Involvement of NF-AT in Type I Human T-cell Leukemia Virus Tax-mediated Fas Ligand Promoter Transactivation*

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Isis Rivera, Edward W. Harhaj†, and Shao-Cong Sun§

From the Department of Microbiology and Immunology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

Human T-cell leukemia virus type I (HTLV-I)-infected T-cells constitutively express surface Fas ligand (FasL), which may serve as a mechanism of viral pathogenesis. HTLV-I induces transcription of FasL gene through the viral transactivator Tax, although the underlying molecular mechanism remains unclear. In the present study, we have analyzed both the cis-activating element and transactivating factors involved in Tax activation of the FasL promoter. We show that the 486-base pair upstream region of the human FasL gene is sufficient for Tax-mediated transactivation in Jurkat T-cells. Interestingly, a palindromic DNA sequence (GGAAACTTCG) covering the consensus NF-AT binding site (GGAAA), is required for Tax activation of FasL promoter. The involvement of NF-AT in this transactivation event is suggested by the finding that Tax fails to activate the FasL promoter in a Jurkat T-cell line defective in capacitative calcium entry, a signaling mechanism involved in NF-AT activation. Furthermore, activation of FasL promoter by Tax is largely attenuated in the nonlymphoid F9 embryonal and COS kidney cells deficient in NF-ATp expression. DNA-protein interaction assays reveal that the NF-AT-like motif in the FasL promoter is bound by both NF-ATp and NF-AT4 in Jurkat T-cells expressing Tax. The binding of NF-ATp, although not NF-AT4, to this enhancer also occurs along with HTLV-I-mediated infection of human peripheral blood T-cells. Furthermore, exogenously transfected NF-ATp binds to the NF-AT-like enhancer and participates in Tax-mediated FasL promoter transactivation. These results suggest an important role for proteins of the NF-AT family in HTLV-I Tax-mediated FasL gene transactivation.

The human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of a T-cell malignancy termed adult T-cell leukemia (1, 2) and a number of nonmalignant diseases (3–5). HTLV-I encodes a 40-kDa regulatory protein, Tax, which serves as a key viral component in the induction of host cell transformation (6–11). Tax not only serves as the HTLV-I viral transactivator but also induces transcriptional expression of a large number of cellular genes, such as those encoding interleukin 2 (IL-2) (12, 13), the α-chain of IL-2 receptor (13–15), and the granulocyte-macrophage colony-stimulating factor (16). The central role of these genes in normal T-cell activation and growth suggests that this specific action of Tax may provide an important mechanism for HTLV-I-induced T-cell transformation.

Although Tax clearly plays a key role in HTLV-I-induced T-cell growth and transformation, recent studies have suggested additional functions for this viral transactivator. Specifically, Tax is shown to transactivate the gene encoding Fas ligand (FasL) (17), a member of the tumor necrosis factor family specifically interacting with a cell surface receptor, Fas (18, 19). Binding of FasL to Fas triggers programmed cell death or apoptosis of the Fas-bearing cells (18, 20, 21). The FasL/Fas-mediated apoptosis is largely controlled by the expression of FasL. Although Fas is constitutively expressed in a large variety of cell types and tissues (21), FasL is predominantly expressed in activated T-cells (20). The induced expression of FasL in activated T-cells provides an important mechanism for the immune system to limit the expansion of antigen-stimulated T-cells when they are no longer needed (22). In addition to its inducible expression in T-cells, FasL is constitutively expressed in stroma cells of retina and Sertoli cells of testis (23, 24). At these sites, FasL appears to induce apoptosis in infiltrating activated lymphocytes, a mechanism involved in the maintenance of immune privilege (23–25). Interestingly, recent studies have shown that certain tumor cells, including those of nonlymphoid origin, constitutively express FasL, which contributes to immune privilege of tumors (26, 27). Similarly, viruses may induce the expression of FasL in host cells, providing a route for escaping the immune destruction. In this regard, the Nef protein of simian immune deficiency virus has been shown to up-regulate FasL, leading to apoptotic death of virus-specific cytotoxic T-cells (28). As mentioned above, the HTLV-I Tax protein also induces expression of FasL, although it remains to be examined whether the Tax-expressing cells participate in the inactivation of anti-HTLV immune response. Nevertheless, Tax has been shown to induce cell death in vitro (29, 30).

Previous studies have suggested that Tax induces the viral and cellular genes by targeting two major cellular transcription factor pathways, the NF-κB/Rel and CREB/ATF (31, 32). Specifically, Tax activates long terminal repeat (LTR) of HTLV-I by physical interaction with the CREB/ATF family of proteins, which specifically bind to the 21-base pair repeats present in HTLV-I LTR (33–36). On the other hand, Tax-mediated activation of many cellular genes, such as IL-2 receptor and granulocyte-macrophage colony-stimulating factor, is mediated by
the NF-κB/Rel transcription factors (16, 37–39). Additionally, we and others have recently shown that Tax induces the transcription of the IL-2 gene through activation of cellular transcription factors that bind to an IL-2 gene enhancer termed CD28-responsive element (CD28RE) (40–43). Interestingly, in addition to NF-κB/Rel, the CD28RE is also bound by members from the NF-AT family (42–46). NF-AT represents a family of enhancer binding proteins that participate in the regulation of IL-2 as well as a large number of other cytokine genes (47–49). The NF-AT family includes at least four structurally related proteins, NF-ATp, NF-ATc, NF-AT3, and NF-AT4 (50–54). The biological activity of NF-AT is regulated by a calcium-dependent phosphatase, calcineurin (55–58). Treatment of T-cells with stimuli of the T-cell receptor complex or the calcium ionophore ionomycin results in the rapid nuclear expression and activation of NF-AT (55, 57–59). We have previously shown that NF-ATp appears to be an important factor regulating the CD28RE enhancer of IL-2 gene (42).

In the present study, we have investigated the mechanism by which the FasL gene is transcriptionally induced in Tax-expressing cells. We provide evidence showing the involvement of NF-AT in Tax-mediated FasL promoter transactivation.

METHODS AND MATERIALS

Cell Lines, Plasmid Constructs, and Antisera—The Jurkat leukaemic T-cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. Jurkat cells stably transfected with a Tax cDNA expression vector (Jurkat-Tax (60)) were maintained in the same complete medium supplemented with 400 μg/ml G418 antibiotic to select for expression of the neomycin resistance gene. The mutant cell line M108 and its parental wild type control (Jurkat-Trunc) were kindly provided by Dr. G. Crabtree (61). MT-2 is an IL-2-independent HTLV-I-transformed human T-cell line (62) and was cultured in RPMI medium. Monkey kidney COS7 cells were cultured in Iscove’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. The cDNA expression vectors encoding NF-ATpF1, NF-ATpX5, and Tax and its mutants have been described previously (63, 64). The luciferase reporter plasmids driven by a 486-bp upstream sequence from the wild type (FasL-luc, previously named CD95L-486) or mutant form (FasLMut-luc, previously named CD95L-486) of FasL promoter (77) (the coding strand sequence was 5’-CCC GAA ACT TCC AGG GGT-3’ followed by resolving the DNA-protein complexes on native 5% polyacrylamide gels (78). For antibody supershift assays, 0.5 μl of diluted or undiluted polyclonal antisera was added to the EMSA reactions 15 min before electrophoresis. The anti-NF-ATp antisera (Upstate Biotechnology) was diluted 10 times or as indicated; antisera for all the other proteins were used undiluted.

RESULTS

A 486-bp Promoter Sequence of the FasL Gene Is Sufficient for Tax-mediated Transactivation—To localize the regions of FasL promoter that modulates Tax-mediated transactivation signal, we examined whether a truncated FasL promoter covering a 486-bp sequence upstream of its translational initiation site (ATG) could respond to Tax. For these studies, a control luciferase plasmid (Luc) lacking a promoter or a luciferase reporter driven by the 486-bp FasL promoter sequence (FasL-luc, for sequence information, see Ref. 77) was transfected into Jurkat T-cells either alone or together with Tax. As expected, Tax had no significant effect on the expression of the control luciferase reporter (Fig. 1A, column 2). However, this viral transactivator potently induced the FasL-luc expression, resulting in an marked increase in luciferase activity (column 4). We also examined whether Tax synergized with other FasL-stimulating signals. In this regard, recent studies demonstrated that FasL promoter could be activated by mitogens, including phorbol 12-myristic 13-acetate (PMA) and ionomycin (65), which stimulate the protein kinase C and calcium signals, respectively. We found that stimulation of Jurkat cell with PMA together with Tax at 40–48 h led to a low level (3–4-fold) induction of the FasL-luc reporter gene activity (Fig. 1B, column 4). When the cells were transfected with the Tax expression vector, however, a markedly higher level of reporter gene activity was induced by these mitogens (column 8). Stimulation of the Tax-expressing cells with PMA and ionomycin separately also potentiated certain levels of Tax-mediated transactivation.
Thus, the proximal region of the FasL promoter contains the Tax-responsive regulatory elements, and the mitogenic signals synergize with Tax in the induction of the FasL promoter activity.

**Tax Activation of FasL Promoter Requires Multiple Signals—**

Prior studies have demonstrated that Tax activation of many target genes is mediated by NF-κB or CREB/ATF transcription factors (32), and mutational analyses have led to the identification of Tax mutants defective in either the NF-κB or CREB/ATF pathway (64, 79). For example, the M47 mutant is defective in CREB/ATF activation but remains fully active in the activation of NF-κB (64). On the other hand, the M22 mutant is active in the CREB/ATF pathway but not the NF-κB pathway. To investigate whether Tax-mediated activation of *FasL* gene involves similar pathways, the effect of these two well-characterized Tax mutants on FasL promoter activity was examined by reporter gene assays (Fig. 2). Although the mutations introduced into M22 and M47 Tax mutants almost completely abolished the induction of NF-κB and CREB/ATF pathways, respectively (data not shown, also see Ref. 64), both mutants still retained partial activity in FasL-luc induction (Fig. 2A, columns 5 and 9). More importantly, both M22 and M47 were capable of cooperating with mitogens, PMA and ionomycin, leading to strong induction of the reporter gene expression (columns 6–8 and 10–12). These results suggest that Tax-mediated activation of the *FasL* gene may involve multiple signals and transcription factor pathways.

To further examine the mechanism of *FasL* gene transactivation, reporter gene studies were performed in different cell lines to compare the cellular conditions required for Tax-mediated activation of FasL promoter and the NF-κB and CREB/ATF transactivation pathways. As expected, in Jurkat cells, Tax transactivated the FasL-luc as well as the reporters for NF-κB (kB-TATA-luc) and CREB/ATF (HTLV-I LTR-luc) (data not shown). However, in undifferentiated embryonal F9 cells, although the HTLV-I LTR
was efficiently transactivated by Tax, FasL-luc failed to respond to Tax (Fig. 2B), suggesting that the F9 cells lack critical factors for FasL promoter transactivation, although they could provide the conditions for Tax activation of HTLV-I LTR. Studies performed with COS cells revealed that transactivation of FasL promoter also differed from that of the kB enhancer. In these monkey kidney cells, Tax transactivated the kB-TATA-luc reporter; however, under the same conditions, the expression of the FasL-luc was only modestly enhanced by Tax (Fig. 2C). Thus, Tax-mediated FasL gene activation likely involves a mechanism distinctive from that of activation of the NF-kB or CREB/ATF target genes.

Capacitative Calcium Entry Appears to Be Required for Tax Activation of FasL Promoter—To further explore the signals involved in Tax-mediated activation of FasL gene, studies were performed using mutant Jurkat cell lines defective in known signaling pathways. In this regard, a cell line, M108, has previously been shown to be defective in capacitative calcium entry (80) and, therefore, to have a blockade in mitogen-mediated activation of NF-AT transcription factors (61). In the M108 cells, Tax was still effective in activation of the kB enhancer because similar levels of kB-TATA-luc activity were induced by Tax in both the wild type Jurkat cells (WT) and the mutant cells (M108) (Fig. 3, columns 2 and 6). This result suggests that the Tax-mediated NF-kB activation pathway is not affected by the mutations in the M108 cells. Interestingly, in sharp contrast, activation of the FasL promoter by Tax was markedly diminished in the mutant cells (compare columns 4 and 8). These findings raised the possibility that Tax-mediated activation of the FasL gene might require the calcium regulatory transcription factor NF-AT.

An NF-AT-like Enhancer Is Required for Tax-mediated FasL Promoter Transactivation—To investigate the transcription factors involved in Tax activation of FasL, we examined the cis-acting element required for Tax-mediated transactivation in the 486-bp promoter region of FasL gene (77). In this regard, an NF-AT-like motif was previously shown to be essential for activation of the FasL promoter by T-cell receptor-mediated signals (65). This motif contains a palindromic sequence, GGAAAcTTC, located at nucleotide 267 to 276 upstream of the translational initiation site (ATG). This sequence covers a consensus NF-ATp binding site (GGAAA, Ref. 48), although it lacks an adjacent binding site (AP-1) for Fos/Jun proteins, which serve as nuclear partners of NF-ATp (48). To examine the role of this palindromic sequence in Tax-mediated FasL promoter activation, a mutant FasL-luc reporter harboring a mutation at the palindromic sequence (Fig. 4, column 10) was used in the reporter gene assays. As expected, the mutant reporter failed to respond to the mitogen signals (column 10). More importantly, this mutation also dramatically diminished Tax-mediated activation of the FasL promoter in both untreated and mitogen-stimulated cells (Fig. 4, columns 11 and 12).

NF-ATp and NF-AT4 Are the Major Factors Binding to the FasL NF-AT-like Enhancer in Tax-expressing Cells—EMSA was performed to analyze the transcription factors involved in binding to the NF-AT-like enhancer. Weak constitutive DNA binding activity was detected from the parental Jurkat cells (Fig. 5A, lane 1, arrow). However, in Jurkat cells stably transfected with a Tax cDNA expression vector (Jurkat-Tax, see Ref. 60), much stronger NF-AT-like DNA binding activity was detected (lane 2, arrow). On the other hand, the DNA binding activity of a nonspecific DNA binding protein (ns) was similar in the Jurkat and Jurkat-Tax cells (lanes 1 and 2, ns), suggesting that the induction of NF-AT-like DNA binding activity was specific. Antibody supershift assays revealed that the protein complexes formed with the NF-AT-like enhancer immunoreacted with antisera specific for NF-ATp (Fig. 5A, lanes 4) and NF-AT4 (lane 8). The addition of an anti-NF-ATc antiserum to the EMSA reaction slightly affected the formation of the DNA-protein complex but did not generate obvious supershifts (lane

**Fig. 3.** Transactivation of FasL-luc, but not kB-TATA-luc, is blocked in mutant Jurkat cells defective in capacitative calcium entry. The wild type parental Jurkat cells (WT) or the mutant M108 cells were transfected with the kB-TATA-luc or FasL-luc together with either pCMV4 (Vector) or pCMV4-Tax (Tax) followed by luciferase assay as described in Fig. 1A.

**Fig. 4.** A palindromic enhancer sequence is required for Tax activation of FasL promoter. Jurkat T-cells were transfected with the indicated reporters either alone or together with pCMV4-Tax (0.5 µg), as indicated. The cells were either not treated or treated (+) for 8 h with PMA (10 ng/ml) and ionomycin (Iono, 1 µM) followed by luciferase assays as described in Fig. 1A. The data shown are representative of at least three independent experiments. The FasL-luc and FasLMut-luc reporters are illustrated above the figure. Mutations introduced to the FasL promoter of FasLMut-luc are underlined.
No immunoreactivity was detected with the anti-NF-AT3 antiserum (lane 7) or antisera for the p50 (lane 5) and other NF-κB subunits (data not shown). Thus, NF-ATp and NF-AT4 are the predominant nuclear factors associating with the FasL palindromic sequence element in Jurkat-Tax cells.

To assess the physiologic relevance of our findings with Jurkat-Tax cells, we next examined whether binding of NF-ATp and NF-AT4 to the FasL enhancer also occurs in HTLV-I-infected cells. We prepared both normal (uninfected) and HTLV-I-infected human peripheral blood T-cells from the same blood sample (see “Materials and Methods”) and then analyzed their nuclear factors binding to the FasL palindromic sequence by EMSA. No DNA-protein complexes were detected from normal T-cells (Fig. 5B, lane 1). In sharp contrast, a strong complex was detected in the HTLV-I-infected T-cells (lane 2). As demonstrated in Jurkat-Tax cells, the DNA-protein complex detected in these virus-infected cells was supershifted by the anti-NF-ATp antibody (lane 4) but did not react with antiserum for various NF-κB subunits, including p50, p52, RelA, c-Rel, and Bcl-3 (lanes 5–9). However, parallel supershift assays revealed that the DNA-protein complex formed in these HTLV-I-infected T-cells did not appear to contain NF-AT4 because no obvious immunoreactivity was detected between the complexes and the anti-NF-AT4 antiserum (Fig. 5C, lane 6). These cells also lack the binding activity of NF-ATc and NF-AT3 (lanes 8 and 9). Of note, all the antiserum used in these assays were proved to be efficient in specifically supershifting their corresponding proteins (data not shown and Refs. 63 and 68). We also examined the FasL enhancer binding activity in an IL-2-independent HTLV-I-transformed cell line (MT-2, Fig. 5C, lanes 10–16) and obtained similar results. Parallel immunoblotting assays confirmed the expression of Tax in Jurkat-Tax cells as well as the HTLV-I-infected cells (Fig. 5D). Together, these findings suggest that NF-ATp is a major factor regulating the NF-AT-like enhancer of the FasL gene in both Tax-transfected and HTLV-I-infected T-cells, whereas NF-AT4 may participate in the enhancer regulation under certain cellular conditions.

Transfected NF-ATp Binds to the NF-AT-like Enhancer and Participates in Tax-induced FasL-luc Transactivation—To further examine the role of NF-ATp in FasL gene induction by Tax, studies were performed to test whether transfected NF-ATp can bind to the FasL NF-AT-like sequence. Expression vectors encoding either the full-length (NF-ATpFL) or a constitutively active form (NF-ATXS, Ref. 63) of NF-ATp were transfected into COS cells, and whole-cell extracts of the transfec-
activation of Fas ligand promoter by HTLV-I Tax

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Figure 6. Transfected NF-ATp binds to the FasL enhancer and participates in Tax-mediated FasL-luc transactivation. A, both the full-length and a truncated form of NF-ATp bind to the FasL palindromic enhancer. COS cells were transfected in 6-well plates with either the pCMV4 parental vector or cDNA expression vectors encoding the full-length (FL) or a truncated form (XS) of NF-ATp (0.25 μg). Whole-cell extracts were subjected to EMSA. The DNA-protein complexes formed between the probe and NF-ATp molecules are indicated by arrows. ns indicates a nonspecific band. B, NF-ATp synergizes with Tax in FasL-luc transactivation. Jurkat (column 1–4) or COS (columns 5–8) cells were transfected with FasL-luc (2.5 μg for Jurkat and 0.5 μg for COS cells) together with 0.25 μg of either pCMV4 (Vector) or the indicated expression vectors, followed by luciferase assays as described in the legend of Fig. 1A.

no detectable effect on the expression of the FasL-luc reporter gene (Fig. 6B, column 2), suggesting that NF-ATp alone was insufficient for activation of the FasL promoter. However, when NF-ATpXS was coexpressed with Tax in the cells, a marked synergy was detected in the reporter gene induction (column 4). These results strongly suggest that NF-ATp functionally participates in Tax-mediated FasL gene transactivation. We also performed similar studies using COS cells. As expected, Tax only modestly induced the reporter in these nonlymphoid cells (Fig. 6B, column 7). However, as seen in Jurkat cells, expression of NF-ATpXS significantly enhanced Tax-mediated reporter gene activation (Fig. 6B, column 8).

DISCUSSION

The HTLV-I Tax protein is remarkable for its ability to transactivate a large variety of transcription units, including both the HTLV-I LTR and various cellular genes (32, 71). Many Tax target genes encode proteins involved in cell-cell interaction and the regulation of cell growth (81). Aberrant expression of these cellular genes has been implicated in HTLV-I-induced T-cell proliferation and subsequent transformation. Recent studies have also identified various Tax target genes with novel functions (81), most interestingly the gene encoding FasL (17). Induction of this death-related cell surface molecule may contribute to Tax-mediated apoptosis observed in cell culture (29, 30). It may also provide a potential mechanism by which HTLV-I-infected T-cells evade immune destruction in vivo, because FasL-expressing cells can trigger antigen-specific cytotoxic T-cells to undergo apoptosis (26–28).

In the present study, we have investigated the transcription factor pathways participating in Tax-mediated induction of FasL promoter. Our data suggest that Tax activation of FasL gene may involve different transcription factor pathways. Consistent with a prior study (17), we have shown that Tax mutants defective in either the NF-κB (M22) or CREB/ATF (M47) transcription factor pathways exhibit markedly diminished FasL transactivation activity compared with the wild type Tax. However, both Tax mutants are able to synergize with mitogens, PMA, and ionomycin, resulting in significantly higher levels of transactivation (Fig. 2A). Furthermore, when coexpressed in Jurkat cells, TaxM22 and TaxM47 also synergize in FasL-luc activation (data not shown and Ref. 17). Although these results indicate that activation of FasL promoter by Tax may require both NF-κB and CREB/ATF (17), the fact that the 486-bp promoter region of FasL lacks binding sites for these factors suggests the involvement of a different mechanism. Indeed, we have shown that NF-ATp is a major transcription factor involved in Tax activation of the FasL gene. A palindromic sequence (GGAAACTTCC) covering the consensus NF-ATp binding site (GGAAA, Ref. 48) is important for Tax-mediated FasL promoter activation. Mutation of the NF-ATp binding site in this sequence dramatically affected the ability of the FasL promoter to respond to Tax-mediated transactivation. Furthermore, both endogenous and transfected NF-ATp bind to this sequence, which contributes to Tax-mediated FasL promoter activation. Consistent with this result, activation of the FasL-luc reporter by Tax is largely attenuated in a mutant Jurkat cell line (M108) defective in calcium entry (Fig. 3), a mechanism required for NF-AT activation (61). Similarly, Tax only weakly transactivates the FasL-luc in the nonlymphoid COS cells, which lack endogenous NF-ATp. Expression of exogenous NF-ATp in both COS and Jurkat cells significantly elevated Tax-mediated FasL promoter activation (Fig. 6B). Taken together with the recent biochemical and gene knockout studies (65, 82), these results strongly suggest that NF-ATp plays a critical role in the activation of FasL gene by both mitogens and HTLV-I Tax protein.

A recent study shows that activation of FasL by DNA damaging agents is mediated by NF-κB and AP-1 transcription factors (83). The FasL promoter contains binding sites for NF-κB and AP-1 in its upstream region (−1080 and −1048, respectively), and mutations in either site completely abolishes the signaling response to DNA damaging (83). Of note, Tax-mediated FasL activation differs from that induced by the DNA-damaging agents or the mitogens PMA and ionomycin. As shown in Fig. 1B, a FasL promoter lacking these upstream enhancer elements still responded to Tax, although this truncated form of FasL promoter was only weakly induced by mitogens. These findings strongly suggest that both the NF-κB and the AP-1 sites are dispensable for Tax-mediated FasL activation. Of course, our data cannot exclude the possibility that NF-κB or AP-1 are involved in functional cooperation with NF-AT in transactivating the proximal NF-AT enhancer in the FasL promoter (Fig. 4). In this regard, we have found that the transfected NF-ATp alone is unable to induce the FasL-luc reporter gene expression (Fig. 6B). When it is coexpressed with Tax, however, strong FasL promoter transactivation activity is produced. This result is reminiscent of our previous finding on Tax-mediated activation of the CD28RE enhancer (42). Thus, it seems likely that the function of NF-ATp may indeed require cofactors that can be induced by Tax. On the other hand, it is also possible that Tax induces posttranslational modification of NF-ATp, resulting in the activation of its transactivation activity. Nevertheless, our data clearly demonstrate that NF-ATp and probably also NF-AT4 contribute to Tax-mediated FasL gene transactivation.
