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Review Article

Aberration in Signal Transduction Pathway in Human T-Cell Leukemia Virus Type I-Infected T Cells

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Adult T-cell leukemia (ATL) is a fatal T-lymphoproliferative disorder, and its development is associated with infection by human T-cell leukemia virus type I (HTLV-I). The molecular mechanism of leukemogenesis has not yet been elucidated. However, several studies have suggested that aberrations in signal transduction in virus-infected T cells are involved in the development of the disease. For instance, NF-κB/Rel, AP-1, and Jak/STAT signaling pathways are transiently activated in normal T cells by growth-signals, whereas they are constitutively deployed in HTLV-I-infected T-cell lines. The HTLV-I viral transactivator Tax has oncogenic properties, and is a key molecule in ATL development. Tax activates several transcription factors, including NF-κB/Rel and AP-1 in HTLV-I-transformed T-cell lines, and induces the expression of STAT proteins. Unlike HTLV-I-transformed T-cell lines, primary ATL cells express low levels of Tax protein. Nevertheless, the NF-κB/Rel, AP-1, and Jak/STAT signaling pathways in primary ATL cells are also constitutively activated. Thus, the aberration in signal transduction pathway may be a common key factor to prolonged survival and proliferation of HTLV-I-infected T cells *in vitro* and *in vivo*, but the mechanisms seem to be different. Aberration in signal transduction could be targeted for the therapeutic control of HTLV-I-associated disease.

Key Words: ATL, HTLV-I, Tax, NF-κB/Rel, AP-1, Jak/STAT

Introduction

Human T-cell leukemia virus type I (HTLV-I) is the causative agent of two diseases; the adult T-cell leukemia (ATL)[1,2], a fatal CD4+ T lymphoproliferative disorder, and HTLV-I-associated myelopathy/tropical spastic paraparesis[3,4], a chronic progressive disease of the central nervous system. HTLV-1 is also associated with other disorders such as uveitis[5], arthropathy[6], and Sjören's syndrome[7].

Only a small proportion (3 to 5%) of individuals infected with HTLV-I develop ATL, about 40 to 50 years after the infection[8]. This long latency suggests that multiple genetic events which accumulate in HTLV-I-infected cells are involved in the development of ATL. On the other hand, T-cell transformation mediated by HTLV-I appears to involve at least two distinct stages[9]. In the first stage, HTLV-I-infected T cells enter an initial period of polyclonal proliferation, which is dependent on the growth factor interleukin (IL)-2. In turn, these clonally expanded cells become immortalized after a few weeks in culture as defined by their continuous growth in the presence of IL-2. The second stage of T cell transformation usually occurs over a period of months to years, during which time a subpopulation of immortalized T cells acquire chromosomal abnormalities and the capacity for IL-2-independent growth.

Unlike most oncoviruses, HTLV-I does not contain a viral oncogene. The virus-encoded transactivator, Tax, has been the subject of intense investigation. In addition to its ability to activate HTLV-I gene expression, Tax can transform primary human T lymphocytes probably by increasing the expression of a unique set of cellular genes that are involved in T cell proliferation. Thus, activation of the specific signal transduction pathway by Tax could be critical for the induction of ATL by HTLV-I. An understanding of the mechanisms involved in Tax activation of cellular gene expression should increase our knowledge of how alterations in cellular gene expression correlate with cellular transformation. This review will discuss recent advances in our understanding of Tax function. In addition, we present a possible alternative mechanism involved in

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Tax-independent activation of signal transduction pathway in ATL cells *in vivo*, which might be involved in the late stage of leukemogenesis.

NF-κB/Rel pathway

Regulation of NF-κB/Rel activation

The prototypical nuclear factor (NF)-κB complex corresponds to a heterodimer of the p50 (NF-κB1) and RelA (p65) members of the NF-κB/Rel family of transcription factors[10-12]. In addition, this family includes RelB, c-Rel, and p52 (NF-κB2). Prior to activation, NF-κB exists in the cytoplasm tightly associated with inhibitory proteins, including IκBα and related proteins (IκBβ, IκBε, p105 [NF-κB1], and p100 [NF-κB2])[10-12]. The activity of NF-κB is stimulated widely by cytokines, oxidative stress, phorbol esters, and viral and bacterial infections. Activation of NF-κB is mediated by signal transduction cascades, which lead to activation of IκB kinases, IKKα and IKKβ[13-17]. These two catalytic subunits phosphorylate the IκBs, a modification of which triggers their ubiquitination and proteolysis, allowing the released NF-κB dimers to enter the nucleus and activate target genes[18]. How IKK is activated by the diverse signals remains unknown. Several upstream kinases, such as two mitogen-activated protein kinase kinase kinases (MAP3K), MEK kinase 1 (MEKK1) and NF-κB inducing kinase (NIK), have been proposed to serve as IKK kinases (IKKKs)[19-22]. Recent studies have identified IKK γ/NEMO (NF-κB Essential Modulator) in an IKK complex[23,24], which is thought to act as a mediator that connects IKK proteins with upstream activators.

Mechanisms of Tax action on NF-κB/Rel

Through NF-κB, Tax modulates the expression of several cytokines, chemokines, immune receptors, adhesion molecules, transcription factors, proto-oncogenes, and anti-apoptotic genes (Fig. 1). In HTLV-I-infected T-cell lines or cells expressing the Tax protein, NF-κB is constitutively localized in the nucleus[25]. Tax in part colocalizes with NF-κB in subnuclear regions that contain specific RNA transcripts from a promoter containing NF-κB binding sites[26-28]. Tax has been reported to interact physically with many members of the NF-κB/Rel family[29,30]. These findings suggest that Tax functions as a transcriptional cofactor acting cooperatively with NF-κB in the nucleus. However, Tax-mediated NF-κB activation is thought to be primarily regulated at the level of NF-κB nuclear translocation[31-34]. In fact, Tax in the cytoplasm can induce NF-κB nuclear translocation. For example, a Tax

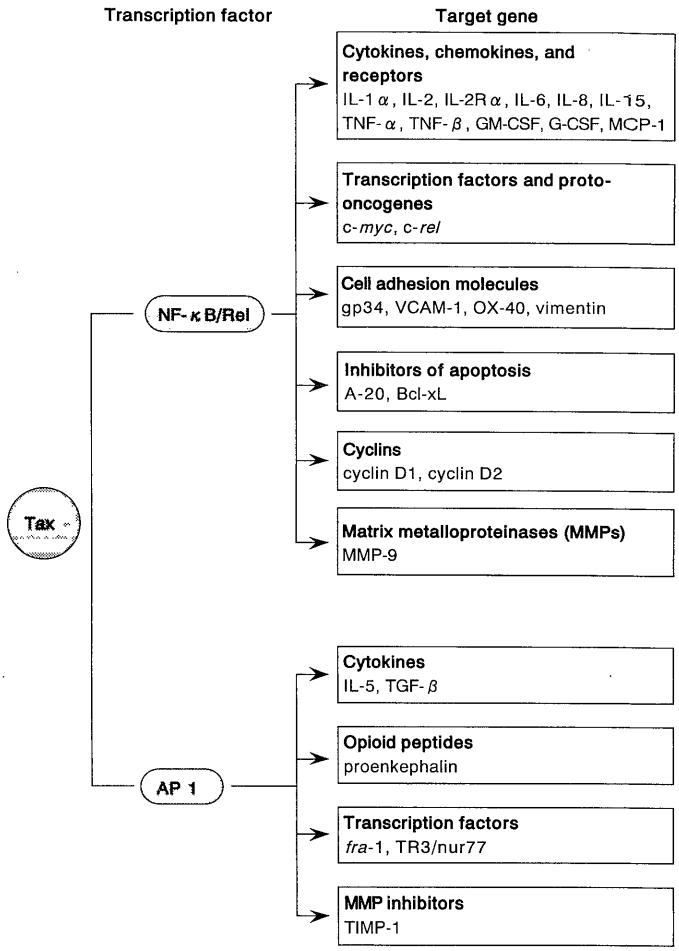

Fig. 1. Tax target and potential transcription factors NF-κB/Rel and AP-1.

mutant defective in nuclear localization can still activate NF-κB-dependent transcription[35]. A series of convincing studies have shown that Tax induces the phosphorylation and degradation of IκBα and IκBβ, suggesting that Tax may induce nuclear translocation of NF-κB by acting prior to or at the level of IκB phosphorylation[31-34, 36,37]. Resolution of this important missing link in the Tax/NF-κB signaling axis was achieved in conjunction with the identification of IKK. Tax triggers activation of both IKKα[38-40] and IKKβ[38-41], and this action requires NIK[39,40] and MEKK1[41]. Yamaoka *et al.*[24] established an IKKγ/NEMO-deficient rat fibroblast line that fails to constitutively express active forms of NF-κB in the presence of Tax. This NF-κB signaling defect could be rescued by overexpression of IKKγ/NEMO, suggesting that IKKγ/NEMO is required for the proper assembly of the Tax-responsive IKK complex. Several recent studies have indicated that Tax physically interacts with IKKγ/NEMO, which facilitates the recruitment of Tax to the catalytic subunits IKKα and IKKβ and promotes Tax-mediated activation

of IKK[42-44]. Recently, Sun and Ballard have proposed two models for Tax-directed activation of IKK (Fig. 2)[45]. In the first model, Tax recruits an upstream IKKK to the IKK signalsome, which triggers phosphorylation and activation of the IKKα and IKKβ. Attractive candidates for this Tax-responsive IKKK are MEKK1 and NIK. In the second model, Tax displaces a cellular IKK inhibitor (IKKI) from the holoenzyme, yielding a more favorable conformation for IKK autoactivation or for the action of a Tax-responsive IKK. Thus, Tax/IKKγ interaction may serve as a molecular trigger for IKK activation.

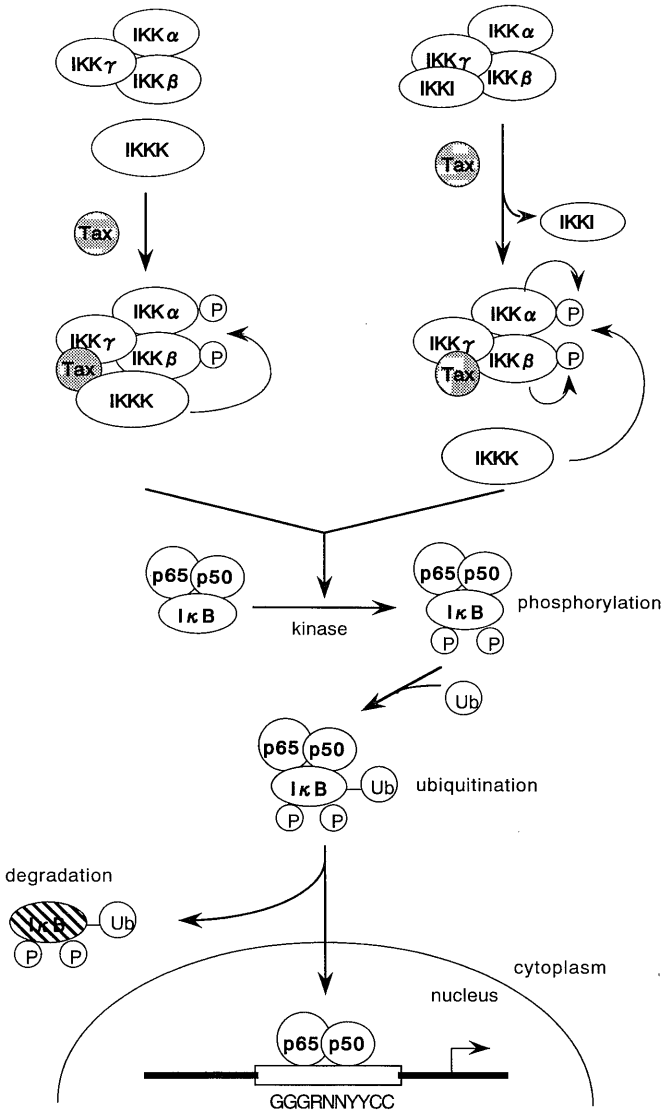

Fig. 2. Hypothetical schemes for Tax-directed activation of IKK. Tax recruits an upstream IKKK to the IKK sugnalsome, which triggers phosphorylation and activation of the IKKα and/or IKKβ catalytic subunit(s). Alternatively, removal of IKKI may render the IKKα and/or IKKβ catalytic subunit(s) more susceptible to activation by the upstream Tax-responsive IKKK. Attractive candidates for Tax-responsive IKKK are MEKK1 and NIK. IKK activation triggers subsequent signal transduction cascades that result in hyperphosphorylation of IκBs, ubiquitination and degradation by the 26S proteasome. After IκBs degradation, NF-κB translocates to the nucleus and activates NF-κB-dependent cellular genes.

Nuclear NF-κB DNA-binding activity, consisting of p50 and RelA (p65), is rapidly induced in Tax-expressing cells via a post-translational mechanism[32]. In contrast, DNA-binding complexes composed of c-Rel and/or p52 are activated with relatively delayed kinetics, suggesting a requirement for *de novo* protein synthesis[32]. Consistent with these studies, the c-Rel-containing complexes have also been shown to be the major NF-κB binding complexes in HTLV-I-infected T-cell lines that constitutively express high levels of Tax[34,46-49]. Tax-mediated phosphorylation and degradation of IκB may contribute to the early-phase induction of the p50/RelA (p65) heterodimer, while the sustained nuclear expression of c-Rel heterodimers appears to be mediated by additional mechanisms. The genes encoding c-Rel and p52 are under the transcriptional control of RelA (p65)[34,50], implying that activation of NF-κB is a prerequisite for the late-phase induction of c-Rel/p52 heterodimers.

Constitutive NF-κB/Rel activation in ATL

Leukemic cells of ATL patients express very low levels of viral genes including Tax, suggesting that the expression of viral proteins is not necessary for leukemic proliferation at the late stage of the disease. However, several genes that can be transactivated by Tax are also constitutively expressed in leukemic cells of ATL patients, probably by a Tax-independent mechanism. For instance, ATL cells *in vivo* display surface IL-2 receptor α chain (IL-2Rα) and express mRNA for cytokines and chemokines including IL-1α, IL-6, IL-8, tumor necrosis factor β, and monocyte chemoattractant protein-1[51]. Tax activates the transcription of these cellular genes through the NF-κB binding site (Fig. 1). Recently, we found that leukemic cells from ATL patients *in vivo*, like HTLV-I-transformed T-cell lines *in vitro*, display a constitutive NF-κB binding activity and increased degradation of IκBα[49]. Thus, the NF-κB/Rel pathway is activated in leukemic cells of ATL patients *in vivo*, which may account for the persistent expression of certain cellular genes. We also noticed that the components of the activated NF-κB/Rel in primary ATL cells are different from those in HTLV-I-transformed cell lines expressing Tax *in vitro*. Gel-shift analysis demonstrated that NF-κB complexes are composed of p50/p50 and p50/RelA (p65) dimers in both primary ATL cells and ATL-derived T-cell lines, which do not express Tax at all, whereas the complexes consist mostly of p50/c-Rel in Tax-expressing HTLV-I-transformed T-cell lines. Our studies suggest that con-

stitutive expression of Tax does not appear to be necessary for maintenance of NF-κB/Rel activation in ATL cells *in vivo* and this activation occurs through a Tax-independent mechanism, possibly due to activation of a different NF-κB subunit.

Role of NF-κB/Rel in Tax oncoprotein function

Several studies have suggested that activation of NF-κB/Rel by Tax is essential for the transformation of rodent cells and human primary T lymphocytes[52-55]. Inhibition of NF-κB activity by antisense NF-κB p65 oligonucleotides interferes with the growth of Tax-transformed cell lines both *in vitro* and *in vivo*[56]. Furthermore, recent experiments conducted with infectious molecular clones of HTLV-I DNA indicate that activation of the NF-κB pathway is critical for the immortalization of T cells by HTLV-I[57]. Recent results obtained with CTLL-2, a mouse IL-2-dependent T-cell line, have suggested that NF-κB activation is the key to increasing the survival of HTLV-I-infected T cells. CTLL-2 cell lines expressing wild-type Tax or a Tax mutant, which is still an activator of NF-κB, are resistant to apoptosis induced in the absence of IL-2[58]. On the other hand, a Tax mutant, which is inactive for NF-κB, enhances apoptosis in the absence of IL-2. The molecular mechanism of Tax-induced anti-apoptotic pathway could involve Bcl-x_L, one of the molecules protecting cells against apoptosis. We and others have recently demonstrated that Tax upregulates the Bcl-x_L gene through NF-κB pathway[59-61].

AP-1 pathway

Regulation of AP-1

The AP-1 complex is formed by three Jun-family members (c-Jun, JunB, and JunD) and four Fos-family members (c-Fos, FosB, Fra-1, and Fra-2), giving rise to a large variety of homo- and hetero-dimers binding to the same DNA consensus sequence, and are responsible for the activation of various target genes involved in the control of cell proliferation, tumorigenesis, and metastasis[62,63]. A causal role of AP-1 in neoplastic transformation has been demonstrated: a dominant negative c-Jun is able to suppress the ras-mediated transformation[64-66]. In addition, it has been reported that cell lines established from c-Jun knock out mice are resistant to ras-mediated transformation[67]. AP-1 proteins are inactive in quiescent cells but are rapidly activated by a variety of extracellular stimuli, including growth factors, cytokines, and cellular stress signals[68,69]. Activation of AP-1 is regulated at the transcriptional and posttranscriptional level. Activation of c-Jun N-terminal kinases (JNK, MAP kinase family member), also known as stress-activated protein kinases (SAPKs), phosphorylates c-Jun at Ser 63 and Ser 73 within the N-terminal activation domain, and stimulates its transcriptional activity[70-72]. The stimulation of transcriptional activity of c-Jun after phosphorylation appears to be due to a higher affinity for the co-activator CBP. In addition, the phosphorylation of c-Jun by JNK reduces ubiquitination and degradation, thereby increasing its stability[73].

Increase in AP-1 activity is a general event at a late stage in T-cells transformed by HTLV-I

T-cell lines transformed by HTLV-I express high levels of mRNA encoding c-Fos, Fra-1, c-Jun, JunB, and JunD[74,77], and have high levels of AP-1 activity[74-76]. Some of these genes are induced by Tax[74,78-80]. Indeed, Tax activates several cellular genes via an AP-1-like site (Fig. 1). Recently, we analyzed the AP-1 activity in peripheral leukemic cells from ATL patients. All fresh peripheral blood leukemic cells prepared from eleven patients exhibited increased AP-1 activity containing JunD, while the activity was not increased in normal peripheral blood mononuclear cells[81]. JunD homodimer generally forms unstable complexes with DNA[82,83]. However, our gel-shift analysis indicated that none of the characterized Fos family members (c-Fos, FosB, Fra-1, or Fra-2) was included in AP-1 binding complex of primary ATL cells. Thus, a factor, different from any currently characterized AP-1 transcription factor, is likely to be a component of the AP-1-binding complex in ATL cells. In addition to primary ATL cells, all T-cell lines derived from ATL patients also displayed constitutive AP-1 activity, but expressed little Tax protein. These findings suggest that Tax is not the only mechanism for constitutive activation of AP-1 in HTLV-I-infected T cells *in vitro* as well as *in vivo*. On the other hand, the JNK kinase activity was constitutively activated in human lymphocytes transformed *in vitro* by HTLV-I[84]. Furthermore, such activation occurs only when HTLV-I-transformed lymphocytes become IL-2 independent. However, Tax does not directly activate JNK because JNK activation is not observed in cells transfected with Tax expression vector. Constitutive JNK activation was also detected in leukemic cells of ATL patients. Taken together, these findings suggest that the acquisition of constitutive JNK activation and subsequent AP-1 activation represent an important late event in HTLV-I leukemogenesis.

Jak/STAT signaling pathway

Inappropriate Jak/STAT activation in HTLV-I-transformed T-cell lines and primary ATL cells

The major growth-stimulatory cytokine for T cells is IL-2. The IL-2R is composed of at least three chains. The α chain is involved in increasing ligand-binding affinity, and the β and common γ (γ_c) chains are necessary and sufficient for transduction of the IL-2 signal[85]. IL-2R β and γ_c chains are members of the cytokine receptor superfamily. None of IL-2R chains has a catalytic kinase domain, but like interferon receptors, IL-2R β and γ_c chains use the Janus (Jak) family of cytoplasmic tyrosine kinases and the signal transducer and activator of transcription (STAT) proteins as one important mechanism to transduce their signals. Upon binding of IL-2 to its receptor, Jak3 is recruited to the γ_c chain, while Jak1 is coupled to the IL-2Rβ chain[86-88]. These kinases are activated upon IL-2 signaling[89] and phosphorylate STAT3, STAT5A, and STAT5B proteins[90,91], allowing these proteins to form homo- and hetro-dimers, translocate to the nucleus, and bind DNA in a sequence-specific manner to initiate transcription of IL-2 responsive genes. Constitutive activation of Jaks and/or STATs is associated with cell transformation. For instance, in Drosophila, dominant activation of Jak leads to malignant neoplasia of hematopoietic cells[92]. Inappropriate activation of STAT has been described in many solid and hematologic malignancies. The role of Jak/STAT activation has been examined in T-cell lines transformed with HTLV-I. Jak1, Jak3, STAT3, and STAT5 proteins were constitutively activated in some IL-2-independent HTLV-I-transformed T-cell lines, whereas no constitutive Jak/STAT activation was observed in HTLV-I-uninfected T-cell lines and IL-2-dependent HTLV-I-transformed ones. Thus, activation of the Jak/STAT pathway in HTLV-I-transformed T-cell lines correlates with the transition from an IL-2-dependent to an IL-2-independent growth[93,94].

Jak/STAT activation has been examined in primary peripheral lymphocytes obtained from ATL patients[95]. Eight out of the twelve patients exhibited constitutive tyrosine phosphorylation of STAT proteins as well as constitutive STAT binding to the sis-inducible element from the c-*fos* promoter and the prolactin responsive element from the β-casein promoter. STAT3 activation was found in six patients (50%), and four exhibited activation of both STAT3 and STAT1. Activation of STAT5 was identified in four patients (33%), and two patients (17%) exhibited activation of both STAT3 and STAT5. Phosphorylation of Jak3 in leukemic cells was associated with activation of STAT3. In contrast, Zhang *et al.*[96] demonstrated no detectable basal phosphorylation of Jak3, STAT3, and STAT5 in leukemic cells of all 8 ATL patients. The reasons for the discrepancy between these two sets of data are unclear but may be related to the relatively small group of samples tested (12 and 8, respectively) and/or differences in methodology. Recently, experiments by Tsukada *et al.*[97], using a probe for the GAS (γ interferon activation site)-like element termed LILRE (LPS/IL-1-responsive element), which is found in the human proIL-1β gene, revealed constitutive activation of LIL-STAT (LPS/IL-1-induced STAT) in all 7 ATL patients, although the precise significance of this activation in leukemogenesis remains unclear at present.

Mechanism of constitutive activation of Jak/STAT pathway in HTLV-I-transformed T cells

HTLV-I-transformed T-cell lines express higher amounts of STAT1, STAT3, and STAT5 mRNA and proteins than HTLV-I-negative cells[98]. Tax induces the expression of STAT1 and STAT5 in a T-cell line, however, it does not induce DNA binding activity of these STAT proteins and Jak3 activation[93,99]. Indeed, Tax-expressing HTLV-I-infected T-cell lines do not always express constitutive DNA binding activity of STAT[99]. Thus, the mechanisms by which HTLV-I transformation induces activation of the Jak/STAT pathway remains to be determined. Some HTLV-I-transformed T-cell lines such as HUT-102 can produce IL-15[100], a T-cell growth factor that activates STAT3 and STAT5[90]. However, antibodies against IL-15 do not substantially decrease the activation level of Jak/STAT proteins[93,95]. Interestingly, conditioned medium from HTLV-I-transformed T-cell lines could induce STAT5 activation in HTLV-I-uninfected T-cell lines (Yamaoka *et al.*, unpublished results). Thus, activation of Jak/STAT pathway in HTLV-I-infected cells may be due to an autocrine or paracrine stimulation.

Concluding remarks

In our current model of Tax induced leukemogenesis (Fig. 3), Tax causes proliferation of HTLV-I-infected T cells by activation of transcription factors including NF-κB and AP-1. Because Tax does not appear to be expressed in primary ATL cells, HTLV-I-infected T cells with constitutive activation of both NF-κB and AP-1 in a Tax-independent manner, are selected during late stages of leukemogenesis probably by mutations or alterations of cellular genes. This may result in reduced requirements of growth factors and may be a crucial step in a leukemic proliferation in vivo. The finding that ATL cells utilize a variety of mechanisms to activate

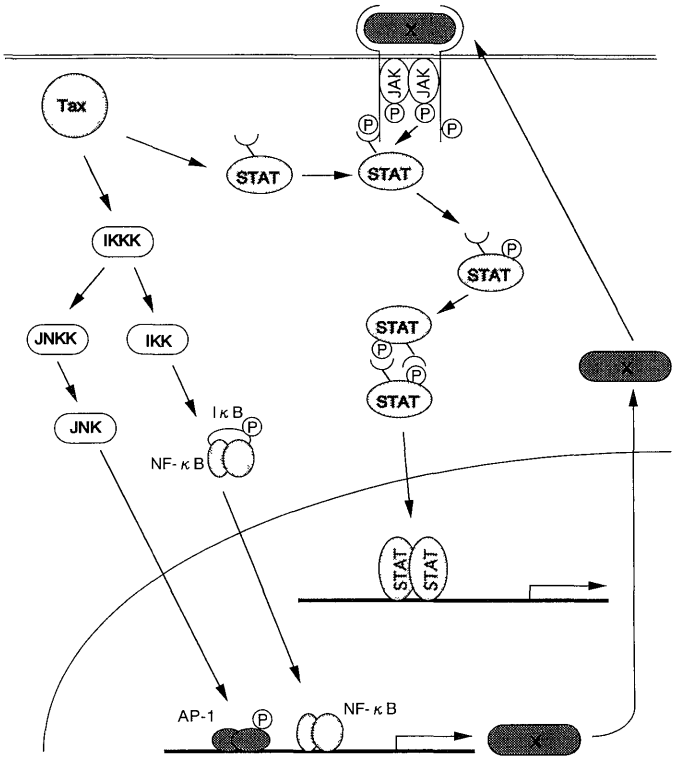

Fig. 3. Schematic diagram of hypothesized role of Tax in transformation.

several signal transduction pathways suggests that such property may be critical for leukemogenesis. We are beginning to understand how HTLV-I interacts with cellular factors to activate and modulate cellular signal transduction pathways leading to the leukemic proliferation of virus-infected cells. However, we do not know which upstream genetic changes in HTLV-I-infected cells result in the activation of these signaling cascades. Further characterization of these signaling cascades in HTLV-I-infected cells *in vitro* as well as *in vivo* should contribute to our understanding of ATL oncogenesis and identify a unique target for the treatment of this unusual leukemia.

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