNAs. The larger of the two proteins, 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 (13, 24). An internal methionine codon in p30^{II} can be used to produce a smaller protein, p13^{II}, which contains the C-terminal 87 amino acids of p30^{II}. Alternatively, p13^{I} can be produced from a singly spliced message by splicing of the first Tax exon directly to the splice acceptor at position 6875 (13, 24, 71). Initial studies suggested that ORF II was dispensable for Tax, Rex, or Env expression, as well as viral replication and immortalization of primary lymphocytes in vitro (37, 107, 108). In addition, Chou et al. (21) described the isolation of a viral clone from leukemic cells that contained a premature stop codon in pX ORF II. They concluded that pX ORF II was not necessary for the outgrowth of leukemic clones in vivo but could not rule out a function for pX ORF II during early infection. 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) (12). 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 that declined towards the end of the study. Most strikingly, using quantitative competitive PCR, we demonstrated a dramatically reduced (up to 100-fold) viral load for the ACH.p30/13-infected animals (4, 12). Taken together, these data suggested that pX ORF II is indeed necessary for maintenance of high viral loads in vivo.

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 (71, 130). Amino acids 71 to 98 of p30^{II} are able to functionally substitute for the NLS of Rex (32). Furthermore, p30^{II} contains serine/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 (24) (Fig. 2). Functionally p30^{II} behaves like a transcription factor and differentially modulates CREB-responsive (CRE) promoters in transient-transfection reporter gene assays (130). Preliminary mutational analysis implicated a central core region within p30^{II} (amino acids 62 to 132) that may mediate the transcriptional enhancement observed. Interestingly, while repressing CRE-mediated transcription, at low concentrations p30^{II} activated viral Tax-responsive-element-dependent transcription independently of Tax expression (130). Recent molecular analyses of transcriptional regulation by p30^{II} showed that the viral protein colocalizes with p300 in cell nuclei and regulates gene expression by binding to the KIX domain of CBP/p300 (129). Furthermore, p30^{II} was able to disrupt CREB-Tax-CBP/p300 complexes bound to the viral 21-bp repeats. Taken together, these data suggest that p30^{II} acts as a repressor of transcription by sequestering CBP/p300 from the pool of available transcription factors. Therefore, at higher concentrations p30^{II} may serve to promote viral persistence by reducing viral gene expression and thus reducing immune recognition of infected cells. 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 (87). In addition, further structure-function analyses will help define the roles of five lysine residues within the transactivation domain of p30^{II}. Intriguingly, these five residues are all preceded by at least one serine residue (SK motif) and thus present potential acetylation sites for CBP/p300. As CBP/p300-mediated acetylation has become a common theme for regulation of protein function (16), 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}. While p30^{II} acetylation remains to be evaluated, p30^{II} may function to directly inhibit acetylation of histone H3 and H4, as well as lysine 320 (K_{320}) of the cell cycle regulator p53 through the p300/CBP-associated factor (R. Harrod and G. Franchini, unpublished observation).

MITOCHONDRIA: TARGET OF pX ORF II P13^{II}

Less is known about the function of the smaller protein, p13^{II}, encoded by pX ORF II. Initial studies demonstrated p13^{II} localization to the nucleus (71), but more-recent reports show mitochondrial localization of the protein (25, 33, 34). This localization is mediated by an atypical mitochondrial targeting sequence (MTS) in the N terminus of p13^{II} (Fig 2). The 10-amino-acid MTS also targets green fluorescent protein to mitochondria when fused to the N terminus of green fluorescent protein (25). 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 (34). Functionally, expression of p13^{II} alters mitochondrial morphology and disrupts the mitochondrial inner membrane potential, suggesting a role for p13^{II} in induction of apoptosis (25). Intriguingly, proteins that localize to mitochondria have been described for other human viruses including Vpr and Tat of HIV, vMIA of human cytomegalovirus, and BHRF-1 of Epstein-Barr virus (11, 40). 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* (58). At this point the biological significance of p13^{II} mitochondrial localization and disruption of membrane potentials remains unclear. Thus far, it has not been demonstrated that p13^{II} indeed induces apoptosis, leaving open the possibility for other mitochondrion-based functions of the viral protein. Such functions could simply include an increased respiratory activity of mitochondria, which is often accompanied by swelling. Thus, p13^{II} may facilitate later stages of HTLV-1 infection such as assembly and release. Furthermore, while screening a cDNA library from an HTLV-1-infected rabbit cell line by *Saccharomyces cerevisiae* two-hybrid assay, Hou et al. (57) discovered the association of p13^{II} with two novel cellular proteins designated C44 and C254. While C254 appears to be rabbit actin-binding protein 280, C44 shares homology with archeal ade-

nylate kinases, the eukaryotic homologues of which localize to mitochondria. Interestingly, the human homologue of C44 is expressed in the Jurkat T-cell line and proliferating, but not resting, PBMC (57). The implications of this finding remain unclear but allow speculation about a potential role of $p13^{II}$ in cellular activation. Furthermore, Mahana et al. (80) 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 (92). 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.

ACCESSORY GENE PRODUCTS OF RELATED DELTARETROVIRUSES: PARALLEL ROLES IN INFECTIVITY AND PATHOGENESIS

Examination of the genome sequence of HTLV-2, simian T-cell lymphotropic virus, and BLV reveals genes and conserved organizational structure similar to those of HTLV-1 (17, 51, 89, 109, 111, 113, 115, 121). While recent molecular and epidemiological studies have expanded knowledge of the number of strains of each of these viruses, HTLV-1, HTLV-2, and BLV belong to a class of complex pathogenic retroviruses all associated with lymphoproliferative diseases (7, 67, 118, 122). Each of these viruses encodes conserved regulatory and accessory genes from pX region ORFs in the 3′ portion of the viral genome. The availability of infectious molecular clones of HTLV-2 and BLV has provided important findings that substantiate the role of homologous gene products in the pathogenesis of these other members of the deltaretroviruses.

HTLV-2 shares 60% amino acid identity with HTLV-1, and infection by this highly related virus is associated, albeit less frequently than HTLV-1, with leukemia and neurologic disease (116). Because each of these viruses shares genome structures and in vitro biological properties, HTLV-2 remains an important model for the dissection of HTLV pathogenesis. Cockerell et al. (27) reported the first successful infection of rabbits with a molecular clone of HTLV-2. Like work in the HTLV-1 system, it was first reported that deletion of genes between *env* and the last exon of *tax*/*rex* of this HTLV-2 molecular clone had no effect on infectivity of the virus in cell culture systems (50). Subsequently, it was reported by this same research group that this clone had reduced infectivity in the rabbit model system, further verifying the importance of this gene region in infectivity (26). The analogous protein in HTLV-2, compared to HTLV-1 $p12^{I}$, appears to be $p10^{I}$, also encoded by pX ORF I. Johnson et al. (60) reported the common property of pX ORF I gene products of HTLV-1 and HTLV-2 to bind MHC-I molecules and perhaps down regulate this important surface protein on infected cells. Thus, like HTLV-1, proteins encoded in the pX region of HTLV-2 are likely to be essential for viral replication 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.

BLV infection of sheep offers a reliable model of disease associated with deltaretrovirus infections. Similarly 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 in cell culture systems but reduced the ability of the virus to replicate in sheep (6, 123, 125). 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 $p12^{I}$ (L. Willems, personal communication). BLV wild type and mutant proviruses that contained deletions in the G4 or R3 genes infected B lymphocytes and permitted the infected cell to resist apoptotic signals (36). To test the functional properties of the viral proteins, Kerkhofs et al. (62) 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. A yeast two-hybrid system, as well as confocal microscopy, was used by Lefebvre et al. (76) to demonstrate that G4 interacts 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. 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 is yet to be determined, but this report illustrates 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 (62). More importantly, while wild-type BLV typically produces 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 (62). 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 potential role of the regulatory and accessory genes in the pathogenesis of the deltaretroviruses.

CONCLUSIONS AND FUTURE DIRECTIONS

Much of the work on HTLV-1-mediated T-cell activation and transformation has focused on the role of the transcriptional activator Tax, which potently activates numerous cellular genes involved in host cell proliferation (59). The oncogenic potential of Tax has been demonstrated in animal models, as well as in vitro transformation assays (9, 14, 41, 98). Therefore, Tax apparently may be responsible for many of the required events necessary for HTLV-1-mediated lymphocyte immortalization. However, it is uncertain whether Tax aids the virus in establishing persistent infection within a cell, a prerequisite for basal transcription of Tax itself. Emerging evidence indicates that while potentially dispensable for viral replication under activation conditions in vitro, expression of the accessory proteins encoded by pX ORFs I and II is critical for efficient HTLV-1 infection in vivo.

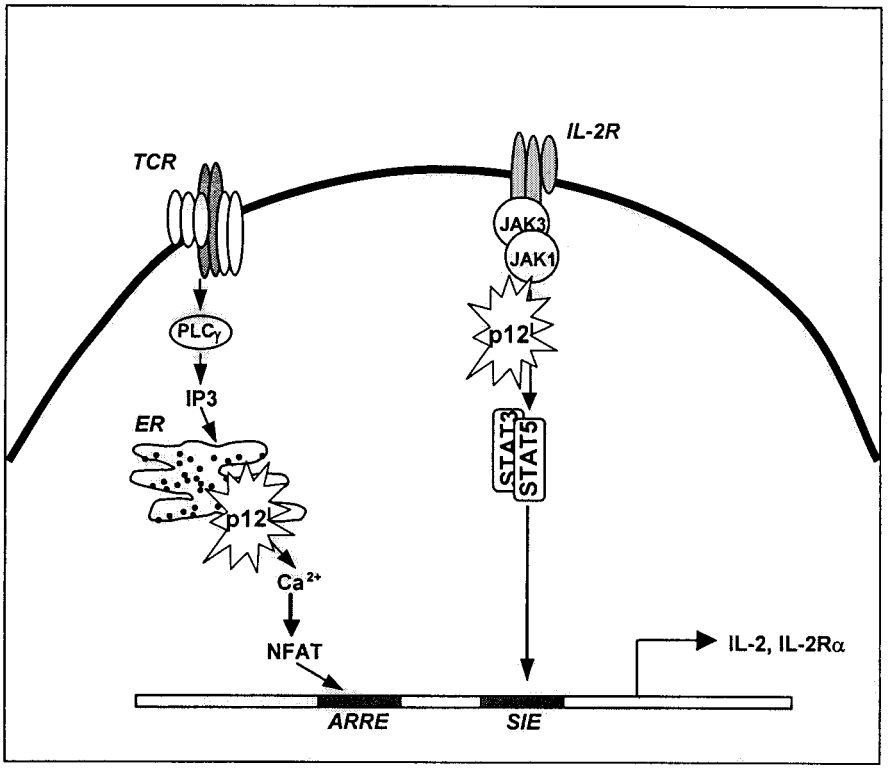

FIG. 3. At left is shown a model of $p12^{I}$ function during T-cell receptor-mediated activation through calcium-mediated enhancement of NFAT responsive promoters. Abbreviations: ARRE, NFAT/AP-1, AP-1 responsive element-nuclear component; PLCγ, phospholipase Cγ. At right, the schematic shows that in the presence of $p12^{I}$, IL-2-mediated signaling through STAT 5 (STAT responsive element [SIE]) may lower the threshold to trigger T-cell activation through the IL-2R.

Based on recent findings from our own laboratory and others, we propose a molecular function for the pX ORF I-encoded $p12^{I}$ in HTLV-1-induced T-cell activation (Fig. 3). This calcium-dependent mechanism is independent on and most likely precedes Tax expression during a natural infection. Through this mechanism, $p12^{I}$ could enhance viral transmission to nondividing lymphocytes, most likely by activating target cells during the very early stages of infection through induction of NFAT-dependent gene expression.

Perhaps the most fundamental question about the contribution of $p12^{I}$ to HTLV-1 replication is whether the accessory protein indeed influences events early in the life cycle of the virus, e.g., prior to integration. Alternatively this accessory protein could enhance viral expression after integration. It has been difficult to address this question due to the lack of a reliable single-round infection assay for HTLV-1. If $p12^{I}$ does indeed increase viral infectivity rather than replication, it will be critical to determine whether $p12^{I}$ is present in newly infected cells prior to transcription of viral genes. Interestingly, HIV Nef, which appears to be functionally homologous, enhances infectivity during the early stages of the virus replication cycle (2, 79, 86, 112, 127). In addition, it has been shown that about 10 to 100 copies of HIV Nef protein are contained in HIV particles (2, 82). If $p12^{I}$ influences replication rather than infectivity, this would add another level of complexity, namely, the seemingly overlapping functions of $p12^{I}$ and Tax. Both proteins have been shown to activate pathways involved in T-cell activation. While $p12^{I}$ activates NFAT2, Tax has been reported to induce IL-2 and IL-2Rα expression partly through NFAT1 transcriptional activity (14, 48). This apparent redundancy could be resolved if $p12^{I}$ and Tax expression were temporally regulated during the viral replication cycle. For example, $p12^{I}$ could be expressed before Tax and lower the threshold for full, Tax-mediated cell activation. This is an attractive model, as Tax has been shown to up regulate expression from the serum response element (59). This leads to synthesis of the AP-1 subunits c-Fos and c-Jun and would thus provide the synergistic signal required for $p12^{I}$-mediated activation of NFAT-dependent gene expression. Therefore, a kinetic analysis of the synthesis of the HTLV-1 regulatory and accessory proteins is necessary.

While less is known about the function of $p30^{II}$ and especially $p13^{II}$ in the viral life cycle, emerging evidence suggests that these proteins may act during later stages of infection to promote viral persistence and potentially aid in virus assembly. It will be interesting to evaluate the effect of single $p13^{II}$ or $p30^{II}$ knockout mutations on the replicative potential of HTLV-1 viral clones in vivo and in vitro. In addition, future studies on the $p13^{II}$ protein will be designed to elucidate whether the mitochondrial swelling observed in the presence of the protein is indicative of $p13^{II}$-induced apoptosis or increased mitochondrial activity, which may aid during the assembly process of the virus. More detailed structure-function analysis of the $p30^{II}$ protein will help identify the minimal

region mediating its transcriptional effects and those involved in regulation of $p30^{II}$ function itself. Findings resulting from such studies will aid in the design of specific $p12^{I}$, $p13^{II}$, and $p30^{II}$ functional mutants, which can subsequently be reintroduced into infectious molecular clones. Such detailed mutational analyses will be important in order to test the effect of specific mutations on protein function in the context of the whole virus in vitro or in vivo. In this regard, the BLV system offers the opportunity to test specific mutations of analogous gene regions in a disease model.

In conclusion, emerging evidence indicates that the accessory proteins of HTLV-1, which were once thought to be dispensable for viral replication, are critically involved in viral transmission and propagation and may in fact be multifunctional proteins.

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