RESEARCH ARTICLE

Tax is Involved in Up-regulation of HMGB1 Expression Levels by Interaction with C/EBP

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Abstract

The high mobility group box 1 (HMGB1) protein is a multifunctional cytokine-like molecule that plays an important role in the pathogenesis of tumors. In this study, real-time polymerase chain reactions and Western blot assays indicated that HMGB1 transcriptional activity and protein level are increased in Tax$^+$-T cells (TaxP). To clarify the mechanisms, a series of HMGB1 deletion reporter plasmids (pHLuc1 to pHLuc6) were transfected into Tax$^-$-T cells (TaxN, Jurkat) and Tax$^+$-T cells (TaxP). We found that promoter activity in Tax$^+$-T cells to be higher than that in Tax$^-$-T cells, indicating a significant increase in pHLuc6. Bay11-7082 (NF-κB inhibitor) treatment did not block the enhancing effect. Chromatin immunoprecipitation assays revealed that Tax was retained on a HMGB1 promoter fragment encompassing -1163 to -975. Bioinformatics analysis showed six characteristic *cis*-elements for CdxA, AP-1, AML-1a, USF, v-Myb, and C/EBP in the fragment in question. Mutation of *cis*-elements for C/EBP reduced significant HMGB1 promoter activity induced by Tax. These findings indicate that Tax enhances the expression of HMGB1 gene at the transcriptional level, possibly by interacting with C/EBP.

Keywords: Human HMGB1 promoter - Tax - transcriptional regulation - C/EBP

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Introduction

Adult T cell leukemia (ATL), an aggressive T-cell malignancy without an effective cure (Yoshida et al., 1982), is found to be caused by human T-cell leukemia virus type I (HTLV-I) infection and occurs in HTLV-I-endemic areas, such as southwestern Japan, Central and South America, the Caribbean islands, Middle East, Aboriginal regions in Australia, and Intertropical Africa (Uchiyama et al., 1977; Vidal et al., 1994). The prognosis is poor, with less than 1 year survival for patients (Goncalves et al., 2010). However, investigators have also shown that low prevalence of HTLV-1 infection in Iranian gastric cancer patients in comparison to controls (Tahaei et al., 2011). HTLV-1 encodes Tax protein that participates in viral replication, viral infectivity, persistence and transformation (Kannian and Green, 2010). Oncoprotein Tax exhibits diverse functions in host cells, resulting in persistent activation of NF-κB and deregulation of its responsive gene expressions for T cell survival (Patrick et al., 2001; Easley et al., 2010; Alfonso et al., 2012). Tax protein predominantly localizes to the nucleus, However, Tax alone does not bind DNA; moreover, it functions as a transcriptional activator through interactions between the Tax and a large array of transcriptional regulators to regulate cellular processes (Boxus et al., 2008). Notably, the viral-transforming protein Tax is an important part of the oncogenic mechanism of HTLV-I in enhancing transcription by interacting with transcriptional factors.

High mobility group box 1 protein (HMGB1), a chromatin-binding nuclear protein and damage-associated molecular pattern molecule, is a multifunctional cytokine-like molecule that has a critical role in the regulation of transcription. Various studies have shown that HMGB1 has pleiotropic effects outside and inside cells. In the nucleus, HMGB1 functions as a non-histone nucleosomal protein that binds DNA, contributes to stabilization of nucleosomes, and promotes DNA repair and replication (Bustin, 1999). In the extracellular milieu, HMGB1 is a pro-inflammatory cytokine that acts as an alarmin via passive release from damaged or necrotic cells (Beyer et al., 2012; He et al., 2012; Yi et al., 2013) or by active secretion from innate immune system cells in response to LPS, TNF-a or IL-1β stimulation to induce T-cell activation, cytokine production, and inflammatory responses by the transduction of cellular signals through its receptors (Akirav et al., 2012; Kang et al., 2013; Mohammad et al., 2013). Aside from these pro-inflammatory functions, HMGB1 protein also promotes regeneration processes and accelerates cell cycle progression. This paradoxical

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function of HMGB1 protein has been revealed in the growth and spread of many types of tumors such as hepatocellular carcinoma, colon cancer, breast cancer, and leukemia (Kostova et al., 2010; Ohmori et al., 2011; Jube et al., 2012; Lee et al., 2012; Liu et al., 2012; Xing et al., 2012; Yu et al., 2012; Stoetzer et al., 2013). Thus, HMGB1 has become the focus of recent cancer research and is currently a relevant target for cancer treatment (Ohmori et al., 2011; Yu et al., 2012).

Some studies have demonstrated that HMGB1 protein binds virus nucleoprotein, modulates viral replication, and promotes retrovirus HIV dissemination and latency, which indicate that the alarmin HMGB1 contributes to the activation of the immune system (Barqasho et al., 2010; Marius et al., 2011; Moisy et al., 2012; Piotr et al., 2012). However, the pathogenic mechanisms for persistent immune activation remain unknown. HTLV-I, similar to HIV retrovirus, is the etiological agent of an aggressive malignancy of the $CD4^+$ T-cells. Its virally encoded Tax oncoprotein is a key factor in HTLV-I pathogenicity by up-regulation of its responsive gene expressions. To date, the role of HMGB1 in the pathogenesis of HTLV-I infection has not been clearly defined. In this work, we demonstrated that HTLV-I virally encoded Tax protein enhanced HMGB1 transcription levels, and that Bay11-7082 (NF-κB inhibitor) did not repress Tax-mediated transcription activation of the HMGB1 gene. Tax protein was recruited at the -1103 HMGB1 site. In order to elucidate further the association of Tax and HMGB1, and the involvement of putative transcription factor-binding motifs targeted by Tax in the regulation of the HMGB1 promoter activity, site-directed mutation was introduced into these putative ciselements within the pHLuc6 (-1163/+83). Mutation in the C/EBP-motif reduced the Luc activity to 52% of the pHLuc6 in TaxP cells. Taken together, our results revealed that Tax protein, a transcription regulator, subtly regulated HMGB1 transcription by interacting with transcription factor C/BEP.

Materials and Methods

Reagents

Plasmid pGL3-neo-luc was donated kindly by Prof. Guoqiang Zhao of Zhengzhou University, pCMV-Tax and pCMV-Neo were from Dr. Sho ji Yamaoka of Kyoto University, Tax M22 and Tax M47 were given by Prof. Edward W. Harhaj of Miami University, and HMGB1 reporter vectors (pHLuc1 to pHLuc6) were constructed and preserved in the immunity research center of Xinxiang Medical University. RNAiso Plus and reverse transcriptase M-MLV (RNase H-) were purchased from Takara (Dalian, China). Plasmid pSV-β-galactosidase, β-galactosidase enzyme assay system, reporter gene detection kit, and GoTaq® qPCR master mix were purchased from Promega (USA). Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) and mouse anti-beta actin monoclonal antibody were purchased from Zhongshan Golden Bridge (Beijing, China). The anti-Tax mouse monoclonal antibodies used in the chromatin immunoprecipitation (ChIP) assay and Western blot assay were bought from ProSpec (Israel) and Santa Cruz (USA), respectively. The anti-HMGB1 mouse monoclonal antibody was bought from Abcam (USA). Lipofectamine 2000, G418, and RPMI 1640 medium were purchased from Invitrogen (USA). BAY11-7082 and all reagents used in Western blot were obtained from Beyotime (Shanghai, China). The ChIP kit was bought from Millipore (USA). The pHLuc6 mutants were processed by Sangon (Shanghai, China).

Cell culture, transfections and luciferase (Luc) assays

Jurkat cells were purchased from ACTT (USA). pCMV-Tax and pCMV-Neo were stably transduced into Jurkat cells and were selected with 600 μg/ml G418 to obtain TaxP and TaxN cells, respectively. TaxP and TaxN cells were preserved in the immunity research center of Xinxiang Medical University. All cells were cultured in RPMI 1640 medium supplemented with FBS (10%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (10 mM), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and L-glutamine (2 mM) in a humidified atmosphere incubator with 5% CO_2. For Luc assays, Jurkat, TaxP, and TaxN cells were each plated at 5×10^5 cells per well on 24-well plates and transfected with 0.2 μg reporter plasmids (pHLuc1 to pHLuc6); Jurkat cells were cotransfected with 0.2 μg reporter plasmids (pHLuc1 to pHLuc6) together with 0.3 μg expression vectors (pCMV-Tax or its mutants) or with increasing doses of pCMV-Tax vectors. For Western blot assay, Jurkat cells were plated at 2×10^6 cells per well on 6-well plates and transfected with 1.2 μg expression vectors (pCMV-Tax or its mutants) or cotransfected with 0.8 μg reporter plasmid pHLuc6 together with increasing doses of pCMV-Tax vectors. For reverse transcription PCR assay, Jurkat cells were transfected with each 0.3 μg expression vectors (pCMV-Tax and its mutants) and 0.3 μg empty vector (pCMV-Neo). The amount of transfected DNA was equalized by the addition of empty vector (PCMV-Neo or pGL3-neo-luc). After 24 hours, cells were harvested and lysed with reporter lysis buffer, and Luc activity in the resulting supernatants was measured using a 20/20n luminometer (Turner BioSystems, USA) according to the manufacturer's instructions. All cells were cotransfected with pSV-β-gal as a control for transfection efficiency, and galactosidase activity was measured using a β-galactosidase enzyme assay system.

RNA extraction and cDNA synthesis

Total RNA from TaxP and TaxN cells, and Jurkat cells after transient transfection with pCMV-Neo, pCMV-Tax, and two mutants (M22, M47), was extracted using RNAiso Plus reagent according to the manufacturer's instructions. The extracted RNA was dissolved in diethylpyrocarbonate-treated water. The quality and concentration of RNA were verified using denaturing gel electrophoresis and by determining the A260/A280 absorbance ratio. Total RNA was then reversely transcribed in a total volume of 10 μL, containing 0.5 μL of 10 mM dNTP, 1 μL of 50 μM Oligo d(T)18 Primers, 0.25 μL of 40 U/μL RNase Inhibitor, 1 μL of 200 U/μl RTase M-MLV ($RNase\ H^-$), 2 μL of 5×M-MLV buffer, and 1 μg of RNA. The response conditions were as follows: 70 °C for 10 min, ice for 2 min, 42 °C for 60 min, 70 °C for 10 min, and 4 °C for 5 min. The first-strand

cDNA was synthesized and stored at -20 °C.

Reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Primers for the human HMGB1 promoter and GAPDH were synthesized by Invitrogen. The sequences of the primers were as follows: 5'-GCGACTCTGTGCCTCGCTGA-3' (sense), 5'-ACATGGTCTTCCACCTCTCTGAGCA-3' (anti-sense) for HMGB1 and 5'-TCAACAGCGACACCCACTCC-3' (sense), 5'-TGAGGTCCACCACCCTGTTG-3' (anti-sense) for GAPDH. RT-PCR parameters were 30 cycles of 94 °C for 1 min, 57 °C for 30 s, and 72 °C for 1 min, after which an additional extension step at 72 °C for 10 min was performed. After the amplification protocol was completed, 10 μL aliquots of the PCR products were subjected to electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, photographed, and then scanned using Band Leader software for gray-scale semi-quantitative analysis. Subsequently, qRT-PCR amplification was performed by using GoTaq® qPCR master mix with cycling conditions of 94 °C for 2 s, 57 °C for 10 s, and 72 °C for 10 s. After the amplification protocol was completed, the PCR product was subjected to melting curve analysis to identify primer dimer formation. Relative gene expression data were calculated using the $2^{-\Delta\Delta CT}$ method. All samples were measured in triplicate.

Western blot

According to the manufacturer's instructions, the protein was extracted with ice-cold radioimmunoprecipitation assay buffer supplemented with 1 mM phenylmethylsulfonyl fluoride for 30 min, sonicated with 4 to 5 sets of 1 s pulses on wet ice using a 100 watt-model high-intensity ultrasonic processor/sonicator, and then boiled for 10 min. The lysates were separated by electrophoresis on polyacrylamide gels containing 8% sodium dodecyl sulfate (SDS) and then transferred to the nitrocellulose membranes. The membranes were blocked with blocking buffer and incubated with anti-HMGB1 (1:1000), anti-Tax (1:2000) and mouse anti-beta actin monoclonal antibody (1:2000). This procedure was followed by incubation with anti-mouse IgG (H+L). Membranes were exposed to X-ray film, and each specific band that recorded the targeted proteins was quantified and analyzed by the Band Scan 5.0 system.

ChIP

TaxP cells (1×10^7) were collected and fixed with 1% formaldehyde to crosslink proteins to DNA for 10 min, then quenched with 10×glycine for 5 min. After washing with ice-cold phosphate buffered saline, cells were resuspended in SDS lysis buffer. Then, the chromatin was sheared to a manageable size with 5 sets of 10 s pulses on wet ice using a 100 watt-model high-intensity ultrasonic processor/sonicator. ChIPs were performed using the ChIP kit. Protein–DNA complexes were immunoprecipitated with 6 μg anti-Tax antibody overnight followed by protein G-conjugated agarose beads. Then, protein/DNA complexes were reversed by incubating samples at 65 °C overnight for 12 h. Associated DNA were eluted and purified according to the manufacturer's instructions, and sequences of the HMGB1 target gene and the negative control (HM-mock) were analyzed by PCR and qRT-PCR using specific primers. The primers were as follows: 5'-CCACTACAGCCAGCACATTT-3' (sense), 5'-TAGATGCAGGCAGAGAGTGC-3' (anti-sense) for HMGB1 target gene and 5'-TTCTGGGGAGGGTCTTTTCT-3' (sense), 5'-GAATCCATTCTGCCTTTCCA-3' (anti-sense) for HM-mock. One microliter of precipitated and purified DNA was subjected to standard PCR, and DNA fragment sizes were analyzed by 2% agarose gel electrophoresis. The detection of specific DNA sequences was performed by qRT-PCR analysis of ChIPed samples using the GoTaq® qPCR master mix. The HMGB1 gene site bound by Tax protein was determined by qRT-PCR analysis relative to the Tax signal at the HM-mock site. The $2^{CT(Input)-CT(IP)}$ value at each site targeted by Tax was normalized to that at HM-mock, which was set to 1.

Statistical analysis

Data were representative of at least three independent experiments and expressed as mean±SD with one-way ANOVA. An unpaired Student's t-test was used to evaluate significant differences between groups. A value $p<0.05$ was considered statistically significant.

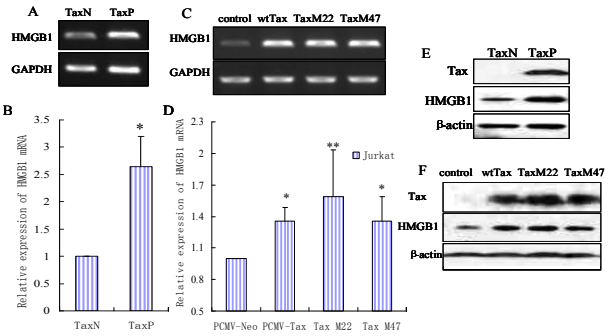

Figure 1. Tax Protein Induces HMGB1 Expression in Different T Cells. (A and C) Results from conventional RT-PCR; (B and D) results from real-time RT-PCR; (E and F) results from Western blot. (A and B) According to the manufacturer's protocol, 5×10^6 cells (TaxN, TaxP) were directly collected, after which total RNA was extracted. Then, total RNA was reversely transcribed into cDNA to conduct RT-PCR and real-time PCR, respectively. HMGB1 expression level relative to a calibrator was calculated using the formula $2^{-\Delta\Delta CT}$. A value of 1 indicates that the expression level is equivalent to calibrator sample. Value was normalized to the expression of GAPDH gene. Data represent mean±S.D. of three different analyses. Error bars indicate SD. (C and D) Jurkat lymphocytes were transiently transfected with pCMV-Neo (control group), pCMV-Tax, and two mutants (M22, M47). After 24 h, total RNA was reversely transcribed into cDNA to conduct RT-PCR and real-time PCR, respectively. A value of 1 indicates that the expression level is equivalent to calibrator sample. Values were normalized to the expression of GAPDH gene. Data represent mean±S.D. of three different analyses. Error bars indicate SD. (E and F) To analyze the expression level of HMGB1 protein in TaxN and TaxP cells, and Jurkat cells after transient transfection with pCMV-Neo, pCMV-Tax, and two mutants (M22, M47), Western blot analysis was performer using anti-HMGB1. β-actin was used as a control. Compared with the control group (TaxN or pCMV-Neo), *denotes $p<0.05$ and **denotes $p<0.01$

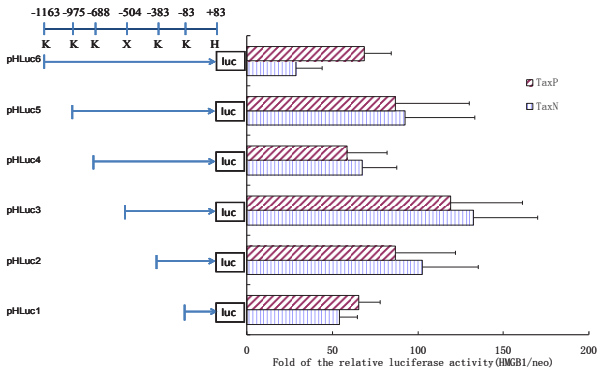

Figure 2. Schematic Representation of Luciferase Reporter Vectors and Relative Luc Activity of the HMGB1 Gene in Different T Cells. The HMGB1 gene regulatory sequences 166 bp (-83 to +83), 466 bp (-383 to +83), 587 bp (-504 to +83), 771 bp (-688 to +83), 1058 bp (-975 to +83), and 1246 bp (-1163 to +83) were inserted upstream of reporter gene pGL3-neo-luc to form pGL3-HMGB1-luc (pHLuc1 to pHLuc6) according to the length of the HMGB1 regulation gene (short to long). Relevant restriction enzyme sites are shown: H, HindIII; X, XhoI; K, kpnI. All Luc reporter constructs (pHLuc1 to pHLuc6) and pGL3-neo-luc were transiently transfected into TaxN and TaxP cells for 24 h, along with the β-galactosidase reporter gene (pSV-β-gal) as an internal control. Then, the cells were lysed with reporter lysis buffer and centrifuged at 12,000×g for 2 min. Luc activity was assayed according to the manufacturer's instructions. The ratio (HMGB1/neo) of the Luc activity obtained from each construct was plotted as bars against each construct from different T cells. The extent of deletion was indicated in the name of the constructions

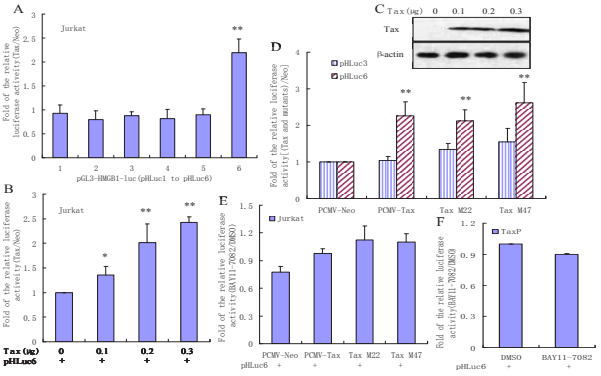

Figure 3. The Effect of Tax on HMGB1 Gene in Jurkat Cells and BAY11-7082 (NF-κB inhibitor) on the Transcription of HMGB1 Gene. Representative results of Luc assay as relative percentages are shown with mean±S.D. of triplicate experiments. (A) Jurkat cells were transiently cotransfected with pCMV-Tax (pCMV-Neo as the control) and pGL3-HMGB1-luc (pHLuc1 to pHLuc6). After 24 h, the cells were lysed for Luc activity and relative Luc activity (Tax/Neo). The ratio of the Luc activity of pCMV-Tax and pCMV-Neo is shown in bars (bars 1 to 6). (B and C) pHLuc6 were transiently cotransfected into Jurkat cells with increasing doses of Tax expression vectors. Relative Luc activity is shown in bars, in which the Tax- group is set to 1 and no standard deviation is shown (B). Protein was extracted from cells transfected with increasing doses of Tax vectors and subjected to Western blot assay with anti-HMGB1, β-actin as a control (C). (D) Jurkat cells were transiently transfected with pCMV-Neo (the control), pCMV-Tax, and two mutants (M22, M47), together with each plasmid pHLuc3 and pHLuc6, containing -504 to +83 HMGB1 fragment and -1163 to +83 HMGB1 fragment, respectively. After 24 h, Luc was measured. The Luc ratio of pCMV-Tax or its mutants and pCMV-Neo was calculated, in which pCMV-Neo activity is set at 1 and no standard deviation is shown. (E) Jurkat cells were transiently cotransfected with pCMV-Neo, pCMV-Tax, and two mutants (M22, M47), together with pHLuc6. After 24 h, the cells were treated with 4 μmol/L BAY11-7082 (NF-κB inhibitor) for 12 h. Its counterpart with DMSO was used as a control. The cells were then lysed, Luc was detected, and relative Luc (BAY11-7082/DMSO) was assayed. (F) TaxP cells transfected with pHLuc6 were treated with 4 μmol/L BAY11-7082 for 12 h. Its counterpart with DMSO was used as a control. The cells were lysed and the relative Luc (BAY11-7082/DMSO) was assayed, in which DMSO group is set at 1 and no standard deviation is shown. Error bars indicate SD. Compared with its control group, *denotes $p<0.05$ and **denotes $p<0.01$

Results

Tax protein enhances HMGB1 transcription levels and promotes the synthesis of HMGB1 protein

Previous study has demonstrated that Tax protein may interact with some transcription factors and/or other transcriptional regulators and influence gene expression (Boxus et al., 2008). To elucidate whether Tax inflences HMGB1 expression in the $CD4^+$ T-cells, in the current study, we directly extracted total RNA and protein from Tax^+-T cells (TaxP) and Tax^--T cells (TaxN) by using RT-PCR, qRT-PCR, and Western blot assays. We found that HMGB1 transcriptional activity increased by 2–3-fold and HMGB1 protein also significantly increased in TaxP cells (Figure 1). Additionally, the results of transient transfection with Tax and its mutations also showed that Tax protein induced HMGB1 expression in Jurkat cells.

The regulation of HMGB1 transcription in Tax^+ and Tax^--T cells

As shown in the above results, HMGB1 expression increased in Tax^+-T cells. To clarify the basal promoter activity of the human HMGB1 gene and observe how Tax affects the regulation of HMGB1 transcription in T lymphocytes, the available HMGB1 regulatory genes were subcloned upstream of the reporter gene pGL3-neo-luc to construct a series of deletion reporter vectors (pHLuc1 to pHLuc6) (Figure 2). Using transient transfection with reporter vectors (pHLuc1 to pHLuc6) and pGL3-neo-luc into Tax^--T cells (TaxN) and Tax^+-T cells (TaxP), we found that the regulation trend of HMGB1 gene was slightly similar, but not identical. We also observed the maximal promoter activity in pHLuc3 containing -504 to +83 fragment. Interestingly, a significant increase in Tax^+-T cells was observed in the pHLuc6 group (Figure 2). These findings suggested that the up-regulation expression of HMGB1 gene in pHLuc6 was responsible for the interaction of Tax protein and some transcriptional regulators.

Tax mediates HMGB1 transcription via non-NF-κB pathway

The above analysis indicated that HMGB1 transcription activity from reporter vector pHLuc6 in Tax^+-T cells was higher than that in Tax^--T cells. To further confirm the involvement of Tax in HMGB1 promoter activation, we transiently cotransfected pHLuc1 to pHLuc6 individually

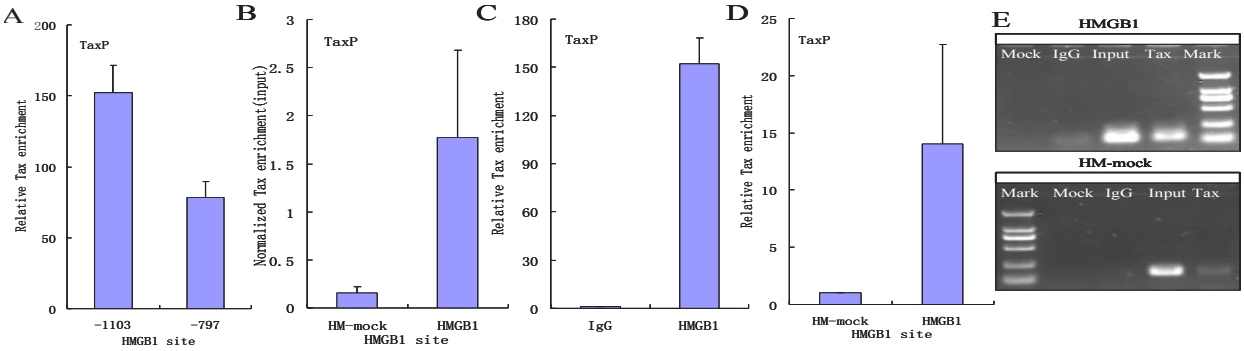

Figure 4. The Levels of Tax Enrichment at HMGB1 Gene Sites. (A) The levels of Tax enrichment at HMGB1 gene sites centered at -1103 and -797 were determined by qRT-PCR analysis of ChIPed samples from TaxP cells. For each ChIP assay, the $2^{CT(Input)-CT(IP)}$ values from each amplicon of a ChIP were multiplied by the normalization factor for that ChIP. Fold enrichment ($2^{-\Delta\Delta CT}$) values for the two sites were added. (B) Recruitment of Tax to HMGB1 gene was assessed by comparing level of Tax enrichment at HM-mock site. For each ChIP assay, Adjusted Averaged Input [$100\times2^{CT(Input)-CT(IP)}$] data are shown in bars. (C and D) Enrichment signals for Tax protein at HMGB1 gene were compared with those of the negative control. Fold enrichment ($2^{-\Delta\Delta Ct}$) values obtained at the HMGB1 site were normalized to the value obtained at the HM-mock site (D) and IgG (C). No standard deviation was shown for HM-mock and IgG, as values were set to 1 to normalize data among ChIP assays. (E) PCR analysis of ChIPed samples showed specific Tax protein enrichment at the HMGB1 gene site. Coimmunoprecipitation using normal mouse IgG and amplification of HM-mock site were performed as negative control. Each panel shows amplification of 1% total input chromatin (Input). The graph shows the data averaged from three independent ChIP assays. Error bars show standard deviations

with pCMV-Tax. The results of relative Luc activity (Tax/Neo) showed that HMGB1 transcription activity increased by 2–3-fold in the pHLuc6 group. By contrast, minimal effect was observed in the other pHLuc groups (Figure 3A). To further examine this result, pHLuc6 was cotransfected into Jurkat cells with increasing doses of Tax expression plasmids. As shown in Figs. 3B and 3C, the co-expression of Tax significantly increased HMGB1 promoter activity in a dose-dependent manner.

Previous studies have demonstrated that nuclear factor NF-κB and CREB/ATF activation have a critical function in the oncogenesis of ATL by HTLV-I, and its viral protein Tax is indispensable for maintenance of the malignant phenotype by transactivation through the CREB/ATF or NF-κB pathway (Patrick et al., 2001; Alfonso et al., 2012). Tax mutants (M22, M47) are inactive in the CREB/ATF (M47) or NF-κB (M22) pathway (Kwon et al., 2005). To determine whether Tax alters the level of HMGB1 via CREB/ATF or NF-κB pathway in T lymphocytes, we transiently transfected pCMV-Neo (empty vector), pCMV-Tax (expression vector), and Tax mutants (M22, M47) into Jurkat cells with pHLuc3 and pHLuc6, containing 587 bp HMGB1 fragment (the strongest HMGB1 promoter activation) and 1246 bp HMGB1 fragment (the longest clone), respectively. Compared with the control group, nearly 2–3-fold increase in HMGB1 transcriptional activity was observed in the pHLuc6 group due to Tax and mutants. This phenomenon did not occur in the pHLuc3 group (Figure 3D). To further validate whether Tax protein promotes HMGB1 transcription through the NF-κB pathway by the mediation of BAY11-7082 (NF-κB inhibitor), we treated Jurkat cells transfected with Tax and mutants (M22, M47) together with pHLuc6 and TaxP transfected with pHLuc6. Surprisingly, no significant inhibition phenomenon was observed in Jurkat cells transfected with Tax and mutants (Figure 3E) and in TaxP cells (Figure 3F). Therefore, BAY11-7082 could not inhibit HMGB1 transcriptional activity up-regulated by Tax protein. Taken together, these results suggest that the up-regulated effect of Tax on cellular HMGB1 gene is mediated by interactions between the viral Tax protein and some transcriptional regulators in the -1163 to -975 region through the non-NF-κB pathway.

Tax is Enriched in the HMGB1 Gene

The above findings indicate that Tax protein is a potential transcriptional regulator for up-regulation of HMGB1 gene in the -1163 to -975 region. To identify HMGB1 genomic sites directly targeted by Tax, we isolated DNA fragments associated with protein-DNA complexes containing protein Tax using a Tax-specific antibody through ChIP method from the Tax^+-T-cell line (TaxP). Using the specific primer, amplification was conducted with ChIPed DNA to probe the HMGB1 gene region bound by Tax protein. Small amounts of nonspecific protein-DNA complexes may be co-purified to lead to a background signal detected by qRT-PCR in ChIP assays. To compare the levels of retained Tax, we quantified the fold enrichment levels of Tax protein in two separate portions of the HMGB1 gene through qRT-PCR amplification. The regions were centered at -1103 and -797 with respect to the site of Tax-enhancing HMGB1 transcription. We observed stronger Tax enrichment at the -1103 site (the fragment encompassing -1163 to -1043) than that at the -797 site (the fragment spanning -848 to -746) (Figure 4). Therefore, the overall results suggest that Tax is recruited at the -1103 site, but not for any known Tax-binding partners.

Tax is involved in the potentiation of HMGB1 expression by C/EBP

The above ChIP analysis indicates that Tax is recruited at the region of HMGB1 gene. To clarify this finding, we used bioinformatics and found that the region from -1163 to -975 (pHLuc6) revealed *cis*-elements for the following transcription factors: CdxA, AP-1, AML-1a, USF, v-Myb, and C/EBP (Figure 5A). To further confirm the involvement of these transcription factors in HMGB1

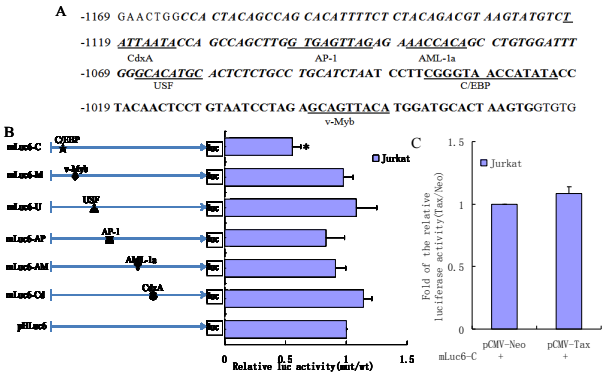

Figure 5. Mutational Analysis of Transcription Factor-binding Sites in the Human HMGB1 Promoter and Luc Analysis to Detect Association of Nuclear Transcription Factors with the Human HMGB1 Promoter Targeted by Tax. (A) The sequence of 188 bp (the region of -1163 to -975 located only in plasmid pHLuc6) is shown in bold uppercase letters. The sequence centered at -1103 site specifically targeted by Tax is shown in bold italic uppercase letters. The putative transcription factor binding sites are underlined beneath the sequence. (B) The individual site-directed mutations were introduced within the pHLuc6 reporter construct. Transient transfection with each mutation construct and wt pHLuc6 together with pCMV-Tax was performed into Jurkat cells, and then Luc activity was determined. Each relative Luc value (mut/wt) is expressed as mean±SD (n=3), in which wt pHLuc6 group is set at 1. Error bars indicate SD. Statistical significance was determined as $*p<0.05$ compared to the control. (C) Jurkat cells were transiently co-transfected with pCMV-Tax (pCMV-Neo as the control) and mLuc6-C, Luc activity was detected, and relative Luc activity (Tax/Neo) was added, in which wt pHLuc6 group is set at 1. Error bars indicate SD

promoter activation, we each performed a deletion mutation by using wt pHLuc6 to generate six mut reporter vectors for Luc analysis, each deleting the corresponding *cis*-element designated as mLuc6-AM for AML-1a, mLuc6-AP for AP-1, mLuc6-Cd for CdxA, mLuc6-U for USF, mLuc6-M for v-Myb, and mLuc6-C for C/EBP. As shown by the Luc assays, mutation in the C/EBP-motif (mLuc6-C) reduced the Luc activity to 52% of the pHLuc6 in TaxP cells (Figure 5B). Tax did not affect mut HMGB1 (mLuc6-C) transcription in Jurkat cells (Figure 5C). Taken together, these findings have demonstrated the novel mechanism through which Tax is involved in the potentiation of HMGB1 expression inside the nucleus by C/EBP.

Discussion

HMGB1 is a chromatin-binding non-histone protein that participates in chromatin-modulating processes in eukaryotes (Bustin, 1999), such as transcriptional regulation, DNA repair, and recombination by binding to the chromatin site in the nucleus (Bianchi and Agresti, 2005; Gerlitz et al., 2009). Generally, HMGB1 is expressed at a basal level as an architectural chromatin-binding protein, but at a slightly elevated level via passive release from damaged or necrotic cells (Beyer et al., 2012; Yi et al., 2013) or active secretion (Akirav et al., 2012; Kang et al., 2013; Mohammad et al., 2013). Moreover, overexpression of HMGB1 protein is observed in breast, colon, and gastrointestinal cancers, as well as in leukemia and other diseases (Kostova et al., 2010; Ohmori et al., 2011; Jube et al., 2012; Lee et al., 2012; Liu et al., 2012; Xing et al., 2012; Yu et al., 2012; Stoetzer et al., 2013). ATL is an acute T-cell malignancy modulated by an oncogenic retrovirus, and the virally encoded Tax protein is believed to be critically involved in the development of ALT. To initially understand the regulatory mechanism of HMGB1 gene in ATL and to observe whether oncoprotein Tax affects HMGB1 regulation in T lymphocytes, we extracted the total RNA and HMGB1 protein from Tax^--T cells (TaxN) and Tax^+-T cells (TaxP). qRT-PCR and Western blot assays showed that HMGB1 transcriptional activity and protein level increased in TaxP cells, which demonstrated that virally encoded Tax protein enhanced HMGB1 expression.

To further analyze the transcription of HMGB1 gene in different T cells, we constructed various recombinant Luc reporter vectors (pGL3-HMGB1-luc) containing the 5'-upstream region of the human HMGB1 gene. We transiently transfected these reporter vectors into TaxN and TaxP cells. The Luc assay showed that the transcription regulation trend was slightly similar but not identical in diverse T cells, with an increase of 30 to 130-fold that of the pGL3-neo-luc and maximal promoter activity in pHLuc3 containing -504 to +83 fragment. Interestingly, the Luc assay of pHLuc6 showed that HMGB1 promoter activity was strengthened in TaxP cells, and the result was confirmed by transient transfection with pCMV-Tax together with pHLuc vectors into Jurkat cells. Tax (active in CREB/ATF and NF-κB pathways), mut M22 (inactive in NF-κB pathway), and mut M47 (inactive in CREB/ATF pathway) (Kwon et al., 2005) also enhanced HMGB1 promoter activity by 2-fold between -1163 to +83. However, BAY11-7082 (NF-κB inhibitor) could not inhibit the enhanced effect by Tax protein. These findings suggest the existence of genomic sites targeted by Tax is between -1163 to -975, and that the enhancement effect mediated by Tax is through the non-NF-κB pathway.

As described in numerous papers, various transcription reactions occurring in the chromatin are mainly triggered by gene specific regulatory factors and chromatin remodeling factors in the transcriptional regulation of genes via their interaction with transcription regulators and chromatin (Bianchi and Agresti, 2005; Nicholas et al., 2011). These properties enable the Tax protein to associate with specific DNA-bound protein complexes and influence HMGB1 gene expression. To verify the above hypothesis, we performed ChIP assays using Tax-DNA complexes from TaxP cells and found that the viral Tax protein was enriched at the -1103 site of the HMGB1 gene (Figure 4). The overall results suggested that the region of the HMGB1 gene that recruited stronger Tax signal was a possible Tax-binding region, but not for any known Tax-binding partners. To further clarify this finding, we used TRANSFEC database search to identify six putative *cis*-elements between -1163 to -975 for the following transcription factors: AM-1a, CdxA, AP-1, USF, v-Myb, and C/EBP. Previous study showed that transcription factor C/EBP could regulate Tax-mediated transactivation of the HTLV-1 long-terminal repeat (Christian et al., 2006). Significantly, in the current study, we found that mLuc6-C for C/EBP could effectively reduced the HMGB1 transcription up-regulated by Tax protein. These data demonstrate a novel mechanism through which Tax is involved in the potentiation of HMGB1 expression inside the nucleus by C/EBP.

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