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Lymphoid Enhancer-Binding Factor-1 Binds and Activates the Recombination-Activating Gene-2 Promoter Together with c-Myb and Pax-5 in Immature B Cells

Zhe-Xiong Jin,2* Hiroyuki Kishi,2* Xing-Cheng Wei,* Tadashi Matsuda,3* Shigeru Saito,† and Atsushi Muraguchi4*

The recombination-activating gene (RAG)-1 and RAG-2 are expressed specifically in immature lymphoid cells undergoing the recombination of Ag receptor genes. We studied the regulation of murine RAG-2 promoter and revealed that −41/−17 RAG-2 promoter region, which was indispensable for the RAG-2 promoter activity in B cell lines, contained binding sites for lymphoid enhancer-binding factor-1 (LEF-1), c-Myb, and Pax-5. We showed that these three transcription factors bound the promoter region in vitro and in vivo. Cotransfection assays using a human embryonic kidney cell line (293T) showed that LEF-1, c-Myb, and Pax-5 cooperatively activated the RAG-2 promoter, via their synergistic DNA binding. We also showed that LEF-1, c-Myb, and Pax-5 physically interact in the cells. Finally, we demonstrated that a dominant-negative LEF-1 protein, which lacks the binding site for β-catenin, suppressed the RAG-2 promoter activity as well as the endogenous RAG-2 expression in a pre-B cell line (18.81). These results suggest that LEF-1/β-catenin complex regulates the RAG-2 promoter activation in concert with c-Myb and Pax-5 in immature B cells. The link between LEF-1/β-catenin and Wnt signaling in B lineage cells will be discussed. The Journal of Immunology, 2002, 169: 3783–3792.

Immunoglobulin and TCR V genes consist of germline V, D, and J segments and are assembled during lymphocyte development by V(D)J recombination. The recombination-activating gene (RAG)1 and RAG-2 encode the essential and lymphocyte-specific components of V(D)J recombination machinery. Their products are sufficient for the recognition and initial cleavage of DNA containing recombination signal sequences that flank each coding segment (1, 2). During lymphocyte development, expression of RAG-1 and RAG-2 is tightly regulated. RAG genes are expressed in immature B or T lineage cells undergoing Ig or TCR gene rearrangements (3–6). The failure of functional expression of RAG causes defect in the formation of functional Ag receptor of lymphocytes, and hence causes the block of lymphocyte development in mouse (7–10).

The transcription of RAG is regulated at different levels. At the chromatin level, Fuller and Storb (11), and we (12) have demonstrated that alteration of chromatin structure detected by DNase I hypersensitivity was noted in the promoter region of mouse and human RAG-1 only in RAG-expressing lymphocytes, indicating that chromatin remodeling is one of the mechanisms for regulating RAG expression. At the cis element level, Yu et al. (13) have demonstrated that the −10-kb 5′-upstream region of RAG-2 is necessary for the expression of RAG in B lineage cells and in CD4+CD8+ thymocytes, and that further upstream region is required for the expression of RAG in CD4+CD8+ thymocytes. Monroe et al. (14) have demonstrated that the human RAG-2 promoter is activated not only in lymphoid cells, but also in nonlymphoid cells (17). Concerning the mouse RAG-2 promoter, Schlissel and his colleagues and we have demonstrated that core promoter of mouse RAG-2 confers lymphoid specificity and may be regulated with distinct transcription factors: Pax-5 (18, 19) or c-Myb (20) in T cells. In the previous report, we have demonstrated that c-Myb interacts with Pax-5 and synergistically activates the mouse RAG-2 promoter in B cells (21).

In this study, we examined the possible involvement of lymphoid enhancer-binding factor-1 (LEF-1), a member of HMG box family, on the RAG-2 promoter activation in immature B cells. LEF-1 was originally cloned as a pre-B and T lymphocyte-specific gene encoding a protein that binds to a CTTTGA motif in the TCRα gene enhancer and that stimulates the activity of this enhancer in transient transfection assays (22, 23). Later, LEF-1 was found to bind DNA through the minor groove and bend the DNA helix, facilitating the assembly of a high-order multiprotein enhancer complex together with other lymphoid-specific proteins (24, 25). In addition, LEF-1 protein has been shown to interact
with β-catenin, an important effector in the Wnt signaling pathway (26–30). It was reported that LEF-1 protein associates with β-catenin through amino-terminal sequences, and together these proteins mediate a transcriptional response to Wnt signaling. Regarding the function of LEF-1 on lymphocyte development, LEF-1-deficient mice exhibited defects in pro-B cell proliferation and survival in vitro and in vivo, although no abnormalities in thymocyte differentiation were seen in these mice (31, 32). By a computer search, we found a possible binding site for LEF-1 at the core RAG-2 promoter region (18). In the present study, we tested whether LEF-1 binds to this site and regulates transcriptional activity of the RAG-2 promoter in immature B cells. We also tested the effect of ectopic expression of the dominant-negative (DN) LEF-1, which lacks the binding sites for β-catenin, on the RAG-2 promoter activity or the endogenous RAG-2 transcription in immature B cells. Our results indicate that LEF-1 participates in the regulation of the RAG-2 promoter together with the hemopoietic transcription factor, c-Myb, and the B cell-specific transcription factor, Pax-5.

Materials and Methods

Cells and cell culture

The 18.81 pre-B cell line (18) and BAL17 B cell line (18), both of which express endogenous murine RAG-1 and RAG-2, were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2 atmosphere. A human embryonic kidney cell line, 293T, and murine retrovirus packaging cell line, Phoenix Amphi (generous gift from G. P. Nolan, Stanford University, Stanford, CA), were grown in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2.

Plasmid constructs

For the promoter assay, the RAG-2 promoter fragments were generated, and inserted into PicoGene basic vector 2 (Nippon Gene, Tokyo, Japan), as previously reported (18). The mouse c-Myb expression vector (pAct-c-Myb) was kindly provided by S. Ishii (RIKEN, Tsukuba, Japan). The mouse Pax-5 expression vector (pEF-BOS-Pax-5) was generated by transfecting the p-5-Bgal vector with RT-PCR using oligonucleotides 5′-GGATACTGTTCCATTTCATCAAGTCC-3′ and 5′-CCGCTGAGTCAGTGGCCTTTGAAGCTCTCC-3′ and cloning it to the pEF-BOS vector (33). To prepare the hemoglobinin (HA)-tagged Pax-5 (pEF-HA-Pax-5), 5′ portion of the HA-tagged Pax-5 was produced by PCR using oligonucleotides 5′-GGATCTGTTCCATTTCATTC3′-3′ and 5′-CCGCTGAGTCAGTGGCCTTTGAAGCTCTCC-3′, and 5′-CCGCTGAGTCAGTGGCCTTTGAAGCTCTCC-3′ and subcloned into the corresponding part of pEF-BOS-Pax-5. The mouse LEF-1 cDNA was generously donated from S. Fujimoto (Kyoto University, Kyoto, Japan) and substituted with c-Myb cDNA in pAct-c-Myb. The DN LEF-1 (pRc/CMV-DN-LEF-1) was kindly provided by S. Shibahara (Tohoku University, Sendai, Japan). A DN LEF-1-containing retrovirus vector (pMX-DN-LEF-1-ires-GFP) was constructed by insertion of DN LEF-1 into a retrovirus vector pMX-ires-GFP, which is kindly provided by T. Kitamura (Tokyo University, Tokyo, Japan).

Transfection and luciferase assay

For transfection into B cell lines, luciferase constructs were transfected using DEAE-dextran method, as described previously (18). pSRα-lucZ gene was used as an internal control. Twenty-four hours after transfection, cells were harvested, and luciferase activity and β-galactosidase activity were measured, as described previously (18). For transfection into 293T cells, calcium/phosphate method was used, as described previously (18). Forty-eight hours posttransfection, cells were harvested, and luciferase activity and β-galactosidase activity were measured. When LEF-1, c-Myb, or Pax-5 expression vector was transfected with different doses, total amount of DNA was adjusted by adding either pAct vector DNA, pEF-BOS vector DNA, or pRc/CMV vector DNA.

Precipitation of LEF-1, c-Myb, and Pax-5 proteins by DNA-Sepharose

Precipitation of transcription factors with −41/−17 mouse RAG-2 promoter sequences conjugated with Sepharose beads was performed, as described before (21). Briefly, nuclear extracts were prepared, precleared with Sepharose 4B, and then incubated with −41/−17 oligonucleotides coupled to Sepharose beads in the presence of poly(dI-dC) at 4°C for 3 h. As a control, oligonucleotides, containing STAT3 binding site, coupled to the beads were used. Bound complexes were eluted from the beads with 1 M NaCl. All samples were resolved in SDS-PAGE, transferred to PolyScreen polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA). The membrane was incubated with c-Myb mAb (clone 1.1; Upstate Biotechnology, Lake Placid, NY) or polyclonal Pax-5 or LEF-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by peroxidase-conjugated anti-mouse IgG or anti-goat IgG (EY Laboratories, San Mateo, CA), and finally developed using Renaissance Western blot chemiluminescence reagent (NEN Life Science Products).

In vitro transcription and translation of transcription factors

For transfection into 293T cells, luciferase constructs were transfected with LEF-1, c-Myb, and HA-Pax-5 expression vectors. Two days later, the cells were harvested and the nuclear extract was prepared. The protein complexes in the nuclear extract were immunoprecipitated with either anti-LEF-1 Ab, anti-c-Myb Ab, or anti-HA Ab (Santa Cruz Biotechnology), or control Abs using protein G-Sepharose beads. Control Abs were used for anti-Run Ab (goat polyclonal IgG Ab purchased from Santa Cruz Biotechnology) for anti-LEF-1 and anti-Pax-5 Ab, and 928 Ab for anti-HA Ab. Immunoprecipitates were eluted in SDS-sample buffer and resolved in 1% SDS-PAGE, transferred to PolyScreen polyvinylidene difluoride membrane; probed with either anti-LEF-1, anti-c-Myb, or anti-HA Ab; and detected using Renaissance Western blot.
chemiluminescence reagent. As a positive control, the nuclear extract was directly applied to SDS-PAGE.

Ectopic expression of the DN form of LEF-1 in 18.81 cell line and primary bone marrow B lineage cells, and analysis of RAG expression

Production of retrovirus carrying the green fluorescent protein (GFP) alone or both GFP and DN form of LEF-1 as well as infection of the recombinant virus were performed according to the method previously reported (36), with some modifications. Briefly, the packaging cell line (Phoenix Amphi) was transfected with 15 µg of retrovirus vector containing GFP (pMX-IRES-GFP) or the DN LEF-1 (pMX-DN-LEF-1-IRES-GFP) using the calcium/phosphate precipitation method. Cells were cultured in 4 ml DMEM culture medium containing 10% FCS. After 24 h, cells were reseeded with fresh medium and allowed to grow for further 48 h. After cell culture, the supernatant containing the recombinant virus was recovered and used for infection. For infection, 18.81 cells (2 x 10^5) were incubated with 0.5 µl of retrovirus-containing supernatant in the presence of 20 mM HEPES (pH 7.6) and 10 µg/ml polybren (hexadimethrine bromide; Sigma-Aldrich, St. Louis, MO). After 8 h, 0.5 ml of fresh medium was added to the culture, and the incubation was extended for 16 h. Twenty-four hours after infection, cells were washed and reseeded with growth medium and allowed to grow for 24 h before being subjected to flow cytometry analysis and cell sorting. GFP-positive cells were sorted by EPICS ELITE (Beckman Coulter), and by cloning the limiting dilution method. At least 10 clones were obtained that contain expression vector for GFP alone (pMX-IRES-GFP) or expression vector for both GFP and DN LEF-1 (pMX-DN-LEF-1-IRES-GFP). Cells lysates were obtained from these transfectants or the wild-type 18.81 cell line (ori), and examined for the expression of DN protein or intact LEF-1 protein by Western blot. To analyze the RAG-2 or RAG-1 transcripts, total cellular RNA was prepared from the transfectants. A quantity amounting to 20 µg of total RNA was separated on a 1% agarose/formaldehyde gel, transferred to Gene Screen hybridization transfer membrane (NEN Life Science Products), and hybridized with a 32P-labeled full-length cDNA of either mouse RAG-2 or RAG-1 according to the manufacturer’s instruction. The hybridized membrane was exposed to x-ray film (Fuji Film, Tokyo, Japan). The bands were stripped by boiling in 0.1% SDS in 0.015 M sodium chloride, 0.0015 M sodium citrate, and 1% SDS, and reprobed with a mouse β-actin cDNA.

For the infection of recombinant retroviruses to primary bone marrow B lineage cells, bone marrow cells were prepared from ICR mice (Sankyo Labo Service, Tokyo, Japan) and cultured on ST2 stroma cells (kindly provided by S. Nishikawa, Kyoto University, Kyoto, Japan) in 0.5 ml culture supernatant containing the recombinant virus and 25% conditioned medium containing mouse rIL-7. After 8-h culture, 0.5 ml fresh medium was added, and the cells were further cultured overnight. Then the cells were harvested, washed, and reseeded on ST2 stroma cells in RPMI 1640 including 10% FCS and 25% conditioned medium containing rIL-7. Post-48-h culture, cells were harvested and stained with biotinylated B220 mAb and streptavidin-conjugated PE. B220-positive and enhanced GFP (EGFP)-positive cells were sorted by EPICS ALTRA cell sorter. RNA was prepared from the sorted cells, and primary bone marrow B lineage cells, and analysis of RAG expression

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Results

The −41/−17 region is essential for murine RAG-2 promoter

To determine the minimal region for activation of murine RAG-2 promoter, luciferase reporter gene connected to −1.1 kb to +147 region of the murine RAG-2 or its serial deletion was transfected into 18.81 pre-B cell line or BAL17 B cell line (Fig. 1). Luciferase constructs linked to −1.1 kb/+147 showed the luciferase activity ~8- to 10-fold higher than that of the promoterless construct. Deletion of the RAG-2 promoter from −1.1 kb to −86 did not affect the promoter activity in both cell lines. Deletion till −41 reduced the promoter activity to about half, and deletion till −16 completely abolished the promoter activity. These data show that −41 to −17 region is essential for murine RAG-2 promoter in B lineage cells. They also show that −86 to −41 region may play a regulatory role in murine RAG-2 promoter activity in B lineage cells.

LEF-1, Pax-5, and c-Myb bind the −41/−17 region of the RAG-2 promoter

We have previously shown that the −41/−17 is highly conserved between human and mouse, and that c-Myc and Pax-5 cooperatively bind this element and activate the RAG-2 promoter in immature B cells (21). Database search for the putative binding sites for transcription factors in the −41/−17 region showed the putative LEF-1-binding element (5'−TCACG−3') at −38/−33 as well as c-Myb and Pax-5 binding sites (Fig. 2A). To examine whether LEF-1 as well as c-Myc/Pax-5 bind the −41/−17 RAG-2 promoter, the DNA-Sepharose precipitation assay was performed. As shown in Fig. 2B, the −41/−17 fragment, but not a control DNA fragment, precipitated LEF-1 from nuclear extract of 18.81 cells. In accordance with the previous report (21), the −41/−17 fragment also precipitated Pax-5 and c-Myb. These results show that LEF-1 binds the −41/−17 region of RAG-2 promoter, just as Pax-5 and c-Myb do.

To determine the site-specific binding of the transcription factors, EMSA was performed using the nuclear extract from 18.81 cells and 32P-labeled −41/−17 fragment in the presence or absence of oligonucleotides containing consensus or mutated binding sites for each of the proteins. As shown in Fig. 3A, a specific band (C) was detected in the gel, and the complex formation was completely inhibited by an excess of the oligonucleotides containing the consensus binding site for LEF-1, c-Myb, and Pax-5. The complex formation was not inhibited by an excess of oligonucleotides

FIGURE 1. Promoter activity of 5′ flanking region of mouse RAG-2 in B cell lines. A, Diagram of promoter/luciferase construct. Mouse RAG-2 gene spanning −1.1 kb to +147 bp or its serial deletion (open box for −1.1 kb to −1 and shaded box for +1 to +147) was linked to 5′ of luciferase reporter gene (closed box). +1, Major transcription initiation site. B, Promoter activity in B cell lines with serially deleted RAG-2 promoter region. A total of 10 µg luciferase constructs linked to serially deleted mouse RAG-2 promoter region (indicated on left) was transfected into 18.81 cells or BAL17 cells. pSRE-lacZ was included as an internal control. Twenty-four hours later, luciferase and β-galactosidase assays were performed on cell extracts. The activity of luciferase construct without promoter in each cell is set to 1. Error bars indicate deviation of three experiments.
containing the mutant LEF-1, c-Myb, or Pax-5 binding site, or those containing GATA binding site. This result shows that LEF-1, c-Myb, and Pax-5 cooperatively activate the RAG-2 promoter region in vivo in the RAG-2-expressing immature B cells. The results suggest that LEF-1, c-Myb, and Pax-5 bind the RAG-2 promoter region in vivo in the RAG-2-expressing immature B cells.

**LEF-1, c-Myb, and Pax-5 cooperatively activate the RAG-2 promoter activity**

To determine the effect of LEF-1 on the RAG-2 promoter activity, 293T cells were transfected with luciferase construct connected to −86/+147 RAG-2 promoter alone or together with expression vector(s) for LEF-1, c-Myb, Pax-5, or their combinations, and the luciferase activity was determined. As shown in Fig. 5, expression of LEF-1 dose dependently increased the luciferase activity and augmented the c-Myb- or Pax-5-induced luciferase activity in a dose-dependent manner. Furthermore, LEF-1 augmented the luciferase activity that was induced by combination of expression vectors for c-Myb and Pax-5 in a dose-dependent fashion. These results show that LEF-1, c-Myb, and Pax-5 cooperatively activate...
the RAG-2 promoter. The synergy of LEF-1 with c-Myb and Pax-5 was enhanced by 1.5-fold with the concomitant ectopic expression of β-catenin (data not shown), indicating that β-catenin is involved in the full transcriptional activation of RAG-2 promoter by LEF-1. We chose −86/+147 RAG-2 promoter instead of −41/+147 RAG-2 promoter to determine the effect of LEF-1 on the promoter activation, because −41/+147 RAG-2 promoter showed very low luciferase activity in 293T cells in the presence of c-Myb, Pax-5, and LEF-1. The DNA sequence between −86 and −41 did not contain the putative binding site(s) for these transcription factors (21), indicating that effect of LEF-1, c-Myb, and Pax-5 on −86/+147 RAG-2 promoter directs −41/−17 region.

FIGURE 4. In vivo binding of LEF-1, c-Myb, and Pax-5 to −41/−17 region of RAG-2 promoter. Chromosomal DNA was purified, and existence of −86/+147 sequences in precipitated DNA was examined by PCR. After release from cross-linking, chromosomal DNA was purified, and existence of −86/+147 sequences in precipitated DNA was examined by PCR. As immunoprecipitation control, Sepharose beads alone (no Ab in all panels) or irrelevant isotype-matched control Abs (Cont Ab; anti-Ran in upper and middle panels or 928 Ab in lower panel) were used. DNA size marker was shown on the left. Input indicates PCR product with chromosomal DNA without immunoprecipitation.

FIGURE 5. LEF-1, c-Myb, and Pax-5 cooperatively activate RAG-2 promoter in 293T cells. Luciferase construct (5 μg) linked to −86/+147 RAG-2 promoter region (pmR2L) was transfected into 293T cells with (indicated as +) or without (indicated as −) 0.2 μg pAct-c-Myb or 1 μg pEF-BOS-Pax-5, along with or without various amounts (1–8 μg) of pAct-LEF-1. pSRα-lacZ was included as an internal control. Total amount of DNA was adjusted with either pAct vectors or pEF-BOS vector. Luciferase and β-galactosidase activities were assayed 48 h later. The luciferase activity with none of c-Myb, Pax-5, and LEF-1 was set to 1. Error bars indicate deviations of three experiments.

LEF-1, c-Myb, and Pax-5 synergistically bind the RAG-2 promoter region

To delineate the molecular mechanism in which LEF-1, c-Myb, and Pax-5 cooperatively activate the RAG-2 promoter activity, the ability of each or combinations of LEF-1, c-Myb, and Pax-5 to bind the −41/−17 region of the RAG-2 promoter was examined by EMSA. The 32P-labeled −41/−17 fragment was incubated with various amounts of rPax-5, rc-Myb, or rLEF-1, or combination of the suboptimal concentrations of these proteins and analyzed in the gel. As shown in Fig. 6, A and B, each of low amounts (less than 0.1 μl) of the rPax-5, rc-Myb, and rLEF-1 produced hardly detectable complex formation, while their combinations markedly augmented the complex formation. The cell lysate prepared from the mock-transfected 293T cells did not augment the complex formation (data not shown). These complex formations by the recombinant proteins were specifically inhibited by the oligonucleotides containing either consensus Pax-5, c-Myb, or LEF-1 binding site (data not shown). The mobility of the complex formed with combinations of rPax-5, rc-Myb, and rLEF-1 was the same as that of the complex formed with each of the recombinant proteins.

The cell lysate of 293T transfectant contains many mammalian DNA-binding proteins other than rPax-5, rc-Myb, or rLEF-1, posing a possibility that some other mammalian factors may participate in their interaction. To rule out this possibility, we prepared the rPax-5, rc-Myb, and rLEF-1 by in vitro transcription/translation system using the wheat germ extract and used them for EMSA. As the wheat germ extract does not contain endogenous mammalian transcription factors, the analysis with the in vitro translated recombinant transcription factors excludes the possibility of the involvement of other mammalian factor(s) in Pax-5/c-Myb/LEF-1 complex. As shown in Fig. 6C, combination of the three recombinant transcription factors generated by in vitro transcription/translation system greatly enhanced the DNA-binding capacity compared with that of each factor. The result demonstrates that the mammalian transcription factors other than Pax-5, c-Myb, and LEF-1 were not prerequisite for their cooperative binding to the RAG-2 promoter, although the possible involvement of transcription factors in the wheat germ extract is not yet excluded.
Physical interactions of LEF-1, Pax-5, and c-Myb

In the previous study, we have demonstrated that c-Myb interacts with Pax-5 and activates the mouse RAG-2 promoter in B cells (21). To explore a possibility as to whether LEF-1 physically interacts with c-Myb, Pax-5, or both in vivo, the nuclear extract prepared from 293T cells that had been cotransfected with expression vectors for LEF-1/c-Myb/Pax-5 was immunoprecipitated with either anti-LEF-1 Ab, anti-c-Myb Ab, or anti-Pax-5 Ab, and the precipitates were immunoblotted with each of these Abs. As shown in Fig. 7, anti-LEF-1 Ab coprecipitated Pax-5 and c-Myb together with LEF-1. Similarly, anti-c-Myb Ab coprecipitated LEF-1 and Pax-5 together with c-Myb. Furthermore, anti-Pax-5 Ab coprecipitated LEF-1 together with anti-Pax-5. When the cell lysate prepared from the mock-transfected 293T cells was used, no complex was detected (data not shown). These results together with that of Fig. 6 demonstrate that LEF-1, c-Myb, and Pax-5 may interact and form a tertial complex in vivo, and the formation of tertial complex may increase the affinity for binding the target DNA. However, the data cannot exclude the possibility of the involvement of the other unknown factor(s) in the assembly of these complexes.

Nucleotide sequence requirement for assembly of LEF-1, c-Myb, and Pax-5 complex

In −41/−17 sequences, binding sites of c-Myb and LEF-1 were overlapped with that of Pax-5. To delineate the nucleotide sequence requirement for assembly of these transcription factors, −41/−17 sequences containing mutant binding sequences for each factor were prepared (−41/−17 Pax-5M, −41/−17 c-MybM, and −41/−17 LEF-1M in Fig. 8A) and used as probes for EMSA. When nuclear extracts of 293T cells transfected with either Pax-5, c-Myb, or LEF-1 were used, −41/−17 Pax-5M could bind c-Myb and LEF-1, but not Pax-5; −41/−17 c-MybM could bind Pax-5 and LEF-1, but not c-Myb; and −41/−17 LEF-1M could bind Pax-5 and c-Myb, but not LEF-1 (Fig. 8B). When nuclear extract of 18.81 cells was used as a source of the LEF-1/c-Myb/Pax-5 complex (Fig. 3), it bound to −41/−17 c-MybM and −41/−17 LEF-1M, but not to −41/−17 Pax-5M (Fig. 8B). We further tested the binding of LEF-1/c-Myb/Pax-5 complex to mutated −41/−17 fragment using the in vitro translated transcription factors. Mixture of suboptimal dose of LEF-1, c-Myb, and Pax-5 cooperatively bound wild-type −41/−17 fragment, −41/−17 c-MybM, and −41/−17 LEF-1M, but did not bind to −41/−17 Pax-5M (Fig. 6C). The results indicate that Pax-5 in the complex is mainly involved in DNA binding, and that c-Myb and LEF-1 may function as cofactors to augment the DNA-binding capacity as well as the transcriptional activity of the complex.

DN LEF-1 suppresses the RAG-2 promoter activity and endogenous RAG-2 expression in a pre-B cell line

To examine in vivo effect of LEF-1 on the RAG-2 promoter activity, 293T cells were transfected with luciferase construct with suboptimal amount of rPax-5 (0.03 μl) with or without various suboptimal doses (0.03–0.1 μl) of r-c-Myb along with or without various doses (0.03–0.1 μl) of rLEF-1. Radioactivity of complexes seen in the gel was measured, and relative binding activity to control (without proteins) is shown in lower panel. C. Synergistic binding of in vitro translated rLEF-1, r-c-Myb, and rPax-5 to −41/−17 region. rLEF-1, r-c-Myb, and rPax-5 were prepared using in vitro transcription/translation system, as described in Materials and Methods, and either 6 or 0.5 μl of the product was used for EMSA, as described above. Radioactivity of complexes seen in the gel was measured, and relative binding activity to control (without proteins) is shown at the bottom.
−86/ +147 RAG-2 promoter in the absence or presence of a cocktail of Pax-5/c-Myb/LEF-1 expression vectors, along with various amounts of the expression vector for the DN LEF-1 that lacks the β-catenin binding domain (37). It was shown that the DN LEF-1 inhibited Pax-5/c-Myb/LEF-1-induced RAG-2 promoter activity in a dose-dependent fashion (Fig. 9A). When RAG-2-expressing 18.81 cells were transfected with the luciferase construct with −86/+147 RAG-2 promoter together with various doses of the expression vector for the DN LEF-1, the DN LEF-1 dose dependently suppressed the RAG-2 promoter activity (Fig. 9B). These results show that the expression of DN form of LEF-1 suppresses the RAG-2 promoter activity in vivo.

To examine the effect of DN LEF-1 on RAG-2 mRNA expression, stable 18.81 transfectants expressing the DN LEF-1 were prepared using retrovirus carrying the DN LEF-1 expression vector. The DN LEF-1 proteins and endogenous LEF-1 were examined by Western blot, and the expression of the endogenous RAG-2 was examined by Northern blot. As shown in Fig. 10, the RAG-2 expression in the DN LEF-1-expressing 18.81 clones was significantly suppressed compared with that in the control (vector alone) clones or the wild-type 18.81 cells. To further analyze the role of LEF-1 in RAG-2 expression in primary B cells, freshly prepared mouse bone marrow cells were infected with recombinant retroviruses carrying either control vector or the DN LEF-1 expression vector by culturing the cells on ST2 stroma cells in the presence of mouse rIL-7. Forty-eight hours postinfection, cells were harvested, and either B220+/EGFP+ (infected) cells or B220−/EGFP− (uninfected) cells were separated by cell sorting. Total RNA was prepared from the sorted cells, and expression of RAG-2 mRNA was analyzed by RT-PCR. As shown in Fig. 10C, the amount of RAG-2 transcripts was significantly reduced in EGFP+ cells in DN LEF-1 vector-transfected population. The expression level of RAG-2...
mRNA was not altered in EGFP− cells of DN LEF-1 vector-transfected population, or EGFP+ cells and EGFP− cells of control vector-transfected population. Taken together, these results show that in vivo expression of the DN LEF-1 inhibits the transcription of RAG-2 in a B cell line as well as a primary B cell culture. At the same time, the effect of DN LEF-1 on the expression of RAG-1 was also analyzed. The expression of RAG-1 was significantly reduced in 18.81 cells or primary bone marrow B lineage cells expressing DN LEF-1 (Fig. 10, B and C), indicating that LEF-1 may also play a role in the expression of RAG-1.

Discussion

This study demonstrates that LEF-1, a member of HMG box family, plays a regulatory role in the RAG-2 gene expression in immature B cells. In the previous study, we have shown that c-Myb and Pax-5 bind to the −41/−17 mouse RAG-2 promoter region and synergistically activate the RAG-2 promoter activity in immature B cells (21). However, the maximum response of the RAG-2 promoter activity induced by combination of c-Myb and Pax-5 was limited, indicating that there may be other transcription factor(s) or coactivator(s) participating in the regulation of the RAG-2 promoter activation. In this study, we showed that there is a putative binding element for LEF-1 in −41/−17 RAG-2 promoter region, which was demonstrated to be indispensable for the RAG-2 promoter activity in B lineage cells (Figs. 1 and 2A). This finding
prompted us to investigate the effect of LEF-1 on the regulation of the RAG-2 expression. We have shown that LEF-1 binds to the RAG-2 promoter region and activates the RAG-2 promoter activity together with c-Myb and Pax-5 via their physical interactions and synergistic DNA binding. The data strongly indicate that LEF-1 participates in the regulation of the RAG-2 expression in immature B cells.

Regarding the binding mechanism of the trimolecular complex to the –41/–17 fragment, mutation experiment for each binding site clearly demonstrated that the mutation for Pax-5 binding site abolished the binding of the complex of LEF-1/c-Myb/Pax-5 to the mutant –41/–17 fragment, but the mutation for either LEF-1 or c-Myb binding site did not (Fig. 8). The result indicates that Pax-5 plays a major role in the binding of the trimolecular complex to the RAG-2 promoter, and that LEF-1 and c-Myb function as cofactors to augment the binding capacity and enhance the RAG-2 promoter activity.

LEF-1 was originally reported to bind TCRα enhancer and activate the enhancer efficiently (22). Subsequently, additional binding sites for LEF-1 have been identified in transcriptional control regions of several other T lymphocyte-specific genes, including those encoding adenosine deaminase (38, 39), CD4 (40), TCRβ, and TCRδ (41, 42). It has been reported that LEF-1 is preferentially expressed in T cells and pre-B cells (22). Despite the extensive evidence for the role of LEF-1 in regulation of the T lymphocyte-specific genes during T lymphocyte development, the target genes of LEF-1 in B lymphocytes have remained obscure. In this study, we have shown that the RAG-2 core promoter is a target for LEF-1 and that LEF-1 regulates the RAG-2 expression in B lineage cells.

Concerning in vivo effect of LEF-1 on B cell maturation, it has been recently reported that the LEF-1-deficient mice exhibited a defect of proliferation and survival of pro-B cells (43). However, there was no abnormality in V(D)J recombination and the B lymphocyte development (43). With this regard, it is conceivable that T cell-specific factor-4 (TCF-4), which is a member of the LEF-1/TCF family and expressed in B lineage cells (32, 44), may act redundantly with LEF-1 in the regulation of V(D)J recombination and/or B cell differentiation. Analysis of the double knockout mice of LEF-1 and TCF-4 may prove this assumption. As to T cell development and the RAG expression, LEF-1/TCF-1 double-deficient mice, but not LEF-1 single-deficient mice, exhibited a block of T cell differentiation in thymus at intermediate CD4−/CD8+ differentiation stage (45). It should be noted that the level of RAG transcripts in the thymocytes in the double knockout mice was drastically decreased, suggesting the involvement of LEF-1/TCF-1 in the expression of RAG in T cell development.

LEF-1 has been shown to have no transcriptional activation potential by itself, but act as an architectural protein in the assembly of multiprotein enhancer complexes (25). For example, LEF-1 was shown to regulate transcription in association with Aly and in collaboration with other enhancer-binding proteins in the TCRα enhancer (46). In this study, we have shown that LEF-1 physically interacted and formed a tertiary complex with c-Myb and Pax-5 in the absence of the target DNA. With this regard, it has been recently shown that Smad3 and estrogen receptor physically interacted with HMG box domain of LEF-1, and TCF-1, respectively (47, 48). These results suggest that the physical interaction of LEF-1/TCF family with other transcription factors may be one of the important mechanisms for the activation of the LEF-1/TCF target genes.

How does LEF-1 function as a coactivator for the RAG-2 promoter in vivo? LEF-1 proteins were reported to activate LEF-1 target genes in association with β-catenin pathway (26–30). However, recent studies showed that LEF-1 activates the target genes in the absence of β-catenin under some situations (49, 50). In this study, we examined the effect of DN form of LEF-1, which is deficient with β-catenin binding domain, on the RAG-2 promoter activity as well as the RAG-2 expression in an immature B cell line. The data showed that both the RAG-2 promoter activity and the endogenous RAG-2 expression in these cells were significantly suppressed by the ectopic expression of the DN form of LEF-1 (Figs. 9 and 10), strongly indicating that LEF-1/β-catenin functions as a coactivator in vivo. However, it should be emphasized that whether this inhibitory effect of the DN LEF-1 on the RAG-2 transcription (Fig. 10) is due to its binding to the RAG-2 promoter or binding to other regulatory element(s), such as RAG-2 enhancer, has been undetermined. With this regard, we have recently identified the candidates of the RAG-2 enhancer at the upstream of RAG gene, but the putative binding site for LEF-1 was to date not found in this region (X.-C. Wei et al., manuscript in preparation).

Concerning the LEF-1/β-catenin proteins and Wnt signaling, it has been reported that β-catenin is an important effector in the Wnt signaling pathway that influences multiple processes in vertebrate and invertebrate development (51). Wnt signals stabilize β-catenin in the cytosol (52) and result in the accumulation and nuclear translocation of β-catenin (53). LEF-1/TCF proteins associate with β-catenin and mediate a transcriptional response to Wnt signaling. Although Wnt functions have been studied in many different tissues, little is known about Wnt signaling in the B cell development. Recently, Reya et al. (43) demonstrated that soluble Wnt proteins act directly on pro-B cells to induce entry into the cell cycle and cell proliferation, and that the absence of LEF-1 impairs the ability of pro-B cells to respond to Wnt signaling. In the present study, we have shown that the ectopic expression of LEF-1 mutant, which lacks the β-catenin binding site, suppressed the RAG-2 transcription in pro-B cells, strongly indicating that the RAG-2 expression may be regulated by LEF-1/β-catenin that localize downstream of Wnt signaling. Wnt signaling pathway in immature B cells leading to association of LEF-1/β-catenin followed by the activation of the RAG-2 transcription must be clarified.

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