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Transcriptional Activation of the Pirb Gene in B Cells by PU.1 and Runx3

Kojo Arita,*1 Shota Endo,*1 Tomonori Kaifu,* Kohji Kitaguchi,* Akira Nakamura,* Hidetaka Ohmori,† Kazuyoshi Kohu,† Masanobu Satake,† and Toshiyuki Takai*

Cells in the immune system are regulated positively or negatively by sets of receptor pairs that conduct balanced, activating, or inhibitory intracellular signaling. One such receptor pair termed paired Ig-like receptor (PIR) is composed of the inhibitory PIR-B and its activating isoform, PIR-A. Upon binding to their shared ligand, MHC class I molecules, these receptors control the threshold for immune cell activation. Gene-targeting studies on PIR-B in mice revealed the importance of the inhibition mediated by the PIR-B–MHC interaction in the immune system. Recent studies also revealed the significance of the interaction of PIR-B with neurite outgrowth inhibitors, including Nogo. The coordinated regulation by PIR-B and PIR-A is considered to be primarily dependent on their expression balance in cells. However, the mechanism underlying transcriptional control of the genes for PIR-B and PIR-A (Pirb and Pira, respectively) remains to be clarified. In this study, we identified the major cis-acting promoter segment for Pirb and Pira in B cells as the −212 to −117 region upstream from the translation initiation codon. PU.1 and Runx3 were found to bind to this Pirb promoter. Truncation of the PU.1-binding motif significantly reduced the promoter activity, whereas the influence of elimination of the Runx3 site was marginal in B lymphoma BCL1-B20 cells. Unexpectedly, PU.1, but not Runx3, knockdown reduced the levels of both the Pirb and Pira transcripts. We conclude that the major promoter of Pirb, and probably Pira as well, is activated dominantly by PU.1 and marginally by Runx3 in B cells. The Journal of Immunology, 2011, 186: 7050–7059.

Receptors expressed on immune cells play pivotal roles in the activation or downmodulation of the immune system, whose functional failures due to various defects are directly linked to provocation of immunological disorders such as allergy and autoimmune diseases. Among these regulatory receptors, paired receptors with shared ligands, but opposite functions, namely those delivering activating or inhibitory signals, provide an important means of regulation in terms of fine-tuning of the activation threshold of cells (1, 2). For instance, the low-affinity FcγRIII receptor, FcγRIIB, have been firmly demonstrated in vitro and in vivo to be crucial in immune cell control upon binding to the shared ligand, IgG immune complexes, and in maintaining peripheral tolerance (2, 3). Thus, the balanced expression of these receptors is considered to be crucial for determination of the activation threshold of the cells: upregulation of FcγRIIB would be protective as to inflammation (2, 3), whereas its downregulation in B cells and myeloid cells could be harmful to the host in terms of prevention of inflammation, allergy, and autoimmunity (4–6).

Paired Ig-like receptors (PIRs) have been attracting the interest of many researchers (7, 8). PIRs are expressed on B cells and myeloid-lineage cells, and are composed of inhibitory or braking PIR-B and a multiple activating isoform, PIR-A (8–11), both of which can bind to MHC class I molecules (MHC-I) expressed ubiquitously (12). These receptors are also considered to control the threshold of cellular activation, based primarily on the balance between the PIR-B and PIR-A expression levels (13, 14). The expression level of PIR-B usually dominates over that of PIR-A, suggesting a benefit in the inhibition dominance as to cell regulation. In mast cells, B cells, and dendritic cells, PIR-B can bind to MHC-I on the same cell surface, which gives rise to constitutive inhibitory signaling mediated by recruitment and activation of cytosolic tyrosine phosphatase, Src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1) (15–18). It has also been shown that PIR-B ectopically expressed on T cells can also regulate the cell activation via constitutive SHP-1 recruitment (19). These observations suggest a crucial role of the constitutive inhibition mediated by the PIR-B–MHC-I interaction and SHP-1 recruitment in maintenance of immune homeostasis. Additional interest in PIR-B has been raised by recent studies, in which PIR-B was demonstrated to be crucial for inhibition of axon regeneration and synaptic plasticity (20, 21) through binding to several myelin-associated neurite outgrowth inhibitors, including Nogo, siRNA, small interfering RNA.
MAG, and OMgp (20), in addition to MHC-I (21, 22). In contrast, the physiological role of PIR-A remains obscure, although it is suggested to be involved in graft-versus-host disease (12) and osteoclastogenesis (23).

Although accumulating evidence suggests the significance of PIR-B’s physiologic and pathologic roles in the prevention of allergy (17, 24), infectious diseases (25, 26), and other immunologic disorders (12, 18, 27–30), as well as in neuronal development and synaptic plasticity (20–22), the mechanism underlying transcriptional control of PIR-B and PIR-A is poorly understood. Although possible binding sequences for transcriptional factors that may control the genes for PIR-B and PIR-A (Pirb and Pira, respectively) were found in the upstream region up to about 1.5 kb and 1.0 kb of Pirb and Pira, respectively (31, 32), the molecule(s) that governs transcription has yet to be identified. In this study, we report identification of the major transcriptional promoter segments of Pirb and Pira and the major transcription factors that bind to this region of Pirb in B cells. This knowledge will provide us with clues for dissecting the precise mechanism underlying coordinated expression of PIR-B and PIR-A in B cells, myeloid cells, and neuronal cells.

Materials and Methods
Cells and mice
BALB/c-derived B cell leukemic cell lines, BCL1-B20 and A20, and a C57BL/6-derived T cell lymphoma cell line, EL-4, were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). RAW264.7, a BALB/c-derived macrophage-like cell line, was purchased from American Type Culture Collection (Manassas, VA). Cells and mice

FIGURE 1. Expression profiles of PIR-A and PIR-B in B cell lines. A, Flow cytometric analyses of cell surface expression of PIR-A/B on B cell lines, BCL1-B20 and A20, and a T cell line, EL4, as a negative control. Cells were stained with PE-conjugated 6C1 (thick line), which recognizes both PIR-A and PIR-B, or the rat IgG1 k isotype control (filled histogram). BCL1-B20 cells showed abundant PIR-A/B expression. B, RT-PCR analyses of mRNA expression of PIR-A and PIR-B in two B cell lines and EL4, as a negative control. To compare the amount of mRNA semiquantitatively, PCR was performed at different cycle numbers. Expression of HPRT served as a loading control. Minus reverse transcription (RT) served as a negative control for PCR. BCL1-B20 cells contained abundant mRNAs for both PIR-A and PIR-B, compared with A20 cells, whereas in EL4 cells the messages were not detectable. C, RT-PCR analysis of FcRγ mRNA in BCL1-B20 cells and macrophage-like RAW264.7 cells. FcRγ mRNA was detected in RAW264.7 cells, but not in BCL1-B20 ones, suggesting that the surface expression of PIR-A is limited in BCL1-B20 cells.

Flow cytometry
For flow cytometry, the following mouse-specific Abs were used: PE-conjugated anti-PIR-A/B (6C1) (9) and a rat IgG1 k isotype control (R3-34) (BD Biosciences, San Jose, CA). Cell surfaces were stained by standard techniques, and flow cytometry was performed with a FACScalibur and CellQuest software (BD Biosciences).

RT-PCR
Total RNA was isolated with RNeasy mini kits (Qiagen, Valencia, CA), according to the manufacturer’s instructions, and 200 ng total RNA was subjected to cDNA synthesis using a PrimeScript RT-PCR kit (TaKaRa Biotechnology, Otsu, Japan). The synthesized cDNA was amplified by PCR for 10–40 cycles (95˚C for 10 s, 60˚C [PIR-A and Runx3], 62˚C [PIR-B], or 58˚C [FcRγ, PU.1, and hypoxanthine-guanine phosphoribosyltransferase (HPRT)]) for 20 s, and 72˚C for 20 s) after denaturation for 2 min at 95˚C. The sequences of the primers used for RT-PCR were as follows: PIR-A, 5'-CTCTGCTGCTTGGTCTGGTCTTCTATCTTCTG-3' and 5'-CCCGAGATGCACGGAGTCAAGATCC-3'; FcRγ, 5'-CAAGATCC-3'; PIR-B, 5'-CTCTGGCTGCTTGGTCTGGTCTTCTATCTTCTG-3' and 5'-CCCGAGATGCACGGAGTCAAGATCC-3'; Runx3, 5'-ATCTGACCAACCTGGAGCTC-3' and 5'-ATCTGACCAACCTGGAGCTC-3'; PU.1, 5'-ACAGTGGAGTGAACATGTGAAGATG-3' and 5'-ACAGTGGAGTGAACATGTGAAGATG-3'; Runx3, 5'-ATCTGACCAACCTGGAGCTC-3' and 5'-ATCTGACCAACCTGGAGCTC-3'; Runx3, 5'-ATCTGACCAACCTGGAGCTC-3' and 5'-ATCTGACCAACCTGGAGCTC-3'; TGTTCAGCTCCACTCCATCCTCAG-3'; PIR-B, 5'-ATCTGACCAACCTGGAGCTC-3' and 5'-ACAGTGGAGTGAACATGTGAAGATG-3'; TGTTCAGCTCCACTCCATCCTCAG-3'; Runx3, 5'-ATCTGACCAACCTGGAGCTC-3' and 5'-ATCTGACCAACCTGGAGCTC-3'; PIR-A, 5'-CTCTGCTGCTTGGTCTGGTCTTCTATCTTCTG-3' and 5'-ACAGTGGAGTGAACATGTGAAGATG-3'; TGTTCAGCTCCACTCCATCCTCAG-3'; Runx3, 5'-ATCTGACCAACCTGGAGCTC-3' and 5'-ATCTGACCAACCTGGAGCTC-3'; PIR-B, 5'-CTCTGCTGCTTGGTCTGGTCTTCTATCTTCTG-3' and 5'-ACAGTGGAGTGAACATGTGAAGATG-3'
AGTTCGAAAGG-3' and 5'-GCATCTGCTTTCTCAAGGCT-3'; and
HPRT, 5'-TGATACGTCAAGGGGACAGA-3' and 5'-TTCAGAGGCTTTCACCA-3'.

Plasmid constructs

The Pirb and Pirb full-length promoter sequences were amplified using TaKaRa Ex Taq (TaKaRa Biotechnology) with the following primer sets: Pirb forward primer, 5'-GAAGATCTCCCAGATCTGACCC-3' and reverse primer, 5'-CCCAAGCTTTCTTCCAGGCTTGA-3'; Pirb forward primer, 5'-GGTTTGACGTCTGGTTGCAAGC-3' and reverse primer, 5'-CCCAAGCTTTCTTCCAGGCTTGA-3'. The PCR product for the Pirb promoter resulted in a 180-bp fragment spanning nt −1187 to −7, in which −1 was 1 base 5' upstream of the translational initiation codon. The product was digested with BglII/HindIII and then cloned into the BglII/HindIII-treated pGL4.10 vector (Promega, Madison, WI), followed by sequence analysis according to the procedures described above.

Luciferase reporter assays

Cells (5.0 × 10^6) were electroporated with 5 μg luciferase reporter plasmid and 0.2 μg pGL4.74 (bRluc/TK) vector (Promega) at 300 V and 950 μF with a Gene Pulser II electroporation system (Bio-Rad, Hercules, CA). The electroporated cells were incubated for 24 h and then assayed with a dual-luciferase reporter assay system (Promega) using LMAX II384 (Molecular Devices, Sunnyvale, CA) and SoftMax Pro v5 (Molecular Devices). Reporter assays were repeated at least three times. Firefly luciferase activity was divided by the Renilla luciferase activity of the pGL4.74 vector plasmid. Mock transfection was conducted with a pGL4.10 promoterless vector.

EMSA

Nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Rockford, IL). Biotinylated and nonbiotinylated oligonucleotides corresponding to positions −153 to −124 of the Pirb gene were generated and used as probes. A total of 4 μg of each probe was incubated with a nuclear extract of BCL1-B20 cells. The binding complexes were resolved by electrophoresis on a 5% acrylamide gel and visualized by autoradiography. The nucleotides were denoted as that with a single underline, consensus sequence is denoted as that with a double underline, respectively. The cloning was carried out according to the procedures described above.

FIGURE 2. Deletion of the segment −146 to −128 of the Pirb gene markedly reduced the promoter activity. A, 5' sequences of the Pirb and Pirb genes and binding motifs for transcription factors found in the −212 to −118 segment. The 5' sequence of the Pirb gene (−212 to +6) is aligned with those of the genes for PIR-A, Piral, 2, 3, 4, 6, and Piral. Boxes indicate the sequences in which nucleotide substitutions or deletions are found. The position of the translational initiation codon is assigned as +1. Smaller dots are plotted every 20 nt below the sequence for Pirb. The larger dot at position −56 indicates a tentative transcriptional start site. Horizontal bars indicate binding motifs for transcription factors found in the −212 to −118 segment based on a database search at Transcription Element Search System, as listed in Supplemental Table 1. B. The segment −146 to −128 of the Pirb gene was important for transcriptional activation of Pirb. Firefly luciferase activities of reporter plasmids with short deletions transfected into BCL1-B20 were measured as in Fig. 3. Assays were repeated three times, and a representative result is shown. Each column represents the mean ± SD of triplicate determinations.
aliquots of nuclear extracts and 100 fmol biotinylated probe was incubated at room temperature in 10 mM Tris, 2.5% glycerol, 1 μg/μl poly

deoxyinosinic-deoxycytidylic acid, 10 mM EDTA (pH 7.5), 1 mM DTT, 100 mM KCl, and 5 mM MgCl2 to 20 min. For competition assays, a 100-
fold molecular excess of the nonbiotinylated probe was also added to the reaction mixture. For supershift assays, nuclear extracts were preincubated with 4 μg Abs for 15 min at 4°C before adding the biotinylated probe. Anti-PU.1 (sc-352X), anti-Elf-1 (sc-631X), anti-Spi1 (sc-14027X), anti-Runx2 (sc-8564X), and anti-Runx3 (sc-8560X) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-Runx3/AML2 (39301) Abs were purchased from Active Motif (Carlsbad, CA). The mixtures were separated by electrophoresis on 6% retardation gels (Invitrogen, Carlsbad, CA) at 100 V for 45 min in 0.5× TBE buffer (0.23 M Tris, 0.23 M boric acid, 1 mM EDTA), transferred to a Hybond-N+ nylon membrane (GE Healthcare, Piscataway, NJ), and then UV cross-linked with UV Stratalinker 1800 (Stratagene, La Jolla, CA) for 5 min. The membrane was treated and visualized with a chemiluminescent nucleic acid detection module kit (Pierce, Rockford, IL).

Knockdown of PU.1 and Runx3 by RNA interference

A total of 2.0 × 106 BCL1-B20 cells was transfected with 4.0 μg PU.1-specific small interfering RNA (siRNA) (ON-TARGETplus SMARTpool, mouse PU.1; Dharmacon, Chicago, IL), Runx3-specific siRNA (ON-TARGETplus SMARTpool, mouse RUNX3; Dharmacon), or control siRNA (ON-TARGETplus Non-Targeting Pool; Dharmacon) using Nucleofector II (Lonza, Basel, Switzerland). The cells were harvested 24 h after transfection, and analyzed for mRNA expression of PIR-A, PIR-B, PU.1, Runx3, and HPRT by quantitative RT-PCR. The cDNA fragments were amplified with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) by using the following primers: PIR-A, 5'-ATTGAATCCTCCACCTG-GGGATGCACAGCTAGAGAGG-3' and 5'-TCCCCATCCTGATGAGATTC-3'; PIR-B, 5'-TCAGTGG-AGGACATGCAAAC-3' and 5'-CTTGGACATGACAGAAGGTGAG-3'; PU.1, 5'-ATGTTACAGGCGTGCAAAATGG-3' and 5'-ATGTCTCATGGCGTCTTCACAAG-3'; Runx3, 5'-CTCCAGCCCGAGACTACAAG-3' and 5'-GGGGCTGTACTGCTTAACCAG-3'; HPRT, 5'-TCAGTCAACCGGG-GACATAA-3' and 5'-GGGGCTGTACTGCTTAACCAG-3'. The ampli
con was detected with DNA Engine Opticone 2 (MJ Research), and the gene expression data were normalized to endogenous HPRT expression.

Statistical analysis

Statistical analysis was performed by means of Student t test. We con
dered p < 0.05 as being statistically significant.

Results

BCL1-B20 cells express PIR abundantly on the surface

B cells and myeloid cells express PIR-B exclusively, and both PIR- B and PIR-A, respectively, on their surface. We first attempted to find a B cell line that is suitable for examination of expression of the mRNA for PIR-B, and to analyze in detail the transcriptional control of PIRb in vitro. To this end, we examined the PIR expression on two B cell lines, BCL1-B20 and A20, and a T cell line, EL-4, as a negative control by flow cytometry using the 6C1 mAb, which recognizes both PIR-A and PIR-B (9). As shown in Fig. 1A, BCL1-B20 cells were found to express PIR abundantly on their surface, whereas A20 cells expressed it only marginally. As expected, PIR expression was not detected on EL-4 cells, a T lymphoma cell line, on flow cytometry.

We next assessed the expression levels of the mRNAs for PIR-A and PIR-B separately by semiquantitative RT-PCR (Fig. 1B). Consistent with the flow cytometric data, BCL1-B20 cells carried easily detectable amounts of mRNAs for both PIR-A and PIR-B. In contrast, in A20 cells, whereas a substantial amount of the mRNA for PIR-B was detected, the expression of the mRNA for PIR-A was minimal. EL-4 did not have a detectable amount of the mRNA for PIR-A or PIR-B. The FcR common γ-chain (FcγR) is

![FIGURE 3. Identification of the major Pirb and PirA6 promoters in BCL1-B20 cells. A, Schematic il

![stration of the firefly luciferase reporter plasmid constructs containing the 5′ upstream region of the Pirb gene and its stepwise 5′ deletions. In the Pirb gene, the 5′ untranslated region (nt positions –56 to –1; National Center for Biotechnology Information RefSeq accession number NM_011095; http://www.ncbi.nlm.nih.gov/genbank/) and exon 1 are shown as open and closed boxes, respectively. Each firefly luciferase (Luc) reporter construct is denoted as its most 5′ nucleotide relative to the Pirb ATG codon. B–E, Firefly luciferase activities of reporter plasmids containing the Pirb promoter region in BCL1-B20 (B), A20 (C), and EL4 cells (D), and of those containing the PirA6 promoter region in BCL1-B20 (E). A control expression vector for the Renilla luciferase gene driven by the SV40 promoter was transfected in parallel. Firefly luciferase activity was divided by Renilla luciferase activity for normalization as to transfection efficiency. Assays were repeated at least three times, and representative results are shown. Transfection of the PGL4 promoterless vector served as a negative control (mock). Each column represents the mean ± SD of triplicate determinations.]}
mandatory for cell surface traffic and signaling of PIR-A (9, 35–37). In BCL1-B20 cells, we did not detect the mRNA for FcRγy, whereas it was easily detected in a macrophage-like cell line, RAW264.7 cells, used as a positive control (Fig. 1C). These results suggest that, on the surface of BCL1-B20 cells, it is PIR-B protein, but not PIR-A, that is abundantly detected by flow cytometry. Based on these results, we selected BCL1-B20 cells as a useful B cell line for further in vitro analysis of transcriptional control of the Pirb gene, referring simultaneously the Pirb gene transcription as a control when necessary.

**Identification of the major transcriptional activating region of Pirb and Pira in BCL1-B20 cells**

We then constructed a series of expression vectors for the firefly luciferase gene driven by a putative 5′-promoter segment of the Pirb gene, as well as of Pira, which is one of multiple Pira genes (see Fig. 2A), spanning nt positions −1187 to −1, and deletion mutants of it with 5′ truncation of the putative promoter to various lengths (Fig. 3A), and performed a luciferase reporter assay by transfecting BCL1-B20 cells with these vectors, followed by measurement of the luciferase activities in cell extracts after 48 h. A control expression vector for the Renilla luciferase gene driven by the SV40 promoter was transfected in parallel for normalization. Based on these results, we selected BCL1-B20 cells as a useful B cell line for further in vitro analysis of transcriptional control of the Pirb gene.

**FIGURE 4.** PU.1 and Runx3 binding to the Pirb promoter region in B cell extracts. Nuclear extracts of BCL1-B20 cells (A, B) and splenic B cells (C) were subjected to EMSA with biotinylated oligonucleotides corresponding to the −153 to −124 segment of the Pirb gene as probes. For competition assaying in A, a 100-fold molecular excess of the non-biotinylated probe was included in the reaction mixture. For EMSA combined with specific Abs to transcription factors (B, C), nuclear extracts were preincubated with Abs specific for PU.1, Elf-1, Sp1, or Runx1, 2, 3 before adding the biotinylated probe. The mixtures were separated by electrophoresis, transferred to membranes, and visualized with streptavidin-HRP.

**FIGURE 5.** PU.1- and Runx3-binding motifs regulate Pirb transcriptional activity. A, Site-directed mutagenesis of PU.1- and Runx3-binding motifs. Luciferase reporter plasmid constructs containing intact binding motifs or deletions of them in the Pirb promoter region are shown. B, Luciferase activities in BCL1-B20 cells transfected with the reporter plasmids. Assays were repeated at least three times, and representative results are shown. Mock transfection was conducted with the pGL4.10 promoterless vector. Firefly luciferase activity was divided by Renilla luciferase activity for normalization as to transfection efficiency. Each column represents the mean ± SD of triplicate determinations.

Most strikingly, further deletion of −212 to −118 almost totally eliminated the promoter activity. Thus, we concluded that a major promoter for the Pirb gene in BCL1-B20 cells could be located within nt segment −212 to −118. For reference, we also conducted reporter assays on A20 cells, another B cell line, and the EL4 T cell line (Fig. 3C, 3D). The Pirb promoter activity in A20 and EL4 cells was much lower than that in BCL1-B20 cells, as expected from the RT-PCR and flow cytometric data shown in Fig. 1. We also performed the reporter assay of the Pirab gene in BCL1B20 cells, and found a major promoter activity in the same segment (−212 to −118) as that of Pirb (Fig. 3E).

Given the −118 to −212 segment contains a major promoter for Pirb and Pira, we searched for binding motifs of known transcription factors in the Pirb promoter with Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess), and finding 19 candidate sequences as to the binding of mammalian transcription factors (Fig. 2A, Supplemental Table 1). Because 5′ sequences of the known Pira genes, that is, Pira1, 2, 3, 4, 6, and Pira11, are very similar to that of Pirb, we also aligned them with that of Pirb for comparison (Fig. 2A). Among them, it is noteworthy in Pirb and in many of Pira that there are several motifs of Ets family molecules such as PU.1, which is known to be critical for various cellular events, including proliferation, differentiation, and survival. It is also interesting to note that the binding motifs of C/EBP and Sp1 expressed ubiquitously are also present in this region. We also searched for binding motifs within segment −407 to −213 and found some, as shown in Supplemental Table 2, many of them being compatible with those reported previously (31). However, we failed to find any PU.1 motifs within the −407 to −213 region.
To narrow down the most critical segment within −212 to −118 of the Pirb promoter, we generated several mutant vectors with small 9–12 nt deletions for luciferase reporter assay in BCL1-B20 cells. As shown in Fig. 2B, we found that only the deletions of −146 to −137 and −136 to −128 strikingly reduced the promoter activity, whereas other deletions did not cause any marked reduction in the activity. Thus, we concluded that a major promoter of the Pirb gene is located within nt segment −146 to −128, where several sequences for possible binding of transcription factors, including PU.1 and Runx, are located.

**PU.1 and Runx3 trigger Pirb transcription**

PU.1 is specifically expressed in B cells and myeloid cells (38), in which Pir-B is abundantly expressed (7–9, 39). Therefore, we were particularly interested in the possibility that PU.1 could trigger transcription of Pirb in B cells. We generated a DNA probe spanning nt positions −153 to −124, which also harbors binding motifs for Elf-1, Sp1, and Runx1 in addition to one for PU.1, and performed EMSA using a nuclear extract of BCL1-B20 cells. As shown in Fig. 4A, EMSA revealed that the labeled DNA probe exhibited a mobility shift when mixed with the nuclear extract, which was not seen in the presence of competitor nonlabeled DNA, indicating that the −153 to −124 segment served as a substrate for specific binding of any factor(s). We next examined which transcription factors described above could bind to this segment by means of gel shift assays involving Abs specific to these four transcription factors. We found that PU.1, but not Elf-1, Sp1, or Runx1, in the BCL1-B20 nuclear extract could bind to the probe, because PU.1-specific Abs induced a marked weakening of the band intensity, as illustrated in Fig. 4B. As Runx2 and Runx3 share the same binding motif with Runx1, we also examined the possible binding of these transcription factors, and found that Runx3, but not Runx2, could bind to the probe (Fig. 4B). To confirm that these binding profiles of transcription factors in BCL1-B20 cells are also the case in B cells from mouse tissues, we performed a similar assay using a nuclear extract of splenic CD19+ B cells. As shown in Fig. 4C, anti-PU.1 and anti-Runx3 Abs induced a disappearance of the specific band, but Elf-1, Sp1, Runx1, and Runx2 ones did not. As already shown in Fig. 2B, the possible PU.1 and Runx3 binding is restricted to only the 19-bp segment spanning positions −146 to −128. Therefore, these results strongly suggest that PU.1 and Runx3 control the transcription of the Pirb gene in B cells by binding to the S’ upstream sites within nt positions −146 to −128.

We next attempted to verify that the predicted binding motifs for PU.1 and Runx3 in fact serve as promoter sequences, and to determine whether these transcription factors actually have an impact on transcriptional activity. To this end, we constructed a series of site-directed mutant vectors for luciferase expression driven by the putative S’-promoter region of Pirb from −407 to −1 with deletion(s) of both or either sequence of the PU.1- and Runx3-binding motifs (Fig. 5A). BCL1-B20 cells were transfected with them and then subjected to luciferase reporter assaying (Fig. 5B). Deletion of the Runx3 site reduced the promoter activity to ∼85%, whereas elimination of the PU.1 site reduced it to ∼20%. Deletion of both the Runx3- and PU.1-binding motifs further reduced the activity to ∼15% (Fig. 5B). Thus, the reduction achieved on deletion of the Runx3 site was much lower than that on deletion of the PU.1 site. These results indicate that PU.1 primarily triggers transcription, whereas Runx3 contributes to it to a lesser extent, and suggest that PU.1 and Runx3 activate cooperatively in the Pirb gene transcription in B cells.

**Knockdown of PU.1, but not Runx3, downmodulates Pirb and Pira mRNA in BCL1-B20 cells**

Next, we examined whether PU.1 or Runx3 knockdown could downmodulate the mRNA levels of Pir-B and Pira in B cells. siRNA specific for PU.1, Runx3, or control siRNA was prepared and transfected into BCL1-B20 cells, and then quantitative RT-PCR was performed after 24 h. As shown in Fig. 6A–D, under...
Runx3-mediated activation of Pirb transcription is marginal in splenic B cells

Finally, we aimed at determining how significantly Runx3 in B cells in vivo controls Pirb transcription. To this end, we used two genetically engineered mouse strains, one of which comprises Runx3 cKO mice that do not express Runx3 in their CD19+ B cells (H. Ohmori, K. Kohu, and M. Satake, manuscript in preparation), and the other strain comprises Runx3 cTg mice in which total Runx3 expression in their CD19+ B cells was accelerated 4- to 5-fold compared with that in wild-type mice (H. Ohmori, K. Kohu, and M. Satake, manuscript in preparation). Splenic CD19+ B cells from these Runx3 cKO or cTg mice and control mice were subjected to RT-PCR analyses of mRNAs for Runx3, Pirb, and Pira. B cells from these Runx3 cKO or cTg mice and control mice were subjected to RT-PCR analyses of mRNAs for Runx3, Pirb, and Pira. In Runx3 cKO mice, truncated Runx3 mRNA was found to be substantially reduced only under the decrease of PU.1, but not Runx3 (Fig. 6C). Interestingly, the amount of Pirb mRNA was also reduced on siRNA transfection of PU.1, but not Runx3 (Fig. 6D). We also monitored the cell surface levels of PIR-A/B by flow cytometry (Fig. 6E). We observed substantial lowering of surface PIR-A/B expression at 24 h after transfection of PU.1 siRNA. Based on the RT-PCR data with a minute amount of mRNA for Feεγγ, which is required for surface expression of PIR-A (Fig. 1C), the reduced surface PIR-A/B level seemed to be due to the reduction of PIR-B. Collectively, these data demonstrate that PU.1 triggers Pirb transcription, and indicate that PU.1 stimulates Pirb transcription as well in BCL1-B20 cells.

Discussion

We have demonstrated in this study that the major promoter region triggering Pirb gene transcription in B cells is located within its 5'-212 to -118 DNA segment, in which PU.1 mainly regulates the transcription through binding to the -146 to -128 segment. PU.1 is an Ets family transcription factor and is expressed specifically in B cells and myeloid-lineage cells (38, 40–43). It has been reported that when CD34+ hematopoietic stem cells have a high level of PU.1, they are prone to develop into macrophages, whereas with insufficient PU.1 expression, they tend to develop into B cells (38), demonstrating PU.1 is a critical transcription factor in B cells and myeloid cells. A study has shown that mice exhibiting reduced expression of PU.1 due to a deletion in its enhancer region lack B-2 cells, whereas the proliferation of B-1 cells in these mice was rather accelerated (44). Interestingly, the B-1 cell compartment enlarges with age in Pirb/- mice, too (39). Thus, downmodulation of Pirb-B could be one of the reasons that the B-1 cell proliferation in vivo was accelerated with reduced PU.1. Downmodulation of Pirb-B would lead to an enhanced TLR9-mediated activation of B-1 cells because our recent study
revealed that the enlarged B-1 cell compartment with PIR-B deficiency is due to the augmented TLR9 signal (27).

Runx3 constitutes the Runx family together with Runx1 and Runx2, and in the immune system it is expressed in myeloid-lineage cells, B cells, and T cells. Various phenotypes have been reported for Runx3 deletion in T cells, such as skewed CD8\(^+\) T cell development due to the suppression of CD4 (45–47), increased production of IFN-\(\gamma\) in Th1 cells, and reduced expression of the IL4 gene (48). In addition, inhibition of the development of dendritic cells by TGF-\(\beta\) was shown to be hampered by Runx3 deletion (49) because stimulation of the TGF-\(\beta\) receptor otherwise enhances the expression of Runx3. Despite these various effects of Runx3 on immune cells, its role in B cells is obscure. In the current study, although we have detected its role in PIR-B expression in BCL1-B20 cells and splenic B cells, Runx3’s contribution was much smaller than that of PU.1 (Figs. 5–7).

Ets family and Runx family transcription factors form a complex, which controls the transcription of mRNAs for TCR \(\alpha\) - and \(\beta\) -chains, and the M-CSFR (50–53). For example, Ets-1 interacts with Runx1, and they collaboratively enhance the abilities of DNA binding in a large complex involving other molecular molecules such as CBP/p300 and GATA-3 at the enhancers of TCR genes (54–56). This mechanism for the PU.1/Runx1 interaction is also the case for the I\(\alpha\)1 germline promoter (57, 58). At myeloid-specific promoters, PU.1 and Runx1 similarly interact and bind to DNA as a large complex containing CBP/p300 and C/EBP (59–61). It is also suggested that Ets-1 may function in collaboration with Runx3 as a CD4 gene silencer (45, 62). Taking these notions altogether, it may be interesting to speculate that PU.1 and Runx3 may form a complex also in B cells for Pirb transcription. Thus, in our tentative model shown in Fig. 8, PU.1 binding to the -146 to -128 region strongly triggers Pirb transcription (Fig. 8A), whereas its transcriptional activation is not eminent on single Runx3 binding to this segment (Fig. 8B). When PU.1 and Runx3 bind together to this region, Pirb transcription will be fully triggered (Fig. 8C). This simplified view of the mechanism for Pirb transcriptional control would be valid for considering the transcriptional regulation of Pirb in B cells and myeloid cells, although we should also consider the possible involvement of other transcription factors that bind to promoter, enhancer, or suppressor sequences more upstream of this region, such as the 5’ -407 to -213 segment of the Pirb gene, as noted in Fig. 3, and Supplemental Tables 1 and 2.

Can similar transcriptional regulation occur for a Pira gene? Pira comprises multicyclic genes, in which we can find six Pir genes in the GenBank, that is, Pira1, Pira2, Pira3, Pira4, Pira6, and Pira11 (Fig. 2A), of which translational products make PIR-A proteins highly homologous to each other. Comparing the 5’ nucleotide sequences up to position -212 of the Pira genes with that of the Pirb gene, we observed >95% sequence homologies (Fig. 2A). Interestingly, every 5’ sequence was found to possess two PU.1-binding motifs at -171 to -176 and -140 to -145 as those of Pirb (Supplemental Table I). Therefore, it is conceivable that the Pira genes are also controlled by PU.1, and we were able to observe PIR-A downmodulation with reduced PU.1 due to RNA interference (Fig. 6A, 6D), suggesting that PU.1 has a significant impact on trans activation of Pira as well. Also, it was shown that the DNA segment corresponding to the major promoter of the Pirb gene (nt -212 to -118) is also employed for Pira transcriptional activation (Fig. 3E). We cannot exclude the involvement of other regulatory mechanisms such as ones controlled by a higher chromatin structure such as histone acetylation, modulation of DNA methylation on CpG motifs, or microRNA for the differential regulation of Pira and Pirb expression.

In resting B cells, only Pirb, that is, not Pira, is expressed on the surface (39). It is speculated that the absence of PIR-A expression on the B cell surface could be due mainly to the absence in B cells of the expression of FcR\(\gamma\), which associates with and is required for the surface traffic of PIR-A (35, 37). Because transcription of the gene for FcR\(\gamma\) (Fcer1g) is induced by deletion of Pax5, a transcription factor crucial for B cell development and function (63), FcR\(\gamma\) expression in B cells is suppressed by Pax5 in resting B cells. We searched for a possible Pax5 binding site in the 5’ upstream region of Pira, but we failed to find such sequence up to position -250 (data not shown), suggesting that regulation of the Pira gene is different from that of Fcer1g. Our observation that BCL1-B20 cells show transcription of Pirb, but not Fcer1g (Fig. 1B, 1C), supports this notion. In addition, splenic B cells have PIR-A mRNA and its protein in the cytosol (9, 64, 65). Therefore, it is most likely that Pira is indeed transcribed and translated in B cells, but is not expressed on the cell surface due to the lack of sufficient FcR\(\gamma\) expression.

Receptor molecules of the leucocyte Ig-like receptor (LILR) family have been considered to be human orthologs or close relatives of murine PIR-A and PIR-B (12, 66–73), wherein LILRA are activating molecules and LILRB are braking ones, like PIR-A and PIR-B. Also, in humans, paired MHC-I receptors, killer Ig-like receptor (KIR), found on NK cells also include activating and inhibitory constituents (74). PU.1 was found to be involved in both LILRA and LILRB transcriptional control, whereas Runx1 and Sp1 were suggested to be related to LILRA2 and LILRB1 transcription, respectively (75). In NK cells, Runx2 is involved in the transcriptional control of KIR2DL4 and KIR3DL3 (76), and its Ets-binding motif is important for KIR3DL1 transcription (77). Thus, also in humans, transcription factors of the Ets and the Runx families are implicated in LILR and KIR transcriptional control like murine Pirb. One may speculate that the fundamental regulatory mechanism for the transcription of mutually cognate receptor pairs, PIR, LILR, and KIR, is conserved beyond species.

In conclusion, we found that the Pirb gene transcription in B cells is controlled mainly by PU.1 and less significantly by Runx3, probably in a cooperative manner, and suggest that PU.1-mediated activation also occurs in Pira transcription. Nonetheless, we cannot exclude the possibility that unidentified, regulatory mechanisms for transcription may still exist for Pirb or Pira. Understanding the whole molecular events that govern the transcriptional control of activating and inhibitory paired receptors in B cells and myeloid cells should provide clues for exploiting activating or inhibitory functions separately to treat allergies and autoimmune diseases, which could be caused by imbalanced regulation of these paired receptors.

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Disclosures

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