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COMPARATIVE STUDIES BETWEEN HTLV-1 AND HTLV-2 FUNCTION AND PATHOBIOLOGY

Topic Editors
Umberto Bertazzoni and Françoise Bex

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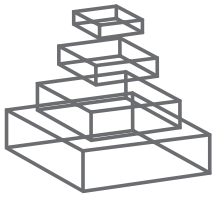

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COMPARATIVE STUDIES BETWEEN HTLV-1 AND HTLV-2 FUNCTION AND PATHOBIOLOGY

Topic Editors:
Umberto Bertazzoni, Università degli Studi di Verona
Françoise Bex, Université Libre de Bruxelles

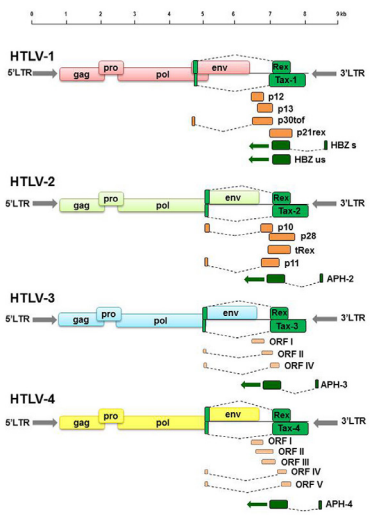

Fig. 1. Schematic representation of HTLV-1, HTLV-2, HTLV-3 and HTLV-4 genomic organization. Romanelli M.G. etal, Front. Microbiol, 9 Sept 2013, doi:10.3389/fmicb 2013.00271

Human T-cell leukemia viruses type 1 and 2 (HTLV-1 and HTLV-2) share a common genetic organization, expression strategy and ability to infect and immortalize T-cells in vitro; however, HTLV-1 and HTLV-2 are strikingly different in terms of clinical impact. HTLV-1 is recognized as the aetiological agent of adult T-cell leukemia/lymphoma (ATLL), and HTLV-associated myeolopathy/tropical spastic paraparesis (HAM/TSP), in contrast, HTLV-2 does not cause hematologic disorders and is only sporadically associated with cases of subacute myelopathy. HTLV-1 and HTLV-2 also exhibit distinct cellular tropisms in vivo: HTLV-1 is mainly found in CD4+T lymphocytes, whereas CD8+T-cells are the preferred target for HTLV-2.

The articles contributed in this Research Topic are covering all the different aspects that characterize HTLV-1 and HTLV-2, by highlighting differences in their biology that might provide clues to their distinct pathogenic properties.

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EDITORIAL
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Editorial to the Research Topic "Comparative studies between HTLV-1 and HTLV-2 function and pathobiology"

Umberto Bertazzoni*

Department of Life and Reproduction Sciences, Section of Biology and Genetics, University of Verona, Verona, Italy
**Correspondence: umberto.bertazzoni@univr.it*

Edited and reviewed by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Keywords: HTLV-1, HTLV-2, expression, proteins, co-infection

Human T-cell leukemia viruses type 1 and 2 (HTLV-1 and HTLV-2) share a common genetic organization, expression strategy and ability to infect and immortalize T-cells *in vitro*; however, HTLV-1 and HTLV-2 are strikingly different in terms of clinical impact. HTLV-1 is recognized as the aetiological agent of adult T-cell leukemia/lymphoma (ATLL), and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), in contrast, HTLV-2 does not cause hematologic disorders and is only sporadically associated with cases of subacute myelopathy. HTLV-1 and HTLV-2 also exhibit distinct cellular tropisms *in vivo*: HTLV-1 is mainly found in CD4+ T lymphocytes, whereas CD8+ T-cells are the preferred target for HTLV-2.

The articles contributed in this Research Topic are covering all the different aspects that characterize HTLV-1 and HTLV-2, by highlighting differences in their biology that might provide clues to their distinct pathogenic properties.

Emphasis was placed on the comparison of mRNA expression (Cavallari et al., 2013), the genetic organization and expression patterns (Ciminale et al., 2014), the structural and functional properties of HTLV-1 and HTLV-2 Tax proteins (Forlani et al., 2013; Ren and Cheng, 2013; Romanelli et al., 2013; Shirinian et al., 2013), the role of accessory proteins and antisense proteins in viral pathogenesis (Anupam et al., 2013; Barbeau et al., 2013), and the mechanisms of HIV-1/HTLV-1 and HIV-1/HTLV-2 co-infections (Pilotti et al., 2013).

The fine tuning of expression of HTLV-1 and HTLV-2 (Cavallari et al., 2013) was focused on the X region that codes for the regulatory and accessory proteins in partially overlapping ORFs. Expression of such compact genomes is accomplished by a combination of ribosomal frameshifting, alternative splicing and polycistronic translation as well as production of negative-strand transcripts that code for the HBZ and APH-2 proteins. Recent studies of the temporal sequence of HTLV-1 gene expression in PBMCs isolated from infected patients revealed a "two-phase" kinetics in which tax/rex mRNA expression precedes that of other viral transcripts. A similar analysis of HTLV-2 mRNA expression indicated a comparable expression pattern, although HTLV-2 is characterized by a more abundant expression of gene products that may favor viral latency (i.e., p28 and tRex).

Given the relevance of Tax in the initial steps of T-cell transformation, their key role as the main viral oncoproteins was discussed in four different articles, each one focusing on a specific aspect of Tax function. Romanelli et al. (2013), highlighted distinctive properties of Tax proteins with emphasis on the activation of the NF-κB pathways and interactions with host factors that participate in signal trasduction. Tax-1 and Tax-2, though sharing many common properties, significantly differ for the presence of a PDZ motif, which is missing in Tax-2. This motif mediates the interaction of Tax with host factors regulating the cell cycle; in addition Tax-2 is unable to activate the non-canonical NF-κB which is attributed only to Tax-1. Shirinian et al. (2013), specifically addressed the subcellular localization and trafficking of Tax-1 and Tax-2, and their effects on cellular regulatory proteins. A special attention was given to Tax-1/Tax-2 post-translational modifications, as well to NF-κB activation and protein-protein interactions involved in oncogenecity both *in vivo* and *in vitro*. Ren and Cheng (2013), discussed their recent observations and views on the differential transforming activity of Tax-1 and Tax-2 in human T cells. Both proteins are unable to immortalize CD8 cells but Tax-2 is more efficient than Tax-1 in immortalizing CD4 cells. Forlani et al. (2013), discussed the pivotal role of the class II transactivator (CIITA) in triggering the adaptive immune response against pathogens. CIITA acts as an endogenous restriction factor against HTLV-1 and HTLV-2 by targeting their viral transactivators Tax-1 and Tax-2. The authors reviewed their findings on CIITA-mediated inhibition of viral replication and discussed similarities and differences in the molecular mechanisms by which CIITA specifically counteracts the function of Tax proteins.

The function of the Rex protein was presented by Ciminale et al. (2014). Although Rex-1 and Rex-2 share a similar domain structure, the truncated forms of Rex-2 are capable of inhibiting Rex function, while their HTLV-1 homolog p21 Rex might lack this activity.

The role in viral replication and viral pathogenesis of accessory proteins HTLV-1 p30 and HTLV-2 p28 were highlighted by Anupam et al. (2013). Both p30 and p28 regulate viral gene expression at the post-transcriptional level whereas p30 can also function at the transcriptional level. Since p30 and p28 have distinct interactome profiles even though they both interact with similar proteins, they must have divergent functions during the lifecycle of the viruses.

The functions of other accessory proteins have been presented by Ciminale et al. (2014). HTLV-1 p13 and p8 are not expressed by HTLV-2 which codes for p11, whose function is still unclear and does not seem to have a homolog in HTLV-1.

The possible role played by antisense proteins HTLV-1 HBZ and HTLV-2 APH-2 in the establishment of pathologies induced by viral infection has been carefully considered by Barbeau et al. (2013). Unlike APH-2, HBZ possesses specific domains which mediate its capacity to enhance its own expression, deregulate several pathways and induce T cell proliferation, providing substantial evidence toward its implication in viral persistence and ATLL cell survival.

Finally the problem of HIV/HTLV co-infection has been discussed by Pilotti et al. (2013). During co-infection with HIV-1, HTLV-2 modulates the cellular microenvironment favoring its own propagation and inhibiting HIV-1 progression. Possible differences between HTLV-1 and HTLV-2 on innate immune mechanisms induction and a particularly impact on NK cells are becoming evident.

In conclusion, in this Research Topic, HTLV-1 and HTLV-2 have been thoroughly compared and major differences outlined and discussed. Of particular interest is that HTLV-2 infection, contrary to HTLV-1, is characterized by an oligoclonal distribution in asymptomatic hosts; this indicates that the etiology of malignant transformation by HTLV-1 cannot be uniquely attributed to this phenomenon and further investigation is needed to understand why HTLV-2 is defective in promoting leukemogenesis.

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HTLV-1 and HTLV-2: highly similar viruses with distinct oncogenic properties

***Vincenzo Ciminale*[1], *Francesca Rende*[1], *Umberto Bertazzoni*[2] *and Maria G. Romanelli*[2] ***

[1] *Department of Surgery, Oncology and Gastroenterology, University of Padua, Padua, Italy*
[2] *Department of Life and Reproduction Sciences, University of Verona, Verona, Italy*

Edited by:
Hirofumi Akari, Kyoto University, Japan

Reviewed by:
Hidekatsu Iha, Oita University, Japan
Fatah Kashanchi, George Mason University, USA

****Correspondence:***
Maria G. Romanelli, Department of Life and Reproduction Sciences, University of Verona, Strada le Grazie 8, Verona 37134, Italy
e-mail: mariagrazia.romanelli@univr.it

HTLV-1 and HTLV-2 share broad similarities in their overall genetic organization and expression pattern, but they differ substantially in their pathogenic properties. This review outlines distinctive features of HTLV-1 and HTLV-2 that might provide clues to explain their distinct clinical outcomes. Differences in the kinetics of viral mRNA expression, functional properties of the regulatory and accessory proteins, and interactions with cellular factors and signal transduction pathways are discussed.

Keywords: HTLV, Tax, Rex, HBZ, APH2, clonality

INTRODUCTION

Four types of HTLV have been described so far, the most prevalent types being HTLV-1 and HTLV-2 (Gessain et al., 2013).

HTLV-1 and HTLV-2 show considerable homology in terms of genome structure, replication pattern, and properties of the structural, regulatory, and accessory proteins. Their transmission follows the same route, by transfer of infected lymphocytes by perinatal transmission and breastfeeding and through blood transfusion, sexual contact, and use of intravenous drugs (Pique and Jones, 2012). Both viruses utilize the GLUT-1 and NRP1 cellular receptors for their entry, although HTLV-1, but not HTLV-2, is dependent on heparan sulfate proteoglycans (Jones et al., 2006). Cell-to-cell transmission is essential for virus replication and occurs through the formation of a virological synapse (Nejmeddine et al., 2005).

Despite these important analogies, HTLV-1 and HTLV-2 are strikingly different in terms of clinical impact, as only HTLV-1 is conclusively associated with neoplasia, namely adult T-cell leukemia/lymphoma (ATLL), which develops in up to 5% of infected individuals. A similar percentage of HTLV-1-infected persons develop a neurological disease termed HTLV-associated myeolopathy/tropical spastic paraparesis (HAM/TSP; Gessain and Mahieux, 2012). In contrast, HTLV-2, though persistently associated with elevated lymphocyte and platelet counts (Bartman et al., 2008) and with an increase in overall cancer mortality (Biswas et al., 2010), does not cause hematologic disorders and is only sporadically associated with myelopathy (Araujo and Hall, 2004).

The two viruses also differ in their geographical distribution. HTLV-1 is endemic in Japan, sub-Saharan Africa, South America, and the Caribbean (Gessain and Cassar, 2012), whereas HTLV-2 is prevalent among the indigenous populations in Africa and the Indian-American tribes in Central and South America as well as among drug users in Europe and North America (Zella et al., 1990; Roucoux and Murphy, 2004). Although their receptor usage allows HTLV-1 and HTLV-2 to be quite promiscuous for different cell types *in vitro*, they exhibit distinct cellular tropisms *in vivo*: HTLV-1 is mainly found in CD4+ T lymphocytes, whereas CD8+ T cells are the preferred target for HTLV-2 (Ijichi et al., 1992).

The following sections highlight differences in the biology of HTLV-1 and HTLV-2 that might provide clues to their distinct clinical outcomes. Emphasis is placed on the comparison of the regulatory proteins, kinetics of mRNA expression, clonal distribution patterns, and interaction with cellular factors and signal transduction pathways.

THE COMPARATIVE ANATOMY OF THE HTLV-1 AND HTLV-2 GENOMES

In their pioneering studies, Seiki et al. (1983) noted the presence of a region in the HTLV-1 genome between the env open reading frame (ORF) and the 3′ LTR that was not present in the previously described oncogenic retroviruses. This region, termed the "X region," is also present in HTLV-2 and codes for the regulatory and accessory proteins in partially overlapping ORFs named x-I through x-V. Expression of the complex arrangement of ORFs in such a compact genome is accomplished by a combination of ribosomal frameshifting, alternative splicing, and polycistronic translation (Ciminale et al., 1992, 1995; Koralnik et al., 1992) as well as production of negative-strand transcripts that code for the HBZ (HTLV-1) and APH-2 (HTLV-2) proteins.

Like other retroviruses, the HTLVs produce an unspliced mRNA which codes for the Gag-Pol-Pro precursor protein and also serves as the viral genome and a singly spliced mRNA that codes for the Env surface glycoproteins. Peculiar to the HTLVs is the expression of the Tax and Rex regulatory proteins that play critical roles in driving expression from the 5′ LTR promoter (Tax) and in enhancing the expression of partially spliced plus-strand mRNAs (Rex). The Tax and Rex ORFs (x-IV and x-III, respectively) are expressed from the same doubly spliced mRNA. In HTLV-1,

all the other X region proteins are coded by individual singly or doubly spliced transcripts, while some HTLV-2 transcripts express more than one ORF.

Recent studies of the temporal sequence of HTLV-1 gene expression in peripheral blood mononuclear cells (PBMCs) isolated from infected patients (Corradin et al., 2011; Rende et al., 2011) revealed a "two-phase" kinetics with tax/rex mRNA expression preceding that of other viral transcripts. A similar analysis of HTLV-2 mRNA expression indicated a comparable pattern, although the relative abundance of some transcripts showed some intriguing differences among the two viruses (Bender et al., 2012; Cavallari et al., 2013).

The major differences between HTLV-1 and HTLV-2 gene products are summarized in **Table 1** and commented in the following sections.

Tax PROTEINS AND THEIR INTERACTIONS WITH CELLULAR PATHWAYS

The principal role of Tax during viral replication is to activate transcription of the LTR promoter through a process that involves the recruitment of CREB/ATF complexes on binding sites in the U3 region. Tax-1 and Tax-2 share this mechanism and are able to cross-activate each others' LTRs (Semmes et al., 1996).

Experiments performed in the early 1990s established a key role for Tax as the main viral oncoprotein necessary for the initial steps of T-cell transformation by HTLV-1 (Tanaka et al., 1990). Transgenic mouse models have consistently demonstrated that Tax expression causes tumors, including lymphoma and leukemia (Ruddle et al., 1993; Hasegawa et al., 2006). Recent comparative studies on the transforming activities of HTLV-1 Tax (Tax-1) and HTLV-2 Tax (Tax-2) demonstrated that, in the absence of other viral proteins, both Tax-1 and Tax-2 immortalize human primary CD4+ T cells, but only Tax-2 is able to immortalize CD8+ T cells (Imai et al., 2013). Furthermore, Tax-2 immortalizes human primary CD4+ T cells more efficiently than Tax-1 (Imai et al., 2013). Although additional viral products have been demonstrated to be relevant for immortalization, the interactions between Tax and host proteins are still considered to be essential for the transformation process. Several studies of the oncogenic properties of Tax-1 highlighted its effects on DNA repair, cell cycle progression, cell death, and p53 function (reviewed by Cheng et al., 2012).

In addition to CREB/ATF proteins, Tax-1 interacts with many other host factors that influence cell proliferation and transformation, including components of the PI3K, AKT, MAPK, TGFβ, SRF, and NF-κB pathways (Romanelli et al., 2013). Tax-2 has been analyzed mainly for its effects on the NF-κB pathway, and was shown to activate the canonical pathway, but, unlike Tax-1, does not activate the noncanonical pathway (Shoji et al., 2009), suggesting that activation of this latter pathway might be a key element in HTLV-1-driven transformation *in vivo*. Although overall highly homologous, Tax-1 and Tax-2 present some distinct structural features. Tax-1 contains a PDZ binding motif (PBM) and leucine zipper domains that are absent in Tax-2. The C-terminal PBM domain is relevant for interaction of Tax-1 with host factors that regulate cell cycle progression and proliferation, such as the human homologue of the *Drosophila melanogaster* disk large tumor suppressor protein (hDLG; Suzuki et al., 1999). The leucine zipper regions present in the central region of Tax-1 are responsible for activation of the non-canonical NF-κB pathway (Higuchi and Fujii, 2009). The nuclear localization and nuclear export signals of Tax-1 and Tax-2 present significant differences which could explain the predominant nuclear localization of Tax-1 compared to Tax-2 (Turci et al., 2006, 2009; Avesani et al., 2010).

An additional difference between Tax-1 and Tax-2 that may play a role in NF-κB activation is the absence of interactions between Tax-2 and TNF receptor-associated factor TRAF6, an E3 ubiquitin ligase that is involved in ubiquitination of effectors of the NF-κB pathway (Journo et al., 2013). Both Tax-1 and Tax-2 are ubiquitinated and SUMOylated, but they differ in the pattern of modification (Zane and Jeang, 2012). The role of ubiquitination, sumoylation, and acetylation of Tax in NF-κB activation and cellular transformation is still an open field of research (Turci et al., 2012; Xiao, 2012; Journo et al., 2013; Lodewick et al., 2013).

Activation of NF-κB by Tax is also connected to the deregulation of autophagy, an additional pathway that is altered in oncogenic signaling. Both Tax-1 and Tax-2 induce autophagosome accumulation, but their interactions with the components of the process differ. In fact, Tax-1 (but not Tax-2) directs the IKK complex to lipid rafts associated with autophagic molecules such as Beclin 1 and Bif-1 (Huang et al., 2009; Ren et al., 2013).

POST-TRANSCRIPTIONAL REGULATION BY Rex

HTLV-1 Rex (Rex-1) and HTLV-2 Rex (Rex-2) share 60% homology at the amino acid level. Rex-1 and Rex-2 are phosphorylated proteins that actively shuttle between the nucleus and the cytoplasm (Palmeri and Malim, 1996; Narayan et al., 2003) and accumulate in the nucleus and nucleoli (Nosaka et al., 1989; Ciminale et al., 1995, 1997; Nosaka et al., 1995; Narayan et al., 2003), a property that is intimately linked to their ability to enhance the nuclear export of incompletely spliced viral mRNAs.

Rex-1 and Rex-2 share a similar domain structure which includes: (i) a nuclear localization signal (NLS; Siomi et al., 1988; Nosaka et al., 1989), which mediates binding to the RXRE (Grassmann et al., 1991) located at the 3′ end of all HTLV-1 transcripts and at the 5′ end of the unspliced HTLV-2 mRNA (Ohta et al., 1988; Black et al., 1991); (ii) multimerization domains; and (iii) a leucine-rich sequence located near the middle of the protein (Rex-1 aa 79–99; Rex-2 aa 81–94), which functions as an activation domain (AD; Weichselbraun et al., 1992) and contains the nuclear export signal (NES; Kim et al., 1996; Palmeri and Malim, 1996) that interacts with CRM1/exportin, which mediates the nuclear export of the Rex-viral mRNA complex (Bogerd et al., 1995; Kim et al., 1996; Palmeri and Malim, 1996). In addition a C-terminal domain unique to Rex-2 is a target for serine phosphorylation and may also contribute to efficient nucleocytoplasmic shuttling (Narayan et al., 2003). Although Rex is not required for cellular immortalization *in vitro*, it is necessary for infectivity and viral persistence *in vivo* (Ye et al., 2003), as it is required for the expression of the virion-associated structural proteins.

The dependence of HTLV mRNAs on Rex function is controlled by positively and negatively acting RNA sequences present

Table 1 | Structural and functional differences between HTLV-1 and HTLV-2 gene products.

Structural and functional properties	Viral proteins		Reference
	HTLV-1	HTLV-2	
	Tax-1	**Tax-2**	
LZR (leucine zipper domain)	+	−	Higuchi and Fujii (2009)
p100 binding	+	−	Shoji et al. (2009)
PDZ domain	+	−	Suzuki et al. (1999)
CD4 + immortalization	+	++	Imai et al. (2013)
	p21Rex	**tRex**	
Inihibition of full-length Rex	−	+	Ciminale et al. (1997) Bai et al. (2012)
	p30Tof	**p28**	
Interaction with the co-activator CBP/p300	+	−	Zhang et al. (2001)
	p13	−	
Increases mitochondrial K+ permeability and ROS production; inhibits Tax function in the nucleus	+	−	D'Agostino et al. (2005) Silic-Benussi et al. (2009) Andresen et al. (2011) Silic-Benussi et al. (2010b)
	p12	**p10**	
Binding MHC-I heavy chains	+	+	Johnson et al. (2000)
Binding to the IL-2R, calreticulin and calnexin and NFAT activation	+	−	Mulloy et al. (1996) Ding et al. (2001) Ding et al. (2002) Albrecht et al. (2002)
	p8	−	
Increases T-cell contacts and intercellular conduits	+	−	Van Prooyen et al. (2010b)
	−	**p11**	Ciminale et al. (1995)
	Antisense viral proteins		
	HBZ	**APH-2**	
Inhibition of Tax-mediated transcription	+++	+	Halin et al. (2009)
p300/CBP interaction	+	−	Clerc et al. (2008)
Transcriptional activity of c-Jun family	−	+	Marban et al. (2012)
Induction of cell proliferation	+	−	Douceron et al. (2012)
Binding to Tax	−	+	Hivin et al. (2005)
bZIP domain	+	−	Marban et al. (2012)

on the primary transcript. Two major types of such RNA *cis*-acting elements have been described: (i) the Rex responsive element (RXRE) which, besides binding Rex, acts as an inhibitory sequence in the absence of Rex, and (ii) *cis*-acting repressive sequences (CRS) that determine poor stability and/or inefficient nucleo-cytoplasmic export. HTLV-1 contains a CRS which maps at the 5′ end of the unspliced mRNA but is spliced out of the other transcripts (Seiki et al., 1990; King et al., 1998). An additional CRS overlaps the RXRE and acts synergistically with the 5′-CRS. In contrast to the 5′-CRS, this 3′-CRS/RXRE is present at the 3′ end of all viral transcripts. Both the 5′- and 3′-CRSs were shown to act mainly as nuclear retention sequences. The 5′-CRS does not bind Rex-1 (Ballaun et al., 1991; Bogerd et al., 1991; Unge et al., 1991), and its inhibitory function is likely to

be mediated by other viral and/or cellular RNA-binding proteins. Other *cis*-acting inhibitory elements (CIEs) were mapped within the gag-pol and env regions of HTLV-1 (Saiga et al., 1997). The inhibitory effect of these regions is counteracted by binding of Rex-1 to the RXRE, although it is not clear whether they function mainly at the level of RNA stability or nucleocytoplasmic export. A 5′-CRS acting as a nuclear retention sequence was also mapped in the R-U5 region of HTLV-2 (Black et al., 1991).

THE ACCESSORY PROTEINS

The "accessory" proteins were labeled as such because their ablation does not have apparent consequences on viral replication *in vitro*. However, studies performed in animal models indicate that some of these proteins are essential for efficient infectivity *in vivo*. Among the accessory proteins, p30Tof/p28, p12/p10, and p21Rex/tRex (in HTLV-1 and HTLV-2, respectively) are considered to be homologous based on their structure and functional properties, while p13 and p8 appear to be unique to HTLV-1, and p11 is peculiar to HTLV-2.

p21Rex and tRex are truncated forms of Rex-1 and Rex-2, respectively, which lack the *N*-terminal arginine-rich NLS and are therefore incapable of binding the RXRE. HTLV-2 tRex is detected as four main isoforms of 22, 20, 18, and 17 kDa which differ in the initiation codon usage and phosphorylation status (Ciminale et al., 1997). The tRex proteins were shown to inhibit Rex-2 function (Ciminale et al., 1997), an activity that might favor latent infection. Although one study indicated that p21rex acts as a repressor of full-length Rex (Heger et al., 1999), this finding was not supported by other studies (Ciminale et al., 1997; Bai et al., 2012).

HTLV-1 p30Tof and HTLV-2 p28 are important for viral propagation in animal models (Silverman et al., 2004; Yamamoto et al., 2008; Valeri et al., 2010). Both proteins sequester the tax/rex mRNA in the nucleus, an effect that may result in reduced viral expression and latency (Nicot et al., 2004; Younis et al., 2004). p30Tof was also shown to interact with the RNA-binding domain of Rex and thereby interfere with its binding to the RXRE (Sinha-Datta et al., 2007; Bai et al., 2010) and to inhibit the expression of Toll-like receptor 4 (Datta et al., 2006), suggesting a role in the innate immune response. These two properties have not been reported for p28. By interacting with the co-activator CBP/p300 (Zhang et al., 2000, 2001), p30Tof also affects Tax-mediated viral expression and transcription of cellular genes involved in T-cell activation and apoptosis (Michael et al., 2004; Taylor et al., 2009). Interestingly, p28 does not appear to affect CBP/p300-mediated transcription. Both p30Tof and p28 are targeted to the nucleus, but only p30Tof shows evident accumulation in the nucleoli (Ciminale et al., 1995; D'Agostino et al., 1997). Interestingly, HTLV-2 p28 and tRex are expressed at higher levels compared to their HTLV-1 counterparts p30/Tof and p21Rex, suggesting a higher propensity of HTLV-2 for latency compared to HTLV-1 (Bender et al., 2012).

p13 corresponds to the C-terminal 87 amino acids of p30Tof (Koralnik et al., 1992), and it is localized mainly in the mitochondrial inner membrane (Ciminale et al., 1999; D'Agostino et al., 2002) and in part to the nucleus (Silic-Benussi et al., 2010b; Andresen et al., 2011). Using a rabbit animal model, Hiraragi et al. (2006) showed that p13 is required for viral infectivity *in vivo*. p13 increases mitochondrial permeability to K^+ and activates the electron transport chain, resulting in increased mitochondrial reactive oxygen species (ROS) production (Silic-Benussi et al., 2009). While p13 has a mitogenic effect in normal resting T cells, which have low ROS levels, the protein induces death of transformed T-cells, which are characterized by a high ROS setpoint (Ciminale et al., 1999; D'Agostino et al., 2005; Hiraragi et al., 2005; Silic-Benussi et al., 2004, 2010a,b). So far no HTLV-2 homologue of HTLV-1 p13 has been identified.

HTLV-1 p12 and its HTLV-2 homologue p10 are coded by the x-I ORF. p12 localizes in the endoplasmic reticulum (ER) and in the Golgi apparatus, where it reduces the expression of the β and γ_c chains of the interleukin-2 receptor (IL-2R, Mulloy et al., 1996) and of MHC-I, thus hindering lysis of HTLV-1-infected cells by CTL (Johnson et al., 2001). p12 also activates STAT-5, which provides a mitogenic signal to T cells (Nicot et al., 2001) and interacts with calreticulin and calnexin (Ding et al., 2001), resulting in increased Ca^{2+} release from the ER (Ding et al., 2002) and activation of NF-AT, a mitogenic pathway in T-cells (Albrecht et al., 2002; Kim et al., 2003). Within the ER, p12 is cleaved into an 8-kDa protein (p8) which trafficks to the immunological synapse and favours T-cell anergy. p8 also increases cell-to-cell viral transmission through the formation of intercellular conduits (Van Prooyen et al., 2010a,b). In analogy to p12, HTLV-2 p10 was shown to bind the MHC heavy chain; however, p10 does not bind the IL2R β chain or the 16-kDa subunit of the vacuolar H+ ATPase (Johnson et al., 2000). No homologue of p8 has been described in HTLV-2.

A recent study from Valeri et al. (2010), showed that p12 is required for *in vivo* propagation in macaques but not in rabbits.

The x-V ORF of HTLV-2 codes for p11 from a doubly spliced transcript that also codes for p10 (Ciminale et al., 1995). Aside from its ability to bind to MHC heavy chain (Johnson et al., 2000), nothing is known about the function of this protein. HTLV-1 also possesses an x-V ORF, but it does not appear to produce a p11 homologue. The function of p11 is still unclear.

HBZ AND APH-2

The structure and function of HBZ and APH-2 were recently reviewed (Barbeau et al., 2013). HBZ is a nuclear transcriptional factor (Gaudray et al., 2002), able to interact with ATF/CREB proteins through its basic zipper (bZIP) domain causing the inhibition of its DNA-binding activity (Hivin et al., 2006); this effect also influences the ability of Tax-1 to activate HTLV-1 transcription (Halin et al., 2009). APH-2 is likewise able to inhibit Tax-2-mediated transcription but its repressive activity is weaker than that of HBZ (Halin et al., 2009). This difference may be ascribed to the presence in HBZ but not in APH-2, of a transcriptional activation domain within its *N*-terminal region that mediates its interaction with the KIX domain of p300/CBP, thus competing for its binding to Tax-1 (Clerc et al., 2008).

HBZ also deregulates several cellular pathways including c-Jun and JunD, FoxP3, NF-κB, TGF-β and Wnt (Barbeau et al., 2013). In addition, HBZ, may enhance its own expression by controlling

the transcriptional activity of JunD (Gazon et al., 2012). Very little is known about the regulation of APH-2 expression in infected cells. However, unlike HBZ, APH-2 enhances the transcriptional activity of c-Jun family proteins (Marban et al., 2012).

The role of HBZ in T-cell transformation is supported by the finding that while Tax-1 expression is often repressed in ATL cells and appears to be dispensable for the late stages of leukemogenesis, HBZ is constitutively expressed in most ATL cases (Satou et al., 2006). Transgenic mice expressing HBZ in CD4+ T cells develop T-cell lymphomas and systemic inflammation that are reminiscent of ATL and HAM/TSP (Satou et al., 2011).

Although HTLV-2 is not causally linked to leukemia or lymphoma, it has been associated with lymphocytosis in infected patients (Bartman et al., 2008). This is consistent with the observation that APH-2 is detected in most PBMCs of HTLV-2-infected patients (Halin et al., 2009) and that its expression is well correlated to proviral DNA load (Douceron et al., 2012). Interestingly, it was observed that the APH-2 mRNA, similarly to HBZ, accumulates in the nucleus (Bender et al., 2012). Barbeau et al. (2013) have recently proposed a model to explain the different effects of HBZ and APH-2 on T-cell proliferation. Both HBZ and of APH-2 can suppress Tax expression, thus favoring evasion of the immune response. In addition, HBZ can stimulate its own expression, inhibit Tax-1-dependent viral expression and induce T-cell proliferation, which can lead to ATL. APH-2 is unable to induce cell proliferation and only partially down-regulates Tax-2 expression.

Although dispensable for the HTLV-1 infection and immortalization of T lymphocytes *in vitro*, studies conducted on a rabbit animal model suggest that HBZ enhances HTLV-1infectivity and persistence *in vivo* (Arnold et al., 2006). Furthermore, HBZ transgenic mice develop systemic inflammation and a CD4+ T-cell neoplasm that is reminiscent of ATL (Satou et al., 2011). Interestingly, in contrast to HBZ, HTLV-2 APH-2 appears to be dispensable for viral infection and persistence in a rabbit animal model (Yin et al., 2012), suggesting a functional divergence of the *in vivo* function of the HTLV-1 and HTLV-2 antisense proteins.

TROPISM AND CLONALITY

The preferential cellular tropism of HTLV-1 for CD4+ T cells and of HTLV-2 for CD8+ T cells is still not clearly understood. It appears that the two viruses make different use of the heparan sulfate proteoglycans to enter T cells (Jones et al., 2006), although recent evidence obtained *in vivo* rabbit model indicates that the apparent tropism for CD4+ or CD8+ cells mainly reflects preferential clonal expansion of infected cells (Kannian et al., 2012). Both HTLV-1 and HTLV-2 are capable of inducing clonal expansion of infected cells *in vivo* (Wattel et al., 1995; Cimarelli et al., 1996). A detailed analysis in asymptomatic carriers, TSP/HAM and ATLL patients revealed that the genomic integration site and transcriptional orientation of the provirus are important factors for determining clonal abundance *in vivo* (Gillet et al., 2011).

A very recent study demonstrated that, contrary to previous hypotheses, HTLV-2-infected individuals have a small number of highly expanded CD8+ T-cell clones, suggesting that HTLV-2 may be subjected to a more strict clonal selection than HTLV-1 in healthy carriers. These data suggest that selective clonal proliferation is more directly responsible for determining the viral burden of HTLV-2 than it does for HTLV-1 (Melamed et al., 2014). In contrast to observations made for HTLV-1, the environment surrounding the integration sites does not seem to have a substantial impact on the expansion of HTLV-2-infected clones (Melamed et al., 2014).

The presence of few very abundant HTLV-2-infected clones is apparently reminiscent of the profile found in ATLL patients. However, the fact that HTLV-2 is not causally linked to a T-cell malignancy suggests that clonal abundance and heterogeneity may not *per se* constitute a determinant of malignant transformation and clinical outcome of HTLV infection (Melamed et al., 2014).

CONCLUSIONS

From the comparative analysis on the functional properties of HTLV-1 and HTLV-2 reported above, the following major differences can be outlined: (i) HTLV-2 is characterized by a more abundant expression of gene products that may favor viral latency (i.e., p28 and tRex); (ii) Tax-1 presents a PDZ binding motif, which is absent from Tax-2 and allows interaction with host factors regulating the cell cycle and proliferation; in addition, Tax-2 is unable to activate the non-canonical NF-κB pathway; (iii) *Cis*-acting inhibitory elements acting at the level of RNA stability are present within HTLV-1 whereas they have not been described in HTLV-2; (iv) while the truncated forms of Rex-2 were shown to inhibit Rex function, the HTLV-1 homologue p21Rex might lack this activity; (v) HTLV-1 expresses p13 and p8, which have not been described in HTLV-2; (vi) HTLV-2 expresses p11, which does not seem to have a homologue in HTLV-1; (vii) unlike APH-2, HBZ presents a basic zipper domain, as well a transcriptional activation domain, which mediate its capacity to enhance its own expression and deregulate several cellular pathways; (viii) HTLV-2 shows *in vivo* tropism for CD8+ T cells and induces expansion of a relatively small number of highly abundant clones.

In spite of the evidence accumulated so far on the similarities and differences between HTLV-1 and HTLV-2, it is not yet clear why only HTLV-1 causes a T-cell malignancy. While Tax-1 and HBZ induce T-cell lymphomas when expressed as transgenes in animal models, the *in vivo* transforming activities of Tax-2 and APH-2 have not been investigated. Aside from the NF-κB pathway, not much information is available regarding the interactions of Tax-2 with cellular pathways known to be engaged by Tax-1. The fact that HTLV-2, contrary to HTLV-1, is characterized by an oligoclonal proliferative distribution in asymptomatic hosts clearly indicates that the etiology of malignant transformation by HTLV-1 cannot be uniquely attributed to this phenomenon.

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HTLV-1/-2 and HIV-1 co-infections: retroviral interference on host immune status

Elisabetta Pilotti [1], ***Maria V. Bianchi*** [1], ***Andrea De Maria*** [2,3,4], ***Federica Bozzano*** [2,3], ***Maria G. Romanelli*** [5], ***Umberto Bertazzoni*** [5] ***and Claudio Casoli*** [1]***

[1] *GEMIB Laboratory, Center for Medical Research and Molecular Diagnostics, Parma, Italy*
[2] *Department of Health Sciences, University of Genova, Genova, Italy*
[3] *Center of Excellence for Biomedical Research, University of Genova, Genova, Italy*
[4] *IRCCS AOU San Martino-IST Genova, Genova, Italy*
[5] *Department of Life and Reproduction Sciences, University of Verona, Verona, Italy*

Edited by:
Françoise Bex, Université Libre de Bruxelles, Belgium

Reviewed by:
Akifumi Takaori-Kondo, Kyoto University Graduate School of Medicine, Japan
Carine M. C. Van Lint, Université Libre de Bruxelles, Belgium

Correspondence:
Claudio Casoli, GEMIB Laboratory, Center for Medical Research and Molecular Diagnostics, Borgo G. Cantelli n.11, Parma 43121, Italy
e-mail: claudio.casoli@gemiblab.com

The human retroviruses HIV-1 and HTLV-1/HTLV-2 share similar routes of transmission but cause significantly different diseases. In this review we have outlined the immune mediated mechanisms by which HTLVs affect HIV-1 disease in co-infected hosts. During co-infection with HIV-1, HTLV-2 modulates the cellular microenvironment favoring its own viability and inhibiting HIV-1 progression. This is achieved when the HTLV-2 proviral load is higher than that of HIV-1, and thanks to the ability of HTLV-2 to: (i) up-regulate viral suppressive CCL3L1 chemokine expression; (ii) overcome HIV-1 capacity to activate the JAK/STAT pathway; (iii) reduce the activation of T and NK cells; (iv) modulate the host miRNA profiles. These alterations of immune functions have been mainly attributed to the effects of the HTLV-2 regulatory protein Tax and suggest that HTLV-2 exerts a protective role against HIV-1 infection. Contrary to HIV-1/HTLV-2, the effect of HIV-1/HTLV-1 co-infection on immunological and pathological conditions is still controversial. There is evidence that indicates a worsening of HIV-1 infection, while other evidence does not show clinically relevant effects in HIV-positive people. Possible differences on innate immune mechanisms and a particularly impact on NK cells are becoming evident. The differences between the two HIV-1/HTLV-1 and HIV-1/HTLV-2 co-infections are highlighted and further discussed.

Keywords: HTLV, HIV-1, co-infection, cytokines, chemokines, JAK/STAT, miRNA, natural killer cells

INTRODUCTION

Microbes that infect the same host may positively influence each other's replication, or fight for supremacy (Margolis, 2003; Kannangara et al., 2005). In recent years, different data have demonstrated that a microbe, in order to favor its own replication, can attenuate or support the infection of other infecting agents by altering the host immune system (Lisco et al., 2009). The role of human T lymphotropic viruses type 1 and type 2 (HTLV-1 and HTLV-2) as determinants of HIV-1 disease during co-infection has been widely studied, but is still a matter of speculation.

Since HTLV-1, HTLV-2, and HIV-1 have common modes of transmission, it is not surprising that co-infection is a frequent condition especially among people with high risk behaviors, as needle sharing and unprotected sexual contact. Though human retroviruses have worldwide distribution (Goubau et al., 1992; Vrielink and Reesink, 2004; Proietti et al., 2005), dually infected subjects have been mainly diagnosed in large metropolitan area or in endemic regions (Briggs et al., 1995; Dezzutti et al., 1998; Araujo et al., 2002; Morimoto et al., 2005; Magri et al., 2012, 2013). Because HTLV screening is not routinely performed in many countries and is not always recommended by physicians to outpatients, the seroprevalence of co-infection is underestimated (Beilke, 2012; Pinto et al., 2012).

HTLV-1, HTLV-2, and HIV-1 share similar genomic organization and tropism for immune cells, in particular $CD4^+$ and $CD8^+$ T cells. However, the finality of their viral cycle is different. In infected patients, HIV-1 is generally present as a virion (either cell-associated or cell-free) and the provirus is detected clearly in a minority of cells (Josefsson et al., 2011), whereas HTLVs are prevalently integrated in their target cells and the propagation of infection occurs by clonal expansion of infected cells (Bangham, 2003).

To understand whether HTLVs may accelerate or attenuate HIV-1 progression, several studies have interpreted HIV-1-associated clinical outcomes, taking into account laboratory records as CD4 mean cell count and HIV-1 viral load. However, conflicting data have emerged on both HTLV-1 and HTLV-2 interactions with HIV-1 and the debate is still open. The lack of uniformity of used criteria may explain this discrepancy, including differences in sampling, variations in outcome surveillance, with other parameters, such as HTLVs and HIV-1 seroconversion time and sex, not always defined. Furthermore, some authors believe that matching patients by immune markers is not a good strategy (Beilke et al., 2004). When not well-defined, also anti-HIV-1 therapy, considering its effect on immunologic host factors and potentially on replication of co-invading agents, could make the comparison of data coming from different studies difficult.

Concerning HTLV-1/HIV-1 co-infection, a more rapid HIV-1 disease progression and a lower mean survival time in co-infected

individuals compared to HIV-1-mono-infected patients (Brites et al., 2001) was seen. Additionally, an increased risk to develop tropical spastic paraparesis/HTLV associated myelopathy (TSP/HAM) and other neurodegenerative conditions was found in dually infected subjects (Tulius Silva et al., 2009). Also, a recent work reported a higher mortality and shortened survival rate in HTLV-1/HIV-1-co-infected children compared to HIV-1-mono-infected patients irrespective of baseline CD4 cell count (Pedroso et al., 2011).

On the contrary, other researchers observed a delayed HIV-1 disease progression in HTLV-1-co-infected patients when compared to HIV-1-mono-infected individuals (Shibata et al., 1989; Page et al., 1990; Chavance et al., 1995; Beilke et al., 2004). In according to these findings, a higher survival rate was reported for HIV-1/HTLV-1-co-infected subjects (Brites et al., 2001, 2005).

It is noteworthy that the impact of HIV-1 on HTLV-1 replication was also investigated. Seroepidemiologic studies showed that HIV-1 positive subjects are more susceptible to HTLV-1 infection, and vice versa (Harrison et al., 1997). Moreover, the HIV-1 Rev protein was found to enhance HTLV-1 gene expression, by interacting with 5'-RU5 region of the HTLV-1 genome (Kubota et al., 1998). A later study, in which the quantification of HTLV-1 and HIV-1 DNA load was determined, suggested that HIV-1 co-infection does not affect HTLV-1 proviral load in peripheral blood compartments (Cesaire et al., 2001).

Similarly to HTLV-1/HIV-1, contrasting results were also reported for HTLV-2/HIV-1 co-infection. However, it is now generally accepted that HTLV-2 exerts a negative effect on HIV-1 replication. In fact, several authors have associated HTLV-2 co-infection with a better outcome for HIV-1 positive individuals (Beilke et al., 2004; Casoli et al., 2007). Earlier studies did not observe this effect and ascribed the lack of significant associations between co-infection and progression to AIDS or death to the absence of clear evidence of HTLV-2 as pathogenic agent (Visconti et al., 1993; Hershow et al., 1996).

HTLVs interfere with HIV infection by mechanisms that appear to be complex and multilayered. More specifically, HTLVs can act on HIV-1 expression directly at molecular levels or indirectly by modulating the expression of immune host factors. This in turn can be induced by HTLVs directly or through cellular activation.

Thus, the intimate relationship between the HIV-1 life cycle and the activation state of cells supporting viral replication results in a dynamic interaction between co-infecting agents and HIV-1 replication in dually infected individuals.

HTLV-1 differs from HTLV-2 in regulating cellular activation of target cells. More specifically, HTLV-1-infected subjects present a prevalence of highly activated cells, while HTLV-2-infected individuals hold up a lower cellular activation status (Nagai et al., 2001; Bovolenta et al., 2002a; Goon et al., 2004). These divergent conditions may contribute to explain the different impact of the two types of HTLV on HIV-1 infection. In particular, for HTLV-2 it was observed that HIV-1/HTLV-2-co-infected patients showed a reduced HIV-1 replication presumably due to lower levels of T cell activation (Bassani et al., 2007).

In this review, we report and discuss recently published data on host immunomodulating factors involved in retroviral interference, and in that regard we also point at other cellular and molecular components that may be considered potentially good candidates.

CYTOKINES/CHEMOKINES

The role of the cytokine/chemokine network as strategic weapon in germ warfare has been extensively discussed (Margolis, 2003). Infecting agents benefit from their ability to drive immune reactions mediated by cytokines and chemokines. This favorable condition for a microbe could be adverse or advantageous for a co-pathogen.

Thus, the perturbation of the immune system, including host cytokine synthesis, induced by HTLV-1 and HTLV-2 infections (Hollsberg, 1999) could have positive or negative impacts on HIV-1 replication. Such immune activation is mainly modulated by HTLV-1 and HTLV-2 Tax proteins (Tax-1 and Tax-2).

Regarding HTLV-1/HIV-1 co-infection, it was reported that Tax-1 up-regulates HIV-1 expression (Bohnlein et al., 1989) by activating the transcriptional factor NF-κB that recognizes two binding sites in the U3 region of the HIV-1 LTR (Leung and Nabel, 1988). In addition, Tax-1 enhances the expression of several cellular proteins, including transcription factors and cytokines as IL-2, tumor necrosis factor α (TNF-α), and others (Banerjee et al., 2007; Boxus et al., 2008). More specifically, some of these cytokines as TNF-α or IL-1β are capable of triggering HIV-1 transcription through a NF-κB-dependent mechanism (Siebenlist et al., 1994). Tax-1 is also responsible for the induction of cytokine receptor expression (Franchini and Streicher, 1995). This state of activation was frequently observed among co-infected subjects who showed a sharp drop in CD4 cell count and rapid progression of HIV-1 disease (Bartholomew et al., 1987; Page et al., 1990; Pagliuca et al., 1990; Gotuzzo et al., 1992; Schechter et al., 1994; Fantry et al., 1995).

However, other studies describe detrimental effects on HIV-1 infections (Harrison et al., 1997; Beilke et al., 2004, 2007). Recently, Abrahao et al. (2012) observed a higher production of IL-2 and IFN-γ in HIV-1/HTLV-1-co-infected individuals than in HIV-1 or HTLV-1-mono-infected individuals. Moreover, while IL-6 and IL-10 levels were similar in all infected groups, IL-4 production was lower in HTLV-1-mono-infected individuals. These findings support the notion that high levels of Th-1 cytokines in co-infected patients provide adverse conditions for HIV-1 infection, suggesting a predominant role of HTLV-1 over HIV-1. This hypothesis is supported by the evidence that IL-2 suppresses HIV-1 replication in some HTLV-1-infected cell lines by inducing APOBEC3G expression (Oguariri et al., 2013).

For what concerns HTLV-2, it was reported that PBMCs derived from HTLV-2 seropositive individuals undergo spontaneous proliferation in short-term cultures in association with the secretion of several cytokines, including TNF-α, IL-5, IL-6, and IFN-γ (Dezzutti et al., 1998).

By studying the cytokine pattern in HTLV-2/HIV-1-co-infected subjects, we determined that HTLV-2 drives immune activation to implement the secretion of cytokines as GM-CSF and IFN-γ (Pilotti et al., 2007), which are capable to induce a "protective" Th1 response against HIV-1 (Creery et al., 2004), since a dominant Th2 profile seems to favor HIV-1 progression (O'Garra, 1998).

Other important immune correlates able to facilitate or suppress HIV-1 infection are the chemokines and their cellular receptors. *In vivo*, HIV-1 prevalently targets immune cells expressing the surface receptor CD4 that mediates virus binding and membrane fusion together with chemokine co-receptors (CCR5 and CXCR4; Kowalski et al., 1987; Lasky et al., 1987). Thus, changes in conformation status or surface availability of these molecules may, in turn, modify HIV-1 disease progression. Beside genetic modifications, the co-receptors expression is mainly affected by the binding of natural ligands. In particular three CCR5 binding chemokines, CCL3, CCL4, and CCL5, act as major HIV-1-suppressive factors.

Because three chemokines are released by both cultured T cells and primary $CD8^+$ T cells in response to HTLV infection (Scarlatti et al., 1997), it was supposed that their up-regulation could explain HIV-1 inhibition observed during co-infection. The fact that HTLV-1-specific $CD8^+$ cytotoxic T lymphocyte (CTL) clones derived from patients with HAM/TSP are actively producing CCL3 and CCL4 chemokines (Cocchi et al., 1995), reinforces the hypothesis that HTLV-1 can influence HIV-1 replication via chemokine expression and release.

As HTLV-2-infected cells become activated, they spontaneously proliferate and produce high levels of various cytokines and chemokines (Casoli et al., 1998, 2000; Lewis et al., 2000; Bovolenta et al., 2002b; Bassani et al., 2007). We observed that up-regulation of CCR5-binding chemokine expression occurs in cultured PBMCs from HTLV-2/HIV-1-co-infected individuals in comparison to HIV-1-single-infected individuals. In particular, we demonstrated that CCL3 secretion is responsible for anti-HIV-1 activity in PBMC cultures from co-infected subjects (Casoli et al., 2000). Lewis et al. (2000) associated the spontaneous synthesis of CCR5 binding chemokines to the ability of HTLV-2 regulatory proteins to transactivate *CCL4* and *CCL5* gene promoters. Also, we found that an isoform of CCL3, namely CCL3L1, which is considered the most potent anti-R5 HIV-1 chemokine (Visconti et al., 1993), was preferentially induced by HTLV-2 (Pilotti et al., 2007). Up-regulation of this chemokine leads to CCR5 down-modulation and subsequent receptor internalization (Townson et al., 2002). Although other groups have shown that the HIV-1 susceptibility is associated with *CCL3L1* gene dose, which is variable among individuals, we demonstrated that HIV-1 inhibition occurred in HTLV-2-co-infected subjects was independent by *CCL3L1* copy number, and that enhanced *CCL3L1* expression was presumably stimulated by Tax-2 protein at the transcriptional level (Pilotti et al., 2007). High levels of GM-CSF and IFN-γ secreted by PBMCs from HTLV-2-infected individuals were found to contribute to HIV-1 interference via CCR5 down-modulation (Pilotti et al., 2007).

Two recent works confirmed the pivotal role of Tax proteins in inducing CC-chemokine synthesis. The first paper reported that both recombinant Tax-1 and Tax-2 induce high levels of CC-chemokines which in turn cause CCR5 down-regulation in cultured PBMCs (Barrios et al., 2011), and the second article demonstrated that Tax-2 transactivates CC-chemokines production in cultured monocyte-derived macrophages (Balistrieri et al., 2013).

JAK/STAT SIGNALING

HTLV-1 and HTLV-2 can efficiently transform human T cells *in vitro* but significantly differ in pathogenicity. The Janus kinase (JAK)/signal transducer/activator of transcription (STAT) signaling pathway (JAK/STAT) is constitutively activated in HTLV-1-transformed cells. This may occur by autocrine stimulation of IL-2, IL-9, and IL-15 cytokines, and IL-2 and IL-15 receptor expression, as a result of Tax-induced NF-κB expression, which in turn stimulate lymphocyte proliferation (Migone et al., 1995; Mariner et al., 2001; Chen et al., 2008). HTLV-1 Tax protein is crucial for viral replication and for initiating malignant transformation and is able to inhibit host antiviral signaling via NF-κB-dependent induction of suppressor of cytokine signaling protein 1 (SOCS1) to evade innate immunity (Charoenthongtrakul et al., 2011). In T cells transformed *in vitro* by HTLV-1, the JAK/STAT activation correlates with the transition from an IL-2 dependent to an IL-2 independent phase of growth (Migone et al., 1995; Xu et al., 1995). In contrast to HTLV-1, the activation status of the JAK/STAT pathway is not constitutively activated in HTLV-2-transformed T cells. However, this pathway could be induced upon IL-2 treatment of the cells. Similarly, the constitutive activation of STAT1, STAT3, and STAT5, as well as the phosphorylation status of JAK kinases (JAK3 and JAK1), observed in HTLV-1-transformed T cell lines, was not detected in HTLV-2-transformed T cells (Mulloy et al., 1998). However, we showed that the ability of human $CD34^+$ IL-3 dependent TF-1 cell line to proliferate after HTLV-2 exposure in conditions of IL-3 deprivation is following the production of the GM-CSF and IFN-γ, through the activation of the JAK/STAT pathway (Bovolenta et al., 2002b). Previously, it was demonstrated that a signature of PBMC freshly derived from HIV-1 infected individuals represents the constitutive activation of a C-terminal truncated STAT5 (STAT5Δ) and STAT1 (Bovolenta et al., 1999).

When analyzing the levels of STATs in HTLV-2 mono-infected and HTLV-2/HIV-1-dually-infected individuals, we observed that these factors are not activated in PBMCs of HTLV-2-mono-infected unless they are cultured *in vitro*, in the absence of any mitogenic stimuli, for at least 8 h (Bovolenta et al., 2002a). The emergence of STAT activation, mainly of STAT1, appears to be related to the secretion of IFN-γ. Of note, this is a characteristic feature of both HTLV-2 and HIV-1-mono-infected individuals. Surprisingly, HTLV-2/HIV-1 co-infection resulted in a low/absent STAT activation *in vivo*, thus correlating with a diminished secretion of IFN-γ in *ex vivo* cultivated PBMCs (Bovolenta et al., 2002a). These findings indicate that HTLV-2 and HIV-1 infection can prime T lymphocytes for STAT1 activation, but they also highlight that an interference is exerted by HTLV-2 on HIV-1-induced STAT1 activation. These results clearly suggest that HTLV-2 may interfere with HIV-1 infection at multiple levels. The observation that PBMCs obtained from both HIV-1- and HTLV-2-infected individuals activate STAT1 as a consequence of the spontaneous release of IFN-γ is supported by previous findings indicating an up-regulation of this cytokine following either HIV-1 or HTLV-2 infection (Dezzutti et al., 1998; Levine et al., 2002). An enhanced transcription of IFN-γ is also induced by the HTLV-2 Tax trans-activator (Nimer et al., 1989; Brown et al., 1991). A large body of evidence points to an increased level of

either IFN-γ or its correlates (such as neopterin or IP-10) in the plasma/serum of HIV-1-infected individuals (Poli et al., 1994), explaining the low but detectable constitutive STAT1 activation observed in HIV-1-infected. This correlation was not seen in HTLV-2-infected individuals (Bovolenta et al., 1999). Therefore, T cells from both HIV-1- and HTLV-2-infected individuals share a constitutive priming for IFN-γ secretion and, consequently, for STAT1 activation; in contrast, only HIV-1 infection is characterized by activation of STAT5Δ *in vivo* (Bovolenta et al., 1999). Because these factors are absent in both HTLV-2 mono-infected and HTLV-2/HIV-1 co-infected individuals, this would reflect the higher pathogenic potential of HIV-1 with respect to HTLV-2, but also highlights a dominant position of HTLV-2 over HIV-1 in terms of maintaining T cells in a primed but not completely STAT5Δ activated state. Since there is evidence that HTLV-2/HIV-1 co-infection is frequently associated with a state of long-term non-progression (LTNP) of HIV-1 disease (Giacomo et al., 1995; Magnani et al., 1995), HTLV-2 infection, and co-infection with HIV-1, represent an important model to better understand the interaction between human exogenous retroviruses and the immune system. IFN-γ-related priming for STAT1 activation may be an alarming signal that biases the immune response toward a Th1-model of containment of HTLV-2 infection, overcome by a peculiar STAT5Δ activation in HIV-1-infected individuals. Concerning the role of JAK/STAT in HTLV-1/HIV-1 co-infection no results have yet been reported.

NATURAL KILLER CELL ACTIVITY

Evidence has been accumulating on the specific targeting of innate immune defenses, and in particular of natural killer (NK) cells, by chronically replicating viruses (Marras et al., 2011). Successful weakening of NK cell response represents a critical step for virus persistence, since NK cells are involved in patrolling peripheral tissues for immediate defense against virus or tumor aggression as well as in cross-talking with critical components of innate immunity, including monocytes and dendritic cells, leading to relevant downstream impact on the shaping of adaptive immune responses (Vivier et al., 2011).

Natural killer cells main cytolytic and cytokine productive function is tightly controlled by a wide array of activating NK cell receptors which alone, or in combination with Toll-like- o cytokine- receptors, are responsible for triggering the NK cell functional program (Vivier et al., 2011). Inhibitory NK cells receptors (i.e., KIRs, NKG2A/CD94, CD85j, IRP60, SIGLEC-7), which are mainly, but not exclusively, HLA class I-specific, provide a negative regulatory signal that is able to override any triggering receptor signaling in the presence of the appropriate cognate receptor. Thus, proper NK cell function may occur only in the presence of activating receptor triggering, with reduced or absent overriding control by inhibirtory receptors sensing the respective ligands on target cells. For example, in the absence of MHC class I expression induced by virus down-modulation, the induction in the infected cells of ligands (e.g., MIC-A, MIC-B, ULBPs, Nectin-2, PVR, etc.) recognized by activating NK cell receptors would result in NK cell activation, cytokine/chemokine production, and cytotoxic activity. Several viruses and mycobacteria exploit altered expression of natural/cytotoxicity receptors in NK cells such as HIV-1 (De Maria et al., 2003), influenza (Arnon et al., 2001; Mandelboim et al., 2001; Gazit et al., 2006), HCV (Bozzano et al., 2011), mycobacterium tuberculosis, and Calmette–Guerin (Vankayalapati et al., 2002, 2004; Bozzano et al., 2009; Marras et al., 2012; reviewed in Bozzano et al., 2012 and in Marras et al., 2011), or skew the NK cell peripheral repertoire inducing expansion of $NKG2C^+$ NK cell subsets as is the case for CMV infection (Guma et al., 2004, 2006a,b; Della Chiesa et al., 2011). Similar interference with inhibitory receptor expression (e.g., NKG2A, KIRs) may be induced by acute infection, or exploited through KIR:HLA class I haplotype interaction, as for example is observed during infection with CMV (Muntasell et al., 2013), HCV (Khakoo et al., 2004; Knapp et al., 2010; Vidal-Castineira et al., 2010), or Chikungunya virus (Petitdemange et al., 2011).

Very little is known on NK cell phenotype and function during HTLV infection, in particular when HTLV-2 is considered.

Characterization of NK cell triggering and inhibitory receptors has been so far poorly addressed to understand the differences underlying HTLV-1 and HTLV-2 diseases.

Early reports on the definition of NK cell receptors point towards a decreased NK cell activity against HTLV-1-infected MT-2 cell lines (Fujihara et al., 1991; Zheng and Zucker-Franklin, 1992). Lysis of cell lines infected with HTLV-1 by *in vitro* activated NK cells was subsequently shown to occur and to depend on viral gene expression, that may be absent in some adult T cell leukemia (ATL) lines (Stewart et al., 1996). HTLV-1 antigen-driven proliferation may result in a ATL form, as shown in mice transgenic for Tax-1 (Grossman et al., 1995) and in humans with expansions in hypofunctional NK cells (Loughran et al., 1997). The proportion of circulating mature NK cells ($CD56^+$ $CD16^+$) is decreased in patients with TSP/HAM (Wu et al., 2000; Brito-Melo et al., 2002; Ndhlovu et al., 2009) and other innate immune cells (NKT) are decreased during TSP/HAM as well. In addition, more recent evidence suggests that NK cells may be targeted and induced to expand by HTLV-1 through a viral load-associated but Tax-independent mechanism (Norris et al., 2010).

Overall, there is need for further insight into NK cell phenotype and function during HTLV-1/-2 infection. More precise characterization of possible changes or modulation of triggering and inhibitory receptor expression on NK cells during chronic infection would help to better understand the mechanism(s) that are exploited by HTLV-1 to divert innate immune responses and downstream $CD4^+$ and $CD8^+$ T cell function. In particular, it is possible that different NK cell regulation induces clinical divergence spanning from the lack of symptoms to TSP/HAM and transformation to ATL, similar to what was observed for HCV infected patients clearing infection (Khakoo et al., 2004; Alter et al., 2011) or for HIV-infected patients who control virus replication (Elite Controllers) or without disease progression in LTNPs (Marras et al., 2013). In addition, in view of the compelling evidence of a remarkable NK cell activation during HIV-1 infection both in untreated patients (Fogli et al., 2004) and in successfully treated combined antiretroviral therapy (ART) patients (Lichtfuss et al., 2012) though belonging to AIDS or non-AIDS clinical groups (Bisio et al., 2013), understanding NK cell activation during HIV-/HTLV-1 co-infection needs improved focusing. These

considerations should be extended to HTLV-2 infection *in vitro* and *in vivo*, since no data have been yet published on NK cells in mono-infected patients.

ROLE OF MicroRNAs

MicroRNAs (miRNAs) are small single-strand non-coding RNAs that repress gene expression by inhibiting translation and inducing mRNA degradation (Ambros, 2004; Bartel, 2004). MicroRNAs can be encoded by both cellular and viral genomes (Berezikov et al., 2005; Grassmann and Jeang, 2008). Furthermore, viral miRNAs have been described not only for DNA viruses but also for RNA viruses as HIV-1 and BLV (Bennasser et al., 2004; Du and Zamore, 2007; Klase et al., 2013). MicroRNAs have been found to regulate up to 92% of the human genes (Miranda et al., 2006), and also to modulate viral gene expression. These alterations could be considered key mechanisms by which the virus imbalances immune system. In fact, as reported by several authors, immune response to invading agents as well as cellular proliferation and differentiation can be affected by host miRNAs (Chen et al., 2004; Fazi et al., 2005; Cobb et al., 2006; Li et al., 2007; Loffler et al., 2007; Ivanovska et al., 2008; Johnnidis et al., 2008; Carissimi et al., 2009; Faraoni et al., 2009; Huang et al., 2009; Lal et al., 2009; Curtale et al., 2010). The importance of RNA interference (RNAi) machinery in retroviral infection outcome was confirmed by recent findings that demonstrated how the miRNAs expression can be controlled by retroviruses (Sampey et al., 2012). Thus, the mechanism of RNAi mediated by miRNAs could be used by a virus to remain hidden from host immune surveillance by generating an advantageous cellular environments, and leading to adverse conditions for a co-invading agent.

MicroRNAs expression was studied during of HTLV-1 or HIV-1 infection but no data were so far reported for the HIV-1/HTLV-1 co-infection. Similarly, studies of miRNAs pattern during HTLV-2/HIV-1 co-infection have not been published up to date.

Concerning HIV-1, changes in host miRNA transcription levels have been detected in $CD4^+$ purified cells from naive and LTNP HIV-1-mono-infected patients. The hypothesis that miRNAs either could directly influence viral RNA sequences, or could affect cellular factors involved in HIV replication were confirmed by several findings (Houzet and Jeang, 2011). More specifically, it was demonstrated that five cellular miRNAs recognize the 3′ end of HIV-1 mRNAs and are up-regulated in resting, but not activated, $CD4^+$ T cells (Huang et al., 2007), providing evidence of a direct inhibition of HIV-1 replication by miRNAs. Two independent groups support this notion with the demonstration that HIV-1 *nef* gene contains a miR-29a targeted site that interferes with the replication of the virus (Ahluwalia et al., 2008; Nathans et al., 2009). Other authors found indirect inhibitory mechanisms miRNAs mediated. Specifically, it was found that miRNAs suppress viral gene expression by decreasing PCAF (P300/CBP-associated factor) expression and interfering with histone acetylation, and leading to HIV-1 latency (Triboulet et al., 2007). However, the deregulation of cellular miRNA expression which was shown to correlate to HIV-1 latency may also favor virus production (Han and Siliciano, 2007; Huang et al., 2007). Other miRNAs were involved in the different monocyte or macrophage susceptibility to the HIV infection (Wang et al., 2009, 2011).

Recently, it was shown that HIV-1 and other retroviruses as bovine leukemia virus can affect the expression of both host miRNAs and small virus-derived interfering RNAs (Klase et al., 2007, 2009; Houzet et al., 2008; Ouellet et al., 2008; Althaus et al., 2012; Kincaid et al., 2012; Schopman et al., 2012).

Similarly to HIV-1, it was demonstrated that HTLV-1 infection is responsible for alteration of host miRNAs profile (Bellon et al., 2009; Rahman et al., 2012). By profiling the expression of miRNAs, known to be involved in differentiation and proliferation of hematopoietic cells (Merkerova et al., 2008), abnormal levels of miR-223, miR-181a, miR-150, miR-142.3p, and miR-155, were detected in both primary ATL cells and in HTLV-1 cell lines (Bellon et al., 2009). Furthermore, these samples showed an altered expression of miR-155, miR-125a, miR-132, and miR-146 that play a role in the regulation of immune response (Bellon et al., 2009). A forced expression of miR-155, induced by Tax with the contribution of NF-κB and AP-1, has been found to enhance the proliferation of HTLV-1-infected cells (Tomita, 2012).

A loss of miR-31 was also observed in primary ATL cells, where the expression of this miRNA is epigenetically regulated, and was correlated with the constitutively activation of NF-κB which contributes to the oncogenic transformation (Yamagishi et al., 2012).

Concerning miRNAs biogenesis, low levels of Drosha enzyme, a key processor of miRNAs synthesis, were found in HTLV-1-infected cell lines and infected primary cells. In addition, *in vitro* studies revealed a nuclear co-localization of Tax and Drosha, and that the interaction between the two proteins leads to absence of cleavage of miRNAs by Drosha (Van Duyne et al., 2012).

MicroRNAs can target complementary sequences of both HTLV-1and HIV-1 transcripts and act as a major line of defense against retroviruses, but on the other hand viral proteins can interfere with RNAi machine directly or by altering the expression of cellular transcription factors. HTLV-1 Tax also interacts with CRE binding (CREB) protein, a key factor to viral transcription, in the presence of the HATs CREB binding protein (CBP), p300, and P/CAF to activate HTLV-1 gene expression while HTLV-2 Tax specifically cooperates with CBP and p300 but not with p300 associated factor to enhance transcription from the viral promoter (Tosi et al., 2006). Another mechanism by which HTLV-1 may influence the host cell miRNA expression profile is through the activation of host transcription factors. Important transcription factors and cellular kinases which interact directly with the viral protein Tax are CREB, serum-responsive factor (SRF), NF-κB, Cyclins D2 and D3, mitotic check point regulators (MAD1), cyclin dependent kinases (CDKs), the CDK inhibitors $p16^{INK4A}$ and $p21^{(WAF1/CIP1)}$, and the tumor suppressor p53 (Caron et al., 1993; Suzuki et al., 1994; Yin et al., 1995, 1998; Clemens et al., 1996; Colgin and Nyborg, 1998; Harrod et al., 1998; Gachon et al., 2000; Nicot et al., 2000; Xiao et al., 2001; Kashanchi and Brady, 2005; Easley et al., 2010). In particular, the NF-κB pathways activation is a hall mark of HTLV-1 infection and may be the result of direct interaction between Tax and the NF-κB regulatory subunit IKKγ (Sun and Yamaoka, 2005; Yasunaga and Matsuoka, 2011). Because numerous miRNA promoter sites are positively regulated by NF-κB, it can be inferred that the activation of NF-κB by Tax increases the expression of several host cell

miRNAs (Li et al., 2012; Lukiw, 2012; Wang et al., 2012). One specific example is given by miR-155 which has been found to be up-regulated in HTLV-1-infected cells, as well as in a TNFα stimulated cell line through an NF-κB pathway (Bellon et al., 2009; Liu et al., 2011). Similar findings have already been described for HIV-1 Tat which activates NF-κB by acting through the same biochemical pathways used by a variety of other NF-κB inducers, reviewed in Baeuerle and Henkel (1994), and for other viral proteins, including Tax of HTLV-2, HBx, and MHBs of hepatitis B virus, and EBNA-2 and LMP of Epstein-Barr virus (Baeuerle and Henkel, 1994) and hemoagglutinin of influenza virus (Pahl and Baeuerle, 1995). Of particular interest is the role of miRNA in HTLV cellular transformation and recent findings demonstrate that alteration of the miRNA profile of infected cells leads to the development of ATL and HAM/TSP diseases (Sampey et al., 2012). In HIV-1 disease, a modification of chromatin by viral proteins and host cell miRNAs can contribute to the dysregulation of host cell miRNA expression and likely provides a key system used by the virus to modify host miRNA profiles (Bignami et al., 2012). Also, it was demonstrated that Tax-1 induces a prompt activation of chromatin remodeling factors as p300 and p/CAF (Rahman et al., 2012). The chromatin reorganization, affected by miRNAs which expression is in turns influenced by Tax proteins, drives the establishment of viral latent status (Aliya et al., 2012).

Recently, we studied miRNA profiles in $CD4^+$ T-cells purified from HTLV-2-mono-infected patients and found evidence for a miRNA signature (miR-329, miR-337-5p, miR-379-5p, miR-503, miR-518d-3p, miR-203, miR-449a, miR-502-5p) that discriminates infected from uninfected subjects, similarly to HIV-1 (**Table 1**; Bignami et al., 2012). Furthermore, by analyzing in detail some functional aspects of the miRNAs belonging to retroviral signature we identified 135 predicted target genes that, on the basis of gene ontology (GO) resources, revealed the presence of three ontology aspects (**Figure 1**). Interestingly, we found that the most significant GO terms, i.e., positive regulation of macromolecule and cellular biosynthetic process, are related to the formation of substances carried out by individual cells.

With specific reference to the deregulated host miRNAs linked to the development of the HTLV-1 oncogenic or neurodegenerative diseases (**Table 2**), only miR-155, a key regulatory component of the innate immune response, is differentially expressed in HIV-1 and HTLV-2 infection. Thus, we speculated that a functional impact of cross-talk between miRNA pattern, and the subsequent multifunctional pathways, may occur during HTLVs/HIV-1 co-infection.

The assessment of how the altered profiles of miRNA expression can influence viral replication and latency, as well as the efficiency of host defenses, may be useful for understanding the basis of the retroviral related modifications of cellular pathobiology and immunologic control.

CONCLUSION

Is the retroviral interference relevant to HIV-1 infection? In this review we discussed how HIV-1/HTLVs co-infection can either positively or negatively affect the course of HIV-1 disease. In particular, co-infection with HTLV-2 seems to confer immunological benefits in patients with HIV-1. By contrast, HTLV-1 is mainly associated to HIV-1 disease progression and to an increased risk of TSP/HAM and ATL.

An overall picture of different effects of HTLVs on HIV infection is emerging. The widely diverging effect of HTLV-1 and HTLV-2 on the clinical course of HIV-1 progression is remarkable, and the findings initially reported for HTLV-2 are particularly surprising. The possibility that specific co-infections may improve the clinical course of HIV-1 infection by directly or indirectly interfering with HIV-1 cell entry, replication and spread, originally proposed by the study of HIV-1/HTLV-2-infected cells (Casoli et al., 2000) has been confirmed by subsequent work on GB virus C which is able to infect B cells and $CD4^+$ or $CD8^+$ T lymphocytes (George et al., 2006).

Modulation of the cytokine/chemokine network represents a major element of shift dynamics that regulates the co-existence of several infections. Thus, cytokines and chemokines might be considered strategic weapons in the bid to gain benefits to the

Table 1 | MicroRNAs equally expressed in HIV-1 and HTLV-2 vs HTLV-1 infection.

	HIV-1 vs healthy Bignami et al. (2012)	**HTLV-1 vs healthy** Pichler et al. (2008), Bellon et al. (2009), Ruggero et al. (2010), Rahman et al. (2012)	**HTLV-2 vs healthy** Bignami et al. (2012)
hsa-miRNA miR-329	Down	nd	Down
hsa-miR-337-5p	Down	nd	Down
hsa-miR-379-5p	Down	nd	Down
hsa-miR-503	Down	nd	Down
hsa-miR-518d-3p	Down	nd	Down
hsa-miR-203	Up	nd	Up
hsa-miR-449a	Up	nd	Up
hsa-miR-502-5p	Up	nd	Up

nd indicates not determined.

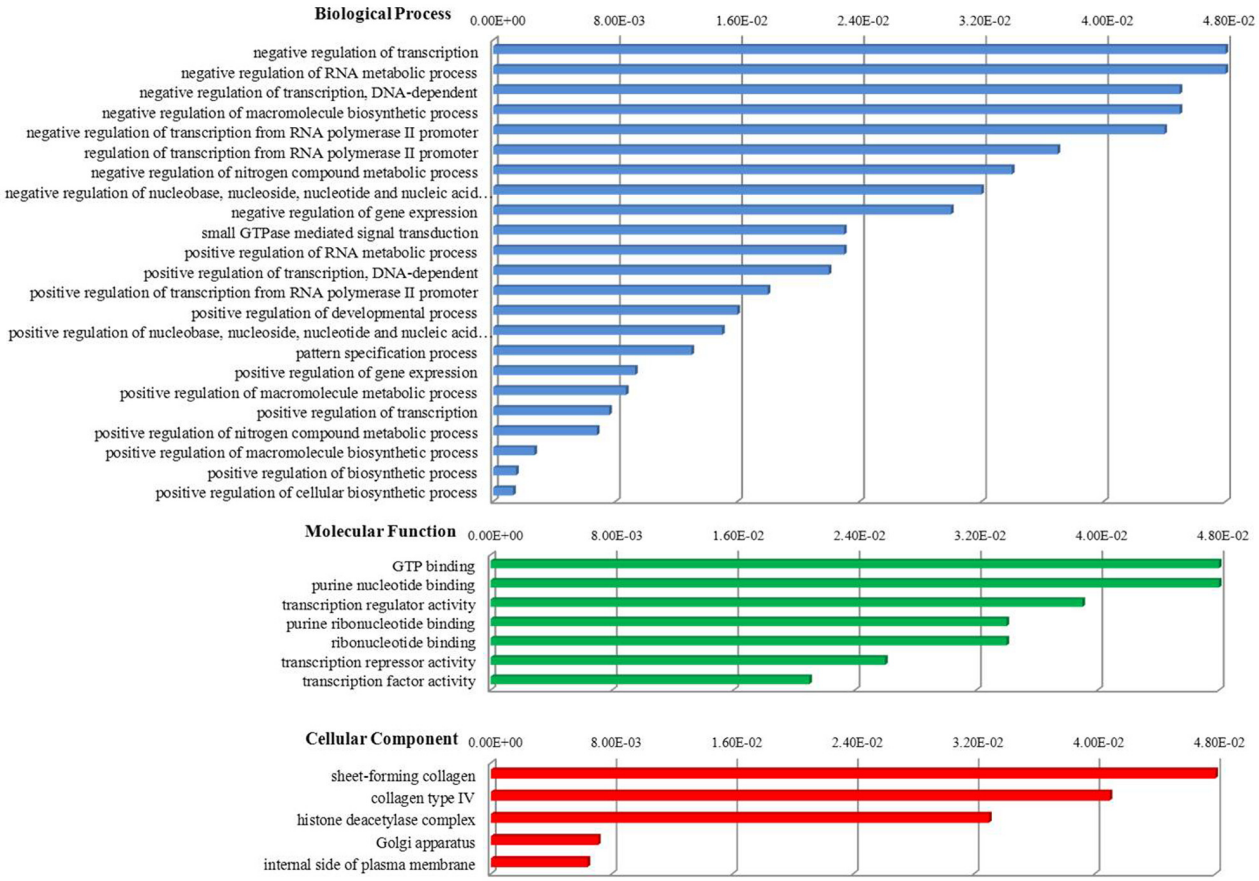

FIGURE 1 | Biological functions of host miRNAs belonging to HTLV-2 and HIV-1 retroviral signature. One hundred and thirty-five predicted target genes were identified by miRDB database (http://mirdb.org). Functional annotations were provided by DAVID bioinformatics resources (http://david.abcc.ncifcrf.gov) on the basis of gene ontology (GO) resources that consider three different aspects of GO: biological process, cellular component, molecular function. The GO term selection was obtained using a significant *P*-value less than 0.05 as a statistical threshold. Functional annotations are sorted by score, from higher (minor significance) to lower (greater significance).

infecting agents. Since a poor Th1 response and a dominant Th2 response have been implicated in the pathogenesis and progression of HIV infection (Clerici and Shearer, 1993, 1994), HTLV-2 priming for a Th1 response via up-regulation of IFN-γ expression may contribute to the "protective" effect of HTLV-2 infection on HIV-1 disease progression. In the case of HTLV-1 co-infection, high frequencies of activated HTLV-1-infected $CD4^+$ T cells can give a boost to HIV-1 replication.

An enhanced secretion of CC-chemokines, in particular of CCL3L1, was ascribed to the transactivating function of Tax-2 and the original studies of HTLV-2/HIV-1 co-infection proposed this as a key mechanism of retroviral interference. The CCL3L1 isoform down-regulates CCR5 co-receptor for HIV-1 entry leading to a LTNP status in co-infected individuals with high HTLV-2 proviral load (Pilotti et al., 2007).

In the same manner, GBV-C acquisition via blood transfusion increases the secretion of CCL5, CCL3, CCL4, and CXCL12 and by means of their NS5A and E2 proteins support the deregulation of co-receptors, thus inhibiting HIV entry and resulting in a reduced mortality in patients with advanced HIV-1 disease (Tillmann et al., 2001; Bhattarai and Stapleton, 2012). Similarly to what occur in HIV-1/HTLV-1 co-infection, HIV-1 disease progression is faster in individuals affected by HCV or HSV co-infection (Van Asten and Prins, 2004; Corey, 2007).

Analysis of JAK/STAT regulation during HTLV-2 infection provides some clues of intervention to interfere with HIV-1 replication by taking advantage of pathway interference instead of enzymatic inhibition of viral enzymes. The lack of knowledge of HTLV-1 mediated activation pathway interference has probably limited efforts in this direction.

Concerning innate immune responses in HIV-1 co-infection with either HTLV-1 or HTLV-2, no data are so far available for NK cell function, and little is known on other innate immune cellular mechanisms. The observed effect of proviral load, but not of Tax-1, on NK cell proliferation during HTLV-1 infection (Norris et al., 2010), is likely to impact also on HIV-1/HTLV-1 co-infected patients, leading to enhanced NK cell activation and possibly disease progression. On the contrary, when considering HTLV-2/HIV-1 co-infection, evidence of HTLV-2 interference

Table 2 | MicroRNAs either up- or down-regulated in HTLV-1 infected cells vs HIV-1 and HTLV-2 infection.

	HIV-1 vs healthy Bignami et al. (2012)	HTLV-1 vs healthy Pichler et al. (2008), Bellon et al. (2009), Ruggero et al. (2010), Rahman et al. (2012)	HTLV-2 vs healthy Bignami et al. (2012)
hsa-miRNA miR-21	ns	Up	ns
hsa-miR-24	nd	Up	ns
hsa-miR-93	nd	Up	ns
hsa-miR-132	nd	Down	ns
hsa-miR-143-3p	nd	Up	nd
hsa-miR-146a	nd	Up	ns
hsa-miR-149	ns	Down	ns
hsa-miR-155	ns	Up	Up
hsa-miR-223	ns	Up	ns
hsa-miR-873	Down	Down	ns
hsa-miR-150	nd	Up	ns
hsa-miR-142-5p	nd	Up	ns
hsa-miR-181a	nd	Down	ns
hsa-miR-125a	Up	Down	Up
hsa-miR-146b	ns	Down	ns

ns indicates not significant; and nd not determined.

with STAT/JAK pathways could possibly be linked to a decreased HIV-1-associated NK cell activation. Further work in this direction is needed to improve our understanding of the mechanism(s) associated with the positive effect of HTLV-2 on HIV-1 disease course, and of the underlying causes leading to AIDS progression by HTLV-1/HIV-1 co-infection and/or to HTLV-1 associated morbidity.

Since miRNAs have been correlated with viral life cycle, they represent good candidates among the top cellular factors to be used by HTLVs to favor their own replication. In the case of co-infection with HIV-1, HTLV proteins were found to interact with cellular chromatin modifying enzymes and with cellular transcription and other immune factors. The interaction of HTLV Tax proteins with cellular factors results in the alteration of miRNAs profile that in turns can activate transcription and consequently viral replication. Furthermore a likely interplay between two competing mechanisms is taking place: the ability of Tax to manipulate chromatin structure and the innate host cellular defense mechanism of RNAi to regulate pathogen gene expression.

HTLV/HIV-1 co-infection can be considered as a useful model for the study of new strategic approaches for HIV-1 vaccine development, as suggested by the finding that the exposure of HTLV-2 infected macaques to SIV_{mac251} was not accompanied by an exacerbation of SIV_{mac251} infection (Gordon et al., 2010).

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Differential transforming activity of the retroviral Tax oncoproteins in human T lymphocytes

Tong Ren*[1] *and Hua Cheng*[2]

[1] *Penn State Hershey Cancer Institute, Hershey, PA, USA*
[2] *Institute of Human Virology, School of Medicine, University of Maryland, Baltimore, MD, USA*

Edited by:
Umberto Bertazzoni, University of Verona, Italy

Reviewed by:
Masanori Baba, Kagoshima University, Japan
Edward Harhaj, Johns Hopkins School of Medicine, USA
Masahiro Fujii, Niigata University Graduate School of Medical and Dental sciences, Japan

***Correspondence:**
Hua Cheng, Institute of Human Virology, School of Medicine, University of Maryland, 655 West Baltimore Street, Baltimore, MD 21201, USA
e-mail: hcheng@ihv.umaryland.edu

Human T cell leukemia virus type 1 and type 2 (HTLV-1 and -2) are two closely related retroviruses. HTLV-1 causes adult T cell leukemia and lymphoma, whereas HTLV-2 infection is not etiologically linked to human disease. The viral genomes of HTLV-1 and -2 encode highly homologous transforming proteins, Tax-1 and Tax-2, respectively. Tax-1 is thought to play a central role in transforming CD4+ T lymphocytes. Expression of Tax-1 is crucial for promoting survival and proliferation of virally infected human T lymphocytes and is necessary for initiating HTLV-1-mediated oncogenesis. In transgenic mice and humanized mouse model, Tax-1 has proven to be leukemogenic. Although Tax-1 is able to efficiently transform rodent fibroblasts and to induce lymphoma in mouse model, it rarely transforms primary human CD4+ T lymphocytes. In contrast, Tax-2 efficiently immortalizes human CD4+ T cells though it exhibits a lower transforming activity in rodent cells as compared to Tax-1. We here discuss our recent observation and views on the differential transforming activity of Tax-1 and Tax-2 in human T cells.

Keywords: HTLV-1/-2, Tax, human T lymphocytes, immortalization, transformation

HTLV-1 and -2 are two highly homologous human retroviruses. Both viruses are blood-borne pathogens, which can be transmitted through perinatal transmission by blood contamination or breast milk feeding, sexual transmission, exposure to contaminated blood products or sharing contaminated needles (Clark et al., 1985; The T- and B-cell Malignancy Study Group, 1988). Although both HTLV-1 and -2 utilize GLUT-1, a ubiquitously expressed cell surface (SU) protein, as the receptor for viral entry (Igakura et al., 2003; Manel et al., 2003; Coskun and Sutton, 2005), they exhibit distinct cell tropism. HTLV-1 preferentially infects and transforms CD4+ T cells, whereas HTLV-2 has tropism for CD8+ T cells, which suggests that there exists a CD8+ T cell-specific co-factor for assisting viral entry and permissive replication of HTLV-2 (Ijichi et al., 1992). HTLV-1 is the etiological factor that causes adult T cell leukemia and lymphoma (Hattori et al., 1981; Hinuma et al., 1981, 1982; Yamaguchi et al., 1984). HTLV-2 was detected in CD8 T cells from a patient with hairy cell leukemia, a rare type of leukemia that affects B lymphocytes (Kalyanaraman et al., 1982), however the causative link between HTLV-2 infection and hairy cell leukemia has not been established. Thus far, HTLV-2 infection has not been etiologically linked to any human disease.

HTLV-1 viral genome is known to encode two oncogenic products, Tax-1 and HBZ (Satou et al., 2006; Satou and Matsuoka, 2012). Tax-1 is the first recognized viral component to display oncogenic potential. Evidence showed that Tax-1 plays an essential role in mediating transformation of T lymphocytes (Tanaka et al., 1990). The transforming activity of Tax-1 in lymphocytes was first demonstrated using HTLV-1 infectious molecular clone. The wild type molecular clone efficiently transformed human primary T cells, while the molecular clone with disrupted *tax* gene failed to do so, defining the role of Tax-1 as a viral transforming protein (Yamaoka et al., 1996, 1998; Akagi et al., 1997; Matsumoto et al., 1997; Iwanaga et al., 1999). However, this experiment did not rule out potential participation of other viral components in HTLV-1-mediated cell transformation. Unexpectedly, when expressed alone, Tax-1 rarely immortalizes or transforms terminally differentiated T cells derived from human peripheral blood (Bellon et al., 2010). In humanized mouse model, Tax-1-expressing human CD34+ blood progenitor cells, when transplanted into NOD/SCID mice, developed T cell lymphoma of human origin (Banerjee et al., 2010). This experimental finding suggests that in some cases, leukemic cells might be evolved from HTLV-1-infected CD34+ blood progenitor cells rather than from infected mature T cells. This assumption is yet to be validated.

A comparative study on two highly homologous Tax proteins from HTLV-1 and -2 is crucial in deciphering the molecular pathogenesis of HTLV-1-associated ATL. We expressed the Tax proteins in human T cells isolated from peripheral blood to imitate the process of HTLV-1 infection. This prospective approach would allow us to evaluate sequential oncogenic events in Tax-mediated proliferation, immortalization and transformation of human primary T cells. Normal lymphocytes isolated from healthy donors are typically at quiescent stage. Stimulation with mitogens such as PHA, recombinant IL-2 or mitogenic anti-CD3/-CD28 antibodies results in rapid proliferation of peripheral blood lymphocytes with displaying lymphoblastic morphology. Activated lymphocytes stop growing 2–3 weeks following stimulation at normal culture conditions by reaching the checkpoint of T cell senescence. HTLV-1 infection overrides replicative senescence of T cells, promotes continuous growth of HTLV-1-infected T cells and induces immortalization, ultimately resulting in IL-2-independent growth of the transformed T cells. At each step of aberrant proliferation,

immortalization or transformation during HTLV-1 infection of T cells, a variety of cellular oncogenic alterations are expected to occur. Thus, studying these processes will provide crucial insights into the pathogenesis of ATL.

We previously hypothesized that in the absence of other viral proteins, Tax-1 is sufficient to immortalize human mature CD4+ T cells while Tax-2 preferentially immortalizes CD8+ T cells. We unexpectedly found that both Tax-1 and Tax-2 failed to immortalize human primary CD8+ T cells, and Tax-2 was able to immortalize human CD4+ T cells more efficiently than Tax-1 in the study with including 12 healthy blood donors. A similar finding was reported later (Imai et al., 2013). In addition, a majority of Tax-2-immortalized CD4+ T cell lines grew *in vitro* at the growth rate similar to some lymphoblastic leukemia cells (Ren et al., 2012). In contrast, Tax-1-immortalized CD4+ T cells were slow growing and exhibited spontaneous cell death at normal culture conditions (Ren et al., unpublished data). These experimental results were unlikely caused by technical variation because Tax-1 and Tax-2, in which their expressions were driven from human elongation factor promoter, were introduced into human primary T cells via VSV-G pseudotyped lentiviruses at similar efficiency.

Tax-2 appears to be more oncogenic than Tax-1 in primary CD4+ T cells when they are expressed alone, however Tax-1 is clearly oncogenic in CD4+ T cells in the context of an intact proviral clone and other expressed viral proteins. Although both Tax proteins are highly homologous, Tax-1 does exhibit distinct structural features in which Tax-2 lacks. Tax-1 contains a PDZ binding motif (PBM) in its carboxyl-terminus that is important for binding to DLG-1 and other PDZ containing proteins, and these interactions were thought to play an important role for cell transformation by Tax-1. In addition, Tax-1, but not Tax-2, undergoes K63-linked polyubiquitination as part of its mechanism to activate NF-κB (Shembade et al., 2007; Journo et al., 2013). It is apparent that these differences do not account for the stronger transforming capability of Tax-2 in primary CD4+ T cells. Tax-1 may acquire a full transforming ability in human CD4+ T cells in cooperation with HBZ, an HTLV-1 antisense gene product that is constitutively transcribed and remains intact in ATL cells. In fact, HBZ itself was proven leukemogenic in mouse model. 30% of HBZ-transgenic (Tg) mice developed T cell lymphoma and most of HBZ Tg mice developed spontaneous inflammatory lesions (Satou and Matsuoka, 2012). Furthermore, increasing evidences showed that the functional interaction between Tax and HBZ is crucial for HTLV-1 oncogenesis in patients. Since high levels of Tax-1 expression lead to hyper-activation of NF-κB, consequently resulting in replicative senescence or even cell death (Zhi et al., 2011), HBZ antagonizes Tax-1's toxic function to facilitate immune evasion and to promote cell cycle progression and proliferation. Interestingly, Tax-2 also induces hyper-activation of NF-κB in CD4+ T cells, and it is capable of promoting T cell proliferation in the absence of the antisense gene product encoded from HTLV-2. This suggests that unlike Tax-1, Tax-2 is a singly important viral component of the HTLV-2 genome to execute its transforming activity in CD4+ T cells.

We also found that the Tax proteins had their own preference on donor selection, and Tax-1 and Tax-2 immortalized CD4+ T cells from different blood donors. In one unusual case, Tax-1 immortalized CD4+ T cells while Tax-2 immortalized CD4+ myeloid dendritic cells (mDCs) from CD4+ cell pools of the same blood donor (Ren et al., unpublished data). These mDCs showed negative lineage markers (CD3−/TCRαβ-/TCRγδ-/CD19−/CD56−/CD16−/CD14−/CD34−) and positive myeloid/dendritic cell markers (CD11c+/CD123+/HLA-DR+/CD2+/CD71+/CD117+/CD33+). Because the number of mDCs in healthy donors is typically less than 1%, the finding that Tax-2 is able to immortalize mDCs from enriched CD4+ pools suggests that the Tax-2-mediated immortalization of blood cells is highly selective. Aside from this finding, genotyping analysis of one Tax-2-established CD4 T cell line showed that these cells were clonal population. Together, our data demonstrate that in the absence of other viral components, Tax-2 alone is sufficient to promote clonal expansion of selected subsets of human primary CD4+ T cells. The potential cellular factor that contributes to Tax's selectivity is currently unclear, which will certainly be an interesting subject for further investigation.

It is known that HTLV-1 has preferential tropism for CD4 T cells in healthy carriers, HAM/TSP and ATL patients. In addition to utilization of GLUT1 as an entry factor for HTLV viruses, both viruses also utilize NRP1. HTLV-1 and -2 have a differential requirement for heparan sulfate proteoglycans (HSPGs). Distinct from HTLV-1, HTLV-2 is not dependent on HSPGs for cell entry (Jones et al., 2006). Because HTLV-1 utilizes a ubiquitously expressed receptor for viral entry, it is expected that this virus would have the capacity to infect cell types other than CD4+ T cells. Indeed, CD8+ lymphocytes, monocytes and B-lymphocytes are found to harbor HTLV-1 (Koyanagi et al., 1993; Eiraku et al., 1998; Nagai et al., 2001). In addition, macrophages, dendritic cells, megakaryocytes as well as glial cells (astrocytes and microglial cells) are also the cell targets for HTLV-1 infection *in vivo* (Macatonia et al., 1992; Koyanagi et al., 1993; Levin et al., 1997; Grant et al., 2002). HTLV-1 isolates can be transmitted to primary human endothelial cells and basal mammary epithelial cells *in vitro* (Ho et al., 1984; Hoxie et al., 1984). Regardless of a broad cell tropism of HTLV-1, this virus exclusively causes malignant transformation of infected CD4+ T cells, suggesting that a panel of CD4 cell-specific cellular factors is crucial in assisting HTLV-1-mediated oncogenesis. Conversely, it is also possible that other types of cells may express cellular repressors to restrict HTLV-1 oncogenesis as seen in other retroviruses. For instance, SAMHD1 is able to restrict HTLV-1 replication in myeloid cells (St. Gelais and Wu, 2011). Unlike HTLV-1, HTLV-2 preferentially infects CD8+ T cells in human. However, Tax-2, when expressed by a pseudotype form of lentivirus, executes its transforming activity in CD4+ T cells. This phenomenon of disintegrated cell tropism (in CD8+ T cells) and disease-causing capacity (in CD4+ T cells) may partially explain why HTLV-2 does not currently cause leukemia in human.

These findings may have important implications on pathogenic virus evolution. Many viruses are considered non-pathogenic, because they do not apparently cause human disease based on current data. These non-pathogenic viruses usually do not receive much attention as known pathogenic viruses do. From an evolutionary point of view, recombination and cell tropism switch among homologous pathogenic and non-pathogenic viruses may occur, potentially generating a more lethal virus. A recent study

demonstrated that cell tropism switch could be initiated by artificially generating chimeric envelope proteins. Switch of the envelope SU domains between HTLV-1 and HTLV-2 alters the tropism of HTLV-1 from CD4 to CD8+ T cells (Kannian et al., 2013). In the case of HTLV-2, the infection rate among IV drug abusers is as high as 30%. Since no treatment is available for HTLV-2 infection and host immunity is not sufficient to eradicate this virus, it is possible that the rate of infection is going to rise due to the presence of accumulative HTLV-2 reservoirs in host CD8+ T cells. Indeed, co-infection of HTLV-2 with HTLV-1 or with HIV-1 has been documented and is increasing (Uchiyama et al., 1977; Shimoyama, 1991), probably because these viruses share similar transmission pathways and risk factors. HTLV-2 could be eventually modified in dendritic cells or monocytes that are co-infected with HTLV-1 or HIV-1 to acquire new cell tropism for CD4+ T cells, leading to its transformation of CD4+ T cells. Although naturally occurring cell tropism switch among homologous viruses may take unpredictable amount of time, this event does occur evolutionarily.

Although Tax-2 does not transform normal CD8 T cells, this viral protein exhibits its transforming activity in CD8+ T cells derived from T cell type of large granular lymphocyte leukemia (T-LGLL). T-LGL leukemia is the malignancy of CD8+ cytotoxic T cells, which usually occurs in elderly patients. The disease course is frequently associated with autoimmune diseases (Rose and Berliner, 2004). Primary T-LGL leukemia cells display the CD3+/CD8+/CD57+ SU markers, representing activated cytotoxic T lymphocytes. Despite T-LGLL cells are leukemic cells, these cells do not grow in culture. By expressing Tax-2 in these cells, long-term growth and clonal expansion of T-LGLL cells could be achieved (Ren et al., unpublished data).

In summary (see **Table 1**), Tax-1 and Tax-2 display differential transforming activities in human T lymphocytes. Tax-2 demonstrates a more potent activity than Tax-1 in immortalizing human terminally differentiated CD4+ T cells. Besides, Tax-2, not Tax-1, is able to transform CD8+ T cells from T-LGL leukemic cells with preexisting oncogenic profile. The ability of Tax-2 to establish specific subsets of T cell lines implicates its utilization in studying human T cell biology and in developing leukemia model. Furthermore, the hypothesis that Tax-2 has a disease-causing capacity in CD4+ T cells needs to be validated in humanized mouse model.

Table 1 | Differential activities of Tax-1 and Tax-2 in human T cells.

	Tax-1	Tax-2
Activation of		
IKK/NF-kB	+	+
Stat3	+	+
PI3K/Akt	+	+
AP-1	+	+
CREB	+	+
Dysregulation of autophagy	+	+
Lipid raft involvement	+[a]	-
Immortalization of		
CD4+ T cells	+[b]	+++[c]
CD8+ T cells	-	-
CD8+ T cells from T-LGLL	-	+

[a]*Tax-1 recruits IκB kinases into lipid raft microdomains for persistent activation of NF-κB signaling, while Tax-2 activation of NF-κB does not appear to involve lipid rafts (Huang et al., 2009).*

[b]*Tax-1 is able to immortalize human primary CD4+ T cells with low efficiency (2 out of 12), and Tax-1-immortalized T cells grow slowly in culture and experience spontaneous cell death.*

[c]*Tax-2 immortalizes human primary CD4+ T cells more efficiently than Tax-1 (4 out of 12). Tax-2-immortalized T cells grow in culture at a rate comparable to some lymphoblastic leukemia cells with healthy growth appearance.*

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The need to accessorize: molecular roles of HTLV-1 p30 and HTLV-2 p28 accessory proteins in the viral life cycle

Rajaneesh Anupam[1,2†], Rami Doueiri[1,2†] and Patrick L. Green[1,2,3,4*]

[1] *Center for Retrovirus Research, The Ohio State University, Columbus, OH, USA*
[2] *Department of Veterinary Biosciences, The Ohio State University, Columbus, OH, USA*
[3] *Comprehensive Cancer Center and Solove Research Institute, The Ohio State University, Columbus, OH, USA*
[4] *Department of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University, Columbus, OH, USA*

Edited by:
Umberto Bertazzoni, University of Verona, Italy

Reviewed by:
Vincenzo Ciminale, University of Padova, Italy
Donna M. D'Agostino, University of Padova, Italy

****Correspondence:***
Patrick L. Green, Department of Veterinary Biosciences, The Ohio State University, 1900 Coffey Road, Columbus, OH 43210, USA
e-mail: green.466@osu.edu

† Rajaneesh Anupam and Rami Doueiri are joint first authors.

Extensive studies of human T-cell leukemia virus (HTLV)-1 and HTLV-2 over the last three decades have provided detailed knowledge on viral transformation, host–viral interactions and pathogenesis. HTLV-1 is the etiological agent of adult T cell leukemia and multiple neurodegenerative and inflammatory diseases while HTLV-2 disease association remains elusive, with few infected individuals displaying neurodegenerative diseases similar to HTLV-1. The HTLV group of oncoretroviruses has a genome that encodes structural and enzymatic proteins Gag, Pro, and Env, regulatory proteins Tax and Rex, and several accessory proteins from the pX region. Of these proteins, HTLV-1 p30 and HTLV-2 p28 are encoded by the open reading frame II of the pX region. Like most other accessory proteins, p30 and p28 are dispensable for *in vitro* viral replication and transformation but are required for efficient viral replication and persistence *in vivo*. Both p30 and p28 regulate viral gene expression at the post-transcriptional level whereas p30 can also function at the transcriptional level. Recently, several reports have implicated p30 and p28 in multiple cellular processes, which provide novel insight into HTLV spread and survival and ultimately pathogenesis. In this review we summarize and compare what is known about p30 and p28, highlighting their roles in viral replication and viral pathogenesis.

Keywords: HTLV-1, HTLV-2, p30, p28, accessory proteins

INTRODUCTION

Human T-cell leukemia virus (HTLV) are complex deltaretroviruses, with HTLV-1 and HTLV-2 causing the most prevalent worldwide infections. HTLV-1 infects approximately 25 million people worldwide and is endemic in Japan, Africa, South America, Iran, and the Caribbean basin (Proietti et al., 2005; Goncalves et al., 2010; Iwanaga et al., 2012). In contrast, HTLV-2 is endemic in Central and West Africa (Goubau et al., 1992; Gessain et al., 1993; Goubau et al., 1993), in native Amerindian populations in North, Central, and South America (Hjelle et al., 1990; Lairmore et al., 1990; Heneine et al., 1991; Levine et al., 1993), and among cohorts of intravenous drug users (IVDUs) in the United States and Europe. Although few HTLV-1 infected individuals (1–5%) develop adult T-cell leukemia/lymphoma (ATL/ATLL) or HTLV-1-associated myelopathy/tropic spastic paraparesis (HAM/TSP), all infected individuals exhibit a persistent antiviral immune response that fails to clear the virus (Poiesz et al., 1980; Yoshida et al., 1982; Takatsuki, 2005; Yoshida, 2005; Lairmore et al., 2012). Furthermore, HTLV-2 infection is not associated with leukemia/lymphoma, although in rare cases it causes a neurodegenerative condition similar to HAM/TSP (Araujo and Hall, 2004).

The genome of HTLV encodes structural and enzymatic genes typical of all retroviruses. In addition, the pX region located between the *env* gene and 3′ long terminal repeat (LTR) encodes four open reading frames (ORF I to IV) with a potential for encoding several proteins (Berneman et al., 1992; Ciminale et al., 1992; Koralnik et al., 1992). As a result of complex splicing, various mRNAs encode regulatory and accessory proteins. Positive regulators of viral gene expression, Tax and Rex, are encoded by a doubly spliced bicistronic mRNA from ORFs IV and III, respectively (Felber et al., 1985; Kiyokawa et al., 1985). Reverse transcription-PCR of mRNA from HTLV-1 infected cell lines and uncultured primary lymphocytes from ATL patients has shown that alternative splicing produces the accessory proteins p12, p30, and p13 (Berneman et al., 1992; Ciminale et al., 1992; Koralnik et al., 1992). A singly spliced mRNA containing ORF-I codes for the accessory protein p12 that can be cleaved to produce a smaller protein, p8 (Van Prooyen et al., 2010). p30 is encoded by a doubly spliced message in which ORF-II is linked to the Tax initiation codon located on exon II, resulting in a 241 amino acid protein. ORF-II also can be singly spliced to produce mRNA that can encode p13 from the internal initiation codon in ORF-II, which corresponds essentially to the last 87 amino acids of p30. Similar studies in the MoT cell line identified accessory proteins in HTLV-2: a bicistronic doubly spliced mRNA encodes p10 and p11 from ORF-I and ORF-V, respectively, and two distinct bicistronic singly spliced mRNAs encoded p28 from ORF II as well as the truncated ORF III isoforms of Rex (Ciminale et al., 1995). p30 and p28 share certain amino acid sequence homology, the last 50 amino acids of p30 share 70% homology with the first 50 amino acids of p28 (Ciminale et al., 1995), and both are nuclear/nucleolar proteins (Koralnik

et al., 1993; Ciminale et al., 1995; D'Agostino et al., 1997; Younis et al., 2004). In addition, newly identified proteins, HTLV-1 basic leucine zipper factor (HBZ) and anti-sense protein HTLV-2 (APH-2), are encoded from the antisense genome strand in HTLV-1 and HTLV-2, respectively (Gaudray et al., 2002; Halin et al., 2009).

Tax transactivates viral gene transcription by recruiting transcription factors p300/CREB binding protein (CBP), CREB and AP-1 to the Tax response element (TRE) in the LTR region (Seiki et al., 1986). Tax drives cellular transformation through its ability to alter cellular gene expression, signaling pathways, and cell cycle (Grassmann et al., 2005). Of the factors targeted by Tax, NFκB clearly plays a prominent role in deregulation of cellular gene expression and cellular transformation (Smith and Greene, 1990; Rosin et al., 1998; Robek and Ratner, 1999; Ross et al., 2000). Although Tax is indispensable for viral transformation, the mechanism by which the virus persists *in vivo* leading to T-cell transformation is not clearly understood (Matsuoka and Jeang, 2007). Studies suggest that HBZ and accessory proteins might play a role *in vivo* in HTLV-1 viral persistence and T-cell malignant transformation (Bartoe et al., 2000; Arnold et al., 2006; Arnold et al., 2008; Valeri et al., 2010). Rex binds to the Rex response element (RxRE) on unspliced and singly spliced viral mRNAs to facilitate their nuclear-cytoplasmic export for translation in the cytoplasm (Younis and Green, 2005). p30 and p28 mRNA species can be identified in infected cells (Li and Green, 2007) and in cells from HTLV-1 infected patients (Rende et al., 2011; Bender et al., 2012), albeit at 10^3–10^4 lower levels than *tax/rex* mRNA.

Reports identifying antibodies and cytotoxic $CD8^+$ T-cells in infected patients with HTLV-1 (symptomatic and asymptomatic) against p30, demonstrate the importance of HTLV-1 accessory proteins in viral persistence and ultimately in the viral life cycle (Jacobson et al., 1992; Chen et al., 1997; Pique et al., 2000). However no studies to date have attempted to identify antibodies or cytotoxic $CD8^+$ T-cells against p28 in HTLV-2 infected patients. In this review we will compare the current knowledge on p30 and p28, highlighting the differences and similarities in their roles in the HTLV life cycle.

IN VIVO ROLE OF P30 AND P28

Initial studies to understand the role of p30 were performed by deleting either ORF-I and –II, or ORF II alone from an HTLV-1 infectious molecular clone, which showed that p30 is dispensable for viral gene expression, infectivity, replication, and T-cell immortalization *in vitro* (Derse et al., 1997; Robek et al., 1998). To examine the role of p30 in viral replication and infectivity *in vivo*, T-cell lines immortalized with a viral clone containing mutations ablating both p30 and p13 reading frames (Bartoe et al., 2000) or p30 alone (Silverman et al., 2004) were generated. It was noted that loss of p30 and p13 or p30 alone resulted in a significantly reduced antibody response and lower proviral loads in rabbits indicating that p30 and p13 are required for maintenance of high proviral loads *in vivo* (Bartoe et al., 2000; Silverman et al., 2004). In addition, when p30 alone was ablated, reversion to the wild type sequence was observed, underlining the *in vivo* importance of p30 in maintaining high level viral infection (Silverman et al., 2004). It is noteworthy that these first experiments were performed before the identification of the HTLV-1 HBZ, which is encoded from the anti-sense viral mRNA that overlaps ORFs in the pX region including p30 ORF-II (Gaudray et al., 2002). It is very likely that the mutations ablating p30 expression interfered with the expression of HBZ, which also was shown to be important for viral infectivity *in vivo* (Arnold et al., 2006). Recently, to investigate the relative contribution of HBZ and p30 to viral infectivity *in vivo*, B cell lines expressing viral mutants with specific ablation of HBZ and p30 were generated and used to infect rabbits and macaques. In contrast to the earlier *in vivo* rabbit studies (Silverman et al., 2004), it was shown that viral mutants lacking p30 had little effect on antibody response, infectivity or proviral loads. However, lack of HBZ resulted in reduced proviral loads in rabbits. In addition, there was no evidence of reversion to wild type sequence by the p30 or HBZ mutants (Valeri et al., 2010). Interestingly, in macaque infections it was noted that lack of p30 and HBZ resulted not only in reduced antibody response and infectivity but also reversion to wild type sequence indicating that p30 and HBZ are important for viral infectivity in macaques (Valeri et al., 2010). The discrepancy regarding the requirement for p30 in rabbit infections may be due to the contribution of HBZ to the viral infection. In the same study it was shown that p30 is also required for productive cell-free infection of dendritic cells *in vitro*. The requirement of p30 in terms of *in vitro* viral infection might be cell type-dependent.

On the other hand, an initial report revealed that deletion of the pX region of HTLV-2 causes a reduction in viral replication but not infectivity *in vivo*, but had no effect *in vitro* (Cockerell et al., 1996). To delineate the role of p28, Yamamoto et al. (2008) created a p28 knockout HTLV-2 molecular clone and stably transfected virus producing cells with the wild type HTLV-2 molecular clone or the HTLV-2Δp28 mutant. The irradiated producer cells were co-cultured with human PBMCs, and viral p19 Gag production and cell survival were measured over time. No difference was observed between HTLV-2 and HTLV-2Δp28 indicating that p28 was not required for *in vitro* primary T lymphocyte infectivity, proliferation, and immortalization. The authors also determined the effect of p28 on viral persistence *in vivo* using a rabbit model and found that rabbits infected with HTLV-2 lacking p28 had lower antibody responses and reduced proviral load compared to those infected with wild type HTLV-2 (Yamamoto et al., 2008). The mutation to delete p28 had no effect on the APH-2 gene providing clear evidence that p28 contributes to viral persistence *in vivo*.

Collectively, it is clear that p30 (HTLV-1) and p28 (HTLV-2) are required for efficient viral replication and persistence *in vivo*. Recent findings discussed below provide various possible mechanisms to explain the role of these accessory proteins in viral infection and/or pathogenesis.

VIRAL GENE EXPRESSION

TRANSCRIPTIONAL REGULATION

It was demonstrated that p30 is a nuclear/nucleolar localizing protein (Ciminale et al., 1992; Koralnik et al., 1992; see **Figure 1** for summary of key p30 domains). Structurally, p30 contains a bipartite nucleolar/nuclear localization signal (NoRS/NLS), which is comprised of two arginine rich domains (amino acids 73–78 and 91–98; D'Agostino et al., 1997). A later study reconfirmed these regions to be a nucleolar retention signal and also identified two other possible nuclear localization signals in the amino (N) and

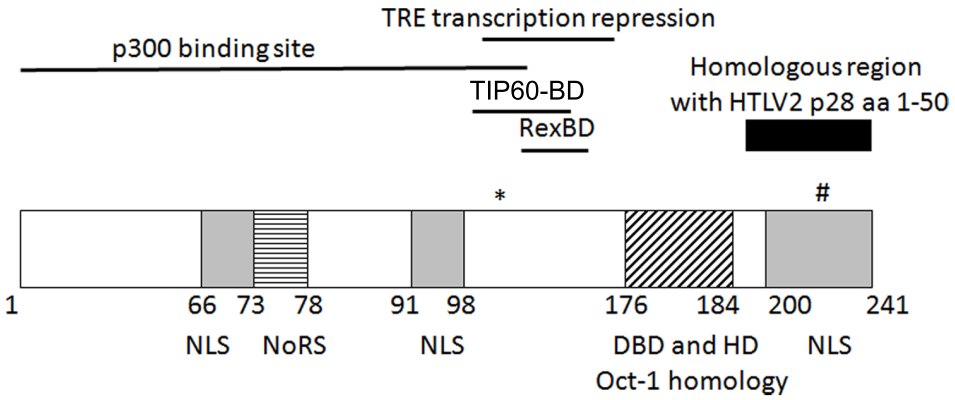

FIGURE 1 | Schematic representation of the known/putative functional domains of p30. (*)K106 required for p30 TRE transcriptional repression. (#)T232 required for nucleolus to nuclear transport upon DNA damage. NLS, nuclear localization sequence; NoRS, nucleolar retention sequence; Rex-BD, Rex binding domain; TIP60-BD, TIP60 binding domain; aa, amino acids.

carboxy (C) termini of p30 (Ghorbel et al., 2006). The latter study also implicated the role of transcription and RNA-interaction in nucleolar/nuclear localization of p30 with the specific interaction of p30 with a 60S ribosomal protein L18a (Ghorbel et al., 2006). Similar to p30, expression of p28 cDNA resulted in nuclear localization of p28 (Ciminale et al., 1995; Younis et al., 2004).

p30 has marked homology with serine and threonine rich transcription factors Oct-1 and-2, Pit-1, and POU-M1 suggesting that p30 could function as a transcriptional regulator (Ciminale et al., 1992). The initial evidence of p30 as a transcriptional regulator was provided by the ability of p30 to differentially modulate CREB responsive promoters by acting as a transactivator when fused to the DNA binding domain of Gal4, and behaving as a repressor when expressed alone. In addition, p30 increased transcription from the TRE at a low concentration but repressed transcription at a higher concentration in the presence and absence of Tax (Zhang et al., 2000). On the other hand, in a similar Gal4 reporter assay, p28 failed to modulate Gal4 DNA binding ability, demonstrating that p28 is devoid of transcriptional activity (Younis et al., 2006). Subsequently, p30 was shown to regulate transcription by binding to CBP/p300. The interaction of p30 and CBP/p300 was evaluated by two hybrid screening, immunoprecipitation, and localization studies. The binding of p30 to p300 was mapped to a highly conserved KIX region of p300, which is also the binding site of Tax. The transcriptional repression of TRE by p30 is a result of competition between p30 and Tax for p300 binding and disruption of the assembly of Tax/CBP/P300 on the TRE (Zhang et al., 2001). The p300 binding region was mapped to amino acids 1–132 whereas the TRE transcription repression function of p30 was mapped to amino acids 100–179. This 80 bp region contains five of the six lysine residues in p30 of which lysine 106 was found to be important for repression. Unlike wild type p300, mutant p300 with impaired histone acetyltransferase activity (HAT) was unable to rescue p30-mediated LTR transcription repression. Moreover, p30 LTR repression was enhanced by histone deacetylase (HDAC-1) and inhibited by Trichostatin A (inhibitor of HDAC-1). These data suggest that the LTR transcription repression function of p30 could be regulated by acetylation on lysine or that these effects are due to global effects of HATs and HDACs on transcription (Michael et al., 2006).

p30 has the ability to transcriptionally activate numerous cellular genes. In one study it was shown that p30 binds and stabilizes the Myc/Tat-interacting protein (TIP60) transcriptional complex (Awasthi et al., 2005). Furthermore, p30 binds and recruits the 60-kDa (TIP60) to the Myc transcription complex to activate Myc responsive genes, which requires the HAT of TIP60. In addition, the transcriptional activity of p30 was dependent on transforming transcriptional activator protein (TRRAP)/p434. The idea that p30 plays a role in transformation by enhancing Myc-responsive genes was supported by the data showing that p30 cooperated with Myc in focus-forming transformation assays (Awasthi et al., 2005). More evidence for regulation of cellular transcription by p30 comes from a study showing that p30 can interact with the PU.1 transcription factor through its DNA binding domain resulting in the suppression of PU.1 DNA binding. Subsequently, the expression of p30 decreased cell surface expression of TLR4 as the expression of TLR4 is directly under the influence of PU.1 transcription activation. Concurrently, reduced TLR4 expression by p30 resulted in reduced pro-inflammatory cytokines TNF-α, MCP-1 and IL-8 whereas the expression of anti-inflammatory cytokine IL-10 was increased. Additionally it was found that p30 increased phosphorylation of GSK3-β leading to an increase in IL-10 production (Datta et al., 2006). These data indicate that p30 can influence innate and adaptive immunity to potentially facilitate viral spread by influencing cellular transcription. Similar experiments to investigate the ability of p28 to regulate transcription of cellular genes demonstrated that unlike p30, p28 has no transcription function, although chromatin immunoprecipitation assays (ChIP) show that p28 is recruited to the LTR along with the transcriptional machinery (Younis et al., 2006).

In order to further characterize the role of p30 in cellular transcription, various groups have performed microarray analysis. The initial microarray analysis showed that p30 altered expression of cellular genes involved in apoptosis, cell cycle regulation, cell adhesion, transcription, and T-cell signaling and activation. It was

found that p30 selectively activates genes involved in T cell signaling/activation and also enhances transcription mediated by NFAT, NF-κB, and AP-1 when stimulated (Michael et al., 2004). The ability of p30 to transcriptionally activate numerous cellular genes was also reported, where p30 binds and stabilizes the Myc/TIP60 transcriptional complex (Awasthi et al., 2005). Recently, similar microarray analysis also showed that p30 was capable of altering transcription of cellular genes involved in apoptosis and transcription (Taylor et al., 2009). Overall, these data indicate that p30 regulates transcription of viral genes as well as cellular genes to create an environment that promotes viral gene expression and survival of infected cells.

POST-TRANSCRIPTIONAL REGULATION

HTLV-1 p30 controls viral gene expression at the post-transcriptional level by interacting with and retaining *tax/rex* mRNA in the nucleus. The expression of p30 results in decreased levels of *tax/rex* mRNA specifically: this effect was not observed with other viral mRNAs. p30 binds to the second splice junction of *tax/rex* mRNA, which then is retained in the nucleus to repress tax expression and, in turn, viral gene expression (Nicot et al., 2004). HTLV-2 p28 mediates nuclear retention of the tax/rex mRNA via a similar post-transcriptional mechanism (Younis et al., 2004). Younis et al. (2006) went on to demonstrate that both p28 and p30 are recruited by Tax to the viral promoter, which then bind to their respective *de novo* transcribed response elements leading to the retention of *tax/rex* mRNA in the nucleus.

Rex and p30 have antagonistic functions where the Rex protein facilitates the nuclear-cytoplasmic export of unspliced and singly spliced mRNA while p30 retains *tax/rex* mRNA in the nucleus. The Rex and p30 interaction is favored when p30 is bound to *tax/rex* mRNA. The interaction of p30 with Rex has no effect on the ability of Rex to export mRNA from the nucleus to the cytoplasm whereas Rex moderately rescues the nuclear retention of *tax/rex* mRNA in the nucleus by p30. This interplay between p30 and Rex may be critical to establishing HTLV-1 latency (Sinha-Datta et al., 2007). Immunofluorescence confocal microscopy showed that p30 and Rex localized with CRM1, suggesting an interaction between Rex and p30 in HTLV-1 post-transcriptional control (Baydoun et al., 2007). On the other hand, p28 inhibited *tax/rex* mRNA export through the TAP/p15 pathway and not CRM1, where exogenous expression of TAP/p15 rescued p28 post-transcriptional repression (Younis et al., 2006).

Recently, microarray studies demonstrated that p30 can regulate cellular gene expression at the post-transcriptional level as well. Cytoplasmic expression of genes involved in cell signaling, transcription, translation, replication, cytoskeleton, DNA, and metabolism was decreased while those involved in repair, apoptosis, cell adhesion, and signaling were increased (Taylor et al., 2009). Studies to evaluate the effect of p28 post-transcriptional regulation on cellular genes have not been performed and are worth investigating.

CELL CYCLE REGULATION

Cell cycle control is an intricate, highly regulated pathway that guarantees proper cellular turnover and involves various cyclin dependent kinases (CDK) and Cyclins that phosphorylate key molecules allowing cell cycle progression. Different studies have shown that p30 also influences cell cycle progression. The G2/M checkpoint is regulated by a tight balance of phosphorylation and dephosphorylation events, where Cdc25c plays a crucial role in the onset of mitosis (Draetta and Eckstein, 1997). p30 activates the G2/M checkpoint by increasing the phosphorylation of Chk1 and subsequently increasing phosphorylation of Cdc25c. Another mechanism of p30-induced G2/M cell cycle arrest would be via reduction of PLK1 levels and phosphorylation resulting in decreased phosphorylation of Cdc25. In support of this mechanism, cells immortalized with an HTLV-1 molecular clone lacking p30 were more susceptible to apoptosis when treated with camptothecin and etoposide (Datta et al., 2007).

In addition to the effects of p30 on the G2M checkpoint, p30 activates Myc-driven genes leading to increased S phase progression and polyploidy (Awasthi et al., 2005). However, a recent study reported that expression of p30 delays S phase entry (Baydoun et al., 2010). The CDK2–Cyclin E complex is known to phosphorylate Rb, which in turn releases the transcriptional factor E2F, thereby controlling the expression of several genes required for S phase entry. It was shown that p30 delays S phase entry by interacting with both Cyclin E and CDK2 affecting their complex formation and resulting in reduced phosphorylation of Rb (Vermeulen et al., 2003). Hence, p30 expression causes a decrease in E2F and Cyclin E levels, and an increase in $p21^{Waf}$ expression. In contrast to p30, the expression of p28 had no effect on S phase entry and p28 was incapable of binding to Cyclin E (Baydoun et al., 2010).

DNA DAMAGE AND REPAIR

The integrity of the genome is essential for cell propagation, and timely repair of DNA damage is equally important to avoid the accumulation of mutations that could result in tumorigenesis. Retrovirus integration elicits the double stranded DNA damage response where cells must repair DNA damage in order to avoid apoptosis. DNA double strand breaks (DSB) are highly toxic to cells and are associated with developmental, immunological, neurological disorders, and various cancers (Jackson and Bartek, 2009; McKinnon, 2009). Anupam et al. (2011) demonstrated that p30 confers a growth advantage to cells that have undergone double-stranded DNA damage. It was shown that p30 interacts with ATM and reduces the levels of ataxia telangiectasia mutated (ATM) and phosphorylated ATM upon double stranded DNA damage. Mass spectrometric studies identified REGγ, a nuclear proteasome activator, as a cellular binding factor of p30 that might target ATM for degradation. This mechanism of p30 targeting ATM to the proteasome for degradation through interaction with REGγ is consistent with co-elution of ATM, p30, and REGγ in the same size exclusion chromatography fractions (Anupam et al., 2011). Lowering the levels of ATM by p30 upon DNA damage would decrease p53-mediated apoptosis thereby facilitating survival of infected cells. Interestingly, the levels of p30 corresponded to the levels of REGγ (Anupam et al., 2011). Another study showed that p30 translocates from the nucleolus to the nucleus upon DNA damage via phosphorylation at threonine 232, which is part of a mitogen-activated protein kinase (MAPK) domain in the carboxy terminus. The expression of p30

inhibits homologous recombination and favors non-homologous recombination in natural and induced DNA damage conditions. p30 inhibits homologous DNA damage by interfering with the assembly of the MRE11/RAD50/NBS1 (MRN) complex by interacting with RAD50 and NBS1. The amino terminal of p28 has a similar MAPK domain except for a proline in the place of threonine. Mutating this proline to threonine to mimic the MAPK domain of p30 did not enable p28 to alter DNA repair (Baydoun et al., 2011). The interaction between p30 and REGγ was confirmed by another study using a similar mass spectrometric technique (Ko et al., 2013). However, this study showed that the ability of p30 to retain *tax/rex* mRNA in the nucleus was dependent on recruiting REGγ to the *tax/rex* mRNA by p30. Further, reduced levels of REGγ resulted in increased HTLV-1 gene expression (Ko et al., 2013). In contrast, another study showed that reduced levels of REGγ had no effect on HTLV-1 gene expression (Doueiri et al., 2012). The discrepancy about the role of p30 and REGγ binding could be explained by the use of different cell lines and techniques.

p30 AND p28 INTERACTOMES

The comparative studies of HTLV-1 and HTLV-2 are useful to highlight the differences that might be responsible for HTLV-1 pathogenesis. Studies investigating host–viral protein interactions have been centered on Tax-1 and Tax-2. Moreover, a large amount of work has focused on p30 because HTLV-1 is pathogenic while similar comparative studies have not been performed on p28. Our group performed a mass spectrometry-based analysis to compare the p30 and p28 interactomes in terms of host–protein interactions. The mass spectrometry results suggested that p30 interacts with a larger and wider range of proteins mainly involved in cell cycle regulation, cell survival, DNA repair, cancer pathways, protein post-translational modifications, and metabolism, whereas p28 interacts with a smaller number of proteins that are involved in mRNA processing and protein post-translational modifications. These data are consistent with the known functions of p30 and p28. Furthermore, we confirmed the interaction between p30 and REGγ and also showed that p28 does not interact with REGγ. Tagging RNA for export is a complex process that involves several proteins involved in splicing such as the SR protein complex and hnRNPs (Caputi and Zahler, 2002; Han et al., 2010; Busch and Hertel, 2012). The exact mechanism utilized by p28 and p30 to retain *tax/rex* mRNA is not known; however, the mass spectrometry analyses identified interactions between p28 and hnRNP H1 and hnRNP F. Conversely, p30 does not have an interaction with hnRNP H1(Doueiri et al., 2012). However, hnRNP K was identified to interact with both p30 and p28. We postulate that p30 and p28 might use these interactions to modulate the role of hnRNPs or other proteins involved in RNA export, which could be a possible mechanism for retention of *tax/rex* mRNA in the nucleus. However the full implication of these interactions warrants further investigation. In addition, a novel interaction between p30 and NEAP interacting protein 30 (NIP30) was also identified. NIP30 is predicted to bind DNA binding/EF hand/Leucine zipper protein (NEFA), which has been shown to localize to the Golgi complex. Preliminary characterization of NIP30 indicated that it is a nuclear protein (Simpson et al., 2000). Since the biological function of NIP30 is not known, further investigation is required to understand the importance of the interaction between p30 and NIP30 (Doueiri et al., 2012).

Interestingly, both p30 and p28 interact with protein arginine methyltransferase 5 (PRMT5). It has been shown that PRMT5 can regulate transcription and also is involved in mRNA processing, mainly splicing (Karkhanis et al., 2011). Moreover, PRMT5 is up-regulated in B cell lymphomas and most transformed cell lines (Wang et al., 2008). The significance of the PRMT5 interaction was investigated by evaluating the effect of PRMT5 knockdown on viral gene expression. Intriguingly, lower levels of PRMT5 significantly reduced HTLV-2 gene expression, whereas no such effect was seen on HTLV-1 gene expression. It remains to be determined whether the p28–PRMT5 interaction regulates HTLV-2 gene expression at the transcriptional or post-transcriptional level. The interaction between p30 and PRMT5 could be important at a stage of viral spread and/or pathogenesis other than gene expression. This study provides a novel hypothesis about the role of p30 and p28 in the different pathological outcomes of HTLV-1 and HTLV-2 (Doueiri et al., 2012). A summary of various molecular process in which HTLV-1 p30 and HTLV-2 p28 are involved is shown in **Figure 2**.

SUMMARY

Human T-cell leukemia virus (HTLV)-1 and HTLV-2 are related retroviruses that have different pathological outcomes. While HTLV-1 infection is linked to cancer, inflammatory and neurodegenerative diseases, HTLV-2 is minimally pathogenic. The differences in the viral oncoprotein Tax-1 and Tax-2 are in part responsible for the distinct outcomes of infection with these viruses. However the absence of Tax expression in most ATL patients and the requirement for the accessory genes in HTLV persistence in *in vivo* models demonstrate a more complex viral regulatory mechanism that allows the virus to infect and persist in the host. HTLVs encode various proteins that affect multiple signaling nodes (Simonis et al., 2012) thereby ensuring tight control of the cellular mechanisms required for infectivity, persistence, and spread, while down-regulating those involved in limiting viral spread. HTLV-1 p30 and its homologue HTLV-2 p28 are accessory proteins required for viral persistence *in vivo*, but are dispensable for *in vitro* viral persistence. p30 and p28 are both post-transcriptional negative regulators of viral replication; they interact with *tax/rex* mRNA and retain it in the nucleus, thereby modulating oncogenic/immunogenic Tax expression allowing the virus to escape immune surveillance while slowly transforming the infected cells. In addition, p30 functions in transcriptional and post-transcriptional mechanisms independent of Tax. p30 causes G2/M cell cycle arrest and delays S phase entry, down-regulates ATM and favors NHEJ, modulates the innate immune response and increases the expression of genes involved in T-cell survival and expansion, while down-regulating genes involved in apoptosis. Interestingly, although the first 50 amino acids of p28 share 77% homology with the last 50 amino acids of p30, this polypeptide domain is devoid of transcriptional activity or effects on the host cell cycle, DNA damage response or immune response. Proteome analysis revealed that

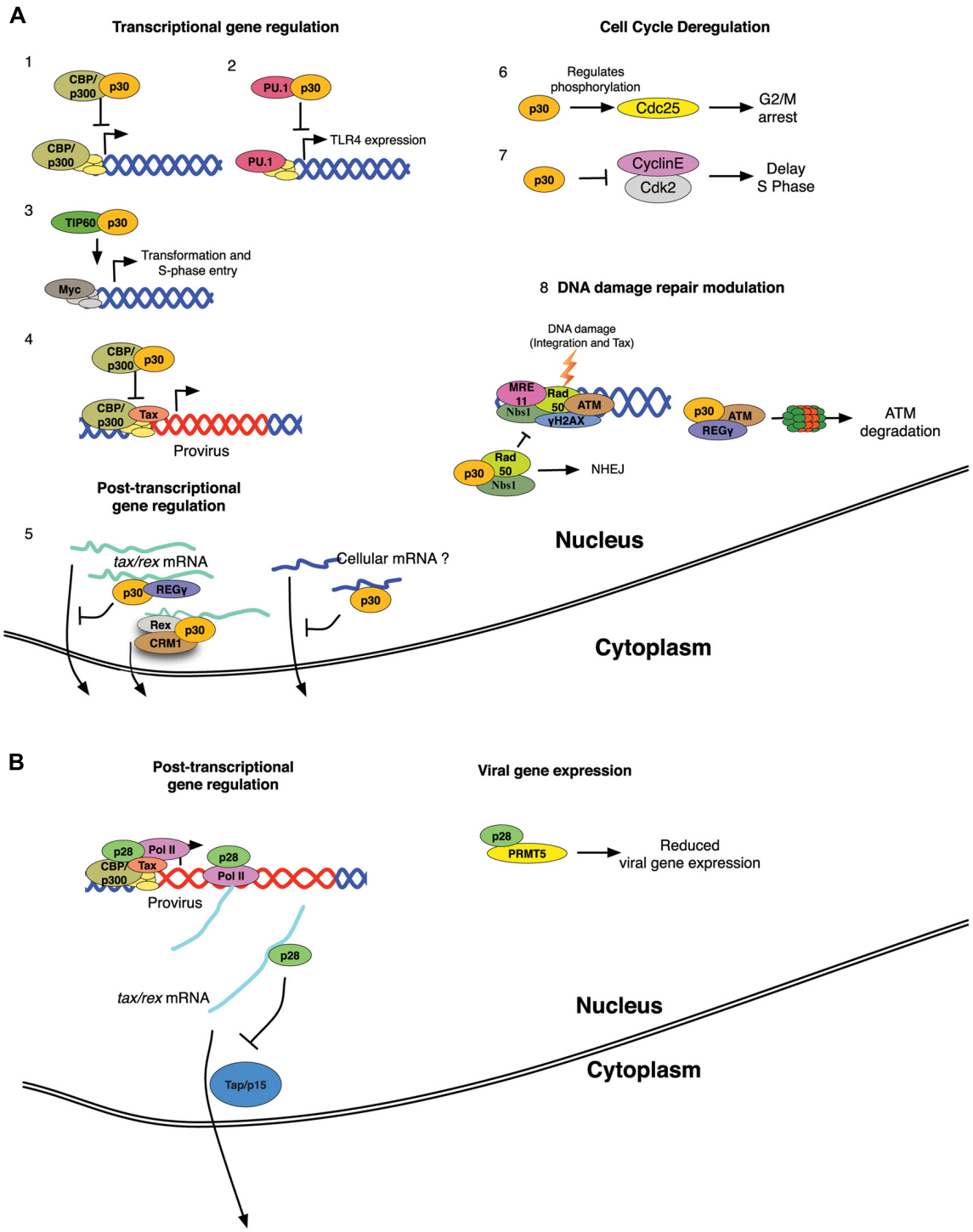

FIGURE 2 | (A) Schematic representation of p30 functions. (1) Interaction of p300 and p30 influences cellular gene expression (2) p30 inhibits PU.1 transcriptional activity, affecting TLR4 expression and ultimately the innate immune response. (3) p30 activates Myc-mediated transcription by recruiting TIP60. (4) p30 disrupts p300 recruitment to the TRE by Tax thereby reducing viral gene transcription. (5) p30 inhibits tax/rex mRNA export possibly through REGγ interaction, Rex counteracts p30 inhibitory function. (6) p30 causes increased phosphorylation in cdc25C on S216 and reduced phosphorylation on S198 leading to G2/M checkpoint activation. (7) p30 disrupts Cyclin E-CDK2 complex formation causing delayed S phase entry. (8) p30 interaction with Regγ down-regulates ATM levels, in addition p30 inhibits MRN complex formation during DNA damage activating NHEJ. **(B)** Schematic representation of p28 functions. p28 is recruited by Tax to the viral promoter where it co-migrates with the transcriptional machinery until the response element is made. p28 interacts with tax/rex mRNA and inhibits its nuclear export by modulating the Tap/p15 pathway. p28 interaction with PRMT5 leads to reduced viral gene expression.

p30 and p28 have distinct interactome profiles even though they both interact with some similar proteins; therefore, p30 and p28 must have divergent functions during the lifecycle of the viruses.

Comparison of the roles of p30 and p28 has provided further insight into HTLV biology. Further studies are needed to determine their distinct roles in viral latency, low penetrance and ultimately the differential pathological outcomes of HTLV-1 and HTLV-2.

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Highlights on distinctive structural and functional properties of HTLV Tax proteins

Maria Grazia Romanelli[1]*, Erica Diani[1], Elisa Bergamo[1], Claudio Casoli[2], Vincenzo Ciminale[3], Françoise Bex[4] and Umberto Bertazzoni[1]

[1] *Department of Life and Reproduction Sciences, University of Verona, Verona, Italy*
[2] *GEMIB laboratory, Center for Medical Research and Molecular Diagnostics, Parma, Italy*
[3] *Department of Surgery, Oncology and Gastroenterology, University of Padua, Padua, Italy*
[4] *Institute for Microbiological Research J-M Wiame, Laboratory of Microbiology, Université Libre de Bruxelles, Brussels, Belgium*

Edited by:
Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:
Jun-ichi Fujisawa, Kansai Medical University, Japan
Makoto Yamagishi, The University of Tokyo, Japan
Yorifumi Satou, Kumamoto University, Japan

****Correspondence:***
Maria Grazia Romanelli, Department of Life and Reproduction Sciences, University of Verona, Strada le Grazie 8, 37134 Verona, Italy
e-mail: mariagrazia.romanelli@univr.it

Human T cell leukemia viruses (HTLVs) are complex human retroviruses of the *Deltaretrovirus* genus. Four types have been identified thus far, with HTLV-1 and HTLV-2 much more prevalent than HTLV-3 or HTLV-4. HTLV-1 and HTLV-2 possess strictly related genomic structures, but differ significantly in pathogenicity, as HTLV-1 is the causative agent of adult T cell leukemia and of HTLV-associated myelopathy/tropical spastic paraparesis, whereas HTLV-2 is not associated with neoplasia. HTLVs code for a protein named Tax that is responsible for enhancing viral expression and drives cell transformation. Much effort has been invested to dissect the impact of Tax on signal transduction pathways and to identify functional differences between the HTLV Tax proteins that may explain the distinct oncogenic potential of HTLV-1 and HTLV-2. This review summarizes our current knowledge of Tax-1 and Tax-2 with emphasis on their structure, role in activation of the NF-κB (nuclear factor kappa-B) pathway, and interactions with host factors.

Keywords: HTLV, Tax proteins, signal transduction, NF-κB

INTRODUCTION

The Human T cell leukemia viruses (HTLVs) are complex retroviruses, belonging to the primate T-lymphotropic virus (PTLV) family. HTLVs are classified as Deltaretroviruses, together with bovine leukemia virus (BLV) and simian T-lymphotropic viruses (STLVs). HTLV-1 was originally described in 1980 (Poiesz et al., 1980) and was the first oncogenic retrovirus discovered in humans (reviewed by Gallo, 2011; Currer et al., 2012). HTLVs originated in Africa around 30,000–40,000 years ago through cross-species transmission of STLVs from monkeys to man. The virus evolved to HTLV and spread to different geographic regions with human migration (Van Dooren et al., 2001). STLVs with high homology to HTLVs are still present in Africa (Hajj et al., 2012). HTLVs are transmitted both vertically and horizontally (reviewed in Watanabe, 2011; Yasunaga and Matsuoka, 2011; Lairmore et al., 2012) but cell-to-cell transmission is essential and occurs through direct contact through the formation of a virological synapse (Nejmeddine et al., 2005; Asquith et al., 2007; Majorovits et al., 2008).

HTLV-1 has received much scientific attention due to its ability to transform primary human T-lymphocytes in cell culture and its association with a neoplasia and a neuropathology (Matsuoka and Jeang, 2007). The most important HTLV-1-associated diseases are the adult T cell leukemia (ATL), a very aggressive form of leukemia, and the HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP), a neurological demyelinating disease (Osame et al., 1986; Bangham and Osame, 2005; Yoshida, 2010; Gessain and Mahieux, 2012; Yamagishi and Watanabe, 2012). Three additional genotypes of HTLV, named HTLV-2, HTLV-3, and HTLV-4, have been isolated and characterized, with HTLV-2 the most common after HTLV-1 (Manns and Blattner, 1991; Mahieux and Gessain, 2009). Although HTLVs share a similar genomic structure, HTLV-2 is much less pathogenic than HTLV-1 since it does not cause neoplastic disorders and is sporadically associated with cases of subacute myelopathy (Feuer and Green, 2005). Understanding the molecular basis of the different pathogenicity of HTLV-1 and HTLV-2 may thus provide important clues to the molecular mechanisms of cancer. All HTLVs possess an open reading frame (ORF) encoding the Tax transactivator, which is essential for proviral gene expression from the viral long terminal repeat (LTR) promoter and also regulates the expression and function of a number of cellular genes and proteins (Feuer and Green, 2005; Wycuff and Marriott, 2005; Calattini et al., 2006; Chevalier et al., 2006). Tax alone is capable of modulating several pathways by activating the transcription factors nuclear factor kappa-B (NF-κB) and cyclic AMP responsive binding protein (CREB; Currer et al., 2012; Tang et al., 2013a). Tax also interacts with proteins controlling cell cycle checkpoints (Akagi et al., 1996; Chlichlia and Khazaie, 2010). HTLV-1 Tax (Tax-1) is necessary and sufficient for T cell immortalization (Akagi et al., 1995) and an ATL-like syndrome has been observed in transgenic mice expressing Tax in the T cell compartment (Ohsugi, 2013). It is noteworthy to mention that in ATL patients, Tax expression is silenced in about 50% of the patients. This observation, along with the fact that Tax is capable of transforming primary T lymphocytes *in vitro*, suggests that Tax might be important for establishing the leukemic phenotype of ATL, but may become dispensable for its maintenance. In addition, recent studies suggest that other viral gene products may play relevant roles in HTLV-1-mediated transformation and may be responsible of the different HTLV-1 and HTLV-2

pathogenicity, including the antisense HTLV-1 genome transcript HBZ (Matsuoka and Jeang, 2011; Lairmore et al., 2012; Satou and Matsuoka, 2012; Zhao and Matsuoka, 2012), the HTLV-1 accessory protein p12 (Kannian and Green, 2010) and p13 (Silic-Benussi et al., 2010a,b).

Tax-1 has been very extensively studied and its interactome includes more than 100 proteins (for a recent review see Currer et al., 2012). To further clarify this point and to better understand the reasons for the difference in pathogenicity between HTLV-1 and HTLV-2 as well as from other HTLVs, in this review we have focused on the structural and functional properties of the different Tax proteins and their relations with other viral and cellular factors. The following sections highlight recent advances in the comprehension of: (i) the transformation potential of Tax-1, Tax-2, Tax-3, and Tax-4 proteins; (ii) Tax's role in the deregulation of signal transduction focusing on studies which describe novel interactions of Tax proteins with host factors and contribute to the understanding of the molecular mechanisms of cell response to viral infection; and (iii) Tax-mediated activation of the NF-κB pathway focusing on differences between Tax-1 and Tax-2 involvement in canonical and non-canonical pathways.

SPECIFIC FEATURES OF DIFFERENT HTLV GENOTYPES

Following the discovery of HTLV-1 in 1980, three additional HTLVs were found: HTLV-2 in 1982 (Kalyanaraman et al., 1982; Vandamme, 2000; Gallo, 2002) and HTLV-3 and HTLV-4 in 2005 (Calattini et al., 2005; Wolfe et al., 2005). These four genotypes show specific geographical areas of distribution. HTLV-1, which includes seven subtypes (HTLV-1A to -1G), is endemic in Japan, sub-Saharan Africa, South America, the Caribbean Islands, and Melanesia. About 5–10 million people worldwide are infected with HTLV-1, most of whom are expected to remain asymptomatic throughout their lifetime (Gessain and Cassar, 2012). An estimated 2–5% of infected people develop clinical complications including ATL, HAM/TSP, infective dermatitis, uveitis, arthritis, and infection by *Strongyloides stercoralis* (Gonçalves et al., 2010). HTLV-2, for which the four subtypes -2A to -2D are known, is endemic within the Amerindian and Pygmy populations, and was found to be epidemic in intravenous drug users (Feuer and Green, 2005). In contrast to HTLV-1, HTLV-2 does not cause proliferative blood diseases. However, HTLV-2 has been linked to neurological disorders, arthritis, pneumonia, and with increased mortality (Araujo and Hall, 2004; Roucoux and Murphy, 2004; Biswas et al., 2010). The two new genotypes, termed HTLV-3 and HTLV-4, were discovered in asymptomatic individuals from Cameroon (Calattini et al., 2005; Wolfe et al., 2005); the pathogenic potential of these viruses is still unknown. HTLV-3 is closely related to the simian virus STLV-3, whereas an STLV corresponding to HTLV-4 has not yet been found (Sintasath et al., 2009).

Comparative studies of the genomic sequences of all four HTLV genotypes have highlighted common as well as unique molecular features. HTLV-1 and HTLV-2 have a similar genomic structure and share approximately 70% nucleotide sequence homology (Feuer and Green, 2005). HTLV-3 and HTLV-4 have a genomic organization which is similar to that of HTLV-1 and HTLV-2 with the presence of *gag, pro, pol*, and *env* ORFs as well as of *tax* and *rex*, whereas ORFs for auxiliary proteins still need to be confirmed

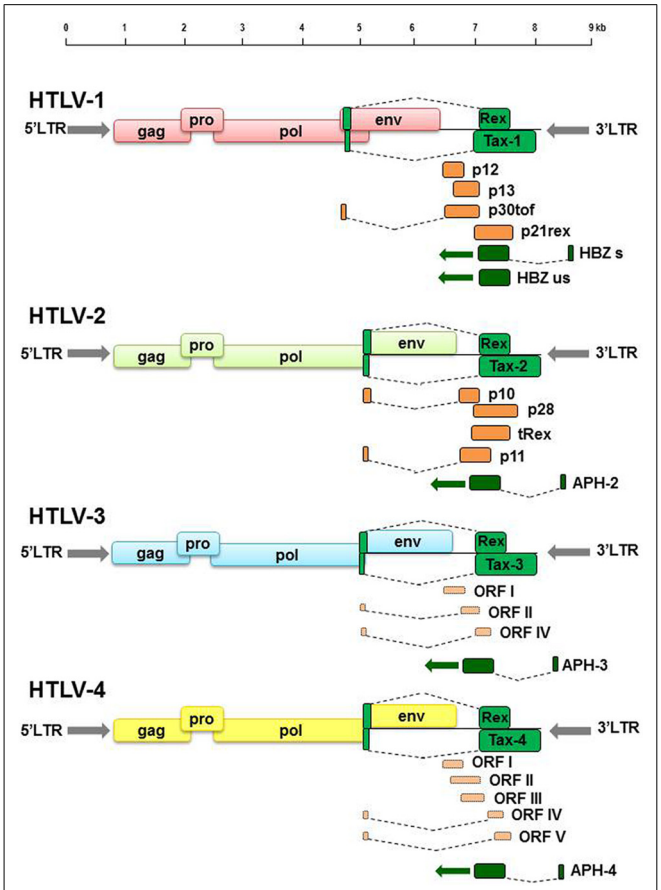

FIGURE 1 | Schematic representation of HTLV-1, HTLV-2, HTLV-3, and HTLV-4 genomic organization. Green colored boxes indicate ORF encoding regulatory proteins. Dark orange colored boxes indicate ORF encoding auxiliary proteins. Light orange colored boxes indicate putative ORF deduced by genomic sequences analyses.

(Gessain et al., 2013; **Figure 1**). HTLV-3 shares about 62% identity with HTLV-1 and HTLV-4 shares 62–71% nucleotide similarity with HTLV-1, HTLV-2, and HTLV-3 (Switzer et al., 2006). HTLV-3 and HTLV-4 present LTRs that lack the distal 21 bp transcription regulatory repeat sequence (Switzer et al., 2006). Both HTLV-3 and HTLV-4 present on the antisense strand a potential ORF named APH-3 and APH-4, respectively (antisense protein of HTLV), analogous to the HBZ gene of HTLV-1 (Larocque et al., 2011) and APH-2 of HTLV-2 (Halin et al., 2009). Sequence alignment indicated that APH-3 and APH-4 are more closely related to APH-2 than to HBZ (Larocque et al., 2011). The proteins also present some differences, as APH-2, APH-3, and APH-4 do not contain a consensus bZIP (basic leucine zipper) domain present in HBZ, and differ in their subcellular localization as compared to HBZ (Halin et al., 2009; Larocque et al., 2011).

COMPARISON OF Tax PROTEINS STRUCTURES

The *tax* gene is encoded within the pX region of HTLV, located between *env* and the 3′-LTR, and is highly conserved among all four genotypes and different subtypes of the virus (Currer et al., 2012). The Tax protein is common to all primate members of

the PTLV family. The primary amino acids (aa) sequence of Tax-1 is composed of 353 residues, and is organized into functional domains that have been extensively investigated (Bertazzoni et al., 2011; Currer et al., 2012). The structural and functional domains of Tax-1, Tax-2, Tax-3, and Tax-4 are shown in **Figure 2**. The N-terminal region of Tax-1 contains a CREB-binding region (Yin et al., 1995) that spans aa 1–60. This region is involved in the interaction with ATF/CREB transcription factors and represents a binding domain required for interaction with proteins involved in transcription, cell cycle progression, and cell signaling regulation (Suzuki et al., 1993a; Goren et al., 1995). All Tax proteins contain the CREB-binding domain within their N-terminus. A nuclear localization signal (NLS) is located within the first 60 aa in Tax-1 (Smith and Greene, 1992), Tax-3 and Tax-4 (Calattini et al., 2006; Switzer et al., 2009). A nuclear localization determinant (NLD) is present within the first 42 aa in Tax-2 (Sheehy et al., 2006; Turci et al., 2006a). An additional localization domain is attributed to Tax-2 at aa position 90–100, which confers to the protein a more abundant accumulation into the cytoplasm as compared to Tax-1 (Meertens et al., 2004a). All four Tax proteins contain a conserved region representing a nuclear export sequence (NES) that has been functionally characterized in both Tax-1 and Tax-2 and is located at aa position 189–202 in Tax-1 (Alefantis et al., 2003; Chevalier et al., 2005). Two leucine zipper-like motif regions (LZRs) are present at aa 116–145 and 213–248 in Tax-1 and conserved in Tax-2, Tax-3, and Tax-4 as well. These regions are required for protein dimerization and binding of cellular factors (Jin and Jeang, 1997; Basbous et al., 2003). Tax-1 and Tax-3 are characterized by the presence of a PDZ-binding motif (PBM) at the C-terminal region (Chevalier et al., 2006) whereas this is missing in Tax-2 and Tax-4. This domain is required for interactions between Tax-1 and cellular factors such as the tumor suppressors hDlg, MAGI-1, and Scribble and the synapse-associated protein DlgI (Rousset et al., 1998; Suzuki et al., 1999; Okajima et al., 2008; Yoshida et al., 2008; Makokha et al., 2013). The absence of the PDZ domain renders Tax-2 unable to interact with these factors. The four subtypes of HTLV-2 (-2A to -2D) code for similar but not identical Tax proteins. Tax-2A is composed of 331 aa and is shorter than Tax-2B, Tax-2C, and Tax-2D (356, 356, and 344 aa, respectively; Feuer and Green, 2005), Tax-2B is the variant that has been studied in greatest detail (Bertazzoni et al., 2011). Tax-1 and Tax-2B share 85% aa similarity, whereas Tax-3 displays 26 and 30% divergence with respect to Tax-1 and Tax-2, respectively. The comparison of Tax-4

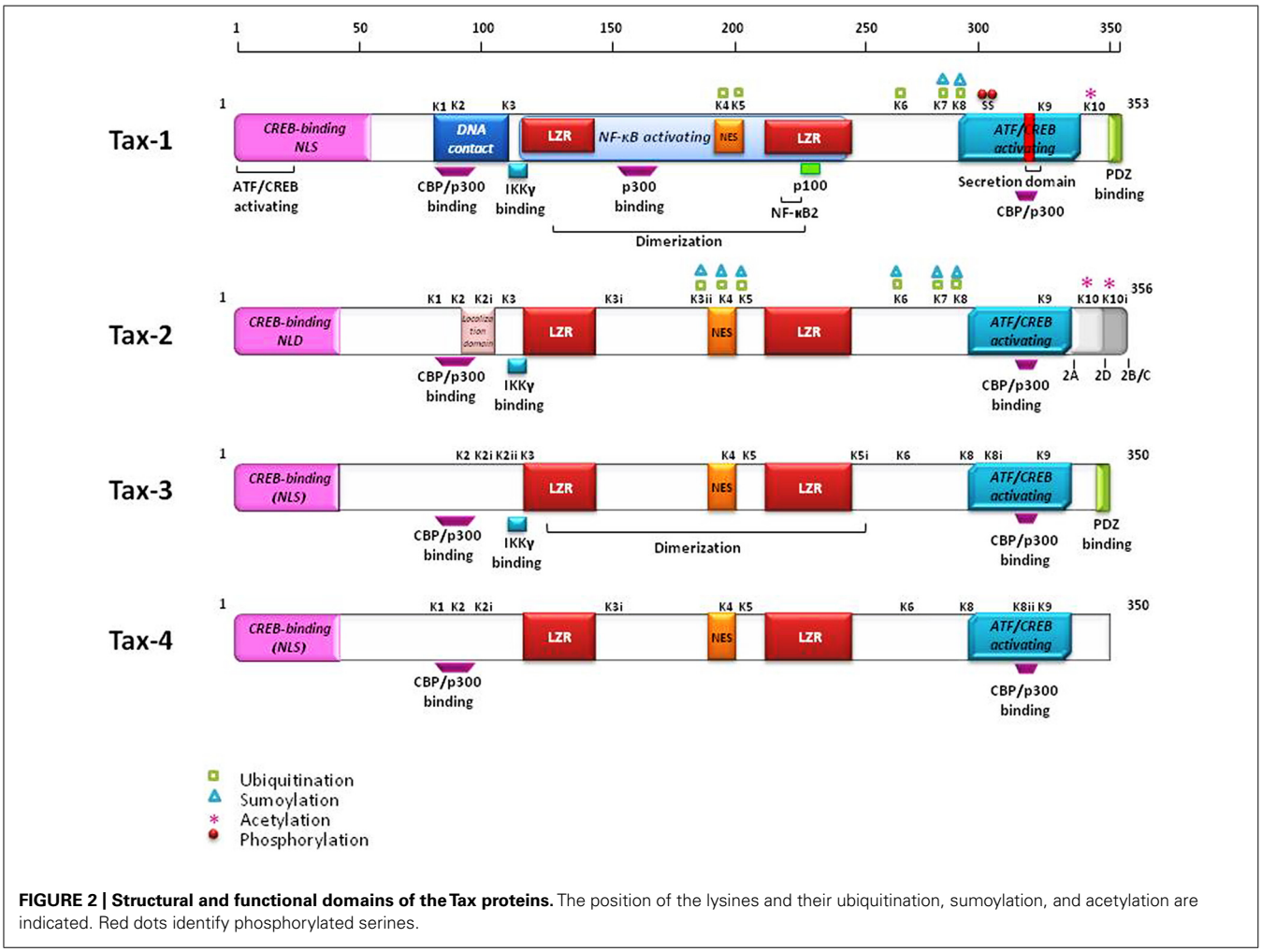

FIGURE 2 | Structural and functional domains of the Tax proteins. The position of the lysines and their ubiquitination, sumoylation, and acetylation are indicated. Red dots identify phosphorylated serines.

with Tax-1, Tax-2, and Tax-3 shows 83, 91, and 85% aa similarity, respectively (Switzer et al., 2009), as outlined in **Figure 3**. The main characteristic that distinguishes Tax-1 from Tax-2 is the presence only in Tax-1 of a motif spanning aa 225–232 that activates the non-canonical NF-κB pathway through interaction with the p100 factor (Shoji et al., 2009). A second relevant difference between Tax-1 and Tax-2 is the presence in Tax-1, but not in Tax-2, of the PBM at the C-terminus (Higuchi and Fujii, 2009; Bertazzoni et al., 2011; Rende et al., 2012). Based on sequence homology, all the Tax proteins possess two functional regions involved in CBP (CREB-binding protein)/p300 binding: a KID-like domain between residues 81 and 95 and a second domain named C-terminal transcriptional activating CR2 domain, between aa 312 and 319. These domains present some differences between Tax-1, Tax-2, Tax-3, and Tax-4. One of the major differences is related to the lysine residue at position 85, which is necessary for Tax-1 to bind CBP/p300 (Hiramatsu and Yoshikura, 1986); this residue is substituted by an arginine in Tax-2, Tax-3, and Tax-4 (Calattini et al., 2006; Chevalier et al., 2006).

Post-translational modifications of Tax-1 and Tax-2 such as phosphorylation, acetylation, ubiquitination, and sumoylation have been extensively described in recent reviews (Bertazzoni et al., 2011; Lodewick et al., 2011; Kfoury et al., 2012). Recent comparative analyses of Tax-1 and Tax-2 post-translational modifications centered on the contribution of ubiquitination and sumoylation to the intracellular localization of Tax and its ability to activate NF-κB (Lamsoul et al., 2005; Kfoury et al., 2006; Nasr et al., 2006; Turci et al., 2009, 2012; Bonnet et al., 2012; Xiao, 2012; Zane and Jeang, 2012; Journo et al., 2013). These studies highlighted the role of specific lysines as targets for sumoylation and ubiquitination in Tax-1 and Tax-2 (lysines K1 to K10 in Tax-1, see **Figure 2**). Lysines K6 and K8 are critical for NF-κB activation and are highly conserved in all Tax proteins. Additional lysines, indicated by additional roman numbers, are present in Tax-2 (K2i, K3i, K3ii, and K10i, **Figure 2**) whereas Tax-3 and Tax-4 contain 11 and 10 lysines, respectively, but not all are conserved at the same position. Alignment of the predicted Tax-4 sequence shows the absence of K10, which is a target of acetylation in Tax-1 and possibly in Tax-2 (Lodewick et al., 2009; Journo et al., 2013).

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Tax-1     MAHFPGFGQSLLFGYPVYVFGDCVQGDWCPISGGLCSARLHRHALLATCPEHQITWDPID 60
Tax-2B    MAHFPGFGQSLLYGYPVYVFGDCVQADWCPVSGGLCSTRLHRHALLATCPEHQLTWDPID 60
Tax-3     MAHFPGFGQSLLYGYPVYVFGDCVQADWCPISGGLCSARLHRHALLATCPEHQITWDPID 60
Tax-4     MAHFPGFGQSLLYGYPVYVFGDCVQADWCPISGGLCSPRLHRHALLATCPEHQITWDPID 60
          ************:************.****:******.***************:******

Tax-1     GRVIGSALQFLIPRLPSFPTQRTSKTLKVLTPPITHTTPNIPPSFLQAMRKYSPFRNGYM 120
Tax-2B    GRVVSSPLQYLIPRLPSFPTQRTAKTLKVLTPPTTPVSPKVPPAFFQSMRKHTPYRNGCL 120
Tax-3     GRVVSSALQYLIPRLPSFPTQRTTRTLKVLTPPTTATTPKVPPSFFHAVKKHTPFRNNCL 120
Tax-4     GRVVGSPLQYLIPRLPSFPTQRTSKTLKVLTPPTTPVTPKVPPSFFQSVRRHSPYRNGCL 120
          ***:.*.**:*************::******** * .:*::**:*::::::::*:**. :

Tax-1     EPTLGQHLPTLSFPDPGLRPQNLYTLWGGSVVCMYLYQLSPPITWPLLPHVIFCHPGQLG 180
Tax-2B    EPTLGDQLPSLAFPEPGLRPQNIYTTWGKTVVCLYLFQLSPPMTWPLIPHVIFCHPRQLG 180
Tax-3     ELTLGEQLPAMSFPDPGLRPQNVYTIWGCSVVCLYLYQLSPPMTWPLIPHVIFCHPEQLG 180
Tax-4     ETTLGEQLPSLAFPEPGLRPQNVYTIWGKTIVCLYIYQLSPPMTWPLIPHVIFCNPRQLG 180
          * ***::**:::**:*******:** ** ::**:*::*****:****:******:* ***

Tax-1     AFLTNVPYKRIEELLYKISLTTGALMILPEDCLPTTLFQPVRAPVTLTAWQNGLLPFHST 240
Tax-2B    AFLTKVPLKRLEELLYKMFLHTGAVIVLPEDDLPTTMFQPVRAPCIQTAWCTGLLPYHSI 240
Tax-3     AFLTRVPTKRLEELLYKIFLSTGAIIILPENCFPTTLFQPTRAPAIQAPWHTGLLPCQKE 240
Tax-4     AFLSNVPPKRLEELLYKLYLHTGAIIILPEDALPTTLFQPVRAPCVQTTWNTGLLPYQPN 240
          ***:.** **:******: * ***:::***: :***:***.***   :.* .**** :

Tax-1     LTTPGLIWTFTDGTPMISGPCPKDGQPSLVLQSSSFIFHKFQTKAYHPSFLLSHGLIQYS 300
Tax-2B    LTTPGLIWTFNDGSPMISGPCPKAGQPSLVVQSSLLIFEKFQTKAFHPSYLLSHQLIQYS 300
Tax-3     IVTPGLIWTFTDGSPMISGPCPKEGQPSLVVQSSTFIFQQFQTKASHPAFLLSHKLIQYS 300
Tax-4     LTTPGLIWTFNDGSPMISGPCPKAGQPSLVVQSSLLIFERFQTKAYHPSYLLSHQLIQYS 300
          :.********.**:********** ******:*** :**.:***** **::**** **:**

Tax-1     SFHNLHLLFEEYTNIPISLLFNEKEADDN-DHEPQISPGGLEPPSEKHFRETEV----- 353
Tax-2B    SFHNLHLLFDEYTNIPVSILFNKGEADDNGDQPPEP---AAQGESSTQKVRPSHTNNPK 356
Tax-3     SFHSLHLLFEEYSTVPFSLLFNEKGANVS-DDEPRG---GPQPPTGGQIAESSV----- 350
Tax-4     SFHHLYLLFDEYTTIPFSLLFKEKEGDDR-DNDPLP---GATASPQGQN---------- 345
          *** *:***:**:.:*.*:**::  .:   *. *     .    .  :
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FIGURE 3 | ClustalW alignments of Tax-1, Tax-2B, Tax-3, and Tax-4 proteins.

Tax is generally defined as a multifunctional protein, since it is able to activate both viral and host gene transcription and to act as mediator of several cellular pathways. Of particular importance was the demonstration that Tax-1 is able to immortalize and transform human primary $CD4^+$ T cells (Grassmann et al., 1992; Franchini, 1995; Yoshida, 2001) and the finding that Tax-1-transgenic mice develop ATL (Hasegawa et al., 2006; Ohsugi, 2013). It is well-established that Tax-1 plays a relevant role in the oncogenesis induced by HTLV infection. Tax-1 acts as a pleiotropic protein conferring proliferative and survival properties to HTLV-1 – infected cells by modulating regulatory factors that induce cell proliferation, cell cycle progression, inhibition of apoptosis, and interference with DNA repair. The main factors include CREB, CBP/p300, NF-κB, cyclin-dependent kinases (CDKs), and Akt (reviewed by Kannian and Green, 2010; Currer et al., 2012).

TRANSFORMATION POTENTIAL OF Tax PROTEINS

Tax-1 is able to transform T-lymphocytes and fibroblasts and to induce tumors in transgenic mice (Azran et al., 2004; Matsuoka and Jeang, 2007; Kannian and Green, 2010; Ohsugi, 2013). The ability to transform primary human T cells was also demonstrated for Tax-2 (Ross et al., 1996; Feuer and Green, 2005). Both proteins are able to inhibit the function of the tumor suppressor p53. A comparative study between Tax-1 and Tax-2 subtypes demonstrated that Tax-2A inhibits p53 function less efficiently than Tax-1 or Tax-2B (Mahieux et al., 2000). Although several *in vitro* studies have investigated the ability of Tax to inactivate p53 (Tabakin-Fix et al., 2006) by acting on the CREB or NF-κB pathway, the mechanism of this inhibition has not yet been completely clarified. A recent study by Zane et al. (2012) identified a cooperative role for the cellular factor Wip1 (wild-type p53-induced phosphatase 1) in Tax-1-mediated inactivation of p53; a subsequent study demonstrated interactions between Tax-1 and Wip1 (Dayaram et al., 2013). In a study that employed transgenic mice expressing Tax-1 which develop mature T cell leukemia and lymphoma, Ohsugi et al. (2013) demonstrated that Tax-1 alters p53 function and that this effect precedes NF-κB activation.

Tax-2 transforms rat fibroblasts less efficiently than Tax-1 (Endo et al., 2002). On the other hand, both viruses immortalizes primary human T cells at a comparable efficiency (Feuer and Green, 2005). Higuchi et al. (2007) demonstrated that the non-canonical NF-κB factor p100 and the PBM present in Tax-1, but not in Tax-2, are essential for the transformation of a T cell. Tsubata et al. (2005) had already shown that the PBM domain is critical for the ability of Tax-1 to induce interleukin-2 (IL-2)-independent growth of the IL-2-dependent T cell line CTLL-2, and that Tax-2 lacks this ability. Xie et al. (2006) showed that the deletion of the PBM in a recombinant HTLV-1 molecular clone (HTLV-1/ΔPBM) alters the requirement for the establishment and maintenance of persistent infection in rabbits. A motif responsible for the distinct transforming activity of Tax-1 and Tax-2 was identified by using a series of Tax-1/Tax-2 chimeric proteins. A region corresponding to aa 225–232 of Tax-1 was shown to play a crucial role in Tax-1's transforming activity, involving stimulation of the non-canonical NF-κB/p100 pathway (Shoji et al., 2009). Imai et al. (2013) recently demonstrated that Tax-2B can immortalize human $CD4^+$ T cells. By infecting peripheral blood mononuclear cells (PBMCs) with lentiviruses encoding Tax-1 or Tax-2B they observed a higher immortalization activity of Tax-2B as compared to Tax-1.

Studies of Tax-2-immortalized T cells demonstrated that Tax-2 causes a dysregulation of autophagy; this may represent a novel survival mechanism in Tax-2-immortalized T cells (Ren et al., 2012). A similar action was attributed to Tax-1, thus suggesting that autophagy may play an important role in the HTLV life cycle (Tang et al., 2013b). Tax-3 was shown to be able to activate the NF-κB pathway and bind CBP in the T cell line CEM, thus suggesting that Tax-3 has *in vitro* transforming activity (Chevalier et al., 2006). The transforming properties of Tax-4 remain to be investigated.

Tax AND SIGNAL TRANSDUCTION DEREGULATION

The role of Tax in HTLV-1-induced oncogenesis has been investigated in large part by analyzing the capacity of Tax-1 to interact with selected cellular factors that play a crucial role in signaling pathways. A list of Tax-interacting proteins is presented in **Table 1**. Tax-1 expression deregulates several signaling pathways involved in the cell cycle, cell proliferation, and cell survival, primarily through the deregulation of two major cellular transcription factor pathways: CREB/ATF and NF-κB (Sun and Yamaoka, 2005; Nyborg et al., 2010). Tax-1 constitutively activates NF-κB by causing a deregulated expression of a variety of cellular genes. Tax-dependent NF-κB activation has been extensively studied and the current state of knowledge will be described in the next section. Tax-1 activation through the cellular transcription factor CREB has been well-characterized at the level of the HTLV-1 promoter located in the LTR region. Within the HTLV-1 promoter three conserved 21 bp repeat enhancer elements called viral CRE elements (vCRE) are present that can be recognized within a complex containing Tax-1 and a phosphorylated form of CREB. The Tax/CREB/vCREB complexes can be associated to other host factors. The best characterized are the cellular coactivators CBP and p300 (Kashanchi and Brady, 2005), which stimulate Tax-mediated transactivation by chromatin remodeling (Nyborg et al., 2010). In addition, Tax-1 interacts with the SWI/SNF chromatin remodeling complexes (Easley et al., 2010) and may be involved in the nucleosome eviction activity mediated by the nucleosome assembly protein 1 (NAP1; Sharma and Nyborg, 2008). Additional host factors that directly interact with Tax-1 and act in the Tax-mediated transactivation are the transducer of regulated CREB (TORC) proteins. TORC-1 and TORC-2 are required for Tax activation whereas TORC-3 enhances Tax-dependent transcription (Koga et al., 2004; Siu et al., 2006). Several cellular factors that interact with Tax and participate to HTLV-1 promoter activation have been identified. The transcriptional activator CIITA affects the functional interaction of the transcription factors CREB, ATF1, and PCAF with Tax-1 (Tosi et al., 2011) and Tax-2 activation of HTLV-2 LTR is strongly inhibited by CIITA (Orlandi et al., 2011). Recently, Tang et al. (2013a) demonstrated that the LKB1 tumor suppressor and the salt inducible kinases (SIKs) act as negative regulatory factors in the activation of HTLV-1 LTR by Tax. They showed that LKB1 and SIK interact with Tax and that this association enables LTR activation by TORCs, CREB, and Tax-1 (Tang et al.,

Table 1 | Tax-1 interacting proteins and deregulated pathways.

Pathways	Factors	Reference
G proteins	Gβ subunit	Twizere et al. (2007)
	Rho GTPases	Wu et al. (2004)
MAPKs	MEKK1	Yin et al. (1998)
	TAK1	Wu and Sun (2007)
JNK	GPS2	Jin et al. (1997)
AP1	p85α	Peloponese and Jeang (2006)
TGFβ	Smad2	Mori et al. (2001)
	Smad3	
	Smad4	
NF-κB	IKKα	Chu et al. (1998)
	IKKβ	
	IKKγ/NEMO	Harhaj and Sun (1999)
	IkBα	Suzuki et al. (1995)
	IkBγ	Hirai et al. (1994)
	RelA	Suzuki et al. (1994)
	p100	Béraud et al. (1994)
	p50	Suzuki et al. (1993b)
	TAK	Wu and Sun (2007)
	TRAF6	Journo et al. (2013)
	NRP/optineurin	Journo et al. (2009)
	USP20	Yasunaga et al. (2011)
	TAX1BP1	Journo et al. (2009)
CREB	CBP/p300	Kwok et al. (1996)
	CREM	Suzuki et al. (1993a)
	ATF-4	Reddy et al. (1997)
	XBP-1	Ku et al. (2008)
	TORC	Koga et al. (2004)
	LKB1	Tang et al. (2013a)
	SIK1	Tang et al. (2013b)
	Paks	Chan et al. (2013)
	CIITA	Tosi et al. (2011)
SW1/SNF	PBAF	Easley et al. (2010)
HDAC	CBP/p300	Ego et al. (2002)
Histone modification	CARM1/SMYD3	Yamamoto et al. (2011)
SRF	SRF	Fujii et al. (1992)
	Elk-1	Shuh and Derse (2000)
	SAP-1	
Microtubule formation	TAB2	Yu et al. (2008)
	hsMAD1/TXBP181	Jin et al. (1998)
	Tax1BP2	Ching et al. (2006)
G1/S transition	Cdk4	Haller et al. (2002)
	Cdk6	
DNA repair	DNA-PK	Durkin et al. (2008)
	ATM	Dayaram et al. (2013)
	CHK2	
	Wip1	
PI3K/Akt1 signaling	Beclin1	Cheng et al. (2012)
	PI3KC3	
Tumor suppression	MAGI-1	Makokha et al. (2013)
	Scribble	Okajima et al. (2008)
	hDlg	Suzuki et al. (1999)

2013a). Additional cellular mediators of Tax-induced activation of HTLV-1 LTR belong to the group I p21-activated kinases (Paks) which physically interact with Tax and CREB-regulating transcriptional coactivators to facilitate HTLV-1 transcription (Chan et al., 2013).

The role of Tax-1 in the expression of cellular genes containing CRE elements was demonstrated for several genes involved in cell cycle and proliferation. Recently Kim et al. (2010) demonstrated that Tax-1 deregulates cyclin D1 gene expression thus determining its overexpression. The mechanism requires an enhanced binding between p300 and phosphorylated CREB and TORC-2. The interaction of Tax-1 with CREB/ATF factors also represses the expression of several genes, including cyclin A (Kibler and Jeang, 2001), p53 (Mulloy et al., 1998), c-myc (Semmes et al., 1996a), and the *ZNF268* gene, which plays a role in the differentiation of blood cells during development and in the pathogenesis of leukemia (Wang et al., 2008).

The main functional and structural differences between Tax-1, Tax-2, and Tax-3 are presented in **Table 2**. It is evident that the Tax proteins differ not only in their transformation abilities, structural properties, and protein interactions, as described in the previous sections, but also in additional aspects of cellular interactions. Tax-2 is distributed both in the nucleus and in the cytoplasm, showing a more diffuse distribution in the cytoplasm compared to Tax-1 (Turci et al., 2009). We have recently demonstrated that Tax-1 and Tax-2 colocalize with TAB2-containing cytoplasmic structures that include RelA and calreticulin (Avesani et al., 2010). Compared to Tax-1, Tax-2 differs in post-transcriptional modification (Lodewick et al., 2011) and we have shown that, in transfected cells, lysine usage for sumoylation differs between Tax-1 and Tax-2 (Turci et al., 2012). When compared to Tax-1, Tax-2 is less efficient in the induction of micronuclei formation (Semmes et al., 1996b), is unable to suppress multilineage hematopoiesis from $CD34^+$ cells *in vitro* (Tripp et al., 2003) and to direct the lipid raft translocation of IκB kinase alpha (IKKα) and IKKβ in transfected cells and in Tax-2-immortalized primary T cells (Huang et al., 2009).

Recent reports have investigated the different role of Tax-1 and Tax-2 in innate immunity. We have previously demonstrated that HIV-1/HTLV-2 coinfection in drug users is associated to a delayed progression of AIDS (Turci et al., 2006b).

Table 2 | Summary of main functional and structural differences between Tax-1, Tax-2, and Tax-3.

	Tax-1	Tax-2[a]	Tax-3	Reference
Transactivating activity	Higher[b]	Lower[b]	n.d.[c]	Semmes et al. (1996a)
Transformation capacity	Higher	Lower	n.d.	Endo et al. (2002)
Micronuclei formation	+	-	n.d.	Semmes et al. (1996b)
Cell cycle arrest	+	-	n.d.	Tripp et al. (2005)
Hematopoiesis suppression	+	-	n.d.	Tripp et al. (2003)
Reduction of histone gene expression	+	-	n.d.	Harrod et al. (2000); Ego et al. (2002)
Inhibition of p53 functions	Higher	Lower	+	Mahieux et al. (2000); Meertens et al. (2004b), Jeong et al. (2005); Calattini et al. (2006)
Total viral mRNA expression	Higher	Lower	n.d.	Li and Green (2007)
Proinflammatory cytokine expression	Higher	Lower	n.d.	Banerjee et al. (2007)
Presence of PDZ motif	+	-	n.d.	Feuer and Green (2005)
Interaction with PDZ-binding proteins	+	-	+	Higuchi and Fujii (2009)
Interaction with p100	+	-	n.d.	Shoji et al. (2009)
Preferential cellular localization	Nucleus	Cytoplasm	n.d.	Turci et al. (2009)
NF-κB transactivation	+	+	+	Chevalier et al. (2012)
NF-κB transactivation (lipid raft translocation of IKK)	+	-	n.d.	Huang et al. (2009)
In vitro CK2 phosphorylation	+	-	n.d.	Bidoia et al. (2010)
Oligo-sumoylation	+	-	n.d.	Turci et al. (2009)
Nuclear bodies	Larger	Smaller	n.d.	Turci et al. (2009)
Ubiquitination and sumoylation	+	+	n.d.	Turci et al. (2012); Zane et al. (2012)
Nuclear localization	+	+	+	Calattini et al. (2006)
T cell immortalization	+	+	+	Chevalier et al. (2006); Imai et al. (2013)

[a] *The properties of Tax-2 include those described for Tax-2A and/or Tax-2B reported in the literature.*
[b] *Higher and lower refers to a comparison between Tax-1 and Tax-2.*
[c] *n.d.: not determined.*

CC-chemokines are produced spontaneously by T lymphocytes of HIV-1/HTLV-2 coinfected subjects (Lewis et al., 2000). Recently, it has been demonstrated that Tax-1 and Tax-2 induce the expression of the CC-chemokines MIP1-α/CCL 3 MIP-1β/CCL4, and RANTES/CCL5 and that downregulate CCR5 in monocytes and PBMCs (Barrios et al., 2011; Balistrieri et al., 2013). Furthermore, a significant decrease of HIV-1 replication has been reported in cultures of PMBCs infected by HIV-1 and treated with Tax-1 or Tax-2 (Barrios et al., 2013). In this *in vitro* cell system the effect of Tax-2 on HIV-1 replication is higher than that of Tax-1 and the authors suggest that Tax-2 may act as an immunomodulatory protein during HTLV-2 infection.

An emerging role in HTLV-1 pathogenesis is attributed to the antisense protein HBZ which contains a bZIP motif, required to form heterodimers with cellular transcription factors. HBZ inhibits viral and cellular expression by interacting with CREB and additional transcription factors and, in contrast to Tax-1, is consistently expressed in ATL cells (Matsuoka and Green, 2009). Compared to HTLV-1, HTLV-2 expresses an antisense protein named APH-2, which is structurally different from HBZ, lacking the classical bZIP domain. APH-2 is able to interact with CREB and to repress the activation of HTLV-2 gene expression mediated by Tax-2 (Halin et al., 2009). A recent study has shown that APH-2 may interact with Tax-2 and when co-expressed with Tax-2, impairs the ability of Tax-2 to activate AP-1 transcription (Marban et al., 2012). AP-1 pathway involves several factors, including Jun, Fos, Maf, and ATF that act on cell proliferation, apoptosis, and oncogenic transformation (Shaulian and Karin, 2001). The distinct structural and functional diversities of HBZ and APH-2 and their interactions with Tax proteins may be relevant for the different pathogenicity of HTLV-1 and HTLV-2 and the mechanisms need to be further investigated.

Tax AND THE NF-κB PATHWAY

Enhanced NF-κB activation is one of the principal consequences of the expression of Tax in the infected cells. The NF-κB family of inducible transcription factors regulate diverse biological processes, including the growth and survival of both T cells and non-lymphoid cells. Activation of NF-κB transcription factors occurs through two tightly controlled signaling processes known as the canonical and non-canonical NF-κB pathways. The principal activators and regulators of these two pathways are illustrated in **Figure 4**. The canonical pathway is activated by different receptor signals including inflammatory cytokines, genotoxic stress, antigens, and toll like receptors (TLRs), whereas the activation

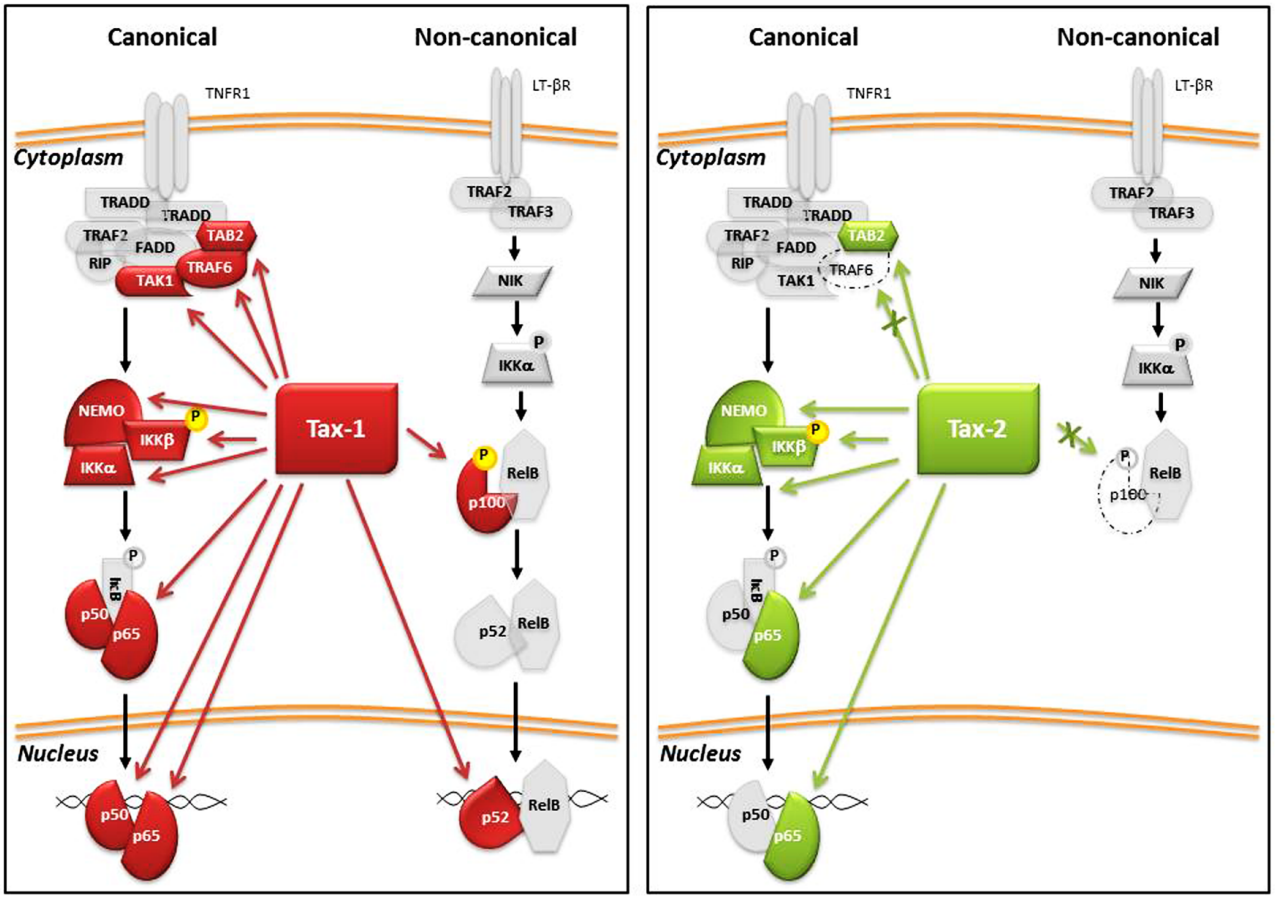

FIGURE 4 | Tax-1 and Tax-2 interactions with factors of NF-κB pathway. Red arrows indicate Tax-1 interaction and the green arrows indicate Tax-2 interactions. Dashed proteins indicate the absence of interaction with Tax-2.

of the non-canonical pathway involves signaling molecules that are recognized only by a specific subset of tumor necrosis factor receptors (TNFRs), such as lymphotoxin-β, BAFF, RANKL, and TWEAK (Sun, 2011). The NF-κB transcription factors family includes five members: RelA/p65, c-Rel, RelB, p50, and p52. The p50 and p52 proteins are expressed as precursor proteins named p105 and p100, respectively. The processing of these precursors to mature forms requires proteasome activity. The five members form dimers with one another and can bind to a variety of target DNA sequences called κB sites to modulate gene expression. p50 and p52 can activate transcription by forming heterodimers with RelA/p65, c-Rel, or RelB. In the cytoplasm, the NF-κB complexes are inactive since they are bound to inhibitory IκB proteins (IκBα, IκBβ, IκBϵ, etc.). Activation of the pathway requires IκB protein degradation and translocation of NF-κB dimers to the nucleus. The common step of activation is mediated by the IKK complex, which phosphorylates IκB and targets it to proteosomal degradation. The IKK complex consists of two active kinases, IKKα and IKKβ, and the regulatory scaffolding protein NEMO (IKKγ). Tax directly interacts with these factors, leading to a persistent activation of NF-κB-mediated transcription. Tax-1 stimulates the activation of both the canonical and non-canonical NF-κB pathway thought the interaction with the IKK factors; the Tax/IKKγ interaction is required for recruiting Tax to the IKK catalytic subunits and for Tax-mediated IKK activation (Sun and Yamaoka, 2005).

Other cellular proteins are important for Tax-mediated NF-κB activation. Tax-1 interacts with NRP/optineurin and TAX1BP1 (Journo et al., 2009; Shembade et al., 2011), and with the ubiquitin-specific peptidase USP20 (Yasunaga et al., 2011). A recent study demonstrated that Tax-1 promotes Bcl-3 expression and nuclear translocation of RelA/p65 (Gao et al., 2013). Kim et al. (2008) provided evidence that Tax-1 induces Bcl-3 expression primarily through activation of the NF-κB pathway. Another recent study has demonstrated that Tax-1 transactivates CD69, a marker of early activation of lymphocytes, through both NF-κB and CREB signaling pathways (Ishikawa et al., 2013).

Tax-1-mediated activation of the non-canonical NF-κB pathway is important in virus-induced tumorigenesis. A region of Tax-1 spanning aa 225–232 is essential for activation of the non-canonical pathway. In contrast to Tax-1, Tax-2 is not able to activate the non-canonical pathway and does not interact with or induce processing of p100 into p52 (Higuchi et al., 2007; Shoji et al., 2009). Furthermore, Tax-2 has been demonstrated to not

be able to interact with TRAF6, a protein with E3 ligase activity that, in the presence of Tax-1, positively regulates the activation of NF-κB pathway (Journo et al., 2013).

Ubiquitination and sumoylation of Tax-1 and Tax-2 are involved in NF-κB activation (Lamsoul et al., 2005; Kfoury et al., 2006; Nasr et al., 2006; Turci et al., 2009, 2012; Journo et al., 2013). Both Tax proteins co-immunoprecipitate and colocalize with IKKγ/NEMO, TAB2, and RelA/p65 in transfected cells (Meertens et al., 2004a; Sun and Yamaoka, 2005; Avesani et al., 2010). Expression of Tax proteins induces IKKα and RelA/p65 nuclear translocation (Higuchi et al., 2007; Ho et al., 2012). An overactivation of NF-κB by Tax induces cellular senescence (Zhi et al., 2011). By knockdown experiments, Ho et al. (2012) demonstrated that chronic activation of NF-κB by Tax-1 results in rapid senescence (Tax-induced rapid senescence, Tax-IRS) that is dependent on IKKα and p65/RelA activation. The Tax-IRS phenomenon constitutes a host checkpoint response to the overactivation of NF-κB that prevents cellular transformation (Zhi et al., 2011; Ho et al., 2012) and represents an interesting mechanism of host cell protection from the deregulating activities of viral proteins.

CONCLUSION

HTLV-1 and HTLV-2 can efficiently transform T-lymphocytes, but only HTLV-1 causes ATL. Although additional viral products play important roles in the HTLV pathogenesis, Tax represents a key factor in the early stage of T cell oncogenesis. In this review we have dissected the structural and functional features of the HTLV Tax proteins, focusing mainly on Tax-1 and Tax-2. These two proteins share many common properties including the capacity of transforming and immortalizing T cells, of transactivating NF-κB pathway and being modified by both ubiquitination and sumoylation. They significantly differ for the presence of a PDZ motif, which is missing in Tax-2, and for the activation of non-canonical NF-κB which is attributed only to Tax-1. The knowledge derived by studying Tax's interactions with cellular factors and their effects on the induction of altered responses in cell pathway regulation confirms the complexity of HTLV oncogenesis. An interesting issue that needs to be explored in the future is the frequent downregulation of the expression of viral genes coded by the plus-strand (including Tax) in circulating leukemic cells from ATL patients. This phenomenon is likely due to epigenetic silencing of the plus-strand promoter, e.g., by methylation and/or expression of repressors of the Polycomb family (Satou and Matsuoka, 2012, 2013; Yamagishi and Watanabe, 2012). A pivotal mechanism that involves a circuit controlled signaling by miRNA has been recently demonstrated by Yamagishi et al. (2012) showing that miR-31 loss, in ATL primary cells, mediated by Polycomb-dependent epigenetic gene silencing, is associated to the overexpression of the NF-κB inducing kinase NIK and leads to constitutive activation of NF-κB oncogenic signaling.

It is likely that further studies aimed at dissecting the functional differences between the Tax proteins will reveal novel functions of host factors that are involved in the signal pathways altered in ATL and may become potential targets for effective therapies against leukemia.

The studies of the differences between Tax-1, Tax-2, Tax-3, and Tax-4 interacting cell factors and transactivating activities will provide useful information to the understating of Tax-1 structural transformation that may open a new approach on HTLV studies based on Tax-1 peculiarities and interactions with addition viral products, that are not present in HTLV-2, HTLV-3, and HTLV-4 Tax proteins.

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Fine tuning of the temporal expression of HTLV-1 and HTLV-2

Ilaria Cavallari[1], Francesca Rende[1], Cecilia Bender[2†], Maria G. Romanelli[2], Donna M. D'Agostino[1] and Vincenzo Ciminale[1,3]*

[1] *Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy*
[2] *Department of Life and Reproduction Science, University of Verona, Verona, Italy*
[3] *Istituto Oncologico Veneto, Istituto di Ricovero e Cura a Carattere Scientifico, Veneto, Italy*

Edited by:
Umberto Bertazzoni, University of Verona, Italy

Reviewed by:
Patrick Green, The Ohio State University, USA
Dustin C. Edwards, National Institutes of Health, USA

****Correspondence:***
Vincenzo Ciminale, Department of Surgery, Oncology and Gastroenterology, University of Padova, Via Gattamelata 64, 35128 Padova, Italy
e-mail: v.ciminale@unipd.it

†Present address:
Cecilia Bender, Istituto Kurz Italia SRL, Parma, Italy

Human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are delta retroviruses that share a common overall genetic organization, splicing pattern, and ability to infect and immortalize T-cells *in vitro*. However, HTLV-1 and HTLV-2 exhibit a clearly distinct pathogenic potential in infected patients. To find clues to the possible viral determinants of the biology of these viruses, recent studies investigated the timing of expression and the intracellular compartmentalization of viral transcripts in *ex-vivo* samples from infected patients. Results of these studies revealed a common overall pattern of expression of HTLV-1 and -2 with a two-phase kinetics of expression and a nuclear accumulation of minus-strand transcripts. Studies in cells transfected with HTLV-1 molecular clones demonstrated the strict Rex-dependency of this "two-phase" kinetics. These studies also highlighted interesting differences in the relative abundance of transcripts encoding the Tax and Rex regulatory proteins, and that of the accessory proteins controlling Rex expression and function, thus suggesting a potential basis for the different pathobiology of the two viruses.

Keywords: HTLV-1, HTLV-2, splicing, Tax, Rex

INTRODUCTION

Human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are genetically related deltaretroviruses (Lairmore and Franchini, 2007). Although both viruses immortalize T-cells in culture and establish a persistent infection *in vivo* (Matsuoka and Jeang, 2007), they differ substantially in terms of pathogenic potential. Unlike HTLV-1, which causes adult T-cell leukemia/lymphoma (ATLL) and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), HTLV-2 has not been linked to lymphoid malignancies. However, HTLV-2 infection is associated with an increase in lymphocytes counts (Bartman et al., 2008) and coinfection with HTLV-2 plays an important role in the progression of HIV-infected patients to AIDS (Casoli et al., 2007).

The two viruses present similar genetic organization and expression strategies (Ciminale et al., 1992, 1995; Koralnik et al., 1992; Cavallari et al., 2011) and share an average 65% identity at the nucleotide level, with higher conservation in the gag, pol, env, and tax/rex genes and lower in the long terminal repeats (LTR), protease and proximal pX region.

The coding potential of the HTLV genomes is greatly enhanced by several gene expression strategies that include ribosomal frameshifting (which generates a Gag-Pro-Pol polyprotein) and alternative splicing (which produces distinct mRNAs coding for Env and non-structural proteins coded by the X region). The pX regions of HTLV-1 and HTLV-2 contain, respectively, four and five major open reading frames (ORFs), termed x-I through x-V. The x-III and x-IV ORFs code for Rex and Tax, which are produced from a dicistronic doubly-spliced mRNA containing exons 1, 2, and 3. Tax and Rex play a critical role in regulating viral expression at the transcriptional and post-translational levels, respectively. Other transcripts produced by the X region code for accessory proteins; these transcripts include singly-spliced mRNAs coding for p21rex, p12, and p13 (HTLV-1), truncated isoforms of Rex (tRex) and p28 (HTLV-2) and doubly-spliced mRNAs coding for p30/tof (HTLV-1), p10 and p11 (HTLV-2) (Ciminale et al., 1992, 1995; Koralnik et al., 1992). While most of the regulatory and accessory proteins of the two viruses share structural and functional homologies, p13 and p8 appear to be unique to HTLV-1 and p11 is unique to HTLV-2. p13 corresponds to the C-terminal 87 amino acids of p30/tof (Koralnik et al., 1992) and is localized mainly in the mitochondrial inner membrane (Ciminale et al., 1999) although it can be partly localized to the nucleus especially when expressed at high levels and in conjunction with Tax (Andresen et al., 2011). p13 increases mitochondrial permeability to K^+ and activates the electron transport chain, resulting in an increased production of mitochondrial reactive oxygen species (ROS) (Silic-Benussi et al., 2009). Increased ROS affect both cell survival and proliferation depending on the cell's inherent ROS set-point. Results of our studies indicated that p13 expression leads to in mitogenic activation of normal resting T-cells, which have low ROS levels; in contrast, p13 induces cell death in cancer cells which are characterized by a high ROS setpoint (Silic-Benussi et al., 2010a,b). p8, which is a cleaved form of p12, traffics to the immunological synapse and favors T-cell anergy. p8 also increases cell-to-cell viral transmission through the formation of intercellular conduits among T-cells (Van Prooyen et al., 2010a,b). p11, which is unique to HTLV-2,

exhibits a partial functional homology to HTLV-1 p12, in that it is able to bind the MHC heavy chain (Johnson et al., 2000).

Recent studies showed that both HTLV-1 and HTLV-2 also produce complementary-strand mRNAs that are transcribed from promoters in the 3′ LTR; these genes were termed, respectively, HBZ (HTLV-1 bZIP factor) (Gaudray et al., 2002) and APH2 (anti-sense protein of HTLV-2) (Halin et al., 2009). HTLV-1 generates two major minus-strand transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006), translated in two proteins that differ by 7 amino acids at their N-terminus (Murata et al., 2006). The negative strand of HTLV-2 generates only one spliced transcript that codes for the APH2 protein (Halin et al., 2009). Both HBZ and APH2 interact with CREB, resulting in the inhibition of Tax-mediated transcription from the 5′LTR (Gaudray et al., 2002; Halin et al., 2009); however, while the hbz mRNA has a growth-promoting effect on T-cells (Satou et al., 2006), no function has been described for the aph2 mRNA. Interestingly, the hbz and aph2 transcripts were detected mainly in the nucleus of infected cells (Rende et al., 2011; Bender et al., 2012), supporting the notion of their possible role as non-coding RNAs.

TEMPORAL ANALYSIS OF HTLV-1 EXPRESSION

An early study by Hidaka et al. (Hidaka et al., 1988) established that HTLV-1 expression is controlled by two key regulatory circuits: a positive feedback provided by the viral transactivator Tax, which drives transcription of the viral genome, and a post-transcriptional regulatory loop provided by Rex, which binds to the Rex-responsive element (RXRE) present at the 3′ end of HTLV-1 transcripts and enhances the nuclear export and expression of a subset of mRNAs coding for the virion-associated proteins Gag-Pol and Env. This study was conducted by transfecting wild type and Rex-mutant proviral clones in fibroblast cell lines followed by isolation of nuclear and cytoplasmic mRNAs and analysis by Northern Blot, a technique that was not capable of distinguishing among different mRNAs of similar size. This approach revealed three major size classes of polyadenylated mRNAs: (i) the 9-kb genomic mRNA coding for Gag-Pro-Pol, (ii) the 4-kb singly spliced mRNA coding for the envelope glycoproteins, (iii) mRNAs of approximately 2.1 kb coding for Tax and Rex (Hidaka et al., 1988). The study did not consider other spliced mRNAs coding for the accessory proteins, as those were discovered several years later. The authors also observed a distinct timing of expression of the transcripts, with the 2.1 kb mRNA preceding the expression of the 9- and 4-kb transcripts, while at a later stage only a trace of the 2.1 kb mRNA, was detectable. Interestingly, a Rex-defective proviral clone only expressed the 2.1 kb mRNAs, demonstrating that Rex is essential for gag and env (but not tax/rex) expression.

A subsequent study by Kimata and Ratner (1991) investigated the temporal regulation of HTLV-1 expression following infection of primary lymphocytes by co-cultivation with the lethally-irradiated HTLV-1-infected cell line MT2. Viral transcripts were detected by RT-PCR using primers spanning the splice-junctions of known viral mRNAs (gag, env, tax/rex). Results showed that tax/rex mRNA appeared at day 4 of co-cultivation, the env mRNA at day 25, and the gag–pol mRNA at day 100 of co-cultivation. The expression of viral transcripts were monitored for 150 days post-infection.

With the aim of investigating the timing of expression of all the transcripts encoded by HTLV-1, Li et al. employed RT-PCR and both an *in vitro* model and a rabbit animal model of HTLV-1 infection (Li et al., 2009). Results showed a general trend toward increased viral gene expression over time both *in vitro* and *in vivo*. However, these studies did not reveal a clear temporal separation between early and late viral transcripts or a switch in the expression of early vs. late viral transcripts as suggested by Hidaka et al. (1988).

To gain further insight into these aspects of HTLV-1 regulation, we recently investigated the timing of spontaneous onset of HTLV-1 expression *in vitro* in primary cells obtained from infected individuals, and measured the levels of individual transcripts by real-time RT-PCR (qRT-PCR) using splice site-specific primers, which allowed measurements of the levels of individual alternatively spliced transcripts. qRT-PCR analysis was also carried out in cells transfected with wild type and Rex-knockout HTLV-1-molecular clones.

We first employed an *ex vivo* viral reactivation model based on the depletion of CD8+ cytotoxic T cells from peripheral blood mononuclear cells (PBMC) of HTLV-1-infected patients, which results in a sharp rise in viral expression (Hanon et al., 2000). Results obtained for 6 TSP/HAM and 3 ATLL patients revealed that the most abundant plus-strand transcripts were tax/rex, gag, and env, followed by p21rex, p30tof, p13, and p12 mRNAs; minus-strand HBZ transcripts were expressed at high levels (Rende et al., 2011).

These studies also revealed a sharp upregulation of viral expression upon *ex vivo* culture which is shown in **Figure 1**, where the time course of expression of all plus-strand viral mRNAs (NCN, Normalized Copy Number) is compared to the NCN of all negative-strand mRNAs (hbz sp1 and us). Results of this analysis in samples examined from the 9 patients revealed 3 main patterns (**Figure 1**). The minus-strand transcripts were slightly up-regulated and their overall NCN values were very similar among the different patients. In contrast, the up-regulation of plus-strand transcripts was more evident and the expression levels of plus-strand transcripts were highly variable in these patients. Patient TSP-4 shows an overall expression of plus-strand NCN of about 2 orders of magnitude greater than TSP1, instead ATLL-3 patient showed a peculiar pattern of expression, with a marked prevalence of minus-strand transcripts compared to plus-strand transcripts (only tax/rex, gag, and env mRNAs were detectable). This patient, whose PBMCs were dominated by monoclonal leukemic cells, might thus represent an example of the subset of ATLL cases in which plus-strand transcription is inhibited by genetic or epigenetic mechanisms (Taniguchi et al., 2005).

The graphs in **Figure 2** illustrate the prevailing pattern of the kinetics of expression of individual HTLV-1 mRNAs revealed by these studies. Tax/rex was the earliest transcript in all patients and p21rex also showed an early peak of expression in the majority of the patients; env, gag, p13, p30tof, and hbz mRNAs revealed a late peak of expression in the majority of the cases. Instead, p12 showed a very variable pattern of expression in the patients. This

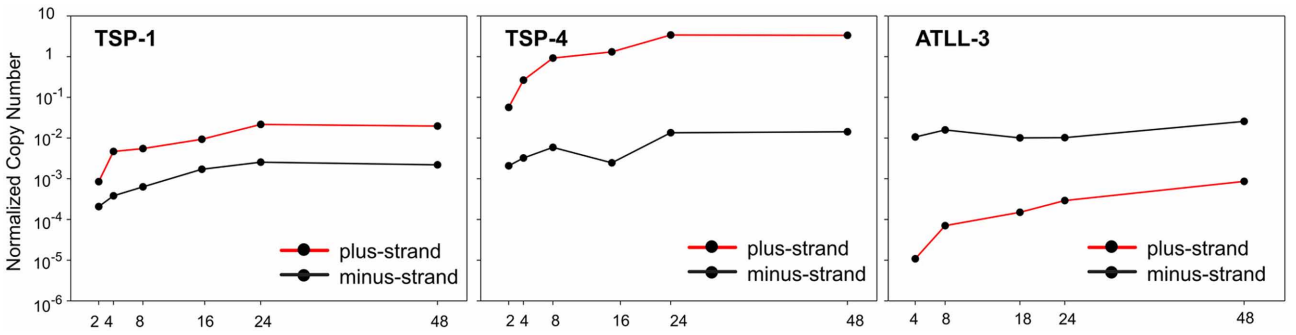

FIGURE 1 | Normalized Copy Number (NCN) of the sum of all plus-strand viral mRNAs (red lines) and of the minus-strand mRNAs, (hbz sp1 and us) (black lines) in PBMCs from 3 infected patients (TSP-1, TSP-4, ATLL-3) over a 48-h time course of culture *in vitro*.

distinct temporal expression pattern is consistent with the tax/rex mRNA acting as an "early" master gene that drives the expression of the other viral transcripts (Rende et al., 2011). Nevertheless, this early-late switch in viral expression is apparently absent in the ATLL-3 patient, while it was evident in all the other patients examined.

This study also tested the HTLV-1 mRNA expression using an *in vitro* model based on the transfection of full-length HTLV-1 molecular clones, which made it possible to investigate the nucleo-cytoplasmic partition of the different transcripts during the time course of expression and to assess the effects of Rex on these processes. Consistent with the results obtained from patient PBMCs, transfection of wild-type HTLV-1 resulted in an "early" nucleo-cytoplasmic export of tax/rex followed by a rise in the export of gag and env mRNAs. Interestingly, this two-phase kinetics was not observed upon transfection of a REX-KO HTLV-1 molecular clone, demonstrating the strict Rex-dependency of this "two-phase" kinetics of expression (Rende et al., 2011). The early expression timing of tax/rex suggests that expression of this transcript is independent of Rex. However, two studies based on the expression of intronless cDNAs derived from the full-length HTLV-1 mature transcripts indicated that Rex may enhance the expression of the tax/rex RNA (D'Agostino et al., 1999; Bai et al., 2012) and binds the RXRE in the context of this transcript (Bai et al., 2012). These apparently conflicting results suggest that the impact of Rex is highly dependent on the processing pathway of the transcripts. It is thus possible that routing the transcripts through the splicing machinery might result in increased efficiency of expression even in the absence of Rex.

TEMPORAL ANALYSIS OF HTLV-2 EXPRESSION

Like HTLV-1, HTLV-2 produces plus- and minus-strand alternatively spliced transcripts that code for virion components and non-structural proteins, including Tax, Rex, and the accessory proteins (Feuer and Green, 2005; Younis and Green, 2005).

The kinetics of expression of the individual HTLV-2 transcripts were described for the first time by Bender et al. (2012). In this paper, the authors investigated the expression kinetics of HTLV-2 mRNAs in a chronically infected cell line and in PBMCs obtained from 3 infected patients using splice site-specific primers and qRT-PCR. The three patients analyzed had similar proviral loads and were HTLV-1- and HIV-1-negative (Bender et al., 2012).

Upon *ex vivo* culture of PBMC the expression of plus-strand transcripts was sharply upregulated in all the examined patients, although the overall expression levels at time zero were highly variable, with patient A showing much lower levels of expression compared to patients B and C (**Figure 3**). On the other hand, the minus-strand transcript (APH2) was transiently upregulated in patient A, not significantly changed in patient B and strongly downregulated (>20-fold) in patient C, who exhibited the highest levels of plus-strand mRNAs expression. The patients thus showed an apparent inverse correlation between changes in minus-strand expression and levels of expression of plus-strand transcripts (**Figure 3**).

Figure 4 shows a synoptic representation of the kinetics of expression of HTLV-2 mRNAs. Patients A and B showed a two-phase kinetics of HTLV-2 gene expression, with an early sharp rise in tax/rex(1-2-3) expression followed by a later expression of gag, env(1-2iso), p28/tRex(1-3), and 1-2-B. One of the two patients also expressed p28/tRex(1-B) as an early transcript, although at a level 1000-fold lower than the "late" 1-3 transcript that also encodes p28 and tRex (Bender et al., 2012). Patient C showed a peculiar kinetics of expression with the APH2 expressed only at the beginning of the time-course followed by a sharp downregulation, while tax/rex(1-2-3), gag, env(1-2iso), and p28/tRex(1-3) were expressed as later transcripts. The p11/p10(1-2-A) mRNA was below the threshold of detection in all patients, suggesting that these proteins might play a marginal role, at least in the context of the infected cells circulating in the peripheral blood.

To verify these findings, we employed an *in vitro* model in which viral expression in the BJAB-Gu HTLV infected cell line was synchronized by controlling the conditions of *in vitro* culture. Diluting a culture of confluent cells, resulted in an overall upregulation of viral gene expression. Results of qRT-PCR confirmed the 2-phase kinetics with the expression of the tax/rex(1-2-3), p28/tRex(1-B), and 1-2-B preceding that of the gag/pol, env, and p28/tRex(1-3) mRNAs.

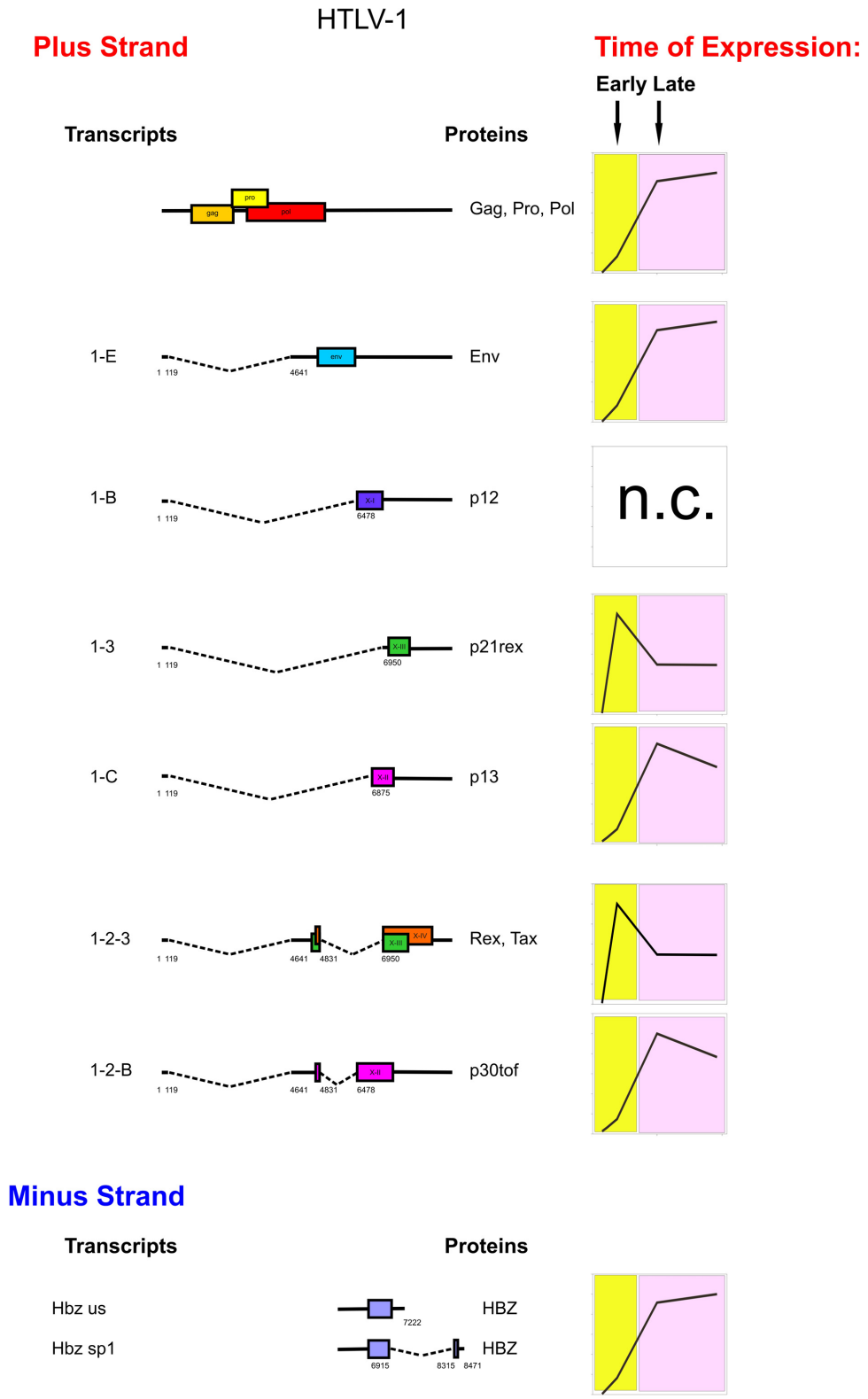

FIGURE 2 | Genetic organization, splicing pattern and kinetics of expression of HTLV-1 mRNAs. On the left, structure and coding potential of HTLV-1 alternatively spliced mRNAs are shown. ORFs are indicated by boxes. Splice sites are indicated by numbers. Right-hand graphs show schematic representations of the prevalent kinetics of expression of HTLV-1 mRNAs in different patients. The p12 mRNA did not show a consistent pattern of expression among the different patients (n.c.).

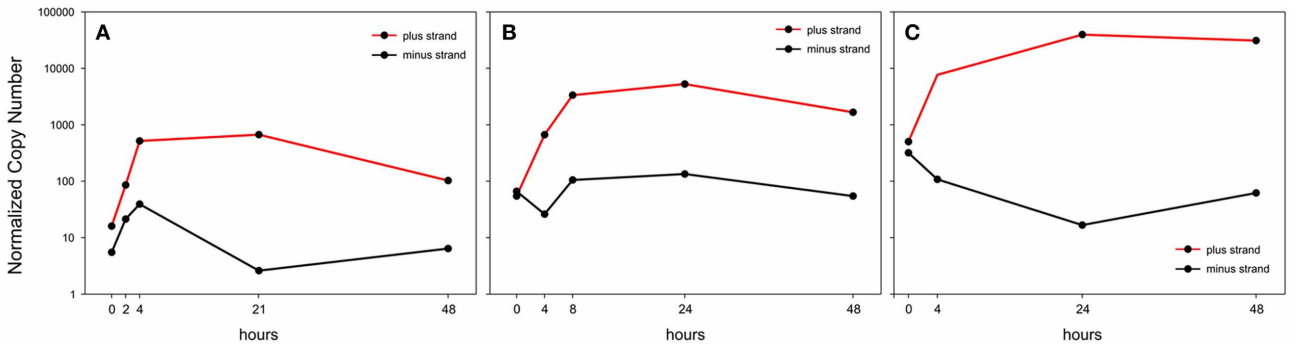

FIGURE 3 | Normalized Copy Number (NCN) of the sum of all plus-strand viral mRNAs (red line) and of the minus-strand mRNA, aph2 (black line) in PBMCs from 3 HTLV-2-infected patients (A,B and C) in a 48-h time course of culture *in vitro*.

MOLECULAR BASIS FOR THE 2-PHASE KINETICS AND REGULATION OF REX FUNCTION BY VIRAL PROTEINS

To find clues to the possible regulatory mechanism underlying the 2-phase kinetics of HTLV-1 expression, we used the expression data to generate a mathematical model (Corradin et al., 2011; Rende et al., 2011). Results underscored the importance of a delay in Rex function compared with Tax in the observed expression kinetics. These considerations led us to investigate the time course of Tax and Rex protein expression from the ACH full-length infectious molecular clone and from a plasmid coding for the mature *tax/rex* mRNA. Flow cytometry analyses showed a relative accumulation of Rex at later time points; consistent with this finding, Rex revealed a slower rate of degradation compared with Tax, suggesting that the activity of these two regulatory proteins might be controlled at the post-translational level. Similar experiments have not yet been carried out for HTLV-2.

A further layer of complexity of the HTLV regulatory networks is revealed by experimental evidence indicating that the function of Rex might be controlled by virus-encoded inhibitors. Among these, truncated forms of Rex lacking the N-terminal, RXRE-binding domain are produced by singly spliced transcripts both in HTLV-1 and HTLV-2 (Ciminale et al., 1996; Heger et al., 1999). These truncated Rex isoform are termed p21Rex (HTLV-1) and tRex (HTLV-2). tRex proteins contain the activation and multimerization domains of full-length Rex and act as inhibitors of full-length Rex-2 (Ciminale et al., 1997), while p21 Rex contains the activation but not the multimerization domains of Rex and its function as a repressor of the full length protein is controversial; some reports suggest that it inhibits Rex (Heger et al., 1999) whereas others do not (Ciminale et al., 1997; Bai et al., 2012).

The x-II ORF products of HTLV-1 (p30/tof) and HTLV-2 (p28) were shown to inhibit both the tax/rex mRNA and the Rex protein (Nicot et al., 2005) (see also the paper by Anupam at al. in this issue).

Interestingly, the relative expression of the tax/rex mRNA compared to the mRNAs coding for potential inhibitors of Tax (p30tof and p28) and Rex (p21rex and tRex) appears to be skewed toward tax/rex in HTLV-1 and toward the inhibitors in HTLV-2 (Rende et al., 2011; Bender et al., 2012). Furthermore, the main trex mRNA was expressed as a "late" transcript in HTLV-2 infected individuals while the mRNA coding for its HTLV-1 ortholog (p21rex) was expressed early in most HTLV-1 infected patients. These findings support the idea that complex feedback regulatory loops control HTLV expression at the post-transcriptional level and suggesting interesting differences in the fine-tuning of Rex function between the two viruses.

Future studies should be aimed at further investigating the functions of these regulatory proteins in HTLV-1 and HTLV-2 and at understanding how they may contribute to the different pathobiology of the two viruses.

CONCLUSIONS AND PERSPECTIVES

The temporal regulation of viral expression has been thoroughly characterized in DNA tumor viruses such as herpes viruses, where different patterns of viral gene expression are associated with latent or productive phases of the viral life cycle and with different diseases [reviewed in Young and Rickinson (2004)]. In the case of EBV, the switch between early and late viral genes is achieved mainly through the genetic and epigenetic regulation of alternative viral promoters. Human papillomaviruses (HPV) exploit a combination of alternative promoter usage, alternative splicing and selection of "early" vs. "late" polyadenylation sites [reviewed in Schwartz (2008)]. The early/late pattern of HPV expression is associated with tissue tropism and restriction of the lytic cycle to differentiated epithelia (Moody and Laimins, 2010).

HTLV-1 and HTLV-2 encode structural, regulatory, and accessory genes from several alternatively spliced mRNAs generated both from the 5′ LTR and 3′ LTR promoters. This complex expression pattern is regulated by two key feedback loops mediated by the two regulatory proteins Tax and Rex which produce a two-phase kinetics in both viruses.

The fact that both HTLV-1 and HTLV-2 show an overall similar two-phase kinetics of expression suggests that this converging expression strategy might reflect important *in vivo* constraints for long term persistence in T-cells and evasion from the immune response which characterize these viruses. On the other hand we also observed some peculiar differences between HTLV-1 and HTLV-2 gene expression, especially concerning the relative expression and timing of the mRNAs coding for proteins that

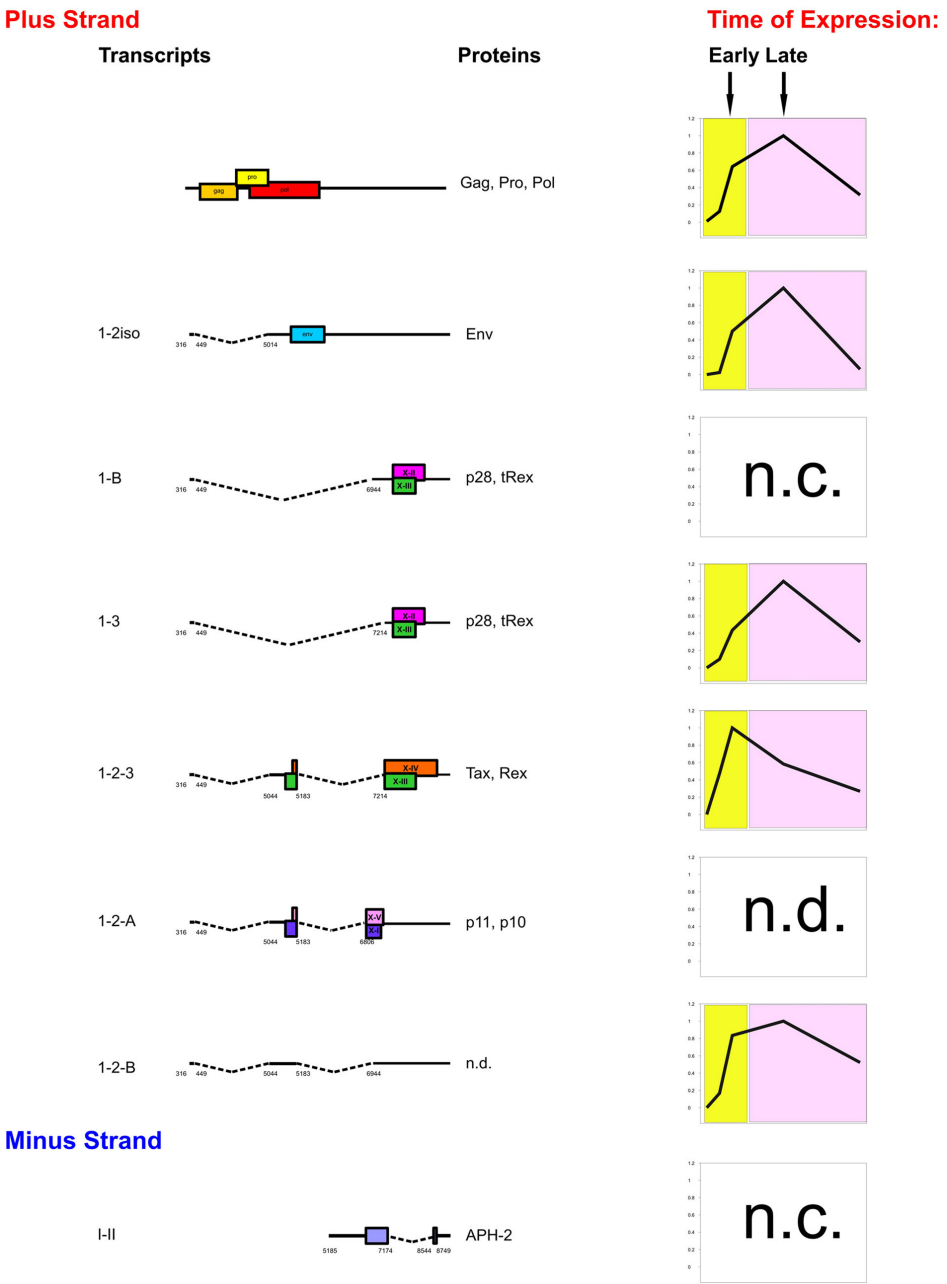

FIGURE 4 | Genetic organization, splicing pattern and kinetics of expression of HTLV-2 mRNAs. The structure and coding potential of HTLV-2 alternatively spliced mRNAs are shown on the left. ORFs are indicated by boxes. Splice sites are indicated by numbers. The graphs depict the prevalent kinetics of expression of HTLV-2 mRNAs in different patients. n.c. indicates transcripts that did not show a consistent pattern of expression in different patients. n.d.: not determined.

control Rex expression and function (see above). These differences might provide the molecular basis for the different tropism and pathogenicity of the two viruses.

However, in contrast to the other tumor viruses mentioned above, neither the levels or the timing of expression of different genes has been linked to a different disease outcome, a topic that should be addressed in future studies. In addition, both the "latent" expression detected in peripheral blood HTLV-infected cells and the "reactivation" upon culture *in vitro* are, at present, poorly understood phenomena. Further investigations are needed to define the pattern (and levels) of HTLV expression in different lymphoid organs.

Interestingly, also in the case of HIV-1, another complex human retrovirus, viral gene expression is controlled by two key regulatory proteins Tat and Rev, which control, respectively, transcription and post-transcriptional processing of alternatively-spliced viral mRNAs.

Early studies suggest that the HIV-1 life cycle is also characterized by a two-phase kinetics with Tat, Rev, and Nef expressed as early genes and Env, Vpu, Vif, Vpr, Gag, Pro, and Pol expressed as late genes. In the context of this expression strategy, Rev acts as a molecular switch controlling the transition between latent and productive infection (Ahmad et al., 1989; Kim et al., 1989). The two-phase kinetics was confirmed by Davis et al. (Davis et al., 1997), using cell-free HIV infection of Hut-78 cells. Using RT-PCR the authors observed the appearance of the tat, rev and nef mRNAs in the cytoplasm 12–16 h after infection, while env and gag expression reached its maximum between 20 and 24 h after infection. In contrast, cell-to-cell transmission of HIV-1 resulted in a late marked increase of env and gag mRNAs without a preceding peak of tat, rev and nef mRNAs.

However, to date, the relative abundance and the time course of expression has not been measured at a single transcript level using qRT-PCR like in the case of HTLV-1 and HTLV-2.

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The MHC-II transactivator CIITA, a restriction factor against oncogenic HTLV-1 and HTLV-2 retroviruses: similarities and differences in the inhibition of Tax-1 and Tax-2 viral transactivators

Greta Forlani, Rawan Abdallah, Roberto S. Accolla* and Giovanna Tosi

Laboratory of General Pathology and Immunology, Department of Surgical and Morphological Sciences, University of Insubria, Varese, Italy

Edited by:
Umberto Bertazzoni, University of Verona, Italy

Reviewed by:
Hideki Hasegawa, National Institute of Infectious Diseases, Japan
David W. Brighty, University of Dundee, UK

****Correspondence:***
Roberto S. Accolla, Laboratory of General Pathology and Immunology, Department of Surgical and Morphological Sciences, University of Insubria, Via Ottorino Rossi 9, 21100 Varese, Italy
e-mail: roberto.accolla@uninsubria.it; accolla.roberto@gmail.com

The activation of $CD4^+$ T helper cells is strictly dependent on the presentation of antigenic peptides by MHC class II (MHC-II) molecules. MHC-II expression is primarily regulated at the transcriptional level by the *AIR-1* gene product CIITA (class II transactivator). Thus, CIITA plays a pivotal role in the triggering of the adaptive immune response against pathogens. Besides this well known function, we recently found that CIITA acts as an endogenous restriction factor against HTLV-1 (human T cell lymphotropic virus type 1) and HTLV-2 oncogenic retroviruses by targeting their viral transactivators Tax-1 and Tax-2, respectively. Here we review our findings on CIITA-mediated inhibition of viral replication and discuss similarities and differences in the molecular mechanisms by which CIITA specifically counteracts the function of Tax-1 and Tax-2 molecules. The dual function of CIITA as a key regulator of adaptive and intrinsic immunity represents a rather unique example of adaptation of host-derived factors against pathogen infections during evolution.

Keywords: restriction factors, CIITA, HTLV-1 Tax-1, HTLV-2 Tax-2, viral replication

INTRODUCTION

Adaptive and innate immune responses represent the most powerful tool used by the host to counteract infectious agents. Additional intrinsic defense systems against viral infections have been recently identified. They include host-encoded restriction factors, initially described for their inhibitory effect on immunodeficiency virus type 1 (HIV-1) infection (reviewed in Wolf and Goff, 2008), such as apolipoprotein B mRNA-editing catalytic polypeptides (APOBECs; Sheehy et al., 2002; Chiu and Greene, 2008; Refsland and Harris, 2013), TRIM (tripartite motif) family members (Stremlau et al., 2004; Ozato et al., 2008; Fletcher and Towers, 2013), tetherin (Neil et al., 2008; Kuhl et al., 2011), and sterile alpha motif (SAM) and HD domain-containing protein 1 (SAMHD1) (Laguette et al., 2011; Sharkey, 2013). Most of these anti-viral proteins were uncovered through the discovery of viral factors that counteract their function, implying that viruses are resistant to the restriction factors of their natural hosts. Although these findings suggested a cross-species restriction, further studies demonstrated that restriction factors may limit pathogenicity *in vivo* even in their specific host (reviewed in Ross, 2009). Besides HIV, the phenomenon of viral restriction has been investigated in other viral infections including oncogenic human T cell lymphotropic virus type 1 (HTLV-1) infection. HTLV-1 was the first human oncogenic retrovirus to be discovered (Poiesz et al., 1980). HTLV-1 is closely related to the less pathogenic HTLV-2 virus. The genomes of these viruses code for similar structural, enzymatic, and regulatory proteins (Franchini, 1995; Nicot et al., 2005). Among them, the transcriptional activators, named Tax-1 (HTLV-1) and Tax-2 (HTLV-2) share roughly 77% amino acid sequence homology and have conserved functional regions. Both viruses infect primarily T lymphocytes, but their infection is associated with different disease manifestations. HTLV-1 is the etiologic agent of an aggressive form of adult T cell leukemia/lymphoma (ATLL), of a neurological disorder designated HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and inflammatory disorders (Yoshida et al., 1982; Uchiyama, 1997; Mahieux and Gessain, 2003). Tax-1 plays a major role in the onset of leukemogenesis by regulating cell cycle progression, cell growth, apoptosis, and DNA repair (Feuer and Green, 2005; Hall and Fujii, 2005; Kashanchi and Brady, 2005; Matsuoka and Jeang, 2007; Yasunaga and Matsuoka, 2011). HTLV-2 has been linked to HAM/TSP "like" cases, whereas no clear epidemiological link to lymphoproliferative malignancies has been demonstrated (Lehky et al., 1996; Roucoux and Murphy, 2004). Comparative studies of Tax-1 and Tax-2 functions brought to light major phenotypic differences in their viral transactivating capacity, transforming activity, modulation of cellular genes expression, and subcellular localization (Semmes et al., 1996; Tanaka et al., 1996; Endo et al., 2002; Sieburg et al., 2004; Shoji et al., 2009; Bertazzoni et al., 2011; Rende et al., 2012; Turci et al., 2012).

Studies on the role of restriction factors in HTLV-1 infection are controversial. HTLV-1 replicates in the same cells as HIV-1 and it does not express an accessory protein analogous to HIV-1 Vif that inactivates hAPOBEC3G. Nevertheless, HTLV-1 seems to be relatively resistant to hAPOBEC3 proteins (Mahieux et al., 2005; Ohsugi and Koito, 2007). Derse et al. (2007) have shown that resistance of HTLV-1 to hAPOBEC3G is mediated by the C-terminus of gag, which seems to exclude hAPOBEC3G

from virions. Other reports have shown that hAPOBEC3G is packaged into HTLV-1 particles, but with opposite effects on virion infectivity (Navarro et al., 2005; Sasada et al., 2005). Interestingly, it has been hypothesized that non-sense mutations in viral genes induced by hAPOBEC3G might allow the virus to escape the host immune response (Fan et al., 2010). Studies related to a possible effect of tetherin on HTLV-1 infectivity indicated that tetherin reduces cell-free infectivity of HTLV-1 with a minor effect on cell-to-cell transmission (Ilinskaya et al., 2013). Finally, evidence of HTLV-1 resistance to SAMHD1-mediated restriction have been recently reported (Gramberg et al., 2013).

Another cellular protein with anti-viral function is the MHC class II (MHC-II) transactivator, also designated CIITA (class II transactivator). The gene encoding CIITA and the elucidation of its function as the master regulator of MHC-II gene transcription and, thus, of antigen presentation to $CD4^+$ T helper cells (TH) were first discovered in our laboratory (Accolla et al., 1986). Upon antigen recognition TH cells coordinate both humoral and cellular immune responses to eradicate pathogen infections and fight tumors (Accolla and Tosi, 2012). This prominent role of CIITA in the homeostasis of the immune system has emerged from the elucidation of the molecular defect at the basis of the bare lymphocyte syndrome (BLS), a severe form of combined immunodeficiency, characterized by the loss of expression of MHC-II molecules (Yang et al., 1988; Steimle et al., 1993; Reith and Mach, 2001). CIITA is a protein of 1130 amino acids localized in both the nucleus and the cytoplasm; it contains four functional domains: the N-terminal transcription activation domain (AD); the proline/serine/threonine-rich region (P/S/T); the GTP-binding domain (GBD), and the C-terminal leucine-rich repeats (LRR) that are critical for the subcellular distribution of the protein (Cressman et al., 2001). The integrity of CIITA domains is critical for the activation function on the MHC-II promoter. CIITA regulates MHC-II gene expression by coordinating sequential steps of the transcription process from the assembly of the general transcriptional machinery and the recruitment of coactivators and chromatin remodeling factors, to the binding of transcription elongation factors (Fontes et al., 1999a). CIITA is recruited to MHC-II promoters via the interaction with DNA-bound factors including the regulatory factor X (RFX) complex and the trimeric NF-Y complex (Caretti et al., 2000; De Sandro et al., 2000; Masternak et al., 2000; Zhu et al., 2000; Jabrane-Ferrat et al., 2002, 2003). Both constitutive and IFNγ-inducible expression of MHC-II is controlled by CIITA, whose gene is regulated at transcriptional level by three distinct promoters driving CIITA expression in different cell lineages (Reith et al., 2005).

Several years ago, we discovered that CIITA restricts HIV-1 infection by acting at the level of viral replication. The molecular mechanism at the basis of this inhibition is the competition between CIITA and HIV-1 Tat transactivator for the cyclin T1 subunit of the elongation complex P-TEFb (positive transcription elongation factor b; Accolla et al., 2002). In this review we summarize our knowledge of the role of CIITA as a restriction factor for HTLV-1 and HTLV-2 viruses. We discuss the results on the inhibition of Tax-1 and Tax-2 functions by CIITA and concentrate on novel insights into the mechanisms through which CIITA operates this suppressive function.

CIITA INHIBITS BOTH HTLV-1 AND HTLV-2 VIRAL REPLICATION

Beside inhibiting HIV-1 transcriptional elongation, CIITA inhibits also the replication of HTLV-1 and HTLV-2. As far as HTLV-1, we demonstrated that exogenously expressed CIITA in 293T cells transfected with the HTLV-1 molecular clone pACH resulted in strong inhibition of HTLV-1 virus production. More importantly, in promonocytic cells endogenously expressed CIITA produced the same effect (Tosi et al., 2011). Two phenotypically and functionally distinct clones of the promonocytic U937 cell line, named *Minus* and *Plus*, previously characterized for their inefficient or efficient capacity to support productive HIV-1 infection, respectively (Franzoso et al., 1994), were studied. Interestingly, we found that the *Minus* clone expresses CIITA and MHC-II, whereas the *Plus* clone does not express either. *Minus* and *Plus* clones were transfected with the pACH plasmid and assessed for viral expression by measuring p19 antigen in the cell supernatants. Remarkably, and similarly to what observed with the HIV-1 infection, we found that the two clones had a different behavior with respect to HTLV-1 infection. The p19 levels were drastically reduced in the supernatants of CIITA-positive *Minus* clone, compared with the CIITA-negative *Plus* clone. Moreover, the stable expression of CIITA in the *Plus* clone after transfection with CIITA cDNA, reverted its permissive phenotype to the *Minus*-like non-permissive one (**Figure 1A**), demonstrating that CIITA is a major restriction factor for HTLV-1. As far as HTLV-2 infection, we found that cells of both the T- and B-lineage are less permissive to HTLV-2 replication in the presence of CIITA. In particular, by using the isogenic B cell system, consisting of CIITA-positive Raji cells and its CIITA-negative derivative RJ.2.2.5 (Accolla, 1983), it was found that RJ.2.2.5 sustained very high levels of virus replication, whereas a profound inhibition of viral replication was observed in Raji parental cells, although both cell lines were equally infected by HTLV-2. Thus, physiologic levels of CIITA were able to strongly inhibit HTLV-2 expression. Consistent with this observation, the permissive RJ2.2.5 cells stably transfected with CIITA cDNA became refractory to HTLV-2 replication showing almost undetectable levels of p19 antigen upon viral infection (Casoli et al., 2004, **Figure 1B**).

Overall, our observations indicated that physiologic amounts of CIITA may inhibit viral expression in cells that are natural target of HTLV-1 and HTLV-2 infection, suggesting that *in vivo* the virus may replicate preferentially in cells lacking CIITA. In this regard, it is interesting that during dendritic cell (DC) maturation induced by different stimuli (LPS, CD40L, Sendai virus, *Salmonella* typhimurium, IFNα, and TNFα), the expression of MHC-II molecules is increased due to an enhanced transport of preformed molecules to the cell surface. In contrast, *de novo* biosynthesis of MHC-II mRNA is shut off because of the epigenetic silencing of CIITA gene (Landmann et al., 2001). Thus, it is conceivable that a similar CIITA silencing might occur in DC infected by HTLV-1 allowing viral replication and spreading to $CD4^+$ T cells (Jones et al., 2008).

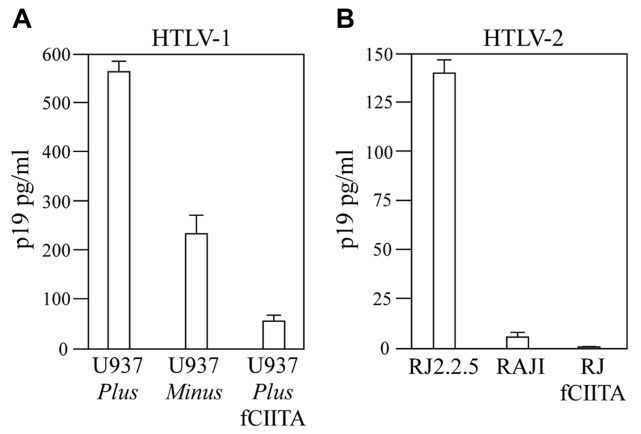

FIGURE 1 | Endogenous CIITA inhibits both HTLV-1 and HTLV-2 gene expression. (A) CIITA-negative U937 *plus* clone, CIITA-positive U937 *minus* clone, and U937 *plus* clone stably expressing fCIITA (U937 *plus* fCIITA) were transfected with the pACH plasmid containing the entire HTLV-1 genome. The amount of HTLV-1 p19 antigen (pg/ml) in cells supernatants was detected by enzyme-linked immunoassay (ELISA) 48 h post-transfection. Error bars indicate standard deviations. Derived from Tosi et al. (2011). **(B)** CIITA-positive Raji cells, their CIITA-negative isogenic mutant RJ2.2.5, and the RJfCIITA cells stably transfected with CIITA were infected with the HTLV-2 Gu strain 2b and the productive infection was evaluated by the presence of HTLV-2 p19 antigen (pg/ml) in cell culture supernatants measured by (ELISA). Error bars indicate standard deviations. Derived from Casoli et al. (2004).

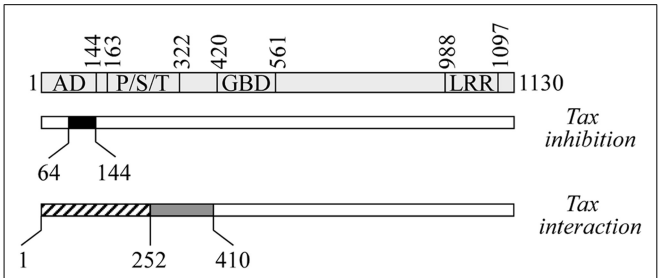

FIGURE 2 | Schematic representation of CIITA regions involved in the suppression of Tax function and in CIITA-Tax association. At the top is a diagram of CIITA with its domains: AD, activation domain; P/S/T, proline/serine/threonine-rich domain; GBD, GTP-binding domain; and LRR, leucine-rich repeats. The black box represents the minimal domain from positions 64–144 that is necessary to block the transcription function of Tax (*Tax-inhibition*, middle). Hatched (positions 1–252) and gray (positions 253–410) boxes represent the two regions of CIITA interacting with Tax (*Tax interaction*, bottom).

CIITA TARGETS Tax-1 AND Tax-2 TRANSACTIVATORS TO INHIBIT VIRAL EXPRESSION

In searching for the molecular mechanisms through which CIITA inhibits HTLV-1 and HTLV-2 viral replication, we found that CIITA targets the viral transactivators Tax-1 and Tax-2. Indeed, we showed that exogenous CIITA could inhibit the Tax-1- and Tax-2-mediated HTLV LTR transactivation in LTR-driven luciferase gene reporter assays (Casoli et al., 2004; Tosi et al., 2006, 2011; Orlandi et al., 2011). By using several CIITA deletion mutants, the N-terminal region 64–144 was found to be minimally necessary to inhibit both Tax-1 and Tax-2 function (**Figure 2**).

Accordingly, HTLV-2 replication was found to be strongly suppressed in RJ.2.2.5 B cells stably transfected with the N-terminal 1–321 fragment of CIITA, which localizes mostly in the nucleus. In contrast, the cytoplasmic mutant CIITA 322–1130, which does not contain the minimal inhibitory domain, did not significantly inhibit HTLV-2 expression (Tosi et al., 2006).

Similarly, cytoplasmic CIITA mutants containing the region 64–144 and partially and /or temporarily accumulating in the nucleus, inhibited Tax-1 transactivation. This was observed with a mutant having the internal deletion of the region 253–410 mediating dimerization of CIITA (Tosi et al., 2002) and accumulating in the nucleus after treatment with Leptomycin B (LMB), an inhibitor of CRM1-mediated nuclear export. Importantly, the study of this mutant in relation to Tax-1 inhibition, provided new insights on the cellular biology of CIITA. For the first time, phosphorylation-dependent dimerization of CIITA has been defined as a critical post-translational modification (PTM) required for CIITA nuclear retention and, thus, transcriptional activity on MHC-II promoters (Tosi et al., 2011). However, because full length CIITA has a dual nuclear and cytoplasmic localization, our findings do not exclude that CIITA might also inhibit Tax in the cytoplasm. Studies are in progress to assess whether cytoplasmic CIITA mutants containing the N-terminal inhibitory region, still inhibit Tax. These experiments will clarify whether CIITA exerts its suppressive function on Tax in both the nucleus and the cytoplasm, potentially revealing a more complex picture, as CIITA might exploit distinct molecular mechanisms to inhibit Tax in the two cellular compartments.

This will be particularly relevant for Tax-2, which, differently from Tax-1, exhibits a predominant cytoplasmic distribution with some accumulation in nuclear bodies (Meertens et al., 2004a; Sheehy et al., 2006; Turci et al., 2009).

A relevant finding of our studies, instrumental in understanding the complex picture of the CIITA-mediated inhibition of Tax-1 and Tax-2 function, was the demonstration of the *in vivo* molecular interaction between CIITA and Tax-1/Tax-2 (Orlandi et al., 2011; Tosi et al., 2011). Since CIITA is localized predominantly in the nucleus but also to a lesser extent in the cytoplasm, one possible scenario is that the cytoplasmic fraction of CIITA could bind Tax itself or a cellular factor crucial for Tax transactivation, inhibiting their nuclear translocation. Intriguingly, our interaction studies revealed that CIITA associates *in vivo* with Tax-1 and Tax-2 by using two adjacent regions at the N-terminus (**Figure 2**). The region 1–252 mediates both the binding to the transactivators and their functional inhibition, whereas the region 253–410 binds to, but does not inhibit Tax proteins (Orlandi et al., 2011; Tosi et al., 2011). We suggested that the two regions form a single Tax-interacting surface in the context of the entire CIITA molecule, but only the presence of the minimal domain 64–144 confers inhibitory properties to this association. Tax-1 region involved in this interaction spans amino acids sequence 1 to 108, including the CREB (cAMP response element-binding protein)-binding domain (Adya and Giam, 1995; Goren et al., 1995; Tosi et al., 2011). In searching for similar or distinct mechanisms of CIITA-mediated inhibition of Tax-1 and Tax-2, future experiments will assess whether CIITA binds the same N-terminal region of Tax-2. Of note, CIITA-Tax interaction was not observed with proteins

produced *in vitro* (data not shown) indicating that a bridging cell factor might play a role in this interaction or that PTM, that do not occur *in vitro*, are crucial to promote the binding. Many studies have shown that sumoylation, ubiquitination, acetylation, and phosphorylation play a critical role in the subcellular localization, protein–protein interaction and function of both Tax-1 and Tax-2, revealing similarities and differences between the two transactivators (Bex et al., 1999; Chiari et al., 2004; Lamsoul et al., 2005; Durkin et al., 2006; Nasr et al., 2006; Gatza et al., 2007; Lodewick et al., 2009; Turci et al., 2009; Bidoia et al., 2010; Bertazzoni et al., 2011; Journo et al., 2013). In addition, dimerization of Tax-1 is necessary for its nuclear localization and interaction with CREB and the 21-bp repeat elements (Tie et al., 1996; Jin and Jeang, 1997; Basbous et al., 2003; Fryrear et al., 2009). Similarly, we previously described that CIITA expressed in cells, but not CIITA produced *in vitro*, forms homodimers in a phosphorylation-dependent manner (Tosi et al., 2002) and this modification is a prerequisite to CIITA nuclear retention (Tosi et al., 2011). Nevertheless, the inability of CIITA to interact with Tax *in vitro* cannot be ascribed to the incapacity of CIITA to self-associate *in vitro*, because, as mentioned above, the dimerization-deficient CIITAΔ253–410 mutant retains the ability to inhibit Tax-1 *in vivo*. Other modifications of CIITA, including acetylation, deacetylation, and ubiquitination (Spilianakis et al., 2000; Wu et al., 2009 and references therein), might have a major role in Tax-1-binding. Such PTMs have been reported to affect the interaction of CIITA with cellular factors involved in MHC-II transcription (Greer et al., 2003) and the recruitment of either corepressors or coactivators on different promoters (Xu et al., 2008; Wu et al., 2009). PTMs-defective forms of both Tax and CIITA will be crucial to determine the potential role of specific modification in Tax-binding and/or functional inhibition. For instance, we have evidence that Tax-1 interacts with both the hypo- and the hyper-phosphorylated forms of CIITA (data not shown).

Overall, our findings suggest that CIITA-mediated inhibition of Tax activity could rely on the physical association between the two factors. We know that this binding occurs off DNA, but it is still unclear whether Tax–CIITA complexes are recruited on the HTLV LTR (**Figure 3A**). If this were case, two hypotheses are equally plausible. Tax bound to CIITA is not assembled on the viral promoter and this correlates with the inhibition of Tax-mediated LTR activation (**Figure 3B**). Alternatively, the binding of CIITA to Tax could still permit its recruitment on the LTR, but not its transcription function. CIITA might prevent the interaction of Tax with components of the transcriptional machinery required for HTLV LTR transactivation (**Figure 3C**). In this context, it is intriguing that, as discussed below, the direct interaction of Tax-1 with PCAF (P300/CBP-associated factor), which cooperates with Tax-1 to activate transcription from the LTR (Jiang et al., 1999), is severely impaired in the presence of CIITA (Tosi et al., 2011). To discriminate between the two above hypotheses, ChIP/EMSA assays are required. However, our subcellular distribution studies of Tax proteins in the presence of CIITA seem to favor the first mechanism. Tax-2 colocalizes with CIITA in the cytoplasm with a characteristic accumulation around the nuclear membrane potentially contributing to Tax-2 loss of function. Interestingly, Tax-2 does not respond by itself to LMB treatment

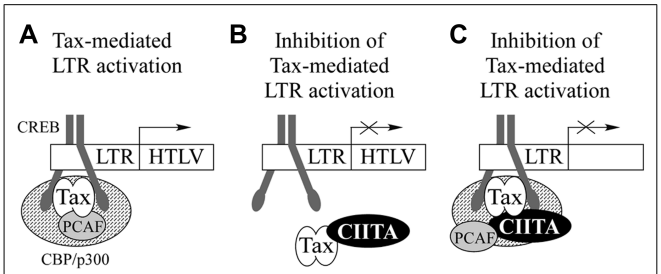

FIGURE 3 | CIITA-Tax interaction might differently affect Tax-mediated activation of the viral LTR promoter. (A) In the absence of CIITA, Tax-1 is recruited by CREB to the viral LTR and promote the formation of an higher order multiprotein complex activating transcription. **(B)** CIITA binds Tax and prevents its recruitment to the LTR promoter. **(C)** Tax bound to CIITA is recruited to the LTR, but its transcription function is impaired.

(Chevalier et al., 2005); however, in cells treated with LMB, CIITA recruits Tax-2 into the nucleus (Orlandi et al., 2011). This suggests that CIITA exerts a driving force on Tax-2 distribution. More recently, extending these studies to Tax-1, we have shown that untagged Tax-1 expressed in 293T cells is trapped by CIITA in the cytoplasm of the majority of cells (Tosi et al., manuscript in preparation). Similarly to Tax-2, the impaired shuttling of Tax-1 into the nucleus, may account for the functional inhibition of Tax-1 by CIITA.

Tax AND CIITA USE COMMON CELLULAR FACTORS TO CONTROL TRANSCRIPTION OF THEIR TARGET PROMOTERS

Tax interacts with a multitude of cellular factors, forming the so called Tax interactome, to modulate the expression of viral and host genes (Boxus et al., 2008; Simonis et al., 2012). Most of these physical and functional interactions derive from studies on HTLV-1 Tax-1. Much less is known about the cellular partners mediating Tax-2 biological functions. Notably, many of these Tax-interacting cellular proteins are used also by CIITA to activate the transcription of MHC-II promoters. These commonly utilized factors include transcriptional modulators, chromatin modifying enzymes, basal transcription factors, and transcription elongation factors (**Table 1**).

In addition, Tax and CIITA share several functional features. Neither are classical DNA-binding transcription factors, but instead interact with a platform of DNA-bound proteins to be recruited to the target promoters. Among them, CREB plays a major role promoting the formation of a multiprotein complex on DNA required for the full transcriptional activation (Kwok et al., 1996; Moreno et al., 1999; Zhu et al., 2000; Lochamy et al., 2007). In particular, Tax interaction with CREB docked at CRE sites of the viral 21-bp repeats, stabilizes the formation of the ternary complex and recruits the coactivators CBP/p300 and PCAF (Harrod et al., 1998, 2000; Kashanchi et al., 1998; Jiang et al., 1999). Another transcription factor that interacts with both Tax and CIITA, is the B subunit of the NF-Y complex, which binds the inverted CCAAT sequence in the Y-box of MHC-II promoters (Pise-Masison et al., 1997; Masternak et al., 2000; Zhu et al., 2000; Jabrane-Ferrat et al., 2003). While many investigations had assessed the contribution of NF-Y to class II transcription, no data regarding the specific role of

Table 1 | Physical and functional interaction shared by Tax and CIITA.

Proteins	Function	Reference
CREB	Transcriptional activators	Kwok et al. (1996), Tosi et al. (2011), Moreno et al. (1999), Lochamy et al. (2007), Zhu et al. (2000).
NF-YB		Pise-Masison et al. (1997), Tosi et al. (2011), Orlandi et al. (2011), Jabrane-Ferrat et al. (2003), Zhu et al. (2000), Masternak et al. (2000).
TFIID	Basal transcription factors	Caron et al. (1993), Fontes et al. (1999a).
CBP, p300	Chromatin remodeling factors	Harrod et al. (1998, 2000), Kashanchi et al. (1998), Tosi et al. (2006, 2011), Kretsovali et al. (1998), Fontes et al. (1999b).
PCAF		Jiang et al. (1999), Tosi et al. (2006), Tosi et al. (2011), Spilianakis et al. (2000).
HDAC1		Ego et al. (2002), Lemasson et al. (2004), Lu et al. (2004), Zika et al. (2003).
BRG1		Wu et al. (2004) Easley et al. (2010), Zhang et al. (2006), Mudhasani and Fontes (2002).
CARM1		Jeong et al. (2006), Zika et al. (2005).
P-TEFb	Transcription elongation factors	Zhou et al. (2006), Cho et al. (2007), Kanazawa et al. (2000).

NF-Y in HTLV transcription were available until recently. We confirmed the association between NF-YB and Tax-1 and extended it to the Tax-2 transactivator, which binds both to transfected and endogenous NF-YB in 293T cells (Orlandi et al., 2011; Tosi et al., 2011). For the first time we have shown that the over-expression of NF-Y significantly inhibited Tax-2-driven, but not Tax-1-driven LTR transactivation (Tosi et al., 2006, 2011). The reasons for this discrepancy are presently unknown and require further investigation. These findings, however, do not conclusively address how endogenous NF-Y might be important for modulating Tax transactivation capacity. It must be stressed that NF-Y is an ubiquitous factor and, seemingly, its physiologic levels do not impair Tax-2 transcriptional activity, as demonstrated by our Tax-dependent gene reporter assays performed in 293T/COS cells and by HTLV-2 productive infection of RJ.2.2.5 cells which constitutively express NF-Y (Tosi et al., 2006, 2011; Orlandi et al., 2011). Nevertheless, it is possible that endogenous NF-Y does not allow maximal Tax-2 transactivation and only its inactivation in cells by the use of dominant-negative NF-Y vectors or by small interfering RNA (siRNA; Mantovani et al., 1994; Dolfini et al., 2009) will provide clear evidence of its negative role in LTR promoter activity.

Several families of proteins binding to the Y-box sequence have been previously identified (Li et al., 1992; Wolffe et al., 1992). Besides NF-YB, two other factors called YB-1 and C/EBPβ have been shown to oppositely regulate HTLV-1 expression. The former increases the basal LTR transcription (Kashanchi et al., 1994), the latter, instead, down-regulates Tax-1-mediated transactivation (Hivin et al., 2004). Thus, distinct family members might exploit alternative mechanisms to modulate HTLV-2 and/or HTLV-1 transcription.

Several chromatin modifying factors, such as Brahma-related gene 1 (BRG1) and histone acetyltransferases (HATs) are commonly used by Tax and CIITA (**Table 1**). While it is well established that the recruitment of HATs to the 21-bp repeats by Tax plays a critical role in transactivation, it is not clear whether the ATP-dependent chromatin remodeling factor BRG1, also participates in Tax-mediated transactivation. Controversial reports have been published in the past on Tax-1–BRG1 functional interplay. In one study BRG1 was shown to interact with Tax-1 physically and functionally and to enhance its capacity to transactivate LTR promoter (Wu et al., 2004; Easley et al., 2010). On the contrary, another report indicated that Tax-1-mediated transactivation does not require BRG1 (Zhang et al., 2006). Interestingly, BRG1 has a dual action on MHC-II gene expression: (i) it induces CIITA pIV promoter activation (Pattenden et al., 2002), (ii) it is recruited by CIITA on MHC-II promoters where, by altering DNA topology, facilitates the access of general transcription factors and coactivators leading to gene expression (Mudhasani and Fontes, 2002). Thus the role of BRG1 in the regulation of HTLV-1 replication in presence of CIITA certainly requires further investigation.

The HATs p300, CBP, and PCAF participate with CIITA to the formation of an active MHC-II enhanceosome (Kretsovali et al., 1998; Fontes et al., 1999b). Moreover, they also catalyze the acetylation of CIITA at two N-terminal lysine residues within a bipartite nuclear localization signal (NLS). Acetylation or inhibition of deacetylation by Trichostatin A leads to increased nuclear levels of CIITA and higher transactivation of class II genes (Spilianakis et al., 2000). Remarkably, CIITA also contains an HAT activity, which is required for IFN-γ-activated MHC-I and MHC-II expression (Raval et al., 2001). While the transcriptional activity of CIITA is linked to the recruitment of HATs on class II promoters, histone deacetylation correlates with transcriptional repression and is mediated by distinct histone deacetylase (HDAC) complexes. HDAC1/HDAC2 stably associated with the mSin3A corepressor bind to CIITA and inhibits its transactivating function through a disruption of MHC-II enhanceosome (Zika et al., 2003). As far as the involvement of HATs in Tax-mediated HTLV transcription, our studies revealed functional differences among the HAT family members interacting with Tax-1 and Tax-2. In particular, we confirmed that HTLV-1 and HTLV-2 gene transcription is synergistically enhanced by the interaction of CBP/p300 with both Tax-1 and Tax-2. In contrast, Tax-1 but not Tax-2 selectively uses PCAF to optimally transactivate HTLV-1 LTR (Tosi et al., 2006, 2011). This effect is independent from the enzymatic activity

of PCAF, which might instead engage other coactivators (Jiang et al., 1999). A selective usage of HATs by the two viral transactivators has been previously demonstrated for the inhibition of p53 by Meertens et al. (2004b). The different requirement for PCAF between Tax-1 and Tax-2 implies that only Tax-1 might affect nuclear PCAF-containing complexes, potentially contributing to the pleiotropic de-regulated expression of cellular genes during T cell transformation. Of note, a reduced transactivation and a defective cellular transformation have been observed with Tax-1 mutants which poorly interact with PCAF (Smith and Green, 1990; Jiang et al., 1999). These observations further support the idea that the higher oncogenic potential of Tax-1 with respect to Tax-2 might be, at least in part, attributed to a peculiar utilization of HATs. Similarly to CIITA, Tax-1 interacts with several HDACs including HDAC1, HDAC3, and HDAC6 (Villanueva et al., 2006; Legros et al., 2011). HDAC1 binding negatively regulates the HTLV-1 gene expression (Ego et al., 2002; Lemasson et al., 2004). Nevertheless, Tax-1 has been shown to replace HDAC1 on LTR promoter allowing transcription initiation (Lu et al., 2004). Thus, both CIITA and Tax may act as a molecular switch, to modulate transcription by coordinating the function of both HATs and HDACs. Besides the roles for HATs and HDAC, more recently arginine-specific methylation of histones has emerged as a critical feature for both MHC-II and HTLV-1 transcriptional regulation. The coactivator-associated arginine methyltransferase 1 (CARM1) has been reported to interact and synergize with both CIITA and Tax-1 to optimally activate transcription of their target genes (Zika et al., 2005; Jeong et al., 2006). Aside from their interactions with specific DNA-bound factors and chromatin modifying proteins, Tax and CIITA bind also component of the general transcriptional machinery, such as TFIID, to direct transcription initiation (Caron et al., 1993; Fontes et al., 1999a). There is also evidence that both transcription factors recruit the P-TEFb to the target promoters by interacting with the cyclin T1 subunit (Kanazawa et al., 2000; Zhou et al., 2006; Cho et al., 2007).

Overall, the findings discussed above highlight the central role of both Tax and CIITA in transcription through the coordination of enhanceosome complex assembly and the control of transcription initiation and elongation.

CIITA EXPLOITS DIFFERENT MOLECULAR MECHANISMS TO INHIBIT THE VIRAL TRANSACTIVATORS Tax-1 AND Tax-2

On the basis of what has been described in the previous section, the hypothesis that the physical interaction between CIITA and Tax may solely account for the observed CIITA-mediated inhibition of Tax-dependent LTR transactivation could reflect only part of the complex molecular interplay between the viral and cellular transactivators. Thus, we hypothesized that CIITA might inhibit Tax-mediated transcription also by sequestering one or more of those factors that are commonly used by CIITA and Tax for their specific transcriptional regulatory functions. This idea was further supported by our previous finding that CIITA inhibits HIV-1 replication by competing with Tat for the binding to cyclin T1 of P-TEFb complex (Accolla et al., 2002). Moreover, it is known that the sequestration of HATs is a common mechanism through which CIITA mediates gene suppression. For instance, CIITA by binding to and sequestering CBP inhibits metalloproteinase-9, collagen α 2(I), thymidine kinase, and cyclin D1 gene expression (Zhu and Ting, 2001; Nozell et al., 2004). In addition, CIITA by competing with NFAT (nuclear factor of activated T-cells) for p300 binding, represses the expression of IL-4 (Sisk et al., 2000). Similarly, CIITA exerts a repressive effect on Cathepsin E expression most likely via interaction with p300 (Yee et al., 2004). For these reasons we assessed whether the over-expression of some of these commonly used factors could rescue Tax function inhibited by CIITA. Whereas the over-expression of HATs did not overcome Tax-2 suppression, the over-expression of PCAF, but not of p300, counteracted the inhibitory function of CIITA on Tax-1 (Tosi et al., 2006, 2011). These data imply that CIITA might inhibit the recruitment of PCAF to the transcriptional complex on the viral promoter simply by sequestering it (**Figure 4A**). Nevertheless, another possibility is that the intrinsic ability of CIITA to interact with Tax-1, could impair Tax-1–PCAF association (**Figure 3C**). Indeed, CIITA decreased the *in vivo* binding of PCAF to Tax-1 (Tosi et al., 2011). CIITA and PCAF bind to two distinct regions of Tax-1 localized at the N-terminus and at the C-terminus, respectively (Jiang et al., 1999; Tosi et al., 2011) indicating that PCAF and CIITA do not compete for the same binding surface of Tax-1. Rather, the binding of CIITA to Tax-1 might alter the conformation of the viral transactivator masking the binding surface to PCAF.

Besides PCAF, the over-expression of CREB and ATF1 transcription factors restored CIITA-inhibited Tax-1 transactivation. Because the N-terminal region of Tax-1 interacting with CIITA includes CREB-binding site (Wu et al., 2004; Tosi et al., 2011), it is not surprising that the two factors compete for Tax-1. In the presence of CIITA, CREB might be no longer available for the recruitment of Tax-1 on the 21-bp repeats, thus preventing the assembly of the multiprotein complex required for optimal HTLV-1 LTR transactivation (**Figure 4A**).

Overall, these results indicate relevant differences in CIITA-mediated suppression of Tax-1 and Tax-2. The sequestration of HATs is not the major mechanism through which CIITA inhibits Tax-2, which, as discussed above, is functionally suppressed by NF-Y, another essential component of MHC-II enhanceosome.

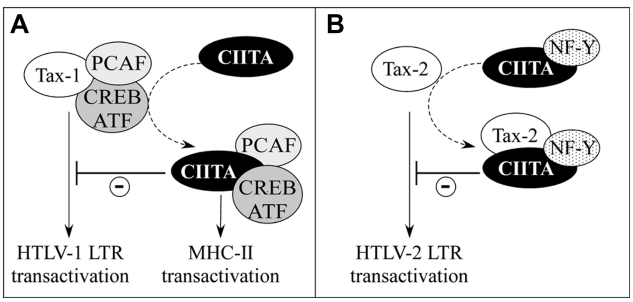

FIGURE 4 | Distinct mechanisms account for CIITA-mediated inhibition of HTLV-1 Tax-1 and HTLV-2 Tax-2. (A) The cellular factors PCAF and CREB/ATF, required for CIITA-mediated MHC-II transcription, are sequestered by CIITA and are no longer available to interact physically and functionally with Tax-1. This results in the inhibition of HTLV-1 LTR transactivation (–). **(B)** CIITA facilitates the interaction between Tax-2 and NF-Y, an inhibitor of Tax-2 transcription function.

Interestingly, NF-Y cooperates with CIITA to inhibit Tax-2 transactivating function suggesting that CIITA might behave as a bridging factor to assemble a defective Tax-2/CIITA/NF-Y transcriptional complex (Tosi et al., 2006; **Figure 4B**). It has been reported that NF-Y interacts with PCAF (Currie, 1998) and it is intriguing that the two factors differently affect the transcriptional function of Tax-1 and Tax-2. Tax-1-mediated LTR transcription is enhanced by PCAF, but is not affected by NF-Y. On the contrary, Tax-2-mediated LTR transcription is inhibited by overexpressed NF-Y, but is not increased by PCAF. In this context CIITA seems to exploit the two players to exert its inhibitory function on the viral transactivators by inhibiting the interaction between Tax-1 and its positive coactivator PCAF, while increasing the binding of Tax-2 to its negative regulator NF-Y (Orlandi et al., 2011).

Future efforts will be devoted to assess whether CIITA inhibits Tax by sequestering other commonly used factors or by inhibiting their physical interaction with Tax-1 and/or Tax-2. At the present we can only exclude competition between CIITA and Tax for cyclin T1-binding. Indeed, the region of CIITA mediating Tat suppression differs from that required to inhibit both Tax-1 and Tax-2, indicating that CIITA blocks HIV-1 and HTLVs through different molecular mechanisms (data not shown).

CONCLUSION

In this review we provided an update on the anti-viral features that make CIITA a fundamental link between adaptive and intrinsic immunity against HTLV infections. By inducing MHC-II expression and, thus, antigen presentation, CIITA triggers the activation of TH cells, which, in turn, orchestrate the adaptive immune responses against pathogens. In addition, CIITA has a direct inhibitory effect on the replication of HTLV-1 and HTLV-2 retroviruses by suppressing the transcriptional function of their viral transactivators. CIITA exploits different ways to exert this latter inhibitory function: it binds Tax-1 and Tax-2; it specifically modulates the interaction of Tax-1 and Tax-2 with relevant cellular factors; and it affects the subcellular localization of the two viral transactivators. Thus, CIITA seems to have evolved multiple strategies to be more effective in the inhibition of Tax function. It is known that other host restriction factors may target different steps of viral life cycle to block HIV-1 infection. Nevertheless, the restriction is more effective in species other than the human species, because HIV-1 has developed countermeasures against these innate defenses. In contrast, CIITA is a peculiar restriction factor because restricts HIV-1, HTLV-1, and HTLV-2 in their natural host (Accolla et al., 2002; Casoli et al., 2004; Tosi et al., 2011). So far, no viral products are known to counteract CIITA-mediated restriction. On the contrary, it is well known that some bacteria (e.g., Mycobacteria and Chlamydia) and some viruses (e.g., human cytomegalovirus and Varicella zoster) cause a reduction of MHC-II molecules on the surface of parasitized cells by inhibiting the pathway leading to the activation of CIITA gene transcription (Accolla et al., 2001). Thus, also HIV-1 and HTLV viruses might have evolved mechanisms to evade the host's immune system based on the suppression of CIITA expression and/or function. At least for HIV-1 infection, our previous results indicated that Tat had no effect on both MHC-II and CIITA expression in T and macrophage cell lines (Tosi et al., 2000). As far as HTLV-1, it has been reported that Tax-1 increases basal MHC-II transcription by interacting with NF-YB (Pise-Masison et al., 1997). Moreover, a putative functional effect of Tax-1 on CIITA-mediated MHC-II gene expression, although unlikely, has not been investigated in detail as yet. Future investigation will thus uncover also the possible complementary role that both Tax-1 and Tax-2 might play a role in CIITA-dependent MHC-II transcription.

HTLV-1 and HTLV-2 viruses preferentially infect and replicate in human T lymphocytes, which express MHC-II molecules upon activation. It must be stressed, however, that whereas the expression of MHC-II molecules on the cell surface last for several days, CIITA expression is time-limited because of its very short half-life (Schnappauf et al., 2003). Thus, sustained expression of CIITA might be required to control HTLV replication. Interestingly, as discussed above, cell surface MHC-II molecule expression in mature DCs is up-regulated, whereas the expression of CIITA is silenced. This might, at least in part, explain why HTLV-1 productive infection is not counteracted in DCs (Jones et al., 2008). These findings disclose the opportunity of developing new therapeutic approaches against HTLV infections based on biological and/or pharmacological strategies aimed at up-regulating, in a controlled manner, the expression of CIITA in cells that are targeted by the virus.

Besides its role as transcriptional activator of HLTV genome transcription, Tax plays a major role in viral pathogenesis and T cell immortalization (Grassmann et al., 1989; Ross et al., 2000). Tax deregulates the expression of cellular genes mostly by the constitutive activation of NF-kB pathway and the inhibition of p53 tumor suppressor (Pise-Masison et al., 1998; Li and Gaynor, 2000; Mahieux et al., 2000; Miyazato et al., 2005; Peloponese et al., 2006; Qu and Xiao, 2011). It is conceivable that CIITA might exert a broader effect on HTLV infection by counteracting Tax oncogenic potential. Future efforts will be devoted to investigate whether CIITA inhibits Tax-mediated NF-kB activation and p53 suppression.

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Tax-1 and Tax-2 similarities and differences: focus on post-translational modifications and NF-κB activation

Margret Shirinian, Youmna Kfoury, Zeina Dassouki, Hiba El-Hajj and Ali Bazarbachi*

Department of Internal Medicine, Faculty of Medicine, American University of Beirut, Beirut, Lebanon

Edited by:
Umberto Bertazzoni, University of Verona, Italy

Reviewed by:
Youichi Suzuki, National University of Singapore, Singapore
Masao Matsuoka, Institute for Virus Research Kyoto University, Japan

***Correspondence:**
Ali Bazarbachi, Department of Internal Medicine, Faculty of Medicine, American University of Beirut, P.O. Box 113-6044, Beirut, Lebanon
e-mail: bazarbac@aub.edu.lb

Although human T cell leukemia virus type 1 and 2 (HTLV-1 and HTLV-2) share similar genetic organization, they have major differences in their pathogenesis and disease manifestation. HTLV-1 is capable of transforming T lymphocytes in infected patients resulting in adult T cell leukemia/lymphoma whereas HTLV-2 is not clearly associated with lymphoproliferative diseases. Numerous studies have provided accumulating evidence on the involvement of the viral transactivators Tax-1 versus Tax-2 in T cell transformation. Tax-1 is a potent transcriptional activator of both viral and cellular genes. Tax-1 post-translational modifications and specifically ubiquitylation and SUMOylation have been implicated in nuclear factor-kappaB (NF-κB) activation and may contribute to its transformation capacity. Although Tax-2 has similar protein structure compared to Tax-1, the two proteins display differences both in their protein–protein interaction and activation of signal transduction pathways. Recent studies on Tax-2 have suggested ubiquitylation and SUMOylation independent mechanisms of NF-κB activation. In this present review, structural and functional differences between Tax-1 and Tax-2 will be summarized. Specifically, we will address their subcellular localization, nuclear trafficking and their effect on cellular regulatory proteins. A special attention will be given to Tax-1/Tax-2 post-translational modification such as ubiquitylation, SUMOylation, phosphorylation, acetylation, NF-κB activation, and protein–protein interactions involved in oncogenecity both *in vivo* and *in vitro*.

Keywords: HTLV-1, HTLV-2, Tax-1, Tax-2, NF-κB

INTRODUCTION

Human T cell lymphotropic virus type 1 (HTLV-1) and type 2 (HTLV-2) are closely related human delta retroviruses. Although currently there are four known types of HTLV retroviruses (Mahieux and Gessain, 2005, 2009), HTLV-1 is the most pathogenic of all and the first oncogenic retrovirus discovered in humans. HTLV-1 infects 15–20 million individuals worldwide. It is transmitted horizontally (sexual transmission), vertically (mother to child), and by blood transfusion (Kaplan et al., 1996). HTLV-1 is the causative agent of adult T cell leukemia/lymphoma (ATL; Poiesz et al., 1980a,b; Gallo, 1981; Hinuma, 1982; Hinuma et al., 1982; Yoshida et al., 1982) and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a distinct neurological disorder with inflammatory symptoms and incomplete paralysis of the limbs (Gessain et al., 1986). HTLV-1 infection is endemic in Japan, Africa, South America, the Caribbean, Melanesia, and certain areas in the Middle East and Eastern Europe (reviewed in Gessain and Mahieux, 2000; Tarhini et al., 2009). The HTLV-1 transactivator protein, Tax-1, has been identified as a significantly potent protein in HTLV-1 pathogenesis. It works as an activator of a variety of transcription factors and has been shown to be sufficient to immortalize T cells *in vitro* and *in vivo* thus playing an important role in cellular transformation (Cereseto et al., 1996; Yao and Wigdahl, 2000; Grassmann et al., 2005; Kashanchi and Brady, 2005; Kfoury et al., 2005; Hasegawa et al., 2006; Mahieux and Gessain, 2007; Matsuoka and Jeang, 2007; Yoshida et al., 2008; Matsuoka and Green, 2009; Yamazaki et al., 2009). HTLV-2, however, was first identified in a T cell line established from a patient with hairy-cell leukemia (Kalyanaraman et al., 1982). In contrast to HTLV-1, HTLV-2 infection has not been linked to the development of lymphoproliferative disorders. However, as in HTLV-1, HTLV-2 infection has been associated with sporadic cases of myelopathy resembling TSP/HAM caused by HTLV-1 (Roucoux and Murphy, 2004). HTLV-2 infection is mainly concentrated in Central and West Africa (Goubau et al., 1990; Gessain et al., 1993), native Amerindian populations in North, Central, and South America (Hjelle et al., 1990; Lairmore et al., 1990; Heneine et al., 1991; Levine et al., 1993), and among intravenous drug users in the United States and Europe (Gazzard et al., 1984; Gallo et al., 1986; Khabbaz et al., 1991; Toro et al., 2005).

Tax-1 AND Tax-2: THEY LOOK SIMILAR BUT ARE QUITE DIFFERENT

SEQUENCE AND STRUCTURAL ORGANIZATION

Both Tax-1 and Tax-2 are required for HTLV-1 and HTLV-2 viral replication and they play an important role in proviral transcription (Landry et al., 2007; Yoshida et al., 2008). In addition, Tax-1 is a key player in immortalization and transformation of infected T cells by enhancing the transcriptional expression of genes that control T cell proliferation, affecting genes involved in mitotic checkpoints and further inactivating tumor suppressor pathways (Peloponese et al., 2007; Boxus et al., 2008; Journo et al., 2009; Chlichlia and Khazaie, 2010).

Tax-1 and Tax-2 share overall sequence homology (**Figure 1A**), but have distinctive differences both at the structural and functional levels (Higuchi and Fujii, 2009; Bertazzoni et al., 2011). Tax-1 is a 353aa (amino acid) residue protein, which is highly conserved in all HTLV-1 serotypes. Of the four serotypes of HTLV-2, Tax-2 subtype A and B are the best characterized (Sheehy et al., 2006) and Tax-2B is the subtype which is represented in **Figure 1**. Tax-2B has 356 amino acid residues, whereas Tax-2A possesses a 25 amino acid truncation at the C-terminus. Tax-1 and Tax-2B share 85% amino acid sequence similarity and have several common domains (**Figure 1A**).

The N-terminal region of both Tax-1 and Tax-2 contain CREB (cyclic AMP responsive element binding)-activating domain and a zinc finger domain (Ross et al., 1997; Feuer and Green, 2005; **Figure 1B**). The CREB domain is required for activation of the viral promoter (Giebler et al., 1997; Boxus et al., 2008). Depending on the cell type, Tax-1 mutants deficient for CREB activation are incompetent for transformation or induction of aneuploidy (Akagi et al., 1997; de la Fuente et al., 2006; Geiger et al., 2008). The zinc finger domain is required for association with a variety of transcription factors including the p62 nucleoporin and mutations in this motif abolishes Tax-1 interaction with p62 and nuclear import (Tsuji et al., 2007). Within the first 60 amino acids of Tax-1, there is a nuclear localization signal NLS (Gitlin et al., 1991; Smith and Greene, 1992) whereas the first 42 amino acid sequence of Tax-2 contain a nuclear localization determinant (Turci et al., 2006)

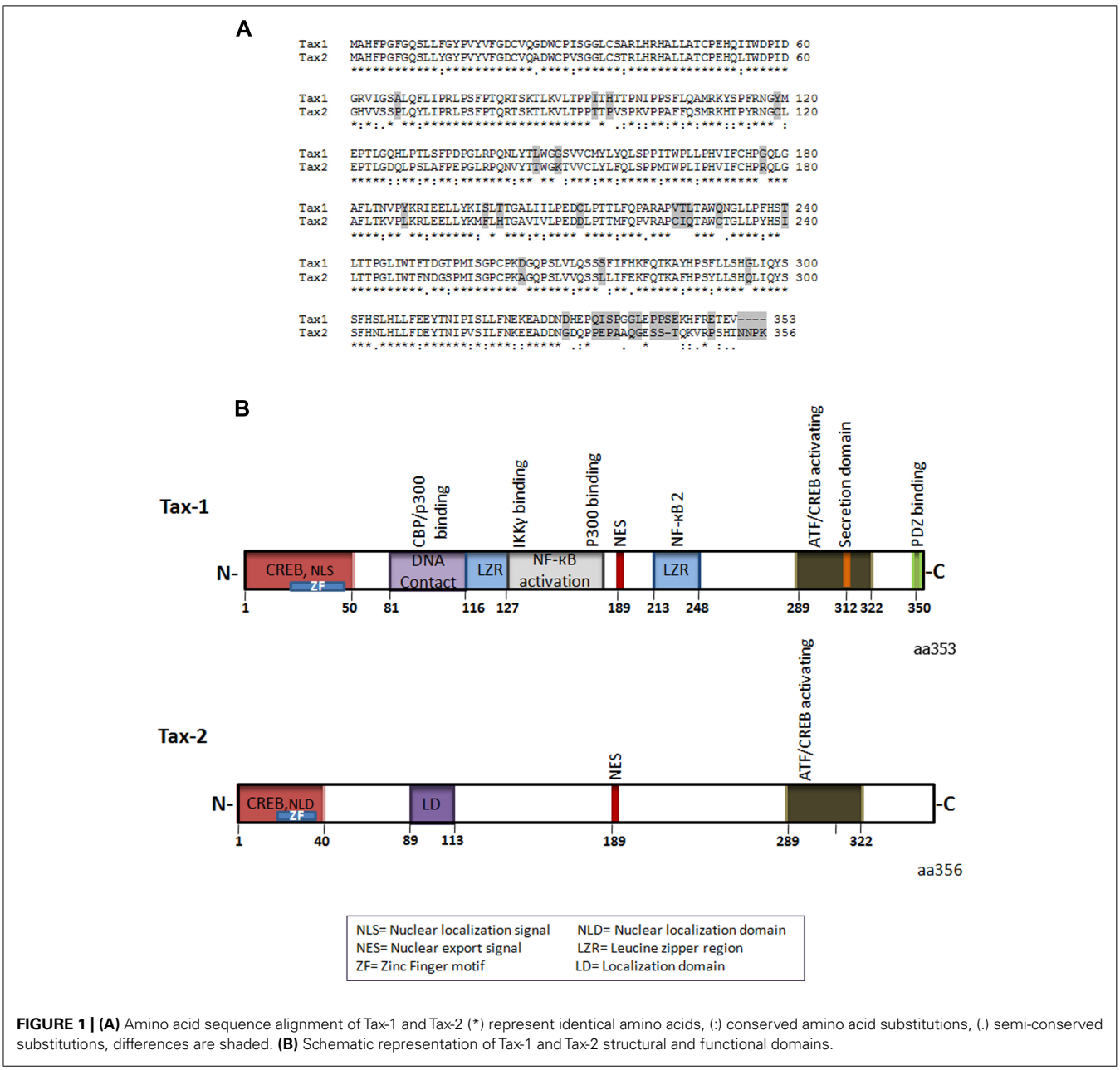

FIGURE 1 | (A) Amino acid sequence alignment of Tax-1 and Tax-2 (*) represent identical amino acids, (:) conserved amino acid substitutions, (.) semi-conserved substitutions, differences are shaded. **(B)** Schematic representation of Tax-1 and Tax-2 structural and functional domains.

required for its nuclear functionality (**Figure 1B**). Furthermore, Tax-2 has an additional cytoplasmic localization domain about 10 amino acids long, situated at amino acid position 89–113 which has been shown to be responsible for its divergent localization compared to Tax-1 (Meertens et al., 2004).

The central region of Tax-1 includes two leucine zipper-like regions (LZR), which are known to be essential for protein dimerization and DNA interaction (Jin and Jeang, 1997; Basbous et al., 2003; Boxus et al., 2008). The first LZR is located at amino acid position 116–145 and is responsible for non-canonical nuclear factor-kappaB (NF-κB) activation and protein dimerization whereas the second LZR is located at amino acid position 225–232 and is responsible for p100 processing and p52 nuclear translocation involved in NF-κB2 activation (Xiao et al., 2001; Higuchi et al., 2007; Shoji et al., 2009; **Figure 1B**). Importantly, Tax-2 lacks these two LZR regions. Both Tax-1 and Tax-2 have nuclear export signal (NES) located at amino acid position 189–202 (Alefantis et al., 2003; Chevalier et al., 2005; **Figure 1B**). Furthermore, Tax-1 and Tax-2 have at the C-terminal region CREB/activating transcription factor (ATF)-activating domain, essential for transactivation of the CREB/ATF and for NF-κB/Rel signaling pathways (Ross et al., 1997; **Figure 1B**).

Tax-1 interacts *in vitro* with a number of proteins of the CREB/ATF family of transcription factors: CREB, CREM (cyclic AMP responsive element modulator), ATF1, ATF2, ATF3, ATF4 (also named CREB2), and XBP1 (X-box-binding protein 1; Zhao and Giam, 1992; Franklin et al., 1993; Bantignies et al., 1996; Reddy et al., 1997). These proteins share a common cluster of basic residues allowing DNA binding, and a leucine zipper (b-Zip) domain involved in homo and hetero-dimerization. Dimer formation modulates their DNA-binding specificity and transcriptional activity (Hai and Hartman, 2001). Tax-1, but not Tax-2, possesses at its C-terminus a PDZ-binding motif (**Figure 1B**). Indeed, PDZ domain-containing proteins play a key role in recruiting and organizing the appropriate proteins to sites of cellular signaling, as well as polar sites of cell–cell communication (Fanning and Anderson, 1999; Harris and Lim, 2001; Sheng and Sala, 2001). The PDZ domain of Tax-1 has been shown to interact with the human homolog of the *Drosophila melanogaster* disc large tumor suppressor protein hDLG (homolog of *Drosophila* disc large), which regulates cellular proliferation and cell cycle phase transition (Rousset et al., 1998; Higuchi and Fujii, 2009). Tax-1 competes with the binding domain of hDLG and APC (The adenomatous polyposis coli) tumor suppressor protein and rescues cells from cell cycle arrest induced by hDLG (Suzuki et al., 1999; Hirata et al., 2004).

Tax-1 but not Tax-2 contain additionally at the C-terminus a secretory signal which is involved in Tax-1 secretion and transport from endoplasmic reticulum to Golgi and in movement from Golgi to the plasma membrane (Alefantis et al., 2003, 2005). The secretory sequence at the C-terminus requires interaction with secretory carrier membrane proteins (SCAMP-1 and SNAP 23) and the coat protein 2 (COPII; Jain et al., 2007). Recently, the C-terminus of Tax-1 has received much attention due to the presence of domains that are unique for Tax-1 and may partially explain the highest transformation capacity of Tax-1 in comparison to Tax-2. Indeed, the C-terminal 53 amino acids of Tax-1 is responsible for increased transformation efficiency in rodent fibroblasts (Majone et al., 1993).

CELLULAR LOCALIZATION OF Tax-1 AND Tax-2

Early studies on Tax-1 and Tax-2 subcellular localization have demonstrated that Tax-1 localizes in the nucleus and Tax-2 in the cytoplasm of HTLV-infected cells (Semmes and Jeang, 1996; Meertens et al., 2004). Both Tax-1 and Tax-2 contain a nuclear localization signal at the N-terminus, however, Tax-2 contains an additional cytoplasmic localization domain at position 89–113. By using series of Tax-1/Tax-2 chimeras, Meertens et al. (2004) have shown that this stretch of sequence indeed contributes to the difference in Tax-2 cytoplasmic localization compared to Tax-1.

In various Tax-1/Tax-2 transfected cells lines, Tax-1 has a punctate nuclear distribution and localizes in nuclear structures named nuclear speckles or bodies (Semmes and Jeang, 1996; Bex et al., 1997), whereas Tax-2 was predominantly present in the cytoplasm (Meertens et al., 2004). In these nuclear bodies, Tax-1 colocalizes with proteins of the splicing machinery such as splicing factors Sm and SC-35, transcriptional components including the largest subunit of RNA polymerase II and cyclin-dependent kinase CDK8 and with important components of NF-κB such as the two subunits p50 and RelA, as well as the regulatory subunit NEMO of IkappaB kinase (IKK; Bex et al., 1997). Furthermore, recent findings indicate that Tax-1 colocalizes within nuclear bodies with small ubiquitin modifiers (SUMO-1, 2, and 3; Lamsoul et al., 2005; Nasr et al., 2006) and with the SUMO E2 ligase Ubc-9 (Kfoury et al., 2011).

Although Tax-1 has been shown to be chiefly abundant in the nucleus, many studies reported cytoplasmic expression of Tax-1 in both Tax-1 transfected and HTLV-1-infected cell lines (Burton et al., 2000; Cheng et al., 2001). In the cytoplasm, Tax-1 targets IκBα and IκBβ for phosphorylation, ubiquitylation, and proteasome-mediated degradation, promoting the nuclear translocation of NF-κB/Rel proteins and the transcription induction of many cellular genes (Nicot et al., 1998). Within the cytoplasm, Tax-1 localizes in organelles associated with secretory pathways, structures associated to the centrosome or microtubule organizing center (MTOC), and in the cell to cell contact regions termed virological synapses (Igakura et al., 2003; Alefantis et al., 2005; Kfoury et al., 2008; Nejmeddine et al., 2009). In contrast, Tax-2 has been shown initially to be mostly cytoplasmic with no clear evidence for localization in nuclear bodies (Meertens et al., 2004). However, a recent study reported Tax-2 punctate distribution in nuclear bodies and colocalization with the Rel A subunit of NF-κB (Turci et al., 2006, 2009).

Interestingly, the post-translational modifications of Tax-1 control its sub cellular localization and its ability to activate the NF-κB pathway. More specifically, Tax-1 is subjected to multiple post-translational modifications such as phosphorylation (Bex et al., 1999), ubiquitylation, SUMOylation (Chiari et al., 2004; Lamsoul et al., 2005; Nasr et al., 2006), and acetylation (Lodewick et al., 2009). Ubiquitylated Tax-1 binds and recruits the IKK subunits at a centrosome-associated signalosome leading to the release of active IKK (Nasr et al., 2006; Kfoury et al., 2008). Using live-cell imaging, Kfoury et al. (2011) also showed that Tax-1 shuttles between nuclear bodies and the centrosome, depending on its ubiquitylation and SUMOylation status.

Finally, Tax-1 interacts with histone methyltransferase (HMTase) SMYD3 which affects its nucleo-cytoplasmic shuttling and regulates NF-κB activation (Yamamoto et al., 2011). Interaction of Tax-1 with the four and a half LIM domain protein 3 (FHL3) also affects Tax-1 sub cellular localization and transactivation capacity (Mccabe et al., 2013).

MODULATION OF CELLULAR PATHWAYS BY Tax-1 AND Tax-2

Tax-1 interacts with various components of the cell signaling system which control cell transformation, proliferation, intracellular protein distribution, cell migration, and virological synapses (Azran et al., 2004; Jeang et al., 2004; Grassmann et al., 2005; Boxus et al., 2008). More than 100 proteins have been reported to interact with Tax-1 (Boxus et al., 2008). Tax-2, however, interacts with a limited number of partners and most of them belong to the NF-κB family of proteins. It is important to note that Tax-1 and to a lesser extent Tax-2 interactome is undergoing a dramatic expansion with additional interaction partners being discovered continuously.

PI3K AND AKT PATHWAY

Phospho-inositol triphosphate kinase (PI3K) and its downstream kinase AKT/PKB (protein kinase B) are activated in T cells by many cytokines including interleukin 2 (IL-2), and provide cell survival and growth signals (Cantley, 2002). PI3K activation results in phosphorylation of AKT at Ser^{473} which in turn activates a broad range of regulatory proteins and transcription factors such as AP1 (Zhang et al., 2007). In both HTLV-1 transformed and ATL cells, the transcription factor AP1 and hence the PI3K/AKT pathway are constitutively active (Fukuda et al., 2005; Peloponese and Jeang, 2006). The PI3K inhibitor (LY294002) or the AKT inhibitor II were shown to induce cell cycle arrest at G1 phase in HTLV-1 transformed cells through p27/Kip1 accumulation, and thus subsequently induce caspase-9-dependent apoptosis (Jeong et al., 2008). Other studies have shown an important role for PI3K/AKT pathway in regulating telomerase activity, and inhibition of PI3K decreased telomerase activity by more than 50% in HTLV-1-infected cells (Bellon and Nicot, 2008). Tax-1 has been also shown to be involved in Forkhead Box O (FoxO) down regulation, an AKT downstream effector and a tumor suppressor, through the ubiquitin–proteasome pathway (Oteiza and Mechti, 2011). Conversely, a recent study demonstrated that Tax-2 efficiently immortalized human primary $CD4^+$ memory T cells by constitutively activating various signaling pathways including the PI3K/AKT pathway and further found that Tax-2 induced autophagy by interacting with the autophagy complex that contains Beclin1 and PI3K class III to form autophagosomes (Ren et al., 2012).

MAPK SIGNALING PATHWAY

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that respond to external mitogen stimuli such as growth factors, cytokines or physical stress. MAPK signaling involves a sequential phosphorylation cascade of MAP kinase kinase kinase (MAP3K). There are at least five distinct MAPK subgroups: the extracellular signal-regulated kinases protein homologs 1 and 2 (ERK1/2), the big MAPK-1 (BMK-1) also referred to as ERK5, the stress-activated protein kinases-1 (SAPK-1) better known as the c-Jun N-terminal kinase homologs 1, 2, and 3 (JNK1/2/3), the (SAPK-2) homologs (p38α/β/d) and finally ERK6 also known as p38γ (Pimienta and Pascual, 2007). Tax-1 binds the MAP3K MEKK1 to stimulate IKK-β kinase activity and NF-κB activation (Yin et al., 1998). TGF-β-activating kinase 1 (TAK1) is the other MAP3K which interacts with Tax-1 and phosphorylates IKK-β and MKK6 (MAP2K6) serine/threonine kinase, thereby activating NF-κB and JNK (Adhikari et al., 2007). Tax-2 interaction with the MAPK signaling pathway leading to its constitutive activation have also been recently reported (Ren et al., 2012).

TGFβ SIGNALING PATHWAY

Transforming growth factor β (TGFβ) inhibits T cell growth in mid-G1 but can also promote tumorigenesis (Pennison and Pasche, 2007). TGFβ binds to a heterodimeric complex composed of type I (TβRI) and type II (TβRII) serine/threonine kinase receptors and activates downstream targets such as Smad proteins. These include receptor-activated R-Smad (Smad1–2–3–5–8) and the common mediator Co-Smad (Smad4). Smad4 containing complexes then translocate to the nucleus and activate transcription of genes under the control of a Smad-binding element (Waterston and Davies, 1993).

Adult T cell leukemia/lymphoma cells produce high levels of TGFβ in the sera of HTLV-1-infected patients due to constitutive activation of AP-1 in the PI3K/AKT pathway (Kim et al., 1990). Tax-1 binds the N-terminus of Smad2, Smad3, and Smad4 proteins, which inhibits their association with Smad-binding elements and competes with Smads for recruitment of CBP/P300. This inhibition will also result in promoting resistance of HTLV-1-infected cells to TGFβ (Mori et al., 2001; Arnulf et al., 2002; Lee et al., 2002). So far, interaction of Tax-2 with Smads has not been reported.

G PROTEINS AND CYTOSKELETAL ORGANIZATION

The guanine nucleotide-binding proteins GTPases (G proteins) are molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states. Tax-1 forms complexes with several members of the small GTPase Rho family G proteins such as RhoA, Rac, Gap1m, and Cdc42 (Wu et al., 2004). Rho GTPases are activated in response to external stimuli such as growth factors, stress, or cytokines. Following activation, they regulate a variety of cellular and biochemical functions such as cytoskeleton organization, regulation of gene expression, and enzymatic activities (Jaffe and Hall, 2005).

Tax-1 binds to proteins involved in cytoskeleton structure and dynamics such as α-internexin, cytokeratin, actin, gelsolin, annexin, and γ-tubulin (Trihn et al., 1997; Reddy et al., 1998; Wu et al., 2004; Kfoury et al., 2008) and through these interactions it might connect Rho GTPases to their targets and affects cytoskeletal organization. Tax-1 binds the Gβ subunit of the G protein-coupled receptor (GPCR) affecting the SDF-1-dependent activation of CXCR4 GPCR chemokine receptor resulting in MAPK pathway over-activation and increased cell chemotaxis (Ohshima, 2007). Additionally, Tax-1 expression at the microtubule assembly center and the Golgi in the cell to cell contact region has been shown to contribute to the intracellular signal which synergizes with

ICAM-1 (intracellular adhesion molecule) to induce T cell microtubule polarization at the virological synapse (Nejmeddine et al., 2005, 2009). Tax-2, however, has not yet been reported to associate with proteins involved in cytoskeletal rearrangement. It is of importance to mention again that Tax-2 lacks a PDZ domain (**Figure 1**). This PDZ domain might contribute to Tax-1 binding to proteins involved in microtubule and cytoskeleton organization, which in turn may play an important role in pathogenicity and transformation capacity (Endo et al., 2002; Ishioka et al., 2006).

ACTIVATION OF CREB SIGNALING

As mentioned previously, both Tax-1 and Tax-2, respectively, act as transcriptional activators of the HTLV long terminal repeat (LTR). Tax-1 and Tax-2 modulate CREB and ATF function (Jeang et al., 1988; Adya and Giam, 1995; Bodor et al., 1995; Brauweiler et al., 1995; Yin et al., 1995b; Bantignies et al., 1996; Tie et al., 1996; Yin and Gaynor, 1996; Bex et al., 1998). Tax-1/Tax-2 activation of the CREB/ATF pathway is critical for efficient viral gene expression and replication (Zhao and Giam, 1992; Wagner and Green, 1993; Adya et al., 1994; Anderson and Dynan, 1994; Yin et al., 1995a; Bantignies et al., 1996). A number of mutants in both Tax-1 and Tax-2 have been described that selectively abrogate the ability of Tax to activate transcription through the CREB/ATF signaling pathway (Smith and Greene, 1990; Semmes and Jeang, 1992; Ross et al., 1997). Tax-1 activates a variety of cellular genes through its interactions with CREB/ATF proteins, for example those encoding IL-17 or c-fos (Alexandre and Verrier, 1991; Dodon et al., 2004). On the other hand, Tax-1 also represses expression of genes like cyclin A, p53, and c-myb by targeting CREB/ATF factors (Nicot et al., 2000; Kibler and Jeang, 2001). Furthermore, Tax-1 has been shown to repress Smad-dependent TGFβ signaling through interaction with CBP/p300 (Mori et al., 2001). Tax-1 has also been shown to abrogate p53-induced cell cycle arrest and apoptosis through its CREB/ATF functional domain (Mulloy et al., 1998). Some bioinformatic analysis of wild type and CREB-deficient Tax-1 protein revealed several cellular genes controlled by CRE elements activated by Tax-1 (de la Fuente et al., 2006) such as Sgt1 (suppressor of G2 allele of SKP1) and p97 (Vcp; valosin containing protein) which have functions in spindle formation and disassembly, respectively.

Both Tax-1 and Tax-2 interact with a series of CREB/ATF factors and modulate expression of viral and cellular genes through CRE elements. However, the specific binding of each CREB/ATF member still needs to be studied, although some in vitro analysis suggest Tax-1 interaction with a number of proteins of the CREB/ATF family of transcription factors: CREB, CREM, ATF1, ATF2, ATF3, ATF4 (also named CREB2), and XBP1 (Zhao and Giam, 1992; Franklin et al., 1993; Bantignies et al., 1996; Reddy et al., 1997).

REPRESSION OF P53 SIGNALING

P53 is a DNA-binding transcription factor, which plays an important role as a tumor suppressor and is primarily involved in cell cycle regulation, apoptosis, and DNA repair (Vousden and Lu, 2002; Zenz et al., 2008). The P53 gene is very often mutated in human tumors and hematologic malignancies (Xu-Monette et al., 2012). Several *in vitro* studies in different cell types have shown that Tax-1 represses p53 activity through different mechanisms including NF-κB activation and/or the CREB pathway (Ariumi et al., 2000; Pise-Masison et al., 2000; Jeong et al., 2004, 2005). Recently, Wip-1 phosphatase protein was shown to interact with Tax-1 and inhibits p53 (Zane et al., 2012). In this study authors have used Tax transgenic mice and found significant differences in Tax-1-driven inactivation of p53 versus p53 inactivation due to genetic mutations. Several studies explored Tax-2 contribution to p53 inactivation. In HTLV-2 subtype A- and B-infected cells, both Tax-2B and to a lesser extent Tax-2A were shown to inhibit p53 in T cells (Mahieux et al., 2000b).

In ATL-derived cell lines, P53 has been shown to be very often inactive and sometimes mutated despite its high expression levels and this activation has been shown to be dependent on Tax-1-induced NF-κB activation through phosphorylation of p53 Ser-15 and Ser-392 (Pise-Masison et al., 2000). Studies by Ariumi et al. (2000) have shown that the phosphorylation of p53 on Ser-15 is not a major cause of the Tax-mediated inactivation of p53. However, Tax with a mutation in the coactivator CBP-binding site (K88A), which activates NF-κB but not the CREB pathway, could not repress the p53 transactivation function. A study dedicated to Tax-2 inhibition of p53 was performed by (Mahieux et al., 2000a) where abundant levels of p53 protein were detected in both HTLV-2A and -2B virus-infected cell lines and p53 was shown to be inactive. Furthermore, they showed that although Tax-2A and Tax-2B inactivate p53, the Tax-2A protein appeared to inhibit p53 function less efficiently than either Tax-1 or Tax-2B. Jurkat cells that constitutively express Tax-1 and Tax-2 showed reduced cellular replication, and Tax-1 inhibition of cellular replication was higher in comparison to Tax-2 (Sieburg et al., 2004).

ACTIVATION OF THE NF-κB PATHWAY

Generalities on NF-κB

Nuclear factor-kappaB is a family of transcription factors that play a crucial role in proliferation, apoptosis, oncogenesis, and immune response. To date, five members of NF-κB have been described: p65 (RelA), c-Rel, RelB, p50/p105, and p52/p100. The precursor proteins p105 and p100 are processed proteolytically to the mature p50 and p52 forms, respectively (Ghosh and Hayden, 2008). All five members share a common Rel homology domain, which is a conserved domain of 300 amino acids that contains a DNA-binding domain, a dimerization domain, a region of interaction with inhibitory proteins IκB, and a NLS (Baeuerle and Henkel, 1994; Baldwin, 1996). These proteins are capable of homo- or heterodimerization using all possible combinations, except for RelB which dimerizes only with p50 or p52 (Ryseck et al., 1992).

In resting cells, NF-κB dimers are trapped in the cytoplasm by inhibitory proteins called IκBs such as p105, p100, IκBα, IκBβ, and IκBγ which mask the nuclear localization signal of NF-κB factors through physical interaction (Siebenlist et al., 1994; Perkins, 2007). NF-κB activation involves phosphorylation of IκB inhibitors by the IKK, which triggers their ubiquitylation and subsequent proteasomal degradation, resulting in nuclear translocation of NF-κB dimers (Karin and Ben-Neriah, 2000; Perkins, 2007).

Nuclear factor-kappaB is activated by a wide variety of signals through two distinct pathways: the canonical and the non-canonical pathways. The canonical pathway is activated by

pathogens, cytokines, and antigen receptors and involves the degradation of one of the three canonical IκB molecules: IκB-α, IκB-β, and IκB-ε and the nuclear translocation of the heterodimers that essentially contain RelA (Silverman and Maniatis, 2001). In response to activating signal, the IκB proteins are phosphorylated by the IKK complex, which is a high molecular weight complex composed of one regulatory subunit IKK-γ (NEMO) in addition to two catalytic subunits IKK-α and IKK-β (Israel, 2010). Upon activation, the IKK complex is able to induce the phosphorylation of the IκB proteins leading to their ubiquitylation and degradation by the proteosome. The non-canonical NF-κB pathway on the other hand primarily involves IKK-α activation upon phosphorylation by NF-κB-inducing kinase (NIK). IKK-α then phosphorylates the C-terminal region of p100 leading to subsequent processing of the p100/RelB complex into p52/RelB and its translocation into the nucleus (Dejardin, 2006). It is important to note that p52/RelB and p50/RelA dimers target distinct NF-κB enhancers thereby activating different subset of genes.

Tax-1 activation of the NF-κB pathway

Tax-1 activates both the canonical and the non-canonical pathways resulting in constitutive activation of NF-κB in HTLV-1-infected cells (Xiao et al., 2001; Higuchi et al., 2007). In the canonical pathway, Tax-1 associates with the IKK-γ/NEMO subunit (Harhaj and Sun, 1999; Jin et al., 1999; Kfoury et al., 2005) and activates upstream kinases such as MAPK/ERK kinase kinase 1 (MEKK1), and TAK1 through TAK1-binding protein 2 (TAB2; Yin et al., 1998; Wu and Sun, 2007; **Figure 2A**). Tax-1 therefore, connects activated kinases to the IKK complex and forces the phosphorylation of IKK-α and IKK-β leading to IKK activation, which results in phosphorylation, ubiquitylation, and proteasome-mediated degradation of IκBα and IκBβ (Harhaj and Sun, 1999; Jin et al., 1999). In addition, Tax-1 binds directly to the IKK-α and IKK-β subunits and activates their kinase activity independently of the upstream kinases (Chu et al., 1998; **Figure 2A**). In fact, silencing of MEKK1 and TAK1 does not impair Tax-1-induced NF-κB activation (Gohda et al., 2007). Within the canonical pathway, Tax-1 can as well bind directly to IκBs and mediate their degradation independently of IKK phosphorylation (Hirai et al., 1994; Suzuki et al., 1995). At the proteosomal level, Tax-1 interacts with the two subunits of the 20S proteasome (HsN3 and HC9), favors anchorage of p105 and accelerates its proteolysis (Rousset et al., 1996; **Figure 2A**). Tax-1 therefore, leads to IκB degradation at multiple levels, thereby allowing nuclear translocation of NF-κB independently of external stimuli. In the non-canonical pathway, Tax-1 interacts with IKK-γ (NEMO) and p100, induces p100 processing and nuclear translocation of the p52/RelB dimer (**Figure 2A**). It therefore appears that IKK-γ is an important Tax-1-binding partner for activation of both pathways (Xiao et al., 2001; Higuchi et al., 2007).

Tax-2 activation of the NF-κB pathway

Many studies have shown the ability of Tax-2 to activate the canonical NF-κB pathway to a level comparable to Tax-1 (Higuchi et al., 2007). The major difference between Tax-1 and Tax-2 lies in the inability of Tax-2 to process p100 (Higuchi et al., 2007; **Figure 2B**). The LZR at amino acid 225–232 of Tax-1, which is missing in Tax-2, is responsible for p100 processing and p52 nuclear translocation

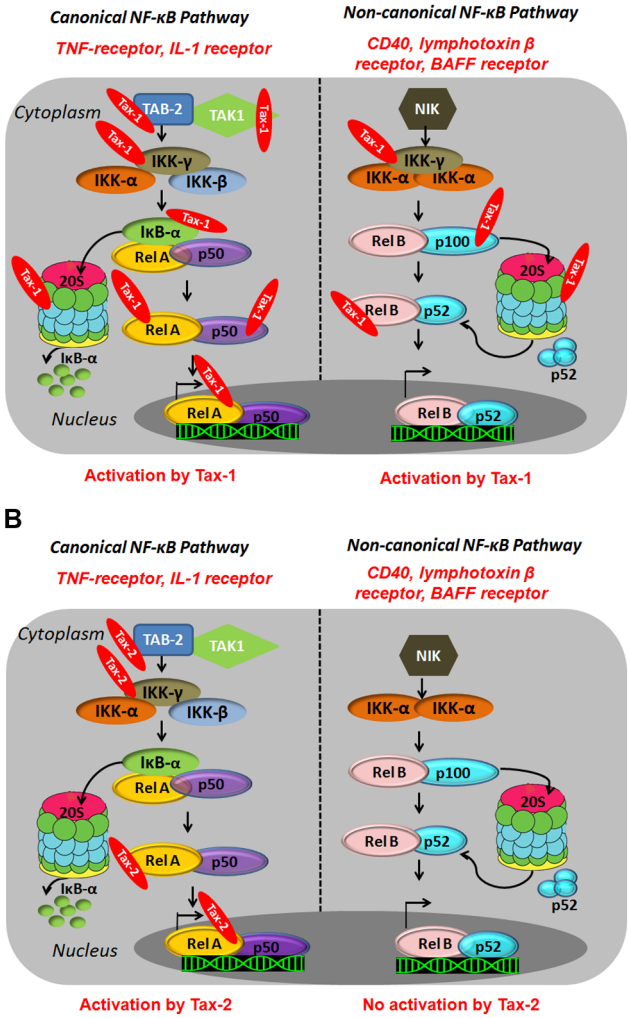

FIGURE 2 | Illustration of canonical and non-canonical NF-κB pathway activation by HTLV-1 Tax-1 (A) and Tax-2 (B). Canonical NF-κB pathway involves a cascade of phosphorylation events by kinases such as TAK1 and the IKK complex (α,β,γ) which results in the proteasomal degradation of the cytoplasmic inhibitor (IκB) and the translocation of the NF-κB dimers to the nucleus and transcriptional activation. The non-canonical pathway involves the NF-κB-inducing kinase (NIK) and IKK-α subunit (α) and results in the proteasomal degradation of p100 and the nuclear translocation of NF-κB dimers p52/RelB and activation of gene expression.

(Shoji et al., 2009). To date, there is no evidence of the ability of Tax-2 to activate the non-canonical NF-κB pathway. In fact, the transforming activity of Tax-1 in CTLL-2 (cytotoxic T-lymphocyte cell lines) cells constitutively expressing the IL-2 receptor is much higher than Tax-2 and this activity has been shown to be partly mediated through the non-canonical NF-κB pathway (Tsubata et al., 2005; Kondo et al., 2006; Higuchi et al., 2007; Shoji et al., 2009). Within the same line, a constitutively active NIK, restores the transforming activity of Tax-2 to a level equivalent to Tax-1 (Higuchi et al., 2007). This inability of Tax-2 to activate the non-canonical NF-κB pathway might partially explain its inability to transform T cells and induce ATL development.

Tax-1 AND Tax-2 POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications of Tax-1 and Tax-2 proteins have been shown to play a critical role in their cellular localization, transactivation, and protein–protein interactions. Furthermore, Tax-1 and Tax-2 pleotropic effects and their structural organization make these proteins a target of many other potential post-translational events which still need to be discovered.

PHOSPHORYLATION

To date, six Tax-1 residues were identified as phosphorylation targets: Thr-48, Thr-184, Thr-215, Ser-300, Ser-301, and Ser-336 (Bex et al., 1999; Durkin et al., 2006; **Figure 3**). Adjacent serine residues at positions 300 and 301 in the carboxy-terminus of Tax represent the major sites for phosphorylation. Indeed, phosphorylation of at least one of these serine residues is required for Tax localization in nuclear bodies and for Tax-mediated activation of gene expression via both the ATF/CREB and NF-κB pathways (Bex et al., 1999). Furthermore, Ser-300 and Ser-301 are required for further post-translational modifications such as ubiquitylation, SUMOylation, and acetylation (Lodewick et al., 2009). On the other hand, the serine/threonine kinase CK2 phosphorylates Tax-1 at three residues: Ser-336, Ser-344, and Thr-351 within its C-terminus, which indirectly affects NF-κB activation (Higuchi et al., 2007; Bidoia et al., 2010). Some indirect evidence of the involvement of Ser-160 phosphorylation in stabilizing Tax-1 has been recently reported (Jeong et al., 2009). Although Tax-1 and Tax-2 share 85% homology in their amino acid sequences, and all the phosphorylated residues are conserved except for Ser-336, the phosphorylation status of Tax-2 is still not well determined. *In vitro* studies showed that CK2 does not phosphorylate Tax-2 as for Tax-1 (Bidoia et al., 2010). A detailed mutational analysis of Tax-2 residues may help in identifying Tax-2 phosphorylated residues and their impact on Tax-2 function.

ACETYLATION

Tax-1 has been shown to be acetylated at Lys-346 (Lodewick et al., 2009). Acetylated forms of Tax-1 were detected in both Tax-1 transfected 293 T cells and T lymphocytes (Lodewick et al., 2009). In the same study it has been suggested that phosphorylation of Ser-300/Ser-301 is essential for its nuclear translocation and hence is a prerequisite for Tax-1 acetylation through interaction with p300 (**Figure 3**). Tax-1 acetylation in turn participates in NF-κB activation (Lodewick et al., 2009). Although there is not much studies yet on Tax-2 acetylation, Lodewick et al. (2009) reported that Tax-2 may also be acetylated.

UBIQUITYLATION AND SUMOylation

Ubiquitylation and SUMOylation have been shown to play an important role in the cellular localization, function, and protein–protein interactions of both Tax-1 and Tax-2 (Chiari et al., 2004; Peloponese et al., 2004; Harhaj et al., 2007; Turci et al., 2009; Avesani et al., 2010). Tax-1 has ten lysines (**Figure 3**). Five of these residues located within Tax-1 C-terminal region were found to be the major targets ubiquitylation [Lys-189 (K4), Lys-197 (K5), Lys-263 (K6), Lys-280 (K7), and Lys-284 (K8)], whereas SUMOylation takes place on Lys-280 (K7) and Lys284 (K8) (Lamsoul et al., 2005; Nasr et al., 2006).

Tax-1 is indeed differentially ubiquitylated by either K-48 ubiquitin chains leading to Tax degradation by the proteasome or by K-63 ubiquitin chains that mediates IKK recruitment to the centrosome and IKK activation (Kfoury et al., 2008). On the other hand, Tax-1 SUMOylation is required for nuclear body formation and recruitment of RelA and IKK-γ to Tax-1-related nuclear bodies, where Tax-driven transcription is promoted (Lamsoul et al., 2005; Nasr et al., 2006; Harhaj et al., 2007; Kfoury et al., 2011). A RING (Really Interesting New Gene) finger domain containing protein RNF4 has recently been shown to bind putative Tax ubiquitin/SUMO modification sites K280/K284 and increase

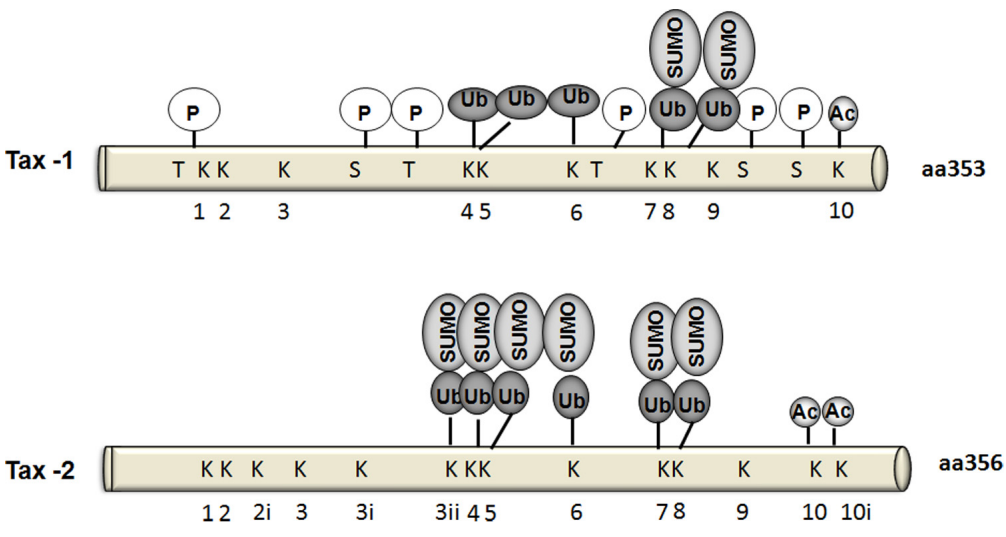

FIGURE 3 | Schematic comparison of Tax-1 and Tax-2 post-translational modifications. Sites of phosphorylation (P), and target lysines for ubiquitylation, SUMOylation, or acetylation are indicated.

Tax cytoplasmic enrichment and NF-κB activation (Fryrear et al., 2012). A recent report added new insights to our understanding of Tax-1 and Tax-2 ubiquitylation- and SUMOylation-dependent NF-κB activation. Bonnet et al. (2012) used Tax-1 mutants (Tax-P79AQ81A) defective for nuclear body formation. Ubiquitylation levels of the mutant and the wild type protein were similar, however, the endogenous SUMOylation levels were lower in the mutant. Despite low SUMOylation levels in the mutants, NF-κB activation was not affected enforcing the possibility that low levels of SUMOylation may suffice for Tax-1-induced NF-κB activation.

The involvement of Tax-2 SUMOylation and ubiquitylation in NF-κB activation remains controversial. Journo et al. (2013) showed that in contrast to Tax-1, Tax-2 SUMOylation and ubiquitylation are not essential to activate NF-κB. In their study, Tax-2 conjugation to endogenous SUMO and ubiquitin was barely detectable, however, Tax-2 was still acetylated. This low level of conjugation to endogenous ubiquitin and SUMO did not prevent Tax-2 activation of an NF-κB-dependent promoter or its interaction with IKK-γ/NEMO. Furthermore, a lysine-less Tax-2 mutant, which is defective for ubiquitylation and SUMOylation but not acetylation, is still able to transactivate an NF-κB-dependent promoter and bind and activate the IKK complex to induce RelA/p65 nuclear translocation. On the other hand, using transfection methods, Turci et al. (2012) have reported that Tax-1 and Tax-2 share a common mechanism of NF-κB activation and that both depend on their ubiquitylation and SUMOylation status. Thus, they show that patterns and levels of ubiquitylation between Tax-1 and Tax-2 are conserved, except for a reduced representation of the Tax-2 mono-ubiquitylated form compared to Tax-1.

INHIBITION OF APOPTOSIS AND INDUCTION OF DNA DAMAGE BY Tax-1 AND Tax-2

Induction of programmed cell death by Tax-1 has been shown in many studies using both *in vitro* Tax-1 inducible cell lines (Ray and Gottlieb, 1993) and *in vivo* transgenic mice. Indeed, Tax-1 transgenic mice are characterized by enhanced apoptosis which is associated with elevated levels of oncoproteins such as Myc, Fos, Jun, and p53 expression (Hall et al., 1998). It is important to mention that ATL malignant transformation involves complex and multi-step mechanisms such as accumulation of DNA damage and aneuploidy. Furthermore, Tax-1 expression sensitizes cells to apoptotic cell death induced by DNA damaging agents (Kao et al., 2000) and by tumor necrosis factor alpha (TNF-α; Saggioro et al., 2001). Upon UV irradiation, Tax-1 localization was increased at the cytoplasm and decreased in the nucleus and Tax-1 NES have been shown to be required for its stress-induced nucleocytoplasmic translocation (Gatza and Marriott, 2006). Caspase activity has been shown to be crucial for Tax-1-induced cell death and apoptosis whereas B cell lymphoma 2 (Bcl-2) expression has been shown to be associated with cell death prevention (Yamada et al., 1994; Chen et al., 1997; Chlichlia et al., 1997, 2002; Rivera-Walsh et al., 2001; Kasai and Jeang, 2004). Interestingly, Tax has been shown by many studies to both induce apoptosis and represses it. Many groups have shown the importance of Tax-1-mediated NF-κB activation in induction of apoptosis (Wheeler et al., 1993; Chen et al., 1997; Chlichlia et al., 1997; Los et al., 1998; Rivera-Walsh et al., 2001). Tax mutants defective in NF-κB activation have reduced apoptosis-inducing activities, and inhibition of Tax-mediated NF-κB transactivation partially inhibited apoptotic cell death (Los et al., 1998; Harrod et al., 2000; Rivera-Walsh et al., 2001). Tax also represses the transcription of the proapoptotic *bax* gene (Brauweiler et al., 1997). In addition, Tax inhibits the caspase cascade in an NF-κB-dependent manner through the induction of the caspase inhibitors X-IAP, cIAP-1, and c-IAP-2 (Kelly et al., 1993).

Previous experiments performed on T cell lines derived from HTLV-2-infected individuals and Tax-2 expressing various cell lines have shown that Tax-2 is capable of inhibiting Fas-mediated apoptosis through the expression of bcl-x(L) messenger and protein (Zehender et al., 2001).

CONCLUDING REMARKS

To date, vast amount of knowledge has been produced regarding the HTLV-1 Tax-1 oncoprotein. Many studies have provided some insights on Tax-1 transcriptional regulation, subcellular localization and post-translational modifications. However, less is known about HTLV-2 Tax-2 although many aspects of its activity and regulation is now being studied. That HTLV-2 is defective in promoting certain steps of leukemogenesis, may indeed serve as a useful comparative tool for understanding the pathogenicity of HTLV-1.

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Functional comparison of antisense proteins of HTLV-1 and HTLV-2 in viral pathogenesis

Benoit Barbeau [1], ***Jean-Marie Peloponese*** [2,3] ***and Jean-Michel Mesnard*** [2,3] ***

[1] *Département des sciences biologiques and Centre de recherche BioMed, Université du Québec à Montréal, Montréal, QC, Canada*
[2] *Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé, Université Montpellier 1, Montpellier, France*
[3] *UMR5236, Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé, Centre National de la Recherche Scientifique, Montpellier, France*

Edited by:
Françoise Bex, Université Libre de Bruxelles, Belgium

Reviewed by:
Hidekatsu Iha, Oita University, Japan
Noreen Martha Sheehy, University College Dublin, Ireland

***Correspondence:**
Jean-Michel Mesnard, UMR5236, Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé, Centre National de la Recherche Scientifique, 1919 Route de Mende, 34293 Montpellier Cedex 5, France
e-mail: jean-michel.mesnard@cpbs.cnrs.fr

The production of antisense transcripts from the 3′ long terminal repeat (LTR) in human T-lymphotropic retroviruses has now been clearly demonstrated. After the identification of the antisense strand-encoded human T-lymphotropic virus type 1 (HTLV-1) bZIP (HBZ) factor, we reported that HBZ could interact with CRE-binding protein (CREB) transcription factors and consequently turn off the important activating potential of the viral Tax protein on HTLV-1 5′ LTR promoter activity. We have recently accumulated new results demonstrating that antisense transcripts also exist in HTLV-2, -3, and -4. Furthermore, our data have confirmed the existence of encoded proteins from these antisense transcripts (termed antisense proteins of HTLVs or APHs). APHs are also involved in the down-regulation of Tax-dependent viral transcription. In this review, we will focus on the different molecular mechanisms used by HBZ and APH-2 to control viral expression. While HBZ interacts with CREB through its basic zipper domain, APH-2 binds to this cellular factor through a five amino acid motif localized in its carboxyl terminus. Moreover, unlike APH-2, HBZ possesses an N-terminal activation domain that also contributes to the inhibition of the viral transcription by interacting with the KIX domain of p300/CBP. On the other hand, HBZ was found to induce T cell proliferation while APH-2 was unable to promote such proliferation. Interestingly, HTLV-2 has not been causally linked to human T cell leukemia, while HTLV-1 is responsible for the development of the adult T cell leukemia/lymphoma. We will further discuss the possible role played by antisense proteins in the establishment of pathologies induced by viral infection.

Keywords: HTLV-1, HTLV-2, antisense transcription, HBZ, APH-2

INTRODUCTION

The human T-lymphotropic virus type 1 (HTLV-1) was the first pathogenic retrovirus to be isolated in humans (Poiesz et al., 1980; Miyoshi et al., 1981). This virus is the etiological agent of adult T cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although an important number of individuals are infected by HTLV-1, only 10% will eventually develop pathologies (Barbeau and Mesnard, 2007; Matsuoka and Jeang, 2007). Leukemic cells in ATLL are mostly $CD4^+$ T cells. One of the best studied associations between HTLV-1 and oncogenesis is the viral Tax protein, as demonstrated in primary T cells and various mouse models (Matsuoka and Jeang, 2007). It likely results from the capacity of Tax to activate multiple transcription factors and to induce genetic instability (Mesnard and Devaux, 1999; Boxus et al., 2008). However, Tax is often repressed in cells from ATLL patients (Takeda et al., 2004; Satou et al., 2006). Selective pressure mediated by strong anti-Tax immune response might justify the lack of Tax expression in ATLL cells (Hanon et al., 2000; Enose-Akahata et al., 2012). HTLV-2 is closely related to HTLV-1 and shares most viral genes such as Tax and Rex but HTLV-2 is clinically distinct from HTLV-1 since it is not associated with any forms of leukemia (Feuer and Green, 2005). However, HTLV-2 can efficiently immortalize and transform T lymphocytes *in vitro* (Ye et al., 2003) and HTLV-2 Tax can immortalize human $CD4^+$ T cells at higher efficiency than HTLV-1 Tax (Imai et al., 2012). Taken together, these data suggest that Tax expression is not sufficient for ATLL development and is thus dispensable at least for the late stage of leukemogenesis. Additional viral proteins obviously are thereby needed to play such a role. Indeed, growing evidence indicate that antisense transcripts produced from the 3′ long terminal repeat (LTR) of the HTLV-1 proviral DNA might fit such a role. These transcripts are involved in the production of the HTLV-1 bZIP (HBZ) factor in infected cells (Cavanagh et al., 2006; Murata et al., 2006). We have recently demonstrated that such antisense transcription also exists in HTLV-2-infected cells and permits the synthesis of the antisense protein of HTLV-2 (APH-2; Halin et al., 2009). This review will discuss the role of antisense transcription and resulting viral proteins in the development of pathologies associated with HTLV infection.

ANTISENSE TRANSCRIPTS ARE PRODUCED FROM THE 3′ LTR OF THE HTLV PROVIRAL DNA

HTLV-1 and HTLV-2 are complex retroviruses sharing a similar genome structure with an approximate 70% nucleotide sequence homology (Feuer and Green, 2005). Like all retroviruses, they

harbor essential genes for their replication, which include *gag*, *pol* and *env*. In addition to these genes, they harbor genes coding for the Tax and Rex regulatory proteins (**Figure 1**). Tax acts in trans to activate transcription initiating from the viral promoter in the 5′ LTR and Rex regulates viral gene expression post-transcriptionally by facilitating cytoplasmic shuttling of incompletely spliced viral mRNAs. HTLV-1 encodes the accessory proteins p12, p13, and p30 whereas HTLV-2 encodes the p10, p11, and p28 accessory gene products (**Figure 1**). Studies have indicated that these proteins are dispensable for *in vitro* infection and transformation of T cells but are important for the ability of the virus to infect, spread, and persist *in vivo* (Albrecht and Lairmore, 2002; Feuer and Green, 2005). Transcription of all these viral genes is dependent on the 5′ LTR region, which is segmented in three regions termed U3, R, and U5. The U3 region harbors important elements like the Tax-responsive elements (TxREs). A Tax dimer interacts with cellular Activating Transcription Factor/CRE-binding (ATF/CREB) proteins bound specifically to TxREs (Basbous et al., 2003b; Nyborg et al., 2010). The formation of such a complex on the 5′ LTR then serves as a binding site for the recruitment of the pleiotropic cellular coactivators p300/CBP through their interaction with Tax. Recruitment of p300/CBP to the viral promoter induces local nucleosome modification by histone acetylation and facilitates stable binding of components of the basal transcription machinery allowing the stimulation of viral transcription (Gachon et al., 2002; Luebben et al., 2010).

For a long time, all retroviral genes have been thought to be transcribed through the U3 region in the 5′ LTR of the provirus. However, the production of antisense transcripts from the 3′ LTR in HTLV-1-infected cell lines (Larocca et al., 1989) and the presence of a conserved open reading frame (ORF) in the complementary strand of the HTLV-1 provirus (Larocca et al., 1989; Gaudray et al., 2002) suggested the existence of viral mRNA of negative polarity. In 2002, we clearly demonstrated the existence of an antisense strand-encoded protein termed HBZ in HTLV-1-infected cell lines (Gaudray et al., 2002). Further experiments by our teams and others have revealed that the antisense HBZ-encoding transcript was spliced and polyadenylated (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006; Yoshida et al., 2008) and that different HBZ isoforms could be produced, with one of them being the most abundant and dependent on a spliced transcript (**Figure 1**). In 2009, we also characterized a spliced antisense mRNA in HTLV-2-infected cell lines, involved in the production of APH-2 (Halin et al., 2009). This transcript is structured similarly to the HBZ transcript, i.e., it is spliced, initiates in the 3′ LTR at multiple sites, and is polyadenylated. The length of the intron and of the 5′ and 3′ untranslated regions is also similar to that of the HBZ transcript (**Figure 1**). These similarities could suggest that the expression of antisense transcription in the HTLV (and STLV) retrovirus family has been conserved among the different viruses. Indeed, we have recently confirmed the synthesis of antisense proteins from HTLV-3 and -4 proviral DNA (Larocque et al., 2011). On the other hand, unlike HBZ mRNA, we did not observe alternative splicing for APH-2, -3, and -4 mRNAs (Halin et al., 2009; Larocque et al., 2011) suggesting that these retroviral antisense genes are likely producing a single isoform.

HBZ AND APH-2 INHIBIT Tax-DEPENDENT VIRAL TRANSCRIPTION

Initial studies highlighted the negative impact of HBZ expression on HTLV-1 replication by virtue of its capacity to inhibit Tax-mediated activation of HTLV-1 transcription (Gaudray et al.,

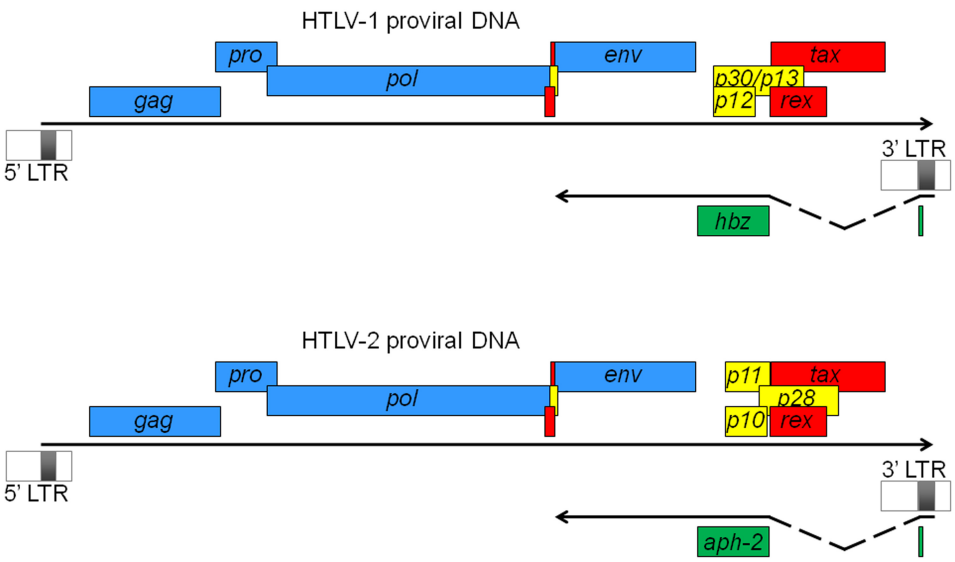

FIGURE 1 | HTLV-1 and HTLV-2 proviral DNA. Viral ORFs are depicted as either common retroviral proteins (blue), regulatory factors (red), or accessory proteins (yellow). Antisense strand-encoded ORFs are also indicated (green). Antisense transcripts are initiated from the 3′ LTR and spliced to produce the antisense proteins, HBZ and APH-2. In both cases, transcripts are initiated at a similar position in the 3′ LTR (which constitutes the first exon) and are spliced to the second exon, which contains the main ORF region as well as the 3′ untranslated region. Their polyA tail is positioned 1450 nucleotides from the ORF stop codon. In HTLV-1, an unspliced transcript initiating downstream of the 3′ LTR (not shown here) has also been described (Yoshida et al., 2008).

2002; Arnold et al., 2006). HBZ acts as a repressor of viral transcription by forming heterodimers with CREB, CREB-2, CREM, and ATF-1 that are no longer capable of binding to TxRE. HBZ is a nuclear transcriptional factor able to interact with ATF/CREB proteins (Gaudray et al., 2002; Lemasson et al., 2007; Hagiya et al., 2011) through its basic zipper (bZIP) domain (**Figure 2**), leading to inhibition of their DNA-binding activity (Hivin et al., 2005, 2006). Consequently, Tax cannot be positioned on the viral promoter and is thereby unable to trans-activate HTLV-1 transcription (Gaudray et al., 2002; Lemasson et al., 2007). Interestingly, APH-2 has also been shown to repress Tax-mediated viral transcription (Halin et al., 2009; Yin et al., 2012) by interacting with CREB. However, unlike HBZ, this interaction is mediated by the LXXLL motif present in the C-terminus of APH-2 (**Figure 2**; Yin et al., 2012). Interestingly, it has been shown that the repressive activity of APH-2 is less strong than that of HBZ. This difference in the inhibitory potential of both proteins might be explained by the additional interaction of HBZ with p300/CBP. Unlike APH-2, HBZ possesses a transcriptional activation domain within its N-terminal region (**Figure 2**) involved in an interaction with the KIX domain of p300/CBP (Clerc et al., 2008; Cook et al., 2011). In the context of viral transcription, we have demonstrated that HBZ could displace p300/CBP from the HTLV-1 promoter by competing with Tax for binding to the KIX domain (Clerc et al., 2008). Moreover, this mechanism appears more efficient than that of the ZIP domain in mediating repression of Tax-dependent viral transcription. On the other hand, additional reports have demonstrated that HBZ can activate cellular gene transcription through its interaction with p300/CBP (Kuhlmann et al., 2007; Polakowski et al., 2010; Macaire et al., 2012).

Analyses of transcription factors involved in the regulation of HTLV-1 antisense transcription showed that the Sp1 transcription factor was critical for its trans-activation (Yoshida et al., 2008; Gazon et al., 2012). We recently demonstrated that Sp1 enhanced antisense transcription by cooperating with the HBZ–JunD heterodimer (Gazon et al., 2012), which implicates the previously reported ZIP-dependent interaction between HBZ and JunD (Thébault et al., 2004; **Figure 2**). Thus, HBZ not only inhibits expression of other viral proteins such as Tax but also stimulates its own expression. While the majority of HTLV-1-specific $CD8^+$ T cells recognize the Tax protein, the frequency of HBZ-specific $CD8^+$ T cells is significantly lower (Macnamara et al., 2010). HBZ-expressing cells could thus escape lysis from a cytotoxic T lymphocyte (CTL) response and would consequently enhance viral persistence in infected people (Suemori et al., 2009; Macnamara et al., 2010). It has also been suggested that Tax can stimulate HBZ expression although this issue remains controversial, as most results were obtained from cell lines transfected with a Tax expression plasmid and reporter vectors containing only one HTLV-1 LTR (Larocca et al., 1989; Cavanagh et al., 2006; Yoshida et al., 2008; Landry et al., 2009). Indeed, such an effect by Tax has not been confirmed in T cells infected with a complete proviral DNA containing two LTRs (Belrose et al., 2011). Moreover, we have observed that activation of sense transcription from the 5′ LTR down-regulated the synthesis of antisense transcripts from the 3′ LTR (Cavanagh et al., 2006; Landry et al., 2007). It should also be emphasized that the regulation of APH-2 expression in infected cells remains currently unknown.

HBZ AND APH-2 REGULATE THE AP-1 PATHWAY IN A DIFFERENT MANNER

In unstimulated T cells, basal protein levels of the AP-1 complex are low but there is a rapid induction of AP-1 activity after T cell stimulation. The AP-1 transcription complex has been shown to

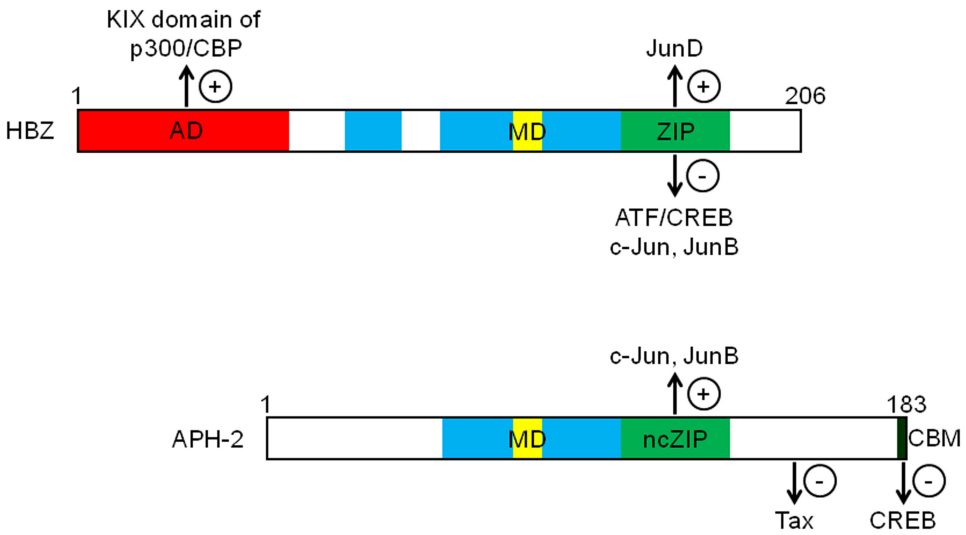

FIGURE 2 | Schematic representations of HBZ and APH-2 functional domains. Both proteins contain basic domains (blue) involved in their nuclear localization. HBZ possesses an N-terminal activation domain (AD in red), a modulatory domain (MD in yellow) and a ZIP (green) controlling the transcriptional activity of Jun and ATF/CREB proteins. While APH-2 does not contain any activation domain, it can interact with c-Jun and JunB through a non-canonical ZIP region (ncZIP in green) and with CREB through a C-terminal CREB-binding motif (CBM in black). Unlike HBZ, APH-2 binds to Tax (+) and (−) respectively indicate an activating or inhibiting effect of viral proteins on targeted proteins.

be involved in the regulation of numerous cellular genes involved in lymphocyte activation. The AP-1 transcription factor consists of heterodimers between the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) family members through their ZIP domains. AP-1 complexes are not limited to Jun and Fos dimers, since Jun and Fos proteins have been shown to dimerize with other bZIP proteins, including members of the ATF/CREB family and the Maf transcription factors. ATLL cells have been shown to contain constitutively activated AP-1 complexes, which are mainly constituted of JunD (Mori et al., 2000). HBZ, via its ZIP domain, forms heterodimers with all the members of the Jun family (Basbous et al., 2003a; Thébault et al., 2004). The interaction of HBZ with c-Jun and JunB results in repression of their transcriptional activity through degradation or sequestration into transcriptionally inactive nuclear bodies (Basbous et al., 2003a; Matsumoto et al., 2005; Hivin et al., 2007; Clerc et al., 2009). On the other hand, the HBZ/JunD heterodimer can activate transcription of cellular genes such as the human telomerase catalytic subunit (hTERT) and the antiapoptotic Bfl-1 (Kuhlmann et al., 2007; Macaire et al., 2012). HBZ can also dysregulate other cell-signaling pathways such as FoxP3 (Satou et al., 2011), NF-κB (Zhao et al., 2009; Zhi et al., 2011; Wurm et al., 2012), TGF-β (Zhao et al., 2011), and Wnt pathways (Ma et al., 2012). The resulting dysregulated pathways suggest that HBZ expression might play a central role in the development of ATLL and HAM/TSP through these altered transcription factors (Mesnard et al., 2006; Satou et al., 2006, 2011; Arnold et al., 2008; Zhi et al., 2011; Sugata et al., 2012).

Unlike HBZ, analysis of APH-2 does not predict a typical bZIP domain. However, APH-2 does interact with c-Jun and JunB through its non-canonical bZIP domain (**Figure 2**; Marban et al., 2012). Although inconsistently, APH-2 does not appear to bind to JunD *in vivo* (Marban et al., 2012). However, given the unique structure of its ZIP domain it is not surprising that APH-2 does not form canonical interactions with human bZIPs (Reinke et al., 2010). An interesting outcome from the interaction between APH-2 and the different Jun family members is that APH-2 potentiates their trans-activation activity (Marban et al., 2012). This is thereby in sharp contrast to the negative or positive modulation of HBZ on the various Jun factors. Interesting results from this study have also inferred a potential complex between Tax2B and APH-2 involving a region from amino acid 102 to 183. As no competition occurred between Jun factors and Tax2B in their binding to APH-2, the region of interaction for Tax2B is likely located outside the bZIP-like domain (**Figure 2**). Furthermore, the modulation of AP-1 activation by Tax2B was greatly reduced when APH-2 was co-expressed. However, the above experiments were conducted in overexpression condition and it will therefore be mandatory to confirm these data with physiologically relevant expression levels. These results highlight a very complex interplay between Tax 2 and APH-2 in relation to other transcription factors and it will be important to determine how this type of interaction is affected in the context of ATF/CREB family members. Furthermore, we have not observed that HBZ interacts with Tax (Hivin et al., 2005) and therefore further experiments will be required to address this issue.

HBZ INDUCES IL-2-INDEPENDENT T CELL PROLIFERATION

Based on the effect of HBZ on AP-1-dependent gene expression, HBZ might contribute to the dysregulation of cell proliferation in infected cells. In fact, two reports using HBZ-specific shRNA expression vectors have demonstrated that HBZ was important for the proliferation of HTLV-1-infected cell lines (Satou et al., 2006; Arnold et al., 2008). In these studies, although HBZ was not found to be required for HTLV-1-induced peripheral blood mononuclear cell (PBMC) immortalization, HBZ was shown to be important for cell proliferation of infected cells and had an impact in infection experiments in rabbits. Furthermore, along with these studies, other reports have determined that HBZ expression correlated with proviral DNA load, likely through its capacity to permit infected cells to proliferate (Arnold et al., 2008; Li et al., 2009). Based on these reports, HBZ has further been associated with ATLL development. Early implication of HBZ in ATLL development has been based on the occurrence of HBZ expression in ATLL cells from most tested patients (Satou et al., 2006; Usui et al., 2008). This is in contrast to Tax, whose expression is often repressed in ATLL cells. A balance between Tax and HBZ expression (Barbeau and Mesnard, 2011) might thereby be important to permit cells to proliferate and, in fact might be determinant in hampering previously reported Tax-mediated cell senescence (Zhi et al., 2011). More recent evidence has also permitted to suggest mechanisms by which HBZ could be implicated in ATLL, such as its induction of hTERT expression via a Sp1-dependent mechanism (Kuhlmann et al., 2007), its interaction with ATF-3 to suppress the ATF-3-induced p53 transcription activity stimulation (Hagiya et al., 2011), and the inhibition of p300/CBP acetyl transferase activity (Wurm et al., 2012). Additional data also argue for a potential oncogenic property of HBZ. Indeed, experiments in transgenic mice expressing HBZ in $CD4^+$ T cells resulted in high incidence of T cell lymphoma (Satou et al., 2011). In addition, NOD/SCID mice engrafted with HBZ-silenced HTLV-1-infected cells were less infiltrating and formed tumors at lesser extent than control infected cells (Arnold et al., 2008). We have also recently demonstrated that proviral DNA-expressed HBZ induced anchorage-independent growth in NIH 3T3 cells and correlated with induced JunD expression (Gazon et al., 2012). An intriguing observation also suggests that the HBZ transcript itself could be essential for induced IL-2-independent T cell proliferation (Satou et al., 2006). Kinetics and intracellular compartmentalization study of HTLV-1 mRNA expression is indirectly favoring the implication of such a transcript in cell proliferation, as HBZ viral transcripts have been shown strongly sequestered in the nucleus when compared to other viral transcripts (Rende et al., 2011). However, these data have been obtained after transfection of HLtat cells with the HTLV-1 proviral clone ACH. When 293T cells were similarly transfected, such a nuclear retention of HBZ mRNA was not confirmed (Li et al., 2012) showing that this observation strongly depends on the transfected cell line and has to be confirmed in physiologically relevant conditions.

Recent studies have compared the proliferation-inducing capacity of APH-2 to this functional characteristic of HBZ (Douceron et al., 2012). Although not associated with any forms of leukemia, HTLV-2 has nonetheless been linked to lymphocytosis in infected patients (Bartman et al., 2008). Certain similar features,

reminiscent of a potential impact on cell proliferation, were noted when similar analyses were performed on APH-2. First, APH-2 expression has been detected in most PBMC samples of HTLV-2-infected patients (Halin et al., 2009; Douceron et al., 2012). Second, a correlation between APH-2 expression levels and proviral DNA load was also noted (Douceron et al., 2012). Furthermore, a typical nuclear sequestration of its mRNA was inferred in both patient cells and infected cell lines (Bender et al., 2012). However, unlike HBZ, APH-2 is not capable of leading to IL-2-independent growth of IL-2-dependent cell lines (Douceron et al., 2012). Moreover, the absence of an impact on proliferation was further suggested in a study demonstrating no impact on proliferation of infected cells lines in addition to no effect on the immortalization capacity of HTLV-2 on infected PBMCs (Yin et al., 2012). In conclusion, no clear correlation between APH-2 mRNA expression, its nuclear retention, and HTLV-2-induced lymphocytosis was observed.

PERSISTENCE OF HTLV-1 VS. HTLV-2 INFECTION IN RELATION TO THEIR RESPECTIVE ANTISENSE PROTEIN

Using the rabbit model, a number of elegant studies have focussed on the consequence of HBZ silencing on HTLV-1 replication. Although no drastic differences were noted in HTLV-1 infection and replication in cell culture conditions, infection of rabbits with irradiated HTLV-1-producing cells highlighted an important role of HBZ on HTLV-1 persistence, as indicated by anti-HTLV-1 antibody titers and proviral DNA loads (Arnold et al., 2006). When similar experiments were conducted with HTLV-2, again APH-2 did not alter viral replication in cell culture experiments. However, inoculation of rabbit with irradiated cells producing HTLV-2 virions deficient for APH-2 led to a higher and more sustained antibody response correlating with higher proviral load at certain time points (Yin et al., 2012). These results thereby argue that, unlike HBZ, APH-2 is not necessary for viral persistence but might instead be related to the reduced capacity of HTLV-2 infection.

Both HTLV-1 and HTLV-2 are able to establish a persistent infection in $CD4^+$ and $CD8^+$ T cells but HTLV-1 establishes a more robust infection in T lymphocytes (Kannian et al., 2012). Moreover, infection results in an increase in proviral loads and viral expression such as Tax, Gag, and Env (**Figure 3**). However, cells highly expressing viral proteins are eliminated by the humoral response and CTL activity of the host

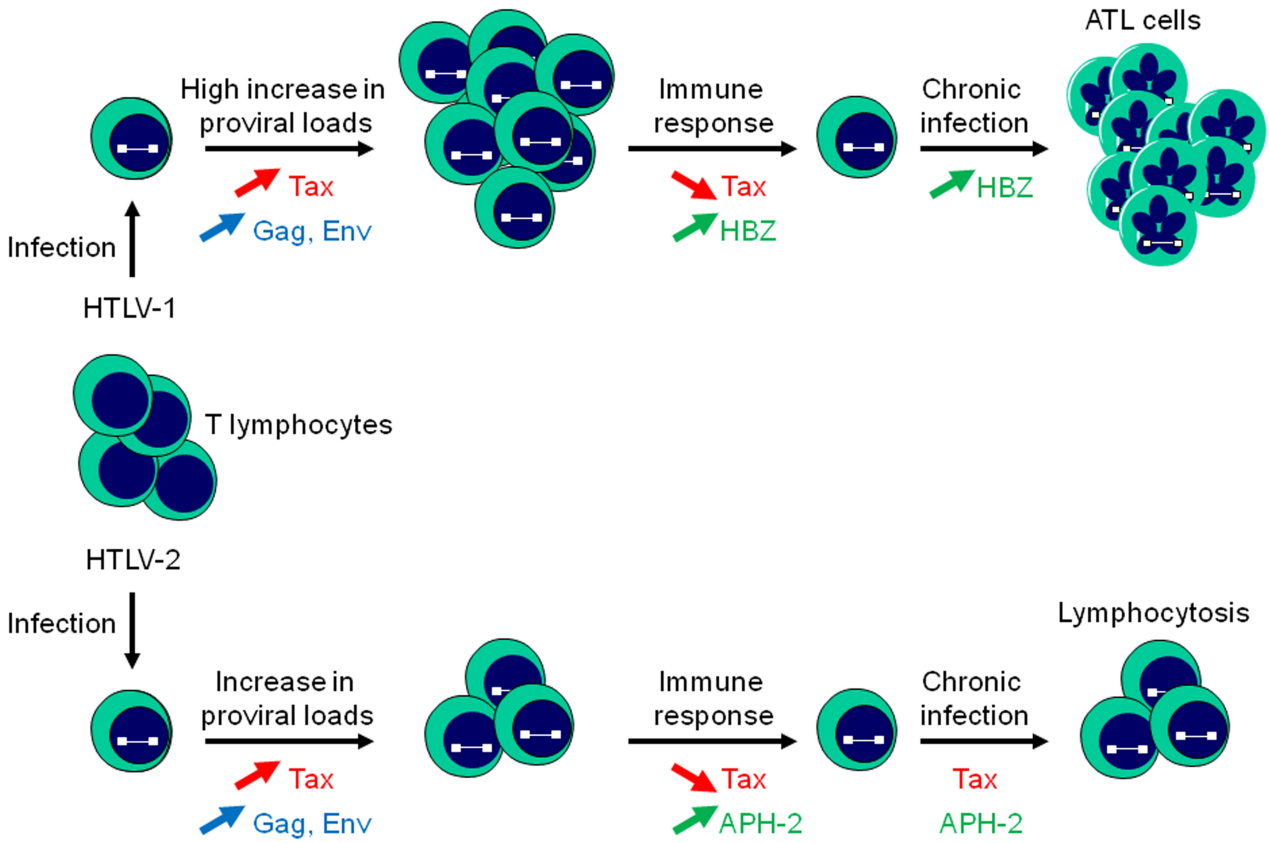

FIGURE 3 | HBZ and APH-2 differently impact T cell proliferation. Infection of T lymphocytes with HTLV-1 or HTLV-2 results in an increase in proviral loads, with a more pronounced effect for HTLV-1 proviral DNA load. An antibody response against Gag and Env and Tax-specific CTL responses induces killing of infected cells. HTLV-1 can evade the immune response by reducing Tax and stimulating HBZ expression. HBZ would subsequently promote the establishment of a chronic infection by inhibiting Tax-dependent viral transcription, stimulating its own expression, and inducing T cell proliferation. APH-2 also down-regulates Tax-dependent HTLV-2 transcription but is unable to induce cell proliferation. A low expression of Tax induced by APH-2 through its capacity to positively modulate c-Jun activation could be responsible for HTLV-2-stimulated lymphocytosis.

(Macnamara et al., 2010; Enose-Akahata et al., 2012; Enose-Akahata et al., 2013). At this stage, HBZ and APH-2 might play a crucial role by down-regulating Tax-dependent viral transcription and may allow infected cells to evade the immune response. In additionally, HBZ promotes the proliferation of infected T lymphocytes (**Figure 3**). This dual action probably confers a survival advantage on HBZ-expressing cells and is consistent with the observation that HBZ favors the establishment of persistent infection in HTLV-1-inoculated rabbits (Arnold et al., 2006). On the other hand, unlike HBZ, APH-2 is not required for viral persistence (Yin et al., 2012) and unable to promote lymphocytosis (Douceron et al., 2012). However, a low expression of Tax from the TxREs of the 5′ LTR induced by AP-1 transcriptional activity stimulated by APH-2 could explain lymphocytosis commonly observed in HTLV-2 carriers (**Figure 3**). Further experiments are definitely needed to better understand the mechanism by which HTLV-2 infection could lead to lymphocytosis.

CONCLUSIONS AND PERSPECTIVES

The discovery of HBZ has led to important new research avenues with potential association with HTLV-1-associated diseases. In fact, ATLL cells from infected patients consistently express HBZ and substantial evidence points toward its implication in viral persistence and ATLL cell survival. Recent evidences further suggest that HBZ lead toward a Treg phenotype. Future experiments will be needed to determine if similar attributes can be given to APH-2. As HBZ and APH-2 also differ mechanistically and functionally in their interaction with transcription factors such as Jun and CREB, it will be important to further expand on these differences and define the exact nature of these interactions. Several transcription factors known to interact with HBZ will also need to be analyzed in the context of APH-2 and in relation to the different cellular localizations of these proteins. In addition, the role played by HBZ on proliferation needs to be clarified as to how its transcript impacts IL-2-independent growth. These studies might also consider variation in the sequence of APH-2 transcripts, which could explain their different ability in inducing proliferation. Finally, these experiments should also be undertaken with equivalent antisense proteins of HTLV-3 and HTLV-4, i.e., APH-3 and APH-4 to determine how these proteins alter the various functions associated to HBZ. Finally, these comparative studies will provide a better understanding as to the mechanism by which HBZ is involved in ATLL development, which might also lead to new potential ATLL treatment.

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