J Med Dent Sci 2003; 50: 155−165

Original Article

Coordinated Regulation of the Promoter and Enhancer Regions of Human CD23 Gene by Signal Through IL-4R and CD40, and the Role of Ku70/80 in the Enhancer Activity

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CD23 has roles in proliferation, antigen uptake and presentation, and the generation of IgE. Signals through IL-4R and CD40 stimulate transcription of *CD23* in B cells and are necessary for immunoglobulin class switch (IgCS). The same signals induce nuclear translocation of Ku, which is also required for IgCS, in human resting B cells, suggesting that these signaling pathways are connected. We examined the regulation of *CD23* gene, and located the minimal promoter at -132+80 region. A pair of 188bp inverted repeats inhibited its activity. The intronic region including EBV responsive element (EBVRE) and the surrounding sequence, required the gene specific promoter to enhance the reporter gene activity. Western blotting and FACS analysis using subclones of DND39 B cells infected with recombinant EBV, revealed that CD23 upregulation did not necessarily correlate with EBNA 2 and LMP 1 expression. Although the specific binding of Ku to EBVRE was not demonstrated, dominant negative Ku80 suppressed IL-4 + anti-CD40-driven CD23 expression. These results suggest that Ku is involved in gene regulation as a signal transducer and gene enhance. Detailed analysis of *CD23* gene regulation would lead to a better understanding of disorders such as allergy and lymphoproliferation.

Key words: B lymphocytes, Cell surface molecules, Transcription factors

Introduction

CD23, also known as the low affinity receptor for IgE, displays wide-ranging roles in the immune response to allergens, and to parasite infections.

CD23 provides a progression signal to primed human B cells[1,2]. This molecule captures antigen-IgE-complex to process the antigen for presentation[3,4]. IgE production also requires CD23 as a surface adhesion molecule for physical interaction with T-cells[5,6], and the CD23-CD21 pairing may control IgE production more selectively than CD40-CD40L[7,8]. Meanwhile, CD23 was proven to be a negative regulator of IgE, as shown in knockout[9] and transgenic mice[10].

In humans, the expression of CD23 is strikingly increased in allergic disorders, and reduction of allergen-induced CD23 expression on B cells has been observed after successful hyposensitization[11,12]. B cells from patients with X-linked severe combined immunodeficiency, fail to increase CD23 in response to IL-4[13]. A study of a patient with non-X-linked hyper-IgM syndrome who had normal CD40L expression, detected an abnormal CD23 regulation in B cells[14]. These lines of evidence give expectation that further investigation of the regulation of CD23 may link to elucidation

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Received January 14; Accepted January 28, 2003

of pathogenesis of many immunological disorders.

EBV infection and growth transformation of B cells induce CD23 expression, and those EBV-infected cells that express CD23 become latently infected and immortalized[15-17].

Despite extensive studies, molecular mechanisms involved in the regulation of *CD23* remain uncertain. Although the importance of STAT6 in IL-4-induced *CD23* upregulation is generally accepted[18,19], it is now obvious that this factor is not exclusive, as shown in studies of STAT6 knock-out mice[20], and B-chronic lymphocytic leukemia cells[21]. NF-κB, is activated by CD40 signaling, and is involved in many immunological functions, however the role in *CD23* up-regulation is still controversy[19,20,22,23]. Other regulatory factors, such as Egr-1 and Egr-2/krox20 also contribute to the regulation of this gene[24,25].

CD23 gene is large, spanning over 13kb long, and is composed of 11 exons[26]. The core promoter is proposed to be located within 190bp upstream of the transcription initiation site[19], and several regulatory regions outside of the core promoter are reported[27-29]. Lacy *et. al*. characterized a short stretch of 37bp DNA in intron 1, designated as EBVRE, and have shown that Ku autoantigen binds to the element in a sequence-specific manner, by comparing nuclear extracts from EBV-negative and EBV-positive cells[29,30].

Ku70/80 heterodimer is important for DNA double-strand break repair, V(D)J recombination, and also for IgCS (reviewed in 31). These activities arise from the DNA end-binding properties of Ku, but several lines of evidence show that Ku70/80 binds to DNA in a sequence-specific manner and acts as a transcription factor[32,33].

We have previously shown that Ku70/80 associates with the cytoplasmic region of CD40, is absent in the nucleus of human resting B cells, and translocates into the nucleus following incubation with IL-4 and anti-CD40 mAb (monoclonal antibody)[34]. These data suggest that Ku70/80 play some role in IL-4R/CD40 signaling as a signal transducer and an enhancer/activator of transcription.

We wished to elucidate the mechanism of *CD23* expression since in-depth analysis may lead to a better understanding of the crosstalk between IL-4R signaling and CD40 signaling pathways and would also provide valuable data for dissecting signaling defects in the patients who fail to mount IgCS upon stimulation. We examined the regulation of CD23 expression under the physiological condition by using *CD23* specific promoter and *CD23* specific enhancer because conflicting data may have arisen from the experimental designs using heterologous promoter or enhancer.

Materials and Methods

Cell cultures

Human EBV-negative Burkitt B cell line, DND39 was obtained from Fujisaki Cell Center and was incubated in RPMI-1640 supplemented with 10% FCS. DND39 cells infected with recombinant EBV was maintained in RPMI-1640 with 20% FCS, 10 mM HEPES and 1500 ng/ml G418 (Life Technologies, Rockville, MD).

Cells were stimulated with 20 ng/ml recombinant human IL-4 (R&D Systems, Minneapolis, MN), 1 μg/ml anti-CD40 mAb G28.5 (a kind gift from Dr. Ed Clark), or both.

Establishment of EBV positive DND39 clones

Recombinant EBV carrying neomycin-resistant gene, originating from Akata B cell line was used for infection of DND39 cells as described previously[35]. Briefly, the cells were first infected with the virus for 48 hours, plated in 96-well flat-bottomed plates at 5,000 cells per well, and selected in the presence of G418 at 1250-1750 ng/ml. EBV infected cells were further expanded from each well in the medium described above.

DNA constructs

The sequence including the 5' terminus and the coding region of *CD23a* (−1286+1821) was amplified from genomic DNA of human peripheral blood lymphocytes using the oligonucleotides; 5'-GCTTGGGAATAAGTCCAGCT-3' and 5'-GGATCCAATGAGATCACAGC-3', cloned into pGEMTeasy vector (Promega, Madison, MA), and was used as a template for PCR to generate fragments depicted in Fig. 1. Five sequentially deleted promoters (−443+80, −243+80, −132+80, −117+80, −89+80) were generated and were cloned into *Xho*I-*Hind* III site of pGL3 vector (Promega).

i455 and i253 are the first 455bp and 253bp of intron 1 of the CD23 gene, respectively. EBVRE: GAAGGGAGGTGGTGGGTGGTGAGGGGGCGGGGGTGGG is a 37bp stretch located 160bp downstream from the 5' end of intron 1. These intronic fragments were cloned into the *Bam*HI site located downstream of the luciferase gene. i253del is a fragment lacking in EBVRE from i253 and was generated with an Exsite

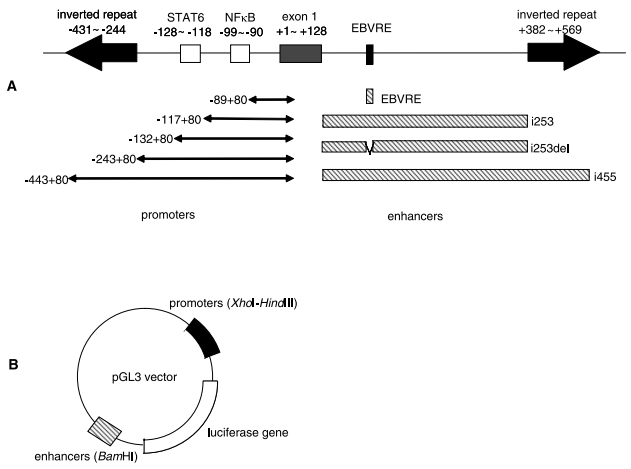

Fig. 1. Constructs used in reporter gene assays.

A, The core promoter and the part of *CD23* intron 1 (intronic enhancer) is located between two 188bp repeat elements. Five different promoter constructs were generated, in which 5' ends were sequentially deleted. -443+80 starts from the beginning of the upstream inverted repeat. -243+80 and -132+80 include both the STAT6 and the NF-κB sites. The STAT6 site is deleted in -117+80, and -89+80 lacks the NF-κB site. These fragments are numbered relative to the transcription start site. The 3' ends of these constructs extend 80bp into exon 1. Four enhancer fragments were generated from intron 1. The 37bp EBVRE is located 160bp downstream from the 5' end of intron 1. i253 is the first 253bp of intron 1, which includes EBVRE. i253del lacks entire EBVRE. i455 contains the downstream inverted repeat.

B, The pGL3 vector possesses two multiple cloning sites (MCSs), one of which is located upstream of the luciferase gene, and the other is located downstream of the reporter gene. We inserted the promoter constructs in the upstream MCS, and the enhancer fragments into the downstream MCS.

PCR-based Mutagenesis Kit (Stratagene) with two oligonucleotides (5'-GGCCGCAGTGTGGACAGAAT-3' and 5'-CTCTGGTTGTGACCTCCTCG-3').

Human Ku80 cDNA was amplified by PCR from human peripheral blood lymphocytes and was cloned into pET11a (Stratagene, La Jolla, CA). Ku80 (449-732) is a fragment lacking in the N-terminal region, generated by amplifying 1372-2223 of Ku80 cDNA, and was cloned into the *Bgl* II site of pEGFP-C1 (BD Clontech Biosciences, Palo Alto, CA). Ku80 (1-684) fragment lacking in the C-terminus was derived by digesting pET11a-Ku80 with *Bcl* I, and were cloned into the *Bgl* II site of pEGFP-C1. Sequences of the fragments generated by PCR or by the mutagenesis kit were verified by using an A310 genetic analyzer with BigDye Terminator DNA Sequencing kit (Applied Biosystem, Foster City, CA).

FACS and Antibodies

Surface expression of Fas/CD95 and CD23 was monitored by a FACScan (Becton Dickinson, San Jose, CA). FITC-labeled anti-human CD95 antibody (UB2) was purchased from MBL (Nagoya). PE-labeled anti-human CD23 (Leu20) is a product of BD Pharmingen (San Diego, CA). Isotype-matched control antibodies from the same species were used for the determination of the background staining.

Cell transfection and luciferase assay

Cell transfection was performed by DEAE-Dextran method. Cells were split 24 hours before transfection and seeded at 1.6×10^5 cells/ml. 4×10^6 cells were collected and washed twice with Solution A (150 mM NaCl, 10 mM HEPES) and suspended in a mixture of 16 μg plasmid DNA, 400 μl DEAE-Dextran (MW 500,000, 2 mg/ml in 150 mM NaCl), and 1400 μl Solution A. The cells were incubated at room temperature for 90 min, washed, divided into four aliquots, and cultured in 1 ml of fasting medium containing 1% FCS. IL-4, anti-CD40 mAb, or IL-4 + anti-CD40 mAb were added 16 to 20 hours after transfection.

Luciferase assays were performed using Dual Luciferase Assay Kit (Promega) with Lumat LB 9501 (Berthold, Postfach) according to the manufacturer's instruction. For each luciferase assay, phRL-TK (Promega) was co-transfected with pGL3 constructs as an internal control.

Each relative luciferase unit was standardized against the co-transfected renilla luciferase activity (RLU). The fold induction was calculated by dividing RLU in stimulated cells by RLU in unstimulated cells.

Western blotting

For Western blotting analysis, 5×10^6 cells of each EBV infected DND39 subclones were lysed in 200 μl lysis buffer (250 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.2% bromophenol blue). 40 μl of the lysates were separated on an 8% SDS polyacrylamide gel. Proteins were transferred onto nitrocellulose filters (Bio-RAD, Hercules, CA), and the filters were incubated with a mixture of anti-EBNA2 (PE2; DAKO JAPAN) and anti-LMP1 (CS1-4; DAKO JAPAN) antibodies. Immunoreactive proteins were detected by HRP-coupled secondary antibodies and enhanced chemiluminescence (NEN Life Science Products, Boston, MA). Parental DND39 cells and B95-8 transformed normal B cells served as negative and positive controls, respectively.

Electrophoretic mobility shift assay (EMSA)

The complementary strands of the DNA probes used in EMSA were synthesized (Amersham Pharmacia, Japan). The EBVRE probe was prepared by annealing a sense strand 5'-GAAGGGAGGTG GTGGGTGGTGAGGGGGCGGGGGTGGGGGC CGCAG-3' and an antisense strand 5'-CCCACCCC CGCCCCCTCACCA-3', leaving 5'- and 3'-overhanging ends at the both sides. The mutated probe (mut1) was prepared by annealing 5'-GAAGGGAGGTGGTGG GTGGTGAGGGTGTAAGGGTGGGGGCCGCAG-3' to the complementary strand 5'-CCCACCCTTACACC CTCACCA-3' (mutated base pairs are underlined). The annealed fragments were labeled by fill-in reaction with Klenow fragment (NEB, Beverly, MA) with [^{32}P]α-dCTP, and then were gel-purified. The probes were also prepared by PCR with [^{32}P]α-dATP using oligonucleotides (5'-GGGACCCGGG**AGATCT**GAAG GGAGGTGGTG-3' and 5'-GGGACCCGGG**AGATCT** CCCACCCCCG-3' for EBVRE and, 5'-GGGACCCG GG**AGATCT**GAAGGGAGGTGGTG-3' and 5'-GGGA CCCGGG**AGATCT**CCCACCCTTA-3' for mut1), and the probes were digested with *Bgl* II for generating 5'-overhanging ends (*Bgl* II sites are indicated in bold). Nuclear extracts were prepared as described previously[34]. For each EMSA, 0.1 μg of nuclear extract was incubated with approximately 50,000cpm of labeled probe in 15 μl of KCl-binding buffer or in 15 μl of NaCl-binding buffer. The compositions of the binding buffers were as follows; KCl-binding buffer: 10 mM Tris, pH7.5, 5 mM $MgCl_2$, 75 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% TritonX-100, 12.5% glycerol and protease inhibitors, NaCl-binding buffer: 30 mM Tris, pH8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 8% glycerol, 0.01% NP-40, 0.5 mM dithiothreitol, 150 μg/ml BSA and protease inhibitors. The binding reaction was allowed to proceed for 30 min at room temperature without non-specific competitor DNA. For supershift assay, the samples were incubated with a mixture of anti-Ku70 mAb and anti-Ku80 mAb (Kamiya Biomedical, Seattle, WA), or with a control isotope-matched mouse IgG for an additional 30 min at 4°C. The entire binding reaction was resolved by electrophoresis through a 5% acrylamide gel in TBE buffer for 150 min at 25 mA. Gels were fixed in methanol-acetic acid, dried and exposed to an X-ray film (Eastman Kodak).

Statistical analysis

Statistical analysis was performed by using F test.

Results

The minimal promoter of CD23 gene is in −132+80.

Five sequentially deleted promoters of *CD23* gene starting with −443+80 were generated, considering the known structure of the 5'-flanking region of *CD23* gene. As shown in Fig. 1, there is a pair of 188bp repeats, one of which located 5' to the core promoter and the other located 3' to exon 1. The sequences are almost completely identical and positioned in the opposite direction. The upstream repeat lying at −431~ −244, is included in −443+80. −243+80 and −132+80 lack this stretch but include both the STAT6 site and NF-κB site. −117+80 and −89+80 are devoid of STAT6 site and both STAT6 and NF-κB sites, respectively. These fragments were cloned into the *Xho* I-*Hind* III site located upstream of the luciferase gene of

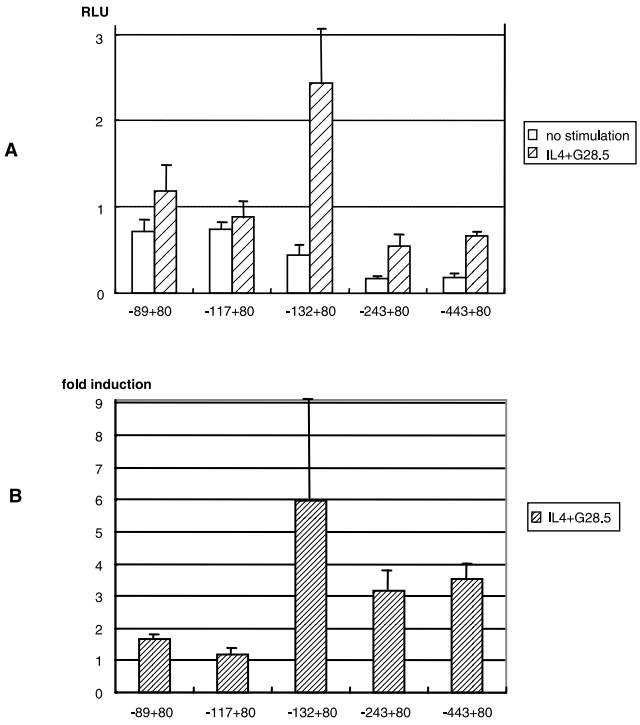

Fig. 2. Activation of the luciferase gene by the five promoters upon stimulation with IL-4 + anti-CD40

A, Reporter constructs containing the promoters were transfected in DND39 cells. Activities at basal level and that in response to IL-4+anti-CD40 were measured. The values of each sample were standardized against the co-transfected renilla luciferase activities and plotted as "relative luciferase units (RLU)". Longer promoters displayed weaker basal activities, however the differences were statistically insignificant. B, The RLUs of stimulated cells were divided by that of unstimulated cells to calculate the enhancement by the stimulation, and were plotted as "fold induction". Promoters that include the STAT6 site exerted enhancing effects. Figure shows a summary of three independent experiments. Error bar indicates ±1 SD.

pGL3 vectors, and basal activities and responses to IL-4+anti-CD40 stimulations were monitored. Longer promoters displayed weaker basal activity although the difference was not significant (Fig. 2A). On the other hand, responses to the stimulations, were only observed with promoters longer than −132+80, and among the three, −132+80 gave us the strongest response (Fig. 2B). These results confirm the importance of STAT6 binding site in the IL-4R signaling, located at −128~−118 in this gene. Previous report defined the minimal promoter at −190~+80, and we now further narrowed the region.

Reporter gene assay using *CD23* gene-specific promoters and enhancers

When i455, the first 455bp of intron 1, was inserted into downstream of luciferase gene in the reporter gene construct driven by −443+80 (−443+80/i455 pGL3), the baseline luciferase activity as well as that in response to IL-4 or IL-4+anti-CD40 were almost completely eliminated (Fig. 3A). The reporter gene activity driven by −132+80 promoter, lacking the upper inverted region, was not suppressed by i455 enhancer region (Fig. 3B). Similarly i253 without downstream inverted repeat was unable to suppress the −443+80 promoter activity (Fig. 3C). These results indicate that the combination of upstream and downstream inverted repeats inhibit the transcription of the *CD23* gene.

We next dislodged both the upstream and downstream inverted repeat to make the model simpler. −243+80 and −132+80 were utilized as gene specific promoters, and EBVRE and i253 which includes EBVRE were examined whether they serve as enhancers. When i253 or EBVRE was incorporated with −243+80 promoter these elements failed to enhance the responses to IL-4 and IL-4+anti-CD40 stimulations above that exerted by −243+80 promoter alone (Fig. 4A). Under the control of −132+80 promoter, i253 enhancer amplified the transcription of luciferase by approximately 1.7 fold when DND39 cells were stimulated with IL-4. Combined stimulation with IL-4 and anti-CD40 further augmented the luciferase activity above the IL-4 stimulation despite the presence or absence of i253 enhancer. EBVRE alone was unable to exert an enhancing effect (Fig. 4B).

We then examined whether EBVRE is critical for the enhancement by generating i253del that lacks the whole EBVRE. The results illustrated in Fig. 4B show that i253del matches i253 in high enhancer activity in the IL-4 stimulated cells. In contrast, the response to IL-4 + anti-CD40 stimulation was significantly reduced

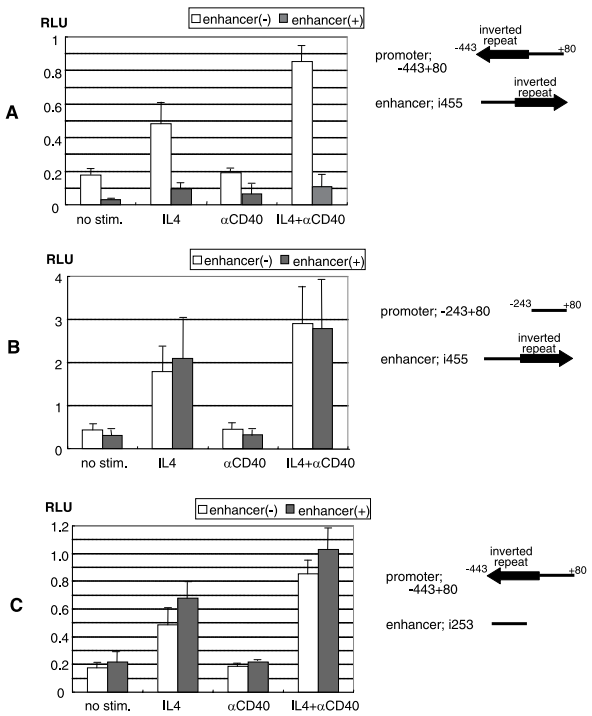

Fig. 3. Presence of two 188bp inverted repeats strongly suppress the transcription of the *CD23* gene.

A, The reporter gene activity of the construct containing -443+80 promoter (white bars) is increased when stimulated with IL-4, and further enhanced by incubation with IL-4+anti-CD40. Anti-CD40 stimulation alone showed minimal effect. Insertion of i455 in the downstream MCS under -443+80 promoter (hatched bars) led to the elimination of the basal activity as well as responses to IL-4 and IL-4+anti-CD40 stimulations.

B, The reporter activity of the construct with -132+80 promoter (white bars) which is without the upstream 188bp repeat, is similar to those shown by the -443+80 promoter when the enhancer region is empty. i455 insert (hatched bars) does not suppress the activities of luciferase activity driven by -132+80 promoter.

C, The construct with -443+80 promoter (white bars) and the i253 insert which lacks the downstream 188bp repeat (dotted bars), shows the similar reporter gene activity to that with promoter alone or that with -132+80/i455 construct. Data are summary of three independent experiments. Error bar indicates ±1 SD.

comparing to that with i253 construct. This suggests that the intronic enhancer region without EBVRE still possesses the potential to enhance transcription of *CD23*. The data also show that EBVRE plays some role, together with the flanking region, to enhance the transcription of the *CD23* gene driven by a specific promoter. The effect of EBVRE was detectable only when the cells were stimulated with IL-4 and anti-CD40, suggesting that the binding factor is mainly induced by concomitant signals via IL-4R and CD40.

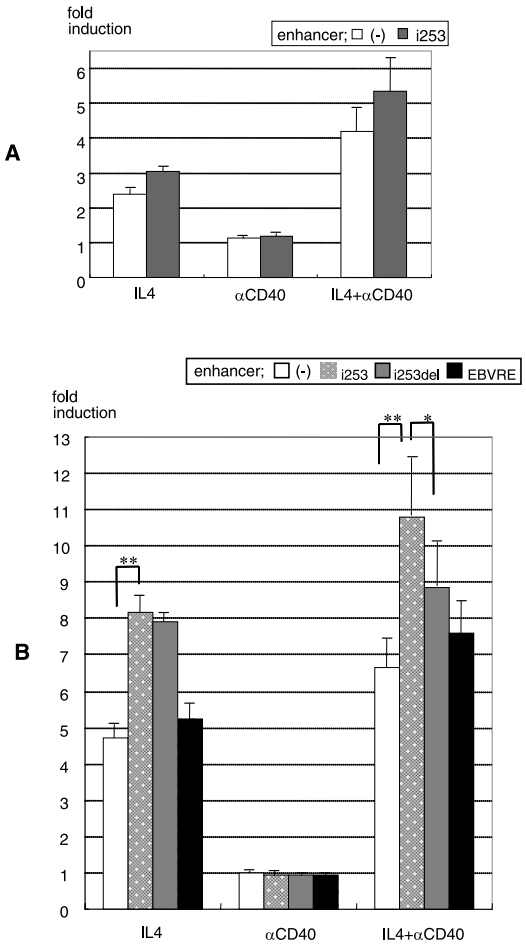

Fig. 4. Enhancement of the gene specific transcription by intronic enhancer is partly dependent on the presence of EBVRE.

A, The core region deprived of the surrounding inverted repeats were introduced for detailed analysis of the EBVRE encompassing region. i253 insert under control of the -243+80 promoter, shows negligible effect on the reporter gene activities.

B, i253 under the control of -132+80 promoter augmented the luciferase activities in response to IL-4 and IL-4+anti-CD40 stimulation (dotted bars). The i253 enhancer without EBVRE (i253del), retains capacity to augment the response to IL-4 (fine dotted bars). The enhancement by IL-4+anti-CD40 was weakened with i253del in comparison to that with i253 (fine dotted bars). EBVRE alone shows minimal effect on the activities of the promoter (black bars).

The fold inductions were calculated by dividing RLU of the stimulated cells with RLU of unstimulated cells. Figure shows summary of three or four independent experiments. Error bar indicates ±1 SD. **＜0.01, *＜0.05

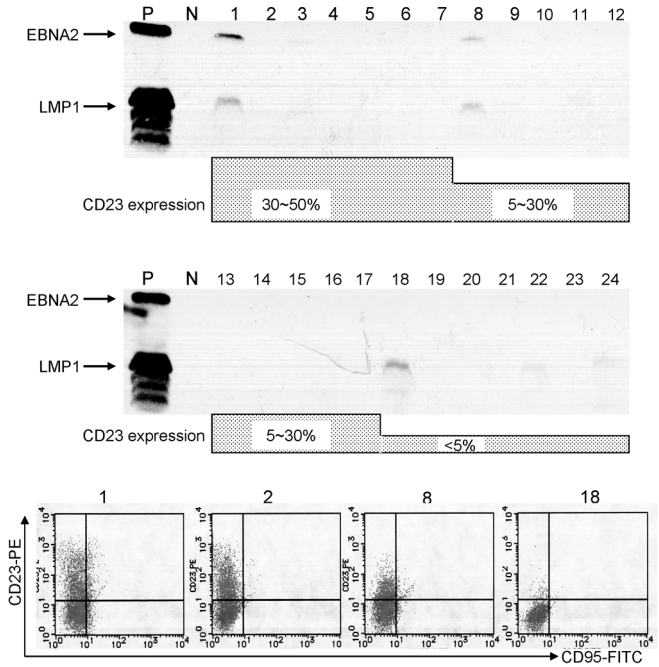

Fig. 5. Expression of EBNA2, LMP1 and CD23 in EBV-infected DND39 cell subclones

DND39 cells were infected with gene manipulated EBV and selected with G418 as mentioned in "Materials and Methods". Western blotting analysis of EBNA2 and LMP1 was performed using cell lysates prepared from $5x10^6$ cells. EBNA2 and LMP1 were not necessarily expressed in every clones. CD23 expression was variable amongst the clones and was not necessarily correlated with the level of EBNA2 or LMP1. Protein expression of EBNA2 and LMP1, and FACS analysis of representative subclones, 1, 2, 8 and 18 are shown.

Lack of correlation between EBNA2/LMP1 expression and CD23 induction

EBV infection is another well-known inducer of CD23 expression. By infecting DND39 cells with recombinant EBV carrying a neomycin resistant gene, we obtained 24 subclones and carried out FACS analysis for CD23 and Fas expression, and Western blotting for the presence of EBNA2 and LMP1 (Fig. 5). In 7 clones (1-7), more than 30% of cells were CD23 positive, but among these, only clone 1 (CD23 positive: 45%) expressed significant amount of EBNA2. Clone 3 (30%) expressed EBNA2 very weakly. Clone 8 expressed both EBNA2 and LMP1, however, CD23 positive cells were 17%, and Fas was negative. Clone 8 to 16 expressed CD23 in 5~30% of cells, and clone 17 to 24 were less than 5% CD23 positive. Although clone 18 expressed a fair amount of LMP1, CD23 positive cells were few.

Protein which specifically binds to EBVRE is not Ku70/80.

We asked whether Ku70/80 is detected at the enhancer region encompassing EBVRE in response to IL-4 and anti-CD40 by using EMSA assay. We followed the already reported method including the probe

design, the KCl-binding buffer, and the running buffer, to detect specific Ku binding[30]. Bands corresponding to Ku70/80 were detected in a binding buffer containing KCl and in a NaCl-binding buffer that has lower ionic strength (Fig. 6). These bands seemed not to be sequence-specific since the bands were also detected with a mutated EBVRE probe. These findings could be ascribed to the binding of Ku to the overhang ends. It is also possible that we have missed a sequence specific Ku70/80 because of abundant nonspecific Ku70/80 bound to the probe.

To improve the sensitivity, we labeled the same probe with a PCR method, which would incorporate 10-13 [^{32}P]α-dATP comparing three [^{32}P]α-dCTP in a fill-in method with Klenow fragment. Fig. 7 shows the presence of another band that migrates faster than Ku70/80. The protein seems to bind EBVRE in a sequence-specific manner, since the band disappears with the mutated probe. The intensity of the protein was unaltered upon stimulation with IL-4, anti-CD40, or with IL-4 + anti-CD40, and the band did not supershift with anti-Ku70/80 mAb (Fig. 7 A and B). The intensity was not augmented with nuclear extract obtained from CD23-expressing EBV-positive-DND39 (Fig. 7B). The data suggest the presence of a factor targeting EBVRE which is not induced by stimulation with IL-4, anti-CD40, or IL-4 + anti-CD40. Characterization and elucidation of physiological significance of the factor await further study.

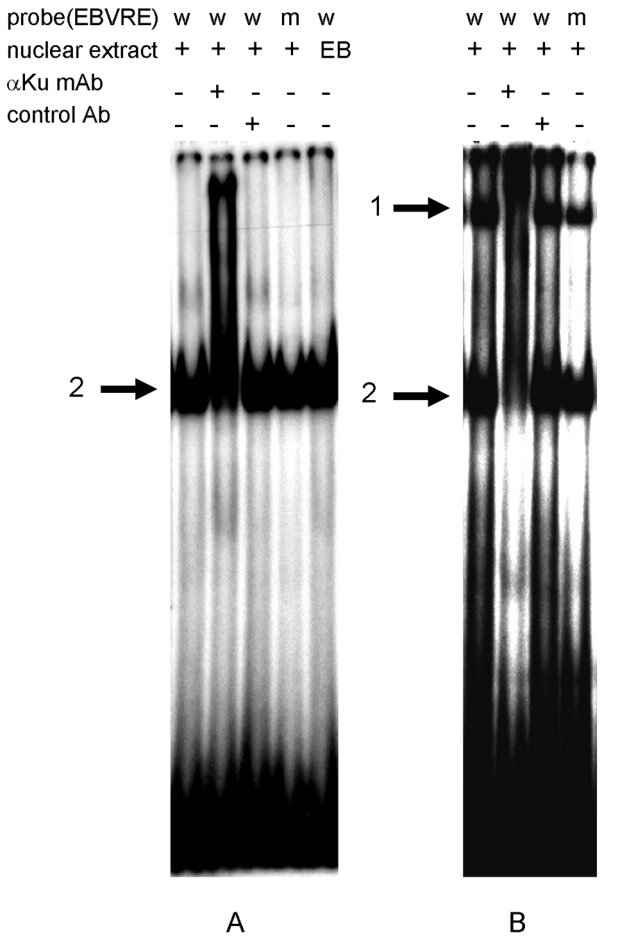

Fig. 6. Ku70/80 was depicted as protein which bound to EBVRE probe in a non-specific manner.

Probes were prepared by labeling with [^{32}P]α-dCTP by Klenow fragement, incubated with 0.1 μg of nuclear extracts, and the complex was resolved in 5% acrylamide gel.

A, Binding reaction was carried out in KCl-binding buffer. A single band corresponding to Ku70/80 was detected with EBVRE probe (w), but the band was also depicted with the mutant probe (m). The intensity remained the same when the nuclear extract from EBV-infected DND39 cells (EB) was applied compared to that with the nuclear extract from parental cell line (+).

B, The ionic strength of the binding buffer was lowered with using NaCl-binding buffer. Upper band which probably contains multiple Ku70/80 was detected in this binding condition. Results are representative of three similar experiments. Arrows indicate the positions of Ku70/80.

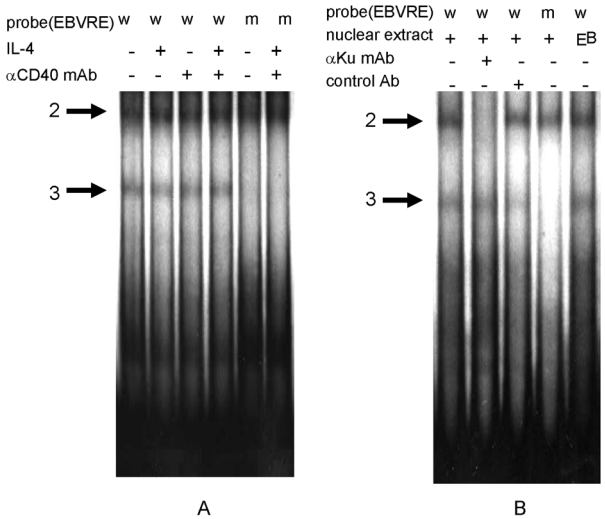

Fig. 7. A sequence specific band which was constitutively expressed is not Ku70/80.

Probes were labeled with [^{32}P] α-dATP by PCR to incorporate more isotopes than labeling with Klenow fragment. Binding reactions were carried out in NaCl-containing binding buffer.

A, An additional band (3) was detected with the EBVRE probe labeled in PCR method. Band 2 corresponds to band 2 in Fig. 6. The band 3 was not reproduced with the mutated probe (m).

B, The sequence specific band (3) is not Ku70/80. Nuclear extract from EBV infected DND39 cells (EB) contained the similar amount of protein.

Data are representative from three independent experiments. A film was exposed for a shorter time because of a high signal intensity of a free probe.

Effect of mutant Ku80 in CD23 response against IL-4 + anti-CD40 stimulation

To seek if Ku exerts any effect on CD23 induction, we have generated two Ku80 mutants, which confer dominant negative effects, according to the previous reports[36,37]. Ku80 (449-732) is a fragment, which imparted a radiosensitive phenotype to the radioresistant CHO-K1 cell line[36]. Ku80 (1-684) was generated after a mutant Ku80 that preferentially suppressed transcription re-initiation in an *in vitro* system[37]. Both of these fragments retained the central region of Ku80 which is required for heterodimerization with Ku70. Full length and the mutant Ku80 constructs were expressed as fusion proteins with EGFP. DND39 cells transfected with these constructs were then incubated in medium alone or incubated with IL-4 + anti-CD40 for 24 hours, and checked for the CD23 expression in the EGFP positive fraction using FACS analysis. Overexpression of full-length Ku80 itself did not alter the CD23 response when unstimulated (data not shown). Fig. 8 shows that neither DND39 cells transfected with full-length Ku80 cDNA nor the cells transfected with Ku80 (449-732) influences CD23 expression in response to IL-4 + anti-CD40 when compared with DND39 cells transfected with empty pEGFP-C1 vector. In contrast, CD23 induction after the treatment with IL-4 + anti-CD40 was suppressed in DND39 cells transfected with Ku80 (1-684) although transfection efficacy of the construct was consistently low. This was not due to the damage to the cells harboring the mutant construct since CD80 expression was induced in the cells when incubated with IL-4 and anti-CD40 (data not shown). These results suggest that Ku70/80 participates in the regulation of CD23 expression.

Discussion

In this study, we have found several novel findings; 1) the gene regulation of *CD23* is affected by the distinctive gene structure as well as binding of several transcription factors, 2) the minimal promoter of *CD23* gene to upregulate the expression in response to IL-4 is confined to −132+80, 3) the EBVRE element residing in intron 1, helps the superinduction of *CD23* in response to IL-4+anti-CD40 stimulation, and 4) EBNA2/LMP1 expression and CD23/Fas expression in EBV-transformed B cells are not invariably correlated.

The 5'-flanking region of the *CD23* gene is constituted of a striking feature of four Alu sequences and two 188bp repeat elements that form an extended, nearly symmetrical structure which frames the core promoter and exon 1[26,27]. Although the Alu sequences are highly redundant throughout the genome of primate cells[38,39], the 188bp repeat element is restricted to the *CD23* gene, however, its functional role remains unknown. By incorporating fragments including both of the repeats into up- and downstream of the luciferase gene in a nearly physiological manner, we have shown that these inverted repeats work together to restrict the expression of the gene. The effect was canceled when either of the partners was removed, suggesting that this repetitive structure may possibly form a large stem-loop *in vivo*.

Previous study on human *CD23a* has demonstrated that the core promoter region resided at −190 to +80 in the 5'-flanking region. The core promoter of *CD23a* gene is now identified at −132 to +80 from our study, showing that the presence of STAT6 binding site is enough for transcription. NF-κB site containing

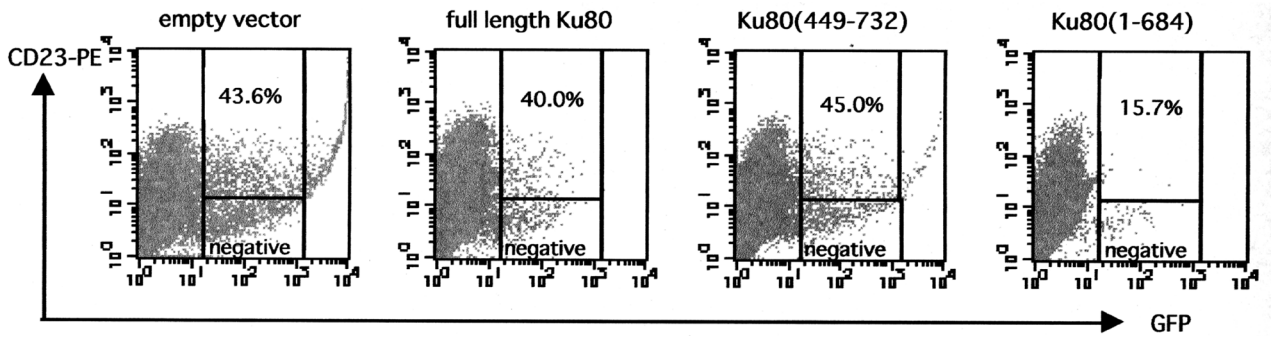

Fig. 8. A dominant negative Ku80 cancels the CD23 expression triggered by IL-4 + anti-CD40 stimulation.

DND39 cells were transfected with pEGFP-C1 (empty) plasmid or pEGFP-C1 carrying wild or mutant Ku80 as described in "Materials and Methods". Transfection efficiencies varied among the plasmids; 5-6% with empty vector and Ku80(449-732), 2% with a full length Ku80, and less than 1% with Ku80(1-684). The transfected cells were detected as EGFP positive cells and minimal of 50,000 events were counted. More than 500 EGFP positive cells were analyzed in each experiment. The ratios of CD23 positive cells in EGFP positive cells were shown in the figure. FACS patterns are representative of three comparable experiments.

sequence (-117 + 80) was not enough for inducing *CD23* expression. Function of NF-κB site possibly working in concert with STAT6 awaits the study on the minimal promoter without NF-κB region.

We have shown that the fragment, i253, supported the enhanced transcription of *CD23* gene in response to IL-4R signal under control of the gene specific core promoter, -132+80. The enhancement was subtle as it was not observed when the promoter was heterologous or even gene specific, was extended beyond the core region. EBVRE, included in i253, failed to exert enhancer activity alone, and i253del which lacks the whole EBVRE segment, still retained the enhancing effect in response to IL-4. This indicates the presence of yet-unidentified sequence in this enhancer site. But for the superinduction of this gene by IL-4+anti-CD40 stimulation, EBVRE was indispensable together with its surrounding area. The delicate and restricted function of EBVRE seems to be discordant from the findings of Lacy *et. al*. who have demonstrated more general enhancer activity with this element[30]. In their system, the *CD23* inducing factor was the presence of EBV in the B cells, the cell lines used were not the same, and the reporter gene was chloramphenicol acetyltransferase instead of luciferase. These may account for the different results. The influence of Ku70/80 autoantigen in the *CD23* gene regulation may not be clearly detectable in DND39 and other B cell lines, because the cytoplasmic distribution in unstimulated cells and the nuclear translocation after IL-4+anti-CD40 stimulation was clear only in peripheral blood B-lymphocytes (PBB). Other assay systems, such as chromatin immunoprecipitation assay, may be another approach to monitor the Ku binding to the regulatory region.

Our attempt to examine the effect of EBV on our reporter construct, failed because the transfection efficiency was extremely poor when DND39 cells were infected with an EBV strain originating from Akata cells. Through the process of selecting CD23-expressing DND39-subclones, we were led to an unexpected finding that surface expression of CD23 and the presence of EBNA2 and LMP1 in the infected cells are not necessarily correlated. It has been accepted that EBNA2 transactivates LMP1, and EBNA2 and LMP1 cooperatively induce *CD23* gene[40-43], furthermore, LMP1 alone may be sufficient for *CD23* up-regulation[44-46]. As we have demonstrated in our Akata cell-derived EBV infection system, CD23 upregulation didn't always require these viral proteins, and LMP1 sometimes failed to enhance cell surface expression of CD23. However, in the study of Wang *et. al*., the function of these proteins were not constant among cell lines, suggesting some other factors involved[41,47].

The direct participation of Ku70/80 on EBVRE remained undetermined. In our EMSA assay, the Ku containing band was detected with EBVRE probe as well as mutant EBVRE probe, and the amount of Ku70/80 did not change between EBV positive cells and EBV negative parental cells, or between stimulated and unstimulated PBB. These results suggest that Ku70/80 might have bound to the overhanging ends. We might have missed a small amount of Ku70/80 that specifically binds to EBVRE because of a large amount of nonspecific binding Ku70/80 present in nuclear extracts. Studies of Ku70/80 as a transcription factor are hampered by the natural properties of abundance as well as stickiness. Utilizing closed microcircles[33] or biotin-streptavidin conjugation at the ends of linear substrates[48] are possible ways to avoid nonspecific end binding of Ku70/80.

We enrolled another approach of transfecting mutant Ku80 that was expected to confer dominant negative effect, to examine the role of Ku70/80 on IL-4R/CD40 signal. Our study has shown that Ku80(1-684) possesses inhibitory effect on CD23 induction.

Ku70/80 as a multifunctional molecule remains an attractive candidate for a signal transducer and gene enhancer in many cellular events. Modification of Ku70/80 and its translocation may lead to the formation of enhancer complex and thus to control gene expression. Dissection of each subject may ultimately lead to a better understanding of the complex regulations.

Acknowledgement

We thank Dr. Ed Clark for G28.5 antibody, and Dr. Norio Shimizu for recombinant EBV of Akata cells.

This work was supported by a Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science, and Technology to T.M.

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