A Human T-cell Leukemia Virus Tax Variant Incapable of Activating NF-κB Retains Its Immortalizing Potential for Primary T-lymphocytes*

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The human T-cell leukaemia virus type 1 (HTLV-1) transactivator (Tax) has been shown to interfere with regulated cellular proliferation. Many studies have focused on the ability of Tax to transform rodent fibroblasts; however, none has defined the molecular requirements for Tax transformation of human lymphoid cells. We show here that tax induces permanent growth of human primary T-lymphocytes by using a transformation/immortalization defective rhadinovirus vector. The cells phenotypically resemble HTLV-immortalized lymphocytes and contain episomally persisting recombinant rhadinoviral sequences, which stably express functional Tax protein. As Tax can activate major cellular transcription factors, we asked for the relevance of these routes in the immortalization of human lymphocytes. By using Tax mutants that either activate exclusively CREB/activating transcription factor or are defective in activating this signaling pathway, we delineated that Tax can induce immortalization of primary human T-lymphocytes through a mechanism independent of NF-κB activation.

HTLV-1, a complex human retrovirus (1), is the etiological agent for adult T-cell leukemia (ATL) (2, 3) and a chronic neurodegenerative disease (4, 5). The virus has little cytotoxic effect on T-lymphocytes, its target cells, but changes their growth properties. In contrast to normal T-cells, HTLV-1-infected lymphocytes can proliferate permanently without antigen stimulation (for review see Ref. 6). Cells freshly immortalized by HTLV-1 initially require exogenous interleukin (IL)-2 for proliferation (7–9); however, prolonged cultivation of these cells eventually results in IL-2 independent growth and loss of T-cell receptor and T-cell functions (7, 8). The IL-2 independent growth can thus be caused by a constitutive stimulation of the IL-2 receptor-associated Jak-Stat pathway (10, 11).

The HTLV-1-encoded transactivator protein p40Tax has many properties typical for oncoproteins. (i) It immortalizes primary rodent fibroblasts and cooperates with the ras oncogene in the transformation of these cells (12); in addition it transforms established rat fibroblast lines (13, 14). (ii) It induces leukemia and neurofibromas in transgenic mice (15, 16). (iii) It has a potential for immortalizing primary human T-lymphocytes (17–20). Tax potently stimulates viral transcription and acts on an array of cellular promoters. Mechanistically, Tax can stimulate transcription either directly through an intrinsic activation domain (21) or through activation of signal transducing pathways such as NF-κB. Despite much investigation, it currently still remains unclear whether NF-κB activation by Tax is linked with its property to transform human lymphocytes.

To define the Tax-stimulated pathway, which mediates the immortalization of human T-cells, we expressed two types of Tax mutants in primary lymphocytes. One mutant activates CREB/ATF but not NF-κB. The second mutant activates neither CREB nor NF-κB. Here we report that the transduction of a Tax mutant that is active for CREB/ATF but inactive for NF-κB resulted in permanent growth of human primary T-lymphocytes. These results, although not directly addressing the role of NF-κB activation in lymphocyte growth, show that the NF-κB activating function of Tax is dispensable for the immortalization of primary human T-cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—The T-cell lines Kati-40, TRI-1, and TAXI-1 (17, 18) were immortalized in vitro from cord blood by rhadinoviral transduction of Tax+, Mondi, an interleukin (IL)-2-dependent HTLV-1-infected T-cell line, was derived from peripheral blood lymphocytes of a South African tropical spastic paraparesis patient (kindly provided by Dr. Walter B. Becker, University of Stellenbosch). Mondi, Kati-40, TRI-1, TAXI-1, and NATH-1–5 were grown in 40% RPMI 1640 and 40% CM medium (Vitrexom, Vilshofen, Germany) supplemented with 20% fetal calf serum and 20 units/ml IL-2 (Boehringer Mannheim). The same medium was used for the HTLV-1-negative human IL-2-dependent T-cell line SSSSBPT (22). The HTLV-1-negative T-cell lines Jurkat, Molt-4, and HuT-78 and the HTLV-1-infected lines HuT-102, C91PL, and MT-2 were cultured with RPMI 1640 supplemented with 10% fetal calf serum. To determine Tax activity, lymphoid cultures were transduced with chloramphenicol acetyltransferase (CAT) indicator plasmids containing the HTLV-1-LTR (17) or four NF-κB binding sites fused to a minimal promoter (23). To discriminate between NF-κB and IL-2 effects on the IL-2Ra receptor, CAT indicator plasmids pIL2RaPpCAt1 and pML2RaPpTKCAt8 (24) were obtained from Dr. Markus Naabholz (Epalinges, Switzerland). Plasmid pML2RaPpCAt1 contains a complete IL-2Ra promoter (~2470 to +162). The plasmid pIL2RaPpTKCAt8 contains the thymidine kinase of herpes simplex virus basal promoter fused to a segment of the IL-2Ra promoter containing the IL-2 response elements. Plasmid DNA (10 μg) was electro-
porated, and CAT reactions were performed as described (25, 26). Results were quantified by phosphorimaging (BAS2000, Fuji-X). As an internal standard, luciferase activity from a co-transfected plasmid pRSVLuc (5 μg) was determined.

**Fluorescence-activated Cell Sorter Analysis**—Flow cytometry analyses were done as described (25). Antibodies recognizing CD2 (Leu-3a), CD3 (Leu-4), CD4 (Leu-3T, 3d), CD8 (Leu-2T), CD19 (Leu-12), CD25 (Anti-IL-2Rα), CD45 (Anti-HL-1), CD69 (Leu-TU-23), CD95, HLA-DR (HLA-DR), and B7-1 (BB1) were purchased from Becton Dickinson (Heidelberg). The CD28 (9.3) and CD58 (AICD58) antibodies were obtained from Squirb (Seattle) and Dianova (Hamburg), respectively.

**Construction of Recombinant Rhadinoviruses and Transduction of Primary Human Lymphocytes**—Recombinant viruses were generated as described previously (17, 18). The DNAs of the Tax mutants M7 (27) and S258A (28) were assembled into the same expression cassette (pHISL) used previously for the construction of Tax+,-expressing rhadinoviruses (17, 18). Upon transfection these open reading frames yielded the same amount of protein as wild type, if they were expressed under the control of the same promoter (data not shown and Ref. 27). This indicated similar stabilities of wild type and mutant Tax proteins. The phenotypes of the Tax mutants were verified in co-transfection experiments using LTR-CAT and NF-κB CAP indicator plasmids. The expression cassettes were inserted into a recombinam plasmid containing a neo* selection marker and flanking rhadinoviral sequences. These sequences permitted recombination with the rhadinovirus Herpesvirus saimiri A11S4 (29) and directed the Tax expression cassettes into the right junction of unique and repetitive sequences of its genome. The recombinant rhadinoviruses were purified by geneticin selection and density gradient centrifugation, stimulated by phytohemagglutinin and infected as described (18).

**DNA and RNA Analyses**—To detect episomally persisting recombinant rhadinovirus DNA, cells were lysed in situ on top of an agarose gel; the DNA was separated by electrophoresis into chromosomal, episomal, and linear fractions (18). The Southern blot was analyzed with a Tax-specific hybridization probe. To verify unarranged tax sequences, total cellular DNA was subjected to PCR analyses using tax-specific primers (sense 5'-CAGCCCACTTCGCCAGGTTGAC-3' or 5'-CCATCATCATACTACACCCCCA-3' and antisense 5'-GTGGTGAGATGGAAGAGTTGGG-3'). These primers resulted in PCR products of 627 and 880 nucleotides. To confirm the presence of the mutation within tax, purified PCR products were sequenced. Cytokine RNAs were detected by standard reverse transcriptase-PCR using primers described in Platzer et al. (30). Tax-, IκB-α, IL-1α, and IL-2α- and γ/β-RNA expression was detected by Northern blot analyses as described previously (18). Hybridization signals were quantified by phosphorimaging.

**Protein Analyses**—Tax was detected on Western blots with monoclonal antibodies against the ECL system (Amer sham Corp., Braunschweig, Germany). The Tax-specific hybridomas 168B17-46- and S258A (28) were T-cell lines expressing CD45, CD3 (LeuTM-4), CD4 (Leu TM-3a), CD8 (Leu TM-2), CD19 (Leu TM-12), and CD2 but not CD19, a B-cell marker (Table I). These cells, however, if itives the protein activity on CREB/ATF-depend-
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like HTLV-immortalized cells, had no or diminished (compared with normal T-cells) CD3 surface expression (data not shown). All cell subtypes of T-cells have been shown to be immortalized by HTLV-1 (9). Consistent with HTLV-1-transformed cells, all Tax-transduced cell lines had an activated phenotype. Qualitative reverse transcriptase-PCR demonstrated that the cells expressed RNAs coding for the inflammatory cytokines IL-1α, IL-6, tumor necrosis factor-α, and leukemia inhibitory factor but not IL-2 RNA (data not shown). In addition the cells expressed a series of activation-specific markers (CD25, CD69, CD95, and B7-1). No major quantitative alteration among the surface markers was detectable with the exception of CD25.

The surface expression of this protein, the α chain of the IL-2 receptor (IL-2Rα), was found significantly reduced in cells transduced by the mutant Tax protein compared with cells containing the wild type Tax (Fig. 2A). Taken together surface markers and cytokine expression profiles reveal significant phenotypic similarities between HTLV-1-immortalized cells and lymphocytes transduced by either Taxwt or its NF-κB activation-deficient S258A derivative.

Reduced Expression of IL-2Rα in Cells Immortalized with the Mutant Tax—Tax stimulates the IL-2Rα promoter via NF-κB (34). To determine whether the reduced surface expression of IL-2Rα could be due to a loss of promoter transactivation by Tax, we investigated the IL-2Rα transcription in cells without Tax, with Taxwt, and with mutant Tax. The mRNAs were detected by Northern blots and normalized to an internal standard mRNA (GAPDH). Fig. 2B shows the average from three independent experiments. As a result the content of IL-2Rα transcripts was clearly reduced in cells containing the Tax mutant thus accounting for the diminished surface expression of CD25. These data suggested that the TaxS258A-expressing cells have lower NF-κB activity compared with the wild type counterpart.

The remaining IL-2Ra mRNA expression detected in the cells could be due to a recently identified upstream element of the human IL-2Ra promoter (IL-2rE), which is not activated by NF-κB (35). This element strongly responds to IL-2, which is a component of the growth medium used. To determine whether the IL-2rE contributes to the activity of the IL-2Ra promoter CAT indicator plasmids were transfected. An IL-2rE containing promoter segment, which is not activated by NF-κB, was found at least as active as the complete promoter in the TaxS258A-expressing NATI cells; in contrast the complete promoter was clearly more active in cells expressing Taxwt. This suggests that the IL-2Ra promoter in TaxS258A-immortalized cells is stimulated by transcription factors different from NF-κB and that in these cells the IL-2rE element accounts for most of the activity of the promoter. Taken together, these data suggested that lower NF-κB activity in the TaxS258A-expressing cells caused reduced expression of IL-2Ra/CD25.

Reduced NF-κB Activity in T-cells Immortalized by TaxS258A—To check whether autocrine activation of NF-κB exists in Tax-immortalized CBLs, the state of NF-κB activation was analyzed. This was measured using the expression level of RNAs initiated from NF-κB site-dependent IL-1α promoter (36). The mRNA content of cells without Tax, with Taxwt, and with mutant Tax was quantitated by Northern blots, which were normalized to an internal standard mRNA (GAPDH). Fig. 3 shows the average from three independent experiments. A clear difference was seen between those cells expressing wild type and mutant Tax. IL-1α transcripts were strongly reduced in the lymphocytes transduced with mutant TaxS258A-expressing cells; in contrast the complete promoter segment, which is not activated by NF-κB, was found at least as active as the complete promoter in the TaxS258A-expressing NATI cells; in contrast the complete promoter was clearly more active in cells expressing Taxwt. This finding likely reflects an intrinsic property of TaxS258A, since four out of four independently immortalized cell lines show the same phenotype.

As a further parameter of NF-κB activity, the amount of IκB-α mRNA was determined. This is a more direct measure of NF-κB activity, since IκB-α is directly involved in the feedback control of NF-κB (37, 38) and because the IκB-α promoter is strongly induced by active NF-κB. Fig. 4A shows that IκB-α mRNA is highly expressed in the cells expressing Taxwt. By contrast, low levels of IκB-α RNA are present in cell lines expressing the TaxS258A and in Tax-negative T-lymphocytic cell lines, consistent with low endogenous NF-κB activity. We also determined the half-life time of the IκB-α protein in cells expressing TaxS258A (NATI-2) and Taxwt. Cycloheximide was

![Expression of the TaxS258A mutant in the immortalized cell lines.](image)

**TABLE I**

| Cells      | Surface markers |
|------------|-----------------|
|            | CD2 | CD3 | CD4 | CD8 | CD19 | CD25 | CD28 | CD45 | CD58 | CD69 | CD95 | B7−1 | HLA-DR |
| TaxS258A   | Nati-1 | +* | + | + | − | − | + | + | + | + | + | + | + |
|            | Nati-2 | + | − | − | + | + | + | + | + | + | + | + | + |
|            | Nati-3 | + | + | + | + | + | + | + | + | + | + | + | + |
|            | Nati-4 | + | + | + | + | + | + | + | + | + | + | + | + |
| Taxwt      | TAXI-1 | + | − | + | − | − | + | + | + | + | + | + | + |
|            | TRI-1 | + | + | + | + | + | + | + | + | + | + | + | + |
| HTLV-1     | Mondí | + | + | + | − | − | + | + | + | + | + | + | + |
|            | MT-2 | − | + | + | + | + | + | + | + | + | + | + | + |
|            | CS1PL | − | + | + | + | + | + | + | + | + | + | + | + |
|            | HuT-102 | + | + | + | + | + | + | + | + | + | + | + | + |

*a* indicates positive.

*b* indicates negative.

*c* NT indicates not tested.
added to block protein synthesis, and the amounts of IκB-α after different time intervals were measured. Fig. 4B shows that IκB-α protein is stable, with a half-life of 2–4 h in TaxS258A cells. The same stability (half-life, 2–4 h) was observed in an uninfected human T-cell line SS8-BPT (data not shown). In contrast, the IκB-α protein has a shorter half-life in the presence of Tax wt (Fig. 3B). These data additionally corroborate that NF-κB activity is not inappropriately elevated in cells expressing the TaxS258A.

As a third measure of the NF-κB activation status, we analyzed the expression of transfected reporter genes. One indicator plasmid used contained four copies of an NF-κB response element positioned upstream of a minimal promoter, and the second contained the promoter from the HTLV-1 LTR. The CAT assays were normalized by co-transfecting a constitutively active luciferase gene. We found that the activity of the HTLV promoter was higher compared with that of the NF-κB test promoter in all cells immortalized by the taxwt and taxS258A genes and in the HTLV-1-transformed controls (Fig. 5A). Consistent with Tax-meditated activation of its own promoter, the activity of the HTLV promoter was much higher in these cells.

**Fig. 2.** IL-2Rα expression in the immortalized lymphocytes. A, flow cytometric analysis of IL-2Rα surface expression of NATI-3 (TaxS258A) and TAXI-1 (Tax wt) and MT-2 (HTLV-1) cells. B, content of IL-2Rα mRNA in the indicated cells. Northern blots were quantitated by phosphorimaging and normalized to the GAPDH signal. The mean of two independent experiments is shown. PSL, phosphor-stimulated luminescence. C, the cells were transfected with CAT indicator constructs containing the unchanged IL2-Rα promoter (black) and a deletion variant containing the IL-2 response element but not the NF-κB response element (gray). A luciferase plasmid (pRSVluc) was used as an internal standard. The mean of two independent experiments is shown. Relative CAT activity normalized to transfections with the deleted promoter is shown.

**Fig. 3.** Reduced IL-1α expression in Tax S258A immortalized lymphocytes. Total RNAs from the indicated cells were analyzed by Northern blot. Specific RNA was quantitated by phosphorimaging and normalized to GAPDH. The mean of two independent experiments is shown. PSL, phosphor-stimulated luminescence.

**Fig. 4.** Reduced IκB-α mRNA and increased IκB-α protein stability in cells immortalized with Tax S258A. A, Northern blot analysis of total cellular RNA derived from the indicated cell lines. The cell lines NATI-1–4 were transduced with TaxS258A, Kati-40, and TAXI-1 with Tax wt, MT-2, and Mondi are infected with HTLV-1. The amount of IκB-α RNA in these cells was quantitated by phosphorimaging of three independent Northern blot experiments. PSL, phosphor-stimulated luminescence. B, stability of IκB-α protein in NATI-2 cells. To block translation, cells were treated with cycloheximide (CHX), and protein was extracted after the indicated times. IκB-α was detected by Western blot. The estimated half-life of the protein is 2–4 h; it is longer than under conditions where NF-κB is activated (10–20 min).
These cells were found to contain greatly reduced levels of NF-κB activity. Our findings support an NF-κB-independent pathway for Tax immortalization of human T-lymphocytes.

Although the NF-κB phenotype of the mutant S258A was thoroughly established by two groups, it is formally not possible to exclude that some unmeasurable low effect on NF-κB could have contributed to the T-cell immortalization. The residual IL-2Rα transcription in the mutant immortalized cells does not indicate residual NF-κB activity, since the promoter is activated by its IL-2 response elements. By using many independent criteria, we found that cells immortalized by the rhadinovirus tax hybrids resemble HTLV-1-immortalized lymphocytes. These cells express virtually identical cytokine patterns and have similar patterns of surface markers. Like HTLV-1-transformed lymphocytes, Tax-immortalized lymphocytes can lose the CD-3 surface marker expression (8). This finding is unique to HTLV-1-associated immortalization since this does not occur with H. saimiri immortalized lymphocytes (32). Three facts support that Tax induced the immortalization of the primary T-cells and that rhadinovirus genes did not contribute to the immortalized phenotype. 1) The only transcription found in human T-cells originated from the genomic region, which is deleted in the vector strain used (40). 2) No herpes viral transcripts were found in T-cells infected and immortalized by the recombinants expressing the Taxε gene (41). 3) The cells infected with vector expressing TaxS258A did not synthesize infectious herpesvirus particles. These findings, besides the observation that the transduction with the H. saimiri vector produces no immortalization, indicate that the cellular growth changes are a direct consequence of HTLV-Tax expression.

The human primary lymphocytes transduced by the Tax mutant S258A are immortalized since they have an extended life span, and they proliferate in the absence of antigen presenting cells. The cells resemble HTLV-1-infected cell cultures derived from patients both in surface marker expression and growth factor requirements. These findings underline the proximity of this model system to ATL lymphocytes. We are not persuaded that rodent fibroblast models for Tax transformation have similar physiological relevance. In this regard we note that in other systems Tax proteins of HTLV-1 and BLV have been shown to exert an immortalizing effect on primary rat fibroblasts (12, 42) and transforming activity in established rat fibroblast cell lines. Thus Yamaoka and colleagues (14) showed that Tax, which is deficient in NF-κB activation, does not transform the Rat-1 fibroblast line. Surprisingly, a different group showed that the Rat-2 cell line could be morphologically transformed by a Tax mutant, which was deficient for NF-κB activation (39). Confusion about the impact of Tax mutations on fibroblast transformation probably stems from system to system variations (i.e. Tax mutants used) and cell type differences. These differences suggest caution in extrapolating results about the role of Tax in transformation from rodent fibroblast cell line systems to human primary lymphocytes.

We believe that our current findings contribute toward the development of a relevant human primary T-cell system for studying HTLV-1-induced ATL. Unlike the findings in rodent cell lines, in primary human T-cells, NF-κB activation by Tax is evidently not a critical component of immortalization. This observation agrees with the finding from Smith and Greene (39). However, our results do not exclude that in other settings activation of NF-κB can be a potent modulator/inductor of cellular proliferation.

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