PDZ domain-binding motif of human T-cell leukemia virus type 1 Tax oncoprotein augments the transforming activity in a rat fibroblast cell line

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Abstract

While human T-cell leukemia virus type 1 (HTLV-1) is associated with the development of adult T-cell leukemia (ATL), HTLV-2 has not been reported to be associated with such malignant leukemias. HTLV-1 Tax1 oncoprotein transforms a rat fibroblast cell line (Rat-1) to form multiple large colonies in soft agar, and this activity is much greater than that of HTLV-2 Tax2. We have demonstrated here that the increased number of transformed colonies induced by Tax1 relative to Tax2 was mediated by a PDZ domain-binding motif (PBM) in Tax1, which is absent in Tax2. Tax1 PBM mediated the interaction of Tax1 with the discs large (Dlg) tumor suppressor containing PDZ domains, and the interaction correlated well with the transforming activities of Tax1 and the mutants. Through this interaction, Tax1 altered the subcellular localization of Dlg from the detergent-soluble to the detergent-insoluble fraction in a fibroblast cell line as well as in HTLV-1-infected T-cell lines. These results suggest that the interaction of Tax1 with PDZ domain protein(s) is critically involved in the transforming activity of Tax1, the activity of which may be a crucial factor in malignant transformation of HTLV-1-infected cells in vivo.

Keywords: HTLV-1; Tax; PDZ; Dlg; Adult T-cell leukemia

Introduction

Adult T-cell leukemia (ATL) is an extremely aggressive human CD4+ T-cell leukemia (Sugamura and Hinuma, 1993; Uchiyama et al., 1977). For instance, ATL is resistant to chemotherapy and most patients die within 1 year of diagnosis. Human T-cell leukemia virus type 1 (HTLV-1) infection of CD4 T cells is the first step in ATL development, but by itself this is not sufficient for leukemia development because a minority of HTLV-1-infected subjects (approximately 5%) develop ATL on average 60–70 years after the infection (Hinuma et al., 1981; Poiesz et al., 1980; Sugamura and Hinuma, 1993).

HTLV type 2 (HTLV-2) was originally isolated from a cell line established from a T-cell variant of hairy cell leukemia (Chen et al., 1983a). HTLV-1 and HTLV-2 can transform primary human T cells in both an interleukin (IL)-2-dependent and -independent manner in vitro, with equivalent efficiency (Chen et al., 1983b; Miyoshi et al., 1981; Yamamoto et al., 1982). However, HTLV-2 is not associated with ATL or leukemias of such a malignant nature (Sugamura and Hinuma, 1993). Thus, elucidation of the mechanisms by which these two viruses determine distinct outcomes will provide fundamental insights into the initiation of multi-step leukemogenesis.

HTLV-1 and HTLV-2 encode Tax1 and Tax2 protein, respectively, and these two proteins exhibit more than 70% amino acid identity (Seiki et al., 1986; Slamon et al., 1984). They are crucial for the transforming activity of HTLV-1 and HTLV-2. For instance, Tax1 by itself immortalizes primary human T cells in an IL-2-dependent manner (Akagi and Shimotohno, 1993; Grassmann et al., 1992). Recombi-
nant HTLV-1 and HTLV-2 carrying an inactive tax1 and tax2 gene, respectively, cannot transform primary human T cells (Robek and Ratner, 1999; Ross et al., 1996).

Tax1 is a multifunctional protein (Yoshida, 2001). It activates the transcription of many cellular genes associated with cell growth, such as genes encoding cytokines (Inoue et al., 1986; Seikevitz et al., 1987), cytokine receptors (Ballard et al., 1988; Cross et al., 1987; Inoue et al., 1986; Maruyama et al., 1987), an anti-apoptotic protein (Tsukahara et al., 1999), cell cycle regulators (Akagi et al., 1996; Iwanaga et al., 2001; Mori et al., 2002; Santiago et al., 1999), and protooncogenes (Fujii et al., 1991; Yoshida, 2001). Moreover, Tax1 inactivates several negative cell-growth regulators such as p53, p16INK, and MAD1 (Akagi et al., 1997; Jin et al., 1998; Pise-Masison et al., 1998; Suzuki et al., 1996). The comparative studies of Tax1 and Tax2 have not, however, elucidated the underlying mechanism for the different pathogenic properties between HTLV-1 and HTLV-2.

Recent results have provided an attractive clue for the different pathogenic properties of these two viruses. Tax1 and Tax2 induce transformed colonies in soft agar (colony formation in soft agar, or CFSA) in a rat fibroblast cell line (Rat-1), and the activity of Tax1 is lower than that of Tax1 (Endo et al., 2002). Their chimeric proteins indicate that the C-terminal 53 amino acids of Tax1 are responsible for the high CFSA activity relative to Tax2. This C-terminal Tax1 fragment, but not the Tax2 counterpart, contains a PDZ domain-binding motif (PBM), C-terminal S/TXV (S/T, serine or threonine; X, any amino acid; V, valine), and it binds to PDZ domain-containing protein discs large (Dlg), a mammalian homologue of Drosophila discs large tumor suppressor (Kiyono et al., 1997; Lee et al., 1997; Rousset et al., 1998; Suzuki et al., 1999). Thus, PDZ domain-containing protein(s), including Dlg, are candidates for mediating the high CFSA activity of Tax1, and thereby the leukemogenic properties of HTLV-1.

In this study, we demonstrate that the Tax1 PBM is indeed involved in the increased CFSA activity of Tax1 relative to Tax2. Tax1, through PBM, interacted with Dlg, whereas Tax2 minimally did so, and moreover, the interaction between Tax1 and Tax2 mutants correlated well with their CFSA activities. We will discuss these findings in the context of the transformation of Rat-1 cells by Tax1 as well as malignant transformation of HTLV-1-infected T cells.

Results

PDZ domain-binding motif in Tax1 is responsible for the high transforming activity

We previously reported that the C-terminal 53 amino acids of Tax1 were responsible for the high transforming activity relative to Tax2, and that this fragment contains a PDZ domain-binding motif (PBM) (Endo et al., 2002). To examine the involvement of the Tax1 PBM in Tax1 transforming activity, we constructed two types of mutant genes. The TaxΔC gene contains a C-terminal four-amino acid deletion in Tax1, thereby removing the PBM motif (T/SXV) (Fig. 1). The Tax2B + C gene has an additional 10 Tax1 amino acids containing PBM at the C-terminus of Tax2B. In addition, we constructed two substitution mutants of Tax1 PBM, at amino acids 351 and 353, in the context of Tax1 and Tax2B + C. These constructs were introduced into a mammalian expression vector (pHβPr-1-neo) carrying the neomycin resistance gene, and the plasmids were transfected into Rat-1 cells, and the cells were selected by neomycin (G418). Anti-Tax1 monoclonal antibody showed that all these transfectants expressed the Tax1 protein or the mutant one. The amount of Tax1 protein in Rat-1 cells was higher than those of the mutants in five (including No2 and No3 in Fig. 2A) out of seven experiments. On the other hand, the amount of Tax2B + C protein in Rat-1 cells, detected by an anti-Tax2B antibody, was higher than that of Tax2B, Tax2B351A, and Tax2B353A in six (including No6, No7, No8 in Fig. 2B) out of seven experiments. Anti-α-Tubulin antibody indicated that the equivalent amount of the proteins was loaded on the respective lane in the Western blot analysis (Fig. 3). These results suggested that Tax1 PBM increase expression of Tax1 protein in Rat-1 cells. Then, these transfectants were seeded into soft agar to measure the transforming activity (colony formation in soft agar, or CFSA). The Rat-1/Tax1 cells formed multiple large colonies in soft agar relative to the control cells [see Mean (%) in Fig. 2A]. The Rat-1/TaxΔC cells also formed more colonies relative to the control, but the number of the colonies was reproduced lower than for Rat-1/Tax1. In addition, the mutation of PBM at amino acids 351 and 353 in Tax1 also reduced the numbers of colonies. The increased transforming activity of Tax1 relative to those of the mutants is not singly due to the increased expression because Tax1 was expressed equivalently or less than TaxΔC or Tax353A in Rat-1 cells in some experiments (TaxΔC in No1, Tax353A in No2, TaxΔC in No4). Tax2B and Tax2B + C also transformed Rat-1 cells to form positive colonies relative to the control, and the number of the colonies induced by Rat-1/Tax2B + C cells was more than that of Rat-1/Tax2B. In addition, the mutation of PBM at amino acids 351 and 353 in Tax2B + C also reduced the numbers of colonies. The almost equivalent expression of Tax2B and Tax2B + C in the experiment No5 suggested that the increased expression of Tax2B + C was not the only reason for
the increased activity of Tax2B + C relative to Tax2B. Taken together, these results indicate that the Tax1 PBM increases the number of the transformed colonies in soft agar, and that amino acids 351 and 353 in Tax1 PBM are critical for the activity.

**Tax1 binds to Dlg through PBM**

The human Dlg protein is a well-known PDZ domain-containing protein, and it was isolated as a mammalian homologue of *Drosophila* discs large tumor suppressor. Dlg interacts with Tax1 through its PBM (Rousset et al., 1998; Suzuki et al., 1999). To examine the involvement of Dlg in the transformation by Tax1, the *tax1* and *tax2B* plasmids and their respective mutant plasmids were transiently transfected into a human embryonic kidney cell line (293T) together with the *Dlg* expression plasmid. Dlg protein in the lysates prepared from 293T cells was immunoprecipitated with anti-Dlg antibody, and then the amount of Tax1 in the precipitates was analyzed by Western blot. Tax1 was

![Fig. 2. The PDZ domain-binding motif (PBM) of Tax1 is responsible for the increased number of Rat-1 transformed colonies. (A, B) The cell lysate was prepared from Rat-1 cells stably transfected with the indicated plasmids, and the amount of Tax in each cell lysate was measured by Western blot analysis using an anti-Tax1 antibody (A) or anti-Tax2B antibody (B). The Rat-1 cells transfected with pHPr-neo plasmid used as a control are indicated as pA. These Rat-1 cells, in which expression of the respective Tax proteins was measured by Western blot analysis, were seeded in soft agar. Three weeks after the inoculation, the number (%) of colonies was determined by light microscopy. The CFSA assay of No1 through No8 was performed in duplicate, and the mean (%) indicate the averages and standard deviations of the activity of the indicated Rat-1 transfectants above. ND: not determined.](image)

The increased activity of Tax2B + C relative to Tax2B. Taken together, these results indicate that the Tax1 PBM increases the number of the transformed colonies in soft agar, and that amino acids 351 and 353 in Tax1 PBM are critical for the activity.

![Fig. 3. The expression of Tax and mutant proteins in Rat-1 cells. The cell lysate was prepared from Rat-1 cells stably transfected with the indicated plasmids, and the amount of Tax and α-Tubulin in each cell lysate was measured by Western blot analysis using an anti-Tax1 antibody, anti-Tax2B antibody or anti-α-Tubulin antibody.](image)
specifically detected in the immune complex of Dlg, whereas TaxΔC was minimally detected in the complex (Fig. 4A). Tax351A and Tax353A were also detected in the Dlg complex, but the amounts were much lower than that of Tax1. The reduced detection of TaxΔC, Ta351A, and Tax353A in the complex was not due to their reduced expression in 293T cells because anti-Tax1 antibody detected the equivalent expression of these mutant proteins to that of Tax1 in 293T cells (Fig. 4C). In addition, anti-Dlg immunoprecipitated the equivalent amount of Dlg protein from the 293T cells transfected with these tax1 plasmids (Fig. 4B). Unlike Tax1, Dlg minimally precipitated Tax2B, whereas it efficiently precipitated Tax2B + C. The Dlg complex also contained Tax2B351A and Tax2B353A, and the amount was much higher than that of Tax2B but still lower than that of Tax2B + C. These results showed that Tax1, but not Tax2B, binds to Dlg, and the interaction correlated with the number of colonies induced by the respective Tax protein in Rat-1 cells (Fig. 2).

**Tax1 alters the subcellular localization of Dlg**

Having demonstrated that Dlg can interact with Tax1 in 293T cells, we next examined the subcellular localization of endogenous Dlg in the absence and presence of Tax1. In the absence of Tax1, co-staining by anti-Dlg (red), anti-Tax1 (green), and Hoechst 33342 (nucleus; blue) showed that endogenous Dlg was mainly localized in the cytoplasm (Fig. 5). In the presence of Tax1, Dlg colocalized with Tax1, and the complex (yellow) was detected mainly in the cytoplasm. In contrast, TaxΔC, which cannot interact with Dlg, did not obviously show the colocalization with Dlg. Exogenous Dlg protein fused with green fluorescent protein (GFP-Dlg) in the absence of Tax1 was also detected mainly in the cytoplasm, and again the localization was altered by Tax1 but not TaxΔC into the Tax1-containing complex (Fig. 6). These results indicated that Tax1 aberrantly sequesters Dlg within the Tax1-containing complex.

**Tax1 translocates Dlg from the detergent-soluble fraction into the insoluble fraction in 293T cells and HTLV-1-infected T-cell lines**

We further delineated subcellular localization of Dlg and Tax1 by cell fractionation. The 293T cells transiently transfected with Tax1 expression plasmid were lysed by mild detergent (1% NP40) or by SDS sample buffer, and the amount of endogenous Dlg and Tax1 in the soluble fraction by NP40, the insoluble fraction and the total fraction lysed directly by SDS-sample buffer, was measured by Western blotting. Dlg in 293T cells transfected with the control plasmid (βA) was detected both in the NP40-soluble and NP40-insoluble fraction (βA in Fig. 7A). However, Dlg in the soluble fraction became undetectable in response to Tax1 expression while that in the insoluble fraction was increased. Since the amount of total Dlg proteins prepared by SDS sample buffer in 293T cells was minimally affected by Tax1, these results indicated that Tax1 translocates the Dlg protein from the detergent-soluble to the detergent-insoluble fraction in 293T cells. It should be mentioned that the detergent-soluble Dlg in the presence of Tax1 was detected in Fig. 4 but not in Fig. 7A. This is likely to be due to the different
Fig. 6. GFP-Dlg colocalizes with Tax1 in 293T cells. The 293T cells were transfected with the GFP-Dlg plasmid together with the tax1, taxΔC, or pHjPr-1-neo (pA) plasmid. At 48 h after transfection, the cells were incubated with mouse anti-Tax1 antibody. After washing, the cells were further incubated with TRITC-labeled anti-mouse IgG. After washing, the cells were stained with Hoechst 33258 for nuclear staining. The cells were then examined for the staining for anti-Tax1 (red), GFP-Dlg (green), and Hoechst (blue) by fluorescent light microscopy (magnification, ×400).

Fig. 5. Subcellular colocalization of endogenous Dlg and Tax1. The 293T cells were transfected with the tax1, taxΔC, or pHjPr-1-neo (pA) plasmid. Then, the cells were stained with anti-Dlg (red), anti-Tax1 (green), and with Hoechst 33258 (blue) for nuclear staining. The stained cells were examined by fluorescent light microscopy (magnification, ×400).

Fig. 6. GFP-Dlg colocalizes with Tax1 in 293T cells. The 293T cells were transfected with the GFP-Dlg plasmid together with the tax1, taxΔC, or pHjPr-1-neo plasmid (pA). At 48 h after transfection, the cells were incubated with mouse anti-Tax1 antibody. After washing, the cells were further incubated with TRITC-labeled anti-mouse IgG. After washing, the cells were stained with Hoechst 33258 for nuclear staining. The cells were then examined for the staining for anti-Tax1 (red), GFP-Dlg (green), and Hoechst (blue) by fluorescent light microscopy (magnification, ×400).
amount of the detergent-soluble fraction used for these experiments, such that much more lysate was used in Fig. 4 than in Fig. 7A. The translocation of Dlg by Tax1 was dependent on functional PBM because TaxΔC, Tax351A, or Tax353A did not induce subcellular translocation of Dlg protein. Tax2B+C but not Tax2B also translocated Dlg protein from the detergent-soluble to the insoluble fraction. The translocation of Dlg protein by Tax2B+C was again dependent on functional PBM because Tax2B351A and Tax2B353A that bind less efficiently to Dlg than Tax2B+C did not induce translocation of Dlg. These results showed that Tax1 but not Tax2B, through the interaction with Dlg, alters the subcellular localization of Dlg from the detergent-soluble to the detergent-soluble fraction in 293T cells.

We next examined whether Tax1 alters the subcellular localization of Dlg in HTLV-1-infected T-cell lines (Fig. 7B). In three HTLV-1-uninfected cell lines, Dlg was detected in the detergent-soluble fraction, although it was undetectable in two HTLV-1-infected cell lines (HUT102, SLB-1). On the other hand, detergent-insoluble Dlg was more abundant in the HTLV-1-infected cell lines than HTLV-1-uninfected cell lines. These results suggested that Tax1 alters the subcellular localization of Dlg from the detergent-soluble to the detergent-insoluble fraction in HTLV-1-infected T-cell lines. In addition, we noticed that the amount of total Dlg protein solubilized by SDS-sample buffer in two HTLV-1-infected cell lines was lower than that in HTLV-1-uninfected cell lines. Thus, Tax1 may reduce the total quantity of Dlg protein in HTLV-1-infected T-cell lines.

Unlike HTLV-1-infected T-cell lines, the amount of detergent-soluble and total Dlg in HTLV-2-infected T cells was equivalent to those of HTLV-1-uninfected ones, although the

Fig. 7. Tax1 alters subcellular localization of Dlg. (A, B) 293T cells transfected with the indicated tax plasmids or pHPr-1-neo plasmid (5A) (A), or the indicated human T-cell lines (B) were divided into two groups. One group was treated with the lysis buffer A. After centrifugation, the supernatant was used as the soluble fraction, and the resultant pellet was further treated with SDS sample buffer and used as the insoluble fraction. The other group was directly treated with SDS sample buffer and used as the total fraction. Three types of samples were size-fractionated by SDS-PAGE, and the amount of Tax and Dlg proteins was measured by Western blot using anti-Tax1 antibody, anti-Tax2B antibody, or anti-Dlg antibody. HTLV-1 infection in the T-cell lines is indicated.

A: 293T cells

B: T-cell lines

Fig. 8. The expression of detergent-soluble Dlg in HTLV-2-infected T-cell line. The indicated human T-cell lines were divided into two groups. One group was treated with the lysis buffer A. After centrifugation, the supernatant was used as the soluble fraction, and the resultant pellet was further treated with SDS sample buffer and used as the insoluble fraction. The other group was directly treated with SDS sample buffer and used as the total fraction. Three types of samples were size-fractionated by SDS-PAGE, and the amount of Dlg proteins was measured by Western blot using an anti-Dlg antibody. HTLV-1 or HTLV-2 infection in the T-cell lines is indicated.
detergent-insoluble Dlg in HTLV-2-infected T cells was undetectable (Fig. 8). These results suggested that the quantitative and qualitative alteration of Dlg is specific to HTLV-1-infected T-cell lines, and such alteration is minimum in HTLV-2-infected ones. In addition, we consistently observed that the molecular weight of Dlg in HTLV-1-infected cell lines was larger than that in HTLV-1-uninfected ones. Thus, the interaction of Dlg with Tax1 may induce the post-transcriptional modification of Dlg protein such as phosphorylation.

Discussion

Once infected, HTLV-1 and HTLV-2 achieve life-long persistent infection in human T cells in vivo (Sugamura and Hinuma, 1993). However, only some HTLV-1-infected individuals, but not HTLV-2 ones, develop an aggressive form of leukemia 60–70 years after the infection. Since HTLV-1 and HTLV-2 equivalently transform T cells in vitro, HTLV-1 but not HTLV-2 presumably encode an activity to initiate leukemogenesis that is distinct from in vitro transformation of T cells. Thus, these two viruses should provide fundamental insights into the initiation of multi-step leukemogenesis. We recently showed that HTLV-1 Tax1 exhibits higher transforming (CFSA) activity in a fibroblast cell line (Rat-1) than Tax2 (Endo et al., 2002). Here, we extended these observations and showed that a PBM at the C-terminal end of Tax1 is a factor responsible for the increased CFSA activity relative to Tax2 (Fig. 2). Thus, our results suggest that the Tax1 PBM could be a factor for initiating the first step of leukemogenesis by HTLV-1.

Tax1, through the PBM, interacted with Dlg, the human homologue of Drosophila tumor suppressor containing PDZ domains, and moreover the interaction correlated well with the high CFSA activity of Tax1. These results suggested that Dlg is involved in the high CFSA activity of Tax1. It should be, however, noted that several PDZ domain proteins have been shown to interact with Tax1 (Rousset et al., 1998). Thus, PDZ domain-containing protein(s) other than Dlg or more than one PDZ domain protein may augment the CFSA activity of Tax1.

The present results suggested that the Tax1 PBM has the activity to augment the expression of Tax1 proteins in Rat-1 cells (Fig. 2). The enhancement of Tax1 protein expression by the PBM was observed in stable Rat-1 transfectants, whereas such enhancement was not apparent after transient overexpression of Tax1 in 293T cells (data not shown). Thus, the interaction of Tax1 with the limiting amount of PDZ domain-containing protein(s) in Rat-1 cells may mediate the increased protein expression.

Through the interaction, Tax1 altered subcellular localization of Dlg from the detergent-soluble to the detergent-insoluble fraction (Fig. 7). Since Tax1 is detected in both the detergent-soluble and the detergent-insoluble fraction, the Tax1 complex with Dlg is likely to move preferentially into the detergent-insoluble fraction. This aberrant sequestration of Dlg within the cells correlated well with the high CFSA activity of Tax1. Exogenous expression of Dlg in a mouse fibroblast cell line, NIH3T3, inhibits the cell cycle progression from GO/G1 to S phase (Ishidate et al., 2000), and Tax can release the cell cycle arrest (Suzuki et al., 1999). Thus, the present results suggest that aberrant sequestration of Dlg within the insoluble fraction by Tax1 is involved in the inactivation of Dlg function towards the cell cycle, as well as the increased CFSA activity.

Tax1 was detected both in the detergent-soluble and the insoluble fraction (Fig. 7). Trihn et al. (1997) showed that Tax1 interacts with a class of intermediate filaments, the cytokeratins (Ker), and discussed that the interaction of Tax1 with the cytokeratins may be involved in the morphologic changes observed in Tax1-expressing cells. Thus, the detergent-insoluble Tax1 may represent the protein interacting with cytoskeleton component(s), and may be responsible for the selective function(s) of Tax1.

The molecular weight of Dlg in HTLV-1-infected cell lines was larger than those in HTLV-1-uninfected ones (Fig. 7B). By using immunoprecipitation analysis, Suzuki et al. (1999) also showed that the Dlg complex with Tax1 migrated slower than Dlg in the absence of Tax1, and this slow migration of Dlg induced by Tax1 was canceled by treating the complex with bacterial alkaline phosphatase. These results indicated that Tax1 induces the phosphorylation of Dlg. Since Tax1 by itself has no kinase activity, Tax1 might activate putative Dlg kinase(s). In normal cells, phosphorylation of Dlg was mainly detected in G2/M phase (Suzuki et al., 1999). Thus, the characterization of phosphorylated Dlg and the significance in cell cycle regulation would elucidate the mechanism of Dlg regulation in normal cells as well as HTLV-1-infected ones.

Several viral oncoproteins such as adenovirus 9 E4ORF1 and the human papilloma virus (HPV) E6 contain a PBM. Interestingly, only E6 proteins that are derived from high-risk HPV's but not low-risk ones have a functional PBM, and interact with PDZ domain proteins such as Dlg (Kiyono et al., 1997; Lee et al., 1997). Furthermore, E6 and E4ORF1 mutants that lose the ability to interact with Dlg can no longer transform fibroblast cell lines (Kiyono et al., 1997; Weiss et al., 1997). In addition, E6 induces the degradation of Dlg by an ubiquitin-mediated proteasome mechanism (Gardiol et al., 1999). Thus, the inactivation of PDZ domain proteins is likely to be a common target for high-risk oncoviruses to induce the malignant transformation of the virus-infected cells.

There are two types of simian T-cell leukemia virus (STLV), STLV-1 and STLV-2. Interestingly, only STLV-1 is associated with the development of leukemia or lymphoma, although there are only few reports of the incidence (Hubbard et al., 1993; Voevodin et al., 1996). STLV-1 and STLV-2 also express Tax1 and Tax2, respectively, and these STLV Tax proteins have more than 70% amino acid identity to HTLV Tax1, and only STLV Tax1 but not STLV-2 Tax2
has a PBM at the C-terminal end (Fig. 9) (Ibrahim et al., 1995). Taken together, our results suggest that the PBM is an evolutionarily conserved factor responsible for the leukemogenic properties of STLV-1 and HTLV-1.

Materials and methods

Cell culture

Rat-1 and 293T cells are a rat fibroblast cell line and a human embryonic kidney cell line, respectively. These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml) at 37 °C in a humidified 5% CO₂ atmosphere. Human T-cell lines used in the present experiments have been characterized previously (Mori et al., 1999; Sugamura et al., 1984). Jurkat, HUT78, and MOLT-4 are HTLV-1-negative human T-cell lines, although Ton-1 is an HTLV-2-positive one. These T-cell lines were cultured in RPMI1640 supplemented with 10% FBS, 4 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml).

Plasmids

The tax1, tax2B, and their corresponding mutant genes were cloned into the plasmid pH[Pr-1-neo] that has a β-actin promoter for protein expression in mammalian cells and neomycin-resistance gene as a selection marker (Matsumoto et al., 1997). The Tax2B plasmid (pCAGGS-Tax2B) was kindly provided by Dr. William W. Hall at University College Dublin, Ireland (Lewis et al., 2000). The mutant tax1 and tax2B genes were constructed by the polymerase chain reaction method. The template to construct mutant tax1 and tax2B cDNA was pbLuescript-tax1 and pCAGGS-Tax2B, respectively. The 3′ primers used were 5′-GAAACACATTCCGAGGATCCCCGCA-3′ for taxAC, 5′-GAGGATCCCTCA-GACCTCGCGTTCCGGGAAATG-3′ for tax351A, 5′-GAGGATCCAGGCTTCTGTGGCATGGGCA-3′ for tax353A, 5′-TTGGATCCGAATTCTGACCTTCTGTGGGAGAAG-3′ for tax2B353A, and 5′-TTGGATCCGAATTCTGACCTTCTGTGGGAGAAG-3′ for tax2B355A, respectively. The common 5′ primers for tax1 and tax2B were 5′-AACAGCTTAGACCTATGACCATG-3′ in the pBluescript-tax1 plasmid and 5′-AAAGATCCGCGT- TAAGGTTGCGACC-3′ in pCAGGS-Tax2B, respectively. DNA sequencing confirmed these mutant plasmids. Plasmid pcDNA3.1 pcDNA+-mycDlg (Dlg) is the expression plasmid for human Dlg (Kohu et al., 2002).

Colony formation in soft agar (CFSAs) assay

A linearized tax plasmid was transfected into Rat-1 cells by the electroporation method using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA) at 200 V and 975 µF. The cells were then cultured for more than 2 weeks in a selection medium containing 500 µg/ml of G418. Pools of resistant cells (1 × 10⁴ cells) were seeded in 0.3% agarose containing 10% FBS/DMEM overlaid on 0.5% agarose in 60 mm plates. After culturing for 3 weeks, the number of colonies was examined under a light microscope, and colony number (%) was calculated as the proportion of colonies measuring more than 100 µm in diameter relative to colonies smaller than 100 µm.

Western blotting

Rat-1 cells expressing Tax1 protein were lysed with RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1.0% Nonidet P-40 (NP40), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2.0 mM phenylmethanesulfonfonyl fluoride, 20 µg/ml aprotinin, 1.0 mM NaF, and 1.0 mM NaF] for 30 min at 4 °C. After centrifugation, the cell lysate (25 µg) was size-separated by electrophoresis under reducing conditions in a 10% polyacrylamide gel with SDS (SDS-PAGE), and the proteins in the gel were electronically transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with Block Ace (Dainippon Seiyaku, Osaka, Japan) for 1 h at room temperature to inhibit nonspecific binding, and further incubated with mouse anti-Tax1 antibody or rabbit anti-Tax2B antibody. After washing with TBS-T [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20], the membranes were further incubated with either anti-mouse or anti-rabbit immunoglobulins conjugated with horseradish peroxidase (Bio-Rad Technologies, Richmond, CA). Proteins recognized by the antibodies in the membrane were visualized using the ECL Western blotting detection system (Amer sham Biosciences, Piscataway, NJ). Antibodies used were mouse anti-Tax1 monoclonal antibody (TAXY-7) (Tanaka et al., 1991), rabbit anti-Tax2B polyclonal antibody (Lewis et al., 2000), mouse anti-Dlg monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA), and mouse mono-
clonal anti-α-Tubulin antibody (Oncogene Research Products, San Diego, CA).

**Co-immunoprecipitation assay**

The tax plasmids were transiently transfected into 293T cells together with the Dlg expression plasmid by the lipofection method (Fugene6, Roche Molecular Systems, Peasanton, CA). At 48 h after the transfection, the cells were treated with lysis buffer A [25 mM Tris (pH 7.2), 150 mM NaCl, 1.0 mM EDTA, 1% NP40, 2.0 mM phenylmethanesulfonyl fluoride, 20 μg/ml aprotinin, 1.0 mM Na3VO4, 1.0 mM NaF], and the cell lysate was immunoprecipitated with anti-Dlg antibody. The amount of Tax1, Tax2B, and Dlg protein in the precipitates was analyzed by Western blotting using anti-Tax1, anti-Tax2B, and anti-Dlg antibody, respectively.

**Immunofluorescence analysis**

To analyze the localization of Tax1, Tax2C, and Dlg proteins in 293T cells, the cells were cultured on 8-well culture slides (Becton Dickinson Labware, Bedford, MA) overnight. The 293T cells on the slides were then transiently transfected with the tax plasmids or the GFP-Dlg plasmid by the lipofection method (Fugene6). At 48 h after transfection, they were fixed with 2% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 2 h at 4 °C. The fixed cells were incubated with serum from a HTLV-1 carrier and a mouse anti-Dlg antibody (BD Biosciences Pharmingen) or mouse anti-Tax1 antibody (TAXY-7). After washing, the cells were further incubated with tetramethylrhodamine-isothiocyanate (TRITC)-labeled anti-mouse IgG (Beckman Coulter, Fullerton, CA) and fluorescein isothiocyanate (FITC)-labeled anti-human IgG (Beckman Coulter). After washing, the cells were further stained with Hoechst 33258. The stained cells were examined under an inverted microscope (Axiovert200) equipped for epifluorescence (Carl Zeiss, Oberkochen, Germany).

**Cell fractionation assay**

The 293T cells were transfected with the tax1, tax2B, and mutant plasmids together with the Dlg plasmid by a lipofection method (Fugene6). At 48 h after transfection, the cells were divided into two groups. One was treated with the lysis buffer A at 4 °C for 15 min. After centrifugation, the supernatant was collected and used as the soluble fraction, and the resultant pellet was further treated with SDS sample buffer [125 mM Tris–HCl (pH 6.8), 2% SDS, 20% glycerol, 0.01% bromophenol blue, and 10% β-mercaptoethanol] 4 °C for 15 min. The lysates were used as the insoluble fraction. The other group was directly treated with SDS sample buffer and used as the total fraction. The three samples were size-fractionated by SDS-PAGE, and the amount of Tax and Dlg was measured by Western blot.

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**References**


