Stimulation of interleukin-13 expression by human T-cell leukemia virus type 1 oncoprotein Tax via a dually active promoter element responsive to NF-κB and NFAT

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INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1), a deltaretrovirus, is the aetiological agent of a severe malignancy of CD4+ T cells, adult T-cell leukaemia (ATL), and of a neurodegenerative, inflammatory disease of the spinal cord, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Poiesz et al., 1980; Yoshida et al., 1984). After primary infection, HTLV establishes lifelong persistence and replicates mainly as integrated provirus. Viral persistence is characterized by long-lasting HTLV-infected T-cell clones, even in non-leukaemic patients. The clonal amplification of infected T cells and the capacity of these cells to establish permanent growth in culture suggest a growth-stimulating viral function that actively supports the replication of infected cells.

Besides structural proteins, the proviral HTLV-1 genome encodes the regulatory proteins Rex and Tax, which are essential for the virus life cycle. The accessory proteins p12, p13 and p30 are important for viral infectivity and replication but are dispensable for transformation. An HTLV-1-encoded minus-strand gene encoding the HBZ protein is probably relevant to the pathogenesis of ATL (Yasunaga & Matsuoka, 2007). HTLV exerts its transforming capacity mainly through the viral oncoprotein Tax. This protein capably immortalizes human T cells (Akagi et al., 1997; Schmitt et al., 1998) and is leukemogenic in transgenic mice (Grossman et al., 1995; Hasegawa et al., 2006). Proliferation of infected cells is enhanced by Tax by stimulating the G1 to S phase transition, which is mediated by directly binding and activating cyclin-dependent kinase Cdk4/6 holoenzymes (Fraedrich et al., 2005; Haller et al., 2002; Neuveut et al., 1998). In addition, Tax exerts anti-apoptotic functions that favour a persistent infection and the survival of infected cells (Krueger et al., 2006; Nicot et al., 2000; Wädele et al., 2006).

To control cellular functions relevant to survival and growth, Tax influences the expression of cellular regulatory proteins by stimulating their promoters. Cellular promoter
elements that are responsive to Tax include binding sites for the cAMP response element (CRE), nuclear factor (NF)-κB, nuclear factor of activated T cells (NFAT), serum response factor (SRF) and activator protein 1 (AP-1) (Fujii et al., 1995; Grassmann et al., 2005; Jeang, 2001). Abnormal NF-κB activation is a hallmark of HTLV-transformed cells and ex vivo samples of ATL patients (Hall & Fujii, 2005; Peloponese et al., 2006). NF-κB signalling is mediated by a family of transcription factors, p65 (RelA), p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel and RelB, that act as homo- and heterodimers in the transcriptional regulation of many genes involved in the control of survival and apoptosis, cell growth, the immune response and inflammation. Inactive NF-κB proteins are sequestered in the cytoplasm by inhibitors of κB (IκBs). They are physiologically activated in response to various stimuli such as inflammatory cytokines, stress inducers and pathogens. Upon stimulation, cytoplasmic NF-κB factors, such as p50 and p65, are released from IκBs, translocate to the nucleus and activate gene transcription. In this process, activated IκB kinases induce the proteasomal degradation of IκBs and thus the release of NF-κB proteins (Sun & Yamaoka, 2005). Several genes relevant to survival and growth (HIAP-1, BCLXL, 4-1BB and CFLIP) are upregulated through Tax-mediated NF-κB activation (Krueger et al., 2006; Nicot et al., 2000; Okamoto et al., 2006; Pichler et al., 2008; Wädele et al., 2006). Another group of promoters that are stimulated by Tax via the NF-κB pathway are those of several cytokines and cytokine receptors (Grassmann et al., 2005; Taylor, 2007).

The NFAT promoter consensus and binding sites have been found to be relevant to the transactivation of several Tax-stimulated promoters (IRF4, FASL and IL-2) (Good et al., 1996; Rivera et al., 1998; Sharma et al., 2000), and constitutively active NFAT1 has been observed in some HTLV-transformed cells (Good et al., 1997). This has raised speculations that Tax could stimulate these and possibly other NFAT target genes with relevance to survival and growth by direct activation of NFAT. NFAT proteins are a family of transcription factors whose activity is tightly regulated by phosphorylation. Inactive cytoplasmic NFAT proteins are activated via dephosphorylation by the calcium-dependent phosphatase calcineurin, resulting in the nuclear translocation of NFAT transcription factors (Crabtree & Olson, 2002; Serfling et al., 2000). NFAT target genes are involved in the control of T-cell activation, in particular many NFAT-stimulated cytokine genes.

The Tax-mediated upregulation of interleukin (IL)-13 in the lymphocytes of HTLV patients (Chung et al., 2003; Wädele et al., 2004) may be of particular importance to HTLV pathogenesis. Among the IL-13 functions described in T cells are inhibition of gamma interferon production, enhancement of cytolytic potential (Yu et al., 1998), inhibition of chemotactic migration of T lymphocytes (Jinquan et al., 1995) and stimulation of IL-13 receptor z1 expression (Gauchat et al., 1997). At least some of these immune-modulating functions could result in perturbation of the host response to HTLV-1. For instance, as IL-13 also acts as an inhibitor of tumour immunosurveillance (Ahlers et al., 2002; Terabe et al., 2000), its overexpression in HTLV-infected cells raises the possibility that it could influence the antiviral immune response (Stuver et al., 1996; Taguchi, 1989). Besides its role in regulating immune functions, IL-13 is able to stimulate proliferation and survival of lymphocytes (Lomo et al., 1997; Manna & Aggarwal, 1998; Relic et al., 2001; Wright et al., 1999) and hence may contribute to persistence and transformation of HTLV-infected T cells. Furthermore, IL-13 is linked to leukaemogenesis of Hodgkin’s lymphoma, where it stimulates the growth of the oncogenic Reed–Sterneberg cells (Skinnider et al., 2002). Similarly, IL-13 could contribute to the pathogenesis of Dalton’s lymphoma (DL). This highly invasive and malignant T-cell lymphoma is characterized by very high IL-13 serum titres, which are critical for the growth and proliferation of DL cells (Deepak et al., 2007). In addition to IL-13 production, the functional IL-13 receptor is also expressed on HTLV-transformed cells and downstream signals are active; thus, an autocrine proliferation stimulus is likely for HTLV-transformed cells (Wädele et al., 2004).

Efficient stimulation of the IL-13 promoter by Tax has been identified as a cause of IL-13 overexpression in HTLV-infected T cells. This transactivation by Tax depends almost completely on a promoter region containing an NFAT-binding P element, which may also account for the activation of IL-13 transcription in B cells and mast cells by NFAT (Monticelli et al., 2004; Skinnider et al., 2001). Here, we analysed the mechanism by which the essential IL-13 promoter site is transactivated by Tax. Testing the isolated Tax-responsive element of the IL-13 promoter (IL13TaxRE) in reporter assays revealed that it was sufficient to mediate both Tax and NFAT responsiveness. However, a specific NFAT inhibitor was unable to interfere with the effect of Tax on the IL-13 promoter and to suppress endogenous IL-13 expression of HTLV-transformed cells. By contrast, NF-κB inhibition completely abolished the Tax responsiveness of IL13TaxRE. This, in conjunction with strong induction of IL13TaxRE by NF-κB factors and the direct binding of NF-κB proteins, indicates that Tax stimulates the element via NF-κB. As NFAT and NF-κB responsiveness was not separable in mutational analyses, IL13TaxRE represents a dually active site responsive to NF-κB and NFAT. In summary, these results indicate that Tax mediates IL-13 upregulation in HTLV-infected cells via NF-κB.

**METHODS**

**Analysis of IL13TaxRE and the wild-type IL-13 promoter by luciferase assays.** To obtain the reporter plasmid containing the isolated IL13TaxRE, a TATA-box motif derived from pGAS-Luc (Stratagene) was cloned into pGL3-Basic (Promega) via Nhel/HindIII using double-stranded oligonucleotides (forward: 5’-GATCCGCTAG-
CTGACTCTAGGGTGATATAAACGTCTAGTCGATGCAGCATC-3', reverse: 5'-GATCAAGCTTATATACCTCTAGTAGTACGATGCAGCATC-3'. Subsequently, oligonucleotides encoding four tandem repeats of the Tax-responsive IL-13 promoter region −119/−130 (pIL13TaxRE), which contain the NFAT-binding P element (−119/−128), were cloned into this plasmid via Nhel restriction sites (forward: 5′-AGTGGCTAGCTGGATTTTCTTTCACGTCGAGTC-3′; reverse: 5′-GACTGCTAGCCATGGAAAATCGAGCTGGAATCGTGGAAAATCGAGCTG-3′), resulting in the luciferase reporter plasmid pIL13TaxREem1 (mt1 mutation) and pIL13TaxREem2 (mt2 mutation) were obtained by inserting oligonucleotides containing four copies of the mutated IL13-TaxRE and pIL13TaxREmt2 (mt2 mutation) were obtained by inserting CAGCTAGCGACT-3′. 

For this plasmid, oligonucleotides encoding four tandem repeats of the Tax-binding element were inserted into the TATA box-containing pGL3-Basic vector via Nhel restriction sites (forward: 5′-AGTGGCTAGCTGGATTTTCTTTCACGTCGAGTC-3′; reverse: 5′-GACTGCTAGCCATGGAAAATCGAGCTGGAATCGTGGAAAATCGAGCTG-3′). This resulted in the luciferase reporter plasmid pIL13TaxRE, which also served as internal standards. 

Chromatin immunoprecipitation (ChiP) assay. To analyse direct binding of NF-xB (p65) to pIL13TaxRE, Jurkat T cells were co-transfected with 20 µg pIL13TaxRE and 20 µg p65 expression vector. Some cells were also stimulated with PMA (0.1 µg/ml) and ionomycin (2 µM) 4 h prior to harvesting. The ChiP assay was performed according to the manufacturer’s instructions (Millipore). Briefly, 3 × 10⁶ Jurkat cells per sample were lysed and DNA–protein complexes were precipitated using 12 µg p65-specific antibodies (Santa Cruz Biotechnology). Bands were visualized with either NF-kB or NFAT1 antibody as specific antibodies. 

Promoter pull-down analysis. Nuclear protein extracts were prepared from 4 × 10⁶ Juana W cells using a Nuclear/Cytosol Extraction kit (Biovision). Aliquots of 70 µg nuclear protein were incubated in 200 µl pull-down buffer [PDB: 0.5% NP-40, 500 mM KCl, 10 mM Tris/HCl (pH 7.5), 1 mM DTT] and 20 µg Poly(dI-dC) (5 µM) added to the remaining samples. After incubation, the samples were washed in 0.5% NP-40, 500 mM aprotinin and centrifuged on a 0.88 M sucrose cushion. The pellet was washed in 25 mM citric acid, 0.5% Triton X-100, and resuspended in 80 µl buffer (25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 50 mM Tris/HCl (pH 8.0), 5 mM DTT) and sonicated. The immunoblot was performed as described previously (Wäldele et al., 2004), except that 80 µg protein was loaded per sample. The nylon membrane was blocked with TBS containing 5% fetal calf serum and 0.05% Tween 20. The blots were incubated with antibodies specific for NF-xB p65, NF-xB p50 (both from Santa Cruz Biotechnology) and NFA1 (ImmunoGLOBE). The purity of the nuclear protein fraction was determined using antibodies for z-tubulin (Sigma-Aldrich) and lamin B1 (Santa Cruz Biotechnology), which also served as internal standards.
RESULTS

Ability of HTLV-1 Tax and NFAT1 to stimulate the same element of the IL-13 promoter

Stimulation of the IL-13 promoter by the oncoprotein Tax has been shown to depend on a promoter region that contains an NFAT-binding P element at position −119/−128 relative to the transcriptional start (Chung et al., 2003; Dolganov et al., 1996; Wäldele et al., 2004). The direct binding to this element raised the question of whether NFAT1 is also involved in the promoter’s transactivation by Tax. To investigate whether this P element-containing region of the IL-13 promoter is sufficient to mediate the Tax effect, a sequence containing four repeats of 12 nt (each consisting of the P element and two flanking nucleotides) was inserted into a luciferase reporter construct (pIL13TaxRE; Fig. 1a). To determine the responsiveness of this isolated promoter element to Tax, luciferase assays were performed in transiently transfected Jurkat T lymphocytes (Fig. 1b). Co-transfection of pIL13TaxRE and wild-type Tax revealed strong activation (~50-fold), whereas a Tax mutant (M7), unable to stimulate transcriptional pathways, had no effect. Thus, the NFAT-binding sequence alone was sufficient to mediate the transactivating Tax effect in the absence of all other regions of the IL-13 promoter and hence constitutes a Tax-responsive element (IL13TaxRE). To investigate whether IL13TaxRE was also responsive to NFAT1, a constitutively active variant of NFAT1 (Macián et al., 2002; Okamura et al., 2000) was co-transfected along with pIL13TaxRE. These experiments revealed strong induction of the promoter (~90-fold). Thus, both Tax and NFAT1 exerted a strong stimulatory effect on IL13TaxRE. To examine whether NFAT1 activity could account for IL-13 expression, we tested an array of HTLV-positive cell cultures for the presence of nuclear NFAT1. To this end, nuclear extracts of an in vitro HTLV-transformed cell line (MT2), ATL-derived cell lines (ATL1, StEd, Champ, JuanaW and PaBe) and a HAM/TSP-derived cell line (Eva) were analysed by immunoblotting (Fig. 1c).

![Fig. 1](http://vir.sgmjournals.org)
These analyses revealed low but detectable levels of NFAT1 protein in the nuclei of all of these cell lines that could potentially contribute to IL-13 transcription.

**No requirement for NFAT signalling in Tax-mediated IL-13 promoter stimulation and IL-13 expression of HTLV-1-infected cells**

To determine the significance of NFAT signalling for the transactivation of IL13TaxRE, an IL13TaxRE/luciferase reporter was analysed in the presence of Tax and the NFAT inhibitor CsA in Jurkat T cells. As a positive control for the induction of NFAT activation, cells were treated with PMA and ionomycin (Fig. 2a). As expected, PMA/ionomycin treatment resulted in a significant increase in IL13TaxRE promoter activity, which could be abolished by CsA. This result provided additional evidence that the Tax-responsive sequence in pIL13TaxRE is stimulated by NFAT signalling. By contrast, CsA did not affect the strong activation of IL13TaxRE by Tax. This observation suggested an NFAT-independent stimulation of IL13TaxRE by Tax. To determine whether a similar NFAT-independent

![Fig. 2.](image-url)

(a) The impact of NFAT signalling on IL13TaxRE was measured in Jurkat cells co-transfected with pIL13TaxRE, pcTax (Tax) or control plasmid. PMA/ionomycin and/or CsA were added for 4 h as indicated. Luciferase activity was calculated as described in Fig. 1(b) and normalized to the untreated control transfection. (b) The impact of NFAT signalling on Tax-mediated IL-13 promoter activation was analysed in Jurkat cells co-transfected with a wild-type IL-13 promoter reporter (pGL3-IL-13P) and pcTax (Tax). Where indicated, samples were treated with CsA for 4 h. IL-13 wild-type promoter activity was measured and calculated as described in (a). (c) To control the function of CsA, IL-13 mRNA expression was induced in Jurkat T cells by an NFAT-dependent mechanism (PMA/ionomycin). IL-13 transcripts were quantified by real-time RT-PCR before and after treatment with PMA/ionomycin or with CsA and PMA/ionomycin. Relative copy numbers were determined by normalizing the IL-13 transcripts to those of ACTB. Bars represent the means ± SEM of at least two independent experiments. (d) To investigate the influence of NFAT inhibition on endogenous IL-13 mRNA expression, patient-derived HTLV-1-transformed cell lines (ATL1, JuanaW, StEd and Eva) were treated with CsA or solvent (ethanol). The amount of IL-13 transcript was measured 0, 16, 24 and 48 h after the addition of CsA by real-time RT-PCR and relative copy number was calculated as described in (c).
 mechanism could account for stimulation of IL13TaxRE in its natural context, a reporter plasmid containing the wild-type IL-13 promoter (Wädele et al., 2004) was co-transfected with Tax and analysed in the presence and absence of CsA (Fig. 2b). The result showed that CsA could not inhibit the efficient Tax transactivation of the IL-13 wild-type promoter. These experiments indicated that NFAT transcription factors are not responsible for the Tax-mediated transactivation of the IL-13 promoter. To determine the role of nuclear NFAT activity for endogenous IL-13 expression of HTLV-infected T lymphocytes, NFAT signalling was inhibited and IL-13 mRNA expression was determined by real-time RT-PCR (Fig. 2d). To this end, IL-13 expression levels of ATL and HAM/TSP patient-derived cell lines (ATL1, JuanaW, StEd and Eva) were analysed after 0, 16, 24 and 48 h of CsA or solvent treatment. Consistently, none of these experiments detected an effect of CsA on IL-13 mRNA expression in any of the cell lines investigated, thus suggesting an NFAT-independent mechanism of IL-13 upregulation in HTLV-1-transformed cells. Taken together, the results obtained from the analyses of the isolated Tax-responsive element, the cloned IL-13 promoter and the endogenous IL-13 mRNA expression in HTLV-transformed cells indicated that NFAT does not mediate the Tax transactivation of the IL-13 promoter.

Activation of IL13TaxRE by NF-κB

While searching for other transcription factors potentially stimulating IL13TaxRE, in silico IL13TaxRE promoter analyses (MatInspector; Genomatix) proposed an overlapping potential binding site for NF-κB factors. In order to investigate whether NF-κB factors directly bind IL13TaxRE, ChIP assays were performed and binding of p65 (RelA) to the four copies of the IL13TaxRE sequence of the luciferase reporter was determined. Jurkat T cells were co-transfected with pIL13TaxRE and the p65 expression plasmid, and either stimulated with PMA/ionomycin or left untreated. After cross-linking, DNA–protein complexes were precipitated using p65-specific antibodies and the amount of recovered plasmid DNA was analysed by PCR. As shown in Fig. 3(a), a 217 bp sequence containing the four copies of IL13TaxRE could be amplified specifically from the precipitated DNA fragments by qualitative PCR, indicating direct association of p65. Specific binding of p65 was further confirmed by quantification of the precipitated DNA fragments by quantitative PCR.

To investigate whether NF-κB activation and binding to IL13TaxRE could account for the stimulatory Tax effect, a dominant inhibitor of NF-κB (IkBaDN) was applied. Jurkat T cells were co-transfected with pIL13TaxRE and expression plasmids for Tax and IkBaDN (Fig. 3b). Here, IkBaDN completely abolished the transactivation of pIL13TaxRE by Tax, indicating a requirement for NF-κB. Furthermore, confirming the functional relevance of NF-κB in IL13TaxRE activity, co-expression of p65 with the IL13TaxRE reporter plasmid resulted in exceptionally high promoter activation that was not inhibited by the addition of CsA (Fig. 3c). In summary, these results clearly demonstrated that the effect of Tax on IL13TaxRE is mediated by NF-κB factors such as p65.

Binding of NF-κB to IL13TaxRE

To analyse whether active NF-κB proteins that could mediate activation of IL13TaxRE were present in our panel of HTLV-transformed cells, the amount of p65 and its heterodimeric binding partner p50 was determined in the nuclei of different HTLV-transformed cells (MT2, ATL1, StEd, Champ, Eva, JuanaW and PaBe). To this end, the immunoblot shown in Fig. 1(c) was reprobed with p65- and p50-specific antibodies (Fig. 4a), revealing a strong presence of p65 and p50 proteins in the nucleus of all cell lines tested. This result extended and confirmed previous observations of constitutive NF-κB activation in HTLV-transformed cells (Jeang, 2001; Silbermann & Grassmann, 2007). The notion that these proteins account for IL-13 expression in HTLV-transformed cells could be corroborated by NF-κB (p65) binding to IL13TaxRE. To address this, nuclear proteins of the ATL-derived cell line JuanaW were used to perform promoter pull-down assays (Fig. 4b). Biotin-labelled probes (52 bp) containing the IL-13 wild-type IL13TaxRE fragment (12 bp) precipitated p65 specifically; competition with unlabelled wild-type probes abolished this effect whereas competition with an unlabelled mutated probe with two point mutations (mt1 mutation) did not affect p65 binding. Furthermore, a mutated biotin-labelled probe (mt1 mutation) precipitated little, if any, p65 protein. In summary, these analyses clearly demonstrated that NF-κB binds the Tax-responsive NFAT1-binding P element of the IL-13 promoter in HTLV-1-transformed cells, which probably accounts for the upregulation of IL-13.

Responsiveness of IL13TaxRE to NFAT1 and NF-κB

The direct association of NF-κB with IL13TaxRE, which acts as an NFAT1-binding P element (Dolganov et al., 1996), raised the question of whether NFAT1 and NF-κB can cooperate or compete in activating this element. To answer this question, the pIL13TaxRE reporter was co-transfected with p65 and constitutive active NFAT1 expression plasmids in variable ratios. Transfection of p65 only resulted in the strongest promoter activity, whereas changing the p65:NFAT ratio in favour of increasing amounts of NFAT1 significantly decreased promoter activity (Fig. 5a). These results clearly contradict additive or cooperative effects of p65 and NFAT1 in IL13TaxRE activation. Rather, the observed results support the notion that NFAT displaces p65 from the promoter element by competing for its binding site. To confirm the postulated competition of NFAT1 and NF-κB for a single binding motif, modified pIL13TaxRE reporters featuring mutations in either the core (pIL13TaxREmt1) or the outer (pIL13TaxREmt2) region of IL13TaxRE were tested. Wild-
type pIL13TaxRE, pIL13TaxREmt1 and pIL13TaxREmt2 were each co-transfected with Tax, NFAT1 or p65 expression plasmid (Fig. 5b). Only wild-type pIL13TaxRE was activated by Tax, NFAT and p65. By contrast, the mutations both in the core and in the outer region of IL13TaxRE completely abolished the responsiveness of the promoters to each of the co-expressed proteins, Tax, NFAT1 or p65. Thus, the experiments demonstrated that the response of IL13TaxRE to NFAT1 and p65 is not separable. In summary, these results characterized IL13TaxRE as a dually active site responsive to NFAT1 and NF-κB.

**DISCUSSION**

Here, we identified an NFAT-binding P element as the Tax-responsive sequence (IL13TaxRE) in the IL-13 promoter. We showed that it is a dually active promoter element responsive to NF-κB and NFAT, which Tax transactivates via NF-κB to cause upregulation of IL-13 in HTLV-1-infected cells.

Investigation of the isolated NFAT-binding P element (Dolganov et al., 1996) revealed that the sequence was sufficient to mediate Tax transactivation in the absence of all other sequences of the IL-13 promoter and thus represents a Tax-responsive element (IL13TaxRE). The same isolated element was also able to mediate a strong response to constitutively active NFAT1. This element may be important for the induction of IL-13 transcription in mast cells, which is regulated primarily by NFAT1 (Monticelli et al., 2004). Although potentially active nuclear NFAT1 protein could be detected, it was irrelevant for the stimulation of IL-13 in HTLV-infected cells. This could be deduced from inhibition experiments with CsA, a potent inhibitor of NFAT activation, which did not affect the level of IL-13 expression and hence suggested an
NFAT-independent mechanism of IL-13 upregulation in HTLV-1-transformed cells. One possible explanation for the inability of NFAT1 to stimulate IL-13 transcription could be its relative low-level activation in HTLV-1-infected cells. Alternatively, competition with other transcription factors for the same site could be an explanation.

The inability of CsA to interfere with Tax transactivation of the isolated IL13TaxRE or the endogenous IL-13 promoter suggests that NFAT is not contributing to the Tax transactivation of the IL-13 promoter. This is supported by observations that HTLV-1 Tax, in contrast to HTLV-2 Tax, can activate NFAT-independent pathways.

**Fig. 4.** Interaction of NF-κB (p65) with IL13TaxRE. (a) Nuclear expression of NF-κB (p65 and p50) of HTLV-transformed cells was determined by immunoblotting. After removal of NFAT1 antibodies from the immunoblot used in Fig. 1(b) the blot was stained with p65- and p50-specific antibodies. Controls of lamin B and α-tubulin were used as indicated. (b) To demonstrate direct binding of nuclear NF-κB protein to IL13TaxRE in HTLV-1-transformed cells, promoter pull-down analyses were performed. Nuclear protein extracts of the ATL-derived cell line JuanaW were incubated with biotin-labelled wild-type or mutated probes containing IL13TaxRE. Unlabelled wild-type or mutated (mt1 mutation) competitor probes were also added as a control for specificity. After precipitation, proteins were detected by immunoblotting with p65-specific antibodies. Relative binding activity was determined as the ratio of proteins precipitated to input protein. A representative result of at least two independent experiments is shown.

**Fig. 5.** Responsiveness of IL13TaxRE to NFAT1 and NF-κB. (a) The response of IL13TaxRE to p65 and NFAT1 was analysed in Jurkat T cells co-transfected with pIL13TaxRE along with constitutively active NFAT1 and p65 expression plasmids in different ratios. Luciferase activity was normalized to protein content and calculated as a multiple of the control (control transfection not shown). Bars represent the means ± SEM of at least three independent experiments. (b) To determine whether sites responding to NFAT1 and p65 were separable within the IL13TaxRE, the indicated mutants were investigated. Jurkat T cells were co-transfected as depicted. Luciferase activity was measured and calculated as a multiple of the particular control.
Tax, has little or no impact on NFAT activation required for the stimulation of an NFAT-dependent promoter (Niinuma et al., 2005). The low but detectable levels of NFAT1 protein in the nuclei of all HTLV-transformed cells tested in this and other studies (Niinuma et al., 2005; Sharma et al., 2002) could be due to expression of the HTLV-1 p12(I) protein, which could enhance NFAT activation by increasing cytoplasmic calcium (Yasunaga & Matsuoka, 2007). Additionally, differentiation or transformation-specific activation of the cells could contribute to NFAT activation.

The following arguments indicate that NF-κB is responsible for the IL-13 promoter upregulation in HTLV-transformed cells: (i) NF-κB activity, indicated by strong expression of p65 and p50 in the nucleus of all HTLV-transformed cells tested, has been observed in this and other studies (Hall & Fujii, 2005; Sun & Yamaoka, 2005); (ii) chromatin immunoprecipitation and promoter pull-down analyses showed NF-κB binding to isolated IL13TaxRE and to this element in the context of the IL-13 promoter in HTLV-1-transformed cells; and (iii) functional analyses in transient reporter assays revealed that IL13TaxRE and the IL-13 promoter are strongly activated by NF-κB.

The strong nuclear expression of p50 and p65 indicated active NF-κB and could account for a major part of the upregulation of IL-13. NF-κB could be directly activated by the viral Tax protein by stimulating IkB kinase-γ. Additionally, Tax-induced co-stimulatory tumour necrosis factor receptors, such as OX40 and 4-1BB, could also have induced NF-κB activity after ligand engagement (Hall & Fujii, 2005; Silbermann & Grassmann, 2007). The requirement for NF-κB to mediate the Tax effect on the IL-13 promoter could be demonstrated by an inhibitor of NF-κB activation, IkBaDN, which completely abolished Tax stimulation of IL13TaxRE. Thus, Tax is highly likely to stimulate the IL-13 upregulation in HTLV-transformed cells by inducing NF-κB. Moreover, Tax-mediated NF-κB activation probably is sufficient for the induction of IL-13 synthesis in T cells. This is suggested by induction of endogenous IL-13 synthesis in Jurkat cells following Tax transfection and in the Tesi Tax-repressible system (Wäldele et al., 2004).

As the functional and binding assays showed, IL13TaxRE binds and responds to both NF-κB and NFAT. However, testing the activity of NF-κB and NFAT1 on mutated IL13TaxRE revealed that the element could not be separated into NFAT- and NF-κB-sensitive parts. Binding to the same sequence may stem from a significant homology of the DNA-binding domains of NFAT1 and NF-κB factors of 20% resulting in similar crystal structures (Northrop et al., 1994; Serfling et al., 2000). Consequently, both factors probably compete for the same binding site, which also explains the lack of cooperation in the stimulation of IL13TaxRE. The notion that both factors compete for the same binding site was further supported by co-transfection experiments of pIL13TaxRE and expression plasmids for both p65 and NFAT1 in varying ratios. These revealed reduced promoter activity when the level of NFAT1 plasmid was increased, which may be explained by displacing p65 from the common binding site. In providing a doubly active site, IL13TaxRE resembles promoter elements in the human immunodeficiency virus long terminal repeat, IL-4 and IRF4 promoters, which are doubly responsive (Li-Weber et al., 2004; Romanchikova et al., 2003; Sharma et al., 2002). NFAT-binding sites are involved in Tax-mediated stimulation of other cellular promoters including those of EASLG and IRF4 (Good et al., 1996; Rivera et al., 1998; Sharma et al., 2000, 2002). These may also be responsive to NF-κB. Thus, it is possible that double-responsiveness and NF-κB signalling by Tax accounts for the Tax sensitivity of some NFAT-binding sites of other target genes.

The demonstration that, due to IL13TaxRE, the IL-13 promoter is highly responsive to NF-κB may have implications for the pathogenesis of other malignant diseases linked to IL-13. For instance, IL-13 overexpression in the malignant Reed–Sternberg (RS) cells of Hodgkin's lymphoma (HL) (Kapp et al., 1999; Skinnider et al., 2001, 2002), which causes the growth-promoting autostimulation, may be triggered by NF-κB via IL13TaxRE. This notion is supported by the strong constitutive activation of NF-κB in HL cell lines (Bargou et al., 1996) and in primary HL/RS cells (Bargou et al., 1997; Jost & Ruland, 2007), as well as by the identification of IL-13 as an NF-κB target in HL cells (Hinz et al., 2002; Jost & Ruland, 2007; Mathas et al., 2003). In summary, these findings on the stimulation of IL-13 by the dually active IL13TaxRE site via NF-κB explain a pathogenesis-relevant pathway in HTLV-infected cells and may provide a novel mechanism of IL-13 upregulation in other diseases in which IL-13 overexpression is pathogenic.

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