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Hijacking the T-cell Communication Network by the Human T-cell Leukemia/Lymphoma Virus Type 1 (HTLV-1) p12 and p8 Proteins

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Abstract

The non-structural proteins encoded by the *orf- I, II, III,* and *IV* genes of the human T-cell leukemia/lymphoma virus type 1 (HTLV-1) genome, are critical for the modulation of cellular genes expression and T-cell proliferation, the escape from cytotoxic T-cells and natural killer cells, and virus expression. In here, we review the main functions of the HTLV-1 Orf -I products. The 12 kDa product from *orf-I* (p12) is proteolytically cleaved within the endoplasmic reticulum (ER) to generate the 8 kDa protein (p8). At the steady state, both proteins are expressed at similar levels in transfected T-cells. The p12 protein remains in the ER and *cis*-Golgi, whereas the p8 protein traffics to the cell surface and is recruited to the immunological synapse. The p12 and the p8 proteins have seemingly opposite effects on T-cells; the ER resident p12, modulates T-cell activation and proliferation, whereas p8 induces T-cell anergy. The p8 protein also increases the formation of cellular conduits, is transferred to neighboring T-cells, and increases virus transmission. The requirement for HTLV-1 infectivity of *orf-I* is demonstrated by the loss of virus infectivity in macaques exposed to an engineered virus, whereby expression of *orf-I* was ablated. Altogether the current knowledge demonstrates that the concerted activity of p8 and p12 is essential for the persistence of virus infected cells in the host.

Keywords

HTLV-1; p12; p8; T-cells; *orf-I*; virus transmission; immunological synapse

1. Introduction

Human T-cell leukemia/lymphoma virus type 1 (HTLV-1) is an oncogenic retrovirus discovered in 1980 from a patient with cutaneous T-cell lymphoma (Poiesz, Ruscetti et al. 1980a;Poiesz, Ruscetti et al. 1980b). It is estimated that 20 million people may be infected by HTLV-1 worldwide, and although the majority of infected individuals remain asymptomatic with 1-2% developing adult T-cell leukemia/lymphoma (ATLL) (Takatsuki,2005;Poiesz, Ruscetti, Gazdar, Bunn, Minna, and Gallo,1980a), or HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) in their lifetime (Gessain,1996).

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In addition to the typical retroviral structural genes *gag, pol,* and *env*, the HTLV-1 genome encodes the regulatory Tax and Rex proteins as well as several other proteins from four overlapping open reading frames (o*rf*) at the 3′end of the viral genome (Franchini and Lairmore, 2007). The Tax and Rex proteins are essential transcriptional and posttranscriptional positive regulators of viral expression. Four additional proteins, p12/p8 and p30/p13, are produced by alternatively spliced *mRNA*s from *orfs I and II*, respectively (Berneman, Gartenhaus et al. 1992;Ciminale, Pavlakis et al. 1992;Koralnik, Gessain et al. 1992). Moreover, an anti-sense RNA, transcribed from the minus DNA strand, encodes the HTLV-I bZIP protein (HBZ) protein (Gaudray, Gachon et al. 2002).

The p12, p13, and p30 proteins are dispensable for *in vitro* human T-cell immortalization (Derse, Mikovits et al. 1997;Robek, Wong et al. 1998). However, when their expression is ablated individually, HTLV-1 remains infectious in rabbits but not in macaques (Valeri, Hryniewicz et al. 2010). Up to date, no direct evidence exists of protein expression from *orf-I* and *orf-II*. However, their importance is suggested by the requirement for these genes in HTLV-1 infectivity, in animal models, the presence of cross-reactive antibodies, and cytotoxic T-cells responses in HTLV-1-infected patients (Dekaban, Peters et al. 2000;Pique, Ureta-Vidal et al. 2000), and the detection of *mRNA* from *ex vivo* samples. The level of *mRNA* expression for these viral genes is 100-to-1000 fold less than the *tax* and *rex* genes in HTLV-1-infected cell lines, in ATLL, HAM/TSP, and asymptomatic carriers (Berneman, Gartenhaus, Reitz, Jr., Blattner, Manns, Hanchard, Ikehara, Gallo, and Klotman ,1992;Ciminale, Pavlakis, Derse, Cunningham, and Felber ,1992;Koralnik, Gessain, Klotman, Lo Monico, Berneman, and Franchini ,1992;Ciminale, D'Agostino et al. 1995;Cereseto, Berneman et al. 1997;Dekaban, Peters, Mulloy, Johnson, Trovato, Rivadeneira, and Franchini ,2000). A recent study, based on a real-time RT-PCR approach, has demonstrated that the *mRNA* level for p12 and p30 is also much lower than that of the structural genes, *gag,* and *env* (Li, Kesic et al. 2009) in the blood of HTLV-1-infected animals.

The p30 protein is a nucleolar protein encoded by a doubly spliced *mRNA* that places the *env* and *tax* genes AUG initiating codon in frame with *orf II* (Ciminale, Pavlakis, Derse, Cunningham, and Felber ,1992;Koralnik, Gessain, Klotman, Lo Monico, Berneman, and Franchini ,1992) and acts as both a transcriptional and posttranscriptional regulator of gene expression(Michael, Nair et al. 2004;Nicot, Dundr et al. 2004). The p13 protein contains the last 87 amino acids of p30 but is encoded by a private singly spliced *mRNA* (Ciminale, Pavlakis, Derse, Cunningham, and Felber ,1992;Koralnik, Gessain, Klotman, Lo Monico, Berneman, and Franchini ,1992). The p13 increases production of reactive oxygen species (ROS) by the mitochondria, and affects cell turnover (Silic-Benussi, Marin et al. 2010). In the presence of Tax, however, a portion of p13 undergoes unconventional ubiquitination and is re-routed to nuclear speckles (Andresen, Pise-Masison et al. 2010). Another negative regulator of Tax-mediated viral expression is the HBZ protein. HBZ *mRNA* also induces T-cell proliferation and is expressed in ATLL cells (Gaudray, Gachon, Basbous, Biard-Piechaczyk, Devaux, and Mesnard,2002;Matsuoka and Green ,2009;Satou, Yasunaga et al. 2006).

2. The proteins encoded by *orf-I*

A singly spliced *mRNA* from *orf-I* (Ciminale, Pavlakis, Derse, Cunningham, and Felber, 1992;Koralnik, Gessain, Klotman, Lo Monico, Berneman, and Franchini ,1992) encodes p12/p8. The Orf-I amino acid sequence is conserved in HTLV-1-infected individuals and is highly hydrophobic (Franchini ,1995;Martins, Soares et al. 2002;Iniguez, Otsuki et al. 2005;Fukumoto, Andresen et al. 2009). Because of the p12 hydrophobicity, it has been difficult to generate antibodies able to recognize the natural Orf-I protein products. Hydropathy and immunogenicity plots predict few soluble regions and two putative transmembrane domains extending from amino acid 12 to 30 and amino acid 48 to 67(Franchini ,1995) (Figure 1). The

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p12 protein contains four putative proline-rich (PXXP) Src homology 3 (SH3)-binding domains, two putative leucine zipper (LZ) motifs, a putative adaptin motif, and a noncanonical ER retention/retrieval motif at the N-terminus (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009;Franchini,1995) (Figure 1). These structural features may contribute to the p12 membrane localization, homodimerization (Trovato, Mulloy et al. 1999), and protein-protein interactions. Immunoprecipitation and immunoblot data have demonstrated that the p12 protein, tagged at the C-terminus with either AU1 or HA1 epitopes, forms dimers in HeLa-Tat cells (Trovato, Mulloy, Johnson, Takemoto, de Oliveira, and Franchini ,1999). Two allelic forms of p12, which differ substantially in their stability, have been described. The p12 allele that carries a lysine at position 88, p12K88, is ubiquitinated and has a half life of 30 minutes, while the allelic variant, p12R88, is not ubiquitinated and has an 8 hour half life (Trovato, Mulloy, Johnson, Takemoto, de Oliveira, and Franchini ,1999).

3. Post-translational cleavage of the Orf-I protein product generates the p12 and p8 proteins that have distinct cellular localization and functions

Early observations have shown that expression of the p12 cDNA consistently yields two proteins (Koralnik, Gessain, Klotman, Lo Monico, Berneman, and Franchini ,1992). The Orf-I protein products were originally described to localize in the ER and the *cis*-Golgi apparatus (Johnson, Nicot et al. 2001;Koralnik, Fullen et al. 1993;Ding, Albrecht et al. 2001). Recently, it has been found that p12 contains two proteolytic cleavage sites: the first, between amino acids 9 and 10 and the second, between amino acids 29 and 30 which produces an 8 kDa protein (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009) (Figure 1). Both cleavage sites appear to be important for the removal of a non canonical ER retention/retrieval signal. These studies, guided by computer-based prediction of amino acid motifs (software PSPORT and ELM[30]), were conducted through site-directed mutagenesis and the construction of fused proteins. Cleavage of the 12 kDa form results in an 8 kDa protein, p8, that localizes to the lipid rafts and is recruited to the immunological synapse upon T-cell receptor ligation (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009). A mutation from glycine to serine at position 29 (G29->S), found in natural HTLV-1 strains, prevents cleavage at the second site and results in ER retention of the full-length p12 protein. In contrast, a mutation at position 26 from glutamate to asparagine (D26N) increases cleavage and results in the predominance of the cleaved the p8 protein (our unpublished data). The enzyme that processes p12 has not been identified to date.

The demonstration that ER retention of p12 is depends on its N-terminus was obtained using transfected constructs, whereby p12 was fused to neuromodulin plasma membrane targeting signal, and green fluorescent protein (GFP-Mem) (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009). Expressed alone, GFP-Mem is targeted to the plasma membrane, however fusion of the first 15 amino acids of p12 with the GFP-Mem protein resulted in localization of the protein to the plasma membrane. Interestingly, when the serine in position 10 was mutated to alanine, the fused protein was retained in the ER. Similarly, the presence of the first 32 amino acids of the wild-type p12 resulted in plasma membrane localization of p8, whereas the 32 amino acids of the p12G29S mutant resulted in retention/retrieval of the uncleaved p12G29S-GFP-Mem protein in the ER. Indeed, the first 5 amino acids of p12 alone (MLFRL), when fused with the GFP-Mem protein, caused retention of the fused protein in the ER, demonstrating that this amino acid stretch functions as a non-canonical ER retention/retrieval motif (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009).

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4. ER function of p12

4.1. p12 binds to the IL-2 Receptor γ and β chains and increases T-cell proliferation

HTLV-1 infects and immortalizes T-cells and full T-cell transformation is associated with T-cell growth in an interleukin-2 (IL-2) independent manner. The switch to IL-2-independence correlates with the constitutive activation of the Jak/signal transducers and activators of transcription (STAT) pathway {Migone, 1995 MIGONE1995 /id}. Tax protein is believed to facilitate T-cell activation and proliferation by the induction of IL-2, the IL-2 receptor (IL-2R) α chain, c-*fos*, and other genes (Johnson, Harrod et al. 2001;Xu, Kang, Heidenrich, Okerholm, O'Shea, and Nerenberg ,1995). The IL-2R is composed of three chains: the α chain, which increases ligand binding affinity, and the signaling molecules β and γ_c chains, which recruit Jak3 and STAT5α and STAT5β (Smith, Jacobson et al. 1999). STAT-5 activation results in IL-2 expression and cell division. The ER resident p12 protein binds to the immature form of the IL-2R β and γ_c chains. It is speculated that p12, by aggregating the two IL-2R chains, mimics the ability of IL-2 to induce STAT5 phosphorylation, thereby lowering the threshold of IL-2 requirement for T-cell growth(Mulloy, Crowley et al. 1996;Nicot, Mulloy et al. 2001) (Figure 2). Thus, p12 expression may favor the entry of T-cells into the S phase even in conditions of suboptimal antigen stimulation and IL-2 production (Nicot, Mulloy, Ferrari, Johnson, Fu, Fukumoto, Trovato, Fullen, Leonard, and Franchini,2001). Therefore, the combined ability of p12 and Tax to promote STAT-5 activation and increase TCR signaling increases T-cell responsiveness to IL-2 and that in turn, increases virus production because IL-2 increases CREB/ATF and Tax transcription (Lin, Hickey et al. 2005).

4.2 p12 modulates calcium release and T-cell activation

Intracellular calcium (Ca^{2+}) release is known to be required for the transcriptional activity of nuclear factor of activated T-cells (NFAT) (Negulescu, Shastri et al. 1994). An increase of intracellular Ca^{2+} concentration activates the phosphatase calcineurin that dephosphorylates NFAT (Zhu and McKeon ,2000)**.** Dephosphorylated NFAT translocates to the nucleus and induces expression of IL-2. p12 protein increases the basal level of cytosolic Ca^{2+} (Ding, Albrecht et al. 2002) and this event results in the dephosphorylation of NFAT, enhancement of IL-2 production, and T-cell proliferation (Ding, Kim et al. 2003) (Figure 3). p12 physically binds to resident chaperones calreticulin and calnexin proteins that facilitate appropriate protein folding, as well as modulate Ca^{2+} storage. Indeed it has been suggested that the binding of calreticulin and calnexin to p12 increased the intracellular Ca^{2+} concentration (Ding, Albrecht, Luo, Zhang, Stanley, Newbound, and Lairmore ,2001). The p12-mediated increase of NFAT activation is observed with agents that stimulate T-cells bypassing the T-cell receptor (TCR), such as phorbol myristate acetate (PMA). PMA stimulation directly activates a protein kinase C (PKC) isoform downstream of the TCR (Albrecht, D'Souza et al. 2002;Ding, Albrecht, Kelley, Muthusamy, Kim, Altschuld, and Lairmore,2002). Therefore, as expected, treatment with a Ca^{2+} chelator (BAPTA-AM or cyclosporine A) or with a dominant negative mutant of NFAT, blocks the p12 mediated Ca^{2+} release and NFAT activation. The effect of p12 on Ca^{2+} release can be recapitulated by the treatment of T-cells with thapsigargin, a drug that depletes intracellular Ca^{2+} stores (Albrecht, D'Souza, Ding, Tridandapani, Coggeshall, and Lairmore ,2002;Ding, Albrecht, Kelley, Muthusamy, Kim, Altschuld, and Lairmore,2002). The p12-mediated increase in NFAT activation is also dependent on inositol triphosphate (IP3) and partially dependent on Ca^{2+} release-activated Ca^{2+} channel (CRAC), a channel that facilitates Ca^{2+} influx across the plasma membrane (Ding, Albrecht, Kelley, Muthusamy, Kim, Altschuld, and Lairmore ,2002). Altogether, these data suggest that in the ER, p12 increases calcium influx, NFAT activation, and IL-2 production.

Surprisingly, the Orf-I protein products also inhibit NFAT through competitive-binding to calcineurin (Kim, Ding et al. 2003). The p12 and p8 proteins carry a highly conserved

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calcineurin-binding motif calcineurin-binding motif PXIXIT that could compete for binding of calcineurin to NFAT (Figure 1)(Kim, Ding, Albrecht, Green, and Lairmore ,2003). Point mutations of the four SH3 like-binding motifs in o*rf-I* demonstrated two positive (SH3-2,4) and two negative regions (SH3-1,3) in NFAT regulation (Figure 1) (Ding, Kim, Nair, Michael, Boris-Lawrie, Tripp, Feuer, and Lairmore ,2003). Thus, the Orf-I proteins regulates positively NFAT transcriptional activity by increasing ER Ca^{2+} release and negatively, by competing with NFAT for calcineurin binding (Figure 4). It is logical to speculate that the modulation of Ca^{2+} release is mediated by the ER resident p12 protein. At present, whether p12 or p8 or both bind to calcineurin remains unclear.

4.3 p12 re-routes the major histocompatibility complex class I to the proteasome for degradation

The immune system has evolved highly sophisticated mechanisms to recognize “self” and “non-self” antigens in order to eliminate pathogens without causing tissue damage. Toll-like receptors on dendritic cells (Fitzgerald and Golenbock ,2007) are able to sense specific pathogens. Dendritic cells engulf microbial antigens and prime T-cells for recognition of pathogen-specific peptides presented by the major histocompatibility complex class I (MHC-I) on the surface of infected cells. Once primed, the cytotoxic T-cells (CTL) continuously scan somatic cell surfaces in search for pathogens. Once a “non-self” antigen from a pathogen is identified, the $CD8^+$ T-cells are activated and rapidly kill the infected cell. The MHC I complex includes a 44 kDa transmembrane glycoprotein heavy chain, the β_2-microglobulin (β_2m), and a short peptide of 8–12 amino acids. In the ER, p12 physically interacts with the MHC I Heavy chain (Hc) and prevents its association with the β_2m (Johnson, Nicot, Fullen, Ciminale, Casareto, Mulloy, Jacobson, and Franchini ,2001;Johnson, Mulloy et al. 2000). This interaction induces the MHC I Hc retro-translocation into the cytosol for degradation by the proteasome (Figure 4). Thus, p12 decreases MHC I on the cell surface. In addition, p12 has been shown to target HLA-A2 and HLA-B7 (Johnson, Nicot, Fullen, Ciminale, Casareto, Mulloy, Jacobson, and Franchini,2001) but other MHC-I types have not been tested. Of importance, peripheral mononuclear lymphocytes isolated from HTLV-1-infected patients, have altered expression of MHC I alleles (Mann, Popovic et al. 1983;Sonoda, Yashiki et al. 1987;Uno, Matsuoka et al. 1995). Likely, the ability of p12 to interfere, with the assembly and trafficking of the MHC I complex to the cell surface, contributes to viral persistence by helping infected cells to evade immune surveillance.

4.4 p12 and natural killer cells

Decreased expression of MHC I on the surface of cells pose the risk of recognition and killing by natural killer cells (NK) cells. Thus, pathogens that reduce MHC-I expression on the cell surface, have evolved strategies that also counteract the function of NK cells and HTLV-1 is no exception. NK cells require ICAM receptors to adhere to T-cells through the leukocyte function antigen-1 (LFA-1) adhesion molecule. The Orf-I proteins have been shown to impair NK killing of infected cells by decreasing the expression of intercellular adhesion molecule ICAM-1 and ICAM-2 on the cell surface (Banerjee, Feuer et al. 2007). This down-modulation is selective since the Orf-I proteins do not alter the expression of ICAM-3 on $CD4^+$ T-cells (Banerjee, Feuer, and Barker,2007). The mechanism of downregulation of ICAM-1 and ICAM-2 by either p12 and/or p8 is unknown. Nevertheless, the down-regulation of MHC class I and ICAM-1 and ICAM-2, induced by the Orf-I proteins, likely helps the HTLV-1-infected cells to escape immune recognition by both CTL and NK cell.

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5. Cell Membrane functions of p8

5.1 p8 downregulates proximal T-Cell Receptor signaling

The immunological synapse (IS) is a highly structured region of contact between the T-cells and the antigen presenting cells (APC), such as dendritic cells. IS is formed following the engagement of the T-cell receptor (TCR), expressed on T-cells, to the MHC-I complex expressed on the APC (Wulfing and Davis ,1998). Such an event leads to the activation of the protein tyrosine kinases Lck and Fyn, which phosphorylates tyrosines located on the cytosolic domains of TCR (Figure 5). The downstream phosporylation of ZAP70 results in activation of LAT, that in turn, recruits Grb2, phospholipase C-1 (PLC-1), and the p85 subunit of phosphatidylinositol 3-kinase and indirectly, Vav, Cb1, and SLP76 (Manz and Groves , 2010). The recruitment of these molecules to the plasma membrane is essential for calcium release from the ER, nuclear translocation of NFAT, enhancement of transcription, IL-2 production, and T-cell proliferation. Interestingly, HTLV-1 infection is associated with a significant degree of immune deficiency (Bunn, Jr., Schechter et al. 1983;Yarchoan, Guo et al. 1986). Indeed, the adaptive immune response to HTLV-1 varies among infected people (Yarchoan, Guo, Reitz, Jr., Maluish, Mitsuya, and Broder ,1986;Jacobson, Shida et al. 1990;Parker, Daenke et al. 1992;Kannagi, Matsushita et al. 1993;Kannagi, Matsushita et al. 1994). The exact nature of the wide variation in levels and quality of the immune responses to HTLV-1 among infected individuals is unclear. Insights regarding these early findings were provided by the realization that the HTLV-1 p8 protein is recruited to the lipid rafts within the IS upon engagement of TCR and causes T-cell anergy (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009). Lipid rafts are a heterogeneous mixture of sphingolipid and cholesterol that are tightly packed and form microdomains in cell membranes and serve as organizational centers for the clustering of signaling molecules (Taner, Onfelt et al. 2004). p12 binds to LAT and decreases LAT phoshorylation, that in turn, results in decreased phosphorylation of the downstream T-cell signaling molecules PLC-γ1 and Vav and dowregulation of NFAT activity (Figure 5) (Fukumoto, Dundr et al. 2007). This contrasts with p12-mediated NFAT activation following PMA stimulation, which, by bypassing TCR, is independent of LAT (Figure 3). The opposing effects of p12 and p8 on NFAT may occur at different stages of the cell cycle since TCR stimulation drives T-cells from a resting state to early G_1 phase, while responsiveness to IL-2 is linked with the S phase. The effect of p8 on the immunological synapse has been recently confirmed by imaging techniques that demonstrate a reduction in the strength of the immunological synapse in the presence of p8 {Van Prooyen N, 2010 362 /id}. An important finding is that p8, by inhibiting NFAT, also inhibits viral replication (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009), since NFAT activation increase Tax activity (Lin, Hickey, Hsu, Medina, and Rabson ,2005). Collectively, these data suggest that HTLV-1, through the timely expression of the Orf-I proteins, may finely modulate T-cell activation as well as its own expression.

5.2 p8 promotes T-cell contact and HTLV-1 transmission

HTLV-1 infects dendritic cell and T-cells and virus transmission is more efficient through direct cell-to-cell contact (Jones, Petrow-Sadowski et al. 2008). Upon cell contact HTLV-1 mediates rapid reorientation of the microtubule organizing center (MTOC) towards the cell-cell junction (Igakura, Stinchcombe et al. 2003). The formation of the virological synapse requires the polarization of cytoskeleton and adhesion receptors, such as LFA-1 and ICAM receptors (Majorovits, Nejmeddine et al. 2008). Prior data demonstrated that the Orf-I protein products increased LFA-1 clustering on T-cells (Kim, Nair et al. 2006) and more recent evidence, demonstrates that the increased clustering of LFA-1 in lipid rafts is mediated by p8, and not by p12 protein (Van Prooyen N, Gold H, Andresen V, Schwartz O, Jones K, Rucetti F, Lockett S, Prabhakar G, Venzon D, and Franchini G ,2010) (Figure 6).

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p8 increases also the number and length of cellular conduits (Watkins and Salter , 2005;Sowinski, Jolly et al. 2008) that favor communication among several types of cells, and p8 is transferred, within minutes, to neighboring T-cells {Van Prooyen N, 2010 355 /id}. Importantly, p8 augments the envelope-dependent transfer of HTLV-I to neighboring T-cells. These data are consistent with recent reports demonstrating that the Orf-I protein products increases viral transmission in an IL-2-dependent manner (Taylor, Brown et al. 2009).

6. p12/p8 and the H^+ vacuolar ATPase

H^+ vacuolar ATPase (V-ATPase) regulates pH in cellular organelles and affects enzymes activity, membrane fusions, and the dissociation of internalized ligand-receptor complexes in endosomes (Toei, Saum et al. 2010). Acidification is achieved through regulation of V-ATPase activity by its isoform composition, as well as the specific targeting and trafficking of V-ATPase to organelles. Activation of V-ATPase causes the gradual acidification of compartments along the secretory pathway, ranging from the ER at pH 7.2 to secretory granules at pH 5.2. In addition, V-ATPases acidify the vesicles of the endocytic pathway from early endosomes at pH 6.3 to lysosomes at pH 5.5 (Marshansky and Futai ,2008).

Co-immunoprecipitation experiments demonstrated that both the p12 and p8 proteins bind to the 16 kDa component of the V-ATPase, the pore forming subunits of the enzyme complex (Franchini, Mulloy et al. 1993;Koralnik, Mulloy et al. 1995). Further work demonstrated that the two putative transmembrane domains of p12 do not participate in this interaction. The proline-rich region amino acid located between amino acid 36 and 48, present in both p12 and p8 (Figure 1), is important of V-ATPase binding (Koralnik, Mulloy, Andresson, Fullen, and Franchini ,1995).

The interaction between p12 and the 16 kDa protein of the V-ATPase likely occurs in the ER and may prevent the assembly of the mature form of the V-ATPase and acidification of the secretory pathway. This effect may have important functional implications. For example, late endosomal compartments require a low compartmental pH in order for MHC II to bind a peptide. MHC II is expressed on professional antigen presenting cells, such as dendritic cells that are infected by HTLV-1 (Jones, Petrow-Sadowski, Huang, Bertolette, and Ruscetti , 2008). MHC II presents exogenous foreign peptides to $CD4^+$ helper T-cells. Therefore, by inhibiting the V-ATPase, p12 could block MHC II presentation to T-cells and reduce immune surveillance (Chow and Mellman ,2005). A similar effect could be exerted on MHC-I antigen presentation.

Recent work shows that the p8 protein traffics to the cell surface viable secretory pathway (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009) and also localizes at early endosomes and recycling endosomes (our unpublished results). Thus, p8 association with the V-ATPase could alter, not only the secretory and the endocytic pathway, but also affect receptor recycling on the cell membrane. Functional data on the effect of p8 or p12 on these pathways are not available at present. It is likely that the biological consequences of p12 and p8 interaction with the V-ATPase vary in different cellular compartments as well as in T-cells versus dendritic cells.

7. Role of Orf-I in viral infectivity *in vivo*

The *orf*-I *mRNA* is detected *in vivo* in HTLV-1 in infected patients as well as in asymptomatic carriers (Furukawa, Furukawa et al. 1991;Berneman, Gartenhaus, Reitz, Jr., Blattner, Manns, Hanchard, Ikehara, Gallo, and Klotman ,1992;Ciminale, Pavlakis, Derse, Cunningham, and Felber ,1992;Koralnik, Gessain, Klotman, Lo Monico, Berneman, and Franchini , 1992;Ciminale, D'Agostino, Zotti, Franchini, Felber, and Chieco-Bianchi ,1995;Cereseto, Berneman, Koralnik, Vaughn, Franchini, and Klotman ,1997). Thus, o*rf-I* expression appears

to be important throughout the course of natural HTLV-I infection. Initial studies that ablated the *orf-I* splice acceptor site from an HTLV-I molecular clone, resulted in a significant loss of HTLV-1 infectivity in the White New Zealand rabbit model (Collins, Newbound et al. 1998). However, that mutation also affected HBZ, whose ablation alone decreases virus levels (Arnold, Yamamoto et al. 2006). Therefore, the results of the early work using this *orf-I* mutant could have been in part due to the alteration of the HBZ protein. Indeed, a more recent study, whereby the expression of the o*rf-I* gene was ablated by a single point mutation in the initiation codon without truncating HBZ, demonstrated that this mutant virus, defective in *orf-I* expression, was able to replicate in rabbits as the wild type HTLV-1 (Valeri, Hryniewicz, Andresen, Jones, Fenizia, Bialuk, Chung, Fukumoto, Parks, Ferrari, Nicot, Cecchinato, Ruscetti, and Franchini ,2010). Of interest, the same o*rf-I* mutant virus was unable to establish infection in primary human dendritic cell *in vitro* and in macaques *in vivo* (Valeri, Hryniewicz, Andresen, Jones, Fenizia, Bialuk, Chung, Fukumoto, Parks, Ferrari, Nicot, Cecchinato, Ruscetti, and Franchini ,2010). This data demonstrates the expression of *orf-I* gene is essential in nonhuman primates and suggests that HTLV-1 infection of macaques may be a more relevant model for humans. This animal species is infected with HTLV-1 in the wild and develop leukemia, whereas rabbits do not (Beilke, Traina-Dorge et al. 1996;Traina-Dorge, Martin et al. 2007;Lairmore, Silverman et al. 2005;Allan, Leland et al. 2001). Collectively, these data support an important role for Orf-I protein products in the establishment and the maintenance of HTLV-1 infection *in vivo*.

8. Naturally Occurring genetic Orf-I polymorphism and Disease Associations

Lysine is a known target for covalent binding of ubiquitin, allowing lysine-containing proteins to be targeted for degradation by the proteasome (Varshavsky ,1996). HTLV-1 Orf-I contains a rare genetic polymorphism that results in mutation of arginine at position 88 to a lysine (p12K88). This allelic variant is ubiquitinated and rapidly degradated whereas the more frequent p12R88 is more stable (Trovato, Mulloy, Johnson, Takemoto, de Oliveira, and Franchini ,1999). The p12K88 variant was initially found in HAM/TSP patients (5 out of 8 patients), raising the hypothesis that the presence of the p12K88 mutation could be associated with HAM/TSP. (Trovato, Mulloy, Johnson, Takemoto, de Oliveira, and Franchini,1999). However, further sequence analysis of proviral DNA from Brazilian and Japanese patients (Martins, Soares, Ribas, Thorun, Johnson, Kroon, Carneiro-Prioetti, and Bonjardim,2002) (Furukawa, Usuku et al. 2004) did not support this hypothesis. Thus, because the p12K88 variant is rare (2.7% of 37 patients and 1.4% of 144 patients, respectively) in HAM/TSP patients, the significance of this allelic polymorphism in TSP/HAM, if any, remains unclear.

A large study performed on 231 HTLV-1 infected individuals demonstrated the preservation of the *orf I* genetic sequence in 46 asymptomatic patients. Interestingly however, premature termination that truncated the carboxy terminus of 17 or of 12 amino acids was found in 0.7% and 4.97%, respectively, of TSP/HAM patients from Japan. In the same study, a female TSP/HAM was also found to have a mutation in the initiating methionine to isolencine (M->I) (Furukawa et al., 2004) and the provirus of the infected sister carried the same mutation.

These data “perse” do not suggest that *orf I* is not necessary for infection. Rather they indicate that the last 17 amino acid may not be necessary to maintain HTLV-I infection. Similarly, the finding of a substitution of M->I in the three HTLV-I infected siblings does not necessarily prove that the *orf I* products are dispensable for viral infectivity, since doubly spliced mRNA *pX-Rex-orf I* encodes the *orf I* product that undergoes cleavage as demonstrated by Koralink et al. 1992.

Sicca syndrome is described as an autoimmune disorder that affects the ability of the lachrymal and the salivary glands to produce tears and saliva (Wu and Fox ,1994). The sicca syndrome

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has been frequently associated with HAM/TSP and it has been suggested that it may be an early event in progression to HAM/TSP (Vernant, Buisson et al. 1988;Hajjar, Sainte-Foie et al. 1995;Nakamura, Eguchi et al. 1997). Sequence analysis of Caribbean or Guianese HTLV-1 infected patients with sicca syndrome demonstrated that p12 was identical in several isolates from patients regardless of their different clinical statuses. Several amino acid changes were identified within the putative transmembrane domains of p12, located between amino acid 12-32 and amino acid 48-68 (Beby-Defaux, Frugier et al. 1999). However, the functional significance of these mutations and their relevance to disease, if any, remains to be established.

More recent studies, suggest that some natural *orf-I* alleles may differ functionally. Reverse genetic studies on natural variants of the HTLV-1 *orf-I* have demonstrated mutations that alter the proteolytic cleavage of the Orf-I protein product. Proviruses with o*rf-I* that expresses mainly p12 are much more common than those that predominantly express p8 (Fukumoto, Andresen, Bialuk, Cecchinato, Walser, Valeri, Nauroth, Gessain, Nicot, and Franchini ,2009)(our unpublished data). Few patients have been shown to carry an allelic polymorphism that favors cleavage to the 8kDa form. Further studies will be necessary to determine whether there is a correlation between the presence of specific *orf-I* alleles and virus levels or disease progression in HTLV-1-infected individuals.

9. Future work and conclusions

Although significant progress has been made in our understanding of the functional importance of p12/p8 *in vitro* and *in vivo*, several questions remain unanswered. Future studies need to identify cellular partners that mediate the effect of p12 and p8 on MHC-I, STAT5, ICAM-1 at ICAM 2, and NFAT proteins. The identification of these cellular proteins will help to address, mechanistically the functions of p12 and p8 and also to uncover other pathways affected by the Orf-I products. The timing of p8 and p12 expression within T-cells and dendritic cells is also of great interest because these proteins have opposing effects on T-cell growth and may differently affect virus replication *in vitro* and *in vivo*. However, this represents a difficult goal. The *mRNA* for *orf-I* is expressed at very low levels and because of the hydrophobicity of p12 and p8, it has been difficult to generate specific antibodies to the natural protein products of *orf-I*.

Very little is known regarding the functional effect of p12 and p8 interactions with the V-ATPase enzymatic complex. The V-ATPases are enzymes composed of multiple subunits that acidifies intracellular organelles, secretory pathway and regulate receptor recycling and degradation(Toei, Saum, and Forgac ,2010). The p12 and p8 proteins might have different effects on the V-ATPase located in endosomal compartments or in the cellular membrane. Modulation of the V- ATPase, activity might be particularly important for virus entry and egress in T-cell and dendritic cells.

The use of reverse genetic, in combination with functional studies of natural *orf-I* mutants of HTLV- 1 infected individuals, will hopefully elucidate the impact of the p12 and p8 functional domains that affect the level of provirus DNA in the host. This information is of great importance because HTLV-1 provirus levels predict disease progression in humans. Thus, the understanding of the underlying mechanisms of p12 and p8 function may facilitate the development of therapeutic approaches interfere with virus persistence in the infected host.

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Figure 1. Amino acid sequence and domains within the p12 and p8 proteins

HTLV-1 p12 is a highly hydrophobic protein that contains an amino terminus noncanonical ER retention/retrieval motif (MLFRL), four putative proline-rich (PXXP) Src homology 3 (SH3)-binding domains, two putative leucine zipper (LZ) motifs, an adaptin motif, and a calcinuerin binding motif. The arrows indicate the two cleavage sites between amino acid positions 9 and 10; 29 and 30, respectively.

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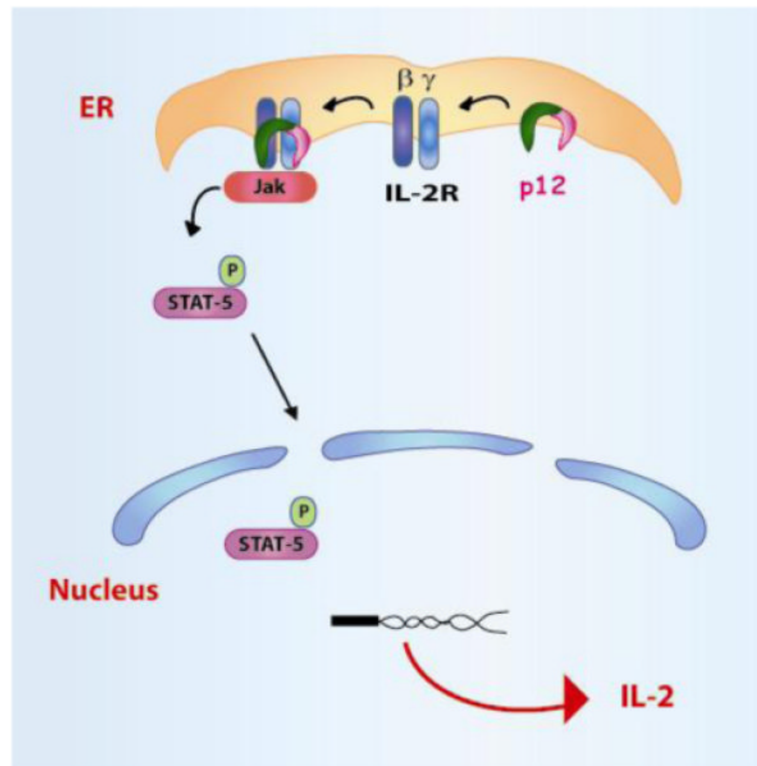

Figure 2. p12 induces the dimerization of the ILR β and γ chains and increases STAT5 activation

In the ER, p12 binds to both the IL-2 the β and γ_c chains and decreases the trafficking of the two IL-2R chains to the cell surface. p12 increases STAT5 phosphorylation and IL-2 gene transcription. This leads to the decreased requirement for IL-2 in HTLV-1-infected cells.

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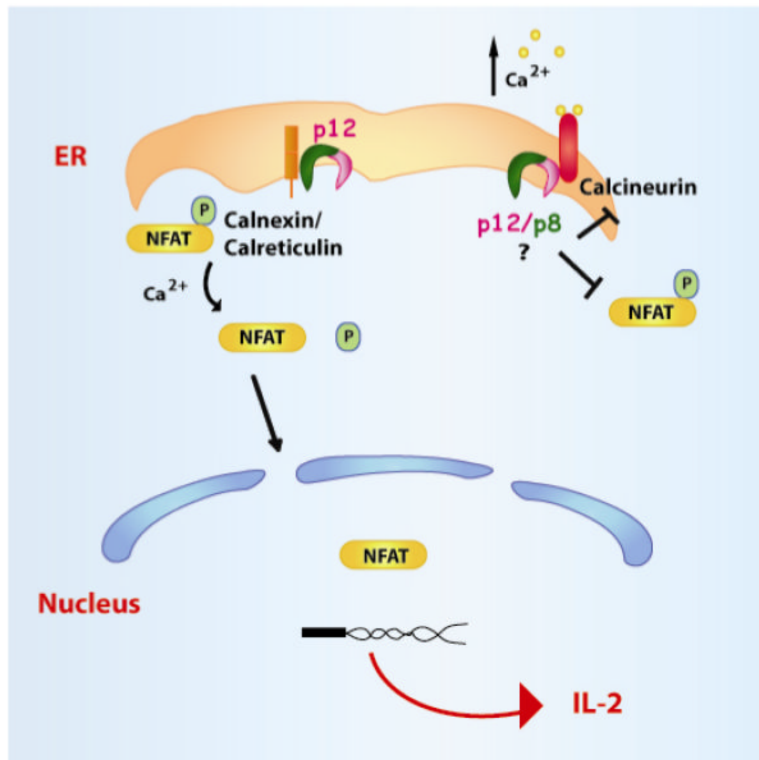

Figure 3. Opposing effects of the Orf-I products on NFAT activation

Upon phorbol myristate acetate (PMA) stimulation, which releases ER calcium-stores in a TCR-independent mechanism, p12 increases NFAT dephosphorylation. The dephosphorylated NFAT is translocated into the nucleus, where it activates IL-2 production and favors T-cell growth (left part of the Figure). The Orf-I product(s), however, also bind(s) to calcineurin, competes with calcineurin for binding to NFAT and decrease T-cell activation (right part of the Figure).

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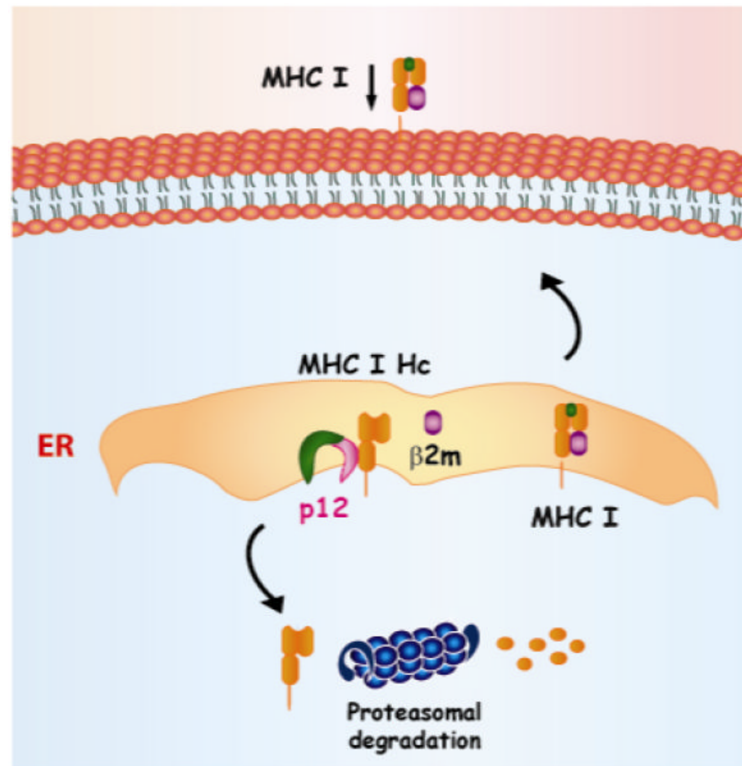

Figure 4. p12 binds to and reroutes the MHC I Heavy chain for degradation to the proteasome
The p12 protein interacts with the MHC I heavy chain (Hc) in the ER and prevents its association with the beta-2-microglobulin (β_2m). The lack of association of MHC I HC with the β_2m causes its retrotranslocation into the cytosol and degradation by the proteasome. This ultimately leads to a decreased MHC I cell surface expression.

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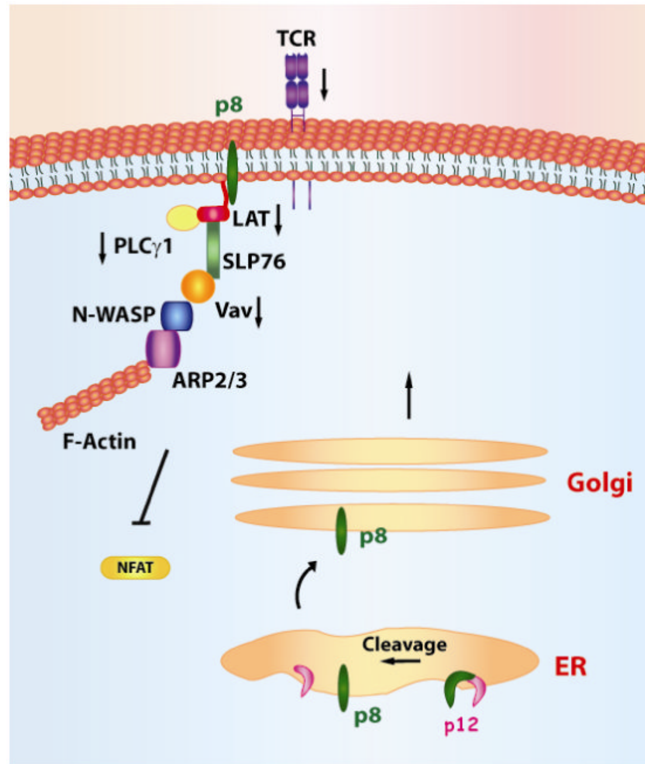

Figure 5. p8 localizes at the immunological synapse and decreases proximal T-cell signaling

p8 decreases T-cell activation after TCR-stimulation through its interaction with LAT, which causes the decrease in phosphorylation of LAT, PLC-γ1, Vav, and Lck and decreased activity of NFAT.

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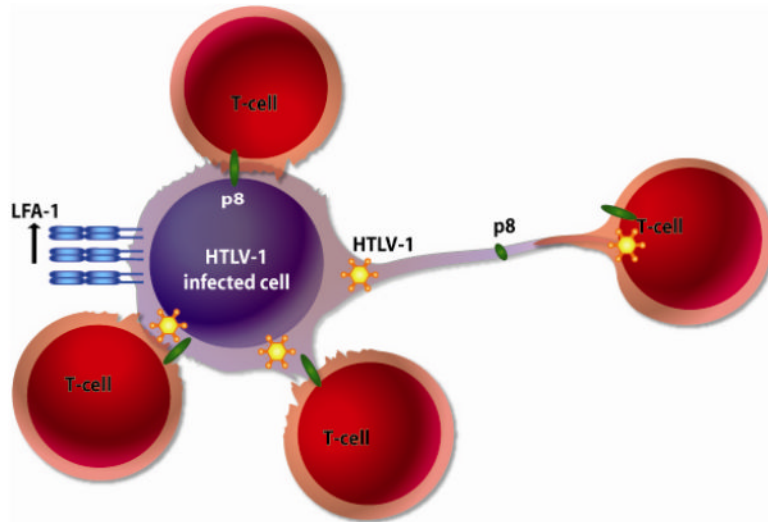

Figure 6. p8 enhances viral spread by increasing cell-cell contact

The p8 protein increases LFA-1 clustering at the cell surface leading to increased cell-cell contact. In addition, p8 stimulates the formation of cellular conduits. These modes of cell contact lead to increased p8 transfer to neighboring cells and ultimately viral transmission.