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Human T-cell leukemia virus type 1 Tax transactivates the *matrix metalloproteinase 7* gene via JunD/AP-1 signaling

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ABSTRACT

Adult T-cell leukemia (ATL) is a T-cell malignancy associated with human T-cell leukemia virus type 1 (HTLV-1) and characterized by visceral invasion. Degradation of the extracellular matrix by matrix metalloproteinases (MMPs) is a crucial process in invasion of tumors and metastasis. MMP-7 (or matrilysin), is a "minimal domain MMP" with proteolytic activity against components of the extracellular matrix. To determine the involvement of MMP-7 in visceral spread in ATL, this study investigated MMP-7 expression in ATL. MMP-7 expression was identified in HTLV-1-infected T-cell lines, peripheral blood ATL cells and ATL cells in lymph nodes, but not in uninfected T-cell lines or normal peripheral blood mononuclear cells. MMP-7 expression was induced following infection of a human T-cell line with HTLV-1, and specifically by the viral protein Tax. Functionally, MMP-7 promoted cell migration of HTLV-1-infected T cells. The MMP-7 promoter activity was increased by Tax and reduced by deletion of the activator protein-1 (AP-1) binding site. Electrophoretic mobility shift assay showed high levels of AP-1 binding proteins, including JunD, in HTLV-1-infected T-cell lines and ATL cells, and Tax elicited JunD binding to the MMP-7 AP-1 element. Tax-induced MMP-7 activation was inhibited by dominant negative JunD and augmented by JunD/JunD homodimers. Short interfering RNA against JunD inhibited MMP-7 mRNA expression in HTLV-1-infected T-cell lines. These results suggest that the induction of MMP-7 by Tax is regulated by JunD and that MMP-7 could facilitate visceral invasion in ATL.

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection causes adult T-cell leukemia (ATL) [1]. ATL is an aggressive malignancy of mature activated T cells characterized by frequent visceral invasion by tumor cells [2]. Vascular infiltration of tumor cells through the endothelial cells is a critical step of cancer invasion and metastasis, which is an important prognostic factor of malignant diseases. In this process of neoplasm infiltration, there is increased production of matrix metalloproteinases (MMPs) from tumor cells [3]. Degradation of the extracellular matrix by MMPs is a crucial step in tumor invasion and metastasis [4]. Indeed, HTLV-1-infected T-cell lines and primary ATL cells secrete high levels of MMP-9, and a significant elevation of plasma MMP-9 was observed in some ATL patients, particularly in the patients with ATL cell infiltration [5]. In addition, ATL cells express extracellular MMP inducer, emmprin, a cell surface inducer of MMPs that stimulates nearby fibroblasts to produce MMP-2 [6].

The MMP family consists of at least 25 zinc-dependent endopeptidases. Almost all MMPs share homologous protein sequences and domain structures. In addition to these conserved domains, MMPs have diverse domains that are related to substrate specificity and recognition of other proteins. However, MMP-7 (matrilysin), MMP-23 and MMP-26 lack the C-terminal hemopexin domain common to other MMPs members, and they have distinctively smaller molecular weights [3]. Although the majority of MMPs are produced predominantly by stromal cells, such as stromal fibroblasts, macrophages and endothelial cells, only a few MMPs, including MMP-7, are expressed

Abbreviations: AP-1, activator protein-1; ATL, adult T-cell leukemia; CRE, cyclic AMP response element; ATF, activating transcription factor; ERK, extracellular signal-regulated kinase; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; HTLV-1, human T-cell leukemia virus type 1; IL, interleukin; IL-2Rα, IL-2 receptor α chain; JNK, c-Jun NH_2-terminal kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA

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by tumor cells themselves, indicating that MMP-7 is expressed in a tumor-associated fashion. MMP-7 is overexpressed in various epithelial tumors and mesenchymal tumors. The production by tumor cells themselves could be useful as a biological marker of an aggressive phenotype and as a target of therapeutic intervention [3].

The expression of MMP-7 in ATL has not been examined previously. In this study, we investigated the expression of MMP-7 in various T-cell lines and clinical samples from ATL patients. We explored whether MMP-7 expression could be induced by the HTLV-1 oncogene Tax. The results demonstrated that JunD, a member of the activator protein-1 (AP-1) family [7], plays a major role in MMP-7 expression in ATL cells.

2. Materials and methods

2.1. Cell lines

HTLV-1-infected T-cell lines, MT-2 [8], SLB-1 [9], MT-1 [10], TL-OmI [11] and HLN-ATL-O [12], and HTLV-1-uninfected T-cell lines, Jurkat, MOLT-4, CCRF-CEM and TY8-3 [13], were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel), 50 U/ml penicillin and 50 μg/ml streptomycin. MT-2 and SLB-1 are HTLV-1-transformed T-cell lines and constitutively express viral genes. MT-1 and TL-OmI are T-cell lines of leukemic cell origin that were established from patients with ATL, and do not express viral genes. HLN-ATL-O was also established from a patient with ATL, but its clonal origin is unclear. TY8-3/MT-2 was established from the interleukin (IL)-2 dependent human T-cell line TY8-3 cocultured with mitomycin C-treated MT-2 cells and was capable of growth completely independent of IL-2 [13]. JPX-9 is a subline of Jurkat that expresses Tax, under the control of the *metallothionein* gene promoter [14]. Peripheral blood mononuclear cells (PBMCs) were isolated from 3 healthy volunteers and 9 patients with acute type ATL, using Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). To use activated T cells, PBMCs were stimulated with 10 μg/ml phytohemagglutinin (PHA) for 48 h. All samples were collected at the time of admission to the hospital before the commencement of chemotherapy. The diagnosis of ATL was based on clinical features, hematological findings and the presence of anti-HTLV-1 antibodies in the serum. Monoclonal HTLV-1 provirus integration into the DNA of leukemic cells was confirmed by Southern blot hybridization in all patients (data not shown). This study was approved by the Institutional Review Board of the University of the Ryukyus. Informed consent was obtained from all blood and tissue donors according to the Helsinki Declaration.

2.2. Reagents

Rabbit polyclonal antibody and mouse monoclonal antibody to MMP-7 were purchased from Chemicon International (Temecula, CA, USA) and Daiichi Fine Chemical (Takaoka, Japan), respectively. Mouse monoclonal antibody to actin was from NeoMarkers (Fremont CA, USA). Rabbit polyclonal antibodies to JunD, phospho-c-Jun (Ser73), phospho-JunD (Ser100), c-Jun NH_2-terminal kinase (JNK) and phospho-JNK (Thr183 and Tyr185), and rabbit monoclonal antibodies to extracellular signal-regulated kinase (ERK)1/2 and phospho-ERK1/2 (Thr202 and Tyr204) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody to Tax, Lt-4, was described previously [15]. JNK inhibitor SP600125 and mitogen-activated protein kinase/ERK kinase1/2 inhibitor PD98059 were obtained from Calbiochem (La Jolla, CA, USA). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Reverse transcription-PCR (RT-PCR)

Total cellular RNA from cells was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1 μg total cellular RNA using an RNA-PCR kit or a PrimeScript RT-PCR kit (Takara Bio Inc., Otsu, Japan) with random primers. The primers used were 5′-GATCTCAAATGAGTCACCTAT-3′ (forward) and 5′-GATCATAGGTGACTCATTTGA-3′ (reverse) for MMP-7, 5′-CCGGCGCTGCTCTCATCCCGGT-3′ (forward) and 5′-GGCCGAACATAGTCCCCCAGAG-3′ (reverse) for Tax, 5′-CGCAGCCTCAAACCCTGCCTTTCC-3′ (forward) and 5′-CAAACAGGAATGTGGACTCGTAGC-3′ (reverse) for JunD, and 5′-GTGGGGCGCCCCAGGCACCA-3′ (forward) and 5′-CTCCTTAATGTCACGCACGATTTC-3′ (reverse) for β-actin. The length of the semiquantitative RT-PCR for each gene was as follows; 35 cycles for MMP-7, 30 cycles for Tax, 40 cycles for JunD, and 28 cycles for β-actin. The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining. The obtained bands of amplified DNA were quantified using AlphaEase FC software (Alpha Innotech Corporation, San Leandro, CA, USA). Data were normalized to β-actin loading controls.

2.4. Western blot analysis

Cells were lysed in a buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, followed by transfer to a polyvinylidene difluoride membrane and sequential probing with the specific antibodies. The bands were visualized with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

2.5. Immunohistochemical analysis

Biopsy samples were taken from the lymph nodes of 6 patients with ATL. In addition, 2 specimens of normal lymph nodes were included. Serial sections were deparaffinized in xylene and dehydrated using graded ethanol solutions. For better detection, sections were pretreated with ready-to-use proteinase K (Dako Inc., Carpinteria, CA, USA) for 10 min at 37 °C. This procedure increased the number of antigenic sites available for binding by the antibody. Sections were washed 4 times in phosphate-buffered saline (PBS) for 5 min each. In the next step, the tissues were placed in 3% hydrogen peroxide and absolute methanol for 5 min to reduce endogenous peroxidase activity, followed by 4 washes in PBS, 5 min each. The tissue sections were incubated with the polyclonal rabbit anti-MMP-7 antibody for 3 h at 37 °C. After 4 washes with PBS, 5 min each, the sections were covered with EnVision plus (Dako Inc., Santa Barbara, CA, USA) for 40 min at 37 °C and washed 4 times in PBS, 5 min each. Antigenic sites bound by the antibody were identified by reacting the sections with a mixture of 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris–HCl buffer and 0.01% hydrogen peroxide for 7 min. Sections were washed 3 times in distilled water, 5 min each, and then counterstained with methyl green for 10 min, hydrated in ethanol, cleaned in xylene and mounted. The stained cells were examined using a light microscope.

2.6. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 20 min at 37 °C. Fixed cells were washed with PBS containing 7% of FBS twice and permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature. The cells were washed with PBS containing 7% of FBS once and resuspended in PBS/7% FBS containing mouse anti-MMP-7 monoclonal antibody or rabbit anti-MMP-7 polyclonal antibody for 20 min at room temperature. The cells were washed with PBS/7% FBS twice and resuspended in PBS/7% FBS containing Alexa Fluor 488-labeled goat anti-mouse IgG or Alexa Fluor 546-labeled goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for 20 min at room temperature. The nuclei were stained with Hoechst 33342

(Wako Pure Chemical Industries, Osaka, Japan). Finally, the cells were washed with PBS twice and observed under a confocal microscope.

2.7. Transfection and luciferase assay

A series of expression vectors for Tax (pβMT-2 Tax) and its mutants (Tax M22 and Tax 703) were described previously [16]. The expression vector (DN-JunD) for dominant negative JunD lacking amino acids 1–162 and the mutant JunD expression vector (JunD/S100A) encoding a modified JunD protein in which the Ser100 residue has been replaced by Ala have been described previously [17,18]. In the JunD/EB1 vector, the dimerization domain of wild type JunD is replaced by the heterologous homodimerization domain of the Epstein–Barr virus transcription factor EB1 and binds DNA exclusively as a homodimer [19]. The −296HMAT-Luc parental construct was generated by inserting the MMP-7 promoter region (−296 to +35 bp surrounding the transcription start site) into the pGL2-basic vector (Promega, Madison, WI, USA) [20]. Inactivating mutations were introduced into two Tcf sites of the −296HMAT-Luc reporter to create Mut(−194/−109Tcf) reporter [20]. The Del(−67AP-1) reporter was prepared by deletion of the AP-1 site of the −296HMAT-Luc reporter. We also used the 2× AP-1-Luc constructed by introducing two copies of IL-8 AP-1 site in front of minimal IL-8 promoter [21]. Jurkat and SLB-1 cells were transfected with the appropriate reporter and effector plasmids using electroporation. After 24 h, the cells were washed in PBS and lysed in reporter lysis buffer (Promega). Lysates were assayed for reporter gene activity with the dual luciferase assay system (Promega). Luciferase activities were normalized relative to the *Renilla* luciferase activity from phRL-TK.

2.8. Small interfering RNA (siRNA)

To repress JunD, predesigned double-stranded siRNA (ON-TARGET plus SMART pool; Dharmacon Inc., Lafayette, CO, USA) was used. A siCONTROL non-targeting siRNA pool (Dharmacon Inc.) was used as a negative control. All siRNA transfections were performed using a MicroPorator MP-100 (Digital Bio Technology, Seoul, Korea) according to the instructions supplied by the manufacturer for optimization and use.

2.9. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cell pellets were swirled to a loose suspension and treated with lysis buffer [0.2 ml, containing in 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM AEBSF and 1 mM DTT] with gentle mixing at 4 °C. After 10 min, NP40 was added to a final concentration of 0.6% and the solution was centrifuged immediately at 1000 rpm for 5 min at 4 °C. The supernatants were removed carefully and the nuclear pellets were diluted immediately by the addition of NP40-free lysis buffer (1 ml). The nuclei were then recovered by centrifugation at 1000 rpm for 5 min at 4 °C. Finally, the remaining pellets were suspended on ice in an extraction buffer [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mM AEBSF, 33 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml E-64 and 10 μg/ml pepstatin A] for 30 min to obtain the nuclear fraction. All fractions were cleared by centrifugation for 15 min at 15000 rpm. Transcription factors bound to specific DNA sequences were examined by EMSA as described previously [22]. To examine the specificity of the probe, we preincubated unlabeled competitor oligonucleotides with nuclear extracts for 15 min before incubation with probes. The probes or competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides as follows: for the AP-1 element of the *MMP-7* gene, 5′-GATCTCAAATGAGTCACCTAT-3′; for the nuclear factor-κB (NF-κB) element of the IL-2 receptor α chain (*IL-2Rα*) gene, 5′-GATCCGGCAGGGGAATCTCCCTCTC-3′; and for the AP-1 element of the *IL-8* gene, 5′-GATCGTGATGACTCAGGTT-3′. The oligonucleotide 5′-GATCTGTCGAATGCAAATCACTAGAA-3′, containing the consensus sequence of the octamer binding motif, was used to identify specific binding of the transcription factor Oct-1. The underlined sequences above are the NF-κB, AP-1 and Oct-1 binding sites, respectively. To identify transcription factors in the DNA–protein complex shown by EMSA, we used antibodies specific for various AP-1 family proteins, including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD (Santa Cruz Biotechnology, Santa Cruz, CA, USA), to elicit a supershift DNA–protein complex formation. These antibodies were incubated with the nuclear extracts for 45 min at room temperature before incubation with radiolabeled probe.

2.10. Cell invasion assay

ACEA electrosensing×16 microtiter plates were coated with Matrigel (BD Biosciences, San Jose, CA, USA) diluted 1:25 in starvation media. SLB-1 cells were seeded at 3×10^5 cells/ml in 100 μl of serum-free medium with or without a goat polyclonal MMP-7 neutralizing antibody or a normal goat IgG (R&D systems, Inc., Minneapolis, MN, USA) in the upper chamber with 8 μm pore size, and the lower chamber contained 10% serum. The ACEA plate was connected to the ACEA Device Station at 37 °C, and SLB-1 cells that invaded through Matrigel were monitored every 15 min in real time by an ACEA Sensor Analyzer for 24 h and quantitated using ACEA RT-CES Integrated Software.

2.11. Statistical analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the unpaired Student's *t*-test. A *p* values less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. MMP-7 expression in HTLV-1-infected T-cell lines and primary ATL cells

First, we examined the expression of Tax in various human T-cell lines (Fig. 1A). HTLV-1-transformed T-cell lines (MT-2 and SLB-1) expressed high levels of Tax protein. In ATL-derived T-cell lines, MT-1 and TL-Oml cells did not express Tax protein. HLN-ATL-O cells expressed Tax but less than MT-2 and SLB-1 cells. We next examined the expression of MMP-7 at mRNA level in various human T-cell lines by RT-PCR. As shown in Fig. 1A, compared to uninfected T-cell lines, HTLV-1-infected T-cell lines consistently expressed MMP-7 mRNA. Especially, HTLV-1-transformed T-cell lines expressed MMP-7 mRNA at high level. We also examined the localization of MMP-7 protein in SLB-1 and MT-2 cells by immunofluorescent staining and confocal microscopy (Fig. 1B). MMP-7 was detectable in the cytoplasm of immunopositive SLB-1 and MT-2 cells.

To substantiate HTLV-1 control of MMP-7 expression, studies were performed using a parental TY8-3 cell line and TY8-3 cells infected with HTLV-1 (TY8-3/MT-2). As shown in Fig. 1C, TY8-3/MT-2 cells demonstrated strong expression of Tax mRNA. We next examined the expression of MMP-7 in these cells. Cognate MMP-7 expression was significantly enhanced in TY8-3/MT-2 cell line, while MMP-7 expression in the parental line was negative (Fig. 1C). These results suggest that infection of TY8-3 with HTLV-1 induces expression of MMP-7.

Furthermore, we examined the mRNA expression of MMP-7 in primary ATL cells freshly isolated from patients in comparison with normal PBMCs in resting and activated conditions (Fig. 2A). Primary ATL cells consistently expressed MMP-7 at levels much higher than normal PBMCs. MMP-7 expression was induced in response to PHA. To investigate the expression of MMP-7 *in situ*, we performed

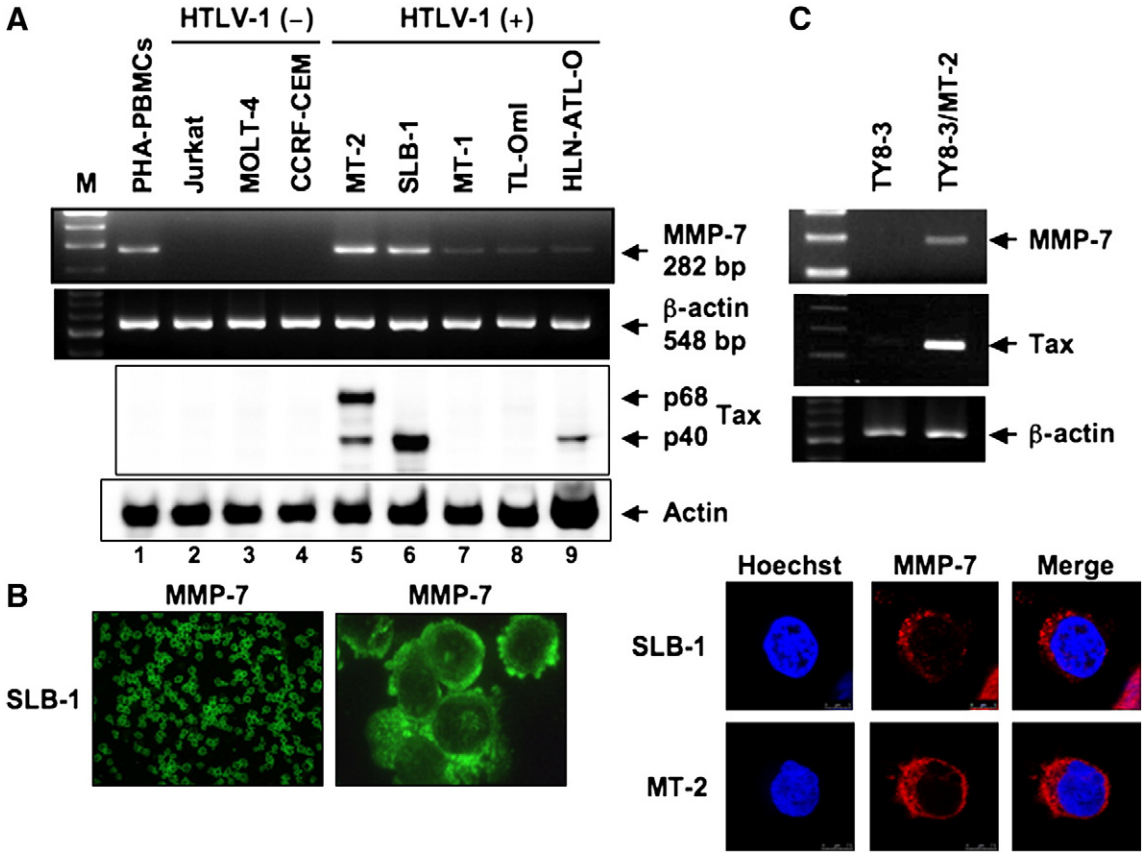

Fig. 1. Expression of MMP-7 in HTLV-1-infected T-cell lines. (A) Expression of MMP-7 and Tax in HTLV-1-infected T-cell lines. RT-PCR analysis was carried out for MMP-7 and β-actin (loading control). Western blot analysis was performed for Tax and actin. Lt-4 monoclonal antibody detected a 40 kDa molecule (p40) in SLB-1 and HLN-ATL-O cells, and p40 and p68 in MT-2 cells. (B) Immunofluorescence images of SLB-1 and MT-2 cells for MMP-7. SLB-1 and MT-2 cells were immunostained with antibody that specifically recognize MMP-7, followed by staining with Alexa Fluor 488-labeled goat anti-mouse IgG (green) or Alexa Fluor 546-labeled goat anti-rabbit IgG (red), and counterstained with Hoechst 33342 (nuclei stained in blue). MMP-7 is detectable *in situ* in the cytoplasm of SLB-1 and MT-2 cells. (C) Comparison of TY8-3 cells and a virally-transformed cell line. RT-PCR analysis for mRNA expression of Tax and MMP-7 in TY8-3 and TY8-3/MT-2 cells. Expression of β-actin was used as a control.

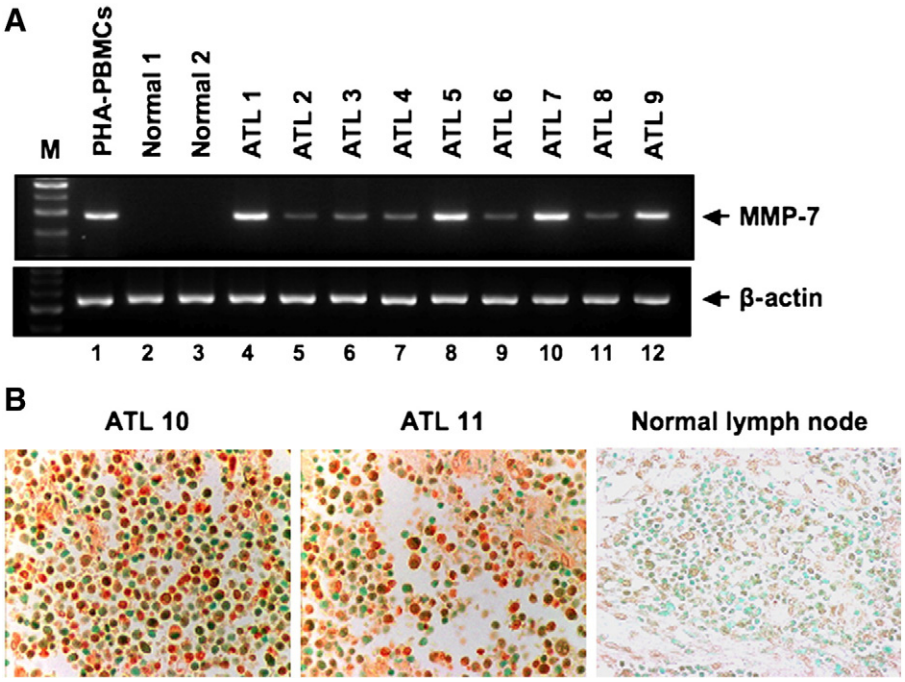

Fig. 2. Expression of MMP-7 in primary ATL cells. (A) RT-PCR analysis for expression of MMP-7 in normal PBMCs and primary ATL cells. Normal PBMCs were treated with PHA for 48 h (PHA-PBMCs). Normal PBMCs from healthy donors ($n=2$), PHA-PBMCs ($n=1$) and freshly isolated primary ATL cells (>90% leukemic cells) from patients with acute type ATL ($n=9$) were examined as indicated. (B) Immunohistochemical staining of MMP-7 in ATL lymph nodes. Tissue sections from ATL lymph nodes ($n=6$) and normal lymph nodes ($n=2$) were stained with anti-MMP-7 antibody. Tissue sections were counterstained using methyl green. The primary antibody shows red staining of MMP7 in image whilst nucleus was stained green with methyl green. Representative results from two ATL patients and one normal lymph node. Original magnification: ×400.

immunohistochemical staining of MMP-7 in ATL cells invading lymph nodes ($n=6$). Representative results are shown in Fig. 2B. ATL cells were positive for MMP-7 in all the tissue samples examined (Fig. 2B), although the staining intensity varied from case to case. MMP-7 expression was weak in normal lymph nodes ($n=2$) (Fig. 2B).

3.2. Tax-dependent expression of MMP-7

The *tax* gene product is the primary viral transactivator protein that modulates the expression of both viral and cellular genes. To examine whether Tax induces MMP-7 expression, we used JPX-9 cells; this is a Jurkat subline that carries the *tax* gene under the control of the *metallothionein* gene promoter [14]. As shown in Fig. 3A, treatment of JPX-9 cells with $CdCl_2$ induced the expression of Tax. MMP-7 mRNA was also induced in $CdCl_2$-treated JPX-9 cells. In contrast, this treatment did not show any effect in Jurkat cells (Fig. 3B).

To test the effect of Tax on MMP-7 expression at the transcriptional level, we further performed the luciferase reporter assays in Jurkat cells using a MMP-7 promoter-luciferase reporter plasmid [20]. There are one AP-1 site and two Tcf sites in the MMP-7 promoter (Fig. 4A). As shown in Fig. 4B, cotransfection of an expression vector for Tax strongly activated the MMP-7 promoter in Jurkat cells after 24 h, indicating that Tax can directly activate the MMP-7 promoter.

To narrow down the transactivation-relevant signaling pathways, Tax mutants M22 and 703 [16] were cotransfected along with the MMP-7 promoter construct, followed by determination of luciferase activities. Tax M22 has an amino acid substitution at codons 130 and 131 from Thr–Leu to Ser-Ala. Tax 703 has an amino acid substitution at codons 319 and 320 from Leu–Leu to Arg–Ser, which is equivalent to mutant M47 [23]. Tax M22 effectively activates cyclic AMP response element (CRE), which mediates the Tax-dependent activation of the HTLV-1 long terminal repeat, but not the NF-κB element. In contrast, Tax 703 activates the NF-κB element but does not affect CRE. Tax M22 slightly activated the MMP-7 promoter (Fig. 4B). Tax 703 clearly activated the MMP-7 promoter, but the levels were less than wild type Tax (Fig. 4B). On the other hand, the two mutants together activated MMP-7 promoter. These results suggest that Tax activates the MMP-7 promoter in NF-κB- and CRE-dependent manners. In this context, it was intriguing to examine whether exogenous expression of subunits of NF-κB or CRE binding protein (CREB) activates the MMP-7 promoter. However, introduction of the NF-κB p65 subunit or CREB failed to activate the MMP-7 promoter (data not shown), and the MMP-7 promoter sequence between −296 and +35 bp did not include sequences suggestive of sites for binding to NF-κB and CREB, suggesting that activation of MMP-7 promoter by Tax is not directly mediated by the NF-κB and CRE pathways.

We have previously reported that Tax activates the AP-1 site via a mechanism different from the NF-κB and CRE-like elements in a T-cell line and the coexpression of Tax M22 and Tax 703 can activate the AP-1 site [24]. We confirmed these results. Tax 703 activated the AP-1 site, but the levels were less than wild type Tax, and Tax M22 slightly activated it (Fig. 4C). The MMP-7 promoter has a canonical AP-1 site (5′-TGAGTCA-3′) located between −67 and −61 [25]. To determine the precise regions of the MMP-7 promoter necessary for Tax-mediated activation, AP-1 site deletion mutant was constructed (Fig. 4D). Deletion of the AP-1 site abolished the Tax-mediated activation of the MMP-7 promoter (Fig. 4D). In contrast, mutation of both the −194 Tcf site and the −109 Tcf site had no effect on the Tax-mediated activation of the MMP-7 promoter (Fig. 4E).

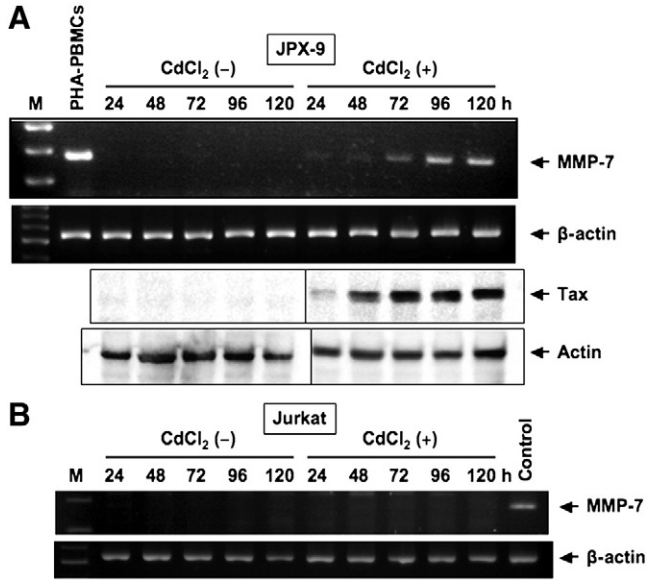

Fig. 3. Tax induces MMP-7 expression. JPX-9 cells (A) and Jurkat cells (B) were treated with or without 20 μM of $CdCl_2$ for the indicated time periods. RT-PCR was carried out for MMP-7 and β-actin (loading control). Control indicates MT-2 cells (positive control). Western blot analysis was also performed for Tax and actin (loading control).

3.3. HTLV-1 and Tax elicit JunD binding to the MMP-7 AP-1 element

In the next step, we determined whether HTLV-1 infection induced AP-1 binding to the AP-1 element in the MMP-7 promoter. EMSA was performed with double-stranded oligonucleotides representing the AP-1 element. Protein complexes bound to the AP-1 site were detected with nuclear extracts derived from all HTLV-1-infected T-cell lines examined, but not the control uninfected T-cell lines (Fig. 5A). Of note, no differences in binding to the octamer motif on DNA were noted in the absence or presence of HTLV-1 infection.

The specificity of DNA–protein complex formation was determined by competition studies with unlabeled competitors. As expected, a "cold" MMP-7 AP-1 double-stranded oligonucleotide or an AP-1 site from the IL-8 promoter effectively competed with the labeled probe and eliminated binding of nuclear extracts from SLB-1 cells in a dose-dependent manner (Fig. 5B). The unlabeled consensus NF-κB site from the IL-2Rα promoter could not compete with the labeled probe (Fig. 5C, lanes 1 and 4). The exact composition of the transcription factor DNA–protein complexes in HTLV-1-infected T-cells was ascertained by supershift analysis. Supershift reactions performed using SLB-1 nuclear extracts revealed that JunD was the predominant component of the AP-1 complexes in SLB-1 (Fig. 5C, lane 11).

Antibodies against c-Fos and c-Jun induced supershifted complexes in Jurkat cells stimulated by PMA plus ionomycin, using the AP-1 site of *MMP-7* gene as a probe (Fig. 5D, lanes 6 and 10). This indicated that at least c-Fos and c-Jun antibodies are also functional in this assay when the complexes contain sufficient amount of appropriate protein.

We also examined the binding activity to an AP-1 site in PBMCs. Protein complexes bound to the AP-1 element were detected in nuclear extracts from PBMCs stimulated with PHA, but not normal PBMCs (Fig. 6A). Antibodies against Fra-2 and JunD induced a supershifted complex in PHA-stimulated PBMCs (Fig. 6A, lanes 11 and 14). The AP-1 complexes were also detected in nuclear extracts from primary ATL cells (Fig. 6B). In all tested nuclear extracts, the addition of antibody against JunD induced a new slow-migrating complex as observed in SLB-1 cells (Fig. 6B, lanes 11, 16 and 21). In addition, antibody against c-Jun induced a weak supershifted complex in ATL 1 (Fig. 6B, lane 9).

We also observed that Tax expression alone elicited binding to the MMP-7 AP-1 probe in nuclear extracts from JPX-9 cells (Fig. 7A). Induction of Tax expression resulted in the formation of complexes with the MMP-7 probe within 48 h, but no differences in binding to the Oct-1 on DNA were noted in the absence or presence of $CdCl_2$. Supershift analyses performed with JPX-9 cells indicated that JunD was the major AP-1 factor induced after 72-h treatment with $CdCl_2$

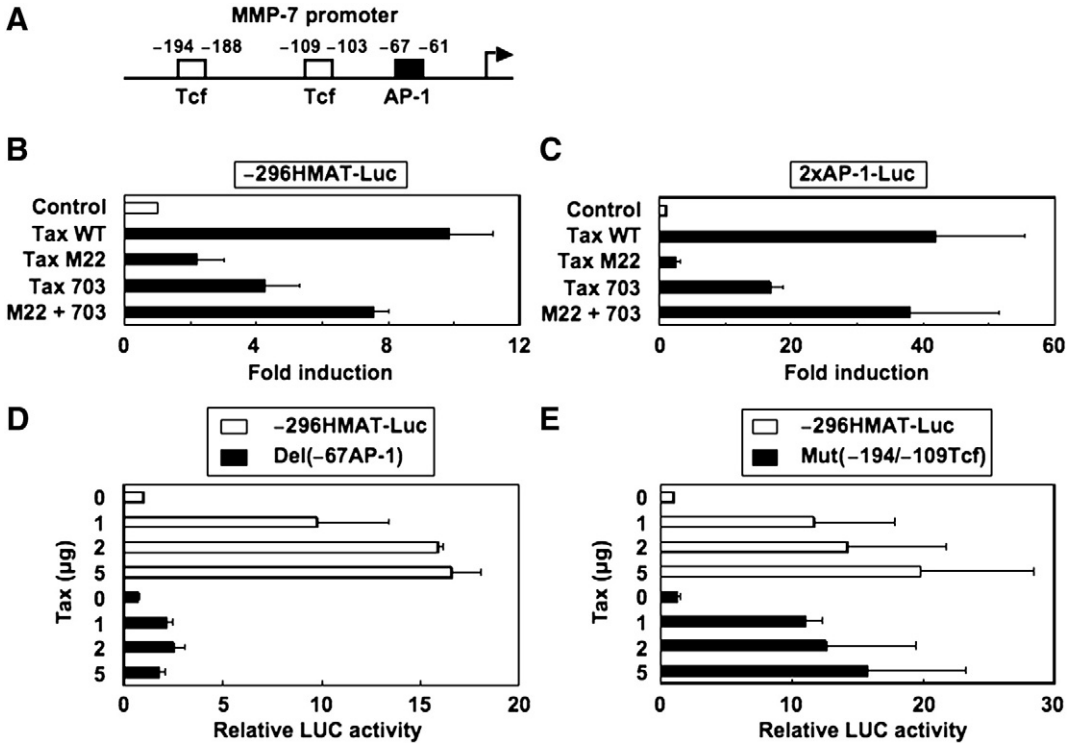

Fig. 4. Tax activates the MMP-7 promoter via AP-1. (A) A diagram of the human MMP-7 promoter is shown. Indicated are positions relative to the transcription start site of the two consensus Tcf binding sites as well as the position of the AP-1 site. Jurkat cells were transfected with either −296HMAT-Luc (B) or 2× AP-1-Luc (C) together with expression vector for HTLV-1 Tax (Tax WT), Tax M22, Tax 703 or empty vector alone or the combination of Tax M22 and Tax 703 using electroporation. Tax transactivation of the AP-1 site deletion (D) or the two Tcf sites mutation (E) of the MMP-7 promoter. The indicated promoter constructs were transfected in the presence of the indicated amounts of Tax. Cells were harvested 24 h post-transfection and luciferase activity was measured with a luminometer. The results are expressed as fold induction by Tax or Tax mutants relative to the vector alone (B and C) or expressed relative to activity of cells transfected with −296HMAT-Luc and empty vector, which was defined as 1 (D and E). Data are mean ± SD of three independent transfection experiments.

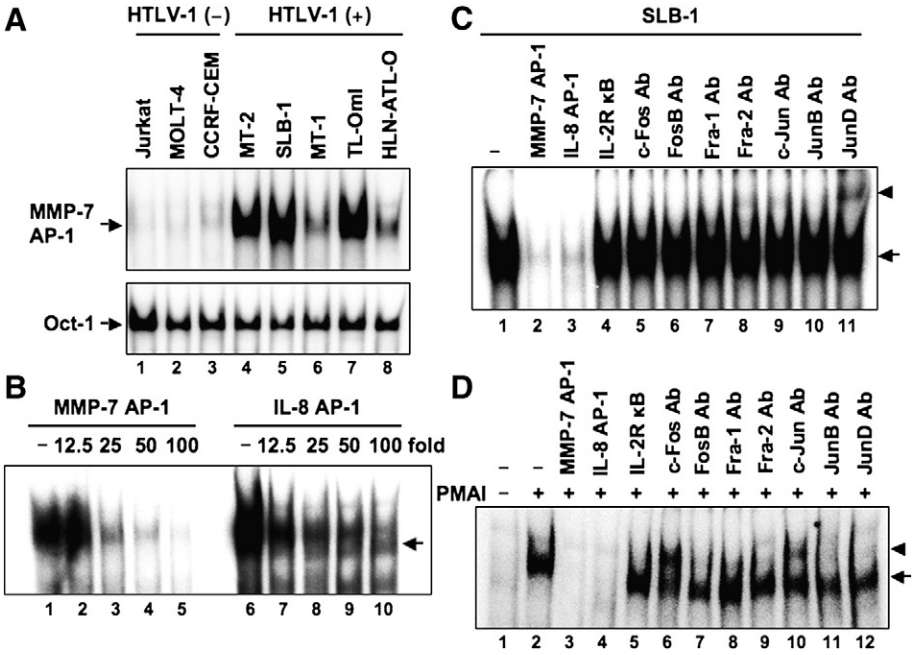

Fig. 5. HTLV-1 infection is associated with binding of JunD to the AP-1 site in MMP-7 promoter. (A) Nuclear extracts (5 μg) from control uninfected and HTLV-1-infected T-cell lines were incubated with the indicated labeled double-stranded DNA probes in EMSA reactions. (B) Nuclear extracts from SLB-1 cells were subjected to competition analysis with a 100-, 50-, 25-, 12.5-fold molar excess of unlabeled double-stranded oligonucleotides representing the MMP-7 AP-1 site (lanes 2–5) or an AP-1 site from the IL-8 promoter (lanes 7–10). (C and D) Nuclear extracts from SLB-1 cells (C) and Jurkat cells stimulated by PMA (20 ng/ml) plus ionomycin (2 μM) (PMAI) for 4 h (D) were subjected to competition analysis with a 100-fold molar excess of unlabeled double-stranded oligonucleotides representing the MMP-7 AP-1 site (lane 2 in C and lane 3 in D), an AP-1 site from the IL-8 promoter (lane 3 in C and lane 4 in D) or a consensus NF-κB site from the IL-2Rα promoter (lane 4 in C and lane 5 in D). The indicated unlabeled oligonucleotides were incubated with nuclear extracts for 15 min prior to binding reactions. Nuclear extracts were also subjected to supershift assays with either no antibody (lane 1 in C and lane 2 in D) or the indicated antibodies (Ab) (lanes 5–11 in C and lanes 6–12 in D). The antibodies were incubated with nuclear extracts for 45 min prior to binding reactions. Arrows: specific complexes, arrowhead: the DNA binding complex supershifted by the antibody.

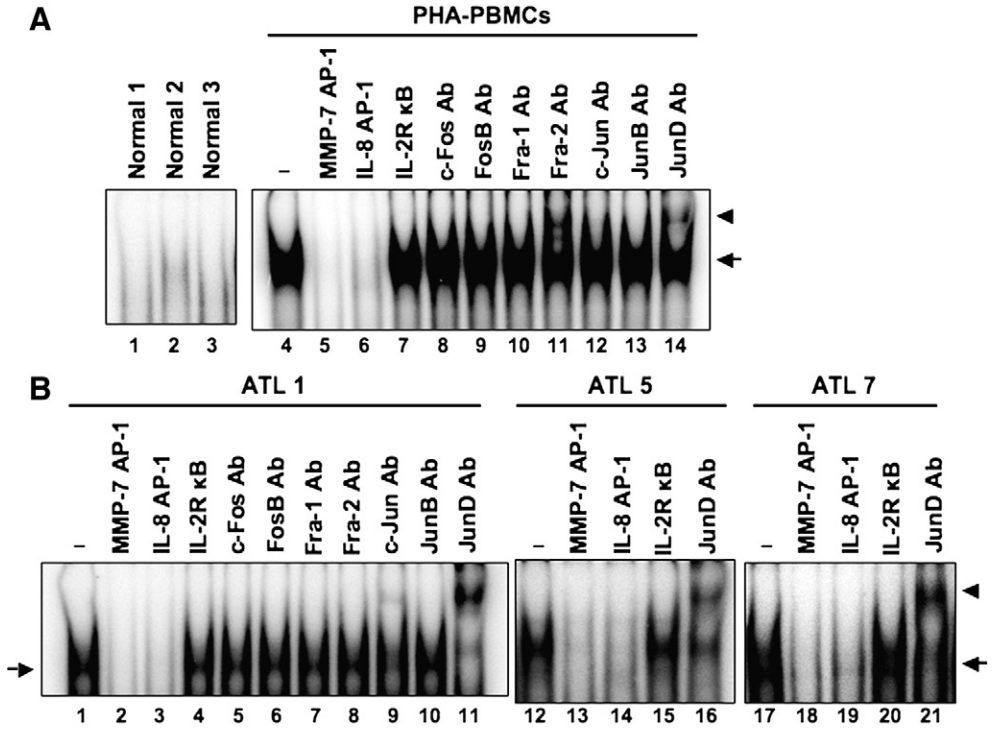

Fig. 6. Components of AP-1 in PBMCs stimulated with PHA and PBMCs from patients with ATL. Normal PBMCs were treated with PHA for 48 h (PHA-PBMCs). Nuclear extracts were prepared from PHA-PBMCs and PBMCs from 3 healthy volunteers (A) or 3 patients with ATL (B). The extracts from PHA-PBMCs and primary ATL cells were treated with antibodies (Ab) against the indicated AP-1 proteins and incubated with an AP-1 site probe derived from the *MMP-7* gene in the absence or presence of the indicated oligonucleotides. Arrows: specific complexes, arrowhead: the DNA binding complex supershifted by the antibody.

(Fig. 7B, lane 11). Considered together, these results suggest that HTLV-1 infection and Tax expression resulted in binding of JunD protein to the AP-1 element of the MMP-7 promoter.

3.4. JunD mediates MMP-7 activation by Tax

To confirm the participation of JunD in the induction of MMP-7 by Tax, we examined whether the activation of MMP-7 promoter could be altered by a dominant negative mutant JunD (DN-JunD). This DN-JunD protein lacks a functional transactivation domain [17]. Jurkat cells were cotransfected with the MMP-7 reporter construct and with either the DN-JunD expression vector or empty vector in the presence or absence of Tax expression vector. As shown in Fig. 8A, activation of the MMP-7 promoter by Tax was inhibited by DN-JunD. Furthermore, SLB-1 cells were cotransfected with the MMP-7 reporter construct and the DN-JunD expression vector. As shown in Fig. 8B, DN-JunD inhibited the MMP-7 promoter activity in a dose-dependent manner.

To further investigate the role of JunD protein in the Tax-mediated stimulation of MMP-7, Jurkat cells were cotransfected with the MMP-7 reporter construct and a vector (JunD/EB1) that drives the expression of a JunD protein in which the JunD dimerization domain is replaced with the dimerization domain from the Epstein–Barr virus EB1 transcription factor [19]. This mutant JunD protein can form transcriptionally active homodimers, but cannot form dimers with endogenous wild type JunD, Fra-2 or FosB. The expression of JunD/EB1 substantially enhanced MMP-7 promoter function, generating a 3-fold

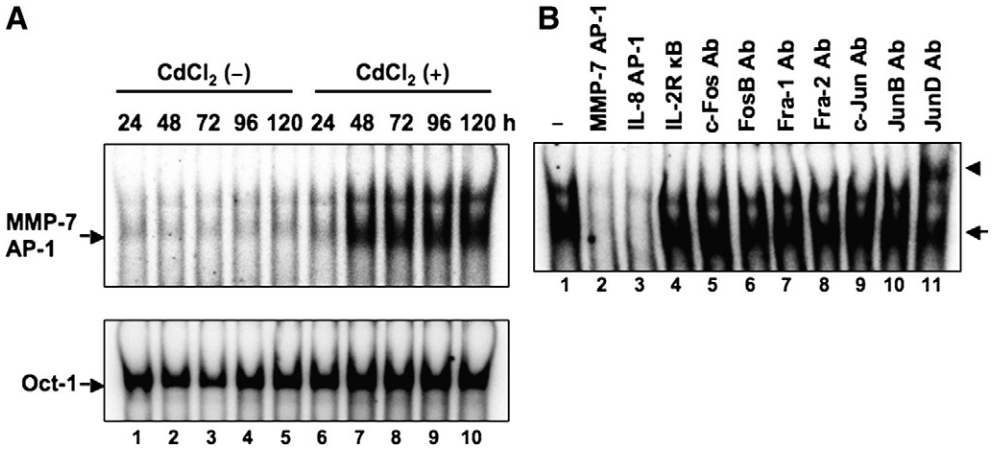

Fig. 7. Tax-induced AP-1 binding activity. (A) Nuclear extracts from JPX-9 cells treated with or without $CdCl_2$ (20 μM) for the indicated time periods were incubated with the indicated probes. (B) The extracts from JPX-9 cells treated with $CdCl_2$ for 72 h were treated with antibodies (Ab) against the indicated AP-1 proteins (lanes 5–11) and incubated with an AP-1 site probe derived from the *MMP-7* gene in the absence (lanes 1 and 5–11) or presence (lanes 2–4) of the indicated oligonucleotides. Arrows: specific complexes, arrowhead: the DNA binding complex supershifted by the antibody.

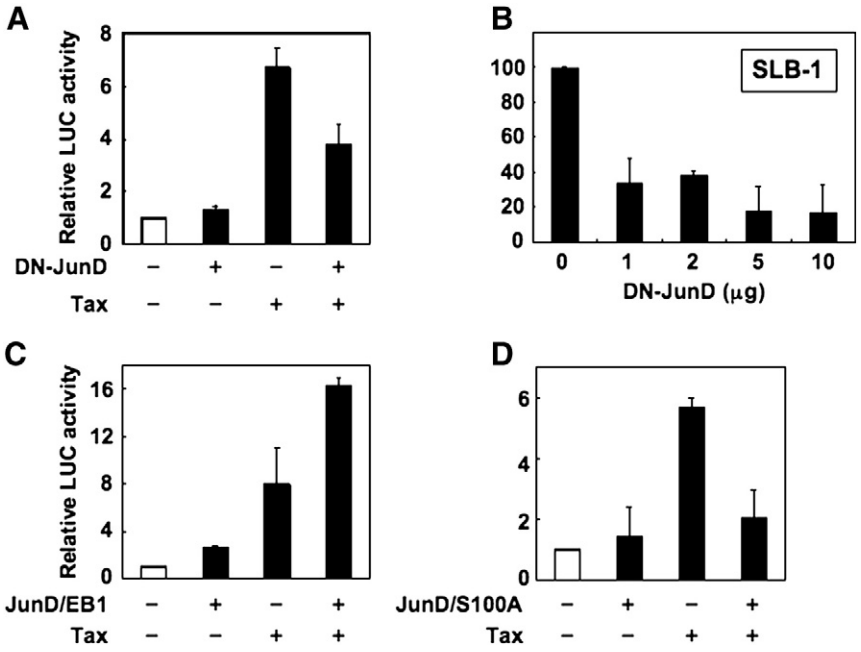

Fig. 8. JunD mediates Tax-induced MMP-7 activation. (A) Jurkat cells were cotransfected with the −296HMAT-Luc together with Tax expression, a dominant negative JunD expression (DN-JunD) or an empty vector. (B) SLB-1 cells were cotransfected with the −296HMAT-Luc together with various amounts of DN-JunD. (C) Jurkat cells were cotransfected with −296HMAT-Luc together with Tax expression vector, a pCMV5-derived vector carrying a JunD/EB1 fusion gene or an empty vector. (D) Jurkat cells were cotransfected with −296HMAT-Luc together with Tax expression, a JunD coding sequence carrying a Ser to Ala mutation at Ser100 (JunD/S100A) or an empty vector. Cells were harvested 24 h post-transfection and luciferase activity was measured with a luminometer. The results are expressed as fold induction by Tax or JunD mutants relative to the vector alone (A, C and D) or expressed relative to activity of cells transfected with −296HMAT-Luc alone, which was defined as 100 (B). Data are mean ± SD of three independent transfection experiments.

higher level of luciferase activity relative to cells transfected with a control empty expression vector (Fig. 8C). Furthermore, we examined whether JunD modifies the Tax-mediated MMP-7 promoter activity. The MMP-7 reporter construct was cotransfected with a JunD/EB1 expression vector in the presence of Tax coexpression. As expected, the expression of JunD/EB1 increased the activity of the promoter in the presence of Tax coexpression (Fig. 8C).

The transcriptional activity of JunD can be regulated by phosphorylation of Ser residues located in the NH_2-terminal domain [26,27]. Most notable in this regard are regulatory phosphorylations occurring on Ser90 and Ser100 within the transactivation domain [28]. Therefore, we next tested whether Tax induces the phosphorylation of the JunD protein in JPX-9 cells. Western blot analysis of the cell lysates identified the phospho-JunD protein in $CdCl_2$-treated JPX-9 cells (Fig. 9A). To further study the functional role of JunD Ser100 phosphorylation in the Tax-stimulated MMP-7 induction, a plasmid encoding a modified JunD protein, in which the Ser100 residue was replaced by Ala (JunD/S100A), was introduced in the presence or absence of Tax into Jurkat cells. Mutation of Ser to Ala in AP-1 proteins prevents phosphorylation of the mutated residue [29]. As shown in Fig. 8D, overexpression of JunD/S100A had no effect on the activity of the MMP-7 reporter construct. However, when cells were cotransfected with JunD/S100A and Tax, JunD/S100A protein inhibited the Tax-induced MMP-7 reporter construct activity.

Since JNK1/2 and ERK1/2 were reported previously to phosphorylate JunD [30], we examined whether JNK1/2 and ERK1/2 mediate the Tax stimulation of MMP-7. We therefore assessed JNK1/2 and ERK1/2 phosphorylation by Western blot analysis with antibodies that specifically recognize the phosphorylated Thr183 and Tyr185 of JNK1/2 and Thr202 and Tyr204 of ERK1/2. The results in Fig. 9A show a strong activation of JNK1/2 and ERK1/2 in JPX-9 cells treated with $CdCl_2$, as evidenced by strong phospho-c-Jun, JunD, JNK1/2 and ERK1/2 signals. The amount of total JNK1/2 and ERK1/2 remained the same regardless of treatment, as shown by the use of pan JNK1/2 and ERK1/2 antibodies, which recognize total JNK1/2 and ERK1/2. These findings indicate that Tax activates JNK1/2 and ERK1/2. We used SP600125, an inhibitor of JNK1/2 and PD98059, an inhibitor of mitogen-activated protein kinase/ERK kinase1/2, an upstream activator of ERK1/2. Treatment with SP600125 and PD98059 inhibited the phosphorylation of JunD in MT-2 cells. MMP-7 expression was also inhibited when the cells were treated with SP600125 and PD98059 (Fig. 9B). These results indicate that activation of JNK1/2 and ERK1/2 is required for MMP-7 expression. SP600125 and PD98059 are chemical inhibitors, and thus their target specificity may be questionable. Therefore, siRNA for JunD was used to examine the role of JunD activation in the signal transduction pathway leading to MMP-7 expression by Tax. siRNA specifically inhibited the expression of JunD at mRNA and protein levels (Fig. 9C). Fig. 9C also shows that MMP-7 mRNA expression was inhibited by siRNA for JunD, confirming that JunD is important in Tax-induced MMP-7 expression.

The functional significance of MMP-7 and JunD was finally examined using invasion assay. In this model, SLB-1 cells markedly invaded through Matrigel-coated plates compared with Jurkat cells (data not shown). A role for MMP-7 in invasion was shown by the inhibition of invasion by addition of neutralizing MMP-7 antibody in a dose-dependent manner (Fig. 10A). In contrast, normal goat IgG had no effect on SLB-1 cell invasion (Fig. 10A, bottom panel). JunD knockdown also caused a marked decrease in SLB-1 cell invasion (Fig. 10B), suggesting that JunD-mediated MMP-7 induction was involved in invasion.

4. Discussion

In the present study, we examined MMP-7 expression in HTLV-1-infected T-cell lines and primary ATL cells, and compared the expression level with that in uninfected T-cell lines and normal PBMCs. The results showed that HTLV-1-infected T-cell lines and ATL cells expressed significantly higher levels of MMP-7 than did any other cell. The results also showed that the viral oncoprotein Tax induced MMP-7 expression in T cells. The interest in the regulation of this MMP is based on its implications in cancer progression [31]. Based

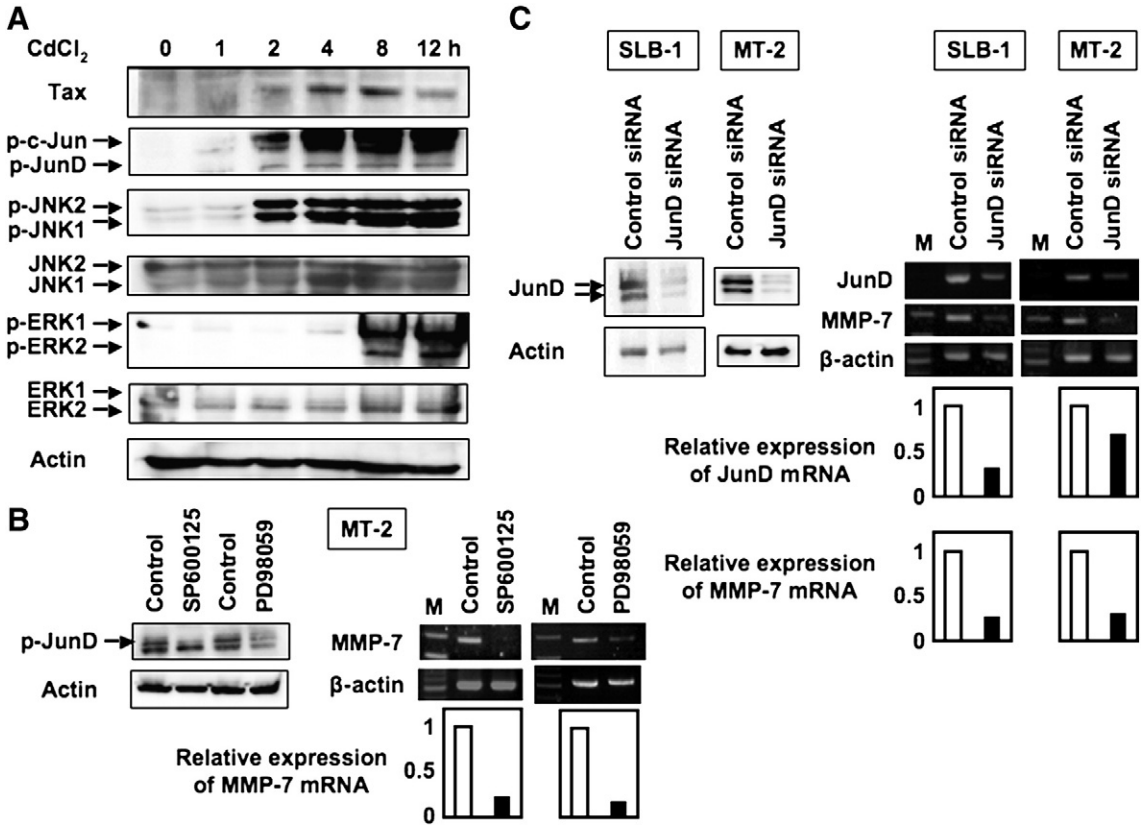

Fig. 9. Activation of JNK1/2 and ERK1/2 is required for MMP-7 expression. (A) Western blot of cell extracts from JPX-9 cells treated with 20 μM of $CdCl_2$ for the indicated time periods using the indicated antibodies. (B) MT-2 cells were treated with SP600125 (20 μM) or PD98059 (10 μM) for 48 h. Total RNA was isolated from each cell and the expression levels of MMP-7 and β-actin (loading control) mRNA were measured by RT-PCR (right panels). Histograms indicate the relative density data of MMP-7 mRNA by densitometric analysis of the bands shown in the top panel normalized to β-actin mRNA (bottom panel). Cell lysates were also analyzed by Western blot using the indicated antibodies (left panels). (C) RT-PCR analysis of JunD, MMP-7 and β-actin (loading control) mRNA expression levels 48 h after transfection with either siRNA for JunD or siCONTROL non-targeting siRNA pool (control) (right panels). Histograms indicate the relative density data of JunD and MMP-7 mRNA by densitometric analysis of the bands shown in the top and middle panels normalized to β-actin mRNA (bottom panel). JunD protein levels were determined by Western blot analysis (left panels).

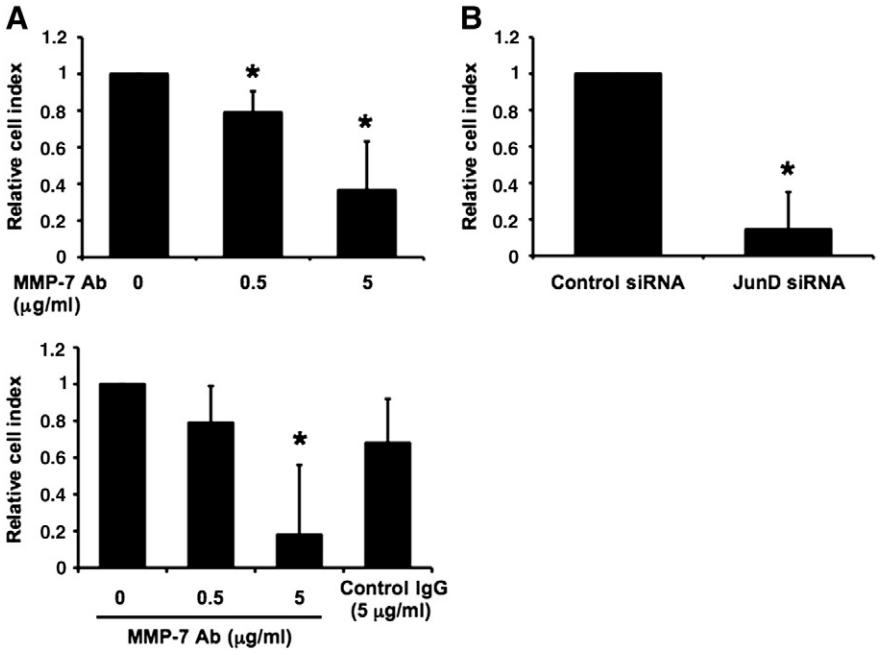

Fig. 10. Neutralization of MMP-7 using an MMP-7 polyclonal antibody (A) and JunD knockdown (B) inhibited SLB-1 cell invasion. (A) SLB-1 cells were seeded with or without a goat polyclonal MMP-7 neutralizing antibody (top and bottom panels) or with normal goat IgG (bottom panel) in the upper chamber for 24 h. (B) SLB-1 cells were transfected with either JunD or control siRNA and then incubated for 24 h. Thereafter, SLB-1 cells were seeded in the upper chamber for 24 h. Cell invasion was monitored using RT-CES system for SLB-1 cells. Data are mean ± SD of three independent invasion experiments in a period of 24 h. *$P<0.05$ vs. control.

on transfection studies, factors of Lef/Tcf family, Stat3, AP-1 and members of the ETS family of proteins are known to regulate *MMP-7* gene expression. For example, cotransfection of β-catenin, c-Jun and PEA3 ETS transcription factor induces promoter activity [20,32].

We also explored the mechanism of Tax-mediated MMP-7 expression in T cells. Induction of high levels of MMP-7 promoter activity by Tax was dependent on an intact AP-1 site. Tax can activate AP-1-responsive genes with different function. Unlike the wild type Tax, neither of the mutant M22 and 703 was able to fully activate the promoter, suggesting that either NF-κB or CREB pathway is insufficient for the transactivation of MMP-7 promoter. Recently, Tax has been reported to transactivate the osteopontin promoter through AP-1 site [33]. In this case, Tax-mediated osteopontin activation was also partially abrogated either by mutation introduced in NF-κB or CREB pathway. Thus, Tax could indirectly control the expression of AP-1-responsive genes through NF-κB or CREB dependent AP-1 activation. Tax induced binding of JunD to the AP-1 element of the MMP-7 promoter. It has been previously reported that JunD is the predominant Jun family protein expressed in HTLV-1-infected T-cell lines and primary ATL cells [34]. In this study, we provide evidence that JunD functions in T cells to regulate the transcription of *MMP-7* gene.

We demonstrated that overexpression of the homodimeric JunD/EB1 enhanced MMP-7 promoter activity in the absence or presence of Tax; in contrast, the expression of a dominant negative JunD protein in Jurkat cells inhibited MMP-7 promoter function in the presence of Tax. We also observed that a siRNA for JunD could substantially reduce MMP-7 expression in HTLV-1-infected T-cell lines. Taken together, these data indicate that JunD is a vital component of the transcriptional machinery that regulates MMP-7 expression in HTLV-1-infected T cells.

The NH_2-terminus of JunD contains 3 mitogen-activated protein kinase target residues (Ser90, Ser100 and Thr117) phosphorylated by both the JNK1/2 and the ERK1/2 pathways [18,30,35]. The transcriptional activity of JunD can be regulated by phosphorylation of Ser residues [26,27]. In the present study, Tax activated JNK1/2 and ERK1/2, leading to phosphorylation of Ser100 in the JunD protein. Phosphorylation of JunD and expression of MMP-7 could be reduced by inhibitors of JNK1/2 and ERK1/2 in an HTLV-1-infected T-cell line. JunD phosphorylation at Ser100 was necessary for stimulation of Tax-mediated *MMP-7* gene transcription. We therefore propose that Tax-mediated *MMP-7* gene transcription in T cells requires both JNK1/2 and ERK1/2 phosphorylation of JunD.

The AP-1 complex induced by Tax and in an HTLV-1-infected T-cell line contained JunD, but the antibody against JunD did not completely induce the supershifted complex. Therefore, the involvement of transcription factors other than JunD is likely to be essential for the transcription of *MMP-7* gene through an AP-1 site. The activating transcription factor (ATF) family represents a large group of basic-region leucine zipper transcription factors. ATF family members form homodimers or heterodimers with other basic-region leucine zipper containing proteins such as the AP-1 [36]. We are investigating the involvement of ATF family members in Tax-induced MMP-7 transcription.

It is known that Tax expression diminishes after the early phase of infection in human, probably due to immune surveillance. It is possible that MMP-7 induction persists after the initial Tax activation. Indeed, Tax-negative ATL-derived T-cell lines, MT-1 and TL-OmI, and primary ATL cells expressed MMP-7, suggesting that Tax does not fully explain ATL pathobiology. Thus, cells no longer expressing Tax continue to show constitutively activated AP-1, resulting in aberrant MMP-7 expression.

The HTLV-1 basic leucine zipper factor gene *HBZ* is located in the 3′ portion of the provirus and is maintained in ATL cells [37,38]. HBZ plays an important role in HTLV-1 infectivity and persistence [39]. HBZ heterodimerizes with either JunD or JunB and transactivates expression of AP-1 target genes [40]. JunD/HBZ heterodimers may induce upregulation of MMP-7 expression in ATL.

What would HTLV-1 gain from upregulation of MMP-7 expression? It is possible that HTLV-1-induced activation of MMPs enhances the spread of the virus or virus-infected cells and ATL cells, through the actions of these proteins on reducing the matrix barrier of tissues. In other words, MMP-7 may underlie the invasive nature of ATL. A role for MMP-7 in invasion was shown by the inhibition of SLB-1 cell invasion by addition of neutralizing MMP-7 antibody.

In summary, we reported in this study that HTLV-1 Tax induces MMP-7 expression in T cells and that this process is regulated in an AP-1-dependent fashion. The *in vivo* relationship between disseminated ATL organ involvement and MMP-7 expression remains an interesting question awaiting further research.

Acknowledgments

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