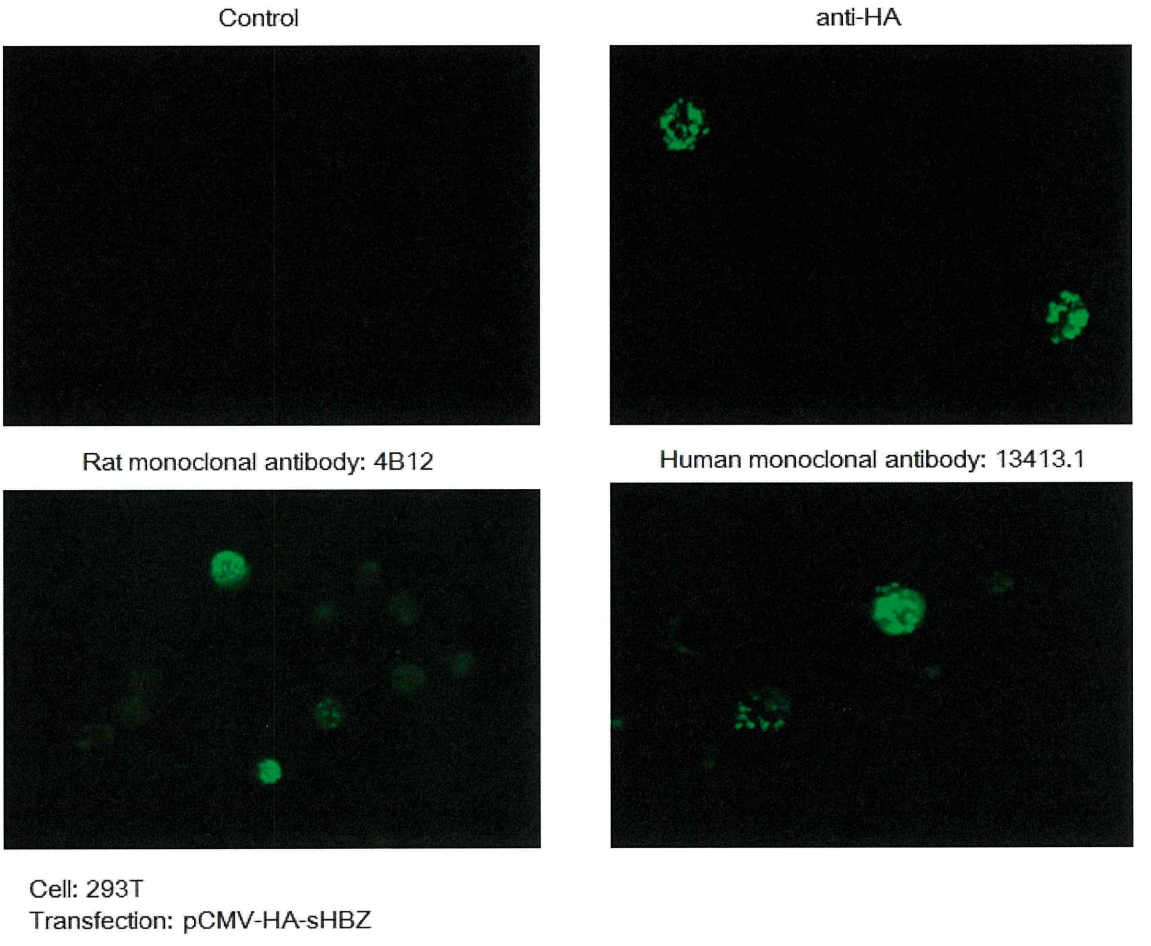
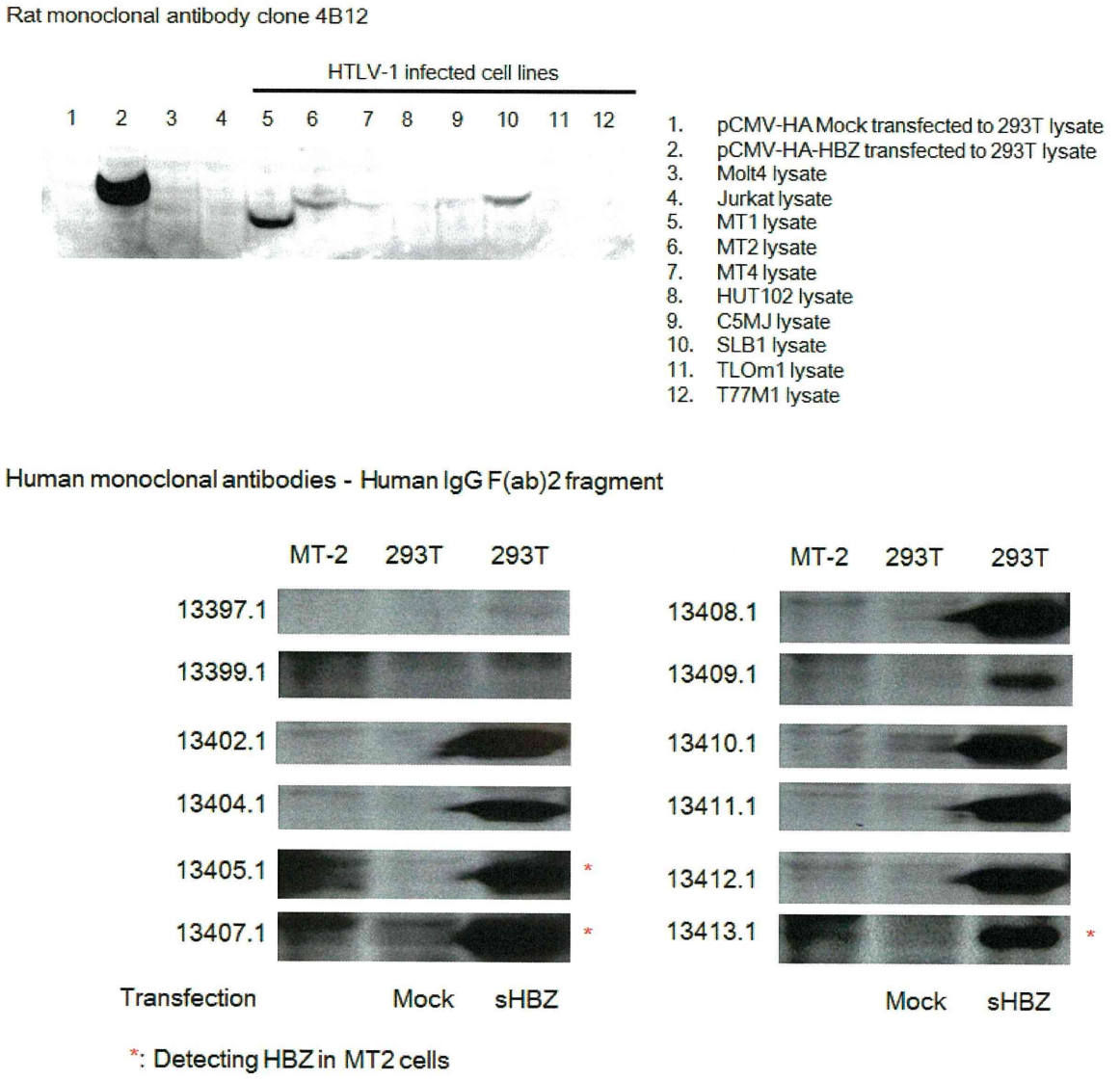
図 8: 新規抗 HBZ モノクローナル抗体を用いた蛍光抗体法による HBZ 蛋白質の検出



○HBZ の C 末端ペプチド抗原（HBZ 193-206：CVNYWQGRLEAMWLQ）を WKA ラットに免疫し、通常のハイブリドーマ 法により HBZ 蛋白を特異的に検出可能なラット抗 HBZ モノクローナル抗体を作製した（clone:4B12, Rat IgG2b）。
○大腸菌で発現させ精製した recombinant HBZ（HBZ 96-206）を抗原として、ファージディスプレイ法により Myc-His タグ付き 2 価ヒト抗 HBZ Fab 抗体を得た。
○ HA タグを付加した全長の spliced HBZ（sHBZ）*をコードする発現プラスミド（pCMV-HA-sHBZ）を 293T 細胞に導入して強制発現させ、ラット抗 HBZ モノクローナル抗体（4B12）とヒト抗 HBZ Fab 抗体（13413.1）を用いて、間接蛍光抗体法により細胞内の HBZ 蛋白質を検出した。
○ いずれのモノクローナル抗体においても、HBZ は既報の通り核小体に似た核内構造に一致して、密度の高いスポットとして検出された。

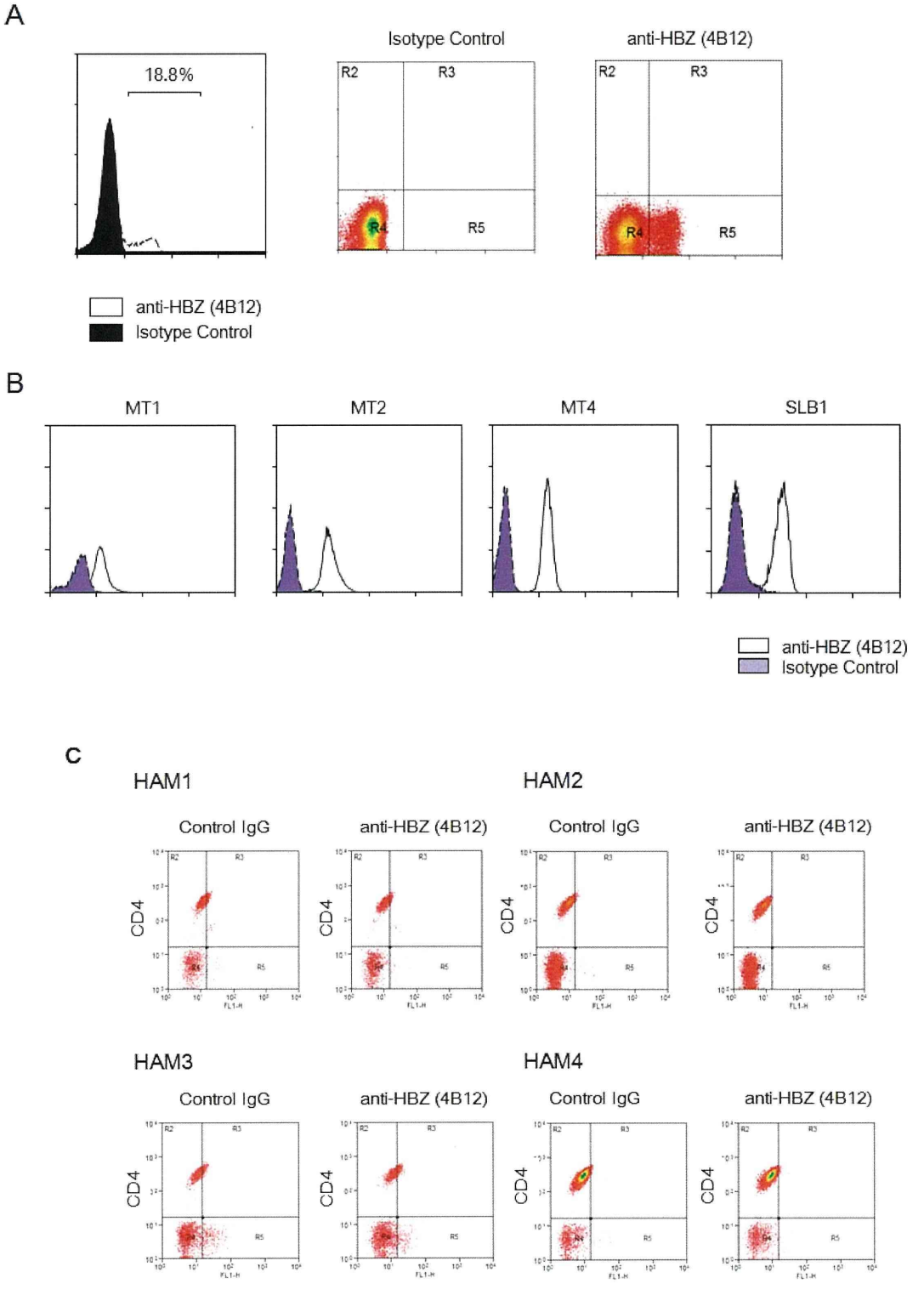
*: HBZ 遺伝子転写産物には spliced と unspliced HBZ が存在するが，spliced HBZ タンパク質は半減期が長く、発現量も多い。

図 9：新規抗 HBZ モノクローナル抗体を用いたウエスタンブロットによる HBZ 蛋白質の検出



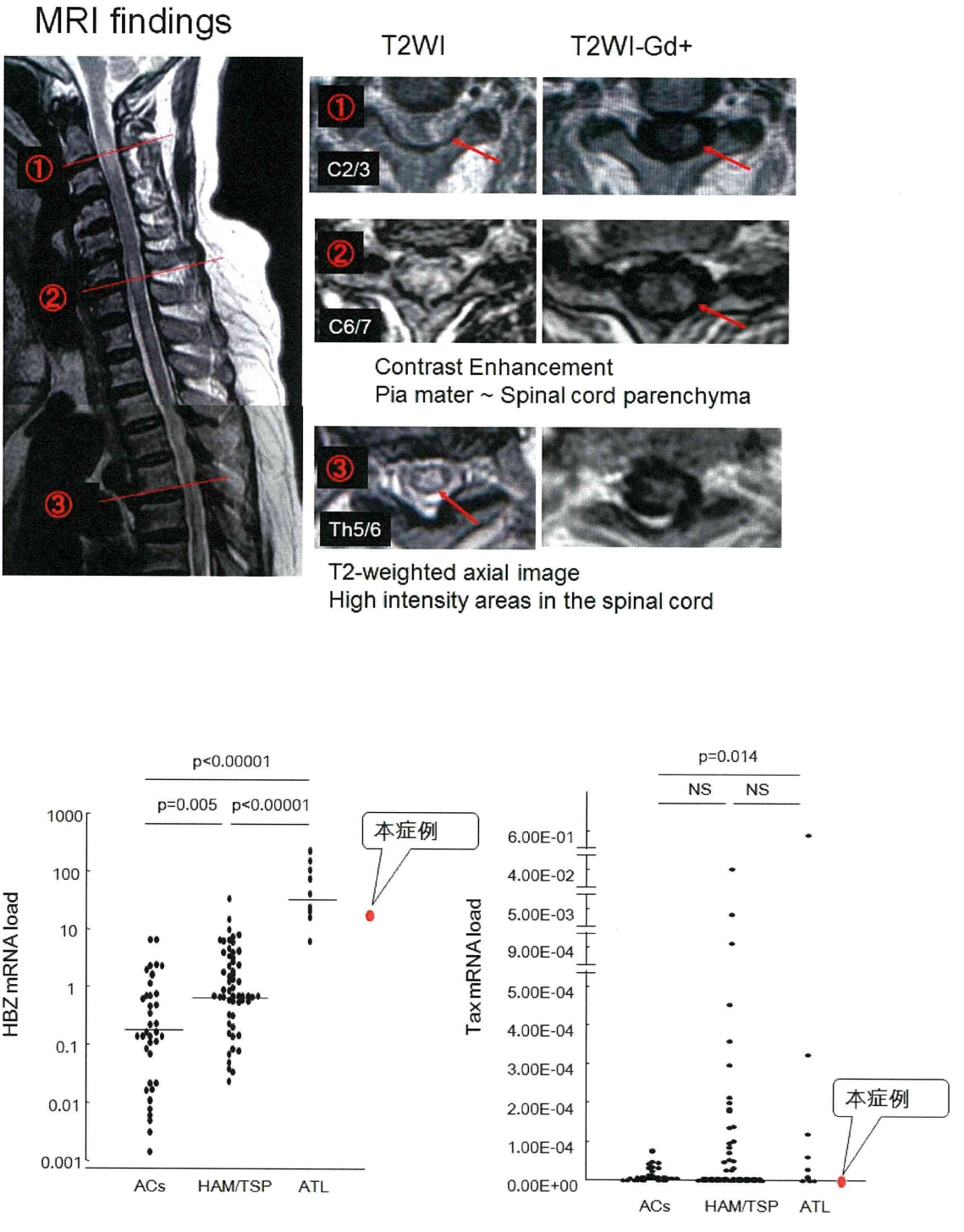
○ ラット抗 HBZ モノクローナル抗体（clone:4B12, Rat IgG2b）、Myc-His タグ付き 2 価ヒト抗 HBZ Fab 抗体を用いて、ウエスタンブロット法により細胞内の HBZ 蛋白質を検出した。
○ ラット抗 HBZ モノクローナル抗体は、HTLV-1 感染細胞株 MT-1, MT-2, MT-4, HUT102,C5MJ, SLB1 から調整した細胞抽出液に含まれる HBZ 蛋白を検出可能であった。
○ MT-1 細胞中の HBZ 蛋白質は、他の HTLV-1 感染細胞株由来のものと移動度が異なり、分子量の違いを反映している可能性が示唆された。
○ ヒト抗 HBZ Fab 抗体のうち、13405.1, 13407.1, 13413.1 の 3 クローンは感染細胞株 MT-2 から調整した細胞抽出液に含まれる HBZ 蛋白を検出可能であった。

図 10：新規抗 HBZ モノクローナル抗体を用いたフローサイトメトリーによる HBZ 蛋白質の検出



○ ラット抗 HBZ モノクローナル抗体は、HBZ を過剰発現させた 293T 細胞（A）および HTLV-1 感染細胞株（B）中の HBZ 蛋白質をフローサイトメトリーにより検出可能であったが、HAM 患者 PBMC（C）中の HBZ 蛋白質は検出できなかった。

図 11:脊髄に多巣性病変を認めた重症例における HBZ mRNA の高発現



脊髄に多巣性病変を認め、発症後 1 か月以内に歩行不能に至った重症例において、ATL 患者レベルの極めて高い HBZ mRNA 発現を認めたが、Tax mRNA は検出感度以下であった。(Saito et al. Retrovirology 6:19, 2009 を改変)

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分担研究報告書

ヒトＴ細胞白血病ウイルス１型関連疾患における感受性遺伝子多型の同定と発症危険群へのアプローチ

研究分担者：熊本大学医学部附属病院　講師　野坂　生郷

研究要旨
ヒトＴ細胞白血病ウイルス(HTLV-1)は、成人Ｔ細胞白血病（ATL）およびHTLV-1関連脊髄症（HAM/TSP）の原因ウイルスであるが、その発症頻度は、全HTLV-1感染者の数%にすぎない。発症には、その他の因子、特に宿主側因子の関与が考えられる。遺伝的背景の解析を行い、遺伝子の多型による因子の同定および発症の危険群を模索することを目的とした。ATL と無症候性キャリアの臨床情報と検体収集の解析、および免疫機序への影響を検討し、発症へ関与している制御性Ｔ細胞の動向や腫瘍解糖系との関与も示した。

Ａ．研究目的

ATL はHTLV-1感染を契機に発症するＴ細胞リンパ腫瘍である。しかしその発症頻度は、全HTLV-1感染者の５％程度であり、非常に少なく、さらに家族内での発症も報告されていることから、宿主側因子の背景も発症機序に関与している可能性が考えられる。本研究では、宿主側因子の遺伝子的背景を多型の違いやその機能を解析することにより､HTLV-1 関連疾患への発症のメカニズムの解明また、病態解明に結びつくと考えられる。本研究に関しては、成人Ｔ細胞白血病、無症候性キャリアに焦点をあて、臨床病態の解析を含め､免疫機構への関与、さらに糖代謝についても解析した。

Ｂ．研究方法

無症候性キャリア、成人Ｔ細胞白血病患者の各病型を対象にした。臨床情報収集解析および検体収集、抽出を行った。制御性Ｔ細胞の検出、ウイルス蛋白であるTaxの発現の解析、サイトカインとの関連、糖代謝について検討した。

（倫理面への配慮）

研究を行うに当たり、ヘルシンキ宣言、遺伝子治療臨床研究に関する指針、疫学研究に関する倫理指針および倫理研究における倫理指針を遵守し、当施設の倫理委員会の承認を受け、患者様に説明を十分行い、同意を得て実施した。

Ｃ．研究結果

無症候性キャリア182例､成人Ｔ細胞白血病258例について、臨床情報収集解析および検体収集、抽出を行った。制御性Ｔ細胞に発現するFoxP3の発現頻度を検討し、成人Ｔ細胞白血病症例においては様々な発現頻度であった。長期間経過を症例の解析では、CD4陽性、FoxP3陽性細胞は一定であった。一部ATL細胞とは異なる集団が存在し、CCL22により制御されていることがわかった。また、細胞株において好気下においても乳酸産生を起こす腫瘍解糖系に関連するPDK1やLDHAの発現の亢進が認められた。

Ｄ．考察

ATLは、HTLV-1感染により起こされる疾患であり、ウイルス蛋白であるTaxやHBZなどにより､細胞の不死化､腫瘍化が in vitro や in vivo で証明されているが、その発症頻度の低さや発症までの期間を考えると宿主側因子の関与が考えられる。多くの症例を網羅的に遺伝子背景に関与する多型を解析することにより、発症に関与する因子の同定に近づいていったと考える。またその宿主側因子の関与に免疫学的な機序の解明が必要であると考えられ、FoxP3 陽性細胞の成人Ｔ細胞白血病に関する役割は、症例間によっても違いがあり、これらの違いが免疫学的な制御に関与している可能性もあると思われる。また解糖系の異常も腫瘍化に関与している可能性を考える。

E．結論

多くのATL症例、無症候性キャリア症例を解析することにより、宿主側の因子の役割を検討でき、複数関与すると思われる遺伝子多型を同定できた。また免疫学的背景の違いもあることがわかった。

F．健康危険情報

収集検体は、数mLの末梢血であり、さらに、通常検査の際に同時に行うため、採取に関する危険性はないものと考えている。また、量も少しであるため、採取に伴う貧血等の可能性もないと考える。

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H．知的財産権の出願・登録状況

1．特許取得

特になし

2．実用新案登録

特になし

3．その他

特になし

研究成果の刊行に関する一覧表

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雑誌

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Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-κB

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Adult T-cell leukemia (ATL) is a highly aggressive T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1). The activation of NF-κB by Tax has been reported to play a crucial role in HTLV-1–induced transformation. The *HTLV-1 bZIP factor* (*HBZ*), which is encoded by an mRNA of the opposite polarity of the viral genomic RNA, is involved in both T cell proliferation and suppression of Tax-mediated viral gene transcription, suggesting that HBZ cooperates closely with Tax. In the present study, we observed that HBZ specifically suppressed NF-κB–driven transcription mediated by p65 (the classical pathway) without inhibiting the alternative NF-κB signaling pathway. In an immunoprecipitation assay, HBZ bound to p65 and diminished the DNA binding capacity of p65. In addition, HBZ induced p65 degradation through increasing the expression of the *PDLIM2* gene, which encodes a ubiquitin E3 ligase for p65. Finally, HBZ actually repressed the transcription of some classical NF-κB target genes, such as *IL-8*, *IL2RA*, *IRF4*, *VCAM-1*, and *VEGF*. Selective suppression of the classical NF-κB pathway by HBZ renders the alternative NF-κB pathway predominant after activation of NF-κB by Tax or other stimuli, which might be critical for oncogenesis. (Blood. 2009;113:2755-2764)

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus that causes adult T-cell leukemia (ATL).[1-4] A unique sequence was found in the viral genome between *env* and the 3′ long terminal repeat (LTR). Denoted the pX region, this sequence encodes the regulatory and accessory genes: *tax*, *rex*, *p12*, *p13*, and *p30*.[5] Among the proteins encoded by these genes, Tax has been thought to play a central role in the leukemogenesis of ATL because of its pleiotropic actions.[6,7] Another gene, the *HTLV-1 bZIP factor* (*HBZ*) gene, is encoded by an mRNA of the opposite polarity of the viral genomic RNA.[8-10] Two major isoforms of the *HBZ* gene transcript have been reported: spliced *HBZ* (s*HBZ*) and unspliced *HBZ* (us*HBZ*).[11-13] Only s*HBZ* is abundantly expressed.[14] HBZ was found to inhibit Tax-mediated transactivation of viral transcription from the 5′LTR by heterodimerizing with c-Jun and CREB2.[15-17] In addition, s*HBZ* RNA promotes proliferation of ATL cells.[11]

Tax-mediated nuclear factor-κB (NF-κB) activation plays a pivotal role in the transforming activity of HTLV-1.[18] Activation of NF-κB by Tax involves both the classical and alternative pathways, which use the NF-κB precursor proteins p105 (NF-κB-1) and p100 (NF-κB-2), respectively. These proteins are processed to the mature p50 NF-κB-1 and p52 NF-κB-2 proteins that heterodimerize with other members of the NF-κB family, RelA (p65), c-Rel, or RelB. Tax activates the classical pathway by interacting with IKKγ,[19,20] which stimulates the phosphorylation of IKKα and IKKβ; phosphorylated IKKα and IKKβ then induce the degradation of IκB by the proteasome,[21] leading to the release of p50/p65 from IκB. Tax also triggers activation of the alternative pathway downstream of NF-κB–inducing kinase (NIK) by activating IKKα via IKKγ and recruiting IKKα to p100, resulting in enhanced processing to p52.[22] In both pathways, the mature dimeric NF-κB proteins translocate to the nucleus and activate genes involved in anti-apoptosis, cell proliferation and angiogenesis. Although NF-κB-1 and NF-κB-2 have similar structures, recent studies strongly suggest that the classical and alternative pathways are involved in the expression of different genes: the classical pathway is mostly involved in innate immunity and inflammatory responses, while the alternative pathway is involved in adaptive immunity and the organogenesis of peripheral lymphoid tissues.[23,24]

NF-κB is activated in many viral infections, and is thought to be important in the protective response of the host to the viral pathogens. Thus, many viruses have evolved distinct strategies to attenuate NF-κB activation. Known mechanisms of NF-κB suppression in virus-infected cells include (1) inhibition of the IKK activity and IκB phosphorylation,[25] (2) inhibition of the NF-κB nuclear translocation,[26] (3) induction of the caspase-mediated cleavage of the RelA subunit,[27] and (4) inhibition of NF-κB RelA transcriptional activation through protein-protein interaction.[28]

Although NF-κB activation by Tax has been reported, it remains unknown whether other viral proteins act on the NF-κB pathway. In this study, we report that HBZ inhibits NF-κB activity by inhibiting p65 DNA binding capacity and promoting expression of PDLIM2 E3 ubiquitin ligase, which results in p65 degradation. This HBZ mediated suppression of the classical NF-κB pathway results in decreased expression of some genes associated with innate immunity and inflammatory responses.

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Methods

Cell culture

Jurkat cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Jurkat and Kit 225 cells stably expressing sHBZ were maintained as described previously.[11] 293FT and Hela cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS and 500 μg/mL G418.

Plasmids

The κB-Luc construct contains 5 tandem repeats of an NF-κB binding site from the IL-2R α-chain gene linked to the firefly luciferase gene. The AP-1-Luc construct contains 2 copies of the IL-8 AP-1 binding site upstream of the IL-8 enhancer-less core promoter linked to the luciferase gene.[29] pSRF-Luc was purchased from Stratagene (Heidelberg, Germany). phRL-TK was purchased from Promega (Madison, WI). Expression vectors for usHBZ and sHBZ deletion mutants were generated by PCR using pME18Sneo-HBZ as a template.[11] These fragments were subcloned into pME18Sneo and pcDNA3.1/*myc*-His(-) (Invitrogen, Carlsbad, CA). The coding region of IKKγ and p50 were amplified by RT-PCR from total RNA derived from Jurkat cells and cloned into the vector pCMV-HA. To construct vectors expressing wild type and deletion mutants of p65, we amplified the coding sequence from Jurkat cell cDNA and subcloned it into the pCMV-Tag 2 vector. The pEF-p52 expression vector, pCGN-HA-ubiquitin, pCG-Tax, pCG-Tax M47 (Tax mutant unable to activate the CREB/ATF pathway), and pCG Tax M22 (Tax mutant defective for NF-κB activation) were described elsewhere.[30-32]

Luciferase assay

Jurkat cells were plated on 6-well plates at 3.5×10^5 cells per well. After 24 hours, cells were transfected with the indicated luciferase reporter plasmid and expression plasmid, and/or empty expression vector (to normalize the DNA dose) mixed with Transfectin (Bio-Rad, Hercules, CA). After 48 hours, cells were collected and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Luciferase values were normalized to renilla luciferase and expressed as the mean of a triplicate set of experiments plus or minus SD.

Measurement of phosphorylation of IκBα

The FunctionELISA IκBα assay was performed according to the manufacturer's instructions (Active Motif, Carlsbad, CA) using 100 μg freshly prepared cytoplasmic protein extract from the samples.

Immunoprecipitation and immunoblotting

To examine protein-protein interaction in 293FT cells, subconfluent cells were transfected with the indicated combinations of expression vectors. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer 48 hours later (50 mM Tris-HCl, pH8.0, 1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, 0.5% Nonidet P-40, and protease inhibitors). Lysates were precleared by incubation with 20 μl of 50% slurry of protein G-agarose (GE Healthcare Life Sciences, Piscataway, NJ) for 30 minutes at 4°C. Precleared cell lysates were incubated with anti-HA (clone 3C2) (MBL, Nagoya, Japan), anti–c-Myc (clone 9E10, Sigma-Aldrich, St Louis, MO) or anti-FLAG M2 (Sigma-Aldrich) antibodies for 1 hour at 4°C, and immune complexes were collected by incubation for 1 hour at 4°C with protein G-agarose. After extensive washing, immunoprecipitated proteins were resolved by 5% to 20% SDS-PAGE and analyzed by western blotting with HRP-conjugated anti-FLAG (Sigma-Aldrich), anti–His-Tag (PM002, MBL), anti-Tax or anti-HA (Sigma-Aldrich) antibodies. Membranes were developed with enhanced chemiluminescence (GE Healthcare Life Sciences). Other antibodies used were as follows: anti–mouse ImmunoglobulinG (IgG) and anti–rabbit IgG were from GE Healthcare Life Sciences; anti-Sp1 and anti–lamin B were from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunofluorescence analysis

Hela cells were transfected with expression vectors using Lipofectamine LTX (Invitrogen). Thirty-six hours after transfection, cells were stimulated with phorbal myristate acetate (PMA) plus ionomycin for 8 hours. sHBZ protein was detected using anti–c-MYC Cy3 (clone 9E10′ Sigma-Aldrich). p65 was detected using anti–Flag-biotin (Sigma-Aldrich) and secondary Streptavidin-Alexa 488 antibody (Invitrogen). Fluorescence was observed with a 63×/1.4-0.60 HCX PL APO objective on a DMIRE2-TCS SP2 AOBS confocal microscope system (Leica, Wetzlar, Germany) as described.[17] Images were acquired and analyzed using LCS 2.61 (Leica) and processed using Photoshop CS2 (Adobe Systems, San Jose, CA).

NF-κB DNA-binding activity assay

Nuclear extracts were prepared using the NucBuster Protein Extraction Kit (Novagen, Madison, WI) according to the manufacturer's instructions. The DNA-binding activity of NF-κB was assayed colorimetrically, using the NoShift Transcription Factor Assay Kit and NoShift NF-κB (p65) reagents (Novagen) according to the manufacturer's instructions. To assess sequence-specific binding activity, we incubated 15 μg of nuclear extract with various combinations of biotinylated NF-κB wild-type dsDNA, specific NF-κB competitor dsDNA lacking biotin end labels, and nonspecific, nonbiotinylated dsDNA with a mutant NF-κB consensus binding motif. All assays were performed in triplicate.

Measurement of apoptotic cell death

For detection of apoptosis, the annexin V-binding capacities of the treated cells were examined by flow cytometry using an annexin V–PE Apoptosis Detection Kit (Biovision, Mountain View, CA), according to the manufacturer's instructions.

Ubiquitination assay

To analyze the ubiquitination of p65, we transfected 293FT cells with expression plasmids encoding FLAG-tagged p65, HA-tagged ubiquitin and mycHis-tagged wild type or mutant sHBZ. Extracts were incubated with anti-FLAG antibodies plus protein G-agarose, washed 5 times, and analyzed by immunoblot with anti-HA antibody.

Synthesis of cDNA and semiquantitative RT-PCR

Total RNA was isolated using Trizol Reagent (GIBCO, Grand Island, NY) according to the manufacturer's instructions. We reverse transcribed 1 μg of total RNA into single-stranded cDNA with SuperScript II reverse transcriptase (Invitrogen). Using the forward (F) and reverse (R) primers specific to the target genes, the cDNA was amplified by increasing number of PCR cycles. The primers used for this study are shown in Table S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The intensity of PCR-amplified band was quantified using ATTO densitography 4.0 (Atto Instruments, Tokyo, Japan). The mRNA level of each product was normalized to the level of *GAPDH* and calculated as a ratio to the level of the control. Representative results of 3 independent experiments are shown.

Small interfering RNA (siRNA) transfection

siRNA targeted to human *PDLIM2* was synthesized according to a previous report.[33] 293FT cells were transfected with expression vectors and siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Western blotting with anti-PDLIM2 antibody (Abnova, Taipei, People's Republic of China) detected PDLIM2 protein 48 hours after transfection.

sHBZ transgenic mice

Transgenic mice expressing the spliced *HBZ* gene under control of CD4 specific promoter/enhancer/silencer have been reported previously.[11] $CD4^+$ cells were isolated from mice thymus using anti–mouse CD4 particles-DM (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. The experiments and protocols used were approved by the

Animal Use and Care Committee of the Institute for Virus Research at Kyoto University.

Statistical analyses

Statistical analyses were performed using the unpaired Student *t* test.

Results

HBZ inhibits the classical NF-κB signaling pathway

To analyze the possible involvement of HBZ in several signaling pathways in ATL, we first evaluated whether HBZ could influence the AP-1, SRF and NF-κB pathways, which are activated by the Tax protein,[34,35] using a transient transfection assay. There are 2 isoforms of the *HBZ* gene: spliced and unspliced *HBZ*. In most of the experiments, we used s*HBZ*, because the protein derived from s*HBZ* is more abundant and potent than that of us*HBZ*.[14] As shown in Figure 1A, the expression of sHBZ did not affect Tax-mediated effects using SRF reporter. Consistent with the previous report, sHBZ has ability to suppress Tax-induced AP-1 activation, and its inhibitory effect was more significant than that of usHBZ (Figure 1B). In addition, sHBZ suppressed p65 mediated NF-κB activation in a dose-dependent manner (Figure 1C).

Tax has been reported to activate NF-κB by several mechanisms.[36] sHBZ might have a negative regulatory effect on Tax-mediated NF-κB activation. When co-expressed, sHBZ dramatically repressed κB-Luciferase expression activated by wild type Tax and Tax M47 mutant (Figure 2A,B). However, because Tax M22 mutant did not activate NF-κB, HBZ expression had no effect. The NF-κB suppressive effect mediated by usHBZ was much weaker than that mediated by sHBZ. Although we previously reported that s*HBZ* RNA was responsible for growth-promoting activity,[11] s*HBZ* RNA did not influence NF-κB (Figure 2A, TTG-HBZ), indicating that HBZ protein has the suppressive function on NF-κB. On the other hand, overexpression of Tax overcame the sHBZ-mediated suppression of NF-κB transcriptional activation (Figure 2C).

It has been reported that activation of the classical NF-κB pathway by Tax is mediated by its ability to physically bind to IKKγ, which results in enhanced phosphorylation and subsequent degradation of IκB.[21] We analyzed whether sHBZ could modulate the Tax-driven phosphorylation of IκBα using a FunctionELISA kit (Figure 2D). No effects on the Tax-up-regulated phosphorylation level of IκBα were observed when cells were cotransfected with sHBZ. In addition, we investigated whether HBZ influenced the interaction between Tax and IKKγ. 293FT cells were transfected with various combinations of Tax, HA-tagged IKKγ and mycHis-tagged sHBZ expression vectors. As shown in Figure 2E, sHBZ did not interfere with the formation of Tax/IKKγ complexes, and there was no obvious binding of sHBZ to IKKγ or Tax.

Two main pathways control the nuclear translocation of NF-κB: the classical and alternative NF-κB pathways.[36] Recently, constitutive activation of the alternative NF-κB pathway has been reported in several lymphoid malignancies, where it may play a critical role.[37] We next analyzed the effect of sHBZ on the alternative (p52 mediated) pathway of NF-κB activation. Figure 3A demonstrates that sHBZ was not capable of suppressing p52-mediated NF-κB activation. In addition, sHBZ had no remarkable effect on Tax-mediated up-regulation of p52 and its precursor protein p100 (Figure 3B). Thus, HBZ-mediated suppression of NF-κB activation occurs via inhibition of the classical pathway.

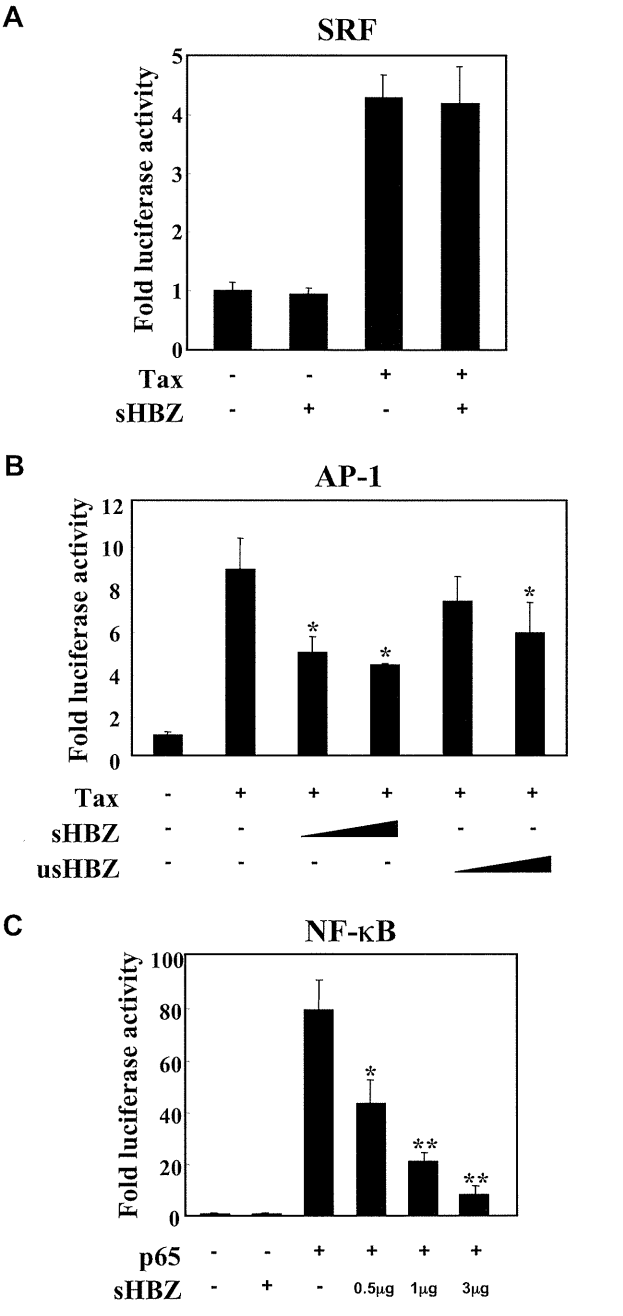

Figure 1. sHBZ inhibited NF-κB and AP-1 activation but did not influence the SRF pathway. Jurkat cells were cotransfected with phRL-TK and reporter plasmid pSRF-Luc (A), AP-1-Luc (B), or κB-Luc (C), respectively, with or without 1 or 3 μg pME18Sneo-sHBZ and 1 μg pCG-Tax (A,B) or 1 μg pCMV-Tag 2-p65 (C). The total amount of DNA for transfection was equalized by adding empty vectors. After 48 hours, a dual luciferase reporter assay was preformed as described in "Methods." All the data shown are relative values of firefly luciferase normalized to Renilla luciferase and expressed as mean of a triplicate set of experiments (± SD). $^*P < .05$; $^{**}P < .01$.

Domains of sHBZ responsible for suppression of NF-κB p65

Next, we sought to identify the region of sHBZ responsible for the repression of NF-κB activation. To this end, we tested the sHBZ deletion mutants shown in Figure 4A. As shown in Figure 4B, wild-type sHBZ dramatically down-regulated p65-mediated NF-κB activation. Compared with other mutants, only the sHBZ-AD+bZIP mutant exhibited weak suppressive activity.

Accumulating evidence shows that the interaction between bZIP proteins and Rel family proteins affects their subcellular

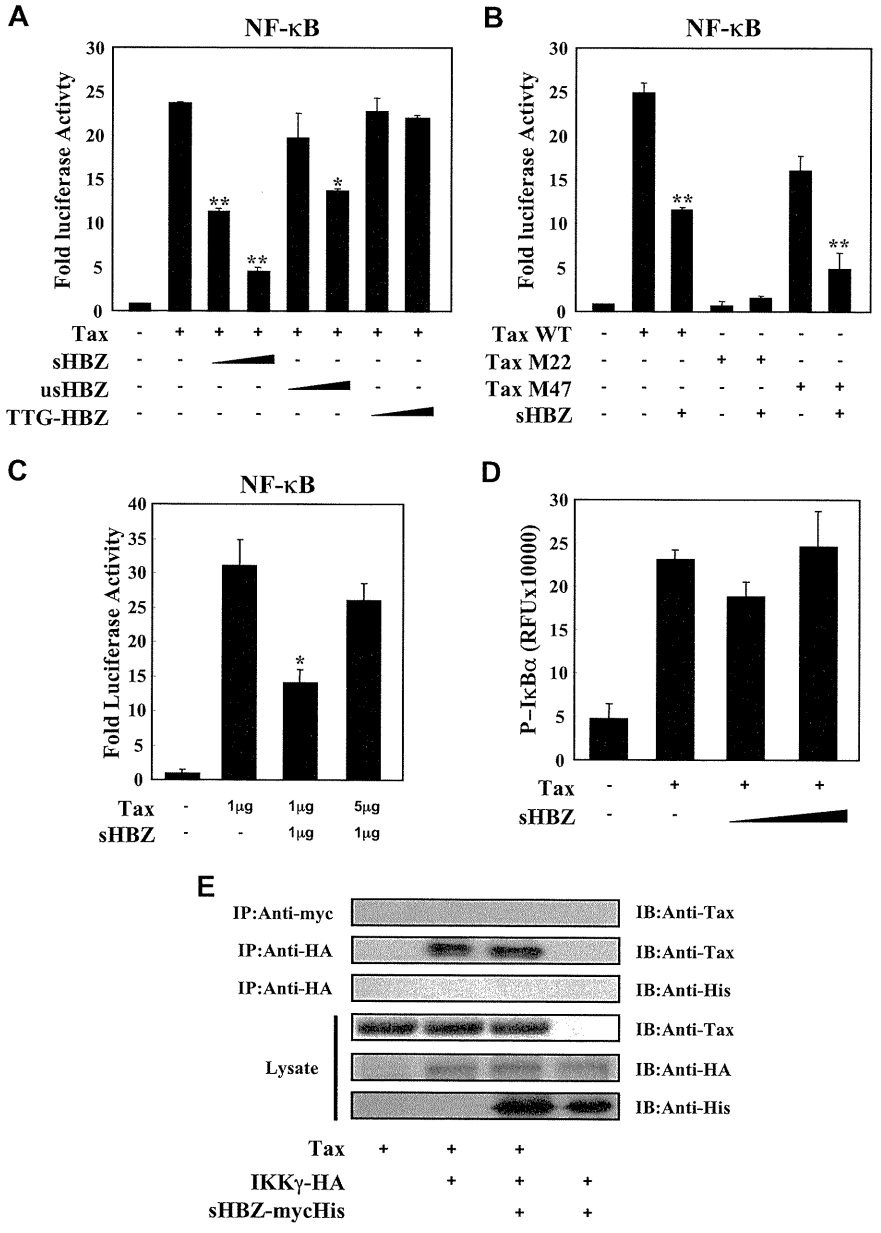

Figure 2. sHBZ suppressed Tax-mediated NF-κB activation but did not interfere with the Tax/IKKγ complex. (A,B) sHBZ repressed Tax-induced NF-κB activation. Jurkat cells were cotransfected with κB-Luc and phRL-TK, with or without 1 μg pCG-Tax, pCG-Tax M47, pCG Tax M22, and with 1 or 3 μg pME18Sneo-sHBZ, usHBZ, or TTG-HBZ. After 48 hours, luciferase activity was measured. (C) Tax overexpression overcame sHBZ-mediated suppression of NF-κB activation. Jurkat cells were cotransfected with κB-Luc and phRL-TK, with or without pCG-Tax and pME18Sneo-sHBZ. The total amount of DNA was equalized by adding empty vectors. (D) sHBZ could not modulate the Tax-driven phosphorylation of IκBα. Jurkat cells were transfected with pCG-Tax and pME18Sneo-sHBZ. Cell lysates were subjected to FunctionELISA IκBα assay. (E) sHBZ did not influence the interaction between Tax and IKKγ. 293FT cells were transfected with the indicated cDNA expression constructs. Cell lysates were subjected to immunoprecipitation (IP) with anti–c-Myc and anti-HA followed by immunoblotting (IB) using anti-Tax or anti-His, respectively. The expression levels of Tax, IKKγ and sHBZ were analyzed. $^{*}P < .05$; $^{**}P < .01$.

localization and modulates transcription activation.[38,39] Therefore, we investigated whether sHBZ can physically interact with NF-κB p65. 293FT cells were transfected with vectors expressing sHBZ and p65. Figures 4C through E illustrate the physical binding between sHBZ and p65 or p50. HBZ has 3 domains, an activation domain (AD), a central domain (CD) and a basic leucine zipper domain (bZIP). To determine which portion of sHBZ is necessary for the binding with NF-κB p65 protein, we performed a coimmunoprecipitation assay. Three mutants (sHBZ-ΔAD, sHBZ-ΔbZIP, and sHBZ-AD+bZIP) could bind to p65 (Figure 4C), indicating that at least 2 of 3 main domains in sHBZ were necessary for the binding between sHBZ and p65. However, only sHBZ-AD+bZIP could suppress NF-κB activation as shown in Figure 4B, indicating the significance of AD and bZIP domains for binding with p65.

To determine which part of p65 protein is necessary for binding with sHBZ, we tested the ability of a series of FLAG-tagged truncated p65 proteins to interact with the full length of sHBZ (Figure 4D). The p65 (313-551) mutant, which did not contain the Rel homology domain, was incapable of interacting with the sHBZ protein. The truncated p65, 1 to 320, which lacked the transactivation domain but remained the Rel homology domain, still interacted efficiently with sHBZ. We conclude that sHBZ interacts with the Rel homology domain of p65. Next, we studied the binding of sHBZ to p50 by immunoprecipitation. As shown in Figure 4E, HBZ did not bind to p50, and did not interfere with the binding between p65 and p50.

The HBZ-p65 interaction was further investigated by confocal microscopy. HBZ exhibited a granular speckles pattern as previously reported[15] (Figure 4Fi). After stimulating with PMA/ionomycin, the cotransfected cells showed nuclear spots representing colocalization of sHBZ and p65 (Figure 4F iii-v, vi-viii).

Because sHBZ interacts with p65, we studied whether sHBZ inhibits the ability of p65 to bind the κB binding site. As expected, sHBZ dramatically decreased p65 DNA binding capability (Figure 4G). Furthermore, analysis using deletion mutants of sHBZ revealed that sHBZ-AD+bZIP could also decrease the binding of

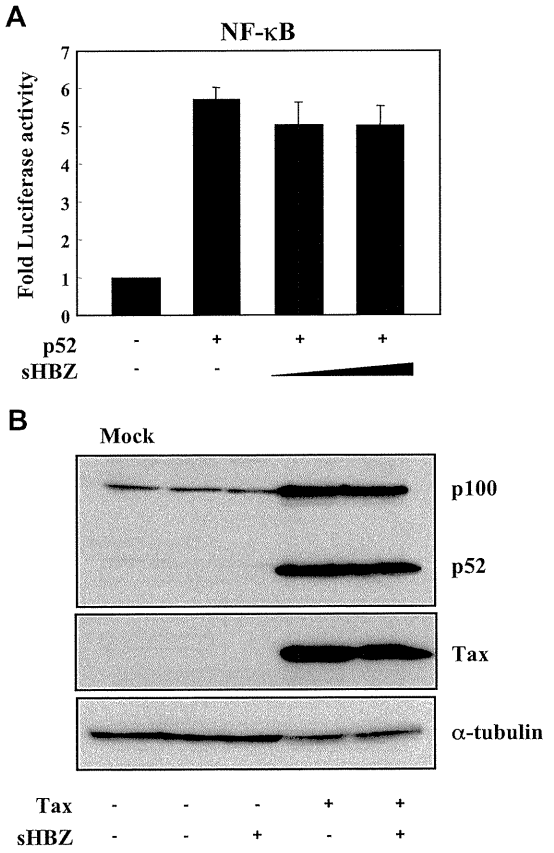

Figure 3. sHBZ did not affect alternative NF-κB pathway. (A) The effect of sHBZ on p52-mediated alternative NF-κB activation. Jurkat cells were cotransfected with κB-Luc, phRL-TK, pEF-p52, and pME18Sneo-sHBZ. Luciferase levels were measured after 48 hours. (B) sHBZ did not influence p52 and p100 expression. Jurkat cells were cotransfected with vectors that express Tax and sHBZ. After 48 hours, cell lysates were then subjected to immunoblot with anti-p52, Tax, and α-tubulin.

p65 protein to DNA. The suppressive effects of deletion mutants lacking AD or bZIP were not statistically significant. This result suggests that both AD and bZIP domains are important for the inhibition of p65 binding to its target sequence, and coincides with the results of the luciferase assay.

Taken together, these observations demonstrate that sHBZ represses p65-induced transcription through direct physical association between sHBZ and p65 via the AD and bZIP domains, and this interaction inhibits the binding of p65 to target sites in DNA.

HBZ promotes p65 degradation through a ubiquitination-dependent pathway

A previous report showed that HBZ could bind to c-Jun, and decrease c-Jun protein via proteasome dependent mechanism.[17] Therefore, we analyzed whether sHBZ could also affect the turnover of NF-κB p65 protein. Expression of sHBZ repressed the level of p65 protein in a dose-dependent manner (Figure 5A). To determine which domain of sHBZ is responsible for the down-regulation of p65 protein levels, we analyzed the level of p65 when co-expressed with each of the sHBZ mutants (Figure 5B). The level of p65 was slightly decreased when the sHBZ-AD+bZIP mutant was expressed, again suggesting that the AD and bZIP domains of sHBZ are required for suppression of p65. By semiquantitative RT-PCR, we confirmed that the level of p65 mRNA did not differ when co-expressed with sHBZ and its mutants (Figure 5B). Taken together, these results demonstrated that sHBZ reduced the level of p65 protein by acting at a posttranscriptional level.

To further elucidate the mechanism by which sHBZ reduces the amount of p65, we measured p65 levels after blocking caspase activity because caspase-mediated cleavage of the NF-κB p65/RelA subunit has been reported as a mechanism of decreasing p65 levels.[27] The activity of z-VAD-fmk caspase inhibitor was confirmed by the finding that z-VAD-fmk could block apoptosis induced by TNFα (Figure 5C). Treatment with z-VAD-fmk caspase inhibitor did not change the down-regulation of p65 by sHBZ (Figure 5C top panel), indicating that caspase-mediated cleavage of p65 was not involved in this inhibitory effect.

Protein ubiquitination is a crucial modification, which induces degradation of proteins in the proteasome and controls the activity of many signaling molecules, including transcription factors such as p53, c-Jun, and p65.[40-42] We therefore studied whether expression of sHBZ could induce the polyubiquitination of p65 and trigger the degradation of p65 protein. Immunoprecipitation studies showed that expression of sHBZ induced heavy ubiquitination of p65 (Figure 5D). In the presence of a proteasome inhibitor, sHBZ remarkably accelerated the ubiquitination of p65 (Figure 5D). This effect was far stronger than that of usHBZ (Figure 5E). Analysis of sHBZ deletion mutations showed that only sHBZ-AD+bZIP induced polyubiquitination of p65, while other mutants had no effect on p65 ubiquitination (Figure 5E).

Several ubiquitin E3 ligases have been shown to specifically induce the ubiquitination of p65, including *SOCS-1*, *Cul2*, *Elongin B/C*, and *PDLIM2*.[41,43-45] To identify the ubiquitin E3 ligase responsible for polyubiquitination of p65 in Kit 225 cells, we analyzed transcriptional profiles of these ubiquitin E3 ligase genes in sHBZ-transfected and control cells. Expression of these candidate genes was analyzed by semiquantitative RT-PCR with different cycles of amplification, which indicated that transcription of the *PDLIM2* gene was up-regulated in sHBZ-transfected Kit 225 and 293FT cells (Figure 6A).

To confirm whether increased PDLIM2 expression is associated with degradation of p65, we suppressed PDLIM2 expression by siRNA. When PDLIM2 expression was inhibited, p65, decreased by sHBZ, partially recovered, indicating that PDLIM2 acts in the degradation of p65 induced by sHBZ (Figure 6B). A previous report showed that PDLIM2 targets p65 to discrete intranuclear compartments (insoluble nuclear fraction) where polyubiqutinated p65 is degraded by the proteasome.[41] We measured p65 levels in soluble and insoluble nuclear fractions of 293FT cells transfected with p65 and sHBZ expression vectors. The down-regulation of p65 protein in the insoluble nuclear fraction was more significant than that in the soluble fraction. Moreover, p65 levels in the insoluble fraction, but not in the soluble fraction, were partially restored by MG132 treatment (Figure 6C). These observations suggested that the insoluble p65 mainly underwent proteasomal degradation and were consistent with the idea that sHBZ acts on p65 levels via PDLIM2. It remains to be elucidated whether HBZ accelerates the ubiquitination of p65 by PDLIM2 in addition to increasing the expression of the *PDLIM2* gene.

sHBZ partially represses selected classical NF-κB target genes

It is well established that the activation and translocation of NF-κB is associated with increased transcription of genes encoding chemokines, cytokines, adhesion molecules, and inhibitors of apoptosis. Recent studies showed that the classical and alternative pathways of NF-κB influence the expression of different sets of genes.[24] Because HBZ suppresses the classical NF-κB pathway, we checked the effect of sHBZ on the expression of p65-specific target genes using Jurkat cells stably expressing sHBZ. Expression of

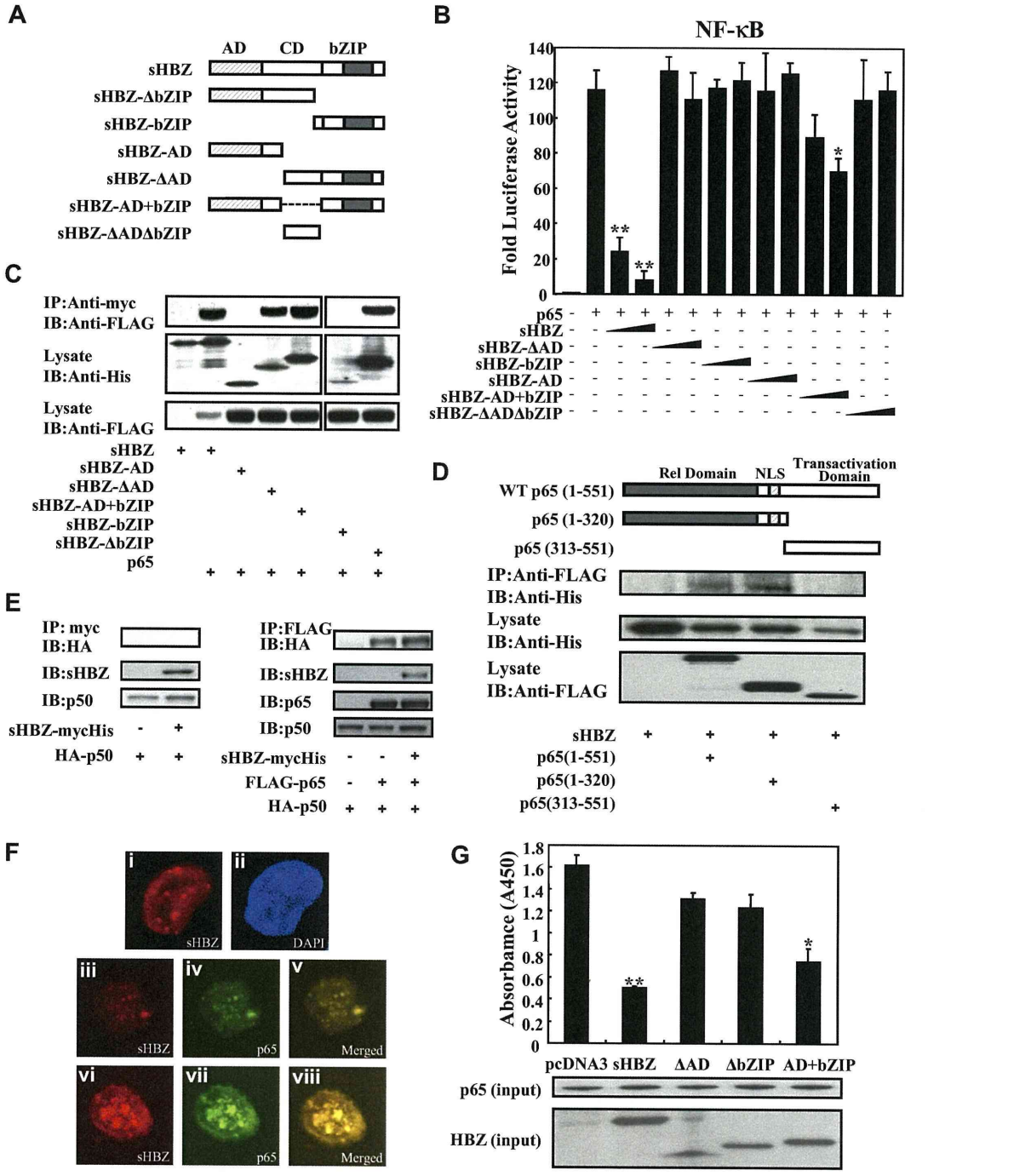

Figure 4. Domains of sHBZ responsible for suppression of NF-κB p65. (A) Schematic diagram of HBZ and its mutants used in this study. Characteristic domains of HBZ are indicated as follows: activation domain (AD), central domain (CD) and basic leucine zipper domain (bZIP). (B) Analysis of HBZ deletion mutants for the effect on p65-mediated NF-κB activation. Jurkat cells were cotransfected with κB-Luc and phRL-TK, with or without 1 μg of pCMV-Tag 2-p65, and with 1 or 5 μg pME18Sneo-sHBZ or sHBZ mutant. After 48 hours, luciferase levels were measured. $^*P < .05$; $^{**}P < .01$. Error bars represent SD. (C) Determination of the region of HBZ responsible for the interaction with p65. 293FT cells were transfected with the indicated mycHis-sHBZ mutants along with the FLAG-p65 vector. Cell lysates were subjected to immunoprecipitation (IP) using anti–c-Myc followed by immunoblotting (IB) using anti-FLAG. The expression levels of p65 and sHBZ mutants were detected. (D) Mapping the region of the p65 protein necessary for the interaction with sHBZ. The schema of p65 deletion mutants has been shown. The locations of the Rel homology domain, the nuclear localization signal (NLS), and the transactivation domain are indicated. 293FT cells were transfected with mycHis-sHBZ along with full-length or mutant FLAG-p65. At 48 hours after transfection, total cell lysates were subjected to IP using anti-FLAG followed by IB using anti-His. (E) HBZ did not influence p65/p50 interaction. 293FT cells were transfected with the indicated expression vectors. Cell lysates were subjected to IP using anti–c-Myc or anti-FLAG followed by IB using anti-HA. The expression levels of p65, p50, and sHBZ were detected. (F) sHBZ colocalizated with p65 in the cell nucleus. HeLa cells were transfected with mycHis-sHBZ together with (panels iii-viii) or without (panels i,ii) FLAG-p65. sHBZ was detected using anti-MYC Cy3 antibody (panels i,iii,vi). p65 was detected using anti–Flag-biotin and secondary Streptavidin-Alexa 488 antibody (panels iv,vii). The overlay of sHBZ and p65 is shown (panels v,viii). DAPI (4,6 diamidino-2-phenylindole) was used to counterstain the nucleus (panel ii). (G) sHBZ decreased p65 DNA binding capability. 293FT cells were transfected with FLAG-p65 together with either mycHis-sHBZ or one of its mutants. Cell lysates were subjected to the enzyme-linked immunosorbent assay (ELISA)–based NoShift assay to measure the DNA binding capability of p65. The absorbance at 450 nm indicated the binding ability of p65 (top panel). The bottom panel shows the amount of p65 and sHBZ in the 20% of input for analysis. $^*P < .05$; $^{**}P < .01$. Error bars represent SD.

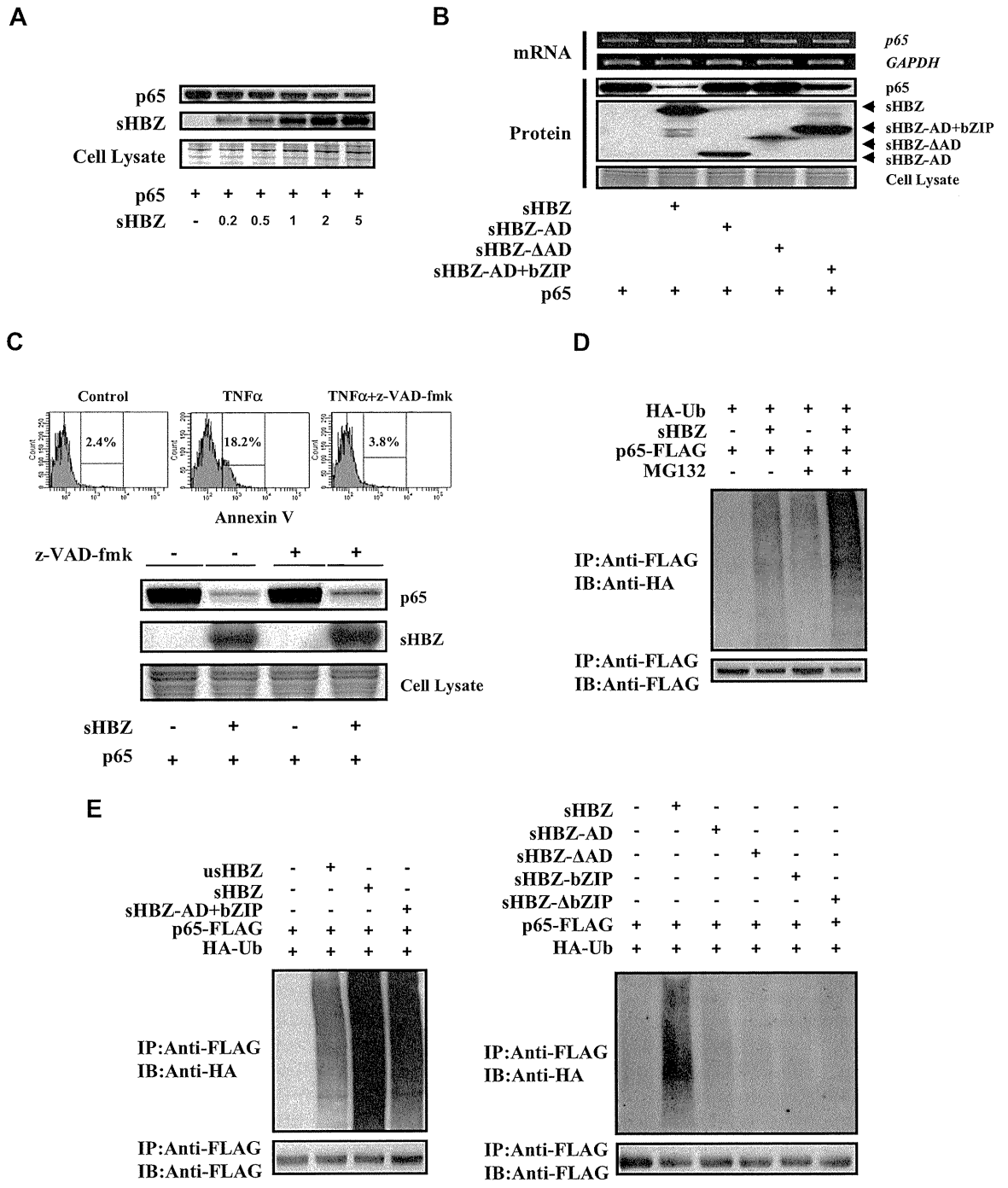

Figure 5. sHBZ promotes p65 degradation through a ubiquitination-dependent pathway. (A) sHBZ repressed the level of p65 in a dose-dependent manner. 293FT cells were transfected with 1 μg pEF-p65 and various amounts of mycHis-sHBZ (0.2, 0.5, 1, 2, and 5 μg). After 36 hours, the cell lysates were subjected to immunoblotting. (B) Activation and leucine-zipper domains of sHBZ were necessary for suppression of p65. 293FT cells were transfected with 50 ng pEF-p65 and 250 ng either mycHis-sHBZ or its mutants. At 36 hours after transfection, the level of *p65* mRNA was analyzed by semiquantitative RT-PCR. The levels of *GAPDH* mRNA are shown as internal control (top panel). Whole cell lysates were subjected to immunoblotting (bottom panel). (C) sHBZ-mediated suppression of p65 protein is caspase independent. Top panel: 293FT cells were transfected with FLAG-p65 together with mycHis-sHBZ. The caspase inhibitor z-VAD-fmk was added 2 hours before transfection. At 48 hours after transfection, cell lysates were subjected to immunoblotting. Bottom panel: Jurkat cells were cultured in the presence of indicated drugs for 24 hours. Cell death was analyzed by annexin V staining. (D,E) sHBZ accelerated the ubiquitination of p65. 293FT cells were transfected with FLAG-p65, HA-ubiquitin, and either mycHis-sHBZ or its mutants. After 24 hours, cells were treated with or without MG132 for 12 hours. Cell lysates were subjected to IP using anti-FLAG followed IB using anti-HA.

genes was analyzed by semiquantitative RT-PCR (Figure 7A). Expression of sHBZ was associated with suppression of some selected target genes, which are normally up-regulated after PMA/ionomycin treatment, such as *IL-8*, *IFN-γ*, *IL2RA*, *IRF4*, *VCAM-1*, and *VEGF*. Because PMA/ionomycin treatment activates NF-κB mainly by the classical pathway, this observation indicates that HBZ expression modulates the transcription of genes activated by the classical pathway. However, overexpression of *sHBZ* gene might influence these results. Indeed, the level of sHBZ mRNA in sHBZ expressing Jurkat cells was much higher than that in an ATL cell line, MT-4 (Figure 7B). However, the level of sHBZ expression in MT-4 cells is comparable to that of $CD4^+$ T cells from

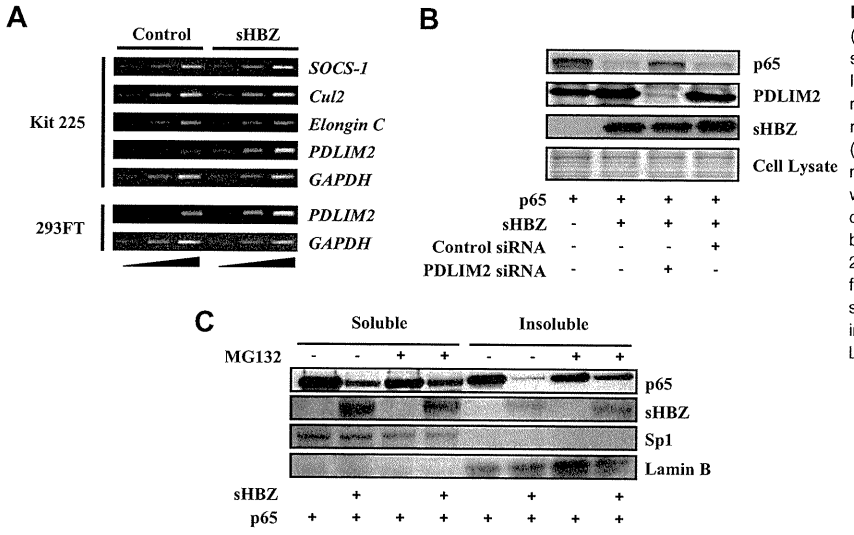

Figure 6. HBZ up-regulates expression of *PDLIM2* gene. (A) sHBZ up-regulates *PMLIM2*. Total RNA was extracted from sHBZ-expressing or control Kit 225 and 293FT cells. The levels of *SOCS-1*, *Cul2*, *Elongin C*, *PDLIM2*, and *GAPDH* mRNA were measured by semiquantitative RT-PCR. The ramp on the left represented an increasing PCR cycle number. (B) Reducing PDLIM2 expression by siRNA recovered sHBZ-mediated suppression of p65. 293FT cells were transfected with expression vectors together with PDLIM2 siRNA or control siRNA. Protein expression was analyzed by western blotting. (C) sHBZ induced the degradation of insoluble p65. 293FT cells, untreated or treated with MG132, were transfected with mycHis-sHBZ along with FLAG-p65. After 48 hours, soluble and insoluble nuclear fractions were subjected to immunblotting. The expression levels of p65, sHBZ, Sp1, and Lamin B were detected.

HBZ-transgenic mice. In this transgenic mouse, sHBZ gene is expressed by a mouse CD4-specific promoter/enhancer/silencer. To investigate HBZ-mediated suppression of genes activated by the classical NF-κB pathway in vivo, we studied the expression of p65-specific target genes in thymus CD4$^+$ cells from sHBZ transgenic mice. After stimulating the cells with PMA/ionomycin, expressions of *IL2RA*, *IRF4,* and *VCAM-1* genes were suppressed in sHBZ transgenic mice as observed in sHBZ transfected Jurkat cells (Figure 7C).

Discussion

Activation of the NF-κB signaling pathway, which has been reported in various cancer cells, plays an important role in the development and progression of tumor cells.[46,47] In oncogenesis by HTLV-1, the *tax* gene has been extensively studied. Tax can activate various transcription pathways and functionally inhibit p53 and MAD1.[7] Furthermore, various tumors have been observed in *tax* gene transgenic animals, depending on the promoter used.[48,49] These findings show the oncogenic potential of Tax in vivo. Although Tax can transform Rat-1 cells in vitro, a Tax mutant lacking the ability to activate NF-κB lost its transforming activity, indicating that NF-κB activation is indispensable for Tax mediated transformation.[18] Tax can activate NF-κB by both the classical and alternative pathways via its interactions with IKKγ[20] and p100.[22] Although they are often activated concurrently, the classical and alternative NF-κB pathways have distinct regulatory functions. Accumulating evidence suggests that the alternative NF-κB pathway is more important in several cancers.[37] It has been reported that the classical and alternative NF-κB pathways differentially control genes with anti-apoptotic functions in lymphoma cell lines.[50] In transformation by Tax, it has been reported that the alternative pathway is critical.[51] This study demonstrates the selective suppression of the classical NF-κB pathway by HBZ, a phenomenon that selectively modulates NF-κB activation by Tax. In many ATL cells, Tax is not expressed, while the *HBZ* gene is expressed in all ATL cases. Even in ATL cells without Tax expression, NF-κB is constitutively activated.[52] Recently, elevated expression of NIK has been reported in ATL cells.[53] Because NIK activates both the classical and alternative NF-κB pathways, HBZ might modulate the classical NF-κB pathway even in the absence of Tax, leading to predominant activation of alternative pathway, and perhaps to oncogenesis.

The classical NF-κB pathway is still potently activated by Tax in the HTLV-1 transformed T-cell lines, such as MT-4 and MT-2, regardless of HBZ expression. Because these cell lines express large amount of Tax, it is likely that HBZ does not have strong suppressive effect on classical pathway of NF-κB due to excess Tax expression. However, the effect of HBZ on the classical NF-κB pathway may be more pronounced when the level of Tax expression is down-regulated or silenced as in chronically infected T cells in infected people and ATL cells.

Many viruses have developed strategies to manipulate NF-κB signaling through the use of multifunctional viral proteins. For example, the HIV-1 encoded Tat protein enhances NF-κB mediated LTR activation while HIV-1 Nef induces the expression of NF-κB inhibitor, IκBα, to suppress this pathway.[54] In Epstein-Barr virus, the LMP-2 viral protein activates the NF-κB pathway by the recruitment of cellular adaptor proteins, TNF receptor–associated factor families and TNF receptor–associated death domain, to the C-terminal domain. Like HBZ, the EBV bZIP protein inhibits the classical NF-κB pathway through interacting with p65.[28] Similar suppression of NF-κB has been reported for other viruses, including African swine fever virus, hepatitis C virus, and human herpesvirus-8. These findings show that NF-κB suppressive activities are common among different viruses, suggesting that these activities are important for viral infection. In this regard, it is noteworthy that transcription of the *IFNγ* and *IRF4* genes, which is induced by the classical pathway, is suppressed by HBZ as shown in this study. A virus might facilitate escape from the host immune system by suppressing the classical NF-κB pathway in such a manner.

Viruses have evolved to sneak through the innate and adaptive antiviral response both at the cellular and whole organism levels, for survival and successful spread of infection. One mechanism used by some viruses to avoid immune surveillance is to control the level of cellular transcription factors by sorting them for degradation through ubiquitination. In all instances of which we are currently aware, this modulation process takes place at the level of E3 ligase, that is, at the step where the substrate specificity is critically defined. Some viral proteins act as E3 ligases, and others redirect host ubiquitin E3 ligases to target new substrate proteins. For example, the E6 oncoprotein of human papilloma virus binds

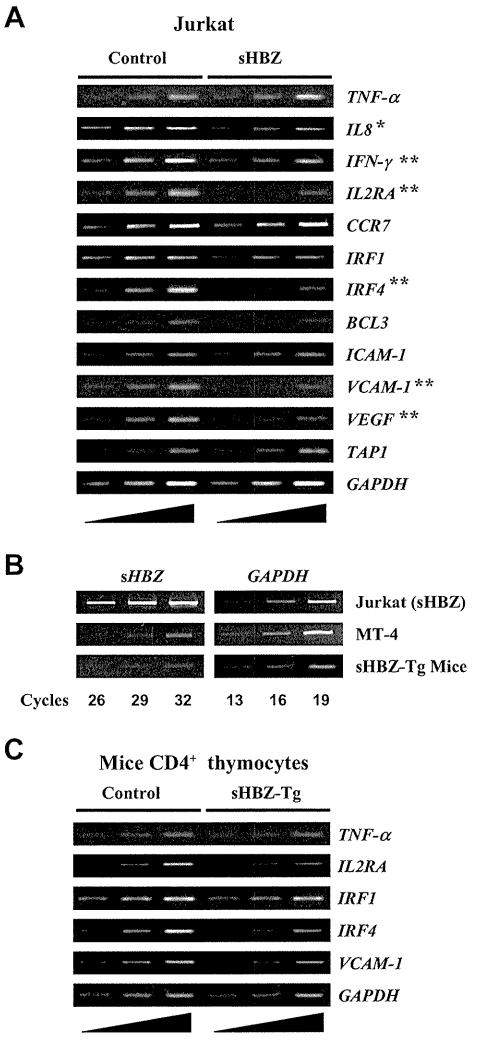

Figure 7. Suppressed expression of selected classical NF-κB target genes in vitro and in vivo by sHBZ. (A) Transcriptional changes of selected classical NF-κB target genes in sHBZ-expressing Jurkat cells. After stimulating the cells with PMA plus ionomycin, the levels of *TNF-α*, *IL-8*, *IFN-γ*, *IL2RA*, *CCR7*, *IRF1*, *IRF4*, *BCL3*, *ICAM-1*, *VCAM-1*, *VEGF*, *TAP1*, and *GAPDH* mRNA were analyzed by increasing cycles of semiquantitative RT-PCR, represented by the ramp on the left. (B) Comparison of the *HBZ* gene transcripts in an ATL cell, MT-4, in HBZ-transfected Jurkat cells, and in CD4+ thymocytes from *HBZ* transgenic mice. All samples were amplified over the same number of PCR cycles as shown. (C) Transcriptional changes of selected classical NF-κB target genes in CD4+ thymocytes from sHBZ transgenic mice. After stimulating the cells with PMA plus ionomycin, the levels of *TNF-α*, *IL2RA*, *IRF1*, *IRF4*, *VCAM-1*, and *GAPDH* mRNA were analyzed by increasing cycles of semiquantitative RT-PCR, represented by the ramp on the left. $^{*}P < .05$; $^{**}P < .01$.

the tumor suppressor p53 through its interaction with another cellular protein, E6-associated protein, leading to the degradation of p53 via the ubiquitin-mediated pathway.[55] VIF encoded by HIV-1 connects APOBEC3G and APOBEC3F as a substrate to the multisubunit E3 ligase for polyubiquitination and degradation.[56] Our study is the first to report that a viral protein enhances expression of the cellular ubiquitin E3 ligase, PDLIM2, resulting in the degradation of p65. In T cells, PDLIM2 can interact with STAT and p65 transcription factors and promote their polyubiquitination and subsequent degradation, thereby negatively regulating STAT and NF-κB–dependent signaling.[41,57] We have not yet clarified whether HBZ can negatively regulate the JAK/STAT pathway. Because PDLIM2 is expressed not only in T cells but also in innate immune cells,[57] we speculate that the positive effect of HBZ on PDLIM2 expression might also influence T-cell proliferation and immune responses.

There are 2 transcripts of the *HBZ* gene. The transcript of the *sHBZ* gene is more abundant than that of usHBZ as reported.[14] Inhibitory effect of sHBZ on Tax mediated transcription from 5′LTR was much stronger than that of usHBZ, and sHBZ has a much longer half-life than usHBZ. Therefore, the protein level of sHBZ is much higher than that of usHBZ,[32] which leads to stronger inhibitory activity of the classical NF-κB pathway as shown in this study. Taken together, sHBZ, rather than usHBZ, is more important in HTLV-1 infected cells.

As shown in this study, HBZ downmodulates the classical NF-κB pathway by 2 mechanisms, (1) inhibition of DNA binding by p65 and (2) enhanced degradation of p65, leading to decreased expression of some RelA specific target genes. Such a function of HBZ in cooperation with Tax-mediated activation might be beneficial for proliferation of infected cells and oncogenesis. Further studies are necessary to clarify the significance of HBZ in proliferation of infected cells and oncogenesis.

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Authorship

Contribution: T.Z., J.Y., Y.S., M.N., M.F., and M.M. designed the research; T.Z., J.Y., Y.S., and M.T. performed the research; T.Z., M.N., M.F., and M.M. analyzed the data; and T.Z., J.Y., M.N., M.F., and M.M. wrote the paper.

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Review

HTLV-1 bZIP factor gene: Its roles in HTLV-1 pathogenesis

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ABSTRACT

The HTLV-1 bZIP factor (HBZ) gene is transcribed as an anti-sense transcript of HTLV-1 from the 3′ long terminal repeat (LTR). Recent studies showed that the *HBZ* gene was expressed in all ATL cases, suggesting its critical role in leukemogenesis. In addition, only the *HBZ* gene sequence remains intact, unaffected by nonsense mutations and deletion. HBZ mRNA promotes proliferation of adult T-cell leukemia (ATL) cells. The HBZ protein has three domains: activation, central, and bZIP domains. HBZ interacts with a variety of cellular factors, and modulates not only cellular functions, but also viral gene transcription from 5′LTR. The complex functions of HBZ modulate T-cells, and promote their proliferation, which is likely indispensable for leukemogenesis by HTLV-1.

Contents

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) was first reported in 1980 (Poiesz et al., 1980). Thereafter, its causative association with adult T-cell leukemia (ATL) was clarified (Hinuma et al., 1981). The whole sequence was reported by Yoshida's group (Seiki et al., 1983), who revealed the unique region between env and 3′ long terminal repeat (LTR), designated the pX region. The pX region encodes regulatory genes, *tax* and *rex*, in addition to accessory genes, *p12*, *p13*, and

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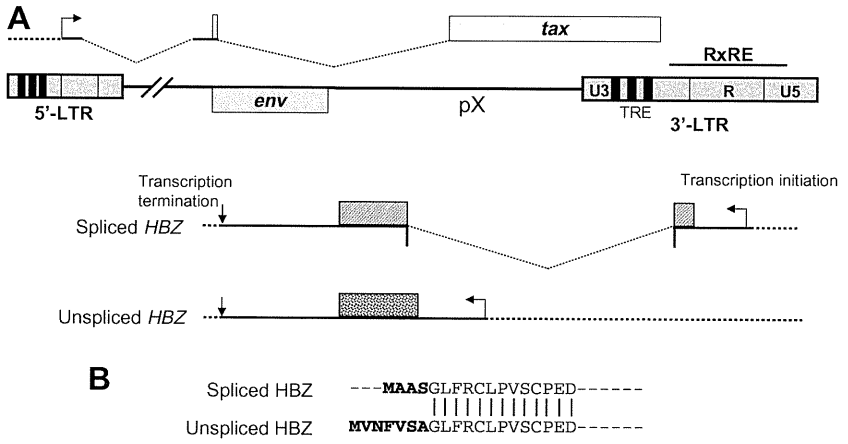

Fig. 1. Spliced and unspliced *HBZ* genes. Schema of the spliced and unspliced *HBZ* genes is shown (A). Structure of *tax* gene is also shown. The coding sequences of the *HBZ* and *tax* gene do not overlap. The N-terminal region of predicted amino acid sequences are shown in (B).

p30. At that time, researchers of HTLV-1 noticed an open reading frame in the minus strand. The presence of an anti-sense transcript of HTLV-1 was first detected by Northern blot in HTLV-1-infected cell lines (Larocca et al., 1989). However, the function and exact structure of this gene product remained unknown. This is because researchers naturally focused on Tax, a fascinating viral protein with pleiotropic actions, and the concept of anti-sense transcription was not so common at that time. In 2002, a viral protein that binds to CREB-2 was found by yeast two-hybrid screening, and named HTLV-1 bZIP factor (HBZ). HBZ was first found to inhibit viral gene transcription of the sense strand (Gaudray et al., 2002). In a separate study, we analyzed HTLV-1 provirus in ATL cells, and reported that Tax expression was frequently disrupted by several mechanisms (Matsuoka and Jeang, 2007). However, the *HBZ* gene sequence, as well as the 3′LTR that is its promoter, remained intact (Fan et al., 2010; Yoshida et al., 2008). In addition, the spliced form of the *HBZ* gene transcript was identified and was expressed in all ATL cases. Importantly, *HBZ* gene expression promoted proliferation of ATL cells (Satou et al., 2006). Thus, HBZ is now recognized as a critical gene for ATL cells. In this review, recent findings on HBZ are summarized, and its roles in HTLV-1 infectivity and oncogenesis discussed.

2. Structure and transcription of the *HBZ* gene

Transcription start sites of the *HBZ* gene were identified by 5′ RACE (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). These studies revealed different transcripts of the *HBZ* gene: a spliced form (sHBZ) and an unspliced form (usHBZ) (Fig. 1). The first exon of the *sHBZ* gene transcript is present in U3 and R regions of the 3′LTR. The difference between sHBZ and usHBZ is only a few amino acids, as shown in Fig. 1B. Transcription start sites of the spliced *HBZ* gene were scattered in the U5 and R regions of the 3′LTR, which is consistent with the finding that the predicted promoter was TATA-less (Yoshida et al., 2008). Analyses of transcription factor binding sites of the promoter region showed that three Sp1 binding sites were critical for transcription of the *HBZ* gene while the third Sp1 site was most important.

Binding of Sp1 to this site was proved by chromatin immunoprecipitation. It has been reported that Sp1 is critical for many TATA-less promoters (Boam et al., 1995; Liu and Cowell, 2000). Since Sp1 is a well-known regulator of housekeeping genes, transcription of the *sHBZ* gene may be relatively constant. Taken together, Sp1 is critical for transcription of the *HBZ* gene. As described below, the *HBZ* gene transcript is better correlated with provirus load than the *tax* gene transcript (Saito et al., 2009), indicating that the *HBZ* gene is constantly expressed in HTLV-1 infected cells. Thus, transcription from the minus strand contrasts with that from the 5′LTR on the plus strand, which is highly inducible by Tax. Viral genes other than the *HBZ* gene are transcribed by the 5′LTR and HBZ suppresses transcription from the 5′LTR. This is an ingenious mechanism to control expression of viral proteins. Tax can activate transcription of the *HBZ* gene through a Tax responsive element (TRE) (Landry et al., 2009; Yoshida et al., 2008). However, its enhancement is not so significant, and Tax expression is usually controlled *in vivo*. Therefore, the significance of Tax mediated upregulation of the *HBZ* gene transcription remains to be determined.

Quantitative analyses of the *HBZ* gene transcripts were reported by two groups (Saito et al., 2009; Usui et al., 2008). Transcripts of the spliced form of the *HBZ* gene were fourfold more abundant than those of the *usHBZ* gene (Usui et al., 2008). This observation correlates with the finding that the promoter activity of the *sHBZ* gene was much higher than that of the us*HBZ* gene (Yoshida et al., 2008). Relative expression level of the *HBZ* gene that was adjusted by provirus load was almost equivalent among HTLV-1 carriers, HAM/TSP patients, and ATL patients (Saito et al., 2009). A previous study reported that HTLV-1 provirus load was correlated with the expression level of the *tax* gene (Yamano et al., 2002). However, the *HBZ* gene transcript was more closely correlated with provirus load than was the level of the *tax* gene transcripts (Saito et al., 2009). Kinetic study of the *sHBZ* gene transcripts in rabbits shows that *sHBZ* gene transcription was detected one week post-infection and