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HTLV-1 Tax protein interacts with cyclin-dependent kinase inhibitor p16^{INK4A} and counteracts its inhibitory activity towards CDK4

Takeshi Suzuki, Saori Kitao, Hitoshi Matsushime[1] and Mitsuaki Yoshida[2]

Department of Cellular and Molecular Biology and [1]Department of Genetics, Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan

[2]Corresponding author

Tax, a regulatory protein of human T-cell leukemia virus type 1 (HTLV-1), is an oncoprotein which immortalizes human T cells and induces tumors in transgenic mice. These effects may be due to its interaction with cellular proteins, consisting of several transcription factors including CREB, NF-κB and SRF, and the transcriptional inhibitor, IκB. Here, we found that Tax binds to a cyclin-dependent kinase inhibitor, p16^{INK4A}, which has ankyrin motifs similar to IκB. p16^{INK4A} binds to the cyclin-dependent kinases, CDK4 and CDK6, and inhibits their activity, resulting in suppression of G_1 phase progression. The binding of Tax to p16^{INK4A} induced a reduction in the p16^{INK4A}–CDK4 complex, with subsequent activation of CDK4 kinase. Tax also suppressed p16^{INK4A}-mediated inhibition of U2OS cell growth. The p16^{INK4A} gene was frequently deleted in many T-cell lines, but not in HTLV-1-infected T-cell lines. Taking these findings together, the functional inactivation of p16^{INK4A} by Tax through protein–protein interaction is suggested to contribute to cellular immortalization and transformation induced by HTLV-1 infection.

Keywords: adult T-cell leukemia/cell cycle inhibitor/HTLV-1/Tax protein/tumor suppressor gene

Introduction

Cell cycle progression in eukaryotes is controlled by a series of cyclin-dependent kinases (CDKs) whose activities depend on their assembly into holoenzymes by binding to specific cyclins. Distinct cyclin–CDK complexes are formed at specific points of the cell cycle and their kinase activities are activated. D-type cyclins (D1, D2, D3) and cyclin E are involved in regulating G_1 phase progression and entry into S phase, and are thus termed G_1 cyclins (Pines, 1993; Sherr, 1994). The cyclin–CDK holoenzyme initially activated in cells after being released from a quiescent state is composed of D-type cyclins and CDK4 or CDK6 (Matsushime *et al.*, 1994; Meyerson and Harlow, 1994). Ectopic expression of cyclin D1 accelerates the entry of rodent fibroblasts into S phase (Quelle *et al.*, 1993; Resnitzky *et al.*, 1994), and microinjection of anti-cyclin D1 antibodies into synchronized fibroblasts during G_1 phase prevents cells from entering S phase (Baldin *et al.*, 1993; Quelle *et al.*, 1993). Thus, cells require D-type cyclins to progress through G_1 phase.

The most well-studied substrate for the cyclin D–CDK complex is the retinoblastoma protein (pRb), one of the tumor suppressor gene products (Dowdy *et al.*, 1993; Ewen *et al.*, 1993). pRb is hypophosphorylated in G_0 and early G_1, and then becomes hyperphosphorylated during mid to late G_1. Hypophosphorylated pRb is a negative regulator of the cell cycle through binding to and inactivating specific transcription factors, such as E2F, whose activities are required for entry into S phase. Phosphorylation of pRb cancels its growth suppressive function through releasing these transcription factors and enabling them to activate transcription (Nevins, 1992; Hinds and Weinberg, 1994).

The activities of these cyclin D–CDK complexes have been shown recently to be regulated by a family of cyclin-dependent kinase inhibitors, such as the INK4 family of proteins which specifically inhibit cyclin D-dependent kinases (Hunter and Pines, 1994; Grana and Reddy, 1995; Sherr and Roberts, 1995); the INK4 family of proteins bind to CDK4 and CDK6 and inhibit their kinase activities. They include p16^{INK4A}, identified as a protein co-immunoprecipitated with CDK4 (Serrano *et al.*, 1993), p15^{INK4B}, identified as an inhibitor induced by transforming growth factor (TGF)-β (Hannon and Beach, 1994), and the more recently described p18^{INK4C} and p19^{INK4D} (Guan *et al.*, 1994; Chan *et al.*, 1995; Hirai *et al.*, 1995). Human p16^{INK4A} and p15^{INK4B} genes are localized tandemly within 30 kb on chromosome 9p21 (Kamb *et al.*, 1994a; Nobori *et al.*, 1994), where chromosomal rearrangement and loss of heterozygosity are frequently observed in tumor cells (Kamb *et al.*, 1994b; Okamoto *et al.*, 1994; Schmidt *et al.*, 1994; Sheaff and Roberts, 1995). Expression of p16^{INK4A} suppressed the transformation phenotypes of cells induced by H-Ras and c-Myc (Serrano *et al.*, 1995), and induced G_1 arrest in pRb-positive cell lines (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995). Thus, it was suggested that p16^{INK4A} acts as a tumor suppressor whose inactivation contributes to the development of human tumors.

The signaling pathway governed by G_1 cyclins, CDK, pRb and E2F plays a major regulatory role during G_1 to S transition in the cell cycle, and this pathway frequently is impaired in many transformed cell lines and tumor cells. These abnormalities include amplification of the CDK4 gene and mutation of the pRb or p16^{INK4A} gene (Khatib *et al.*, 1993; Okamoto *et al.*, 1994; Otterson *et al.*, 1994; Schmidt *et al.*, 1994; Parry *et al.*, 1995). In viral carcinogenesis, several oncoproteins of DNA tumor viruses, such as E1a, E7 and large T, bind directly to and inactivate the tumor suppressor pRb to drive quiescent cells into a replicative state which may lead to cellular immortalization (Dyson *et al.*, 1989; Whyte *et al.*, 1989; Nevins, 1992). Here we report that the Tax oncoprotein of human T-cell leukemia virus type 1 (HTLV-1) binds to

and inactivates another tumor suppressor, $p16^{INK4A}$ protein, in the same regulatory pathway.

HTLV-1 (Poeisz *et al.*, 1980; Yoshida *et al.*, 1982) is a causative agent of adult T-cell leukemia (ATL), and the viral protein Tax is suggested to play a key role in the onset of ATL (Yoshida, 1993). In fact, Tax immortalizes human T cells and induces tumors or leukemia in transgenic mice (Nerenberg *et al.*, 1987; Grassmann *et al.*, 1992; Grossman *et al.*, 1995). Tax interacts with cellular transcription factors and activates specific transcription. These cellular factors are CREB and CREM proteins, NF-κB p50, p65 and c-Rel proteins, and SRF (Fujii *et al.*, 1992; Zhao and Giam, 1992; Suzuki *et al.*, 1993a,b, 1994). In addition to these transcription factors, Tax also binds to NF-κB p105, p100 and IκB proteins which are inhibitors of NF-κB (Hirai *et al.*, 1992, 1994; Watanabe *et al.*, 1993; Beraud *et al.*, 1994; Lanoix *et al.*, 1994; Murakami *et al.*, 1995; Suzuki *et al.*, 1995). The Tax binding domain in the IκB proteins was identified as the ankyrin motifs (Hirai *et al.*, 1994). Ankyrin motifs are also found in other proteins involved in cell cycle control and tissue differentiation (Lux *et al.*, 1990), thus suggesting possible interaction of these proteins with Tax. The CDK inhibitor, $p16^{INK4A}$ protein, is one of these candidates (Serrano *et al.*, 1993).

Here we show that Tax binds to $p16^{INK4A}$ protein and counteracts the inhibitory activity of $p16^{INK4A}$, resulting in activation of CDK4. Furthermore, Tax relieved cells from $p16^{INK4A}$-induced growth arrest. These results provide a novel insight into the role of Tax in cell cycle regulation and cellular transformation.

Results

Interaction of $p16^{INK4A}$ with Tax

Previously, we have demonstrated the binding of Tax protein to the ankyrin motifs of NF-κB p105, IκB-γ and IκB-α (Hirai *et al.*, 1992, 1994; Suzuki *et al.*, 1995). These results prompted us to test whether Tax interacts with other cellular factors that have ankyrin motifs. As a candidate, we were interested in the $p16^{INK4A}$ protein, a CDK inhibitor that contains four tandem repeats of the ankyrin motif (Serrano *et al.*, 1993). To test this possibility, we produced a fusion protein of $p16^{INK4A}$ with glutathione *S*-transferase (GST) and analyzed its binding to Tax protein which was fused to six histidine residues at the N-terminus (His–Tax). GST–$p16^{INK4A}$ was adsorbed on glutathione–Sepharose beads and mixed with purified His–Tax. The complex was collected by centrifugation and subjected to Western blot analysis for detection of Tax (Figure 1A). Tax was detected in the complexes with GST–$p16^{INK4A}$, and the amount of Tax in the complexes increased dose dependently (lanes 4–6). Tax did not bind to GST alone (lanes 1–3). This indicates that Tax directly interacts with $p16^{INK4A}$ *in vitro*.

To demonstrate the interaction of Tax with $p16^{INK4A}$ in cells, expression vectors for Tax and $p16^{INK4A}$ were co-transfected into 293T cells. The total cell extract was treated with anti-Tax antibodies and the immune complexes were analyzed by Western blotting with anti-$p16^{INK4A}$ antibodies to detect the Tax–$p16^{INK4A}$ complexes. As shown in Figure 1B, $p16^{INK4A}$ was detected when wild-type Tax and $p16^{INK4A}$ were co-expressed (lane 2), whereas it was not detected when a Tax mutant d7/16 with a small deletion, which can activate neither the 21 bp enhancer nor the NF-κB site, was used (lane 4) (Hirai *et al.*, 1992). On the other hand, when another Tax mutant d320 with a small deletion, which is inactive on the 21 bp enhancer but active on the NF-κB site through binding to the ankyrin motifs, was used, $p16^{INK4A}$ was co-immunoprecipitated with Tax protein (lane 3). These results demonstrated the direct and specific capacity of Tax to bind to $p16^{INK4A}$ *in vitro* and also *in vivo* when $p16^{INK4A}$ is overexpressed.

To demonstrate that Tax binds to endogenous $p16^{INK4A}$ protein, Tax was transiently expressed in 293T cells and the formation of the Tax–$p16^{INK4A}$ complex was analyzed similarly to the analysis shown Figure 1B. As can be seen in Figure 1C, lane 2, anti-Tax antibodies co-immunoprecipitated $p16^{INK4A}$, although much longer exposure of the autoradiogram was required to detect the band. In a parallel experiment, when a fusion protein (HA–$p16^{INK4A}$) of the hemagglutinin (HA) epitope and $p16^{INK4A}$ was expressed, a band corresponding to HA–$p16^{INK4A}$ was detected in addition to the endogenous $p16^{INK4A}$ (lane 3). The result clearly indicates that once Tax protein is expressed, it can bind to the cellular $p16^{INK4A}$ protein, if it is present in cells.

Tax counteracts $p16^{INK4A}$ function

It has been reported that $p16^{INK4A}$ specifically binds to the cyclin D-dependent catalytic subunits CDK4 and CDK6 and inhibits their kinase activity (Serrano *et al.*, 1993). To examine the effect of Tax binding to $p16^{INK4A}$, the kinase activity of CDK4 was assayed *in vitro* using GST–pRb as a substrate, which contains a fragment of pRb protein carrying the phosphorylation sites. Expression vectors for CDK4, $p16^{INK4A}$ and Tax were co-transfected into 293T cells and the cell extracts were treated with anti-CDK4 antibodies. The immune complexes were subjected to *in vitro* kinase assay by adding GST–pRb and [γ-^{32}P]ATP, and the phosphorylation of GST–pRb was analyzed by SDS–gel electrophoresis (Matsushime *et al.*, 1994). When CDK4 alone was transfected, significant phosphorylation of GST–pRb was detected (Figure 2, lane 1), indicating that CDK4 was activated by forming complexes with endogenous D-type cyclins. Co-transfection of $p16^{INK4A}$ resulted in decreased phosphorylation (lane 2), consistent with the inhibitory effect of $p16^{INK4A}$ on CDK4 activity. Additional expression of Tax in this system restored the CDK4 kinase activity (lane 3), which had been suppressed by expression of $p16^{INK4A}$. These results indicate that Tax counteracts the inhibitory effect of $p16^{INK4A}$ on CDK4 kinase activity. The inactive Tax mutant d7/16, which could not bind to $p16^{INK4A}$, did not show any effect (lane 4), suggesting that this Tax activity is exerted through its binding to $p16^{INK4A}$.

To elucidate the mechanism of the effect of Tax on CDK4 activity, the $p16^{INK4A}$–CDK4 and $p16^{INK4A}$–Tax complexes in the transfected cells were analyzed; the cell extracts were treated with anti-CDK4 antibodies and the immunoprecipitates were analyzed by Western blotting with anti-$p16^{INK4A}$ antibodies to detect the $p16^{INK4A}$–CDK4 complexes (Figure 3, lanes 1–3). When CDK4 and $p16^{INK4A}$ were expressed, $p16^{INK4A}$ protein was co-immunoprecipitated with CDK4, but when Tax was

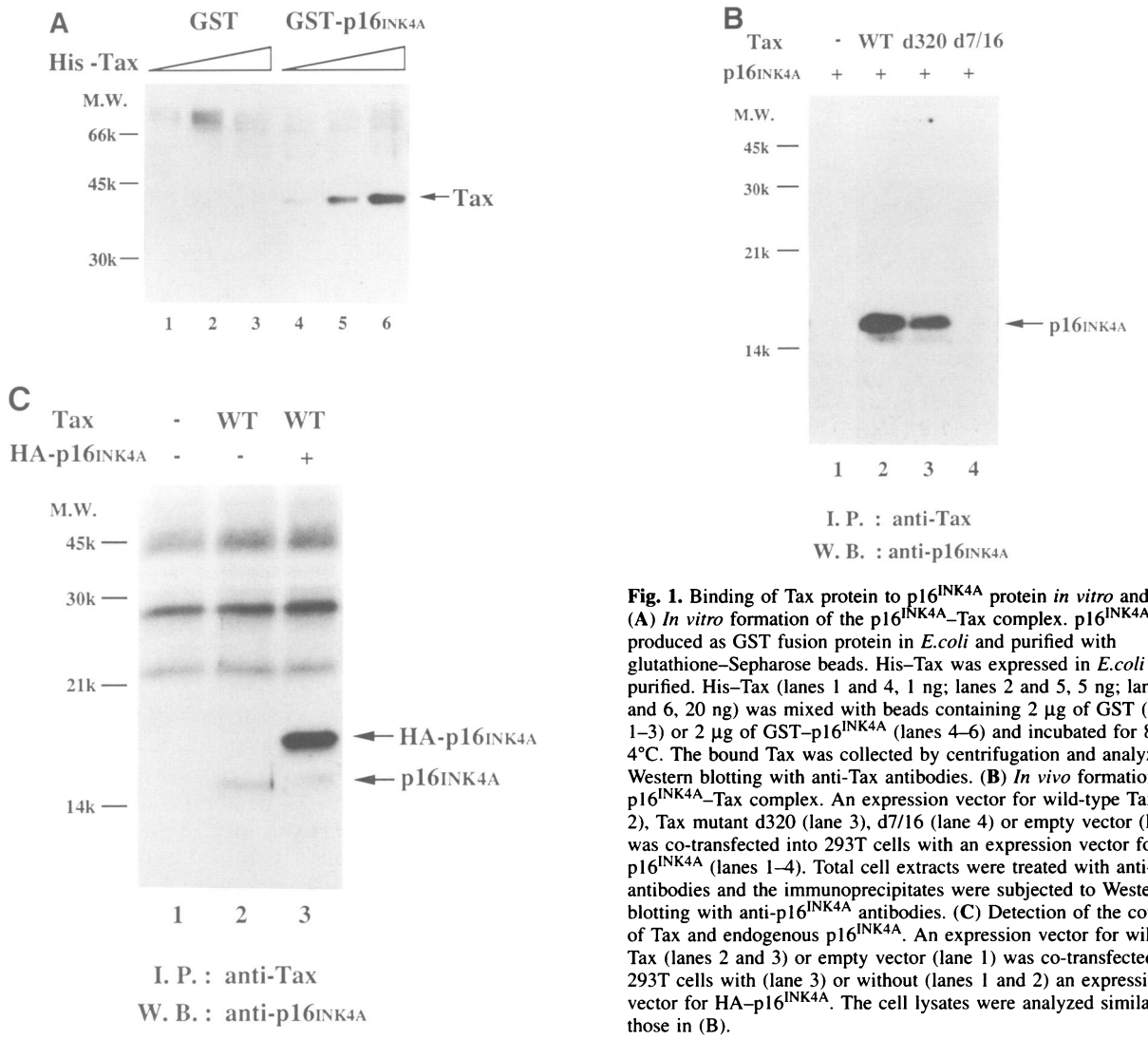

Fig. 1. Binding of Tax protein to $p16^{INK4A}$ protein *in vitro* and *in vivo*. (**A**) *In vitro* formation of the $p16^{INK4A}$–Tax complex. $p16^{INK4A}$ was produced as GST fusion protein in *E.coli* and purified with glutathione–Sepharose beads. His–Tax was expressed in *E.coli* and purified. His–Tax (lanes 1 and 4, 1 ng; lanes 2 and 5, 5 ng; lanes 3 and 6, 20 ng) was mixed with beads containing 2 μg of GST (lanes 1–3) or 2 μg of GST–$p16^{INK4A}$ (lanes 4–6) and incubated for 8 h at 4°C. The bound Tax was collected by centrifugation and analyzed by Western blotting with anti-Tax antibodies. (**B**) *In vivo* formation of the $p16^{INK4A}$–Tax complex. An expression vector for wild-type Tax (lane 2), Tax mutant d320 (lane 3), d7/16 (lane 4) or empty vector (lane 1) was co-transfected into 293T cells with an expression vector for $p16^{INK4A}$ (lanes 1–4). Total cell extracts were treated with anti-Tax antibodies and the immunoprecipitates were subjected to Western blotting with anti-$p16^{INK4A}$ antibodies. (**C**) Detection of the complex of Tax and endogenous $p16^{INK4A}$. An expression vector for wild-type Tax (lanes 2 and 3) or empty vector (lane 1) was co-transfected into 293T cells with (lane 3) or without (lanes 1 and 2) an expression vector for HA–$p16^{INK4A}$. The cell lysates were analyzed similarly to those in (B).

expressed, co-precipitated $p16^{INK4A}$ was drastically reduced. The inactive mutant of Tax, d7/16, was not effective in this assay. As shown in Figure 3, lane 5, when wild-type Tax was expressed, the formation of the $p16^{INK4A}$–Tax complex was demonstrated. These results indicate that Tax binds to $p16^{INK4A}$ and inhibits the formation of the $p16^{INK4A}$–CDK4 complex, allowing the formation of the enzymatically active cyclin D–CDK4 complex.

Tax blocks $p16^{INK4A}$-induced cell growth arrest

Overexpression of $p16^{INK4A}$ *in vivo* was shown to prevent cell proliferation in pRb-positive cells, and this effect can be assayed easily by counting growth-arrested flat cells using U2OS cells (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995). To demonstrate the effect of Tax on cell growth arrested by $p16^{INK4A}$, we examined whether Tax inhibits the flat cell-inducing activity of $p16^{INK4A}$ in U2OS cells. Expression vectors for $p16^{INK4A}$ and/or Tax were transfected with a puromycin resistance gene into U2OS cells. After 2 weeks, the numbers of non-dividing flat cells that were resistant to the drug were counted (Table I). Tax alone, as well as empty vector, had no significant effect on the number of flat cells. Overexpression of $p16^{INK4A}$ increased the number of flat cells, confirming the cell growth inhibition induced by $p16^{INK4A}$. Co-transfection of $p16^{INK4A}$ and Tax resulted in a drastic decrease in the number of flat cells, indicating that Tax suppressed $p16^{INK4A}$-induced cell growth inhibition *in vivo*. A mutant of Tax, d7/16, which did not bind to $p16^{INK4A}$, was not effective in a similar assay (data not shown). Therefore, Tax binding to $p16^{INK4A}$ and the resulting recovery of CDK kinase activity, which were demonstrated by *in vitro* assay, are in fact able to induce cell cycle progression.

Expression of $p16^{INK4A}$ in HTLV-1-infected T-cell lines

It has been reported that the $p16^{INK4A}$ gene is deleted or mutated at high frequency in tumor cell lines and primary tumors (Kamb *et al.*, 1994a,b; Nobori *et al.*, 1994; Okamoto *et al.*, 1994; Schmidt *et al.*, 1994; Sheaff and Roberts, 1995). To analyze the situation of $p16^{INK4A}$ in HTLV-1-infected cells, we examined the genomic DNA and expression of $p16^{INK4A}$ in various T-cell lines infected and not infected with HTLV-1. Southern blot analysis using cDNA as a probe showed homozygous deletions of the $p16^{INK4A}$ gene in all four uninfected T-cell lines tested,

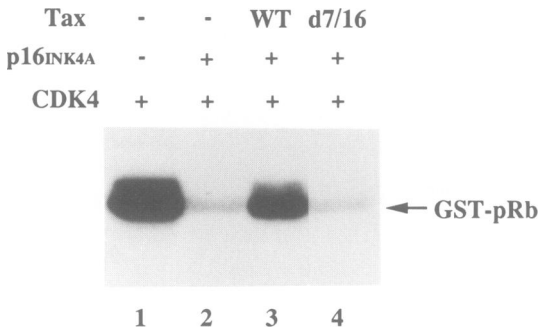

Fig. 2. Tax counteracts the inhibitory effect of $p16^{INK4A}$ on CDK4 kinase activity. Expression vectors for CDK4 (lanes 1–4), $p16^{INK4A}$ (lanes 2–4), wild-type Tax (lane 3) and d7/16 (lane 4) were co-transfected into 293T cells. Total cell extracts were treated with anti-CDK4 antibodies and the immunoprecipitates were subjected to immune complex kinase assay of CDK4 using GST–pRb as a substrate. GST–pRb contained a fragment of pRb protein carrying the phosphorylation site. Phosphorylated GST–pRb was analyzed by SDS–PAGE followed by autoradiography.

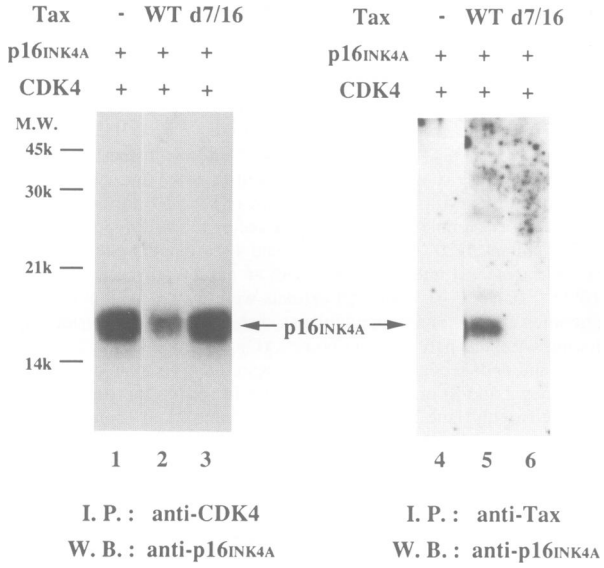

Fig. 3. Tax binds to $p16^{INK4A}$ and reduces the complex formation of $p16^{INK4A}$ and CDK4. Expression vectors for CDK4 (lanes 1–6), $p16^{INK4A}$ (lanes 1–6), Tax (lanes 2 and 5) and its mutant d7/16 (lanes 3 and 6) were co-transfected into 293T cells and the cell extracts were immunoprecipitated with anti-CDK4 antibodies followed by Western blotting with anti-$p16^{INK4A}$ antibodies (lanes 1–3). The same extracts were immunoprecipitated with anti-Tax antibodies followed by Western blotting with anti-$p16^{INK4A}$ antibodies (lanes 4–6).

Jurkat, CEM, HSB-2 and Molt-4 (Figure 4a). However, in HTLV-1-infected T-cell lines, MT-1, MT-2 and Hut102, we detected a normal band at the same intensity as that in HeLa and 293T cells, which express wild-type $p16^{INK4A}$ protein. This indicates that the $p16^{INK4A}$ gene locus was not grossly altered in HTLV-1-infected T-cell lines. To exclude the possibility of a small deletion in the $p16^{INK4A}$ gene, we carried out PCR analysis by amplifying the fragment containing exon 2 of the $p16^{INK4A}$ gene, in which mutations frequently have been found (Figure 4b). The results were consistent with those of the Southern blot analysis. Conservation of the $p16^{INK4A}$ gene in all T-cell lines infected with HTLV-1 was in contrast to the finding in uninfected T-cell lines.

Table I. Flat cell-inducing activity of $p16^{INK4A}$ is inhibited by Tax

Transfected plasmids	No. of flat cells		
	Exp. 1	Exp. 2	Exp. 3
Empty vector	6	30	14
Tax	10	43	19
$p16^{INK4A}$	202	645	344
$p16^{INK4A}$ + Tax	41	92	44

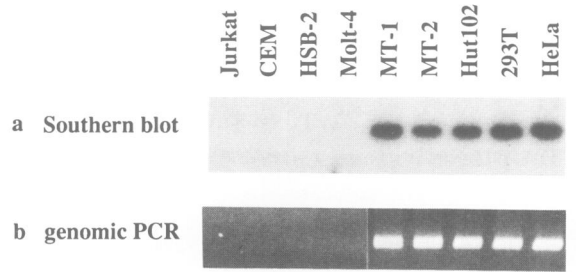

Fig. 4. (**a**) Southern blot analysis of the $p16^{INK4A}$ gene. A ^{32}P-labeled *Eco*RI–*Xho*I fragment of $p16^{INK4A}$ cDNA was hybridized with *Hin*dIII-digested genomic DNA of the indicated cell lines. A strong band of ~12 kb in size was demonstrated. (**b**) PCR amplification of exon 2 of the $p16^{INK4A}$ gene. Specific primers flanking $p16^{INK4A}$ exon 2 (described in Materials and methods) were used to amplify genomic DNA of the indicated cell lines.

Northern blot analysis demonstrated expression of $p16^{INK4A}$ mRNA in HTLV-1-infected T-cell lines, but not in uninfected T-cell lines (Figure 5). The latter finding was consistent with the homozygous deletion of the gene as demonstrated by Southern blot analysis. To examine whether the $p16^{INK4A}$ gene in HTLV-1-infected T-cell lines has a point mutation, the coding region of the gene was amplified by genomic DNA PCR and RT-PCR and sequenced (data not shown). Of three HTLV-1-infected T-cell lines, the wild-type $p16^{INK4A}$ coding sequence was confirmed in MT-2 and Hut102 cells, but a point mutation was found at codon 79, CGA (Arg) to TGA (Stop) in MT-1 cells. The MT-1 cell is a unique T-cell line infected with HTLV-1, in which Tax protein is not expressed, although it is expressed abundantly in MT-2 and Hut102 cells (Kiyokawa *et al.*, 1984). Therefore, these results indicate that the wild-type $p16^{INK4A}$ gene is expressed when Tax protein is expressed in T-cell lines; otherwise, the gene was deleted or mutated. Therefore, it is suggested that the functional inactivation of the $p16^{INK4A}$ gene product by Tax protein has physiological significance in HTLV-1-infected T-cell lines. In fact, the expression of $p16^{INK4A}$ protein was confirmed in HTLV-1-infected cells which have the wild-type $p16^{INK4A}$ coding sequence, although the levels were much lower than those in HeLa or 293T cells (data not shown).

Discussion

In this study, we demonstrated that HTLV-1 Tax binds to $p16^{INK4A}$, an inhibitor of CDK, and decreases the level of the $p16^{INK4A}$–CDK4 complex, resulting in the activation of CDK4 kinase which had been suppressed by $p16^{INK4A}$. Through this interaction, Tax inhibits $p16^{INK4A}$-induced suppression of U2OS cell growth. Since deletion or

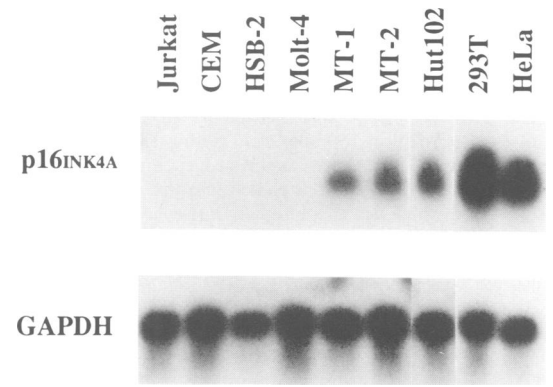

Fig. 5. Expression of $p16^{INK4A}$ mRNA in HTLV-1-infected T-cell lines. Cytoplasmic RNA preparations from the indicated cell lines were analyzed by Northern blotting using ^{32}P-labeled $p16^{INK4A}$ (top) or GAPDH probe (bottom).

mutation of the $p16^{INK4A}$ gene is reported to be involved in tumor development in a wide range of tissues (Kamb *et al.*, 1994a,b; Nobori *et al.*, 1994; Okamoto *et al.*, 1994; Schmidt *et al.*, 1994; Sheaff and Roberts, 1995), the functional inactivation of $p16^{INK4A}$ by Tax was suggested to contribute to the cellular immortalization and transformation induced by HTLV-1 infection.

Regarding our proposal of the physiological significance of Tax binding to $p16^{INK4A}$, one may argue that the expression of $p16^{INK4A}$ is low in T cells, thus $p16^{INK4A}$ might not be the relevant target. The expression of $p16^{INK4A}$ is in fact rather low in HTLV-1-infected T-cell lines and we could not demonstrate Tax binding to the endogenous $p16^{INK4A}$ (data not shown). However, the frequent deletion of the $p16^{INK4A}$ gene in many uninfected T-cell lines (this study) and in T-cell leukemia (Ogawa *et al.*, 1994) strongly indicates that $p16^{INK4A}$ is critical for the normal regulation of T cells, at least at certain stages; thus its inactivation by deletion was observed so frequently after immortalization or transformation. Therefore, the functional inactivation of $p16^{INK4A}$ by Tax binding could play a critical role in immortalization or transformation of HTLV-1-infected T cells at certain stages.

The molecular mechanism of the functional inactivation of $p16^{INK4A}$ and activation of CDK by Tax is analogous to that operating in the IκB–NF-κB regulatory system (Hirai *et al.*, 1994; Suzuki *et al.*, 1995). $p16^{INK4A}$ protein has four tandem repeats of ankyrin motifs (Serrano *et al.*, 1993), and IκB family proteins contain 6–8 repeats of ankyrin motifs (Beg and Baldwin, 1993). These ankyrin motifs are thought to be the site at which the partners of complexes such as $p16^{INK4A}$–CDK4 or IκB–NF-κB interact and thus exert their inhibitory activities. Previously we reported that Tax binds to the ankyrin motifs of IkB and inhibits the formation of the IκB–NF-κB complex, resulting in nuclear translocation of active NF-κB (Hirai *et al.*, 1994; Suzuki *et al.*, 1995). In a similar way, Tax interacts with $p16^{INK4A}$, possibly through the ankyrin motifs, and then inhibits the formation of $p16^{INK4A}$–CDK4, resulting in activation of CDK4 kinase (Figures 1B and 2).

As reported previously, overexpression of $p16^{INK4A}$ induced G_1 arrest in pRb-positive cells but was ineffective in pRb-negative cells (Guan *et al.*, 1994; Koh *et al.*, 1995; Lukas *et al.*, 1995; Medema *et al.*, 1995). This indicates that $p16^{INK4A}$ acts upstream of CDKs, resulting in inhibition of phosphorylation of pRb protein; however, in the absence of pRb protein, overexpression of $p16^{INK4A}$ does not affect G_1 phase progression. An inverse correlation was also reported between the expression of $p16^{INK4A}$ and pRb function in many tumor cell lines; deletion or mutation of $p16^{INK4A}$ appears to be restricted to subsets of tumor cell lines that retain pRb function and vice versa (Okamoto *et al.*, 1994; Otterson *et al.*, 1994; Schmidt *et al.*, 1994; Parry *et al.*, 1995). These reports suggest that this regulatory pathway is critical for normal cell cycle control; thus, loss of either gene function in the regulatory pathway leads cells towards transformation. We showed that the $p16^{INK4A}$ gene was intact and the transcripts were expressed in Tax-expressing HTLV-1-infected T-cell lines, although it was frequently deleted or mutated in HTLV-1-negative T-cell lines. These observations suggest that the functional inactivation of $p16^{INK4A}$ by Tax shown in this study might be a critical step in deregulation of the cell cycle.

In addition to the above prediction, one may argue that the pRb gene might be inactivated or that D-type cyclins are overexpressed in HTLV-1-infected T-cells responding to Tax. However, these arguments would not be correct. It was reported that pRb was expressed in all cases of HTLV-1-infected ATL cells tested so far (Cesarman *et al.*, 1992). We also detected broad bands of pRb protein in HTLV-1-infected T-cell lines tested in this study by Western blotting, indicating expression of pRb protein in both its hypo- and hyperphosphorylated forms. Furthermore, the expression level of CDK4 was not altered significantly by HTLV-1 infection (data not shown). These observations strongly suggest no inactivation of the pRb gene and the intactness of the pRb pathway. Induction of the overexpression of D-type cyclins by Tax was also excluded. Using a T-cell line carrying the inducible Tax gene (Ohtani *et al.*, 1989), we could not observe the augmentation of the expression of cyclin D1, D2 and D3 when Tax expression was induced efficiently (Figure 6). These results clearly indicate that Tax does not affect the pRb pathway in T cells through the expression of D-type cyclins. Taken together, these observations strongly suggest that the functional inactivation of $p16^{INK4A}$ by Tax at the early stage of HTLV-1 infection would be a critical event in HTLV-1-induced oncogenesis.

The other known members of the INK4 family, $p15^{INK4B}$ $p18^{INK4C}$ and $p19^{INK4D}$ all have four repeats of ankyrin motifs and inhibit the activities of D-type CDKs (Guan *et al.*, 1994; Hannon and Beach, 1994; Chan *et al.*, 1995; Hirai *et al.*, 1995). Therefore, we speculate that Tax binds to these INK family proteins in a manner similar to its binding to $p16^{INK4A}$. However, the ankyrin motifs are rather loosely conserved. Moreover, our previous finding that Tax binds to NF-κB-2 p100 in preference to NF-κB-1 p105 (Murakami *et al.*, 1995), both of which have ankyrin motifs, suggests some specificities in Tax binding to ankyrin motifs. Further studies on this line are now in progress. Among them, we have special interest in $p15^{INK4B}$, an inhibitor induced by TGF-β (Hannon and Beach, 1994). It was reported that HTLV-1-infected T-cell clones were resistant to growth inhibition by TGF-β, and this phenomenon correlated with the inability of TGF-β to prevent hyperphosphorylation of pRb (Hollsberg *et al.*,

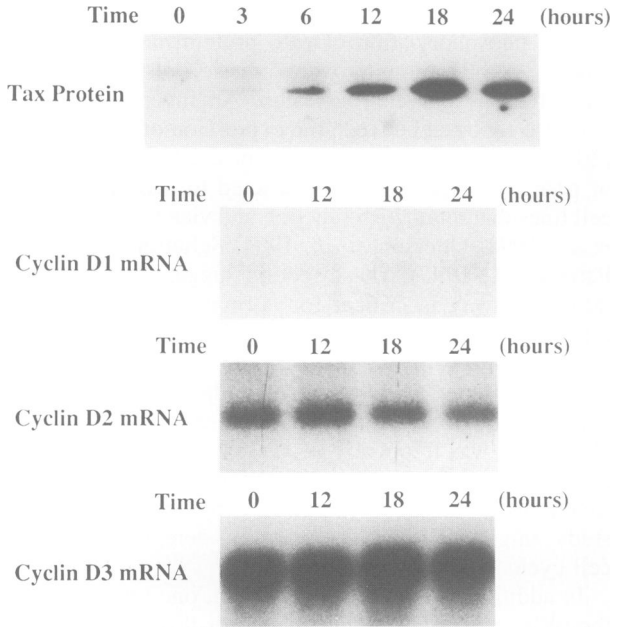

Fig. 6. No induction of D-type cyclin expression by Tax. JPX-9, a T-cell transformant carrying the Tax gene directed by the metallothionein promoter, was treated with 20 μM $CdCl_2$ to induce Tax expression. Cytoplasmic RNA was prepared at the indicated times after treatment, and subjected to Northern blot analysis using ^{32}P-labeled cyclin D1, D2 or D3 probe. Expression of Tax protein was also shown by Western blotting with anti-Tax antibodies.

1994). This suggests that HTLV-1 induces cell cycle progression through a pathway that is insensitive to TGF-β.

Ever since genetic linkage analysis of families with hereditary melanoma localized a putative susceptibility gene to the chromosome 9p21 region including p16^{INK4A} (Cannon-Albright *et al.*, 1992), extensive studies have shown that deletions and mutations involving p16^{INK4A} occur in many different types of human cancers (Kamb *et al.*, 1994a,b; Nobori *et al.*, 1994; Okamoto *et al.*, 1994; Schmidt *et al.*, 1994; Sheaff and Roberts, 1995). Furthermore, several tumor-derived alleles of p16^{INK4A} were reported to encode functionally compromised p16^{INK4A} products (Koh *et al.*, 1995; Lukas *et al.*, 1995). These analyses provide support for the notion that p16^{INK4A} acts as a tumor suppressor whose inactivation contributes to the development of human tumors. Several oncoproteins of DNA tumor viruses, such as E1a, E7 and large T, induced cellular immortalization probably through their ability to bind to and inactivate a tumor suppressor, pRb (Dyson *et al.*, 1989; Whyte *et al.*, 1989; Nevins, 1992). Tax binding to and inactivation of another tumor suppressor, p16^{INK4A}, in the same cell cycle regulatory pathway provides a novel insight into the cell cycle regulation involved in HTLV-1-induced cellular immortalization and transformation.

Materials and methods

Cells, plasmids and antibodies

MT-1, MT-2 and Hut102 are human T-cell lines infected with HTLV-1. Jurkat, CEM, HSB-2 and Molt-4 are human T-cell lines. JPX-9 cells are a transformant of Jurkat cells carrying an inducible Tax gene under the control of a metallothionein promoter (Ohtani *et al.*, 1989). Expression of Tax was induced by adding 20 μM $CdCl_2$ to the culture. These T-cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS). 293T, an adenovirus-transformed human embryonic kidney cell line carrying the SV40 large T antigen, and U2OS cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS.

Human p16^{INK4A} cDNA was donated by Dr David Beach of the Howard Hughes Medical Institute, Cold Spring Harbor Laboratory. A fragment containing the entire coding sequence of human p16^{INK4A} cDNA was isolated by PCR and cloned into the *Xba*I–*Bam*HI site of the pCG vector, which has a cytomegalovirus enhancer/promoter, or its derivative pCG-HA vector, which carries the sequence for the influenza virus HA epitope (Hirai *et al.*, 1994). Mouse CDK4 cDNA was cloned into the pSG vector, which has an SV40 enhancer/promoter. The expression plasmid of the fusion protein GST–p16^{INK4A} was constructed by inserting the p16^{INK4A} coding sequence into a vector, pGEX (Smith and Johnson, 1988). For the production of GST–pRb protein, pGEX-pRb(379–928) plasmid was used (Matsushime *et al.*, 1994).

Rabbit anti-Tax antibodies were prepared against the 11 C-terminal amino acids of Tax protein. Anti-CDK4 antibodies and anti-p16^{INK4A} antibodies were obtained from Santa Cruz Biotech. Inc., CA and Pharmigen, CA, respectively.

In vitro assay of complex formation

pGEX-p16^{INK4A} was introduced into *Escherichia coli* and the production of GST–p16^{INK4A} was induced by 1 mM isopropyl β-D-thiogalactoside (IPTG). The cells were recovered by centrifugation (5000 *g* for 5 min) at 4°C and lysed by sonication in phosphate-buffered saline (PBS) containing Triton X-100. The lysates were mixed with purified histidine-tagged Tax and incubated for 8 h at 4°C. Incubation for 1 h was sufficient for detection of the binding, but the maximum binding was observed after several hours incubation. The GST fusion proteins were collected on glutathione–Sepharose 4B (Pharmacia, NJ) and analyzed by Western blotting with anti-Tax antibodies.

Transfection and immune complex kinase assay

293T cells were transfected with plasmids by the standard calcium phosphate precipitation procedure. Preparation of cell extracts and immunoprecipitation were essentially the same as described previously (Matsushime *et al.*, 1994). Briefly, the cells were suspended in IP buffer consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, 2.5 mM EGTA, 1 mM dithiothreitol (DTT), 0.1% Tween-20, 10% glycerol, protease inhibitor and phosphatase inhibitor, sonicated and then centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant was then precipitated for 8 h at 4°C with protein A–Sepharose CL4B (Pharmacia) pre-coated with anti-CDK4 antibodies. Immunoprecipitated proteins on beads were washed twice with IP buffer and twice with 50 mM HEPES, pH 7.5. The precipitates were suspended in 30 μl of kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) containing 0.2 μg of GST–pRb fusion protein as a substrate and 2.5 mM EGTA, phosphatase inhibitors, 20 μM ATP and 10 μCi of [γ-^{32}P]ATP (NEN Dupont, Boston, MA). After incubation for 30 min at 30°C, the samples were separated in SDS–polyacrylamide gel followed by autoradiography.

To detect protein complexes, the cell extracts were treated with anti-Tax or anti-CDK4 antibodies, and the immunoprecipitates were separated in SDS–polyacrylamide gel followed by Western blotting. The filter was incubated with anti-p16^{INK4A} antibodies and the bands were visualized with protein A–horseradish peroxidase conjugate and an enhancement chemiluminescence detection kit (Amersham, IL).

Growth suppression assay

Expression vectors for p16^{INK4A} and/or Tax were transfected with a puromycin resistance gene plasmid into U2OS cells by the calcium phosphate precipitation procedure. Two days after transfection, the cells were replated at different dilutions and cultured in medium supplemented with 2 μg/ml of puromycin (Sigma, MO). After 2 weeks, drug-resistant cells were examined under the microscope and flat cells were counted in randomly selected small areas (400 mm^2). Results of three independent experiments are presented in Table I.

Southern blot, PCR and Northern blot analysis

For Southern blot, 10 μg of genomic DNA was digested with *Hin*dIII, separated on 0.8% agarose gel, and transferred to a nylon membrane. It was hybridized with ^{32}P-labeled cDNA of p16^{INK4A} at 65°C in a mixture containing 4× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate) and 100 μg of sonicated and denatured salmon sperm DNA and then washed in 0.5× SSC, 0.1% SDS at 65°C for 45 min.

For PCR analyses, we synthesized the specific primers for p16^{INK4A} as follows. Primers for exon 1 were (5′–3′) CGGAGAGGGGGAGAGCAG

(sense) and TCCCCTTTTTCC-GGAGAATCG (antisense). Primers for exon 2 were (5′–3′) TATAAGCTTGGCTCTA-CACAAGCTTCCTT (sense) and TATTCTAGATGAGCTTTGGAAGCTCTCAG (antisense). Primers for RT-PCR amplifying cDNA fragment containing exons 2 and 3 were (5′–3′) CACCAGAGGCAGTAACCATG (sense) and ATGAAA-ACTACGAAA-GCGGG (antisense). PCR conditions consisted of 5 min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 55°C and 90 s at 72°C. The PCR products were analyzed on 1.5% agarose gel and sequenced by the dideoxy chain termination method.

Cytoplasmic RNA was extracted by the vanadyl complex procedure described previously (Murakami *et al.*, 1995). Briefly, cells were lysed in buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$) containing 0.3% NP-40 and 10 mM ribonucleoside–vanadyl complex, and nuclei were removed by centrifugation. Cytoplasmic RNA was isolated from the supernatant by phenol extraction. RNA was denatured with 2.2 M formaldehyde, electrophoresed through formaldehyde–agarose gel and transferred to a nylon filter. It was hybridized overnight with ^{32}P-labeled cDNA of p16^{INK4A}, cyclin D1, D2, D3 or glyceraldehyde phosphate dehydrogenase at 42°C in a mixture containing 50% formamide, 4× SSC, 100 μg of sonicated and denatured salmon sperm DNA and 10 μg of poly(A). The filter was washed in 0.1× SSC, 0.1% SDS at 65°C for 15 min.

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