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*J Immunol* 1999; 163:6371-6377; http://www.jimmunol.org/content/163/12/6371

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Characterization of Paired Ig-Like Receptors in Rats$^{1,2}$

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To explore the phylogenetic history of the murine paired Ig-like receptors of activating (PIR-A) and inhibitory (PIR-B) types, we isolated PIR homologues from a rat splenocyte cDNA library. The rat (ra) PIR-A and raPIR-B cDNA sequences predict transmembrane proteins with six highly conserved extracellular Ig-like domains and distinctive membrane proximal, transmembrane, and cytoplasmic regions. The raPIR-B cytoplasmic region contains prototypic inhibitory motifs, whereas raPIR-A features a charged transmembrane region and a short cytoplasmic tail. Southern blot analysis predicts the presence of multiple Pira genes and a single Pirb gene in the rat genome. Although raPIR-A and raPIR-B are coordinately expressed by myeloid cells, analysis of mRNA detected unpaired expression of raPIR-A by B cells and raPIR-B by NK cells. Collectively, these findings indicate that the structural hallmarks of the Pire gene family are conserved in rats and mice, yet suggest divergence of PIR regulatory elements during rodent speciation. The Journal of Immunology, 1999, 163: 6371–6377.

The mouse paired Ig-like receptor (PIR)$^{1–2}$-B/p91 belongs to an emerging family of type I transmembrane proteins that possess extracellular (EC) Ig-like domains and cytoplasmic regions containing one or more immunoregulatory tyrosine-based inhibitory motifs (ITIM) (1–3). These ITIM-bearing receptors often share EC domain homology with activating receptor counterparts that have distinctive transmembrane and cytoplasmic regions. The cytoplasmic tail of PIR-B contains three ITIM units that can mediate inhibitory signals via interaction with the src-homology phosphatase SHP-1 (1, 2, 4–8). PIR-B shares greater than 90% homology with the EC domain of its PIR-A counterparts that have charged arginine residues in their transmembrane segments and short cytoplasmic tails with no known functional motifs (1, 9). PIR-A proteins associate with dimeric Fc receptor γ chains (FcRγ) containing immunoregulatory tyrosine-based activation motifs (ITAM) to form activation receptors (7, 10, 11).

The genes encoding PIR-A and PIR-B map to the proximal end of mouse chromosome 7 (1, 9). Sequence comparison indicates that the mouse (mo) PIRs share significant homology with several leukocyte-specific Ig-like receptor genes that are tightly clustered in a syntenic region of human chromosome 19 (12–15). This leukocyte receptor complex (LRC) on chromosome 19q13.4 includes a multigene family of Ig-like receptors that have been described as follows: Ig-like transcripts (ILT) (12); leukocyte Ig-like receptors (LIR) (13); monocyte/macrophage Ig-like receptors (MIR) (14); human monocyte cDNA 18 (HM18) (16); human PIR (huPIR) (17); and will subsequently be referred to as the human (hu)ILT/LIR/MIR multigene family. The moPIR and huILT/LIR/MIR families both contain activating and inhibitory members with similar EC regions. However, the respective mouse and human EC regions are quite distinct in that moPIRs have six Ig-like domains, four C2-type, and two V-like, whereas huILT/LIR/MIR have either four or two Ig-like domains, all of which are of C2-type (1, 12–14). Another distinction is that mice possess one inhibitory Pirb gene and approximately eight activating Pira genes, while humans have multiple inhibitory gene members, and relatively few activating receptor genes (13, 15, 18). The hallmark paired expression of PIR-A and PIR-B in mouse myeloid, B, and dendritic cell lineages is not a general rule for their human counterparts, where the cellular distribution of huILT/LIR/MIR expression extends beyond the myeloid, B, and dendritic cell lineages to include subpopulations of NK cells and T cells (12–14).

The evolutionary history of moPIR and huILT/LIR/MIR seems complex in that many structural features are conserved, yet fundamental differences are evident in their genomic organization and cellular distribution. In this report, we characterize rat PIR cDNAs and examine the phylogenetic relationships among rat, mouse, and human relatives.

Materials and Methods

Isolation of cDNA clones

A splenic cDNA Agt11 library derived from a Sprague Dawley rat (Clontech, Palo Alto, CA) was screened as previously described (1). In brief, $5 \times 10^{5}$ plaque plaques were lifted onto duplicate nitrocellulose filters and probed overnight with an [$\alpha^{32}$P]dCTP-labeled, EcoRI-digested cDNA fragment (1.5 kb) of the mouse PIR-B extracellular domain. Posthybridization filters were washed twice in 1× SSC and 0.1% SDS at 55°C and exposed to x-ray film for 48 h. Hybridizing phage cDNA clones were purified with two subsequent rounds of screening followed by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation.

Sequence analysis

DNA sequencing was performed using an automated sequencer (Li-Cor, Lincoln, NE). Each clone was sequenced three or more times on the same or opposite strand by the dideoxy chain-termination method using Thermo Sequenase (Amersham Pharmacia Biotech, Amersham, Arlington Heights,
IL) or SequiTherm Excel (Epicentre Technologies, Madison, WI) cycle sequencing kits. Nucleotide and amino acid sequences were analyzed using the DNAStar (Madison, WI), BLAST (19), PHYLIP (20), and GENEDEV (21) programs.

Generation of full-length raPIR-A and raPIR-B cDNAs

mRNA was purified from a Lewis rat spleen using FastTrack mRNA isolation kits (Invitrogen, Carlsbad, CA), and full-length raPIR cDNAs were generated following the Marathon cDNA amplification kit protocol (Clontech). RaPIR specific reverse primers used in 5′-rapid amplification of cDNA ends (RAE) were 5′-CTCTTGTACACTAGCTCCA-3′ and 5′-GAGCCTTCTGAGTCTCCTCAA-3′. RaPIR-specific forward primers used in 3′-RAE were 5′-GAGCCAGTAACAGATGTGATGT-3′ and 5′-CTCTTCTAGTGATGCCAACC-3′. Primers used in end-to-end PCR to generate full-length PIR-A were forward 5′-CCCTCACGCTACACGCT-3′ and reverse 5′-GGAGGTTAGGAAATGGTAA-3′. Primers used in end-to-end PCR to generate full-length PIR-B were forward 5′-CTACACACTACCAGCT-3′ and reverse 5′-GGGAGCCATGGTAA-3′. The resulting amplification products were cloned into pCR2.1 (Invitrogen).

Abs, cell sorting, and cell lines

mAbs HIS24-PE (IgG2b; anti-CD45R), HIS48-FITC (IgM; anti-granulocyte), and 10/78-PE (IgG1; anti-NKR-P1A) were obtained from PharMingen (San Diego, CA). B cells were identified as CD45R+ (X), and analyzed with probes corresponding to the common extracellular region (left panel) or to the PIR-B specific cytoplasmic region (right panel).

RT-PCR

Total RNA was reverse transcribed into cDNA using random primed SuperScript II (Life Technologies, Rockville, MD). Common forward primers 5′-CCCTCAGTGTGACCTCAA-3′, PIR-A-specific reverse primers 5′-CCATCTGTAGATTCCCG-3′, and PIR-B-specific reverse primers 5′-CTGGGCGTATAGTCACATCC-3′ amplified products of 366 bp and 820 bp, respectively. Control β-actin forward 5′-TACAACCTCCTTGCAGCTCC-3′ and reverse 5′-GGAGGTTAGGAAATGGTAA-3′ primers were used. Each amplification reaction underwent an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 5 s, annealing at 66°C for 15 s, and extension at 72°C for 30 s, followed by a 2-min final extension. Amplified products were visualized in 2% agarose gels containing ethidium bromide and documented with the Bio-Rad Fluor-S Imager (Hercules, CA).

Results

Isolation of rat PIR-A and PIR-B

In experiments aimed toward identifying homologues of the mouse Pir genes, analysis of EcoRI-digested genomic DNA from humans, monkeys, rats, mice, dogs, cows, rabbits, chickens, and yeast indicated cross-hybridization of a moPIR-B EC domain probe to all vertebrate DNA samples, with the highest reactivity to rat DNA (Ref. 1 and data not shown). Screening of a rat splenic cDNA library with a mouse PIR-B extracellular region probe yielded three hybridizing clones (R16, R29, and R34). Sequence analysis indicated that clone R16 was a partial cDNA (1.5 kb) that encoded four Ig-like domains with homology to moPIR. Similarly, analysis of clones R29 and R34 indicated that these were incompletely spliced cDNAs encoding Ig-like domains similar to moPIR. RACE extension of clone R16 in the 5′ and 3′ directions yielded invariant 5′-RACE products, but distinct
PIR-A-like and PIR-B-like 3'-RACE products. Full-length raPIR-A and raPIR-B candidates were then generated by end-to-end PCR using common 5'-primers complementary to the signal sequence and distinct 3'-primers for the PIR-A and PIR-B 3'-untranslated regions. As depicted in Fig. 1A, the full-length raPIR-B cDNA (2.5 kb) is predicted to encode a type I transmembrane protein with six extracellular Ig-like domains, a short EC membrane proximal domain, an uncharged transmembrane segment, and a long cytoplasmic tail with four putative ITIM motifs. The full-length raPIR-A cDNA (2.3 kb) likewise is predicted to encode a type I transmembrane protein with six extracellular Ig-like domains homologous to those of raPIR-B, a distinct EC membrane proximal domain, a charged transmembrane segment, and a short cytoplasmic tail devoid of ITIM.

FIGURE 2. Multiple alignment of rat PIR-B (AF16936), mouse PIR-B (AF038149), human ILT-1 (U82275), MR-10 (AF004231), and LIR-1 (AF009220) extracellular regions. Amino acids are numbered with reference to the start of the signal sequence for each sequence, and one Ig-like domain is aligned per block of amino acid sequence. The top line represents the extracellular portion of raPIR-B; conserved residues are represented as dots (.), and gaps are indicated by dashes (-). Cysteine residues likely to be involved in intradomain disulfide bond formation are highlighted in bold, and potential N-linked glycosylation sites are underlined.

FIGURE 3. (A) Amino acid sequence alignments of the cytoplasmic tails of raPIR-B, moPIR-B (1), mogp49-B (24), and human inhibitory LRC members (25–27) illustrate perfect conservation of the third raPIR-B ITIM. This particular ITIM unit, which contributes to the inhibitory function of moPIR-B via the binding of SHP-1 (4–6), may therefore contribute to an inhibitory function predicted for raPIR-B. (B) The raPIR-A transmembrane domain (Fig. 3B) shares the presence of a charged arginine residue with moPIR-A (1), rat killer cell Ig-like receptor (KILR)-1 (AF082533), mouse activation receptor (MAR)-1 (28), bovine Fc receptor for IgG2 (boFc 

PFR-A-like and PIR-B-like 3'-RACE products. Full-length raPIR-B and raPIR-B candidates were then end-to-end PCR using common 5' primers complementary to the signal sequence and distinct 3' primers for the PIR-A and PIR-B 3'-untranslated regions. As depicted in Fig. 1A, the full-length raPIR-B cDNA (2.5 kb) is predicted to encode a type I transmembrane protein with six extracellular Ig-like domains, a short EC membrane proximal domain, an uncharged transmembrane segment, and a long cytoplasmic tail with four putative ITIM motifs. The full-length raPIR-A cDNA (2.3 kb) likewise is predicted to encode a type I transmembrane protein with six extracellular Ig-like domains homologous to those of raPIR-B, a distinct EC membrane proximal domain, a charged transmembrane segment, and a short cytoplasmic tail devoid of ITIM. Southern blot analysis of rat genomic DNA employing a common raPIR extracellular probe derived from cDNA clone R16 resulted in multiple (6–10) hybridizing restriction fragments (Fig. 1B). Conversely, a specific probe spanning the cytoplasmic tail of raPIR-B resulted in a relatively simple hybridization pattern of one or two predominant hybridizing fragments. This analysis suggests that raPIR-B, like its moPIR-B relative (1), may be encoded by a single gene that shares extracellular region homology with multiple raPIR-A genes. As shown in Fig. 2, comparison of the extracellular amino acid sequences of raPIR-B, moPIR-B (1), huILT-1 (U82275), MR-10 (AF004231), and LIR-1 (AF009220) extracellular regions. Amino acids are numbered with reference to the start of the signal sequence for each sequence, and one Ig-like domain is aligned per block of amino acid sequence. The top line represents the extracellular portion of raPIR-B; conserved residues are represented as dots (.), and gaps are indicated by dashes (-). Cysteine residues likely to be involved in intradomain disulfide bond formation are highlighted in bold, and potential N-linked glycosylation sites are underlined.

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A) Multiple alignment of transmembrane and cytoplasmic amino acid sequences for activating and inhibitory relatives of rat PIR-A and PIR-B.

B) Tissue distribution of rat PIR expression.

Discussion

The rat homologues of mouse PIR-A and PIR-B have been identified in the present study as type I transmembrane proteins that have six highly similar extracellular Ig-like domains and distinctive transmembrane and cytoplasmic regions. The name paired Ig-like receptor was coined in part to designate the coordinate expression of PIR-A and PIR-B transcripts by mouse B cells and myeloid cells (1). Despite the remarkable conservation of structural features for the rat and mouse PIR family members, rat PIR-A and PIR-B do not maintain the hallmark paired expression of activating and inhibitory receptors. Instead, differential, or unpaired, expression of PIR-A and PIR-B was observed for B and NK cells, respectively. The expression of PIR-B by NK cells more closely resembles that of PIR-A, as seen in other tissues. When the expression pattern was examined in the thymus, ovary, and lung, whereas PIR transcripts were not expressed in the bone marrow (Fig. 4A). Hybridizing transcripts were less abundant in other tissues. When the expression pattern was examined using RT-PCR (Fig. 4B), PIR transcripts were detected in B, myeloid, and macrophage cell lines, whereas PIR transcripts were not seen in other tissues. When the expression pattern was examined by RT-PCR (Fig. 4B), PIR transcripts were detected in B, myeloid, and macrophage cell lines, whereas PIR transcripts were detected only in myeloid and macrophage cell lines. When normal B cells (CD45R+/CD3-), NK cells (NK-1-P1A+CD3-), and granulocytes (HIS48+) were isolated and examined, PIR transcripts were seen in B cells and granulocytes, whereas PIR transcripts were found in NK cells and granulocytes. Thus, while PIR transcripts are coordinately transcribed in myeloid lineage cells, PIR-A and PIR-B transcripts are differentially expressed in B cells vs NK cells.

Phylogentic analysis of PIR

When the EC domains of PIR-A, moPIR-A (1), and KILR-1 (AF082533), mmpg49-B (2), moMAR-1 (28), and human LRC members were compared using the PHYLIP and CLUSTAL software packages with amino acid sequences, the implied relationships in the derivative trees largely conform to their percentages of identity (Fig. 5). Results from this phylogenetic analysis suggest that PIR, PIR, and mmpg49 form a group that is closely related to the huILT/LIR/MIR family.

These results also indicate that huLT/LIR/MIRs are more like their rat and mouse counterparts than other human LRC members.
closely resembles the picture in humans, where NK cells may selectively express inhibitory members of the huILT/LIR/MIR multigene family (13). Unpaired expression of raPIR-A by B cells, however, is more unique. The expression pattern in rats thereby resembles that in humans where activating and inhibitory members of the PIR family are coordinately expressed in myeloid cells, yet may be differentially regulated in lymphoid and dendritic cell lineages. The rat may thus prove to be a useful model for the dissection of opposing signaling pathways employed by these activating and inhibitory receptors.

Inhibitory receptors represent a diverse group of recently recognized cell surface receptors that can modulate cellular activation.
via the interaction of their ITIM-containing cytoplasmic tails with the src-homology phosphatases SHP-1, SHP-2, and SHIP-1 (reviewed in Ref. 3). The predicted cytoplasmic tail of raPIR-B contains four potential ITIM motifs that are conserved in moPIR-B and the huILT/LIR/MIR inhibitory receptors. The SHP-1-binding ITIM unit, VTYAQL, is identical in rats, mice, and humans, thereby suggesting that raPIR-B will also bind SHP-1 and serve as an inhibitory receptor.

Many inhibitory receptors, including PIR-B, have a high level of EC domain homology with proteins that have distinct transmembrane and cytoplasmic regions. These noninhibitory counterparts commonly have an arginine residue in their transmembrane segments and have short cytoplasmic tails without recognizable signaling motifs. RaPIR-A is similar to raPIR-B in its EC region, but has a transmembrane segment endowed with an arginine residue. The presence of an arginine in the transmembrane segments of moPIR-As promotes their association with ITAM-containing FcRγ chain dimers to form activating signaling complexes (7, 10, 11). Thus, the raPIR-As, like their mouse counterparts, are likely to form activating receptor complexes in conjunction with ITAM-containing coreceptors.

Human chromosome 19q13.4 encodes a large cluster of Ig-superfamily receptors, the majority of which have not been found to be conserved in mice (12–15, 26, 27, 31, 34). Recent cloning of NKp46 in humans, rats, and mice represents the first example of an LRC member that is conserved in rodents. The huNKp46 homologues KILR-1 (raNKp46) and MAR-1 (moNKp46) have been mapped respectively to syntenic regions of rat chromosome 1 and mouse chromosome 7. Recently, a rat ITIM-bearing receptor, neutrophil Ig-like receptor (NILR)-1, has also been mapped to rat chromosome 1 within 1.4 cM of raNKp46, thereby indicating the existence of a leukocyte receptor complex on rat chromosome 1 (35). The mouse Pira and Pirb genes are located in the same region of mouse chromosome 7 as moNKp46, (1, 28), thereby predicting that raPIR should map within the leukocyte receptor complex of rat chromosome 1. Monoallelic expression of paternal alleles encoding the Pir genes was demonstrated recently in mice (36), extending the number of genes that are genomically imprinted in this region of mouse chromosome 7 (37). It is noteworthy that huILT/LIR/MIR are also in close proximity to paternally imprinted genes on chromosome 19q13.4 (38), raising the interesting possibility that genomic imprinting may be a conserved feature of these syntenic multigene families.

The identification of PIR homologues in rats clearly suggests a shared evolutionary history for the raPIR, moPIR, and huILT/LIR/MIR families. However, several distinctive features complicate attempts to understand their phylogenetic relationships. Individual domain comparisons for the rat, mouse, and human family members indicate that the murine PIRs are highly conserved and, unlike the huILT/LIR/MIRs, share EC regions that possess two extra Ig-like domains at the N-terminal portion, possibly resulting from exon duplication/recombination events. On the other hand, unpaired expression of PIR-A and PIR-B transcripts in rat B and NK cells more closely resembles the expression pattern of huILT/LIR/MIR. Hence, raPIRs combine characteristics of both their mouse and human relatives, suggesting that PIR and ILT/LIR/MIR share a common ancestor that has maintained a high degree of genetic plasticity during mammalian evolution. Discovery of PIR and ILT/LIR/MIR homologues in additional species should shed further light on the evolutionary history of this diverse group of cell surface receptors.
Acknowledgments

We thank Ann Brookshire for helping in preparing the manuscript, and Pete Burrows, Chen-lo Chen, Vincent Hurez, Stephanie Blandin, and Gregory Ippolito for helpful advice and criticism.

References


