TABLE 5. COMPARISON OF GENES DIFFERENTIALLY EXPRESSED BETWEEN HUMAN T LYMPHOTROPIC VIRUS TYPE I-ASYMPTOMATIC CARRIERS AND HUMAN T LYMPHOTROPIC VIRUS TYPE I-ASSOCIATED MYELOPATHY/TROPICAL SPASTIC PARAPARESIS CD8+ T CELL LIBRARIES FOR SELECTED TERMS OF GENE ONTOLOGY

		Normalized frequencies			
Tag	*FC*	*HAM/TSP*	*HAC*	*Gene symbol*	*Description*
Immune response					
CTGATCTGTG	54.0	1	54	HLA-C	Major histocompatibility complex, class I, C
CTGGCGCGAG	14.3	4	57	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta
CTGACTTGTG	7.4	21	156	HLA-C	Major histocompatibility complex, class I, C
TGACCCACCA	8.0	1	8	HLA-C	Major histocompatibility complex, class I, C
TGGGAGCTCA	8.0	1	8	NCR3	Natural cytotoxicity triggering receptor 3
CTGACCTGTG	−2.0	483	240	HLA-C	Major histocompatibility complex, class I, C
GGCTCCTCGA	−2.1	19	9	TAPBP	TAP binding protein (tapasin)
GCTTAATGCT	−2.2	24	11	CD8B	CD8b molecule
GTAGCACCTC	−2.3	69	30	CST7	Cystatin F (leukocystatin)
TTCCCTTCTT	−2.4	26	11	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1
CCACTACACT	−3.5	14	4	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
CCTCTAGAGG	−3.5	14	4	CCL5	Chemokine (C–C motif) ligand 5
GCTGAACGCG	−3.0	9	3	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
AGGGGCTGCC	−4.8	19	4	HLA-C	Major histocompatibility complex, class I, C
CGAGCCTGTT	−5.8	23	4	ZAP70	Zeta-chain (TCR)-associated protein kinase 70 kDa
GCGTCCTGCC	−13.0	13	1	LAT	Linker for activation of T cells
Apoptosis					
CAAGATAAAT	9.0	1	9	MAGED1	Melanoma antigen family D, 1
GTGGACCCCA	8.0	1	8	PUF60	Poly-U binding splicing factor 60 kDa
AGACTAACCT	−2.0	32	16	GZMH	Granzyme H (cathepsin G-like 2, protein h-CCPX)
GAGACTTGAG	−2.6	23	9	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
GACTCTGGGA	−2.8	11	4	CLPTM1L	CLPTM1-like
GGGGGCGCCT	−3.0	9	3	SLC25A6	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
TGTGTGGGGC	−3.0	9	3	RHOT2	Ras homolog gene family, member T2
GTGACAACAC	−8.0	8	1	VDAC1	Voltage-dependent anion channel 1
TGAAGCAGTA	−8.0	8	1	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
TGCCCCTGAA	−8.0	8	1	XAF1	XIAP-associated factor 1
Cell adhesion					
GAAGAGTTCC	5.7	3	17	LEF1	Lymphoid enhancer-binding factor 1
ATAGGTCAGA	4.0	3	12	CLSTN1	Calsyntenin 1
TGGAACTGTG	−2.0	16	8	SIGLEC8	Sialic acid binding Ig-like lectin 8
GAGACTTGAG	−2.6	23	9	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
TTGCCCAGCA	−2.7	16	6	CERCAM	Cerebral endothelial cell adhesion molecule
CCTCTAGAGG	−3.5	14	4	CCL5	Chemokine (C–C motif) ligand 5
CAAAAAAAAA	−13.0	13	1	ADA	Adenosine deaminase
Inflammatory response					
TGGGAGCTCA	8.0	1	8	NCR3	Natural cytotoxicity triggering receptor 3
TACCTGCAGA	−2.2	54	25	S100A8	S100 calcium binding protein A8
GAGACTTGAG	−2.6	23	9	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
CCTCTAGAGG	−3.5	14	4	CCL5	Chemokine (C–C motif) ligand 5
GCTGAACGCG	−3.0	9	3	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
Cytokine activity					
TAGAAAAATA	12.0	1	12	GPI	Glucose-6-phosphate isomerase
TTTATCTGCT	8.0	1	8	HMGB1	High-mobility group box 1
CCACTACACT	−3.5	14	4	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10
GAAATTTAAA	−3.0	9	3	HMGB1	High-mobility group box 1
TCTGCTAAAG	−13.0	13	1	HMGB1	High-mobility group box 1

HAC, HTLV-1-asymptomatic carriers; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; FC, fold change. The tag sequence represents the 10-bp SAGE tag. Normalized frequencies were obtained using SAGE software by calculating the total number of 300,000.

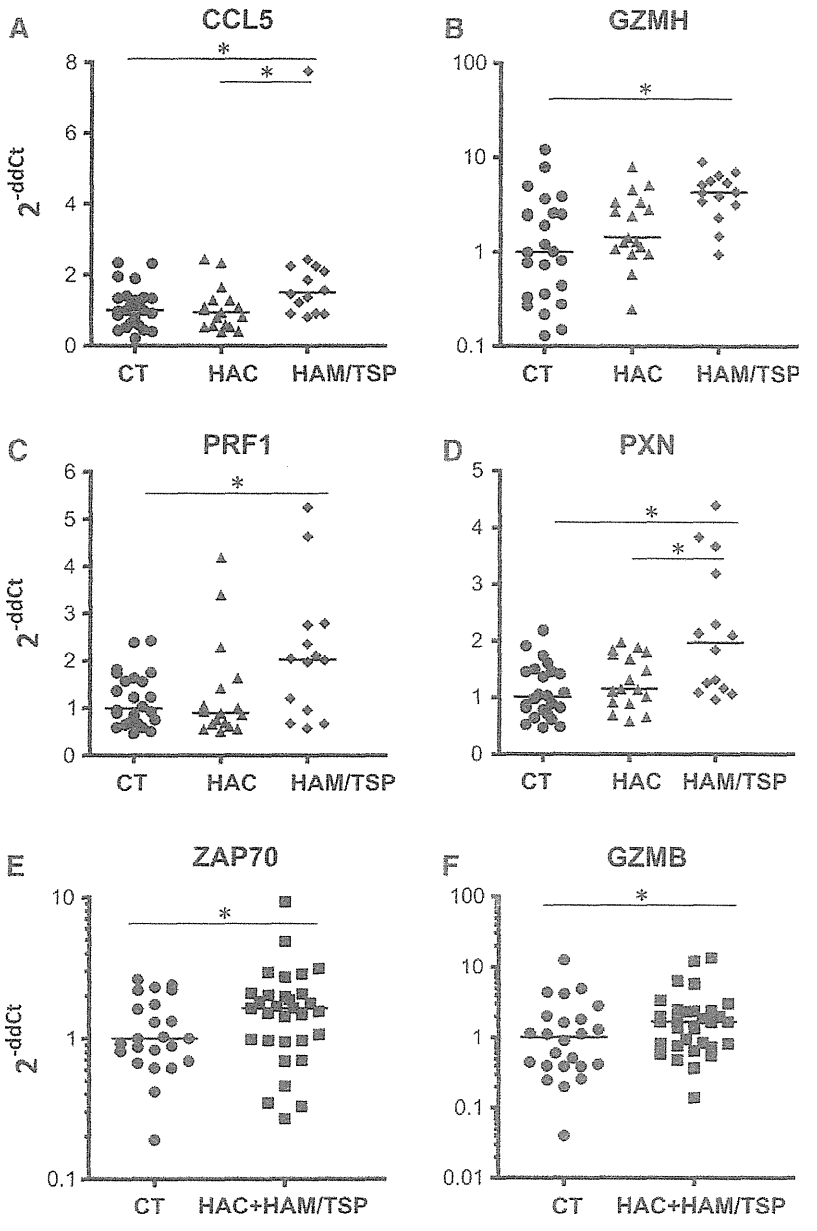

FIG. 2. Gene expression in CT, HAC, and HAM/TSP $CD8^+$ T cells using qRT-PCR. (A) CCL5, (B) GZMH, (C) PRF1, (D) PXN, (E) ZAP70, and (F) GZMB. The relative level of gene expression was obtained from the CT ($n=24$), HAC ($n=17$), and HAM/TSP ($n=14$) groups. Bars indicate the median of each group. The median of the CT group was used as a calibrator. Asterisks indicate p values <0.05 (*) compared with ANOVA. The Mann–Whitney test was used for comparison between the two groups. CT, control group; HAC, HTLV-1-asymptomatic carriers; and HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis.

We also tested expression levels of GZMB and although no difference was observed among the CT, HAC, and HAM/TSP groups, we found a significant increase in whole infected patients (HAC+HAM/TSP) compared to the CT group (Fig. 2F).

PRF1 and GZMB protein levels were differentially expressed among HAC and HAM/TSP groups

To more directly confirm the results of gene expression data, we quantified proteins level of PRF1 and GZMB by quantitative flow cytometry. When we performed intracellular staining of PRF1, we detected significantly higher MFI in the HAM/TSP group compared to the HAC group (Fig. 3A). Moreover, the GZMB intracellular expression also revealed a significant increase of MFI in HAM/TSP patients than the HAC group, as shown in Fig. 3B.

Distinct correlation between gene expression and PVL

We considered if there was an association between PVL and the gene expression data and we saw no correlation among PVL and PRF1, GZMB, ZAP70, or PXN ($p>0,05$). However, PVL correlated positively with GZMH ($r=0.3947$, $p=0.0345$) and CCL5 ($r=0.5257$, $p=0.0060$).

Discussion

$CD8^+$ T cells play an important role in HTLV-1 infection and their frequency and efficiency are related to PVL levels,

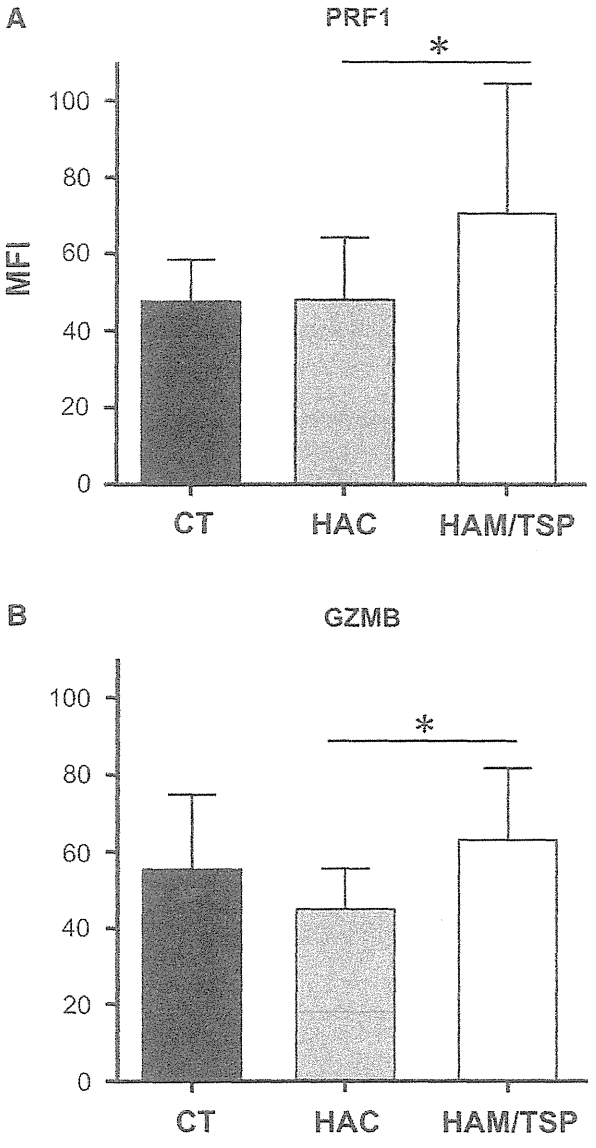

FIG. 3. Quantitative flow cytometry of PRF1 (A) and GZMB (B) in $CD8^+$ T cell populations from HTLV-1-infected patients and controls. MFI indicates the mean fluorescence intensity. Bars indicate the mean of each group with standard deviation. Asterisks indicate p values < 0.05 (*) compared with ANOVA. CT, control group; HAC, HTLV-1-asymptomatic carriers; and HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis.

which in turn might be associated with HAM/TSP development. In our study, we found that a distinct gene expression profile of $CD8^+$ T cells between the HAC and HAM/TSP groups exists. Using SAGE methodology, we screened for some differentially expressed genes associated with a number of functional categories, including apoptosis, cytolysis, cytotoxicity, cellular development, growth and proliferation, immunological and neurological diseases, inflammatory response, and diseases and infection. Due to their potential biological role in HTLV-1 infection, we focus on cytotoxicity and cytokine-related genes and assessed the levels of expression of GZMB, GZMH, PRF1, ZAP70, PXN, and CCL5.

Our results showed that patients with HAM/TSP have high expression levels of genes related to cell-mediated lysis, namely GZMH and PRF1. Moreover, GZMH expression showed a significant positive correlation with PVL. In addition, we found that HTLV-infected patients (HAC + HAM/TSP) expressed higher levels of GZMB than the noninfected group (CT). In agreement with mRNA measurements, we detected higher protein expression levels of PRF1 and also of GZMB in HAM/TSP patients, compared to the HAC group.

$CD8^+$ T cells are responsible for combatting target cells bearing antigens recognized by the T cell receptor (TCR). When $CD8^+$ T cells encounter cells presenting HTLV antigens, they are supposed to release specialized granules containing cytolytic molecules, including perforin and granzymes, and to kill them. During HTLV-1 infection, overexpression of these cytolytic molecules, as we found here, might be explained as an effect of the vast antigen stimulus due to circulating infected cells, which is more prominent in HAM/TSP patients who have a high PVL. These findings suggest that $CD8^+$ T cells are activated during HTLV-1 infection, mainly in HAM/TSP patients, although they are not able to limit viral replication and control disease development.

In contrast to our results, Sabouri *et al.*[32] detected that HTLV-1-infected individuals express lower protein levels of PRF1 as compared to CT. The same study found that GZMB shows higher levels in the HAM/TSP group than in HAC, corroborating our data. Vine *et al.*[33] compared the gene expression of $CD8^+$ T cells from HTLV-1-infected individuals with low and high levels of PVL. They found that a number of genes related to cell-mediated lysis (including PRF1 and GZMB) were overexpressed in patients with low PVL, suggesting that there is an association between a strong cytotoxic $CD8^+$ T cell activity and an effective HTLV-1 suppression, which diverges from our findings.

At this point we cannot explain these apparently contrasting results. In fact, the investigation of PRF1 and granzyme expression is complex. $CD8^+$ T cell degranulation does not require new gene transcription and can occur within 30–60 min of stimulation[34] making mRNA and mainly protein measurement a challenge.

We also detected ZAP70 increased expression in HTLV-infected patients (HAC + HAM/TSP) compared to the noninfected group (CT), suggesting that $CD8^+$ T cells of HTLV-1-infected individuals are activated. ZAP70 is a protein tyrosine kinase associated with TCR, which is involved in signal transduction that leads to cell response upon TCR activation.[35] The high ZAP70 expression can also be an effect of the vast antigens circulating during infection, as we proposed for cytolytic altered genes.

Another cytokine investigated in our study was CCL5 (RANTES), with higher expression in the HAM/TSP group as compared to the HAC and CT groups. This cytokine is associated with immunomodulation and inflammatory processes. In agreement with our findings, several studies have demonstrated that elevation of CCL5 expression is observed in HTLV-1-infected cell lines, in peripheral blood cells, and in lymph nodes from HAM/TSP and ATLL patients.[36–38] High levels of this chemokine were also found in the cerebrospinal fluid from HTLV-1 patients.[38] It is likely that $CD8^+$ T cells from HTLV-1-infected individuals are somehow chronically activated,

probably in response to HTLV-1 antigens. Additionally, mediating leukocyte recruitment and T cell stimulation, CCL5 might play a role in HAM/TSP physiopathology.

At last, we detected an increase of PXN in the HAM/TSP group compared to the CT and to HAC groups. Paxillin is a cytoskeletal adaptor protein that plays an important role in cell adhesion and motility in adherent cells. During HIV-1 infection, PXN was described as a positive regulator of viral infection. In other words, PXN is involved in host cytoskeleton organization that allows viral entry in the target cell during virological synapse.[39] Additionally, in human cytomegalovirus infection, PXN regulation plays a role in the process of viral entry into monocytes and consequently in viral dissemination.[40] It is not clear what role PXN plays in T cells, but there is evidence that PXN is involved in $CD8^+$ T cell degranulation in immunological synapse. Once TCR is activated, there is a microtubule organizing center reorientation, movement, and fusion of the cytolytic granules with the plasma membrane, with PXN involvement.[34] In this way, although there is no report of PXN participation in HTLV infection, as we found a high expression level of ZAP70 and of the cytolytic genes PRF and granzymes, we propose that PXN is overexpressed in HAM/TSP patients due to the intense granules releasing in the immunological synapses. PXN could also be involved in HTVL spread, although $CD8^+$ T cells are not the main infected cells during the infection.

To investigate whether the gene expression differences found between the HAC and HAM/TSP groups were caused by the differences in PVL, we assessed the correlation between PVL and the gene expression data. We could not answer this question since half of the genes showed a correlation with PVL whereas the rest of them did not. The analysis of data with similar PVL and different clinical status would be useful to address the gene expression differences between the HAC and HAM/TSP conditions. However, in our cohort, we could not identify a group of HAC and HAM/TSP patients with similar PVL.

In this study, we analyzed the whole $CD8^+$ T cell population, which comprises specific $CD8^+$ T cells to a variety of antigens, including HTLV-1-specific $CD8^+$ T cells, and also includes $CD8^+$ T cells infected by HTLV-1. To identify genes involved in HTLV-1 infection, it would be preferable to study purified HTLV-1-specific $CD8^+$ T cells; however, these cells were not isolated here because current quantification techniques of antigen-specific cells may modify gene expression. It is known that 10% of total circulating $CD8^+$ T cells are HTLV-1-specific $CD8^+$ T cells.[41] Thus, we believe that the gene expression profile generated here is most likely the result of virus-activated cells.

We also performed Tax protein quantification, which is a marker of proviral expression, to estimate the frequency of HTLV-1-infected $CD8^+$ T cells capable of expressing Tax protein. We found that this frequency, despite being higher in HAM/TSP, was low in both groups, compared to the frequency of HTLV-1-infected $CD4^+$ T cells (data not shown). Since HTLV-1-infected $CD8^+$ T cells are not the main reservoir of HTLV-1 *in vivo*, we believe that the frequency of these cells did not undermine our results.

We found some discordant results among the different employed methodologies: SAGE, qRT-PCR, and flow cytometry. We understand that each methodology has its particularities and some inherent drawbacks, and we believe the main reason for our discordant findings is that we could not perform SAGE, qRT-PCR, and flow cytometry with the same number of samples.

Our findings showed that $CD8^+$ T cells of HAM/TSP patients have an inflammatory and active profile. PXN and ZAP70 overexpression in HTLV-1-infected patients was described for the first time here and reinforces this concept. However, although active and abundant $CD8^+$ T cells exist, they are not able to completely eliminate infected cells and prevent the development of HAM/TSP. Moreover, these active cells might contribute to the pathogenesis of the disease by migrating to the CNS, as we found deregulation of CCL5 expression in infected patients.

Our results provide a large-scale perspective of gene expression that should be further tested with biological functional assays to increase our understanding of the role these molecules play in the development of HTLV-1-related diseases.

Sequence Data

SAGE data have been deposited in the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) (GEO ID: GSM641893 and GSM641894).

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Author Disclosure Statement

No competing financial interests exist.

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Human T-cell leukemia virus type 1 Tax protein interacts with and mislocalizes the PDZ domain protein MAGI-1

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Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL). HTLV-1 encodes the oncoprotein Tax1, which is essential for immortalization of human T-cells and persistent HTLV-1 infection *in vivo*. Tax1 has a PDZ binding motif (PBM) at its C-terminus. This motif is crucial for the transforming activity of Tax1 to a T-cell line and persistent HTLV-1 infection. Tax1 through the PBM interacts with PDZ domain proteins such as Dlg1 and Scribble, but it has not been determined yet, which cellular PDZ proteins mediate the functions of Tax1 PBM. Here we demonstrate that Tax1 interacts with the PDZ domain protein MAGI-1 in a PBM-dependent manner, and the interaction mislocalizes MAGI-1 from the detergent-soluble to the detergent-insoluble cellular fraction in 293T cells and in HTLV-1-infected T-cells. In addition, Tax1-transformation of a T-cell line from interleukin (IL)-2-dependent to IL-2-independent growth selects cells with irreversibly reduced expression of MAGI-1 at mRNA level. These findings imply that Tax1, like other viral oncoproteins, targets MAGI-1 as a mechanism to suppress its anti-tumor functions in HTLV-1-infected cells to contribute to the transforming activity of T-cells and persistent HTLV-1 infection. (*Cancer Sci* 2013; 104: 313–320)

Human T-cell leukemia virus type 1 (HTLV-1) is the etiological agent of adult T-cell leukemia (ATL).[1,2] Among several non-structural genes encoded by HTLV-1, Tax1 is a crucial regulator of viral life cycle as a potent transcriptional activator for its own transcription.[3–5] In addition, Tax1 is a key player involved in T-cell immortalization, transformation, persistent infection, inflammation, and leukemogenesis.[6–11] All these pleitropic functions of Tax1 are believed to be directed by a wide spectrum of interactions with cellular factors. For instance, numerous PDZ domain containing cellular proteins have been shown to complex with Tax1 through the PDZ binding motif (PBM) located at its C-terminus.[12,13] We have previously shown that the PBM plays a key role in Tax1 mediated activities. The motif was critically involved in Tax1-induced transformation of a rat fibroblast cell line and a mouse T-cell line.[14,15] Moreover, an HTLV-1ΔPBM virus with a deletion of the Tax1 PBM in HTLV-1, failed to establish persistent infection in rabbits as measured by lack of antibody response against HTLV-1 and the absence of HTLV-1 proviruses.[16]

Human T-cell leukemia virus type 2 (HTLV-2) is closely related to HTLV-1, but it is unable to cause any malignancy.[17,18] One notable difference between the two is the lack of PBM in Tax2, and thus Tax1 PBM is proposed to be one of the major determinants of HTLV-1 pathogenesis.[14,19] Intriguingly, PBMs have been identified in other viral oncoproteins such as the E4-ORF1 protein of human adenovirus type 9 and the E6 proteins of high-risk human papilloma viruses (HPV),[20–22] suggesting that the oncogenic ability of these viruses may depend in part on interactions involving their PBMs with cellular proteins.

PSD-95/Disc Large/Zona Occludens-1 (PDZ) domain containing proteins form signaling complexes at the inner surface of cell membrane and are involved in a broad range of functions like cell signaling, cell–cell adhesion, tight junction integrity, molecular scaffolding for protein complexes, and tumor suppression.[23,24] The human genome contains hundreds of PDZ domain containing proteins,[25] and some of them have been shown to be targeted by viral oncoproteins.[26–29] Tax1 selectively interacts with Dlg1 and Scribble.[13,30] Of the two, Dlg1 has been widely studied due to its involvement in cell growth signaling and tumor suppression.[31–33] Our previous findings suggested that inactivation of Dlg1 increases the ability of Tax1 to transform a mouse T-cell line (CTLL-2) from interleukin (IL)-2-dependent to IL-2-independent growth, but it did not increase such transforming activity in a PBM defective Tax1 mutant.[34] Hence we concluded that other yet unidentified PDZ protein(s) besides Dlg1 likely inhibit(s) transformation of CTLL-2 by Tax1 and inactivation of these proteins by Tax1 is essential for the transformation of CTLL-2.

MAGI-1 (MAGUK with inverted domain structure 1) is a PDZ protein closely related to Dlg1,[35] and it has also been implicated in tumor suppression in various systems. For instance, MAGI-1 suppressed invasion of tumor cells by recruiting PTEN/β-catenin complex to the adherent junctions and downregulation of phosphatidylinositol 3-kinases (PI3K) signaling.[36] MAGI-1 is a known target of viral oncoproteins adenovirus-9 E4-ORF1 and high-risk HPV E6.[37,38] In the present research, we provide evidence that MAGI-1 is targeted by the HTLV-1 oncoprotein Tax1 in two independent mechanisms. Tax1 interacts with MAGI-1 and alters the subcellular localization. In addition, a low MAGI-1 expression is selected during Tax1-immortalization of human T-cells and IL-2-independent transformation of CTLL-2 cells. Interestingly, unlike Tax1, Tax2-immortalization of human T-cells is not associated with downregulation of MAGI-1 expression. These findings suggest that MAGI-1, a PDZ protein known to be associated with tumour suppression, is an important cellular target of HTLV-1 Tax1 for T-cell immortalization and transformation.

Materials and Methods

Cell lines and cell culture. The human embryonic kidney cell line 293T was cultured in DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. The human T-cell lines used in the present experiments have been

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characterized previously.[14] PBL/HTLV-1 and PBL/HTLV-1ΔPBM are IL-2-dependent HTLV-1-immortalized human T-cell lines, and they were established as previously described.[16] SLB-1, MT-4 and HUT-102 are IL-2-independent HTLV-1-transformed human T-cell lines. HUT78, MOLT-4 and Jurkat are HTLV-1-negative human T-cell lines. SLB-1, HUT-102, HUT78, MT-4, MOLT-4 and Jurkat cells were cultured in RPMI 1640 supplemented with 10% FBS, 4 mM glutamine, penicillin (50 U/mL), and streptomycin (50 μg/mL) (RPMI/10%FBS). PBL/HTLV-1 and PBL/HTLV-1ΔPBM were cultured in RPMI/20%FBS with 1 nM recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan). CTLL-2 is a mouse cytotoxic T-cell line that grows in an IL-2-dependent manner,[6,15] and was cultured in RPMI/10%FBS containing 2-mercaptoethanol (2-ME) and 1 nM IL-2. CTLL-2 cells stably expressing hAkt1ΔmPH were described previously.[39] They were cultured in RPMI/10%FBS containing 2-ME, 1 nM IL-2 and 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA, USA). Tax1- and hAkt1ΔmPH-transformed IL-2-independent CTLL-2 cells were established as described previously,[34,39] and cultured in RPMI/10%FBS containing 2-ME but without IL-2.

Plasmids. The expression plasmids pHβPr-neo-Tax1 and pHβPr-neo-Tax1ΔC that were used for the expression of Tax1 and Tax1ΔC respectively in 293T cells together with the lentiviral vector CSII-EF-IG-RfA used for the generation of recombinant lentiviruses for expression of Tax1 in T-cells have been described previously.[40,41] Tax1ΔC is a PBM-negative mutant, with a four amino acid deletion from the C-terminus of Tax1.[14] pcDNA3.1:FLAG-MAGI-1c (A gift from L. Banks, International Centre for Genetic Engineering and Biotechnology, Italy), was constructed by cloning FLAG-MAGI-1c into the HindIII/EcoRI site of pcDNA3.1 (Invitrogen) with a FLAG-tag at the N-terminus.

Western blotting analysis. Cells were lysed with the SDS-sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol. Protein concentrations of the cell lysates were measured using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The cell lysates (20 μg) were then treated with 50 mM DTT, 0.01% bromophenol blue, and heated at 95°C for 5 min. The resultant lysates were subjected to SDS-PAGE separation. The proteins in the gel were transferred to a nitrocellulose membrane, which was then incubated with 5% skim milk for 1 h at room temperature to inhibit non-specific binding, and further incubated with the primary antibody. After washing with TNN buffer (10 mM Tris–HCl [pH 7.5], 50 mM NaCl, and 0.05% NP40), the membranes were further incubated with either anti-mouse or anti-rabbit immunoglobulins conjugated with horseradish peroxidase (Bio-Rad Technologies) as secondary antibodies. Proteins recognized by the antibodies in the membrane were visualized using the ECL Western blotting detection system (Amersham Biosciences, Piscataway, NJ, USA). Primary antibodies used above were mouse anti-Tax1 mAb (TAXY-7),[42] rabbit anti-Tax2 pAb,[43] mouse anti-FLAG M2 mAb, rabbit anti-MAGI-1 antibody (Sigma-Aldrich, Tokyo, Japan), rabbit anti-Akt mAb (Cell Signaling Technology, Beverly, MA, USA), mouse anti-Syntrophin-β mAb (Affinity Bioreagents, Golden, CO, USA) and mouse anti-α-Tubulin mAb (Oncogene Research Products, San Diego, CA, USA). The above named anti-Tax1 and anti-MAGI-1 antibodies were also used for the immunoprecipitation and immunoflorescence assays.

Immunoflorescence. To analyze the subcellular localization of Tax1 and MAGI-1 in 293T cells, the cells were cultured on glass slides in a six-well culture plate overnight, and transiently transfected with pHβPr-neo, pHβPr-neo-Tax1, or pHβPr-neo-Tax1ΔC plasmid using the lipofection method (FUGENE 6; Roche Diagnostics, Tokyo, Japan). At 48 h after transfection, the cells were fixed with 4% formaldehyde in PBS for 25 min at 4°C and permeabilized by 0.1% TritonX-100. The fixed cells were then incubated with the rabbit anti-MAGI-1 antibody and the mouse anti-Tax1 antibody for 30 min. After washing the slides with PBS, the cells were incubated with Alexa594-labeled anti-mouse IgG, Alexa488-labeled anti-rabbit IgG (Molecular Probes) alongside Hoechst 33258 for another 30 min. The stained cells were then examined by fluorescent microscopy (BZ-8000; KEYENCE, Osaka, Japan) for analysis.

Co-immunoprecipitation. The Tax1 plasmids were transiently transfected into 293T cells with or without the pcDNA3.1:FLAG-MAGI-1c (4 μg) by the lipofection method (FUGENE 6). At 48 h after the transfection, the cells were treated with the 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 Na_3VO_4, 1.0 mM NaF), after centrifugation of the cell lysates, the supernatants were immunoprecipitated with anti-Tax1 antibody. The amount of Tax1 and MAGI-1 proteins in the precipitates was analyzed by a Western blotting analysis using anti-Tax1, and anti-MAGI-1 or anti-FLAG M2 antibodies, respectively.

Cell fractionation. The 293T cells were transfected with Tax1 and its mutant plasmid by the lipofection method (FUGENE 6). At 48 h after transfection, the cells were divided into two groups. One was treated with the lysis buffer A (described above) 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 the SDS-sample buffer (125 mM Tris–HCl [pH 6.8], 2% SDS, 20% glycerol, 0.01% bromophenol blue, and 10% β-mercaptoethanol) at 4°C for 15 min. The lysates were used as the insoluble fraction. The other group was directly treated with the SDS-sample buffer and used as the total fraction. A similar cell fractionation assay was performed for HTLV-1-negative (Jurkat) and HTLV-1-positive (SLB-1) T-cells. The three sets of samples were separately size-fractionated by SDS-PAGE, and the amounts of proteins were measured by a Western blotting analysis.

Immortalization of PBMCs. Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors. They were stimulated with 10 μg/mL phytohemagglutinin (PHA) (Sigma Aldrich) in RPMI/20%FBS supplemented with 55 μM 2-ME for 2 days, and then further cultured in RPMI/20%FBS with 0.5 nM IL-2 and 2-ME for 2 days. A portion of these cells was set aside to be used as Tax untreated PBMCs. Immortalization of PBMCs by Tax1 and Tax2 was carried out as we recently described.[44]

Transient transfection assays. To generate recombinant lentiviruses, 293T cells were transfected with pCAG-HIVgp, pCMV-VSV-G-RSV-Rev (provided by Dr H. Miyoshi) and the respective lentiviral vectors encoding Tax1 or Tax1ΔC using FUGENE 6. At 72 h after the transfection, the supernatant was collected and used to infect CTLL-2 and Jurkat cells at 4×10^5 cells/well in a 12-well plate in a final volume of 2 mL medium. At 48 h after transfection, cell lysates were prepared from the transfected cells and used for a Western blotting analysis.

RNA extraction and quantitative real-time RT-PCR. All reagents used were acquired from TAKARA BIO Japan. The assay was carried out according to the manufacturer's instructions. Total RNA was extracted by the Fast Pure RNA kit and 1 μg was used for cDNA synthesis using the Prime Script RT reagent kit. Two-step 40-cycle quantitative real-time RT-PCR for MAGI-1 expression was carried out by the Thermal Cycler Dice Real Time system and the DNA master SYBR Green using the primers 5′-GCACTGGATGGCAAGATGGA-3′ and 5′-ACCAATGGGAATGGACTGGAAG-3′ for both mouse and human MAGI-1. Amplification of each template was

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conducted in triplicate. The quantity of each transcript was calculated according to the instrument's manual and normalized to the amount of GAPDH mRNA. Amplification without template was included as a control.

Results

Tax1 interacts with MAGI-1 through the PBM. To determine any unidentified Tax1 PBM interacting proteins, a GST fusion protein of Tax1 versus that of its PBM-deletion mutant (Tax1ΔC) were used (Fig. 1A). Proteins bound to Tax1 but not to Tax1ΔC were analyzed by mass spectrometry, and MAGI-1 was identified as one of the Tax1 PBM interacting partners. In order to confirm the interaction of Tax1 with MAGI-1, Tax1 or Tax1ΔC expression plasmids were transiently transfected into the 293T cells or they were co-transfected with the FLAG-tagged MAGI-1 expression plasmid by the lipofection method (FUGENE 6). At 48 h after transfection, cell lysates were immunoprecipitated with anti-Tax1 antibody. The amount of MAGI-1 or FLAG in immunoprecipitates was analyzed by a Western blotting analysis (Fig. 1B,C). MAGI-1 was co-immunoprecipitated only with Tax1 but not with Tax1ΔC (Fig. 1B, IP: Tax1). As will be shown later, the amount of detergent-soluble MAGI-1 protein is reduced by wild type Tax1 but not Tax1ΔC (Fig. 1B). In addition to endogenous MAGI-1, exogenously transduced MAGI-1 was also efficiently co-immunoprecipitated with Tax1 but not with Tax1ΔC (Fig. 1C, IP: Tax1). These results indicate that Tax1 can interact with MAGI-1, and this interaction is dependent on the presence of the PBM in Tax1.

Tax1 alters the subcellular localization of MAGI-1. To further establish the association of Tax1 with MAGI-1 *in vivo*, the subcellular localization of both proteins was examined. The 293T cells were grown on cover slips overnight, followed by transient transfection with Tax1 plasmids. At 48 h after the transfection, the cells were stained with anti-Tax1 and anti-MAGI-1, and their subcellular localization was analyzed by fluorescent microscopy. On its own, MAGI-1 was primarily located in the cytoplasm and on the membrane (Fig. 2, Control). In the presence of Tax1, MAGI-1 colocalized with Tax1 as particular spots (yellow in color), mainly in the perinuclear region of the cytoplasm (Fig. 2, Tax1). In contrast, Tax1 PBM deletion mutant did not obviously show colocalization with MAGI-1 and did not alter the subcellular localization (Fig. 2, Tax1ΔC). We and others have shown a similar behavior of Tax1 with other PDZ proteins such as MAGI-3, Dlg1 and Scribble.[14,45,46] Taken together, our results indicate that Tax1 mislocalizes MAGI-1 by aberrantly sequestering it within Tax1 containing complexes.

Tax1 translocates MAGI-1 from the detergent-soluble to the detergent-insoluble cellular fraction. Several oncoproteins of tumor viruses inactivate functions of cellular tumor suppressor proteins by altering their subcellular localizations.[13,30,37,46,47] We therefore went ahead to investigate the subcellular localization of MAGI-1 in the presence of Tax1 by cell fractionation assay. At first, 293T cells transiently transfected with Tax1 or Tax1ΔC were lysed by a mild detergent (NP40). The NP40-insoluble fraction was further lysed by the SDS-sample buffer, and then the NP40-soluble fraction, the NP40-insoluble fraction and the total fraction directly lysed by the SDS-sample buffer were separately collected. The amounts of endogenous MAGI-1 and transduced Tax1 protein in these three fractions were measured by a Western blotting analysis. MAGI-1 in 293T cells transfected with Tax1ΔC was dominantly detected in the soluble fraction, with a smaller amount in the insoluble fraction, and the amounts were almost equivalent to those in cells transfected with the control plasmid (Fig. 3A, lane 1, 3). On the other hand, while MAGI-1 in the soluble fraction of cells was drastically reduced by transfection of Tax1, MAGI-1 in the insoluble fraction was greatly increased (Fig. 3A, lane 2). Total MAGI-1 protein was unaffected by expression of Tax1 or Tax1ΔC (Fig. 3A, top panel). Similarly, when we examined HTLV-1-infected human T-cells (SLB-1) and uninfected human T-cells (Jurkat), MAGI-1 was detected in the insoluble fraction of SLB-1 with high expression of Tax1 but not that in Jurkat (Fig. 3B). Taken together, our results show that Tax1 induces the translocation of MAGI-1 from the detergent-soluble to the detergent-insoluble cellular fraction. Note worthy MAGI-1 in the insoluble fraction of SLB-1 was detected as a higher molecular weight band than MAGI-1 in the soluble one. This is likely to be due to the post-transcriptional modification of MAGI-1 similar to the phosphorylation reported for Dlg1 in HTLV-1-infected T-cells.[13,14]

Downregulation of MAGI-1 protein during Tax1-induced transformation. We next investigated whether Tax1-induced transformation alters MAGI-1 expression. CTLL-2 is a mouse T-cell line, and it is widely used in HTLV-1 research due to its transformation by Tax1 from IL-2-dependent into IL-2-independent growth.[6,15] Transient expression of Tax1 or Tax1ΔC in CTLL-2 and Jurkat cells barely affected MAGI-1 expression (Fig. 4A). In addition, stable expression of Tax1 in CTLL-2 cells in the presence of IL-2 little affected the expression of MAGI-1 (Fig. 4B, left panel). On the other hand, MAGI-1 expression in Tax1-transformed IL-2-independent

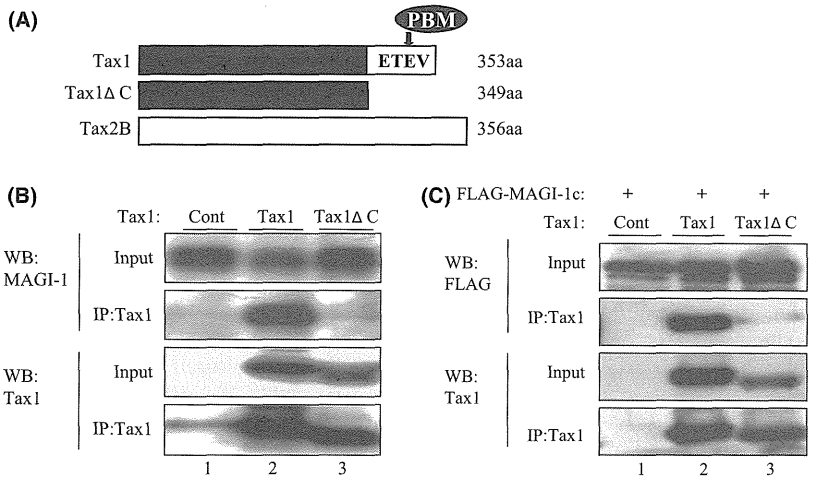

Fig. 1. Tax1 interacts with MAGI-1 in a PBM-dependent manner. (A) Structures of Tax1, Tax1ΔC and Tax2 proteins used in this study. The amino acid sequence of the PBM is shown. (B, C) 293T cells were transiently transfected with pHβPr-neo-Tax1 (lane 2), pHβPr-neo-Tax1ΔC (lane 3) or pHβPr-neo plasmid (lane 1) together with (C) or without pcDNA3.1:FLAG-MAGI-1c (B) by the lipofection method (FUGENE 6). At 48 h post-transfection, the cells were treated with lysis buffer, and cell lysates immunoprecipitated with anti-Tax1 antibody. Total cell lysates (Input) and immunoprecipitates (IP: Tax1) were characterized by a Western blotting analysis using the indicated antibodies.

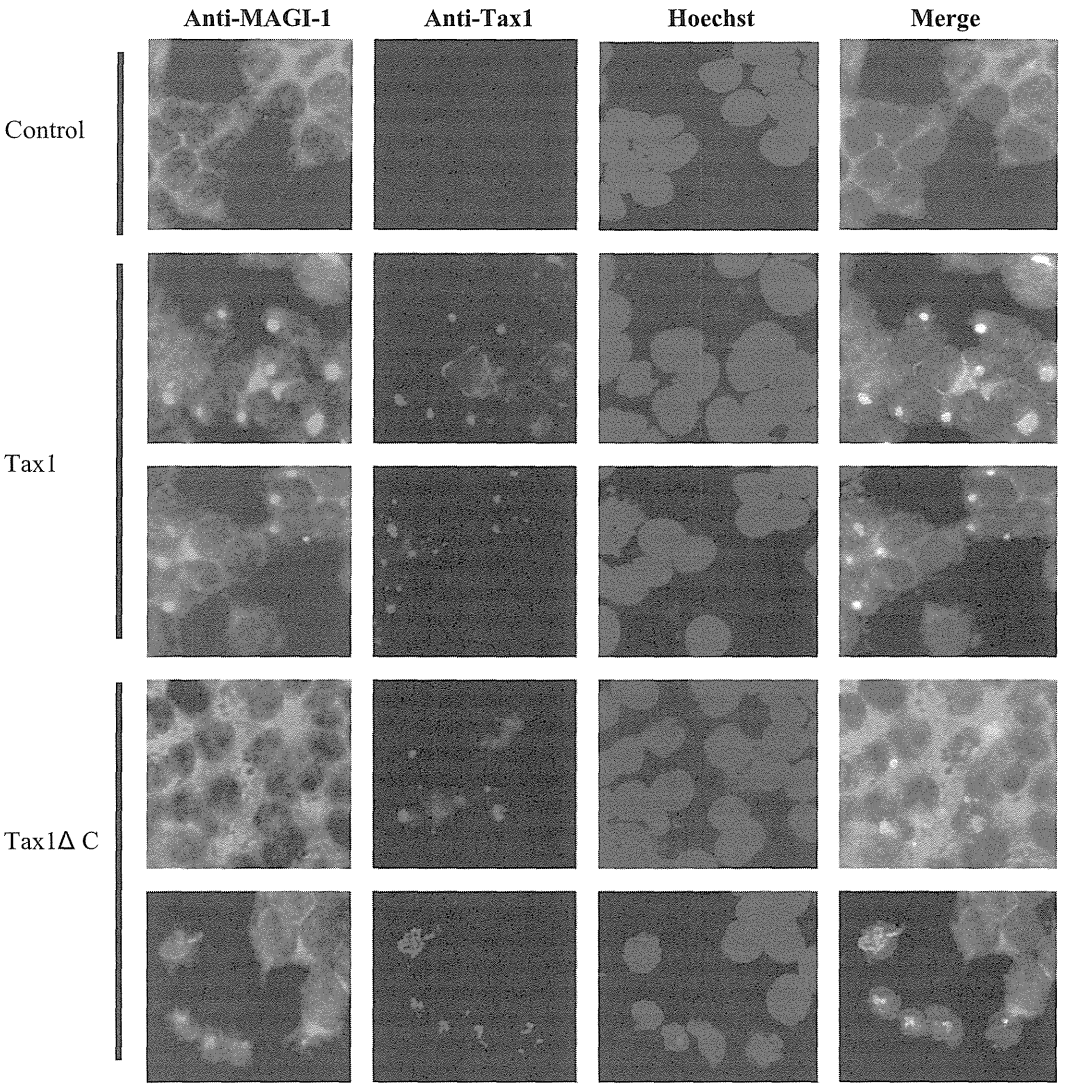

Fig. 2. Subcellular localization of Tax1 and endogenous MAGI-1 in 293T cells. 293T cells were transfected with either the Tax1 or Tax1ΔC plasmid as described in the methods. The cells were stained with anti-Tax1 (red), anti-MAGI-1 (green), and Hoechst 33258 (blue) for nuclear staining. The stained cells were examined by fluorescent light microscopy.

CTLL-2 cells was much lower than that of their untransformed counterparts (Fig. 4B, right panel, Fig. 4C). The expression of another PDZ domain containing protein, syntrophin, with which Tax1 also interacts (Higuchi M, unpublished data, 2006), was intact in Tax1-transformed CTLL-2 cells (Fig. 4C), indicating that MAGI-1 reduction in Tax1-transformed cells was a specific phenomenon. A real-time RT-PCR assay showed that MAGI-1 downregulation occurred at the transcriptional level, as shown by the almost undetectable corresponding mRNA levels (Fig. 4D). We previously reported that Tax1ΔC also transforms CTLL-2, but the activity was much lower than that of Tax1.[15] Contrary to our expectations, Tax1ΔC also induced downregulation of MAGI-1 (Fig. 4C lanes 7–9). To examine if MAGI-1 downregulation in Tax1-transformed CTLL-2 cells is a consequence of IL-2 withdrawal, we restored IL-2 in the cultures of Tax1-transformed cells and checked for the MAGI-1 expression. IL-2 restoration could not, however, rescue the expression of MAGI-1 in two Tax1-transformed cells (Fig. 5A). We recently reported that a constitutively active Akt1 oncoprotein (hAkt1ΔPH) transforms CTLL-2 from IL-2-dependent growth into IL-2-independent growth.[39] Like Tax1, MAGI-1 expression in Akt1-transformed IL-2-independent cells was also downregulated as compared to the untransformed CTLL-2 cells growing in the presence of IL-2, and restoration of IL-2 failed to rescue MAGI-1 expression in these transformed cells like Tax1-transformed cells (Fig. 5B). These results suggest that Tax1-transformation of CTLL-2 cells is associated with an irreversibly downregulated expression of MAGI-1.

Human T-cell leukemia virus type 1 has been shown to immortalize and transform human T-cells in an IL-2-dependent and IL-2-independent manners, respectively.[9,10,16] These findings prompted us to examine the expression status of MAGI-1 in human T-cells. As shown, all five HTLV-1-transformed T-cell lines including one transformed by HTLV-1 with a Tax1 PBM deletion mutant showed lower amounts of MAGI-1 compared to the three HTLV-1-negative cell lines (Fig. 6A). Similar to the observation in Tax1-transformed CTLL-2 cells, a real-time RT-PCR analysis showed that MAGI-1 mRNA was significantly reduced in HTLV-1-infected cells as compared to the HTLV-1-uninfected ones (Fig. 6B). HTLV-2 is a close ally of HTLV-1, exhibiting more than 70% similarity at the nucleotide sequence level. Intriguingly, HTLV-2 does not cause any leukemia or lymphoma in spite of its ability to immortalize

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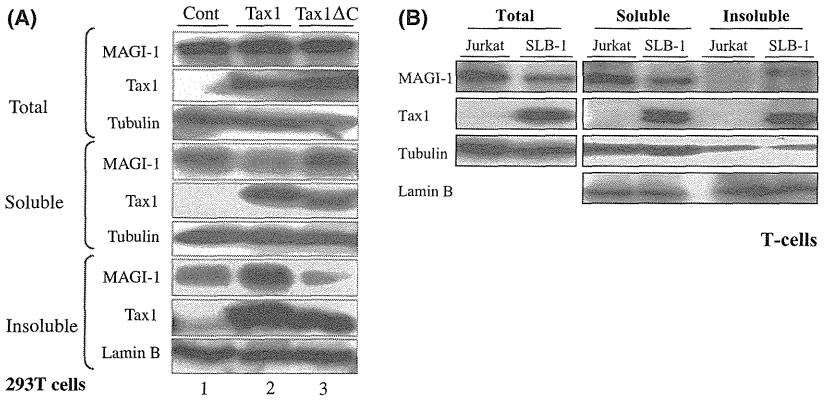

Fig. 3. Tax1 translocates MAGI-1 from the soluble to the insoluble cellular fraction. (A) The 293T cells transfected with Tax1 (lane 2), Tax1ΔC (lane 3) or control (lane 1) were divided into two groups. One group was treated with the NP40 lysis buffer and fractionated as described in Materials and Methods. The other group was directly treated with the sodium dodecyl sulfate (SDS)-sample buffer and used as the total fraction. The three types of samples were size-fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by a Western blotting analysis. (B) Cell lysates from the total, soluble and insoluble fractions of Jurkat and SLB-1 cells were prepared as describe in (A) and the amounts of MAGI-1, Tax1, Tubulin and Lamin B proteins in the lysates were measured by a Western blotting analysis.

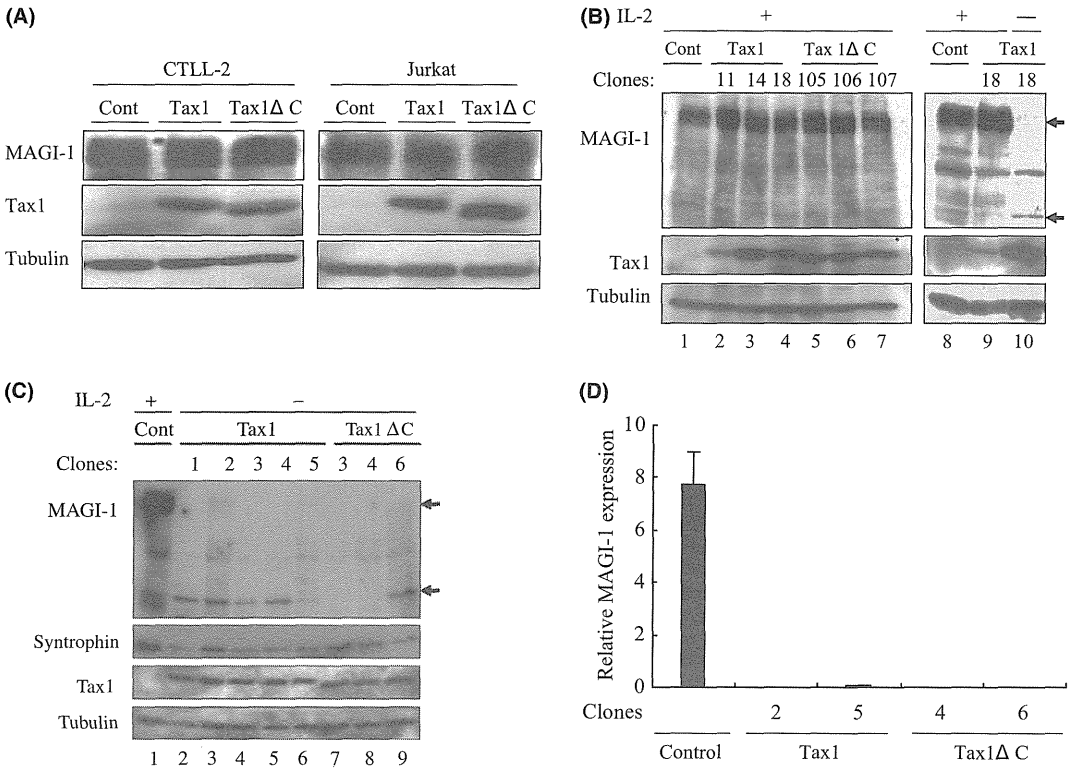

Fig. 4. MAGI-1 downregulation by interleukin (IL)-2-independent transformation of T-cells. (A) CTLL-2 and Jurkat cells were transfected with lentiviruses encoding Tax1. At 48 h after transfection, cell lysates were prepared and the amounts of MAGI-1, Tax1 and Tubulin were determined by a Western blotting analysis. (B) CTLL-2 cells stably expressing Tax1 established as previously described[15] were used as IL-2-dependent cells (lane 2–9). All the above cells were then transferred to IL-2 deficient medium and cultured for more than 1 month. Only one Tax1 expressing clone (Tax1–18) survived in the absence of IL-2 (right panel, lane 10). Cell lysates were prepared from the indicated cells, and protein expression was measured by a Western blotting analysis. (C) CTLL-2 cells transformed by Tax1 and Tax1ΔC were established as described previously.[34] Cell lysates were prepared from these Tax1-transformed (lanes 2–6), Tax1ΔC-transformed (lanes 7–9) and parental CTLL-2 cells (lane 1), and the expressions of MAGI-1, Tax1, Syntrophin-β and Tubulin proteins were measured by a Western blotting analysis. (D) A set of clones each transformed by either Tax1 or Tax1ΔC was selected from (C) above, and the relative gene expression of MAGI-1 was evaluated by the quantitative real-time polymerase chain reaction (PCR) method. The amounts of MAGI-1 were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The quantitative results are expressed as mean ± standard deviation (SD) of three values per sample. The experiment was independently carried out twice to confirm reproducibility.

human T-cells in an IL-2-dependent manner *in vitro* as effectively as HTLV-1.[17,48] HTLV-2 Tax2 protein has also been shown to play a crucial role in the immortalization ability of the virus.[17,18,43,49] We established peripheral T-cells immortalized by either Tax1 or Tax2, and the expression of MAGI-1 in these cells was determined. Our data indicated that while

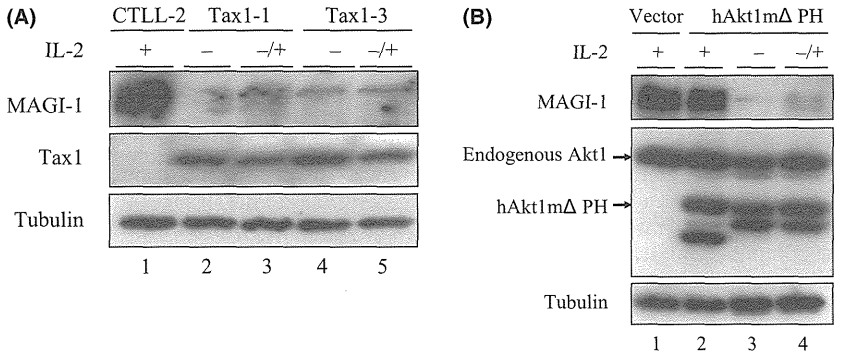

Fig. 5. Restoration of interleukin (IL)-2 cannot rescue MAGI-1 expression in transformed cells. (A) Two distinct Tax1-transformed IL-2-independent CTLL-2 cells (clone 1, 3) were cultured with or without IL-2 for 1 week, and the expression of MAGI-1, Tax1 and Tubulin in the transformed cells with IL-2 (lane 3, 5), the transformed cells without IL-2 (lane 2, 4) and parental CTLL-2 cells with IL-2 (lane 1) were measured by a Western blotting analysis. (B) hAkt1 mΔPH-transformed IL-2-independent CTLL-2 cells were cultured with or without IL-2 for 1 week, and the expressions of MAGI-1, Akt1 and Tubulin proteins in the hAkt1 mΔPH-expressing cells with IL-2 (lane 2), the hAkt1 mΔPH-transformed cells without IL-2 (lane 3), the hAkt1 mΔPH-transformed cells with IL-2 (lane 4) as well as the vector-transduced CTLL-2 cells cultured in the presence of IL-2 (lane 1) were measured by a Western blotting analysis.

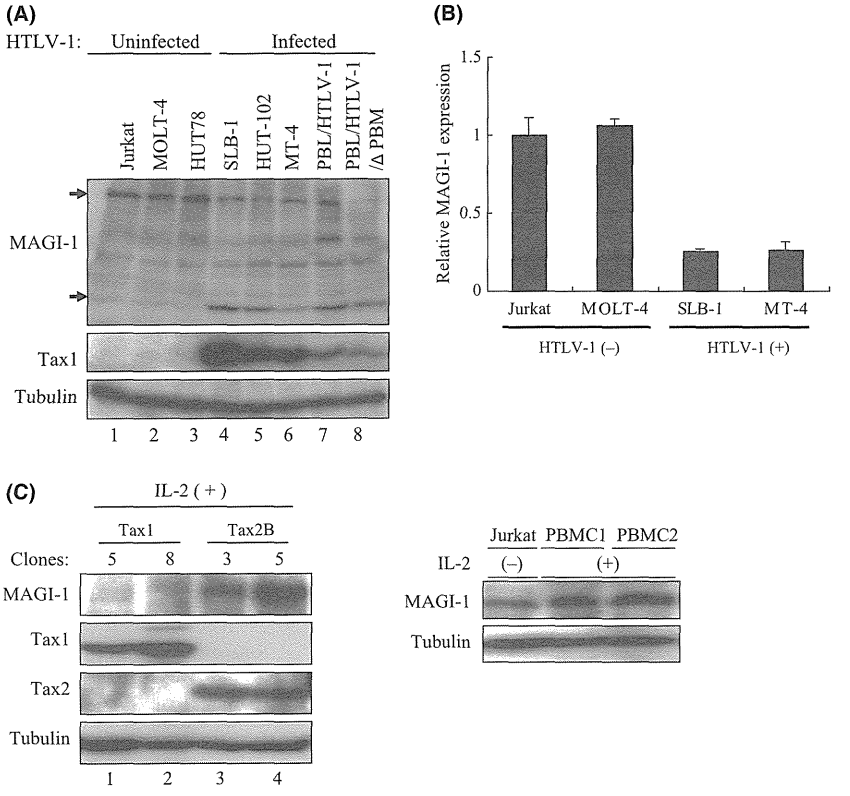

Fig. 6. Expression of MAGI-1 in human T-cell lines transformed by HTLV-1. (A) Cell lysates were prepared from five HTLV-1-transformed (lanes 4–8) and three human T-cell leukemia virus type 1 (HTLV-1)-negative (lanes 1–3) T-cell lines. The lysates were subjected to Western blotting analysis with the antibodies indicated. (B) Expression of MAGI-1 mRNA in two HTLV-1-negative and two HTLV-1-positive cell lines was measured by the quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) method. The amounts of MAGI-1 were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The quantitative results are expressed as mean ± standard deviation (SD) of three values per sample. (C) Cell lysates were prepared from two Tax1 and two Tax2-immortalized T-cells (left panel) and HTLV-1 uninfected Jurkat cells and Tax untreated peripheral blood mononuclear cells (PBMCs) (right panel). Western blotting analysis was performed using the corresponding antibodies.

immortalization of human T-cells by Tax1 abolished their expression of MAGI-1, expression of MAGI-1 was intact in those immortalized by Tax2 similar to HTLV-1-uninfected Jurkat and Tax untreated PBMCs (Fig. 6C). Taken together, these findings suggest that the low level MAGI-1 expression phenotype in HTLV-1-transformed cells is as a consequence of Tax1-mediated immortalization of human T-cells. We observed a lower molecular weight product that was recognized by the anti-MAGI-1 antibody in all Tax1- and HTLV-1-transformed T-cells (Figs 4,6), although the identity and the function remains to be clarified.

Discussion

Recent research has focused on the PBM of HTLV-1 Tax1 as a possible determinant of HTLV-1 pathogenesity in reference to the non-leukemogenic HTLV-2 Tax2, which lacks this motif. Tax1 through the PBM has been shown to interact with cellular PDZ domain containing proteins, some of which are implicated in tumor suppression,[31–33] for instance deficiency of Scribble in mice induced prostate cancer through activating MAP kinase pathway.[49] Nevertheless, it remains unclear whether these PDZ proteins explain any of the Tax1 PBM

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functions or other cellular PDZ protein(s) play(s) a major role in Tax1 functions. The present study identifies MAGI-1 as a novel Tax1 interacting partner and found that Tax1 aberrantly sequesters MAGI-1 into Tax1 containing complexes and translocates it from the detergent-soluble to the detergent-insoluble cellular fraction (Figs 2,3). Since MAGI-1 is a member of the MAGUK family of proteins that function to assemble numerous cellular targets into large signaling complexes in cells,[24] these results suggest that Tax1, through mislocalizing MAGI-1, may disrupt the protein complexes and interfere with their cell signaling activities to promote aberrant cell growth of HTLV-1-infected cells.

Human T-cell leukemia virus type 1-transformed human T-cells displayed a reduced expression of MAGI-1 as compared to their HTLV-1-uninfected counterparts (Figs 6A, B). In addition, while Tax1 expression in CTLL-2 cells in the presence of IL-2 little affected MAGI-1 expression (Fig. 4B), Tax1-induced IL-2-independent transformation of CTLL-2 cells downregulated MAGI-1 expression and the downregulation was not restored by the addition of IL-2 (Figs 4C, 5A). The downregulations of MAGI-1 in Tax1-transformed cells were independent of Tax1 PBM, since Tax1ΔC-transformed cells also displayed a downregulated expression of MAGI-1 (Fig. 4C). Moreover, IL-2-independent transformation of CTLL-2 by another oncoprotein hAkt1ΔPH also downregulated MAGI-1 expression (Fig. 5B). Collectively, these results suggest that Tax1 indirectly downregulates MAGI-1 in CTLL-2 cells during the IL-2-independent transformation process. Thus, it is tempting to speculate that cells with a low MAGI-1 expression are selected during the transformation and immortalization of T-cells by Tax1 or HTLV-1.

Unlike Tax1, Tax2-immortalized human T-cells expressed substantial amounts of MAGI-1 (Fig. 6C). Our recent study showed that Tax2 immortalizes human T-cells much more efficiently than Tax1, and the difference is not mediated by the PBM,[44] indicating that a difference between Tax1 and Tax2 other than the PBM controls T-cell immortalization activities by Tax1 and Tax2. Based on these observations, we present a hypothesis to explain the distinct MAGI-1 expression in Tax1-immortalized cells versus Tax2-immortalized cells; Tax2 can immortalize both MAGI-1-high and MAGI-1-low expressing cells, whereas Tax1 can immortalize only MAGI-1-low expressing cells. Further analysis is, however, required to elucidate the mechanism underlining the differential MAGI-1 expression in Tax1- versus Tax2-immortalized cells. It should be noted that it still remains unclear as to why HTLV-1 is more pathogenic than HTLV-2; however, it has been proposed that such a difference in pathogenesity is attributed to the differences in the respective Tax activities. Our current findings add to this notion that the downregulation of MAGI-1 in Tax1-immortalized T-cells contributes to the pathogenic difference between HTLV-1 and HTLV-2.

Although the function of MAGI-1 in T-cells remains unknown, Tax1 can interfere with the activities of MAGI-1 via altering the subcellular localization and downregulating the mRNA. Further research is necessary to clarify the role of MAGI-1 in T-cell functions and more specifically in HTLV-1 pathogenesis. Nevertheless, MAGI-1 has been proposed to play tumour suppressor functions in other systems. For instance, MAGI-1 but not the related MAGI-2/3 suppressed the transformation of baby rat kidney cells by EJ-ras oncoprotein together with either HPV-16 E6 or adenovirus E1A oncoprotein.[32] Moreover, downregulation of MAGI-1 was associated with poor prognosis of hepatocellular carcinoma.[50] Two other viral oncoproteins, adenovirus-9 E4-ORF1 and high-risk HPV E6 were also shown to sequester MAGI-1 in the cytoplasm and target it for degradation, respectively.[37] It is therefore not surprising to speculate that MAGI-1 plays a tumour suppressor function in T-cells.

In summary, we report herein that similar to other viral oncoproteins, Tax1 can inactivate functions of the PDZ protein MAGI-1 by alteration of its subcellular localization and selection of cells with its downregulated mRNA expression in transformation. Since MAGI-1 has been implicated in tumour suppression in other systems, this can be as a mechanism to suppress its potential anti-tumor functions in HTLV-1-infected cells.

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Disclosure Statement

The authors have no conflict of interest.

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Human T cell leukemia virus type 2 (HTLV-2) Tax2 has a dominant activity over HTLV-1 Tax1 to immortalize human CD4$^+$ T cells

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Abstract While human T cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia, a close relative, HTLV-2, is not associated with any leukemia. HTLV-1 and HTLV-2 encode the Tax1 and Tax2 proteins, respectively, which are essential for the immortalization of human T cells by the respective viruses, thereby causing persistent infection. In this study, we compared Tax1 and Tax2 with respect to their immortalization activity in human T cells. Lentivirus-mediated transduction of the *tax2* gene into human peripheral blood mononuclear cells stimulated with phytohemagglutinin and interleukin-2 in 96-well plates induced outgrowing T cells in most wells, but the cells infected with the control viruses died within 3 weeks. Surprisingly, the number of outgrowing cells induced by Tax2 was much higher than that induced by Tax1, and the appearance of outgrowing cells by Tax2 was earlier than that induced by Tax1. Nevertheless, both Tax2 and Tax1 preferentially immortalized CD4$^+$ T cells, but not CD8$^+$ T cells. Our study showed that HTLV-2 Tax2 can immortalize human CD4$^+$ T cells, and the activity is much higher than that of Tax1. The distinct T cell immortalization activities of Tax2 and Tax1 might therefore play a role in the different pathogeneses observed for these two viruses.

Keywords HTLV-1 · HTLV-2 · Tax2 · CD4 · ATL

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia (ATL) [1–3]. HTLV-1 infection is generally asymptomatic throughout life, but 3–5 % of HTLV-1-infected individuals develop ATL, with an average age of onset at 60 years. These findings indicate that multiple host and environmental factors are associated with the development of the disease [4]. HTLV-1 immortalizes primary human T cells in the presence of interleukin(IL)-2 in vitro, and a fraction of cells, as a rare event, progress to acquire IL-2-independent growth properties [5, 6]. These studies suggest that the immortalization of T cells by HTLV-1 is therefore of critical importance to establish a persistent infection in vivo [7].

In addition to the structural genes, HTLV-1 has at least two oncogenic genes, *tax1* and *HBZ* (HTLV-1 bZIP factor). In transgenic mice, Tax1 and HBZ independently induce T cell lymphoma [8–10]. In addition, Tax1, but not HBZ, is essential for the immortalization of human T cells. For instance, the inactivation of the *tax1* gene in recombinant HTLV-1 abrogates the T cell immortalization activity of HTLV-1 [11].

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Moreover, the transduction of the *tax1* gene without other viral genes into primary peripheral blood mononuclear cells (PBMC) establishes immortalized T cells [12, 13].

HTLV-2 is a close relative of HTLV-1, and it also immortalizes human T cells with equivalent efficiency to HTLV-1 [14]. Nevertheless, there is no etiological association of HTLV-2 with malignancies. Therefore, comparative studies between HTLV-1 and HTLV-2 provide insight into the molecular mechanism of HTLV-1 leukemogenesis. To obtain information about the distinct pathogenesis between HTLV-1 and HTLV-2, we investigated whether HTLV-2 Tax2 can immortalize human T cells, and how strong (relative to Tax1) the effects of Tax2 are on immortalizing human T cells. We found that Tax2 can immortalize T cells in vitro, and surprisingly, that the activity was much higher than that of Tax1. These findings will be discussed in the context of the distinct pathogenesis between HTLV-1 and HTLV-2.

Materials and methods

Cells and culture conditions

Jurkat and SLB-1 cells are HTLV-1-negative and HTLV-1-positive human T cell lines, respectively. These T cell lines were cultured in RPMI1640 medium supplemented with 10 % fetal bovine serum (FBS), 4 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (RPMI/10 %FBS). The 293T cells were derived from a human kidney, and the cells were cultured in Dulbecco's modified Eagle medium supplemented with 10 % FBS, 4 mM glutamine, 0.1 mM MEM non-essential amino acids (aa), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Plasmids

CSII-EF-EGFP, CSII-EF-Tax1, CSII-EF-Tax2B, and CSII-EF-Tax300 are the lentiviral expression vectors encoding EGFP, Tax1, Tax2B, and Tax300, respectively [15–17]. CSII-EF-IG is a lentiviral bicistronic EGFP expression vector and was also used for Tax gene expression [16]. pEFneoTax1, pEFneoTax2B, and pEFneoTax300 were used as the expression vectors encoding Tax1, Tax2B, and Tax300, respectively [16, 17]. The *tax* mutant gene, *tax300*, is a chimeric gene that contains the N-terminal region of Tax2B from aas 1 to 299 and the C-terminal region of Tax1 from aa 300 to 353. *tax300* is the same gene as *tax221* which was described in a previous study [18]. pNFAT-Luc is a luciferase expression plasmid that is regulated by three copies of the NFAT site (−286 to −249 of human IL-2 gene) and the human IL-2 promoter (−64 to −47) [19]. The κB-Luc is a luciferase expression plasmid regulated by the κB element of the IL-2 receptor α-chain gene and the minimal HTLV-1 promoter [20]. pGK/β-gal expresses β-galactosidase under the control of the phosphoglycerate kinase promoter and is used to normalize the transfection efficiency.

Immortalization assay

Human PBMCs were isolated from the blood of a healthy donor. They were stimulated with 10 μg/ml phytohemagglutinin (PHA) (Sigma Aldrich) in RPMI/20 %FBS supplemented with 55 μM 2-mercaptoethanol (2-ME) for 2 days and then further cultured in RPMI/20 %FBS with 0.5 nM IL-2 and 2-ME for 2 days. To generate recombinant lentiviruses, 293T cells were transfected with pCAG-HIVgp, pCMV-VSV-G-RSV-Rev (provided by Dr. H. Miyoshi, RIKEN Tsukuba Institute) and the respective lentiviral vectors encoding Tax1, Tax2B, or Tax300 using FuGENE 6 (Roche). At 72 h after the transfection, the supernatant was collected and used to infect PHA-stimulated PBMC (4×10^5 cells) in a final volume of 1 ml of RPMI/20 %FBS containing 8 μg/ml polybrene, 0.5 nM IL-2, and 2-ME. The virus titer was measured by a Lenti-X qRT-PCR Titration Kit (Clontech), and the viruses containing 1.2×10^8 copies of viral genomic RNA were used for the infection. At 48 h after the infection, the cells were resuspended in RPMI/20 %FBS with IL-2 and 2-ME and cultured in 96-well flat-bottom plates at the density of 1.33×10^3 cells/0.1 ml/well, 4.0×10^3 cells/0.1 ml/well, or 1.2×10^4 cells/0.1 ml/well for 10 weeks. Half of the volume of culture medium was exchanged with new medium once every 4–5 days. The number of wells containing outgrowing cells was counted by light microscopy.

Western blotting analysis

To prepare total cell extracts, the cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (2 % SDS, 62.5 mM Tris–HCl pH 6.8, 10 % glycerol, 50 mM dithiothreitol, 0.01 % bromophenol blue) and heated at 95 °C for 5 min. Then they were size-separated by electrophoresis under reducing conditions in 10 % polyacrylamide gel with SDS. The proteins in the gel were electrotransferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was incubated with TBS-T [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.1 % Tween 20] in 5 % skimmed milk for 1 h at room temperature to inhibit non-specific binding and was further incubated with either an anti-Tax1 mouse monoclonal antibody (Taxy-7) [21] or rabbit anti-Tax2 polyclonal serum (GP3738) [22]. After being washed with TBS-T, the membranes were incubated with either anti-mouse (for Tax1) or anti-rabbit (for Tax2) immunoglobulin G

conjugated with horseradish peroxidase (Bio-Rad Laboratories). Protein bands in the membrane recognized by the antibodies were visualized using the ECL Western blotting detection system (GE Healthcare).

Flow cytometric analysis

The cells were incubated with phycoerythrin (PE)-labeled anti-human CD4 and fluorescein isothiocyanate (FITC)-labeled anti-human CD8 monoclonal antibodies or with isotype matched control antibodies for 30 min at 4 °C. After washing with PBS containing 2 % FBS, the cells were analyzed by flow cytometer (FACScan, Becton–Dickinson) using the Cellquest software program (Becton–Dickinson). PE-labeled mouse anti-human CD8 and FITC-labeled anti-CD4 were purchased from eBioscience.

Transient transfection and luciferase assays

Jurkat cells in RPMI/10 %FBS were seeded at 2×10^5 cells/1.0 ml/well in a 12-well plate. The cells then were cotransfected with the Tax expression plasmid and pGK/β-gal, together with either pNFAT-Luc or κB-Luc by using TransFectin (Bio-Rad Technologies) according to the manufacturer's instructions. At 48 h after transfection, the cell lysates were harvested, and the luciferase and β-galactosidase activities in the lysates were determined using a Luciferase assay system (Promega) and Galacto-Light System (Applied Biosystems). The activity of luciferase was normalized to that of β-galactosidase. The assay was carried out three times to confirm the reproducibility.

Measurement of nuclear NFATc2 and NF-κB p65 proteins

NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) were used to prepare cytoplasmic and nuclear fractions from Tax-immortalized cells and Jurkat cells. The cytoplasmic (10 μg) and the nuclear (5 μg) fractions were characterized by a Western blotting analysis using anti-p65 (F6; Santa Cruz Biotechnology), anti-NFATc2 (4G6-G5; Santa Cruz Biotechnology), anti-Sp1 (PEP2; Santa Cruz Biotechnology), anti-Tubulin (DM1A; Calbiochem), and anti-Tax1 and anti-Tax2 antibodies.

Results

Tax2 immortalizes primary human T cells in the presence of IL-2

The tax2B gene used in this study was derived from the HTLV-2b subtype [15]. The tax300 gene is a chimeric gene that combines tax2B and tax1, and has the N-terminal Tax2B aa (1–299) and the C-terminal Tax1 aa (300–353) (Fig. 1a) [16]. To measure the immortalization activity of the genes in human T cells, we generated lentivirus vectors encoding tax2B, tax300, and tax1 by using 293T cells, and the virus titers in the culture supernatant of 293T cells were adjusted by quantification of the viral genome by real time polymerase chain reaction (PCR). The viruses containing 1.2×10^8 copies of viral genomic RNA were then used to infect two types of cells, human PBMCs stimulated with 10 μg PHA and recombinant IL-2 for 2 days, or a human T cell line (Jurkat). The control lentivirus expressed green fluorescent protein (GFP). The anti-Tax1 antibody detected Tax1 and Tax300 proteins, but not Tax2B, in both PBMCs and Jurkat cells infected with the respective viruses, although the expression level in PBMCs was lower than in Jurkat cells (Fig. 1b, c). An anti-Tax2 antibody detected all three Tax proteins in both cells. However, it should be noted that the anti-Tax2 antibody was raised against Tax2 peptides, and detects Tax2 and Tax300 proteins more efficiently than Tax1 protein [16, 22]. On the other hand, anti-Tax1 antibody detects Tax1 and Tax300 proteins more efficiently than Tax2B protein. Collectively, the results of the Western blotting analyses indicated that the amounts of Tax2B, Tax1, and Tax300 proteins in PBMCs infected with the respective viruses were either almost equivalent or the

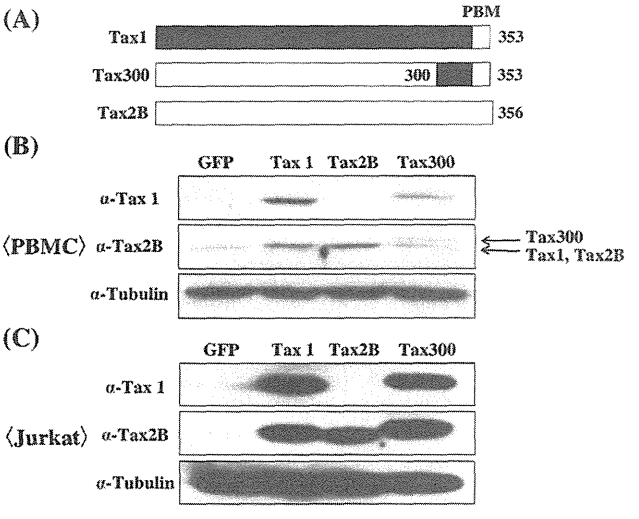

Fig. 1 Lentiviral introduction of *tax1*, *tax2B*, and *tax300* into PBMCs and Jurkat. **a** Tax1 and Tax2B consisted of 353 and 356 aa, respectively. Tax300 has the N-terminal region of Tax2B from aa 1 to 299 and the C-terminal region of Tax1 from aa 300 to 353. PBM indicates the PDZ domain binding motif present only in Tax1 but not in Tax2B, which is essential for the augmented transforming activity of Tax1 in CTLL-2 relative to Tax2 [32, 34]. **b**, **c** The PHA-stimulated PBMCs (**b**) and Jurkat cells (**c**) were infected with the indicated viruses. At 48 h after the infection, the cell lysates were prepared, and the amounts of Tax1, Tax2B, Tax300, and Tubulin in the lysates were measured by a Western blotting analysis using anti-Tax1 (Taxy-7), Tax2, and anti-Tubulin antibodies

amount of Tax1 was more than that of Tax2B. Tax300 was always detected as a higher molecular protein than Tax1 by our Western blotting analysis, although both Tax300 and Tax1 consist of 353 aas. This difference between Tax300 and Tax1 might be caused by their distinct conformation or distinct post-translational modification.

Next, these virus-infected PBMCs were cultured in the presence of IL-2 in a 96-well plate at cell densities of 1.2×10^4, 4×10^3, and 1.33×10^3 cells/well (Fig. 2a, b). At 7- to 10-days post-infection, PBMCs that were infected with Tax2B-viruses showed multiple small clumps of cells in all the wells, and the number of clumps was much higher than that of the cells infected with the control virus. Thereafter, these Tax2B-infected cells continuously proliferated, and 80 out of 96 wells (1.2×10^4 cells/well) contained obvious outgrowing cells at 10-weeks post-infection, whereas all the cells infected with the control virus had died within 3 weeks. At 7- to 10-days post-infection, Tax1 also induced small clumps of cells similar to Tax2B in all the wells, but they then stopped proliferating for 4–5 weeks. Thereafter, some cells started to regrow, and at 10-weeks post-infection, 10 out of 96 wells contained obvious outgrowing cells. Tax300 also induced outgrowing cells from PBMCs, and at 12-weeks post-infection, 36 out of 96 wells contained obvious outgrowing cells, the number of which was lower than that induced by Tax2B. Three independent experiments confirmed the augmented immortalization activity of Tax2B relative to Tax1. We transferred several outgrowing cells to a 24-well plate, and then to a culture flask, and the expression levels of Tax in such cells were characterized by a Western blotting analysis. Three Tax2B-transduced cells express the Tax2B protein, but not Tax1, whereas three Tax1-transduced cells expressed Tax1 protein, but not Tax2B (Fig. 2c). These results indicated that Tax2 is therefore able to immortalize PBMCs, and this activity is stronger than that of Tax1.

Tax1 preferentially immortalizes $CD4^+$ T cells [12, 23]. Therefore, it would be interesting to see whether Tax2 also has

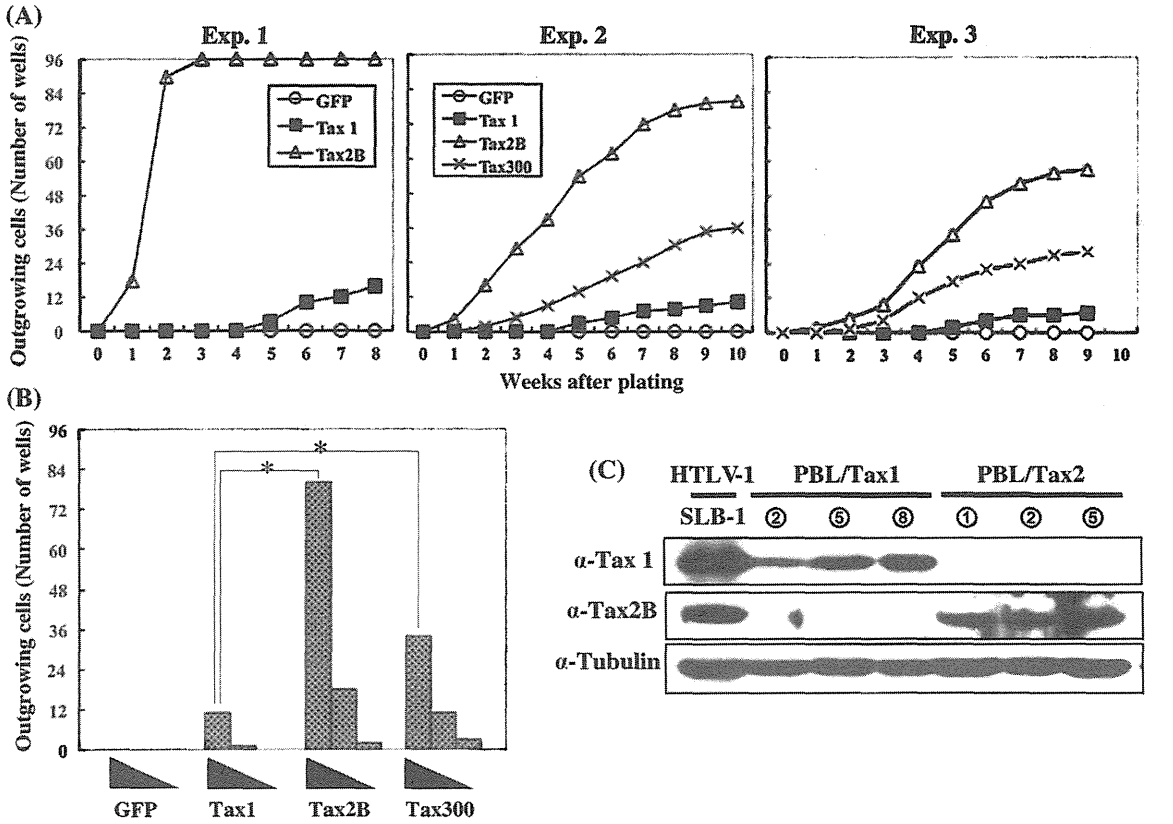

Fig. 2 Tax2B immortalizes human PBMCs. **a**, **b** PHA-stimulated PBMCs infected with lentiviruses encoding *GFP*, *tax1*, *tax2B*, or *tax300* were cultured in RPMI/20 %FBS/IL-2 in 96-well plates at a density of 1.2×10^4, 4.0×10^3, or 1.33×10^3 cells/0.1 ml/well for 10 weeks. The number of wells containing outgrowing cells (seeded at cell density of 1.2×10^4 cells/0.1 ml/well) was counted under a light microscope for 8–10 weeks. The results of three independent experiments are presented in **a**. The number of wells containing outgrowing cells (seeded at cell density of 1.2×10^4, 4.0×10^3, or 1.33×10^3 cells/0.1 ml/well) at 10-weeks post-infection of the experiment 2 in **a** is indicated in **b**. *Asterisk* A significant difference ($p < 0.001$) by the χ^2 test. **c** Three PBL/Tax1 cell lines (PBL/Tax1-2, PBL/Tax1-5, PBL/Tax1-8) and three PBL/Tax2B cell lines (PBL/Tax2B-1, PBL/Tax2B-2, PBL/Tax2B-5) were established from the PBMCs infected with Tax1 and Tax2B lentiviruses as shown above, respectively. The amounts of Tax1 and Tax2B protein in these cells were measured by a Western blotting analysis using anti-Tax1 and Tax2 antibodies

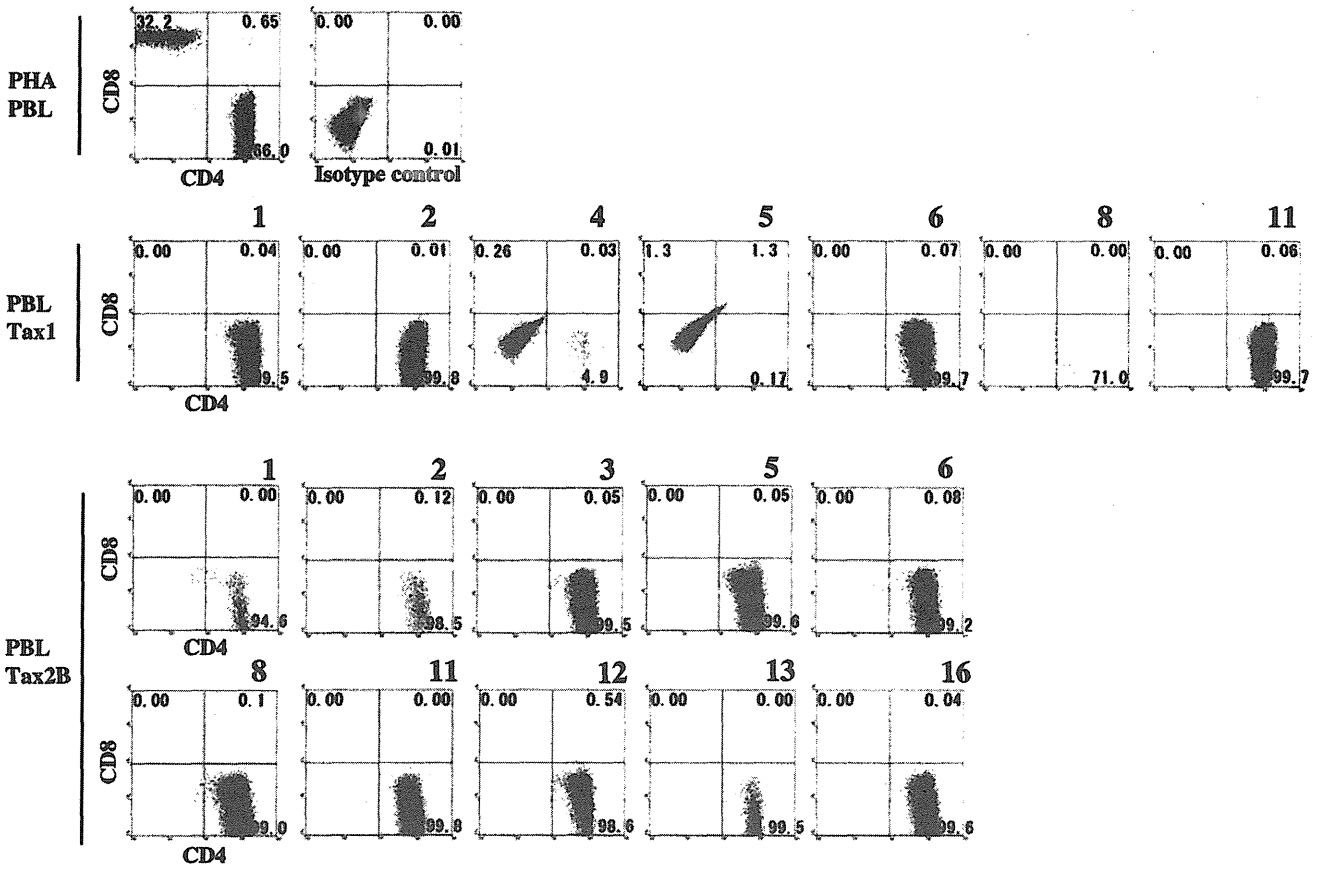

Fig. 3 Tax-immortalized cells express CD4, but not CD8 antigen. The Tax-immortalized T cell lines were characterized for CD4 and CD8 expression using a flow cytometer. Seven PBL/Tax1 cell lines and ten PBL/Tax2B cell lines were established by infection of PBMCs with the Tax1-lentivirus or Tax2B-lentivirus, respectively, and were cultured in the presence of IL-2

an immortalization ability similar to that of Tax1. A flow cytometric analysis showed that all ten Tax2B-immortalized cells were CD4 single positive (Fig. 3). On the other hand, five out of the seven Tax1-immortalized cells were CD4 single positive, one was double negative and the last one (PBL/Tax1-4) was a mixture of double negative cells with a minor population of CD4 single positive cells. These results indicated that Tax2 immortalizes $CD4^+$ T cells, but not $CD8^+$ T cells.

Tax2 activates NFAT-dependent transcription much more efficiently than Tax1

NF-κB and NFAT are transcription factors that control the expression of many genes regulating T cell functions, including cell proliferation and anti-apoptosis, and they are stimulated by both Tax1 and Tax2 [6, 24]. To determine whether the high immortalization activity of Tax2B is related to activation of these transcription factors, we next measured the transcriptional activation by Tax2B, Tax1, and Tax300. The tax expression plasmids and luciferase reporter plasmid under the control of either NF-κB or NFAT were transfected into Jurkat cells using the lipofection method. At 48 h post-transfection, the cell lysates were prepared, and the luciferase activities were measured. Tax2B and Tax300 activated the NF-κB reporter 20- to 25-fold more than the control plasmid, and the activities were half that of Tax1 (Fig. 4). While Tax2B and Tax300 activated the NFAT reporter more than 100-fold, Tax1 only activated NFAT 3- to 10-fold. These results were consistent with those of a previous study [18]. We also examined whether Tax stimulates the nuclear localization of NF-κB p65 and NFATc2, which would indicate the activation of these transcription factors (Fig. 5a). The treatment of Jurkat cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and ionomycin induced an increased nuclear expression of NF-κB p65 and NFATc2 proteins. The transient transduction of Tax1 and Tax2B into Jurkat cells also induced an increased nuclear expression of NF-κB p65 and NFATc2 relative to the control, but the amount of nuclear NFATc2 in Tax1-transduced cells was lower than that of Tax2B-transduced cells, which is consistent with the above described reporter assay.

We then examined the status of NF-κB and NFAT in Tax-immortalized T cells. The amounts of nuclear p65 and NFATc2 in each of three Tax1- and Tax2B-immortalized T cells were more than those of unstimulated Jurkat and they

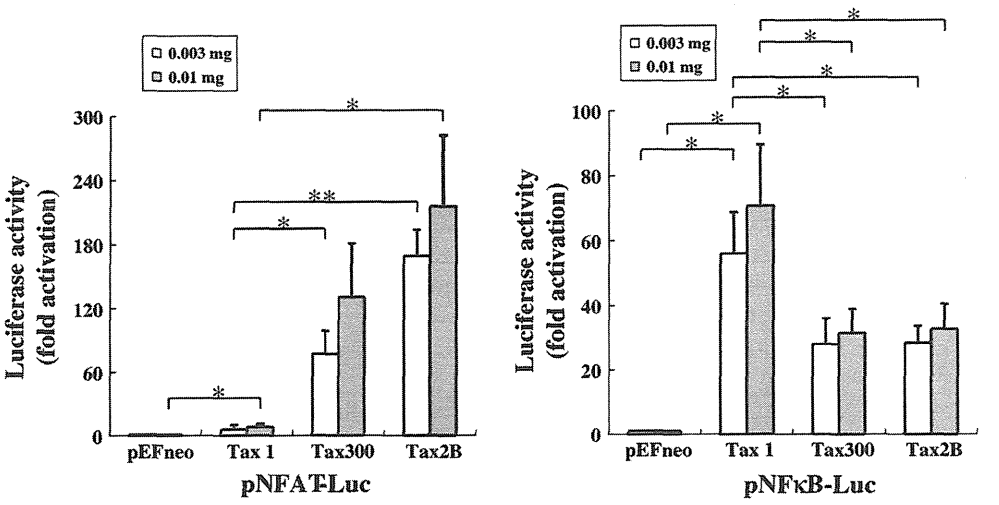

Fig. 4 Tax2B activates both NFAT and NF-κB in a T cell line. Jurkat cells (2×10^5 cells/1.0 ml/well) in a 12-well plate were co-transfected with the Tax expression plasmid and pGK/β-gal, together with either pNFAT-Luc or κB-Luc, using TransFectin. At 48 h after transfection, the luciferase and β-galactosidase activities in the lysates were determined using a luminometer. The activity of luciferase was normalized to that of β-galactosidase, and the fold activations were calculated as the ratio to that of the control transfection with the pEFneo plasmid. The data shown are the averages of three independent experiments with standard deviations. *One asterisk* and *two asterisks* indicate significant differences ($p < 0.05$) and ($p < 0.01$) by the unpaired student's t test, respectively

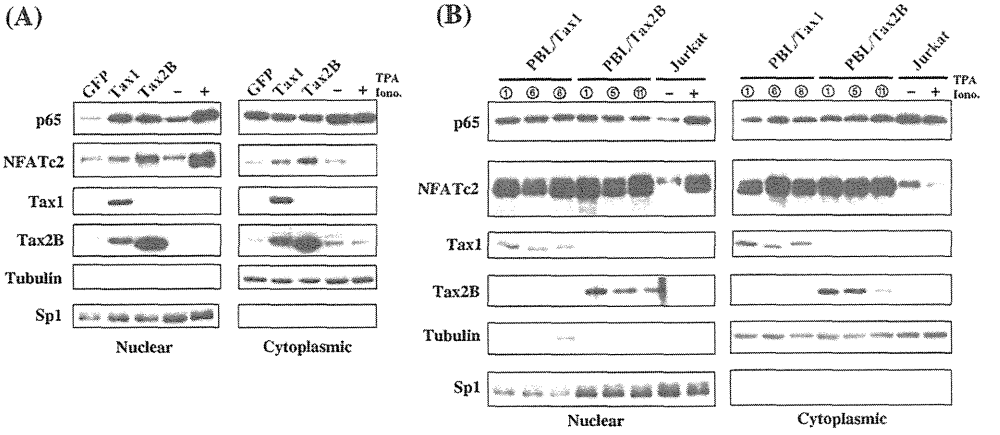

Fig. 5 Tax2B induces the nuclear translocation of NFATc2 and p65 in T cells. **a**, **b** Jurkat cells (4×10^5 cells) were infected with bicistranic Tax1-EGFP lentivirus, Tax2B-EGFP lentivirus or the control virus in 6-well plate and were cultured for 48 h. Jurkat cells (2×10^6 cells) were treated with 20 ng/ml TPA and 1 μM ionomycin for 60 min. Cytoplasmic and nuclear fractions from the infected Jurkat cells (**a**) and Tax-immortalized cells (**b**) were analyzed by a Western blotting analysis using anti-p65, anti-NFATc2, anti-Tax1, anti-Tax2B, anti-Tubulin, and anti-Sp1 antibodies. Tubulin and Sp1 were used as marker proteins that localize in the cytoplasm and the nucleus, respectively

were also equivalent to that of stimulated Jurkat, thus suggesting that Tax1- and Tax2B-immortalized cells have augmented p65- and NFATc2-dependent transcriptional activities. However, it should be noted that the high levels of nuclear NFATc2 in Tax1-immortalized T cells were unexpected, as Tax1 showed a much lower NFAT transcriptional activity than that of Tax2B (Figs. 4b, 5a). Collectively, these results suggested that Tax1- and Tax2B-immortalized T cells have equivalent activations of NF-κB p65 and NFATc2, which did not support the possibility that NFAT contributes to the distinct immortalization activity of Tax2B versus Tax1.

Discussion

In this study, we demonstrated that the HTLV-2b Tax2B protein has an immortalizing activity in human T cells within PBMCs, and such cells could be long-term cultured for at least 3 months (Fig. 2). A previous study showed that

tax2-inactivated HTLV-2 does not immortalize human T cells [25]. Taken together, these findings indicate that Tax2 is a crucial factor required for the immortalization of HTLV-2-infected T cells, thereby leading to persistent infection.

HTLV-2 and HTLV-1 preferentially immortalize $CD8^+$ T cells and $CD4^+$ T cells, respectively, in vitro [26]. Nevertheless, Tax2B selectively immortalized $CD4^+$ T cells, but not $CD8^+$ T cells, in this study (Fig. 3). These results indicate that Tax2 is not a determinant for the immortalization tropism of HTLV-2. Indeed, the chimeric virus composed of HTLV-2 and HTLV-1 showed that the *envelope* gene, but not *tax2*, is a determinant for the selective immortalization of $CD8^+$ T cells by HTLV-2 [27].

The immortalization activity of Tax2B on human T cells was much more efficient than that of Tax1 (Fig. 2). A microscopic examination revealed that Tax1 and Tax2 initially induce the outgrowth of PBMCs for 1 week. Thereafter, while the Tax2-transduced cells continue to grow, Tax1-transduced cells stop growing for several weeks, and then some cells start to regrow. The overexpression of Tax1 in several cell types, including a T cell line, was shown to induce senescence and/or cell cycle arrest [28, 29]. Therefore, such growth inhibitory activity of Tax1 might explain why Tax1-infected PBMCs stop growing for a while in culture. Tax2 might not have these growth inhibitory activities, thereby inducing greater immortalization activity in T cells than Tax1. It should be noted that HTLV-1 HBZ, which shows an oncogenic activity in transgenic mice, alleviates the growth inhibitory activity of Tax1 [10, 29]. Collectively, we herein present a hypothesis that Tax2 alone is sufficient to immortalize T cells by HTLV-2, whereas both Tax1 and HBZ are required to efficiently immortalize T cells by HTLV-1.

The same set of the lentiviruses used here previously showed that Tax1 induces IL-2-independent growth transformation to the originally IL-2-dependent T cell line, CTLL-2, and that the activity was much higher than that of Tax2B and Tax300 [16, 20]. Therefore, it was intriguing that Tax2 and Tax1 have such distinct activities to T cells in the absence and presence of IL-2, despite the fact that they originated from the common ancestor virus. One possible scenario is that Tax2 in the context of HTLV-2 has evolved to immortalize T cells in the presence of a relatively high amount of IL-2, whereas Tax1 in the context of HTLV-1 has evolved to do so in a relatively low amount of IL-2 [30]. A further analysis, however, will be required to establish this hypothesis and to determine how these differences are associated with the leukemogenic activity of HTLV-1.

In addition to NF-κB and NFAT, Tax1 activates the transcription of several cellular genes through the transcription factor CREB, and Rosin et al. [31] presented an evidence that the activation of CREB pathway by Tax1 is important for the immortalization of primary human T cells by Tax1. However, the CREB pathway is unlikely to be a main factor for the reduced immortalization activity of Tax1 relative to Tax2B, as Tax1 and Tax2B showed equivalent CREB activities in a luciferase reporter assay [18]. It should be noted that Tax1 and Tax300, but not Tax2B, have a PDZ domain binding motif (PBM) at their C-terminus (Fig. 4a), and this motif is critical for the high transforming activity of Tax1 to CTLL-2 [32]. However, Xie et al. [33] showed that the deletion of PBM in recombinant HTLV-1 minimally affects immortalization activity to human T cells in the presence of IL-2. Therefore, this motif is unlikely to be responsible for distinct immortalization activities of Tax1 and Tax2B in human T cells. Tax1 and Tax2B have other distinctions from PBM. For instance, Tax1 but neither Tax2B nor Tax300 activates the non-canonical NF-κB2 pathway, which also plays a critical role in the high transforming activity toward CTLL-2 [16, 17]. Therefore, precise comparisons of Tax1 and Tax2B including these differences are required to identify the function(s) responsible for immortalization of human T cells as well as the pathogenesis.

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