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Activation of the IL-2 gene promoter by HTLV-I Tax involves induction of NF-AT complexes bound to the CD28-responsive element

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The *tax* gene product of the type I human T-cell leukemia virus (HTLV-I) is a potent transcriptional activator of various growth-related cellular genes, including that encoding interleukin-2 (IL-2). Tax activation of many of these target genes appears to be mediated by the NF-κB/Rel and CREB/ATF family of cellular transcription factors. However, the mechanism by which Tax transactivates the IL-2 gene remains unclear. In the present study, we demonstrate that neither NF-κB/Rel nor CREB/ATF is sufficient for Tax-mediated activation of the IL-2 promoter. Two novel nuclear protein complexes are induced by Tax and specifically bind to an IL-2 gene enhancer, the CD28-responsive element (CD28RE). Immunobiochemical analyses suggest that these DNA binding complexes contain at least two members of the nuclear factor of activated T cells, NF-ATp and NF-ATc. However, the CD28 binding NF-AT complexes do not contain Jun and Fos family proteins that have been proposed to serve as NF-AT partners in the activation of the IL-2 NF-AT motif. Transient transfection studies demonstrate that the *in vivo* expressed NF-ATp binds to the CD28RE probe and enhances Tax-mediated activation of this critical IL-2 enhancer. We demonstrate further that binding of NF-AT to CD28RE is critical for Tax activation of the IL-2 promoter. Together, these results suggest a novel mechanism of Tax-mediated activation of the IL-2 gene, which involves the induction of NF-AT-containing CD28RE binding complexes.

Keywords: CD28RE/HTLV-I/IL-2/NF-AT/Tax

Introduction

The type I human T-cell leukemia virus (HTLV-I) is an oncogenic retrovirus that is etiologically associated with adult T-cell leukemia (ATL), an aggressive and often fatal malignancy of mature $CD4^+$ T lymphocytes (Poiesz *et al.*, 1980; Hinuma *et al.*, 1981; Kalyanaraman *et al.*, 1982; Yoshida *et al.*, 1982; Franchini *et al.*, 1984). The HTLV-I genome encodes a 40 kDa protein, termed Tax, that is required for transcriptional activation of HTLV-I long terminal repeats (LTRs) (Sodroski *et al.*, 1985). Emerging evidence suggests that this viral transactivator plays a central role in the initiation of T cell transformation by HTLV-I. For example, Tax has been shown to immortalize human primary $CD4^+$ cord blood lymphocytes in culture in the context of a non-transforming herpesvirus saimiri vector (Grassmann *et al.*, 1992). Furthermore, transgenic mice expressing Tax have been shown to develop multiple mesenchymal tumors (Hinrichs *et al.*, 1987; Nerenberg *et al.*, 1987) and lymphocytic leukemia (Grossman *et al.*, 1995).

Although the molecular mechanism by which Tax induces the transformation of T cells is not clear, it appears that cellular factors are critically involved. Tax has been shown to activate transcription of not only the HTLV-I LTR but also various cellular genes, such as those encoding the c-*fos* proto-oncogene (Fujii *et al.*, 1988; Nagata *et al.*, 1989; Alexandre and Verrier, 1991), the T cell growth factor interleukin (IL)-2 (Maruyama *et al.*, 1987; Siekevitz *et al.*, 1987) and the α-subunit of its high affinity receptor complex (IL-2Rα) (Inoue *et al.*, 1986; Cross *et al.*, 1987; Siekevitz *et al.*, 1987). The central role of these genes in normal T cell activation and growth suggests that this specific action of Tax may be an important mechanism underlying HTLV-I-induced T cell transformation (Green and Chen, 1990; Smith and Greene, 1991).

Lacking DNA binding activity, Tax appears to induce the target genes indirectly by modulating the activity or expression of specific host transcription factors (Greene *et al.*, 1989; Smith and Greene, 1991). Tax activates HTLV-I LTR by physical interaction with HEB-1 and the CREB/ATF family of proteins which specifically bind to the 21 bp repeats present in HTLV-I LTR (Béraud *et al.*, 1991; Zhao and Giam, 1991, 1992; Suzuki *et al.*, 1993). Induction of IL-2Rα by Tax, on the other hand, involves the activation of NF-κB/Rel (Ballard *et al.*, 1988; Ruben *et al.*, 1988; Crenon *et al.*, 1993), a family of transcription factors specifically binding to the κB motif in the IL-2Rα promoter (Grilli *et al.*, 1993; Baeuerle and Henkel, 1994; Siebenlist *et al.*, 1994). The molecular mechanism underlying Tax activation of the IL-2 gene remains unclear.

In normal T cells, the transcriptional expression of the IL-2 gene is under tight transcriptional control, and its induction requires two signals that are triggered by the engagement of T cell receptor and the co-stimulation of other cell surface molecules such as CD28 (reviewed by Schwartz, 1990, 1992; Linsley and Ledbetter, 1993). These signals induce a series of intracellular events leading to the activation of a large array of transcription factors, including NF-κB/Rel, AP-1, the nuclear factor of activated T cells (NF-AT), as well as a protein complex binding to the CD28-responsive element (CD28RE), all of which appear to be involved in IL-2 gene expression (reviewed by Ullman *et al.*, 1990; Fraser *et al.*, 1993; Crabtree and Clipstone, 1994). Among these factors, NF-AT appears to be responsible for the cell type-specific expression of IL-2

as well as many other cytokines (Crabtree and Clipstone, 1994). Several NF-AT species have been cloned (McCaffrey *et al.*, 1993; Northrop *et al.*, 1994; Hoey *et al.*, 1995; Masuda *et al.*, 1995), among which NF-ATp and NF-ATc have been best studied (McCaffrey *et al.*, 1993; Northrop *et al.*, 1994). The NF-AT factors normally exist as cytoplasmic phosphoproteins. During T cell activation, they are dephosphorylated rapidly through a calcium signaling pathway and then translocated to the nucleus where they bind to the IL-2 NF-AT sites in cooperation with a nuclear partner, NF-ATn, which contributes to the activation of the IL-2 gene (Crabtree and Clipstone, 1994). Although the molecular nature of NF-ATn has not yet been identified definitively, the Fos and Jun family proteins have been proposed as a candidate (Jain *et al.*, 1993).

Although Tax alone has no significant effect on IL-2 gene expression, it renders the T cells capable of producing IL-2 in response to only one stimulation signal. Indeed, in synergy with mitogens like phorbol 12-myristic 13-acetate (PMA) and phytohemagglutinin (PHA), Tax induces the expression of both endogenous IL-2 and IL-2 promoter-driven reporter genes (Maruyama *et al.*, 1987; Siekevitz *et al.*, 1987; Wano *et al.*, 1988). Previous studies have suggested that Tax activation of the IL-2 promoter is modulated by a κB enhancer, the cognate binding site of NF-κB/Rel (Hoyos *et al.*, 1989). However, since many known NF-κB/Rel inducers, such as the cytokine tumor necrosis factor (TNF)-α and the mitogen PMA, fail to mimic the function of Tax in the activation of the IL-2 gene, additional transcription factors are clearly required. Here we demonstrate that Tax induces NF-AT-containing protein complexes that bind to the CD28RE independently of the c-Jun/c-Fos family factors. Induction of these DNA binding complexes correlates with Tax activation of both the CD28RE enhancer and the IL-2 promoter.

Results

Neither the NF-κB/Rel nor CREB/ATF transcription factor pathway is sufficient for Tax activation of the IL-2 promoter

Prior studies have identified Tax mutants that selectively activate the NF-κB/Rel (e.g. Tax M47) or CREB/ATF (e.g. Tax M22) family of transcription factors (Smith and Greene, 1990; Semmes and Jeang, 1992). While TaxM47 activates the human immunodeficiency virus (HIV)-1 LTR by inducing the nuclear expression of NF-κB/Rel, M22 is able to activate HTLV-I LTR through enhancing the activity of CREB/ATF factors (Smith and Greene, 1990). To explore the mechanism by which Tax activates the IL-2 gene, the effect of wild-type and mutant forms of Tax on the activity of the IL-2 promoter was investigated using luciferase reporter gene assays. For these studies, human Jurkat T cells were transfected with cDNA expression vectors encoding either the wild-type Tax or its mutants TaxM22 or TaxM47 along with a luciferase reporter gene driven by the human IL-2 promoter (IL-2–luc, 575 bp, see Siebenlist *et al.*, 1986). As expected (Wano *et al.*, 1988; Hoyos *et al.*, 1989), in synergy with the mitogen PMA, the wild-type Tax potently activated the IL-2 promoter, leading to an elevated level of luciferase expression (Figure 1A, column 2). However, neither Tax M22 (column 3) nor Tax M47 (column 4) had a significant

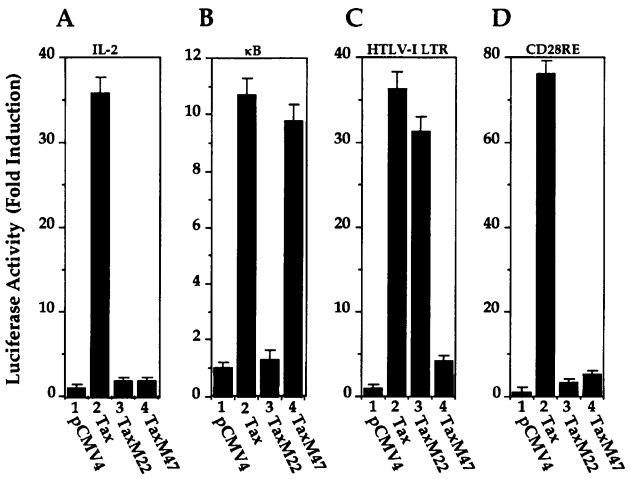

Fig. 1. IL-2 promoter and CD28RE enhancer are activated by the wild-type Tax but not its mutants TaxM22 and TaxM47. Jurkat cells (5×10^6) were transfected with either the parental pCMV4 vector lacking a cDNA insert or 2 μg of the cDNA expression vectors encoding either the wild-type Tax or its mutants M22 or M47 together with 2 μg of the indicated luciferase reporter plasmids [(**A**) pGL2IL-2-luc; (**B**) pGL2κB-TATA-luc; (**C**) pGL2HTLV-I-LTR-luc; (**D**) pGL2CD28RE-luc]. At 40 h post-transfection, the recipient cells were treated with PMA (10 ng/ml) for 8 h (A) or untreated (B–D) and then collected for luciferase assay (Promega). Luciferase activity is presented as fold induction relative to the basal level measured in cells transfected with the parental vector pCMV4. The values shown in this and all the following luciferase assays represent the mean fold induction of at least three independent experiments.

effect on the IL-2 promoter activity. It is noteworthy that, as previously reported (Smith and Greene, 1990), TaxM47 and TaxM22 potently activated the κB enhancer (Figure 1B, column 4) and HTLV-I LTR (Figure 1C, column 3), respectively. Together, these results suggested that neither the NF-κB/Rel nor CREB/ATF family of transcription factors are sufficient to mediate Tax activation of the IL-2 promoter.

To identify the enhancer element in the IL-2 promoter that possibly modulates a novel pathway of Tax transactivation, the effect of Tax and its mutants on the activity of various IL-2 gene enhancers was investigated (data not shown). One enhancer element, the CD28RE, was found to be potently activated by Tax. More importantly, this enhancer mimicked the full-length IL-2 promoter in that it responded to only the wild-type Tax (Figure 1D, column 2) but not the Tax mutants, TaxM22 (column 3) or TaxM47 (column 4). Thus, activation of the IL-2 promoter by Tax appears to involve a novel transcription factor pathway that is modulated by the CD28RE.

Tax-induced CD28RE binding protein complexes contain NF-AT

The Tax-induced CD28RE binding nuclear factors were examined by electrophoretic mobility shift assays (EMSAs) using nuclear extracts isolated from Jurkat T cells stably transfected with a Tax cDNA expression vector (Jurkat–Tax, see Wano *et al.*, 1988). Two major DNA binding complexes, designated C1 and C2, were detected from these Tax-expressing cells (Figure 2A, lane 2) but not from the parental Jurkat cells lacking the Tax protein (lane 1). These complexes resulted from specific DNA binding since their formation was blocked efficiently by

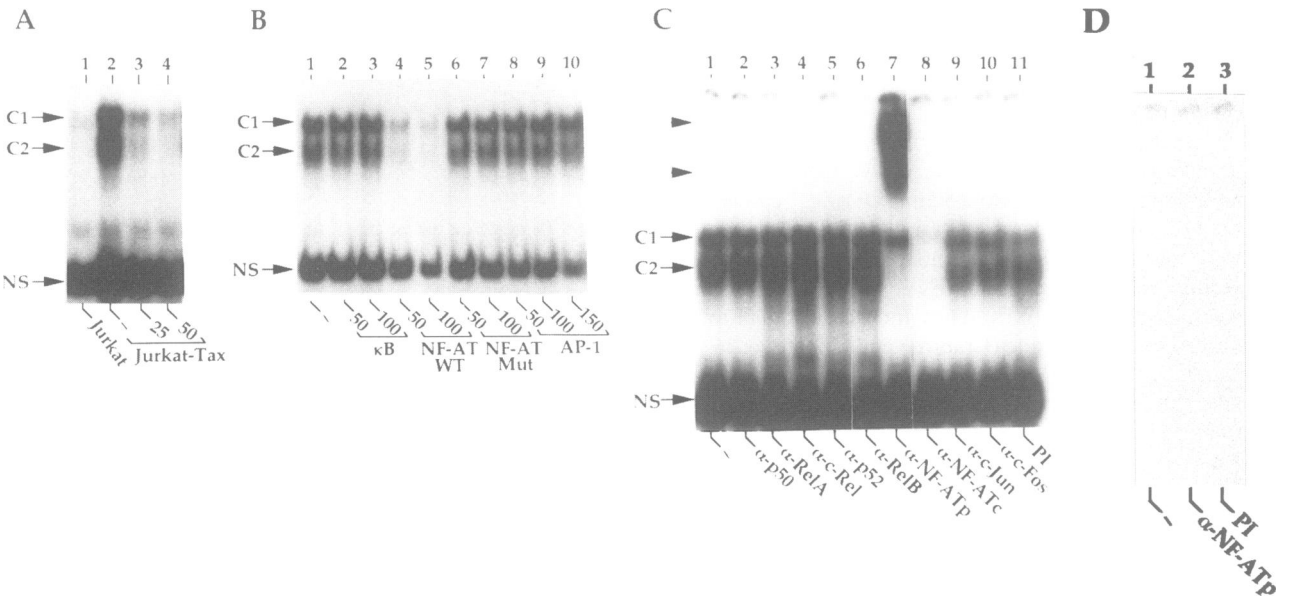

Fig. 2. Tax induces NF-AT-containing nuclear protein complexes specifically binding to CD28RE. (**A**) EMSA of nuclear extracts isolated from Jurkat or Jurkat–Tax cells using ^{32}P-labeled wild-type CD28RE. In lanes 3 and 4, a 25- and 50-fold molar excess of the unlabeled probe was included in the EMSA to show the DNA binding specificity of the complexes C1 and C2. NS indicates a protein complex that binds non-specifically to various different probes (data not shown). The free probe is not shown in the figure. (**B**) Competition assays. EMSA was performed using the radiolabeled wild-type CD28RE probe and Jurkat–Tax nuclear extract either in the absence (–) or presence of the indicated amounts (fold molar excess) of various unlabeled oligonucleotide probes. (**C**) Immunoreactivity assay of the CD28 binding proteins. DNA binding reaction mixtures containing Jurkat–Tax nuclear extract and radiolabeled CD28RE probe were supplemented with a pre-immune serum (PI) or antibodies against the indicated NF-κB/Rel, NF-AT or Jun/Fos family proteins prior to EMSA. The 'supershifted' bands are indicated by arrowheads. (**D**) Radiolabeled CD28RE probe was incubated in the EMSA buffer without cell extracts in the absence (–) or presence of either anti-NF-ATp or a pre-immune antiserum.

an excess of unlabeled wild-type CD28RE probe (lanes 3 and 4), while the unlabeled competitor did not influence the formation of a non-specific protein band (NS) significantly. The molecular nature of the CD28RE binding proteins present in Tax-expressing cells was analyzed by competition assays using various oligonucleotides containing binding sites for known IL-2 regulatory factors, including the κB, NF-AT and AP-1 sites from the human IL-2 gene promoter. As shown in Figure 2B, the presence of excess amounts of the κB probe had no detectable effect on the formation of either C1 or C2 (lanes 2 and 3). On the other hand, inclusion of the NF-AT probe in the EMSA reaction efficiently blocked the formation of both complexes (lanes 4 and 5), suggesting that the CD28RE binding factors had DNA binding specificity similar to NF-AT. In further support of this notion, an NF-AT probe bearing mutations in the core binding site of NF-ATp (GGAA, see Rao, 1994) failed to compete for the CD28RE binding complexes (lanes 6 and 7). However, in contrast to the NF-AT motif that appears to associate with a protein complex containing both NF-AT and Fos/Jun family proteins (Rao, 1994), the CD28RE probe did not bind the latter, since the formation of neither complex was influenced by excess unlabeled AP-1 probe (lanes 8–10).

To characterize further the Tax-induced CD28RE binding factors, immunological 'supershift' was performed using antibodies specific for NF-κB, NF-AT or the c-Fos/c-Jun proteins (Figure 2C). Consistent with the competition assays (Figure 2B), neither C1 nor C2 immunoreacted with antibodies for various members of the NF-κB/Rel family, including p50 (lane 2), RelA (lane 3), c-Rel (lane 4), p52 (lane 5) and RelB (lane 6). Similarly, antibodies recognizing various members of c-Fos and c-Jun proteins [c-Fos (4–10G) and c-Jun/AP-1 (D), respectively (Santa Cruz, Inc.)] failed to react with the CD28RE binding complexes (lane 7 and 8). In contrast, addition of a monoclonal antibody against NF-ATc (Northrop *et al.*, 1994) abolished the formation of both C1 and C2 (lanes 8), suggesting the presence of this component of NF-AT in these CD28RE binding complexes. On the other hand, an antiserum specific for NF-ATp (Upstate Biotechnology, Inc., see also McCaffrey *et al.*, 1993) selectively 'supershifted' the C2 complex (lane 7). It is noteworthy that the observed immunoreactivity between the NF-AT-specific antisera and the CD28RE binding complexes appears to be specific since these antisera did not affect the formation of the non-specific complex (NS). Furthermore, incubation of anti-NF-ATp with the CD28RE probe in the absence of a protein extract did not generate a 'supershift' (Figure 3D), thus confirming that anti-NF-ATp did not 'supershift' the bare CD28RE probe. Together, these results suggested that the HTLV-I Tax-induced novel CD28RE binding protein complexes contained both NF-ATc and NF-ATp but not the Fos/Jun proteins. Further, the fact that complex C2 immunoreacted with both anti-NF-ATc and anti-NF-ATp implied that these two NF-AT members might form a heterodimer on CD28RE.

The transfected NF-ATp binds to CD28RE and enhances its activation by Tax

To investigate whether CD28RE is a functional target of NF-AT, studies were performed to investigate whether NF-AT could bind to the CD28RE probe. For these studies, a cDNA expression vector encoding a truncated form of NF-ATp (NF-ATpXS; McCaffrey *et al.*, 1993) was transiently transfected into human kidney 293 cells.

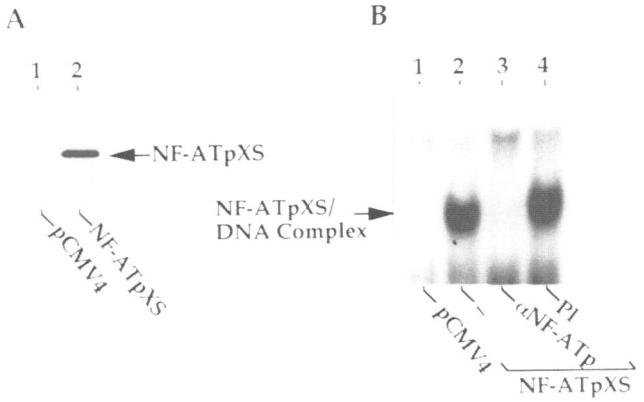

Fig. 3. The transfected NF-ATp binds to the CD28RE probe. (**A**) Immunoblotting assay of transiently expressed NF-ATp. Whole cell extracts isolated from 293 cells transfected with either the parental vector pCMV4 or a cDNA expression vector encoding a truncated form (amino acids 228–639) of murine NF-ATp (NF-ATpXS) were subjected to immunoblot using the NF-ATp-specific antiserum. (**B**) DNA binding assay of NF-ATpXS. Nuclear extracts isolated from 293 cells transfected with either pCMV4 (lane 1) or pCMV4NF-ATpXS (lanes 2–4) were subjected to EMSA using radiolabeled CD28RE probe in the absence (–) or presence of pre-immune (PI) or NF-ATp-specific immune (αNF-ATp) sera.

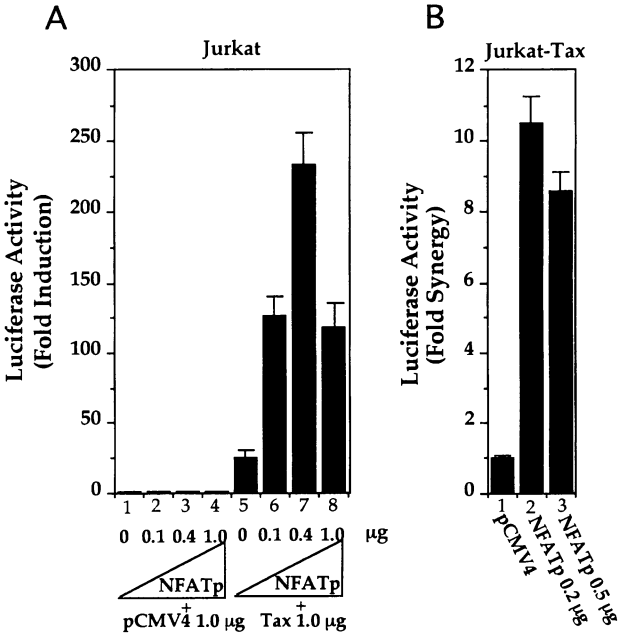

Fig. 4. Synergy between NF-ATp and Tax in the activation of CD28RE–luc. Jurkat (**A**) or Jurkat–Tax (**B**) cells were transfected with the indicated amounts of cDNA expression vectors encoding NFATpXS (NF-ATp) or Tax, together with 1 μg of pGL2CD28RE-luc reporter plasmid. Transcriptional activation of the reporter gene was detected as described in the legend to Figure 1, except that in (B) luciferase activity is presented as fold induction (fold synergy) relative to the control CD28RE–luc activity detected in Jurkat–Tax cells transfected with the parental vector pCMV4. This control activity obtained from Jurkat–Tax cells is usually similar to that detected in Jurkat cells transiently transfected with the Tax cDNA expression vector (~30-fold induction over the negative background).

Immunoblotting analysis using anti-NF-ATp readily detected the expressed NF-ATp protein from cells transfected with this cDNA (Figure 3A, lane 2) but not from mock-transfected cells (lane 1). Parallel EMSA using nuclear extracts isolated from these transfected 293 cells revealed that, as expected, this exogenously transfected NF-ATp formed a complex with the CD28RE probe (Figure 3B, lane 2). The presence of NF-ATp in this DNA–protein complex was confirmed further by the finding that anti-NF-ATp (lane 3), but not a pre-immune serum (lane 4), was able to 'supershift' this complex. Thus, the transfected NF-ATp was constitutively expressed in the nucleus (as well as in the cytoplasm, data not shown) and able to bind to the CD28RE probe.

To examine directly whether NF-ATp could transactivate CD28RE, Jurkat T cells were transfected with the cDNA encoding NF-ATpXS along with the CD28RE luciferase reporter plasmid, either in the absence or presence of Tax (Figure 4A). Although NF-ATpXS alone (columns 1–4) or together with a full-length NF-ATc (data not shown) had no significant effect on the luciferase activity, when co-transfected with Tax it potently enhanced CD28RE–luc expression (columns 5–8), thus suggesting that NF-ATp is involved in Tax activation of CD28RE. These results also suggested that Tax might induce either the modification of NF-ATp or additional factors that cooperate with NF-ATp, leading to the activation of the CD28RE. The latter possibility was supported by the finding that transfection of excessive amounts of NF-ATp plasmid led to a decrease of its synergistic action (column 8). Similar functional results were obtained using Jurkat–Tax cells, where NF-ATp enhanced Tax-mediated CD28RE–luc induction >8-fold (Figure 4B, columns 2 and 3).

Formation of the CD28RE binding NF-AT complexes correlates with Tax activation of the IL-2 promoter

To examine the functional importance of the CD28RE binding NF-AT complexes in the activation of the IL-2 promoter by Tax, selected mutations were introduced into the CD28RE oligonucleotide, and the effects of these mutations on the Tax-induced nuclear factors were evaluated by competition assays. Mutation of only two nucleotides (CC→TT) in the core region of CD28RE (Figure 5A, MUT) was found to abolish completely its binding by NF-AT, since this mutant probe failed to compete for the formation of the NF-AT-containing C1 and C2 complexes (Figure 5B, lanes 5–7). To correlate the binding of these nuclear complexes with the transcriptional activity of the IL-2 promoter, the CC→TT mutation was introduced by site-directed mutagenesis into the CD28RE site of the IL-2 promoter, and the effect of this mutation on Tax-mediated activation of the IL-2 promoter was then analyzed by luciferase reporter assays performed using the Jurkat–Tax cells (Figure 5C). As observed with the transiently transfected Tax (Figure 1A), the wild-type IL-2–luc was potently activated in the Tax-expressing Jurkat–Tax cells (see the legend of Figure 5C). However, introduction of the CC→TT mutation into the CD28RE site of the IL-2 promoter (IL-2–lucMUT) markedly reduced the response of the IL-2 promoter to Tax (~80%, see Figure 5C, column 3), a consequence similar to that seen with the CD28RE deletion mutant of the IL-2 promoter (IL-2Δluc ΔCD28RE, column 4). Thus, the formation of CD28RE–NF-AT complexes appeared to be critical for Tax-mediated activation of both the CD28RE enhancer and the IL-2 promoter.

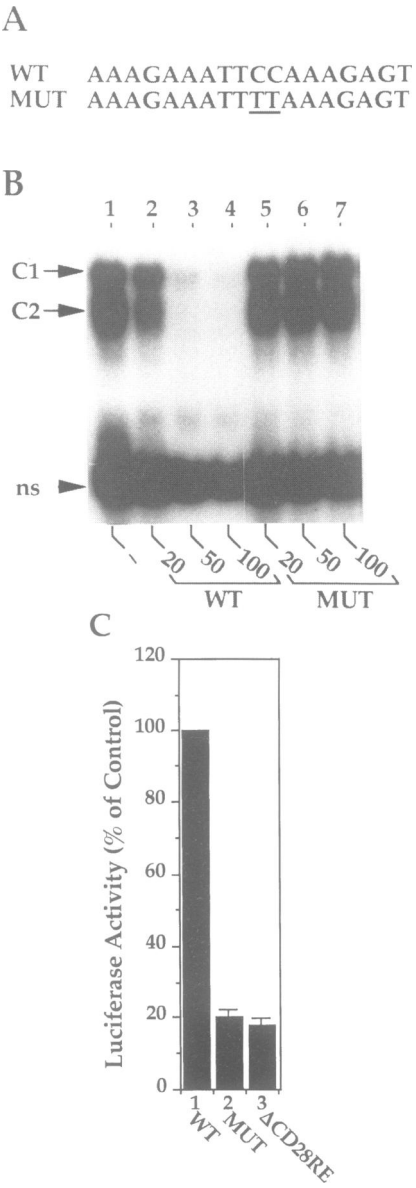

Fig. 5. Formation of the CD28RE–protein complexes correlates with Tax activation of the IL-2 promoter. (**A**) Sequence of the wild-type (WT) and mutant form (MUT) of CD28RE probes used in the competition assays. (**B**) EMSA was performed using Jurkat–Tax nuclear extract and the radiolabeled CD28REWT probe in the absence (–) or presence of the indicated amounts (fold molar excess) of unlabeled wild-type or mutant CD28RE competitors. (**C**) Luciferase reporter gene assays to detect Tax transactivation of the wild-type (WT), CD28RE mutation (MUT) or CD28RE deletion (ΔCD28RE) forms of the IL-2 promoter. The CC→TT mutation shown in (A) and a deletion of CD28RE were introduced into IL-2–luc by site-directed mutagenesis, and the resultant IL-2–lucMUT and IL-2–luc ΔCD28RE, as well as IL-2–lucWT, were transfected into Jurkat–Tax cells followed by luciferase assays. Compared with the basal luciferase activity obtained with parental Jurkat cells, the wild-type IL-2–luc (WT) was induced ~30-fold (data not shown). The luciferase activity for IL-2–lucMUT and IL-2–lucΔCD28RE is presented as percentage of the activity IL-2–lucWT (expressed as 100%).

Discussion

Antigen-stimulated T cell proliferation results from the induction of the T cell growth factor IL-2 and its high affinity receptor complex. While the IL-2 receptor can be induced by various immunological stimuli, the transcriptional activation of the IL-2 gene is under tight control, which requires both engagement of the T cell receptor (TCR) complex and surface ligation of a co-stimulatory molecule such as CD28 (Schwartz, 1990). This regulatory mechanism may account at least in part for the controlled T cell proliferation that occurs transiently as a response to a specific foreign antigen (Schwartz, 1990). Deregulation of such a mechanism may have significant impact on T cell growth and thus serves as a critical step in the initiation of T cell malignancies. In this regard, expression of the HTLV-I *tax* gene product in human T cells not only induces the constitutive expression of the IL-2 receptor but also renders the T cells capable of producing IL-2 in response to TCR stimulation even in the absence of a co-stimulatory signal (Maruyama *et al.*, 1987). Tax also activates IL-2 gene expression in synergy with various T cell mitogens, such as PMA (Wano *et al.*, 1988). This pathogenic action of Tax probably plays a major role in the induction of polyclonal T cell proliferation observed during the early stage of HTLV-I infection (Smith and Greene, 1991).

Prior studies have suggested that the NF-κB/Rel transcription factors play a role in Tax activation of the IL-2 promoter (Hoyos *et al.*, 1989). This viral regulatory protein appears to induce the proteolytic degradation of an NF-κB/Rel inhibitor, IκBα (Sun *et al.*, 1994; Brockman *et al.*, 1995; Maggirwar *et al.*, 1995), leading to the constitutive nuclear expression of active NF-κB/Rel proteins. These nuclear enhancer binding proteins then specifically bind to the κB element present in the IL-2 promoter which appears to contribute to Tax-mediated induction of IL-2 gene expression (Hoyos *et al.*, 1989). However, it has remained a puzzling question why many NF-κB/Rel inducers, such as PMA and TNF-α, fail to mimic the action of Tax in the activation of the IL-2 gene. In the present study, we have shown that in fact the NF-κB/Rel transcription factor pathway is not sufficient for Tax activation of the IL-2 promoter, since a Tax mutant (Tax M47, see Smith and Greene, 1990) capable of κB activation fails to activate the IL-2 promoter. We have identified a novel cellular transcription factor pathway involving members of the NF-AT protein family. Interestingly, the Tax-induced NF-AT proteins specifically bind to CD28RE, an IL-2 gene enhancer that normally modulates the CD28 co-stimulatory T cell activation signal (Fraser *et al.*, 1991). Consistent with a prior study (Li and Siekevitz, 1993), the CD28RE enhancer is potently activated by Tax, as determined by luciferase reporter assays. More importantly, we have shown that this enhancer element functionally modulates the NF-AT transcription factor pathway. The transiently transfected NF-ATp protein specifically binds to the CD28RE and, in synergy with Tax, potently activates this enhancer. Furthermore, mutation of two nucleotides at the CD28RE site, which abolishes the binding of the NF-AT complexes to this motif, markedly inhibited the activation of the IL-2 promoter by Tax, thus further suggesting an important role for these CD28 binding factors in IL-2 gene induction.

NF-AT is a major IL-2 gene regulator known to cooperate with a nuclear partner, possibly the Fos and Jun family proteins, in the activation of the NF-AT enhancer of the IL-2 promoter (Rao, 1994). The NF-AT motif of IL-2, as well as many other cytokine genes, contains

binding sequences for both NF-ATp and Fos/Jun proteins (AP-1). Although an AP-1 site is also present adjacent to the CD28RE motif and has been proposed to be important for the activation of CD28RE by immunological stimuli (Rooney *et al.*, 1995), this motif is clearly not involved in Tax activation of CD28RE of the human IL-2 promoter. Firstly, the CD28RE probe used in our study (for both EMSA and luciferase reporter gene assays) does not contain the intact 3′ adjacent AP-1 motif (see Materials and methods). Secondly, an oligonucleotide probe covering the functional AP-1 binding site fails to compete for the formation of the NF-AT-containing CD28RE binding complexes. Finally, and most importantly, the CD28 binding complexes do not exhibit immunoreactivity with antibodies recognizing various members of Jun and Fos family proteins. However, NF-AT does not seem to be the only factor involved in Tax activation of CD28RE, since transiently expressed NF-ATp alone or together with NF-ATc cannot activate CD28RE (Figure 4A and data not shown), although NF-ATp potently transactivates this enhancer in synergy with Tax. NF-κB/Rel factors have been shown to bind to CD28RE in cells stimulated with mitogens together with a monoclonal antibody against CD28 (anti-CD28) (Ghosh *et al.*, 1993; Lai *et al.*, 1995). With an extended time of autoradiography, we could detect low level of c-Rel/p50 complex with the CD28RE probe (data not shown). At the moment, we do not know whether the low level of NF-κB contributes to Tax-mediated activation of CD28RE. Studies are in progress to examine systematically the potential partners of NF-AT involved in the formation of the CD28RE binding complexes.

Materials and methods

Cell lines, reagents and antibodies

Human Jurkat leukemic 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, see Wano *et al.*, 1988) were cultured in the same medium containing 400 μg/ml of the G418 antibiotic to select for expression of the neomycin resistance gene. Human kidney 293 cells were cultured in Iscove's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. PMA was purchased from Sigma and used at a concentration of 10 ng/ml. The antibodies for c-Fos (4–10G) and c-Jun [c-Jun/AP-1 (D)] were obtained from Santa Cruz Biotechnology, Inc. These antibodies recognize different members of the Fos and Jun family. The antibody against the murine NF-ATp was purchased from Upstate Biotechnology, Inc. and the NF-ATc monoclonal antibody was generously provided by Dr Gerald R.Crabtree (Stanford University). The peptide-specific antisera for various NF-κB/Rel components were gifts from Dr Warner Greene (The Gladstone Institute of Virology and Immunology, San Francisco).

Plasmid constructs

pCMV4NF-ATpXS encoded a truncated murine NF-ATp similar to the NF-ATpXS previously described (McCaffrey *et al.*, 1993) but lacked the N-terminal hexahistidine tag. In brief, a 2 kb *Msc*I–*Cla*I restriction fragment from the murine NF-ATp cDNA (kindly provided by Dr Anjana Rao, see McCaffrey *et al.*, 1993) was inserted into the *Eco*RV and *Cla*I sites of a modified pCMV4 mammalian expression vector, pCMV4HA (Sun *et al.*, 1996). This intermediate construct was digested with *Kas*I and *Xba*I to remove the C-terminal untranslated sequences as well as part of the coding sequences. After filling in the ends with Klenow fragment of DNA polymerase I, the vector was religated, which generated pCMV4NF-ATpXS. The cDNAs encoding Tax and its mutants (Smith and Greene, 1990) were also subcloned into the pCMV4 vector. To construct the reporter plasmid pGL2-CD28RE-luc, a *Hin*dIII–*Xho*I insert (containing four copies of the human IL-2 CD28RE motif linked in front of a thymidine kinase promoter) of the plasmid 4×CD28RE-CAT (gift from Drs Paritosh Ghosh and Howard Young, National Cancer Institute, Frederick, see Ghosh *et al.*, 1993) was transferred into the pGL2-basic luciferase plasmid (Promega). pGL2IL-2-luc was generated by transferring a *Hin*dIII restriction fragment containing 575 bp of human IL-2 promoter (Siebenlist *et al.*, 1986) into the pGL2-basic vector. pGL2IL-2-luc MUT and pGL2IL-2-luc ΔCD28RE were constructed by site-directed mutagenesis (ClonTech), and the oligonucleotides used were 5′-TAAAGAAATTttAAAGAGTCAT-3′ and 5′-GGGTTTAAA-GCTTAAGAGTCATC-3′, respectively. κB-TATA-luc and HTLV-I-LTR-luc were provided by Dr Warner Greene (Ganchi *et al.*, 1992; Sun *et al.*, 1996).

Transient transfection and luciferase assays

Jurkat cells (5×10^6) were transfected using DEAE-dextran (Holbrook *et al.*, 1987) with the indicated amount of reporter plasmids and effector cDNA expression vectors. At 40 h post-transfection, the recipient cells were either left untreated or incubated with 10 ng/ml PMA for 8 h and then subjected to extract preparation using a reporter lysis buffer (Luciferase reagent, Promega) at ~40 μl per 10^6 cells. Luciferase activity was detected by mixing 5 μl of extract with 25 μl of luciferase substrate (Promega) and measured with a single photon channel of a scintillation counter (Beckman).

Immunoblotting

Human 293 cells were seeded onto 12-well plates and transfected using DEAE-dextran (Ganchi *et al.*, 1992). Subcellular extracts were prepared according to Schreiber *et al.* (1989). For immunoblotting analyses, protein samples were fractionated by 10% reducing SDS–PAGE, electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with an antiserum recognizing NF-ATp (Upstate Biotechnology, Inc.) using an enhanced chemiluminescence detection system (ECL; DuPont, NEN).

Nuclear extract preparation and EMSA

Jurkat T cells or Jurkat–Tax cells were collected by centrifugation at 800 *g* for 5 min. Nuclear extracts were prepared as previously described (Schreiber *et al.*, 1989). EMSA was performed by incubating the nuclear extracts (5 μg) with a ^{32}P-radiolabeled wild-type CD28RE probe at room temperature for 10 min followed by resolving the DNA–protein complexes on native 4.75% polyacrylamide gels. For antibody 'supershift' assays, 1 μl of diluted antibodies (5-fold for anti-NF-ATp, 2-fold for anti-NF-ATc and the various anti-NF-κB antisera) was added to the EMSA reaction 5 min before electrophoresis. Competition assays were performed by including excess unlabeled oligonucleotide probes in the reaction.

Oligonucleotide probes used in EMSA and competition assays were as follows: CD28RE WT, AAAGAAATTCCAAAGAGT; CD28RE MUT, AAAGAAATTttAAAGAGT; IL-2 κB, AAAGAGGGATTTCACCTA-CAT; NF-AT WT, GGAGGAAAAACTGTTTCATA; NF-AT MUT, GG-AccttAAACTGTTTCATA; AP-1, TAGTGATGAGTCAGCCG. The lower case letters indicate the mutated nucleotides.

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