Human T Cell Lymphototropic Virus 1 Manipulates Interferon Regulatory Signals by Controlling the TAK1-IRF3 and IRF4 Pathways

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We previously reported that human T cell lymphotropic virus 1 (HTLV-1) Tax oncoprotein constitutively activates transforming growth factor-β-activated kinase 1 (TAK1). Here, we established Tax-positive HuT-102 cells stably transfected with a short hairpin RNA vector (HuT-shTAK1 cells) and investigated the physiological function of TAK1. Microarray analysis demonstrated that several interferon (IFN)-inducible genes, including chemokines such as CXCL10 and CCL5, were significantly down-regulated in HuT-shTAK1 cells. In contrast, Tax-mediated constitutive activation of nuclear factor-κB (NF-κB) was intact in HuT-shTAK1 cells. IFN-regulatory factor 3 (IRF3), a critical transcription factor in innate immunity to viral infection, was constitutively activated in a Tax-dependent manner. Activation of IRF3 and IRF3-dependent gene expressions was dependent on TAK1 and TANK-binding kinase 1 (TBK1). On the other hand, IRF4, another member in the IRF family of transcription factors overexpressed in a Tax-independent manner, negatively regulated TAK1-dependent IRF3 transcriptional activity. Together, HTLV-1 manipulates IFN signaling by regulating both positive and negative IRFs.

The molecular mechanism underlying gene transcription of type I interferon (IFN) and IFN-inducible genes has been studied extensively over the past few decades. The interferon regulatory factor (IRF) family of transcription factors coordinatealy regulates the induction of IFN-inducible genes via an interferon-stimulated response element (ISRE) in their promoters (1). IRF3 and IRF7, especially, have been characterized as key regulators in inducible transcription upon viral infection (2). TANK-binding kinase 1 (TBK1) and IKKe have been shown to phosphorylate IRF3 and IRF7 directly (3, 4). The TBK1/IκKe-IRF3/7 pathway is stimulated by the activation of pattern recognition receptors, including Toll-like receptors (TLRs) (5) and cytosolic viral RNA sensors, such as RIG-I (6) and MDA5 (7). In contrast, IRF4, another member of the IRF family that is preferentially expressed in lymphoid cells, was first identified as a transcription factor that negatively regulates the activity of IFN-regulated genes (8) and TLR signaling (9).

Human T cell lymphotropic virus type 1 (HTLV-1) is known as the cause of adult T cell leukemia/lymphoma (ATLL). HTLV-1-derived oncoprotein Tax is thought to be a key molecule of ATLL onset and has many pathological functions such as virus replication, immortalization of host cells, and activation of several transcriptional factors and signal transduction molecules, including NF-κB, CAMP-response element-binding protein, and phosphoinositide 3-kinase-Akt in host CD4+ T cells (10–12). We have recently found Tax-dependent constitutive activation of the TAK1-MAPK pathway (13). TAK1 is known as a key kinase leading to NF-κB in tumor necrosis factor-α and interleukin-1 signaling pathways (14, 15); however, it is dispensable in Tax-dependent constitutive NF-κB activation (13). TAK1 has been demonstrated to participate in TLR-mediated activation of NF-κB (16) but not the TBK1-IRF3/7 pathway (17, 18); however, the role of constitutive TAK1 activation in IFN-regulatory signals triggered by HTLV-1 is still largely unknown.

In the present study, we demonstrated that Tax-dependent TAK1 activation induces TBK1-IRF3 activation and the expression of several IFN-inducible genes, including CXCL10 and CCL5. Moreover, IRF4, overexpressed in HTLV-1-infected cells in a Tax-independent manner, negatively controls the transcriptional regulation of these genes.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Reagents—Anti-phospho-TAK1 (Thr-187) (19) and anti-Tax antibodies (20) were described previously.*
Antibodies against TAK1, TAB1, TAB2, IRF3, IRF4, JNK, p38, ubiquitin, IKKα, p65, green fluorescent protein, actin, proliferating cell nuclear antigen, lamin B, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-IRF3 (Ser-396), phospho-p65 (Ser-536), phospho-JNK (Thr-183/Tyr-185), phospho-p38 (Thr-180/Tyr-182), phospho-IKKα/β (Ser-176/180), IKKβ, IKKγ, and TBK1 were purchased from Cell Signaling Technology (Danvers, MA). A proteasome inhibitor N-acetylleucylleucylnorleucinal (ALLN; Nacalai Tesque, Kyoto, Japan) was dissolved in dimethyl sulfoxide.

**Cell Culture**—Jurkat, Jurkat-derived JPX-9, and HTLV-1-transformed cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HuT-102 cells were stably transfected with pSUPER.gfp+neo vectors (OrigoEngine, Seattle, WA) to express shRNAs against human TAK1 or firefly luciferase. The target sequence for TAK1 is 5’-ATGACGA-TTCATGAGTGTAG-3’, which is located in 3’-untranslated region. HuT-shTAK1 and HuT-shLuc cells were selected by 0.8 mg/ml G418 and then sorted by green fluorescent protein expression. Stable transfectants were maintained in medium containing 0.5 mg/ml G418. For experiments, G418 was removed 36 h before harvest.

**Immunoblotting**—Whole cell lysates, cytoplasmic extracts, and nuclear extracts were prepared as described previously (21), resolved by SDS-PAGE, and transferred to Immobilon-P nylon membrane (Millipore, Bedford, MA). The membrane was treated with BlockAce (Dainippon Pharmaceutical, Suta, Japan) overnight at 4 °C and probed with primary antibodies as described above. Antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (DakoCytomation, Glostrup, Denmark) and visualized with the ECL system (GE Healthcare).

**DNA Microarray**—Total RNA was extracted from cells using an RNeasy Mini kit (Qiagen, Valencia, CA). Gene expression was analyzed using a GeneChip® system with Human Genome Array U133 Plus2.0 (Affymetrix, Santa Clara, CA) as described previously (22). In this study, a total of four arrays were used: two for HuT-shLuc cells and two for HuT-shTAK1 cells. A fold change value of greater than 1.5 (up-regulated) or less than 1.5 (down-regulated) was considered to be biologically important. The statistical significance of the fold change was calculated for two groups by performing Student’s t test, and p values <0.05 were regarded as significant.

**RNA Interference**—Cells were transfected with each siRNA for Tax, TBK1, RIG-I, MDA5, IRF3, IRF4, firefly luciferase (Luc) using the Amaxa electroporation system. All siRNAs were designed at and purchased from Invitrogen. Luc siRNA with a two-nucleotide overhang at the 3’-end of the sequence was designed at iGENE Therapeutics (Tokyo, Japan) and synthesized by Hokkaido System Science (Sapporo, Japan). The target sequences are summarized in supplemental Table S1.

**Real Time RT-PCR**—Total RNA was prepared using the RNeasy Mini kit (Qiagen). First-strand cDNA was synthesized by SuperScript II reverse transcriptase (Invitrogen). The cDNA was amplified quantitatively using SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Japan). The primer sequences are summarized in supplemental Table S2. Real time quantitative RT-PCR was performed using an ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA). All data were
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RESULTS AND DISCUSSION

Constitutive TAK1 Activation Induces IFN-inducible Genes—We first established Tax-positive HuT-102 cells stably transfected with shRNA expression vectors against TAK1 (HuT-shTAK1) and luciferase (HuT-shLuc). TAK1 protein expression and activating phosphorylation were down-regulated in HuT-shTAK1 cells compared with control HuT-shLuc and parent HuT-102 cells (Fig. 1A). Two TAK1 adaptor proteins, TAB1 and TAB2, were also severely down-regulated at the protein level, although their mRNAs were comparably expressed (Fig. 1, A and B). We reported previously that TAB1 and TAB2 form a stable complex with TAK1 in HuT-102 cells (13), suggesting that TAK1 has a scaffolding function for stabilization of the complex. In contrast, protein expression (Fig. 1A) and ubiquitination (Fig. 1C) of Tax were not affected by the loss of function of the TAK1 complex. In accordance with our previous results, phosphorylation of JNK/p38 MAPKs (Fig. 1D), but not NF-κB (Fig. 1E), was suppressed in HuT-shTAK1 cells. In addition, proliferation in vitro was comparable (Fig. 1E).

We next performed microarray analysis to find TAK1-regulated genes in HTLV-1-transformed cells. Surprisingly, a series of IFN-inducible genes were down-regulated in HuT-shTAK1 cells (Fig. 2A), although IFN-α/β mRNAs were not significantly expressed (data not shown). In contrast, the expressions of NF-κB-regulated genes, including A20 and IκBα, were not changed. Wu and Sun (23) recently reported that TAK1 is involved in Tax-induced NF-κB activation; however, based on our RNAi experiments, we currently believe that TAK1 is not essential for constitutive activation of the canonical NF-κB pathway. The microarray results were confirmed by real time RT-PCR, in which mRNA expressions of CXCL10, IFIT1, and CCL5 were down-regulated (Fig. 2B, C, and D). IFN-inducible genes were examined by RT-PCR. Data are the mean ± S.D. of triplicate determinations (error bars). The statistical significance of differences between groups was calculated by Student’s two-tailed t test. *, p < 0.01.

 normalized to β-actin mRNA. The data shown are representative of at least three independent experiments.

Reporter Gene Assay—HeLa cells were cotransfected with reporter plasmids, including the ISRE-driven firefly luciferase reporter plasmid (Stratagene) and pRL-EF1α Renilla luciferase plasmid and Tax expression plasmid using a Lipofectamine reaction. Lysis was performed with our previous results, phosphor-ylation of JNK/p38 MAPKs (Fig. 1D), but not NF-κB (Fig. 1E), was suppressed in HuT-shTAK1 cells. In addition, proliferation in vitro was comparable (Fig. 1E). We next performed microarray analysis to find TAK1-regulated genes in HTLV-1-transformed cells. Surprisingly, a series of IFN-inducible genes were down-regulated in HuT-shTAK1 cells (Fig. 2A), although IFN-α/β mRNAs were not significantly expressed (data not shown). In contrast, the expressions of NF-κB-regulated genes, including A20 and IκBα, were not changed. Wu and Sun (23) recently reported that TAK1 is involved in Tax-induced NF-κB activation; however, based on our RNAi experiments, we currently believe that TAK1 is not essential for constitutive activation of the canonical NF-κB pathway. The microarray results were confirmed by real time RT-PCR, in which mRNA expressions of CXCL10, IFIT1, and CCL5 were down-regulated (Fig. 2B). Similarly, the expressions of IFN-inducible genes were suppressed by Tax siRNA (Fig. 2C), indicating that Tax-induced TAK1 activation regulates their transcriptional activation. In contrast, Tax-dependent viral gag expression was independent of TAK1 (Fig. 2D). Moreover, the RNAi experiment verified that TAK1-induced activation of JNK1/2 and p38α is not involved in CXCL10 expression (data not shown), suggesting that TAK1 induces IFN-inducible genes via NF-κB- and MAPK-independent pathways.

TAK1 Mediates Tax-dependent Activation of TBK1-IRF3—IRF3 has been characterized as a critical transcriptional activator in IFN-regulated genes upon viral infection (2); however, the role in HTLV-1-infected T cells is still unknown. We set up an RNAi experiment for selective IRF3 knockdown (Fig. 3A). IRF3 siRNA significantly suppressed CXCL10 mRNA expression, but not TAK1-independent viral gag mRNA expression (Fig. 3B). Immunoblot analysis demonstrated that phosphorylation of IRF3 was detected in Tax-positive cell lines (Fig. 4A). The phosphorylation was abrogated by Tax siRNA (Fig. 4B). The ISRE-dependent transcriptional activity and mRNA expression of CXCL10 and CCL5 were induced by ectopic expression of Tax in HeLa and Jurkat cells (supplemental Fig. 1).
addition, IRF3 was inactivated in HuT-shTAK1 cells (Fig. 4 C). Moreover, both TAK1 and IRF3 were activated by Tax in an inducible cell line, JPX-9 (Fig. 4 D).

To investigate upstream regulation of IRF3, we focused on a major IRF3 kinase, TBK1. RNAi experiments demonstrated that TBK1 was essential for phosphorylation of IRF3 (Fig. 5A) and the transcriptional activation of CXCL10 and CCL5 (Fig. 5B). As shown in Fig. 2A, the expression of MDA5 and RIG-I, sensors of viral RNAs that are well known to trigger the TBK1-IRF3 pathway, were down-regulated in HuT-shTAK1 cells. Interestingly, the expressions of these mRNAs were also suppressed by knockdown of Tax (supplemental Fig. 2A). In contrast, IRF3 siRNA did not inhibit the expression of MDA5 or RIG-I (supplemental Fig. 2B). Moreover, knockdown of MDA5 or RIG-I did not inhibit the expression of CXCL10 and phosphorylation of IRF3 (supplemental Fig. 2, C and D). MDA5 and RIG-I recognize viral RNA molecules harboring a 5’-triphosphate end (24); however, transcripts from the integrated HTLV-1 genome lack the RNA structure. These findings indicate that the expressions of MDA5 and RIG-I are targeted by Tax and TAK1, but the expressed RNA sensor proteins are not involved in the IRF3 activation.

Although TAK1 is essential for pathogen-induced activation of NF-κB and MAPKs, TAK1 is dispensable for IFN-α/β expression and IRF3 activation by early vesicular stomatitis virus infection or stimulation with poly(I-C) in mouse embryonic fibroblasts (17). In comparison with these reports, HuT-102 and MT-2 cells are chronically infected with HTLV-1 and also constitutively express viral Tax oncoprotein. It has recently been reported that Tax interferes with the IFN-α-induced JAK-STAT pathway by competition with STAT2 for CBP/p300 binding (25), suggesting that Tax controls two different IFN signaling pathways. In addition, the TAK1-JNK cascade is required for IRF3 function in macrophages (18). In contrast, the RNAi experiment ruled out JNK and p38 in IRF3 phosphorylation in HuT-102 cells (data not shown). Because Tax is associated with IKK/NEMO, an adaptor protein that allows RIG-I to activate both the NF-κB and IRF3 signaling pathways (26, 27), we further tested the effect of IKKγ siRNA on phosphorylation of IRF3. However, IKKγ knockdown did not affect Tax- and TAK1-dependent activation of IRF3 (supplemental Fig. 3). This is consistent with our previous finding that TAK1 is not able to associate with the Tax-IKKγ complex (13). Together, although it is necessary to elucidate a molecular mechanism for how
TAK1 controls the regulation of IRF3 phosphorylation, this is
the first demonstration that Tax activates IRF3 in a TAK1-de-
pendent manner.

**Figure 6.** Suppression of IFN-inducible genes by IRF4. A, total cellular RNAs were extracted from Jurkat and HTLV-1-transformed cells and subjected to RT-PCR using a set of IRF4 primers. B, whole cell lysates were extracted from Jurkat and HTLV-1-transformed cells and subjected to immunoblotting with anti-IRF4 and proliferating cell nuclear antigen (PCNA) antibodies. C, HuT-102 cells were transfected with Tax and Luc siRNA. At 60 h after transfection, IRF4 mRNA expression was examined by RT-PCR. D, HuT-102 cells were transfected with IRF4 and Luc siRNAs. The expressions of CXCL10, IFIT1, IFN-β, and IRF4 mRNA were examined by RT-PCR. E, Tax-negative ED40515(-) cells were transfected with IRF4 and Luc siRNA. At 60 h after transfection, CXCL10 mRNA expression was examined by RT-PCR.

**Figure 7.** Suppression of TAK1-TBK1-IRF3 pathway by IRF4. A, HuT-shTAK1 and HuT-shLuc cells were transfected with IRF4 and Luc siRNAs. At 60 h after transfection, the expressions of CXCL10 and IRF4 mRNA were examined by RT-PCR. B, HuT-102 cells were transfected with IRF4 and Luc siRNA in combination with IRF3 and TBK1 siRNA, and then CXCL10 mRNA expression was amplified by RT-PCR.

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