Induction of IκB-ζ by Epstein-Barr virus latent membrane protein-1 and CD30

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Abstract. Activation of nuclear factor-κB (NF-κB) in Burkitt's lymphoma (BL) and Hodgkin's lymphoma (HL) cells is important in the transformation and development process of these lymphomas. Epstein-Barr virus (EBV) latent membrane protein-1 (LMP-1) and ligand-independent signaling by overexpressed CD30 are known to cause permanent activation of NF-κB in lymphomas. However, hyperactivation of NF-κB triggers cellular senescence and apoptosis. Here, we show that IκB-ζ, an inducible regulator of NF-κB, is constitutively expressed in BL and HL cell lines. In addition, immunohistochemical staining identified nuclear IκB-ζ-positive BL cells, and Hodgkin and Reed-Sternberg cells in lymph nodes. Expression of LMP-1 and CD30 increased IκB-ζ expression at the transcriptional level. IκB-ζ promoter was regulated by activation of the NF-κB-inducing kinase (NIK)/IκB kinase/NF-κB pathway via the carboxyl-terminal tumor necrosis factor (TNF) receptor-associated factor (TRAF)-interacting regions of LMP-1 and CD30. Interestingly, IκB-ζ inhibited NF-κB activation by LMP-1 and CD30. The results suggest that NF-κB-induced IκB-ζ negatively modulates NF-κB hyperactivation, resulting in a fine balance that ultimately endows a net evolutionary benefit to the survival of BL and HL cells.

Introduction

The nuclear factor-κB (NF-κB) plays a key role in several cellular functions, e.g., sustenance of proliferative signaling, evasion of growth suppression, resistance to cell death, ability of replicative immortality, and activation of invasion and metastasis in hematological malignancies (1). The inflammatory process has emerged as a useful marker of cancer progression (2,3). NF-κB is also involved in the induction of inflammation (2,3). Constitutive activation of NF-κB occurs in most malignant lymphomas and plays a major role in lymphomagenesis and clinical aggressiveness (1). Furthermore, Epstein-Barr virus (EBV) latent membrane protein-1 (LMP-1) and CD30 overexpression have been shown to activate NF-κB and induce rapidly progressing lymphomas (1,4).

EBV is associated with the development of lymphomas including Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL), diffuse large B-cell lymphoma and natural killer/T-cell lymphoma (5). LMP-1, a transmembrane protein, is essential for *in vitro* transformation of primary B cells (6). The carboxyl-terminal cytoplasmic domain of LMP-1 contains two carboxyl-terminal activation regions (CTARs); CTAR-1 and CTAR-2. CTAR-1 binds to tumor necrosis factor (TNF) receptor-associated factors (TRAFs) (7), whereas CTAR-2 binds to the TNF receptor-associated death domain (TRADD) (8). NF-κB activation by the CTAR-1 and CTAR-2 domains of LMP-1 is probably mediated by the binding of TRAFs directly or indirectly to both the CTAR-1 and CTAR-2 domains (7-10).

CD30, a member of the TNF receptor superfamily, is also a transmembrane protein and highly expressed in a variety of lymphoma subsets including HL. Overexpression of CD30 was reported to transduce signals independent of CD30 ligand in HL cells (11). A region of ~100 amino acids from the carboxyl-terminal region of CD30 is involved in NF-κB activation (12). TRAFs recognize the carboxyl-terminal D2 and D3 subdomains of CD30 (12).

Activation of NF-κB has often been linked to recurrence, poor survival, tumor progression, aggressiveness and chemoresistance (13). However, there are also studies that found NF-κB or upstream activators rather to act as tumor suppressors. Contributing to its anticancer property, NF-κB has been shown to mediate apoptosis in a variety of cell types (14). Overexpression of RelA (p65) caused a cell cycle arrest followed by apoptosis (15). Premature cellular senescence is a terminal cell cycle arrest that can be induced by oncogenic activation or chemotherapy (16,17). NF-κB also participates in a senescence-associated cytokine response (18). Therefore,

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appropriate regulation of NF-κB is critical for the proper function and survival of the cell.

IκB-ζ is an atypical nuclear member of the IκB family (19). The activity of NF-κB is modulated in a gene-specific manner by IκB-ζ. In contrast to classical IκB proteins that are constitutively expressed and controlled by inducible degradation, IκB-ζ expression is barely detectable in resting cells but is rapidly induced by various pro-inflammatory stimuli, such as lipopolysaccharides and interleukin (IL)-1β (20). IκB-ζ regulates NF-κB signaling, and reporter analyses suggested that IκB-ζ may act as an inhibitor of NF-κB (19). In contrast, other studies have reported that IκB-ζ can induce gene expression of individual NF-κB target genes (21). A recent study identified the nuclear IκB-ζ to be upregulated in activated B-cell-like subtype of diffuse large B-cell lymphoma (22). We have also reported constitutive expression of IκB-ζ in adult T-cell leukemia cells (23). The hypothesis tested in the present study was that IκB-ζ is induced by LMP-1 and CD30 and that it is also involved in regulation of NF-κB.

Materials and methods

Cell culture. Raji and Daudi are EBV-positive BL cell lines. In contrast, BJAB and Ramos are EBV-negative BL cell lines. B95/Ramos is Ramos infected with the B95-8 strain of EBV. L428, KM-H2, HDLM-2 and L540 are HL cell lines. These cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10 or 20% fetal bovine serum (FBS) and antibiotics. Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics.

RNA detection. Total RNA was extracted from various cell cultures by TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The first-strand cDNA was synthesized from 1 μg cellular RNA using a PrimeScript RT-PCR kit (Takara Bio Inc., Otsu, Japan) with random primers. The sequences of the primers used are summarized in Table I.

Plasmids, transfection and luciferase assay. Cells (293T) were transfected by the calcium phosphate DNA coprecipitation method. The expression plasmids pSG5-LMP-1, pSG5-LMP-1Δ187-351, pSG5-LMP-1Δ349 and pSG5-LMP-1Δ194-386 were previously described (24,25). For CD30 expression, the plasmids wild-type human CD30 (pME-hCD30) and its mutant [pCR-hCD30(Δ95)] were used (12). The wild-type and various mutants of IκB-ζ, and pcDNA3-RelA were described previously (26,27). The dominant-negative mutants of IκBα, IκBβ, IκB kinase (IKK) α, IKKβ, IKKγ and NF-κB-inducing kinase (NIK) have been previously described (28-31). The plasmid for truncated TRAF2 protein with retention of only the TRAF domain, ΔTRAF2, has been described previously (32). The human IκB-ζ promoter-luciferase gene constructs have already been described (23,33). The single and combined internal deletion mutants of NF-κB sites were constructed by deletion of the NF-κB sites of the plasmid pGL3-hIκB-ζ(-853) (23). A reporter plasmid, expressing luciferase through a minimal promoter linked to five copies of the typical NF-κB responsive element from the *IL-2 receptor α chain* (*IL-2Rα*) gene (κB-LUC), was used to measure the NF-κB transcription competence (34). Two copies of the IL-8 activator protein-1 (AP-1) binding site were inserted upstream of the IL-8 enhancer-less core promoter linked to luciferase gene (AP-1-LUC) (35). Plasmids containing the IL-8 promoter (-133 to +44 bp) and the IL-6 promoter (-225 to +14 bp) linked to luciferase expression vectors were constructed from luciferase expression vectors (35,36). Bcl-3 luciferase reporter construct was described previously (37). In all cases, phRL-TK was cotransfected to correct for transfection efficiency. After 24 h, luciferase assays were conducted using the dual luciferase reporter system (Promega Corp., Madison, WI, USA), in which the relative luciferase activity was calculated by normalizing transfection relative to the *Renilla* luciferase activities. Data were expressed as mean ± SD of three experiments.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted and transcription factors bound to specific DNA sequences were examined by EMSA, as previously described (38). The top strand sequences of the oligonucleotide probes or competitors were as follows: for the NF-κB element (κB1) of the *IκB-ζ* gene, 5'-GATCCGACGGGAATGTCCGGGACT-3'; for the mutated κB1 sequence, 5'-GATCCGACGtGtATGaCCGGGACT-3'; for the NF-κB element (κB2) of the *IκB-ζ* gene, 5'-GATCGGTCTGGGAATTTCCAGTG-3'; for the mutated κB2 sequence, 5'-GATCGGTCTGtGtATaaCCAGTG; for the NF-κB element of the *IL-2Rα* gene, 5'-GATCCGGCAGGGGAATCTCCCTCTC-3'; and for the AP-1 element of the *IL-8* gene, 5'-GATCGTGATGACTCAGGTT-3'. The above underlined sequences are the NF-κB and AP-1 binding sites, respectively. The sites of mutation are indicated in lowercase letters. In competition experiments, the nuclear extract was pre-incubated with 100-fold excess of unlabeled oligonucleotides for 15 min. To identify NF-κB proteins in the DNA-protein complex shown by EMSA, we used antibodies specific for various NF-κB family proteins, including p50, RelA, c-Rel, p52 and RelB (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). These antibodies were incubated with the nuclear extracts for 45 min at room temperature before incubation with radiolabeled probes.

Immunohistochemical analysis. Lymph node biopsy samples were obtained from patients with BL and HL. IκB-ζ immunohistochemistry was performed using an anti-IκB-ζ antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) after pretreatment of deparafinized tissue sections with ready-to-use proteinase K (Dako, Carpinteria, CA, USA). The sections were counterstained with methyl green, hydrated in ethanol, cleaned in xylene and mounted. Informed consent was obtained from all tissue donors.

Results

Upregulated IκB-ζ expression in BL and HL. To investigate the role of IκB-ζ in the pathogenesis of BL and HL, we assessed IκB-ζ mRNA expression levels in established BL and HL cell lines using reverse-transcription polymerase chain reaction (RT-PCR). We found that IκB-ζ mRNA expression was limited to EBV-infected BL cell lines but not in uninfected

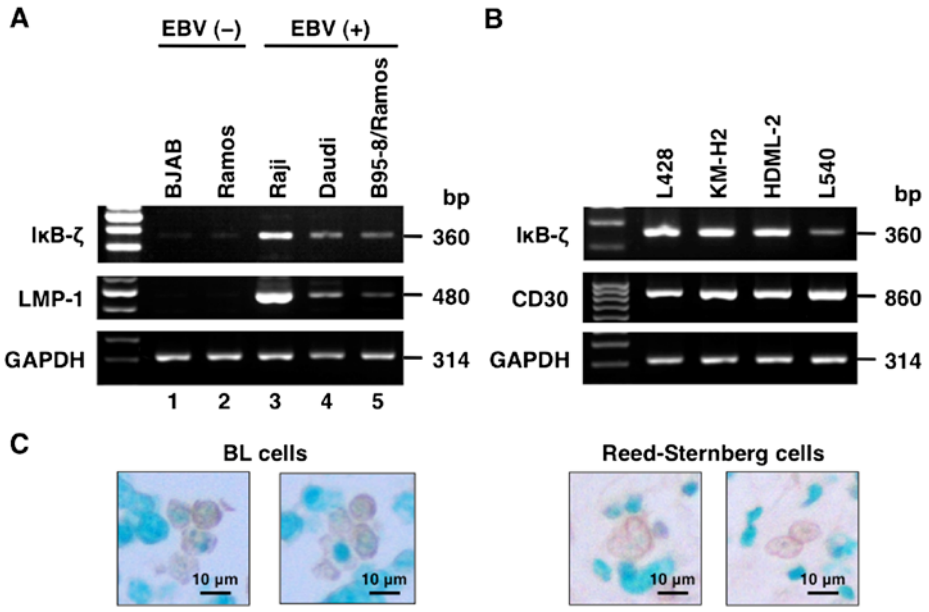

Figure 1. Expression of IκB-ζ in BL and HL cells. (A) RT-PCR analysis of the indicated genes expression in EBV-negative (lanes 1 and 2) and -positive BL cell lines (lanes 3-5). (B) RT-PCR analysis of expression of the indicated genes in HL cell lines. (C) Immunohistochemical staining of IκB-ζ in BL and HL lymph nodes. Tissue biopsy sections were stained with anti-IκB-ζ antibody. Tissue sections were counterstained using methyl green. Magnification, x1,200.

Table I. Primer sequences.

Gene name	Forward (5')	Reverse (3')
IκB-ζ	GGAGCTTTTACTGAAGAATAAGA	ATCTGTTCTCCCACAGGGCCATC
LMP-1	GTGACTGGACTGGAGGAGCC	GAGGGAGTCATCGTGGTGGTG
CD30	CTGTGTCCCCTACCCAATCT	CTTCTTTCCCTTCCTCTTCCA
IL-6	ATGAACTCCTTCTCCACAAGC	CTACATTTGCCGAAGAGCCCTCAGGCTGGACTG
IL-8	ATGACTTCCAAGCTGGCCGTG	TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC
GAPDH	GCCAAGGTCATCCATGACAACTTTGG	GCCTGCTTCACCACCTTCTTGATGTC

cells (Raji, Daudi and B95-8/Ramos) (Fig. 1A). On the other hand, all HL cell lines showed IκB-ζ mRNA levels (Fig. 1B). All EBV-infected BL cell lines and HL cell lines constitutively expressed LMP-1 and CD30, respectively (Fig. 1A and B). Immunohistochemical staining of BL cells, and Hodgkin and Reed-Sternberg cells in lymph nodes showed abundant IκB-ζ protein in the nuclei of these cells (Fig. 1C).

LMP-1 and CD30 induce IκB-ζ mRNA expression. To investigate the induction of IκB-ζ in BL and HL, we performed transient expression assays using mammalian expression vectors for LMP-1 and CD30 in 293T cells. After the transfection, RNA was extracted from the cells and the IκB-ζ mRNA levels were analyzed by RT-PCR. IκB-ζ mRNA induction was observed at 48 and 24 h after LMP-1 and CD30 transfection, respectively. In contrast, IκB-ζ mRNA was hardly detected in cells transfected with empty vectors (pSG5 and pME18S) (Fig. 2A and B).

LMP-1 and CD30 activate the IκB-ζ promoter. To determine whether LMP-1 and CD30 regulate IκB-ζ promoter activity, transient expression assays were performed using the reporter plasmids, pGL3-hIκB-ζ(-11k) and pGL3-hIκB-ζ(-853), and expression vectors for LMP-1 and CD30. LMP-1 and CD30 transactivated the -11 kb and -853 IκB-ζ promoter fragments, but this effect was lost in pGL3-basic. LMP-1 and CD30 induced relative levels of IκB-ζ promoter-directed luciferase expression in a dose-dependent manner, suggesting that LMP-1 and CD30 functionally activate minimum IκB-ζ promoter between -853 and -17 bp (Fig. 2C and D).

Importance of carboxyl-terminal regions of LMP-1 and CD30 for IκB-ζ promoter activation. To map the regions in the LMP-1 and CD30 proteins that mediate activation of IκB-ζ promoter, LMP-1 and CD30 mutants were expressed and their effect on IκB-ζ promoter activity was investigated. The LMP-1 mutants used included LMP-1Δ187-351 (which contains only CTAR-2 in the carboxyl-terminus), LMP-1Δ349 (which lacks CTAR-2) and LMP-1Δ194-386 (in which the entire carboxyl-terminal cytoplasmic region is deleted) (Fig. 3A). In cells that expressed CTARs-free LMP-1Δ194-386, IκB-ζ promoter activity was not increased. In contrast, activation of pGL3-hIκB-ζ(-11k) and pGL3-hIκB-ζ(-853) was observed by both CTAR-1-free LMP-1Δ187-351 and CTAR-2-free LMP-1Δ349, although to a lesser extent than by wild-type LMP-1 (Fig. 3B, lower panel). These results suggest that LMP-1 activates IκB-ζ expression

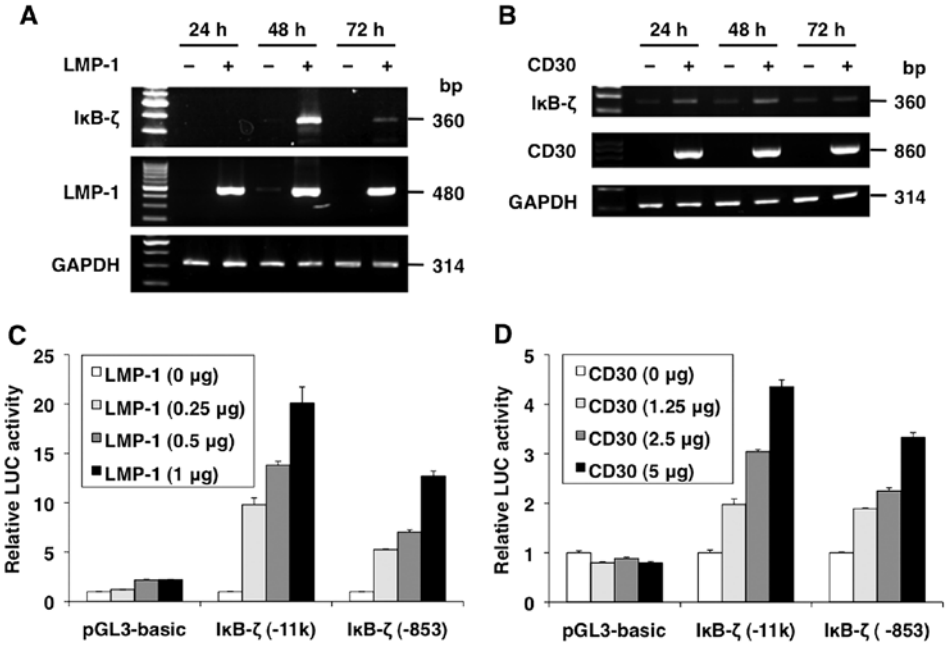

Figure 2. Ectopic expression of LMP-1 and CD30 induces IκB-ζ expression at transcriptional level. (A) LMP-1 and (B) CD30 upregulate IκB-ζ mRNA expression. Cells (293T) were transfected with plasmids encoding LMP-1 and CD30, or empty vectors. RNA was isolated from cells at the indicated times, and RT-PCR was carried out. (C) LMP-1 and (D) CD30 activate the IκB-ζ promoter. Cells (293T) were transfected with the indicated IκB-ζ promoter fragments cloned into pGL3-basic together with increasing amounts of (C) LMP-1 or (D) CD30 expression plasmid. The activities are expressed relative to that of cells transfected with the indicated reporter plasmids and empty vectors, which was defined as 1. LUC, luciferase.

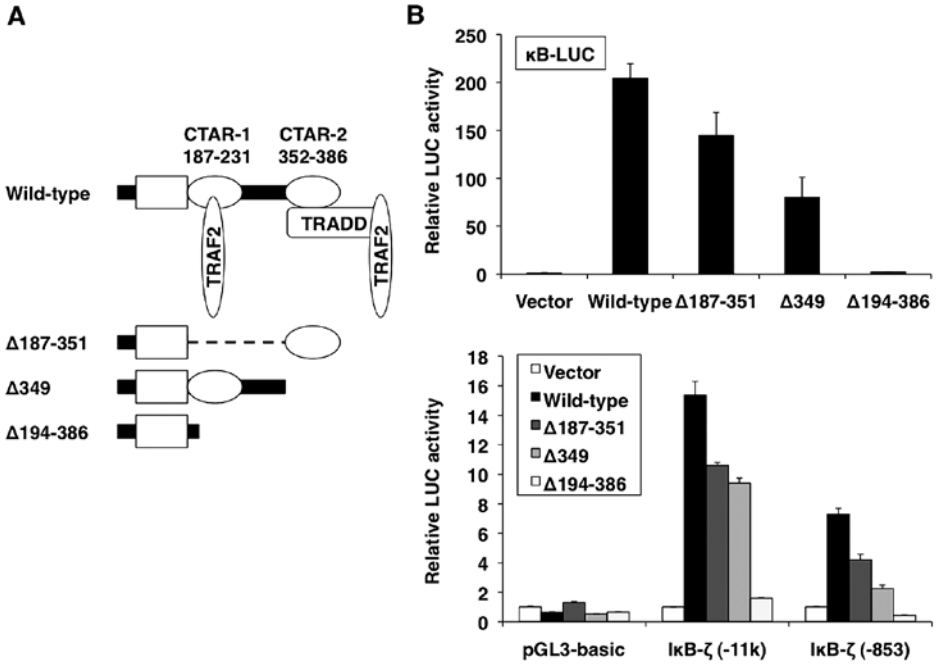

Figure 3. Deletions in CTAR-1 and CTAR-2 abrogate the effect of LMP-1 on IκB-ζ promoter activity. Cells (293T) were cotransfected with plasmids encoding wild-type or various mutants of LMP-1 (A) and with κB-LUC (B, upper panel) or the indicated IκB-ζ promoter reporter plasmids (B, lower panel). The results are represented as fold induction by wild-type or mutants of LMP-1 relative to the vector alone.

via the cooperative activity of CTAR-1 and CTAR-2 signaling motifs. As measured in an NF-κB-dependent luciferase reporter gene assay, LMP-1 increased NF-κB activation via CTAR-1 and CTAR-2 (Fig. 3B, upper panel).

The carboxyl-terminal region of CD30 is also essential for signal transduction (Fig. 4A) (12). Next, we investigated whether the carboxyl-terminal region of CD30 plays a role in the induction of IκB-ζ. As shown in Fig. 4B, lower panel, IκB-ζ-driven reporter gene activity was not increased by CD30Δ95, which lacks the carboxyl-terminal region of CD30. Notably, the effect of the structural context of the carboxyl-terminal region of LMP-1 and CD30 on IκB-ζ promoter activation correlated with NF-κB activation as reporter analyses by mutant constructs (Figs. 3B and 4B, upper

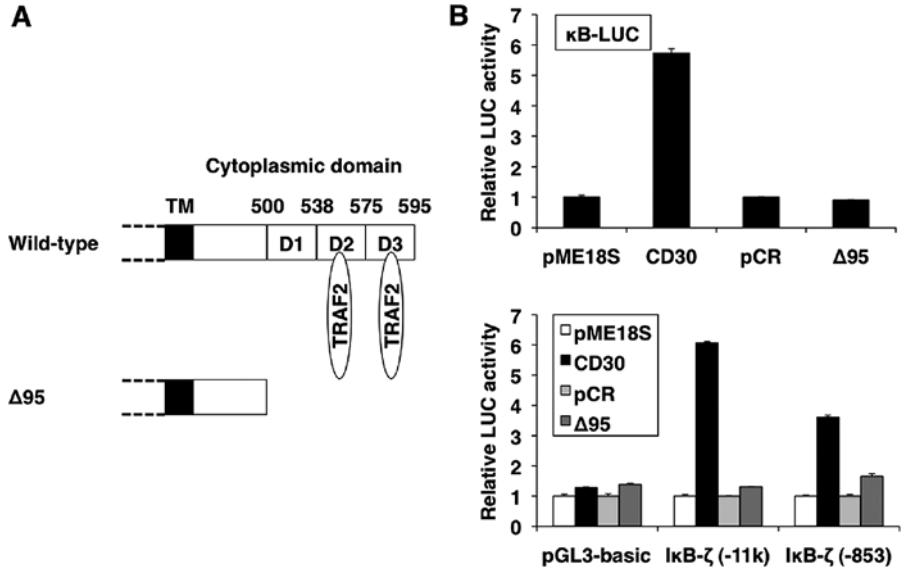

Figure 4. The carboxyl-terminal domain of CD30 is required for its activity. (A) Schematic diagrams of wild-type and mutant CD30 protein. TM, transmembrane domain. (B) Induction of IκB-ζ transcriptional activity by wild-type and deletion mutant of CD30. Cells (293T) were transfected with wild-type or CD30 mutant and κB-LUC (B, upper panel) or the indicated IκB-ζ promoter reporter plasmids (B, lower panel). Negative control vectors pME18S and pCR are used for pME-hCD30 and pCR-hCD30(Δ95), respectively.

panels). These results suggest that LMP-1 and CD30 activate IκB-ζ promoter through the NF-κB signaling pathway.

LMP-1 and CD30 activate IκB-ζ promoter activity via the NF-κB signaling pathway. LMP-1 and CD30 are constitutively aggregated pseudo-TNF receptors that activate NF-κB through their carboxyl-terminal cytoplasmic domains associated with TRAF2 (7-12). Aggregated TRAF2 activates NIK and its downstream target, the IKK complex, which is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (39,40). The IKK complex phosphorylates the inhibitory IκB proteins, which are bound to NF-κB in the cytosol. Their phosphorylation is followed by their degradation, dissociation of NF-κB from the inhibitors, and NF-κB translocation into the nucleus (41). In order to determine the role of TRAF2/NIK/IKK/IκB proteins in mediating IκB-ζ activation induced by LMP-1 and CD30, 293T cells were cotransfected with LMP-1 or CD30 expression plasmid and plasmids expressing dominant-negative forms of TRAF2, NIK, IKKα, IKKβ, IKKγ, IκBα or IκBβ. All dominant-negative mutants reduced IκB-ζ promoter activation by LMP-1 and CD30 (Fig. 5A). These data indicate that the activation of NF-κB through TRAF2/NIK/IKK plays a role in the activation of IκB-ζ promoter by LMP-1 and CD30.

NF-κB sites in the promoter are essential for the transcriptional upregulation of the IκB-ζ gene. The human IκB-ζ promoter contains two NF-κB motifs (κB1 and κB2) (Fig. 5B, top panel) (33). To determine the involvement of κB1 and κB2 in the induction of *IκB-ζ* gene expression by LMP-1 and CD30, we investigated the activity of IκB-ζ promoter with deletions in κB1 and κB2 sites. As shown in Fig. 5B, LMP-1 and CD30 activated the wild-type promoter pGL3-hIκB-ζ(-853) activity. A single deletion of the κB2 site from the IκB-ζ reporter plasmid (κB2D) markedly inhibited LMP-1- or CD30-induced transactivation, whereas a single deletion of the κB1 site (κB1D) resulted in moderate activation. These data indicate that IκB-ζ κB2 site was necessary for transcription of IκB-ζ. Furthermore, double deletions (κB1/κB2D) completely abolished the LMP-1- or CD30-induced transactivation. Expression of RelA is sufficient to induce IκB-ζ expression. The predominant role of κB2 in the induction of IκB-ζ expression was further supported by the finding that a promoter with a deleted κB2 site failed to respond to overexpressed RelA (Fig. 5B, bottom panel). In contrast, a promoter with a deleted κB1 was slightly activated by RelA. Considered together, these data suggest that upregulation of IκB-ζ by LMP-1 and CD30 requires both the κB1 and κB2 sites of the IκB-ζ promoter.

Since the deletional analysis of the IκB-ζ promoter indicated that LMP-1 and CD30 activated transcription through both the κB1 and κB2 sites, it was important to identify the nuclear factors that bind to these sites. Using the κB1 and κB2 sequences in the IκB-ζ promoter as the probes in EMSA, NF-κB binding was detected in 293T cells transfected with LMP-1 (Fig. 6A, lane 2) and CD30 (Fig. 7A, lane 2). In addition, formation of these complexes was competed with excess of unlabeled wild-type κB1, κB2 and consensus IL-2R κB oligonucleotides (Figs. 6A and 7A, lanes 3, 5 and 7). In contrast, mutated oligonucleotides, κB1 mut and κB2 mut, and irrelevant consensus IL-8 AP-1 oligonucleotide, did not compete with the labeled probes (Figs. 6A and 7A, lanes 4, 6 and 8). Supershift analyses demonstrated that the κB1 and κB2 complexes contained both p50 and RelA subunits of the NF-κB family (Figs. 6A and 7A, lanes 9 and 10). To determine the role of LMP-1 and CD30 on endogenous NF-κB binding to DNA, we measured NF-κB binding to respective NF-κB sites in the IκB-ζ promoter in LMP-1-expressing Daudi cells and CD30-expressing L428 and HDLM-2 cells. As expected, protein complexes bound to both κB1 and κB2 sites were detected in nuclear extracts from Daudi, L428 and HDLM-2 cells (Figs. 6B and 7B, lane 1). The specificity of

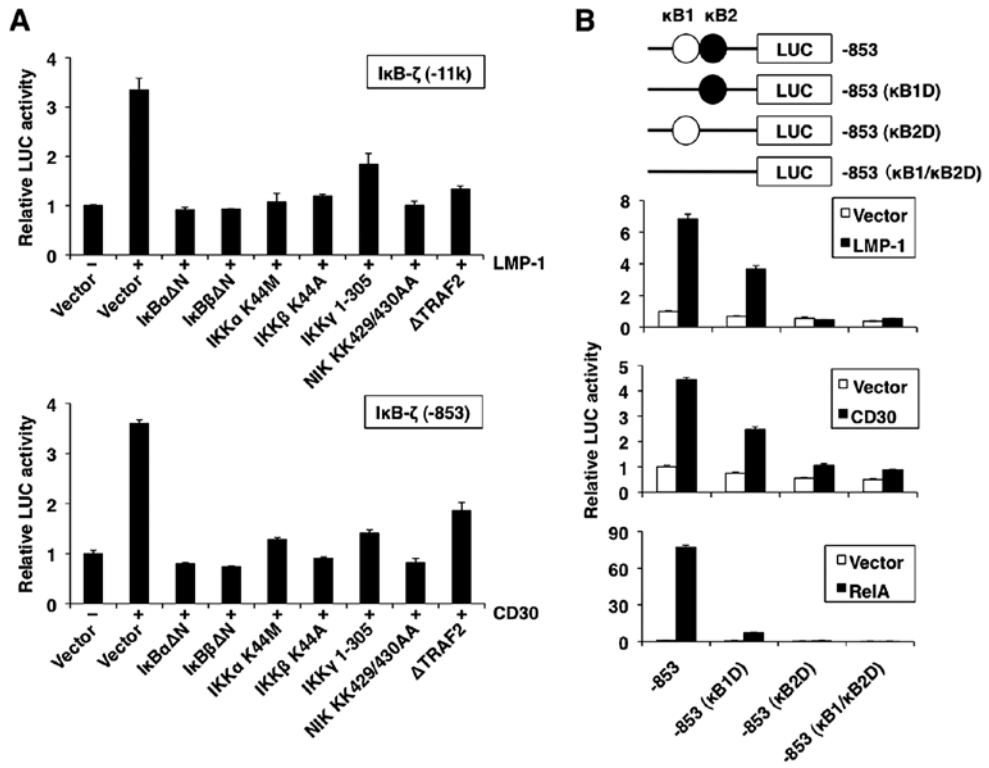

Figure 5. LMP-1 and CD30 activate the IκB-ζ promoter via the NF-κB signaling pathway. (A) Effects of TRAF2-, NIK-, IKKs- and IκBs-dominant-negative mutants on LMP-1- and CD30-mediated activation of the IκB-ζ promoter. Cells (293T) were transfected with pGL3-hIκB-ζ(-11k) or pGL3-hIκB-ζ(-853) together with LMP-1 or CD30 and the indicated dominant-negative mutants or empty vector. The activities are expressed relative to that of cells transfected with pGL3-hIκB-ζ(-11k) or pGL3-hIκB-ζ(-853) and empty vectors, which was defined as 1. (B) Cells (293T) were transfected with pGL3-hIκB-ζ(-853) or the single and combined internal deletion mutants of NF-κB sites, together with either empty vector or expression plasmid for LMP-1, CD30 or RelA. Schematic diagrams of the IκB-ζ reporter constructs containing the wild-type [pGL3-hIκB-ζ(-853)] and internal deletion mutants of κB1 and/or κB2 motifs are indicated on the top of the figure. The activities are expressed relative to that of cells transfected with pGL3-hIκB-ζ(-853) and an empty vector, which was defined as 1.

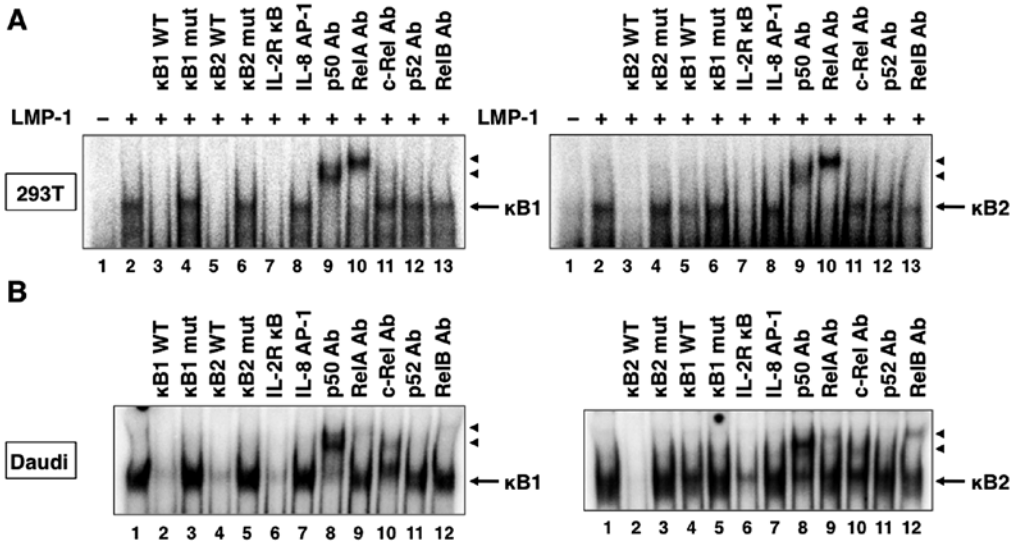

Figure 6. LMP-1 induces NF-κB binding to the NF-κB sites in the IκB-ζ promoter. (A) Cells (293T) were transfected with control or LMP-1 expression plasmid. Nuclear proteins were extracted 48 h after transfection. Nuclear extracts from transfected 293T cells (A) or Daudi cells (B) were incubated with the labeled DNA probes representing the IκB-ζ κB1 and κB2 sites. Nuclear extracts were subjected to competition analysis with an excess of unlabeled oligonucleotides representing the IκB-ζ κB1 and κB2 sites (lanes 3 and 5 in A, and lanes 2 and 4 in B, respectively), IκB-ζ mutated κB1 and κB2 sites (lanes 4 and 6 in A, and lanes 3 and 5 in B), a consensus NF-κB site from the IL-2Rα promoter (lane 7 in A, and lane 6 in B) or an AP-1 site from the IL-8 promoter (lane 8 in A, and lane 7 in B). Nuclear extracts were also subjected to supershift assays with either no antibody (lane 2 in A, and lane 1 in B) or the indicated antibodies (Ab) (lanes 9-13 in A, and lanes 8-12 in B). Arrows, specific complexes; arrowheads, DNA binding complex supershifted by the antibody.

DNA-protein complexes in these extracts was determined by competition studies using unlabeled competitors. As observed in nuclear extracts from 293T cells transfected with LMP-1 and CD30 expression plasmids, unlabeled κB1 and κB2

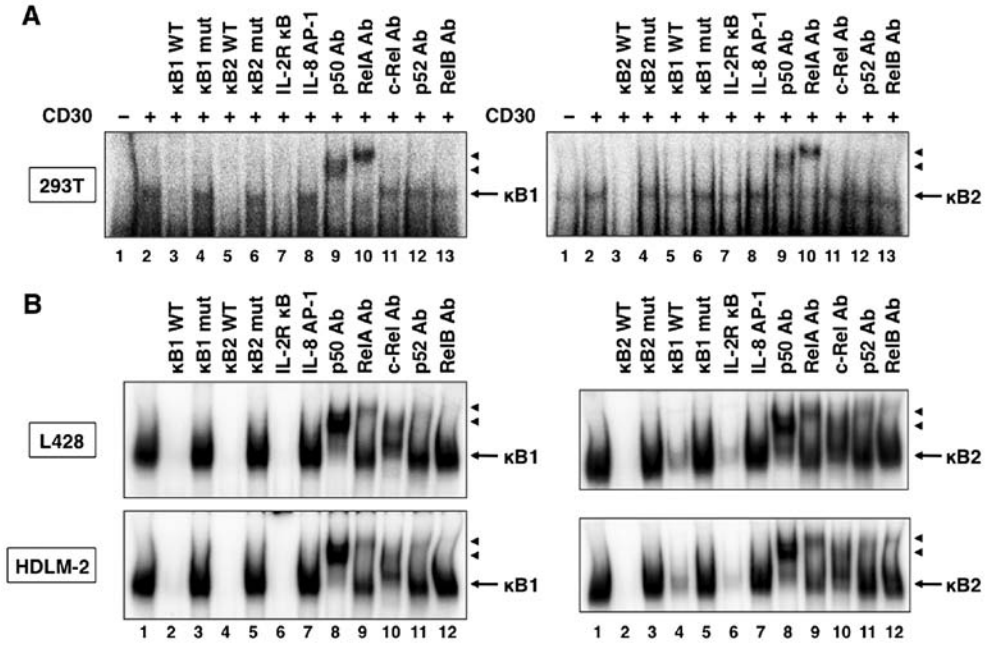

Figure 7. CD30 induces NF-κB binding to the NF-κB sites in the IκB-ζ promoter. (A) Cells (293T) were transfected with control or CD30 expression plasmid. Nuclear proteins were extracted 48 h after transfection. Nuclear extracts from transfected 293T cells (A), L428 or HDLM-2 cells (B) were incubated with the labeled DNA probes representing the IκB-ζ κB1 and κB2 sites. Nuclear extracts were subjected to competition analysis with an excess of unlabeled oligonucleotides representing the IκB-ζ κB1 and κB2 sites (lanes 3 and 5 in A, and lanes 2 and 4 in B, respectively), IκB-ζ mutated κB1 and κB2 sites (lanes 4 and 6 in A, and lanes 3 and 5 in B), a consensus NF-κB site from the IL-2Rα promoter (lane 7 in A and lane 6 in B) or an AP-1 site from the IL-8 promoter (lane 8 in A and lane 7 in B). Nuclear extracts were also subjected to supershift assays with either no antibody (lane 2 in A and lane 1 in B) or the indicated antibodies (Ab) (lanes 9-13 in A, and lanes 8-12 in B). Arrows, specific complexes; arrowheads, DNA binding complex supershifted by the antibody.

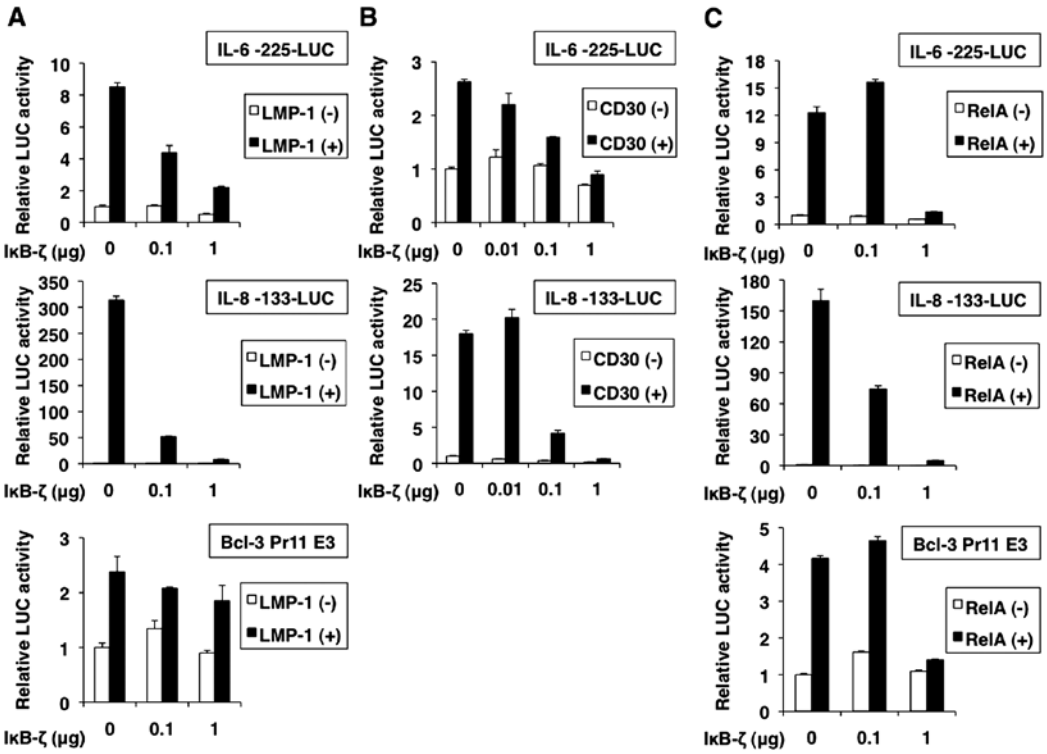

Figure 8. Cotransfection of IκB-ζ inhibits the LMP-1-, CD30- and RelA-mediated transactivation of IL-6, IL-8 and Bcl-3 promoters. Cells (293T) were transfected with IL-6, IL-8 or Bcl-3 promoter reporter plasmid together with the expression plasmid for LMP-1 (A), CD30 (B) or RelA (C), and the indicated amounts of IκB-ζ expression plasmid. The activities are expressed relative to that of cells transfected with the indicated reporter plasmids and empty vectors, which was defined as 1.

oligonucleotides, and consensus NF-κB site from the IL-2Rα promoter, but not the mutated κB1 and κB2 oligonucleotides, and consensus AP-1 element, efficiently competed with the labeled probes (Figs. 6B and 7B, lanes 2-7). Antibodies

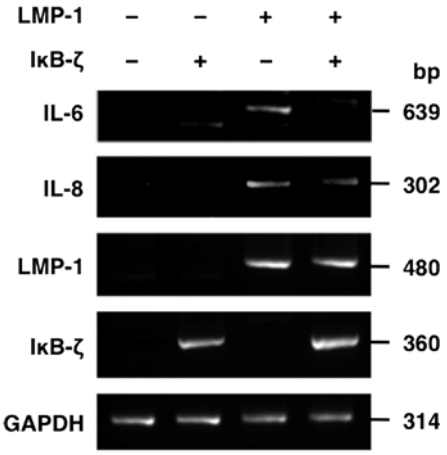

Figure 9. Cotransfection of IκB-ζ inhibits the LMP-1-induced expression of IL-6 and IL-8. Cells (293T) were transfected with the expression plasmid for LMP-1 together with the IκB-ζ expression plasmid. RNA was extracted 48 h after transfection. The mRNA expression level of the indicated genes was analyzed by RT-PCR.

against p50, RelA, c-Rel and RelB induced a supershift of the DNA-protein complexes in nuclear extracts of Daudi cells (Fig. 6B, lanes 8-10 and 12), whereas the κB1 and κB2 complexes contained p50, RelA, c-Rel, p52 and RelB in nuclear extracts of L428 and HDLM-2 cells (Fig. 7B, lanes 8-12). Taken together, the results indicate that NF-κB proteins bind to both κB elements of the IκB-ζ promoter in Daudi, L428 and HDLM-2 cells.

Role of IκB-ζ in the expression of NF-κB target genes. Unlike other IκB family members, IκB-ζ has dual opposite functions on the expression of different cellular genes activated by NF-κB (19-21,27). To identify the genes whose expression is regulated by IκB-ζ, we examined the promoter activities of several NF-κB target genes in 293T cells transfected with LMP-1, CD30 or RelA, and IκB-ζ. *IL-6*, *IL-8* and *Bcl-3* genes are known to be activated by NF-κB (35-37). Luciferase reporter analyses indicated that promoters of IL-6, IL-8 and Bcl-3 were activated by transfection of LMP-1, CD30 and RelA as expected (Fig. 8A-C). Cotransfection of IκB-ζ dose-dependently inhibited the LMP-1-, CD30- and RelA-induced activation of promoters of IL-6, IL-8 and Bcl-3. In addition, we analyzed whether IκB-ζ overexpression leads to downregulation of known NF-κB targets on mRNA level. To this end, we determined the effect of IκB-ζ overexpression on IL-6 and IL-8 mRNA expression in the presence of LMP-1 by RT-PCR. These analyses demonstrated that IL-6 and IL-8 mRNA levels were downregulated after IκB-ζ overexpression (Fig. 9), suggesting that IκB-ζ plays a role in negatively regulating NF-κB targets.

We also analyzed the effect of IκB-ζ overexpression on the activation of its promoter induced by LMP-1, CD30 and RelA. LMP-1-, CD30- and RelA-induced IκB-ζ promoter activation was dose-dependently repressed by IκB-ζ overexpression (Fig. 10A-C). Thus, IκB-ζ can repress its own transcription. IκB-ζ expression itself was regulated by NF-κB, suggesting that its activity is controlled through a negative feedback loop.

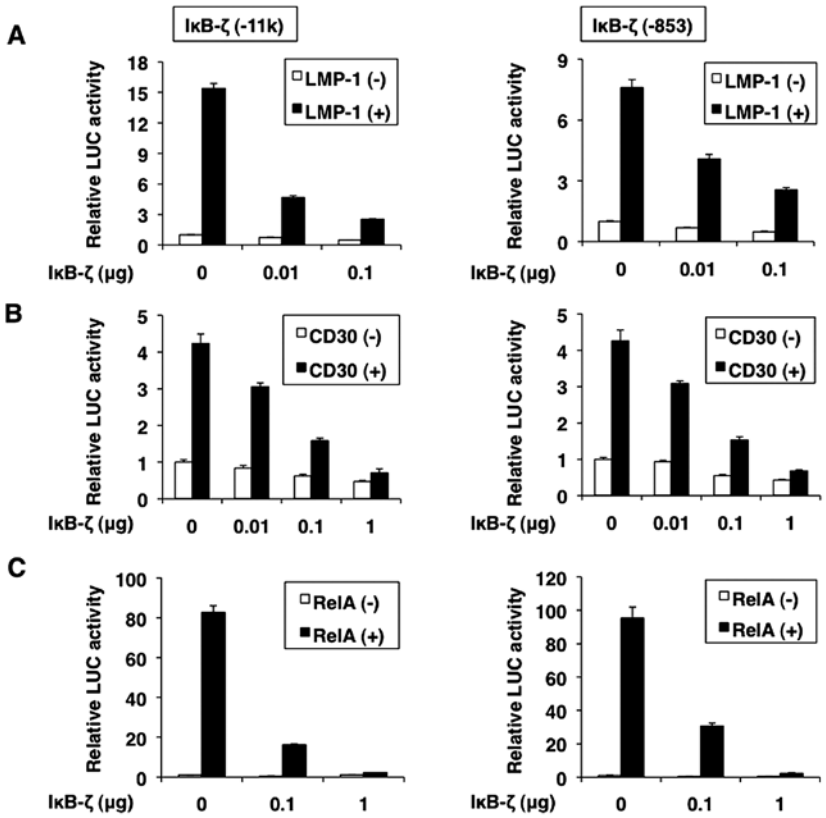

Figure 10. IκB-ζ represses activated transcription of its own promoter. Cells (293T) were transfected with the indicated IκB-ζ promoter reporter plasmids together with the expression plasmid for LMP-1 (A), CD30 (B) or RelA (C), and the indicated amounts of IκB-ζ expression plasmid. The activity is expressed relative to that of cells transfected with the indicated reporter plasmids and empty vectors, which was defined as 1.

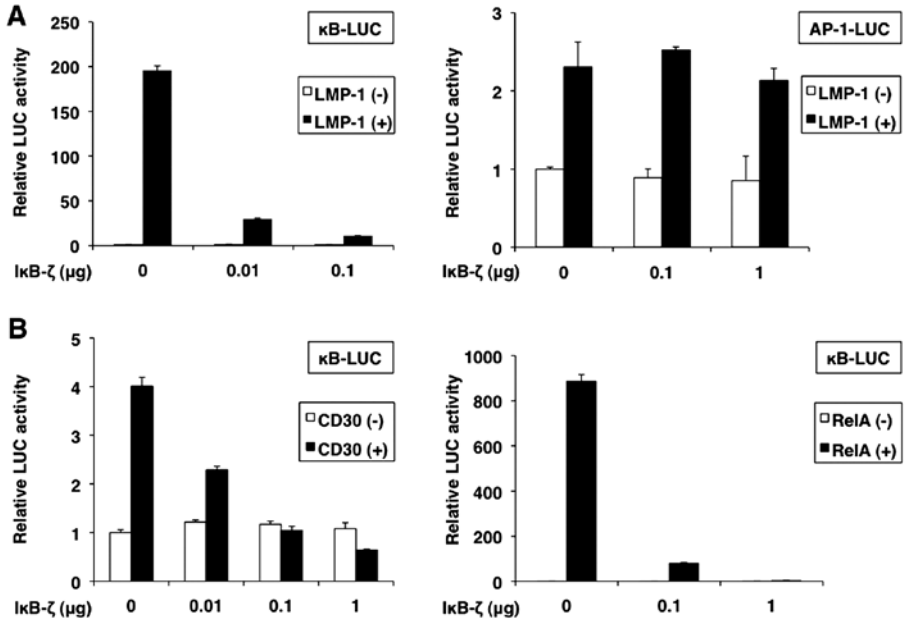

Figure 11. IκB-ζ inhibits NF-κB but not AP-1 promoter activity. Determination of NF-κB- or AP-1-dependent luciferase activity in 293T cells transfected with κB-LUC or AP-1-LUC, together with the expression plasmid for LMP-1 (A), CD30 or RelA (B), and the indicated amounts of IκB-ζ expression plasmid. The activity was expressed relative to that of cells transfected with the indicated reporter plasmids and empty vectors, which was defined as 1.

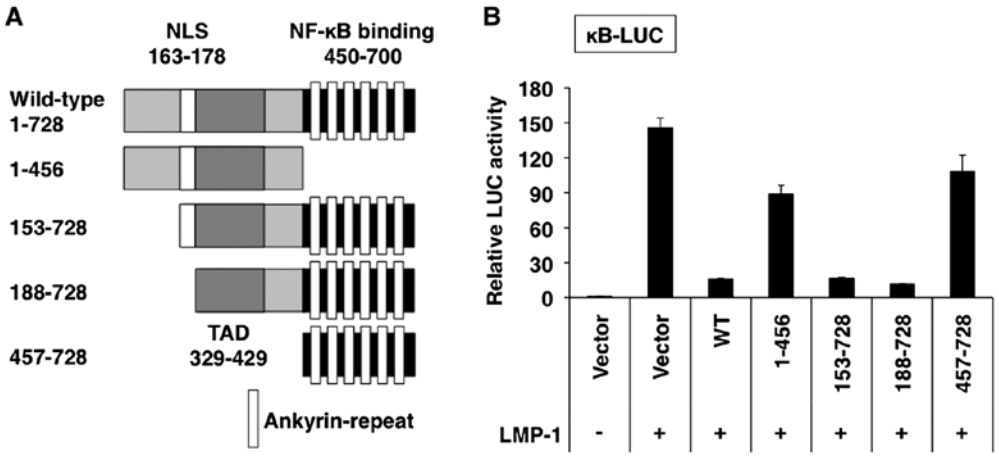

Figure 12. The internal fragments and carboxyl-terminal ankyrin-repeats of IκB-ζ have transcriptional inhibitory activity. (A) Schematic diagram of the various expression plasmids for mouse IκB-ζ. The carboxyl-terminal region of IκB-ζ harbors the ankyrin-repeats, which are responsible for the NF-κB binding. The amino-terminal region contains a nuclear localization signal (NLS) and a transcriptional activation domain (TAD). (B) IκB-ζ functional domains responsible for inhibition of LMP-1-induced NF-κB activity. Cells (293T) were cotransfected with expression plasmid for LMP-1 and an expression plasmid for various fragments of IκB-ζ together with κB-LUC. The activity is expressed relative to that of cells transfected with the reporter plasmid and empty vectors, which was defined as 1.

To confirm the role of IκB-ζ in NF-κB activity, we transfected 293T cells with IκB-ζ, and LMP-1, CD30 or RelA, and measured the activity of κB-LUC, an NF-κB reporter construct. As expected, we found that NF-κB reporter activity induced by LMP-1, CD30 and RelA was repressed by IκB-ζ overexpression in a dose-dependent manner (Fig. 11A, left panel, and 11B). However, the results showed that IκB-ζ did not affect AP-1 reporter activity induced by LMP-1 (Fig. 11A, right panel).

Mutants of IκB-ζ truncated from the amino- and carboxyl-termini were expressed in the presence of LMP-1 in 293T cells, and the NF-κB reporter activity was measured (Fig. 12A). The amino-terminal truncated mutants (153-728 and 188-728) as well as the full-length IκB-ζ (1-728), showed inhibitory activities against LMP-1-induced NF-κB activation, whereas the mutants consisting of the amino-terminus to amino acid 456 (1-456) and the amino-terminal truncated mutant (457-728) exhibited less activity than the full-length IκB-ζ (1-728) (Fig. 12B). These results indicate that the region between amino acids 188-728 harbors a domain with transcriptional inhibitory activity.

Discussion

Constitutive activation of the oncogenic NF-κB pathway is a characteristic hallmark of several lymphoma subtypes (1).

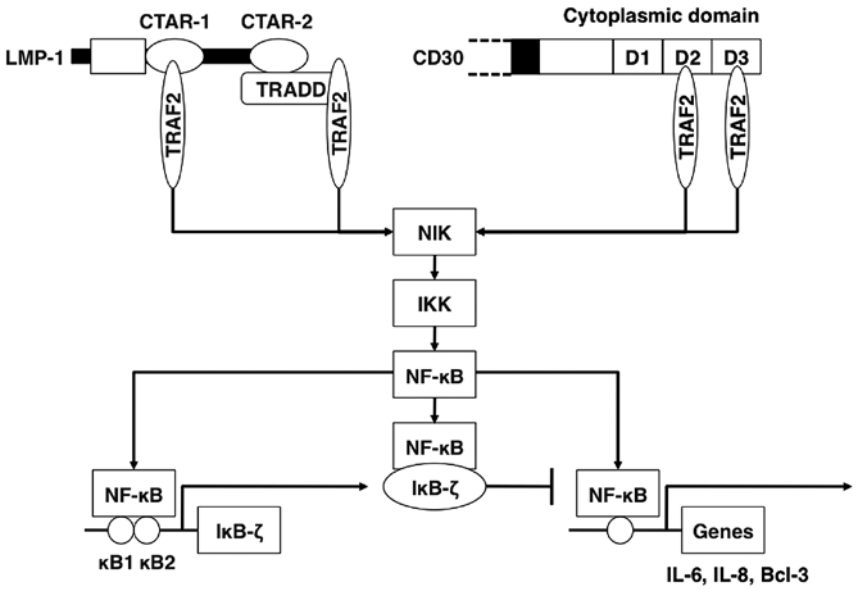

Figure 13. Hypothetical model for NF-κB-IκB-ζ autoregulatory loop in BL and HL.

EBV LMP-1 and CD30 have been demonstrated to activate the NF-κB signaling pathways in lymphomas (1). It has become increasingly clear that activation of NF-κB is not only controlled in the cytoplasm but, presumably even more importantly, also modulated in the nucleus. The nuclear IκB family member IκB-ζ acts a multifaceted modulator of NF-κB activity (20). We demonstrated high IκB-ζ expression in LMP-1-expressing BL and CD30-expressing HL cell lines. Nuclear IκB-ζ expression was also shown in lymph nodes from patients with BL and HL by immunohistochemical staining. In contrast, normal lymph nodes did not express IκB-ζ (23). Due to the potential significance of these observations on the two lymphoma types, we investigated the transcriptional basis for LMP-1- and CD30-induced IκB-ζ expression. Our results demonstrated that LMP-1 and CD30 activate IκB-ζ transcription primarily through two NF-κB sites in its promoter. The TRAF/NIK/IKK pathway also contributed to the activation of the IκB-ζ promoter as shown by the use of dominant-negative constructs. These data provide the molecular basis for the observed LMP-1- and CD30-induced overexpression of IκB-ζ (Fig. 13).

We next considered the consequence of IκB-ζ overexpression in BL and HL cells. The results showed that IκB-ζ potently repressed the LMP-1- and CD30-induced NF-κB activation in a negative feedback loop, suggesting the presence of an NF-κB-IκB-ζ autoregulatory loop (Fig. 13). IκB-ζ associates with the NF-κB subunit p50 and IκB-ζ inhibits the DNA binding of the RelA/p50 heterodimer and the p50/p50 homodimer (19). Negative autoregulatory loop provides an effective mechanism for the control of NF-κB activation. The inhibitory roles of the negative autoregulatory loop on NF-κB-mediated transcription may be critical in fine tuning the balance between activators and suppressors of tumors to maintain lymphoma *in vivo*. The relatively high frequency of expression of another nuclear IκB family protein, Bcl-3, was reported in some lymphoma types (42). Like IκB-ζ, Bcl-3 is a multifaceted modulator of the NF-κB activity and has multiple functions (43). Because the ankyrin-repeats of IκB-ζ are homologous to that of Bcl-3 (20), Bcl-3 may act as a competitor for IκB-ζ, or vice versa. Appropriate cellular responses are regulated by the control of precise balance between accelerators, brakes and steering wheels to maintain homeostasis following environmental change (20). Elucidation of the precise mechanism that determines the atypical nuclear IκB family effects should be paramount to our understanding of the role of NF-κB family in lymphomas.

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