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

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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 in the CNS. 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 Pirα, respectively) remains to be clarified. In this study, we identified the major cis-acting promoter segment for Pirb and Pirα 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 Pirα transcripts. We conclude that the major promoter of Pirb, and probably Pirα as well, is activated dominantly by PU.1 and marginally by Runx3 in B cells. The Journal of Immunology, 2011, 186: 000–000.

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 FcγRIIa, which bind to MHC class I molecules (MHC-I) expressed on the same cell surface, which gives rise to constitutive inhibitory signaling mediated by recruitment and activation of 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.

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Abbreviations used in this article: eKO, conditional knockout; cTg, conditional transgenic; HPRT, hypoxanthine-guanine phosphoribosyltransferase; KIR, killer Ig-like receptor; LILR, leukocyte Ig-like receptor; MHC-I, MHC class I; PIR, paired Ig-like receptor; SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase-1; siRNA, small interfering RNA.

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MAG, and OMP (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 were maintained in RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C under 5% CO2. C57BL/6 mice were purchased from Charles River Japan. Runx3 conditional transgenic (cTg) mice (accession number CDB0421T; RIKEN Center for Developmental Biology, http://www.cdb.riken.jp/arg/TG%20mutant%20mice%20list.html) and Runx3 conditional knockout (cKO) mice (accession number CDB0458K; RIKEN Center for Developmental Biology, http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) were provided by H. Ohmori, K. Kohu, and M. Satake, manuscript in preparation (33, 34). Mice were maintained and bred in the Animal Facility of the Institute of Development, Aging, and Cancer, Tohoku University, an environmentally controlled and specific pathogen-free facility, according to guidelines for experimental animals defined by the University, and the animal protocols were reviewed and approved by the Animal Studies Committee of the University. For separation of splenic B cells from mice, CD19+ cells were purified with a MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). All experiments were performed on 6- to 12-wk-old male and female mice.

**Flow cytometry**

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

**RT-PCR**

Total RNA was isolated with RNasy 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, Ohtsu, 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 [FcRg], RT-PCR analysis of 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'–CTCTGCTGACAGCTCAACTGTCAG–3' and 5'–CCCTGTTGTTAGACCATAT-3'; PIR-B, 5'–GTCGAGCTCCATCTCTCACT–3' and 5'–ATCCTTTGTTGTTGACCTCT–3'; FcRg, 5'–CAAGATCC–3' and 5'–GGTGGCTGATAGGTGACCCACT–3'; Runx3, 5'–ATCGACCAACTCGGACAC–3' and 5'–ATCGACCAACTCGGACAC–3'.
Plasmid constructs

The Pirb and Pira6 full-length promoter sequences were amplified using TaKaRa Ex Taq (TaKaRa Biotechnology) with the following primer sets: Pirb forward primer, 5'-GAAGATCTCTCCAGAGTCTGACCC-3' and reverse primer, 5'-CCCAAGCTTCTCCCTGATGCGTCAACTG-3'; Pira6 forward primer, 5'-GGGTACCGAGCTGTCACTGTCAG-3' and reverse primer, 5'-CCCAAGCTTCTCCCTGATGCGTCAACTG-3'. The PCR product for the Pirb promoter resulted in a 1180-bp fragment spanning nt −704 to −7, in which −1 was 1 base 5' upstream of the translational initiation codon. The product was digested with KpnI/HindIII and then cloned into the KpnI/HindIII-treated pGL4.10 (Luc2) vector (Promega, Madison, WI), followed by sequence analysis to confirm the proper reading frame of the inserted product. The PCR product for the Pira6 promoter resulted in a 697-bp fragment spanning nt −704 to −7, in which −1 was 1 base 5' upstream of the translational initiation codon. The product was digested with KpnI/HindIII and then cloned into the KpnI/HindIII-treated pGL4.10 (Luc2) vector (Promega, Madison, WI), followed by confirmation of the sequence. Promoter constructs of various lengths were generated by PCR amplification, exploiting the full-length promoter constructs as a template, with the combination of the reverse primer for the full-length promoter construct and the following primers: Pirb, 5'-GAAGATCTTCTAGAAAACATCAACTGTTGTT-3' for the −407 construct, 5'-GAAGATCTGGCCATGAGAGCGAC-3' for the −286 construct, 5'-GAAGATCTGGCAGAGGGTAC-3' for the −212 construct, 5'-GAAGATCTTAGAAACATCAAAATGCATT-3' for the −117 construct, and 5'-GAAGATCTAGAAACATCACAATGGGAC-3' for the −66 construct.

Luciferase reporter assays

Cells (5.0 × 10^6) were electroporated with 5 μg luciferase reporter plasmid and 0.2 μg pGL4.74 (hRluc/hTK) 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 II (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 target DNA was incubated with nuclear extracts from RS-5 A7 cells, and EMSA 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/ml poly-
deoxyinosinic-deoxyctydilic acid, 10 mM EDTA (pH 7.5), 1 mM DTT, 100 mM KCl, and 5 mM MgCl₂ for 20 min. For competition assays, a 100-
mol 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-Sp1 (sc-14027X), anti-
Runx2 (sc-856x), and anti-Runx3/AML2 (sc-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 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′-TGTTACAGGCGTGCAAAATGG-3′ and 5′-TCAGTGGCTAGCTATTTTGAGG-3′; PIR-B, 5′-TCAGTGAGGACACATGCAAAC-3′ and 5′-CCTTGACATGACAGAAGGTGAG-3′; PU.1, 5′-ATGGTACAGGGCGTGCAAATGG-3′ and 5′-TGATCGCTATGGCTTCTCCA-3′; Runx3, 5′-CTCCAGCCCGAGACTACAAG-3′ and 5′-GGGATGACGCTAGAGGAGG-3′; HPRT, 5′-TCAGTCAACGGGG-
GACATAA-3′ and 5′-GGGGCTGTACTGCTTAACCAG-3′. The ampli-
con was detected with DNA Engine Opticon 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-
sidered p < 0.05 as being statistically significant.

**Results**

**BCL1-B20 cells express PIR abundantly on the surface**

B cells and myeloid cells express PIR-A 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
PIR 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 (FcRγ) is

**FIGURE 3.** Identification of the major Pirb and
PirA promoters in BCL1-B20 cells. A. Schematic il-
ustration 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 lu-
ciferase (Luc) reporter construct is denoted as its most
5′ nucleotide relative to the Pirb ATG codon. B–E,
Firefly luciferase activities of reporter plasmids con-
taining the Pirb promoter region in BCL1-B20 (B), A20 (C), and EL-4 cells (D), and of those con-
taining the PirA 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
transfer 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 FcRγ, 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 Pira 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 Pira6, 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 of the transfection efficiency. As shown in Fig. 3B, we found that deletion of −1187 to −408 of the Pirb gene did not cause any reduction in luciferase activity in BCL1-B20 cells, but further deletion to −287 or −213 lowered the activity to ∼70 or ∼50%, respectively, indicating the possibility of separate promoter activities for nt segments −407 to −287 and −286 to −213 of the Pirb gene. 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 Pira6 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 Pira6, 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/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, Piral, 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.

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 assay 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. −407, intact motifs; ΔDouble, deletion of both the Runx3- and PU.1-binding motifs; ΔPU, deletion of the PU.1-binding motif; ΔRx3, deletion of the Runx3-binding motif.
To narrow down the most critical segment within -212 to -118 of the \textit{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 \textit{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 \textit{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 \textit{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 \textit{Pirb} gene in B cells by binding to the 5’ 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 5’-promoter region of \textit{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 \textit{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 PIR-A in BCL1-B20 cells by 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

**FIGURE 6.** PU.1, but not Runx3, knockdown reduces mRNA level of PIR-B and PIR-A in BCL1-B20 cells. A–D, Transfection of BCL1-B20 cells with siRNA for PU.1 or Runx3 induced downregulation of the mRNA for PU.1 (A) or Runx3 (B), respectively, after 24 h. The signals of the mRNAs for PIR-B (C) and PIR-A (D) were concomitantly downmodulated only by PU.1 knockdown, but not by Runx3 siRNA. As a control for these quantitative RT-PCR, mRNA for HPRT was monitored. Each column represents the mean ± SD (n = 3). E, Reduced PIR-A/B on the BCL1-B20 cell surface after PU.1 knockdown. BCL1-B20 cells were transfected with PU.1-specific siRNA. Cells were harvested at 24 h after transfection, stained with PE-conjugated anti-PIR-A/B mAb (6C1), and then analyzed by flow cytometry. **p < 0.01, ***p < 0.001.
the condition that PU.1 or Runx3 siRNA transfection reduced specifically the level of mRNA for PU.1 or Runx3, respectively, the amount of Pirb mRNA was found to be substantially reduced only under the decrease of PU.1, but not Runx3 (Fig. 6C). Interestingly, the amount of Pira 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 FcRγ, 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 Pira transcription as well in BCL1-B20 cells.

**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 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

**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-γ in Th1 cells, and reduced expression of the Il4 gene (48). In addition, inhibition of the development of dendritic cells by TGF-β was shown to be hampered by Runx3 deletion (49) because stimulation of the TGF-β 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 α- and β-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 transcriptional 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 Igα 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 multicopy genes, in which we can find six Pira 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 PIR-B, that is, not PIR-A, 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 FcRy, which associates with and is required for the surface traffic of PIR-A (35, 37). Because transcription of the gene for FcRy (FcεR1g) is induced by deletion of Pax5, a transcription factor crucial for B cell development and function (63), FcRy 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 FcεR1g. Our observation that BCL1-B20 cells show transcription of Pira, but not FcεR1g (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 FcRy expression.

Receptor molecules of the leukocyte 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 LILRBR 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 LILRBR transcriptional control, whereas Runx1 and Sp1 were suggested to be related to LILRA2 and LILRBR1 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 PIR-B. 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
The authors have no financial conflicts of interest.

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binding between inhibitory receptors and MHC class I can regulate mast cell
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4. Hayami, K., D. Fukuta, Y. Nishikawa, Y. Yamashita, M. Inui, Y. Ohyama,
3. Hayami, K., D. Fukuta, Y. Nishikawa, Y. Yamashita, M. Inui, Y. Ohyama,
2. Hayami, K., D. Fukuta, Y. Nishikawa, Y. Yamashita, M. Inui, Y. Ohyama,
1. Hayami, K., D. Fukuta, Y. Nishikawa, Y. Yamashita, M. Inui, Y. Ohyama,
Supplemental Table 1. Candidate transcription factors that can bind to the DNA segment from −212 to −118 5' to the Pirb gene.

<table>
<thead>
<tr>
<th>Tr. factor</th>
<th>Consensus sequence</th>
<th>Nucleotide position</th>
<th>Cell specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL4</td>
<td>GAGGA</td>
<td>−130, −154, −176</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>T-Ag</td>
<td>GRGGC</td>
<td>−130</td>
<td>SV40</td>
</tr>
<tr>
<td>MIG1</td>
<td>SYGGGG</td>
<td>−132</td>
<td>Yeast</td>
</tr>
<tr>
<td>Runx1</td>
<td>TGTGGK</td>
<td>−133</td>
<td>Hematopoietic cells, etc.</td>
</tr>
<tr>
<td>PEB1</td>
<td>YDTGCRRW</td>
<td>−142</td>
<td>Yeast</td>
</tr>
<tr>
<td>Elf-1</td>
<td>TTCCTSYY</td>
<td>−143</td>
<td>Hematopoietic cells, liver, kidney, etc.</td>
</tr>
<tr>
<td>PU.1</td>
<td>TTCCTC</td>
<td>−145, −171</td>
<td>B cells, macrophages, neutrophils</td>
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<tr>
<td>Sp1</td>
<td>GGCCA</td>
<td>−149</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>GAGGAGGG</td>
<td>−154</td>
<td>Ubiquitous</td>
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<tr>
<td>GR</td>
<td>CAGAG</td>
<td>−168, −210</td>
<td>Ubiquitous</td>
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<td>GCN4</td>
<td>AAGTCA</td>
<td>−172</td>
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<tr>
<td>MAF</td>
<td>GGAAGT</td>
<td>−174</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>PEA3</td>
<td>AGGAWG</td>
<td>−176</td>
<td>Epidermis, mammary gland, brain, etc.</td>
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<tr>
<td>c-Ets-1/2</td>
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<td>−176</td>
<td>Hematopoietic cells, etc.</td>
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<tr>
<td>TCF-2α</td>
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<td>Liver, etc.</td>
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<td>Ubiquitous</td>
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<td>Adrenal tissue, etc.</td>
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<tr>
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<td>TGSCHDDV</td>
<td>−199</td>
<td>Muscle</td>
</tr>
<tr>
<td>RC2</td>
<td>GGYTYWW</td>
<td>−205</td>
<td>Glia, etc.</td>
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</tbody>
</table>

1The nucleotide sequence spanning from positions −212 to −118 exhibiting a major promoter activity for Pirb was subjected to a search with the Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess) for listing possible binding motifs of known transcription (Tr.) factors. For comprehensiveness, yeast and viral factors are also shown.


3The nucleotide position(s) given for the consensus sequences are the most 5' nucleotide(s) from the ATG initiator codon of Pirb. Note that −171 PU.1 site is on the reverse orientation.

4Expression specificity of each transcription factor in cells.
Supplemental Table 2. Candidate transcription factors that can bind to the DNA segment from −407 to −213 5' to Pirb gene.

<table>
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<th>Tr. factor</th>
<th>Consensus sequence</th>
<th>Nucleotide position</th>
<th>Cell specificity</th>
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<td>SEF1</td>
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<td>Myogenin</td>
<td>CATCTG</td>
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<td>RFX2</td>
<td>CATCTG</td>
<td>−231</td>
<td>Ubiquitous</td>
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<tr>
<td>IAF</td>
<td>GCCATCTG</td>
<td>−233</td>
<td>Pancreas</td>
</tr>
<tr>
<td>NF-InsE2/3</td>
<td>GCCAYCTG</td>
<td>−233</td>
<td>β-Cell</td>
</tr>
<tr>
<td>INSAF</td>
<td>GCCATCTG</td>
<td>−233</td>
<td>β-Cell</td>
</tr>
<tr>
<td>NF-1</td>
<td>GCCA</td>
<td>−233</td>
<td>Neural cell</td>
</tr>
<tr>
<td>UCRF-L</td>
<td>GCCAT</td>
<td>−234</td>
<td>Murine leukemia virus</td>
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<tr>
<td>c-Ets-1/2</td>
<td>AGGAAR</td>
<td>−240</td>
<td>Hematopoietic cells, etc.</td>
</tr>
<tr>
<td>PEA3</td>
<td>AGGAAR</td>
<td>−240</td>
<td>Epidermis, mammary gland, brain, etc.</td>
</tr>
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<td>GAL4</td>
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<td>−241, −2382</td>
<td>Ubiquitous</td>
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<tr>
<td>CAC-BP</td>
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<td>−249</td>
<td>Hematopoietic cells, etc.</td>
</tr>
<tr>
<td>Sp1</td>
<td>GTTGG</td>
<td>−249</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>T-Ag</td>
<td>GRGGC</td>
<td>−261</td>
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<td>Tal-1</td>
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<td>Hematopoietic stem cell</td>
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<tr>
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<td>AP-1</td>
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<tr>
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<td>HNF-3α</td>
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<td>B cell, kidney, etc.</td>
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<td>−358</td>
<td>T cell</td>
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<td>CATAAA</td>
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<td>GAAACATC</td>
<td>−404</td>
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</tr>
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</table>

1The nucleotide sequence spanning from positions −407 to −213 of Pirb was subjected to a search with the Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess) for listing possible binding motifs of known transcription (Tr.) factors. For comprehensiveness, yeast, viral,
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