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PILRα, a Novel Immunoreceptor Tyrosine-based Inhibitory Motif-bearing Protein, Recruits SHP-1 upon Tyrosine Phosphorylation and Is Paired with the Truncated Counterpart PILRβ*

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SHP-1-mediated dephosphorylation of protein tyrosine residues is central to the regulation of several cell signaling pathways, the specificity of which is dictated by the intrinsic affinity of SH2 domains for the flanking sequences of phosphotyrosine residues. By using a modified yeast two-hybrid system and SHP-1 as bait, we have cloned a human cDNA, PILRα, encoding a 303-amino acid immunoglobulin-like transmembrane receptor bearing two cytoplasmic tyrosines positioned within an immunoreceptor tyrosine-based inhibitory motif. Substrate trapping in combination with pervanadate treatment of 293T cells confirms that PILRα associates with SHP-1 *in vivo* upon tyrosine phosphorylation. Mutation of the tyrosine residues in PILRα indicates the pivotal role of the Tyr-269 residue in recruiting SHP-1. Surface plasmon resonance analysis further suggests that the association between PILRα-Tyr-269 and SHP-1 is mediated primarily via the amino-terminal SH2 domain of the latter. Polymerase chain reaction amplification of cDNA in combination with genomic sequence analysis revealed a second gene, *PILRβ*, coding for a putative activating receptor as suggested by a truncated cytoplasmic tail and a charged lysine residue in its transmembrane region. The *PILRα* and *PILRβ* genes are localized to chromosome 7 which is in contrast with the mapping of known members of the inhibitory receptor superfamily.

The initiation of cell signaling pathways relies on a dynamic interaction between activating and inhibiting processes that can include, among other things, changes in the phosphorylation status of certain tyrosine residues within the target proteins. Dephosphorylation of these residues is mediated by such phosphatases as the cytosolic SHP-1 (also known as SHP, PTP1C, SH-PTP1, or PTPN6 (1)) which is expressed in hematopoietic cells, and to a lesser extent in non-hematopoietic cells, and which contains tandem amino-terminal Src homology 2 (SH2)[1] domains. The importance of SHP-1 in cellular signal delivery is underscored by the *motheaten* mouse that carries a natural mutation in the SHP-1 locus and is characterized by widespread autoimmune phenomena resulting from an inability to modulate immune responses (2, 3).

Affinity for the SH2 domains is pivotal for interaction of substrates with SHP-1. The flanking sequence of the phosphotyrosine residue promotes the recruitment of specific SH2-containing phosphatases and, thus, determines the specificity of the signaling pathway. The consensus sequence (S/L/I/V)*XYXX*(L/V), based on sequences originally deduced from several receptors known to bind to the carboxyl-terminal SH2 domain of the protein tyrosine phosphatase SHP-1 (4–6), defines all immunoreceptor tyrosine-based inhibitory motif (ITIM)-bearing receptors (7) including natural killer cell, B cell, and monocyte and dendritic cell inhibitory receptors (reviewed in Refs. 8 and 9). Members of the inhibitory receptor superfamily (10, 11) can be divided into two groups. The immunoglobulin (Ig) superfamily includes luminal amino-terminal (*e.g.* type I) transmembrane glycoproteins with two or more Ig-like domains such as p58/KIR2DL3, FcγRIIB, Ig-like transcripts as well as the mouse PIR-B. The other group is comprised of CD94/NKG-2, CD72, and the mouse Ly49 and NKR-P1 which are cytoplasmic amino-terminal (*e.g.* type II) transmembrane proteins expressing C-type lectin extracellular architecture.

The existence of complementary proteins expressing similar extracellular domains as the inhibitory receptors, while having distinctive transmembrane and frequently truncated cytoplasmic tails, suggests similar ligand-binding specificities but contrasting signaling capabilities for each individual of the protein pair. The truncated protein would have cellular activating properties, in contrast with its ITIM-bearing counterpart which would inhibit cell signaling through recruitment of SHP-1, SHP-2, or SHIP phosphatases (9) seemingly via their respective carboxyl-terminal SH2 domains (12, 13). Human immunoreceptors map to the "complex" or "leukocyte receptor cluster" located on chromosome region 19q13.4 or to the natural killer complex on chromosome 12 (11). Mouse immunoreceptors are located on chromosomes 7 and 6 in regions syntenic to human chromosome 19 and 12, respectively (11), or to band B4 of mouse chromosome 10 (14) which does not appear to have any conserved linkage homology to either human chromosome 19 or 12.

We now present evidence of a novel pair of receptors express-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF161080 and AF161081.

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[1] The abbreviations used are: SH2, Src homology 2; PILRα, paired immunoglobulin-like receptor α; PILRβ, paired immunoglobulin-like receptor β; ITIM, immunoreceptor tyrosine-based inhibitory motif; PCR, polymerase chain reaction; kb, kilobase pair; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; FBS, fetal bovine serum; mAb, monoclonal antibody; pAb, polyclonal antibody; GST, glutathione S-transferase; MHC, major histocompatibility complex; PNGase:F, peptide:*N*-glycosidase F.

ing a single extracellular Ig-like domain that we have designated as "paired immunoglobulin-like receptor" (PILR)α and PILRβ to distinguish their putative inhibitory and activating gene products, respectively. The ITIM-bearing receptor PILRα recruits SHP-1 via its amino-terminal SH2 domain and is likely to have cellular inhibitory potential. The lack of a cytoplasmic tail and the presence of the transmembrane lysine residue in the second receptor PILRβ suggest its potential activating function. Both genes map cytogenetically to human chromosome 7.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—All cell lines were obtained from American Type Culture Collection except where indicated. The human embryonic kidney epithelial cell line 293T (Edge Biosystems) was maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum (FBS). The T cell-derived KG-1, Jurkat and K562 cell lines were maintained in RPMI, 10% FBS. The B cell-derived cell lines WIL2-NS, Namalwa, Daudi, and Raji were maintained in RPMI, 10% inactivated FBS. The 6F11 B cell line was cultured in Iscove's modified Dulbecco's medium containing 2 mM L-glutamine and 15% FBS. The macrophage 28SC cell line was maintained in Iscove's medium supplemented with 0.03 mM thymidine, 0.1 mM hypoxanthine, 0.05 mM β-mercaptoethanol, and 10% FBS. NK-92ci cells (ImmuneMedicine, Vancouver, British Columbia, Canada) transformed with a pCep-IL-2 construct were maintained by selection in interleukin-2-free Myelocult medium (Stem Cell Technologies, Vancouver, British Columbia, Canada).

Antibodies—SHP-1 was precipitated and detected using a monoclonal antibody (mAb; P17320; Transduction Laboratories) or an in-house polyclonal antibody (pAb; 237, see Ref. 15). PILRα cDNA was subcloned in-frame with a carboxyl-terminal triple HA tag. The HA epitope was precipitated with a high affinity rat anti-HA mAb (3F10; Roche Molecular Biochemicals) and detected with a mouse mAb (12CA5; Roche Molecular Biochemicals). Separate membranes were also probed with anti-phosphotyrosine 4G10 mAb (Upstate Biotechnology, Inc.) and anti-ubiquitin pAb (U-5379; Sigma). Secondary antibodies were horseradish peroxidase-conjugated to goat anti-mouse or goat anti-rabbit antibody (Bio-Rad) and detection relied on enhanced chemiluminescence (NEN Life Science Products).

Yeast Two-hybrid Screen—Screening of a human mammary gland cDNA library (CLONTECH) with the SHP-1-C455S catalytic mutant (16) was performed as follows. The full-length SHP-1-C455S was cloned into *Sal*I-restricted pBTM116-Src by standard polymerase chain reaction (PCR) procedures and thus was fused in-frame with the carboxyl terminus of the DNA binding domain of the bacterial activator LexA. pBTM116-Src contains a mutant c-Src kinase expression cassette designed for the phosphorylation of protein tyrosine residues in yeast (17). The pBTM116-Src-SHP-1-C455S construct was then used to transform the yeast reporter strain, L40α (*MAT*a *trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacz*). Following transformation using Li^{2+} acetate (18), L40α expressing pBTM116-Src-SHP-1-C455S was further transformed with the library. Positive interactors were isolated according to the manufacturer's specifications (CLONTECH Laboratories, Inc.). The positive control consisted of a pBTM-116-cnx1+ and pGADGH-hus5+ combination kindly provided by M. Pelletier and D.Y. Thomas[2] (NRC Biotechnology Research Institute, Montreal, Quebec, Canada). cDNA sequences were determined and subjected to the BLAST search of the NCBI data bases. The target sequence of interest was used as a probe for screening of cDNA libraries.

Screening of Human cDNA Libraries—^{32}P-Labeled probes were prepared by random labeling of the PILRα PCR product using Ready-to-Go DNA Labeling Beads (Amersham Pharmacia Biotech) and [α-^{32}P]dCTP. Placenta, acute myelocytoma leukemia (KG-1), bone marrow, and leukocyte cDNA libraries were screened using the labeled probes. Positive clones were subjected to PCR using λ GT11-specific primers.

Mutant Expression, Immunoprecipitation, and Immunoblotting—Single and double tyrosine to phenylalanine mutants of PILRα-HA, *i.e.* Y269F, Y298F, Y269F/Y298F, were generated by PCR-based site-directed mutagenesis. 293T cells were transfected either alone or in combination with SHP-1 by calcium phosphate precipitation. Forty-eight hours after transfection the cells were treated with pervanadate (100 μM, 30 min at 37 °C), a phosphatase inhibitor that induces maximal tyrosine phosphorylation (19), and then washed with phosphate-buffered saline and lysed on ice. Solubilized extracts were immunoprecipitated with the appropriate antibodies followed by protein A- or protein G-Sepharose. The immunoprecipitates were resolved by SDS-PAGE and revealed by standard immunoblotting techniques.

Glycosylation and Ubiquitination Status of PILRα-HA—293T cells were transiently transfected with PILRα-HA. Following immunoprecipitation with an HA-directed antibody, immune complexes were washed and denatured, and *N*-glycosylation status was determined by overnight incubation at 37 °C with 5 milliunits of *N*-glycosidase F (PNGase:F, Roche Molecular Biochemicals) while a parallel series of experiments determined *O*-glycosylation status by incubation with 1 milliunit of neuraminidase (to remove sialic acid residues, Roche Molecular Biochemicals) and/or 1 milliunit of endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase, Roche Molecular Biochemicals). The various reactions were resolved on 10% SDS-PAGE gels. To test for the presence of covalently bound ubiquitin in the 55-kDa expressed PILRα-HA species, transiently transfected 293T cells were treated with pervanadate, and HA-bound immunoprecipitates were collected, resolved by SDS-PAGE, and probed with an anti-ubiquitin pAb.

Preparation and Expression of GST Fusion Proteins Containing the SH2 Domains of SHP-1—A 300-base pair segment encoding either the amino-terminal SH2 domain (SH2(N)) or the carboxyl-terminal SH2 domain (SH2(C)) of SHP-1 were generated by PCR amplification of human SHP-1 cDNA (20) and subcloned into pGEX-2T (Amersham Pharmacia Biotech). The SHP-1.SH2(N) and SHP-1.SH2(C) GST fusion proteins were produced in *Escherichia coli* DH5α cultures transformed with the corresponding plasmid and induced with 25 μM isopropyl-β-D-thiogalactoside for 22 h at 28 °C. The GST fusion proteins were isolated using glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Protein expression and purity were determined by SDS-PAGE and Coomassie Blue staining.

Surface Plasmon Resonance Measurements—Surface plasmon resonance (SPR) was performed on a BIAcore apparatus using CM5-sensor chips (Biosensor AB, Uppsala, Sweden). The tyrosyl-phosphorylated (pY) synthetic peptides, KDDGIV(pY)ASLALSSSTS and PQNETL(pY)-SVLKA, corresponding, respectively, to the Tyr-269- and Tyr-298-based motifs contained in PILRα were immobilized at 0.5 mg/ml on the Biosensor chip. Immobilization efficiency was verified using the anti-phosphotyrosine 4G10 mAb. GST-SH2 domain fusion proteins (see above) were initially dialyzed in 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl and 3.4 mM EDTA (HBS). The GST fusion proteins (0.5–2000 nM), diluted in HBS, 0.05% P20, were injected over the test surfaces at a flow rate of 5 μl/min. Regeneration of the Biosensor chip using 46 mM HCl, 1 M NaCl did not result in loss of subsequent signal. Dissociation constants (K_D) were calculated by saturation (nonlinear regression) analysis as well as by kinetic analysis (*e.g.* the ratio of k_{off} and k_{on} determined using BIAevaluation software; Biosensor AB, Uppsala, Sweden).

Northern Blot Analysis—Human multiple tissue Northern blots (CLONTECH Laboratories, Inc.) containing 2 μg of poly(A)$^+$ RNA per lane were hybridized consecutively with ^{32}P-labeled full-length PILRα cDNA and with ^{32}P-labeled β-actin cDNA probes. The level of expression in a series of human cells was also examined. Total RNAs from the individual cell lines were extracted by the TriZOL method (Life Technologies, Inc.) and enriched in poly(A)$^+$ RNA by passage on oligo(dT) spun columns (Amersham Pharmacia Biotech). Ten μg of poly(A)$^+$ RNA were loaded per lane, transferred to membrane, probed as above, and then washed (maximum stringency being 2 × 15 min, 0.1× SSC, 0.1% SDS, 55 °C).

Long Range PCR—The sequence of the 5′-untranslated region was amplified by 5′-rapid amplification of cDNA ends from Marathon-Ready cDNA (CLONTECH), whereas the genomic organization of *PILRα* and *PILRβ* genes was partially determined using the proofreading *Taq* polymerase GenomeWalker™ kit (CLONTECH).

RESULTS

The SHP-1 Catalytic Mutant SHP-1-C455S Interacts with Several ITIM-bearing Protein Fragments—A SHP-1-C455S catalytic mutant was used as bait in a yeast two-hybrid screening of a human mammary gland cDNA library. 4.5×10^6 clones were screened to reveal 24 interactors able to grow on Trp^-/Leu^-/His^- medium and able to induce β-galactosidase activity (Fig. 1). These positive clones were sequenced and identified as a novel ITIM-bearing protein, subsequently named PILRα, as well as known interactors of SHP-1 namely SHPS-1 (21), EGFRBP-Grb2 (22), and the leukocyte-associated Ig-like recep-

[2] M. Pelletier and D. Y. Thomas, unpublished data.

tor LAIR-1 (23). The sequence of the novel interactor was extended by PCR amplification of the target cDNA library and was ultimately confirmed following its use as a probe in the cDNA library screening which yielded the full-length sequence.

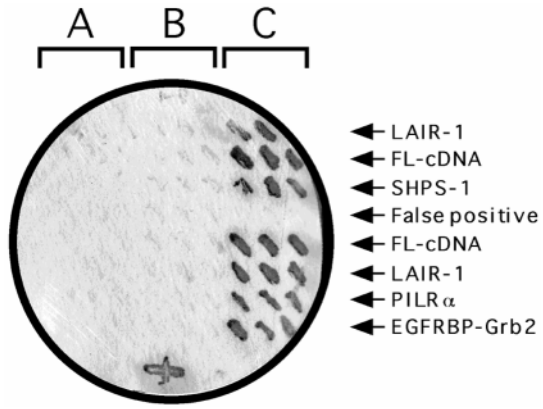

FIG. 1. **SHP-1 interacts with a number of known proteins and a novel inhibitory receptor PILRα.** Interactions were assayed in the yeast strain, L40α, which requires Trp, Leu, and His to grow. pBTM-116-Src-SHP-1-C455S (bait) and pACT2 (target, library) constructs carry Trp and Leu, respectively, as their selective markers. Library plasmids from positive clones were cured and used in combination with irrelevant (*A,* negative control), pBTM-116-Src (*B,* vector control) or pBTM-116-Src-SHP-1-C455S (*C,* the bait) plasmid, to retransform yeast. These yeast transformants were randomly plated in triplicate onto Trp^-/Leu^-/His^- master plates, grown to a visible biomass, and then submitted to colony-lift β-galactosidase assay. The positive control (+) was a pBTM-116-cnx1+ (bait)/pGADGH-hus5+ (target) combination. FL-cDNA is an unknown interactor described in the NCBI dbEST data base as "fetal lung cDNA."

The nucleotide sequence and deduced amino acid sequence, indicating a theoretical mass of 31.8 kDa, are depicted in Fig. 2. Structurally the gene product does not contain an IgG domain *per se*, although the single extracellular cysteine residue (Cys-125) is flanked by a motif, *e.g.* Y*X*C*X*V*X*L, reminiscent of the carboxyl-terminal cysteine-based motif, *e.g.* (F/Y)*X*C*X*(V/A)*X*H, involved in the intradomain disulfide bond of immunoglobulin and major histocompatibility complex (MHC) proteins (PROSITE data base; reference number PS00290). This same segment bears a slight homology to extracellular regions of sialoadhesin (24). In addition, there is a potential *N*-glycosylation site (N*X*(T/S)), numerous serine and threonine residues capable of being *O*-glycosylated, a transmembrane domain, and three potential tyrosine phosphorylation sites, two of which, *e.g.* Tyr-269 and Tyr-298, reside within an immunoreceptor tyrosine-based inhibitory motif.

PILRα-HA Interacts with SHP-1 in Vivo—To examine protein-protein interactions, 293T cells were co-transfected with PILRα-HA and SHP-1 and treated with pervanadate. Fig. 3 demonstrates that PILRα-HA is phosphorylated in 293T cells (Fig. 3*A, top*), that PILRα-HA and SHP-1 co-precipitate (Fig. 3*A, middle*), and that tyrosine phosphorylation was essential for binding to SHP-1 (Fig. 3*A, bottom*). In addition, confirmation that the association occurred *in vivo* was accomplished by co-immunoprecipitation of PILRα-HA as part of an immune complex containing the SHP-1-D421A substrate-trapping mutant (25) (Fig. 3*B*).

PILRα-HA migrated with an apparent molecular mass of 55 kDa rather than the expected theoretical molecular mass of 36 kDa. The 55-kDa molecular mass includes 4.6 kDa contributed

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AATAGGGGAAAATAAGCCAGATGGATAAAGGAAGTGCTGGTCACCCTGGAGGTGCACTGGTTTGGGGAAGGCTCCTGGCCCCCACAGCCC
TCTTCGGAGCCTGAGCCCGGCTCTCCTCACTCACCTCAACCCCCAGGCGGCCCCTCCACAGGGCCCCTCTCCTGCCTGGACGGCTCTGCT
GGTCTCCCCGTCCCCTGGAGAAGAACAAGGCCATGGGTCGGCCCCTGCTGCTGCCCCTACTGCCCTTGCTGCTGCCGCCAGCATTTCTGC  270
                                M  G  R  P  L  L  L  P  L  L  P  L  L  L  P  P  A  F  L  Q   20

AGCCTAGTGGCTCCACAGGATCTGGTCCAAGCTACCTTTATGGGGTCACTCAACCAAAACACCTCTCAGCCTCCATGGGTGGCTCTGTGG  360
  P  S  G  S  T  G  S  G  P  S  Y  L  Y  G  V  T  Q  P  K  H  L  S  A  S  M  G  G  S  V  E   50

AAATCCCCTTCTCCTTCTATTACCCCTGGGAGTTAGCCACAGCTCCCGACGTGAGAATATCCTGGAGACGGGGCCACTTCCACAGGCAGT  450
  I  P  F  S  F  Y  Y  P  W  E  L  A  T  A  P  D  V  R  I  S  W  R  R  G  H  F  H  R  Q  S   80

CCTTCTACAGCACAAGGCCGCCTTCCATTCACAAGGATTATGTGAACCGGCTCTTTCTGAACTGGACAGAGGGTCAGAAGAGCGGCTTCC  540
  F  Y  S  T  R  P  P  S  I  H  K  D  Y  V  N  R  L  F  L  [N  W  T]  E  G  Q  K  S  G  F  L  110

TCAGGATCTCCAACCTGCAGAAGCAGGACCAGTCTGTGTATTTCTGCCGAGTTGAGCTGGACACACGGAGCTCAGGGAGGCAGCAGTGGC  630
  R  I  S  N  L  Q  K  Q  D  Q  S  V  Y  F  C  R  V  E  L  D  T  R  S  S  G  R  Q  Q  W  Q  140

AGTCCATCGAGGGGACCAAACTCTCCATCACCCAGGCTGTCACGACCACCACCCAGAGGCCCAGCAGCATGACTACCACCTGGAGGCTCA  720
  S  I  E  G  T  K  L  S  I  T  Q  A  V  T  T  T  T  Q  R  P  S  S  M  T  T  T  W  R  L  S  170

GTAGCACAACCACCACAACCGGCCTCAGGGTCACACAGGGCAAACGACGCTCAGACTCTTGGCACATAAGTCTGGAGACTGCTGTGGGGG  810
  S  T  T  T  T  T  G  L  R  V  T  Q  G  K  R  R  S  D  S  W  H  I  S  L  E  T  A  V  G  V  200

TGGCAGTGGCTGTCACTGTGCTCGGAATCATGATTTTGGGACTGATCTGCCTCCTCAGGTGGAGGAGAAGGAAAGGTCAGCAGCGGACTA  900
  A  V  A  V  T  V  L  G  I  M  I  L  G  L  I  C  L  L  R  W  R  R  R  K  G  Q  Q  R  T  K  230

AAGCCACAACCCCAGCCAGGGAACCCTTCCAAAACACAGAGGAGCCATATGAGAATATCAGGAATGAAGGACAAAATACAGATCCCAAGC  990
  A  T  T  P  A  R  E  P  F  Q  N  T  E  E  P  Y  E  N  I  R  N  E  G  Q  N  T  D  P  K  L  260

TAAATCCCAAGGATGACGGCATCGTCTATGCTTCCCTTGCCCTCTCCAGCTCCACCTCACCCAGAGCACCTCCCAGCCACCGTCCCCTCA 1080
  N  P  K  D  D  G  I  V  Y  A  S  L  A  L  S  S  S  T  S  P  R  A  P  P  S  H  R  P  L  K  290

AGAGCCCCCAGAACGAGACCCTGTACTCTGTCTTAAAGGCCTAACAATGGACAGCCCTCTCAAGACTGAATGGTGAGGCCAGGTACAGTG 1170
  S  P  Q  N  E  T  L  Y  S  V  L  K  A  *                                                  303

GCGCACACCTGTAATCCCAGCTACTCTGAAGCCTGAGGCAGAATCAAGTGAGCCCAGGAGTTCAGGGCCAGCTTTGATAATGGAGCGAGA 1260
TGCCATCTCTAGTTAAAAATATATATTAACAATAAAGTAACAAATTTA                                           1308
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FIG. 2. **Nucleotide sequence and deduced amino acid sequence of human PILRα.** The signal peptide (*underlined*), the transmembrane region (*bold, underlined*), a potential *N*-glycosylation site (*boxed*), and putative binding sites for SHP-1 SH2 domains (*shaded*) are indicated. The AATAAA polyadenylation signal is also shown (*bold*). The *bold numbers* to the *right* indicate the nucleotide position, and the *italicized numbers* indicate the amino acid position.

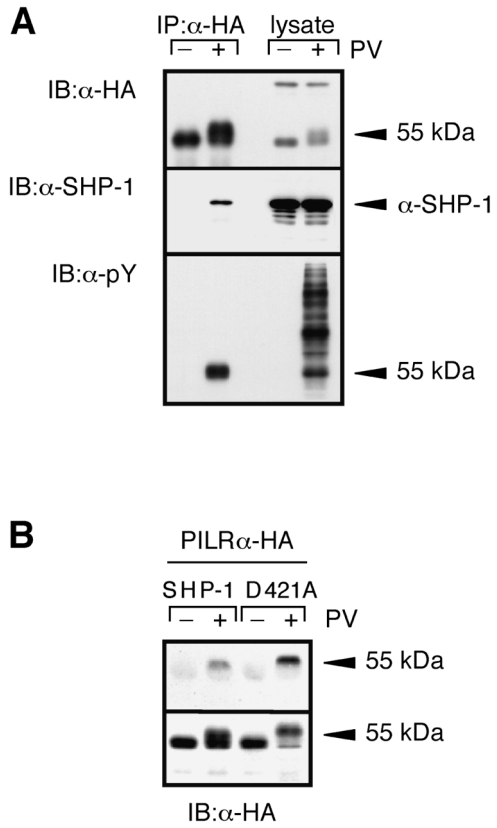

FIG. 3. **The phosphorylation of PILRα-HA is required for *in vivo* association with SHP-1.** *A,* pervanadate (*PV*)-treated (+) and non-treated (−) 293T cells co-transfected with PILRα-HA and SHP-1 were lysed. Total cell lysates as well as proteins co-immunoprecipitated with a mAb directed at the HA tag of the PILRα construct were resolved by SDS-PAGE and immunoblotted with anti-HA mAb (*top*), anti-SHP-1 mAb (*middle*), or anti-phosphotyrosine mAb (*bottom*). *B,* pervanadate-treated (+) and non-treated (−) 293T cells transiently co-transfected with PILRα-HA and either SHP-1 or the SHP-1-D421A (D421A) substrate-trapping mutant were lysed, and immune complexes were immunoprecipitated with anti-SHP-1 mAb (*top*). Bound proteins were resolved by SDS-PAGE, and the association of PILRα-HA with SHP-1 was determined by immunoblotting with anti-HA mAb. Expression of PILRα-HA in these same cells was monitored by immunoblotting cell lysate proteins with anti-HA mAb (*bottom*). *Arrowheads* indicate the molecular mass of the expressed PILRα-HA protein (*e.g.* 55 kDa).

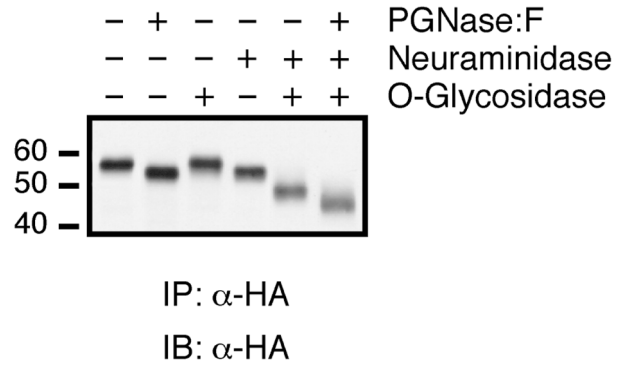

FIG. 4. **Characterization of the glycosylation status of expressed PILRα-HA.** 293T cells were transiently transfected with PILRα-HA. Tyrosine phosphorylation was maintained by treatment with pervanadate. *N*- and *O*-glycosylation status was examined by immunoprecipitation with anti-HA mAb followed by digestion with peptide:*N*-glycosidase F (*PNGase:F*) and/or neuraminidase (to remove sialic acid residues) and/or endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase). The reactions were resolved by SDS-PAGE and immunoblotted with an anti-HA mAb. *Numbers* to the *left* indicate the molecular mass (kDa) of protein standards.

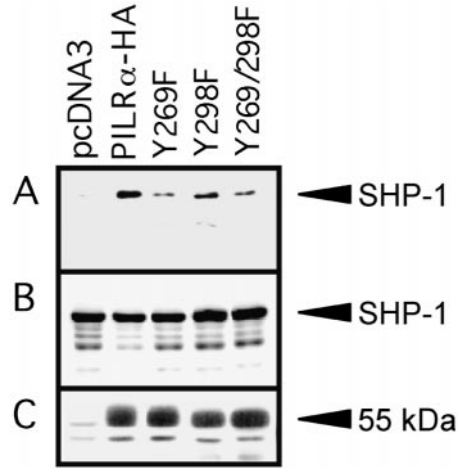

FIG. 5. **SHP-1 interacts with the first tyrosine within the PILRα-HA ITIM.** Standard PCR-based procedures were used to create Tyr to Phe substitutions of the two ITIM-based tyrosine residues either individually or as the Y269F/Y298F double mutant of PILRα-HA. *A,* pervanadate-treated 293T cells transiently co-transfected with the indicated PILRα-HA mutants and SHP-1 were lysed and immunoprecipitated with anti-HA mAb. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-SHP-1 mAb. Expression of the co-transfected constructs was monitored by immunoblotting the lysates from these same cells with (*B*) anti-SHP-1 mAb or (*C*) anti-HA mAb.

by the triple HA tag. The significant difference between the deduced and the apparent molecular mass of PILRα-HA on Western blotting led to the examination of the glycosylation and/or ubiquitination status of this protein. Samples of PILRα-HA immunoblotted for ubiquitin did not reveal any signal (data not shown). Digestion of immunoprecipitates with either PNGase:F or with neuraminidase plus *O*-glycosidase revealed shifts in the migration of expressed PILRα-HA. The combination of PNGase:F and neuraminidase plus *O*-glycosidase reduced the molecular mass to approximately 42 kDa thus revealing that post-translation addition of *N*- and *O*-linked carbohydrate residues accounts for a substantial portion of expressed 55-kDa PILRα-HA (Fig. 4).

Mutation of Tyrosine Residues of PILRα-HA Alters Its Interaction with SHP-1—The role of the ITIM-based tyrosine residues of PILRα-HA in recruiting SHP-1 was investigated by mutational analysis. Immunodetection of SHP-1 following co-immunoprecipitation with an HA-directed antibody indicated that the Tyr-269 motif, *e.g.* IVYASL, was the target for binding with SHP-1 (Fig. 5*A*). Indeed, whereas the Y298F substitution seems to have had only a slight effect on the association with SHP-1, the Y269F mutant and the Y269F/Y298F double mutant significantly decreased the SHP-1 signal. PILRα-HA (Fig. 5*C*) and SHP-1 (Fig. 5*B*) were equally expressed in the corresponding lysates.

Determination of the K_D Values of Interactions between PILRα Phosphotyrosyl Peptides and SH2 Domains of SHP-1 by SPR—The importance of the Tyr-269 motif in recruiting SHP-1 is supported using BIAcore detection. The immobilized phosphorylated peptide corresponding to this motif, *e.g.* KDDGI(pY)-ASLALSSSTS, demonstrated high affinity for the SHP-1-SH2(N) domain (Fig. 6, *top*) and intermediate affinity for the SHP-1-SH2(C) domain (Fig. 6, *bottom*). Interestingly, the SHP-1-SH2(C) domain selectively recognized the Tyr-298 phosphopeptide, *e.g.* PQNETL(pY)SVLKA, but with much lower affinity (Fig. 6, *bottom*). The dissociation constants (K_D) for the various interactions were determined by saturation analysis (nonlinear regression analysis) and supported by those obtained by kinetic analysis (*e.g.* $K_D = k_{off}/k_{on}$: Table I). Saturation analysis revealed K_D values of 167 ± 4 and 1091 ± 196 nM

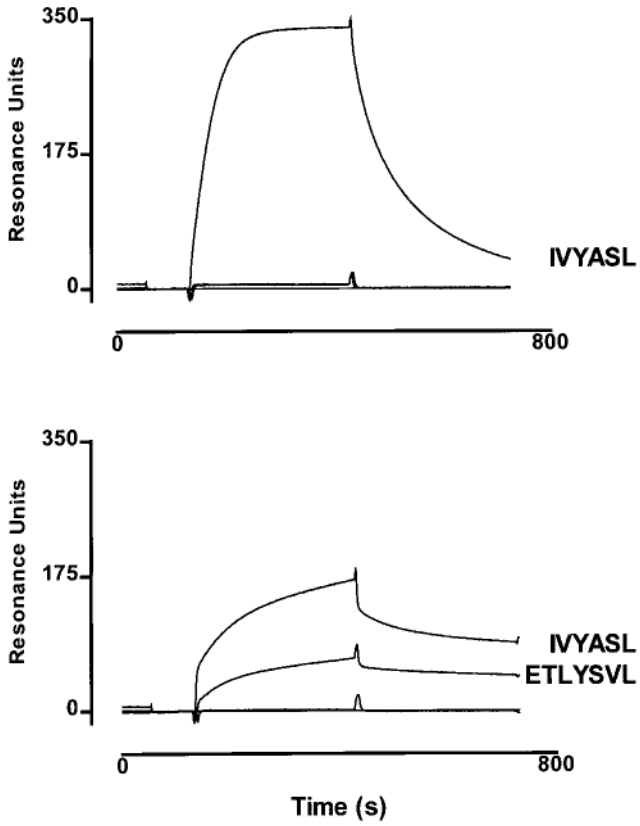

FIG. 6. **The binding potential of candidate tyrosine residues in the PILRα cytoplasmic domain was analyzed by surface plasmon resonance.** The tyrosyl-phosphorylated synthetic peptides, KDDGIV(pY)ASLALSSSTS (IVYASL) and PQNETL(pY)SVLKA (ETLYSVL), corresponding, respectively, to the Tyr-269- and Tyr-298-based Y*XX*(L/V/I) motifs of PILRα were immobilized on separate flow cells of the CM5 sensor chip. GST fusion proteins of the amino-terminal (SHP-1-SH2(N)) and carboxyl-terminal (SHP-1-SH2(C)) SH2 domains of SHP-1 were injected (concentrations ranging from 0.5 to 2000 nM) over the test surfaces. The representative curves shown here correspond to the binding of 400 nM of SHP-1-SH2(N) (*top*) and SHP-1-SH2(C) (*bottom*) to the various test surfaces. Only the tyrosyl-phosphorylated peptide(s) recognized by either SH2 domain are indicated. Curves such as these were used to obtain the kinetic data presented in Table I. The control surface did not bind either of the SH2 domains, and thus the resultant data appear as *straight lines* on both graphs.

TABLE I
Binding potential of the SHP-1 amino- and carboxyl-terminal SH2 domains with human PILRα tyrosyl-phosphorylated peptides

Values represent the mean (±S.E.) of five separate experiments. ND, no detectable binding.

Phosphorylated peptides	KDDGIV(pY)ASLALSSSTS	PQNETL(pY)SVLKA
SHP-1.SH2(N)		
k_{on} (10^4 M^{-1} s^{-1})	5.10 ± 0.53	ND
k_{off} (10^{-3} s^{-1})	2.48 ± 0.65	ND
K_D (nM)	47.8 ± 10.5	ND
SHP-1.SH2(C)		
k_{on} (10^4 M^{-1} s^{-1})	2.42 ± 0.99	0.46 ± 0.05
k_{off} (10^{-3} s^{-1})	3.78 ± 0.96	12.9 ± 0.59
K_D (nM)	214.0 ± 77.7	3000 ± 431

following binding of the SHP-1-SH2(N) and -SH2(C) GST fusion proteins, respectively, to the Tyr-269 phosphopeptide. A K_D value of 3777 ± 447 nM was obtained upon binding of the SHP-1-SH2(C) GST fusion protein to the Tyr-298 phosphopeptide. GST itself did not bind to any of the test surfaces (data not shown) and neither of the GST-SH2 domain fusion proteins bound significantly (*e.g.* less than 5 resonance units) to the control Biosensor chip surface.

The Genomic Organization of PILR Reveals Two Distinct, yet Structurally Related, Receptors—Rapid amplification of cDNA ends designed to clone the 5′ end of the PILRα cDNA revealed the presence of a second cDNA displaying long regions of near sequence identity to PILRα but differing in its 5′ non-coding sequence. The existence of a related gene was also suggested from long range PCR performed on human genomic DNA (results not shown) and was confirmed by the sequence of a 200-kilobase segment of human chromosome 7 deposited subsequently in GenBank™ under the accession number RG161A02. The *PILRα* gene consists of seven exons and six introns spanning approximately 26.7 kb. The second gene, designated *PILRβ,* is located 5.6 kb upstream of the *PILRα* gene and consists of four exons and three introns spanning approximately 9.8 kb (Fig. 7*B*). The nucleotide sequence of the first three exons of the two genes is extremely similar, displaying more than 90% sequence identity (Fig. 7*A*), and suggests that the two genes share a common origin. Tables II and III summarize the nucleotide sequence of the splice sites of the *PILRα* and *PILRβ* genes, respectively, and indicate that in both cases the intron-exon boundaries conform to the GT-AG rule. *PILRβ* codes for a protein with similar extracellular features as those of PILRα but with a short cytoplasmic tail and a charged lysine residue within its transmembrane domain (Fig. 7*A*).

Expression of PILR in Tissues and Selected Cell Lines—Northern blot analysis of selected human tissues revealed a strong signal in peripheral blood leukocytes and lower intensity signals in lung, spleen, and placenta (Fig. 8*A*). Analysis of various human cell lines revealed a signal in a two B cell lines, *e.g.* WIL2-NS and 6F11 (Fig. 8*B*), whereas all other cells tested were negative for *PILR*. Due to the high nucleotide sequence identity of the two genes and the similar length of the two transcripts, the Northern blots do not allow us to discriminate which of the two forms, *e.g. PILRα* or *PILRβ*, is expressed in these tissues and cell lines. To resolve this question, PCR with a forward oligonucleotide primer whose sequence was common to both *PILRα* and *PILRβ* (HUKF1, 5′-GGGAAGCTTATGGGTCGGCCCCTGCTGCTGCCC) and reverse primers specific for each form, HUKR3 for *PILRα* (5′-CCATCTCGAGTTTTGAGAGGGCTG) and HUKR5 for *PILRβ* (5′-TCCTCCGGGGCTAATACACATCC), were performed on reverse transcribed single-stranded cDNA from various tissues including colon, leukocyte, ovary, prostate, small intestine, spleen, testis, thymus, placenta, and mammary gland. The results indicate that both *PILRα* and *PILRβ* were expressed, *e.g.* were paired, at the mRNA level in the individual samples (results not shown).

DISCUSSION

The cytosolic phosphatase SHP-1 contains tandem SH2 domains that bind tyrosine-phosphorylated proteins and, thus, by virtue of its catalytic subunit, gets recruited as an effector enzyme in a signaling pathway initiated by activated tyrosine kinase receptors (26). Specificity in tyrosine kinase signaling pathways is critical and is often dictated by the intrinsic affinity of SH2 domains for the flanking sequences of phosphotyrosine residues. The ITIM consensus sequence (S/L/I/V)-*XYXX*(L/V), via its recruitment of phosphatases, is central to inhibiting the signaling cascade initiated by activating receptors and, thus, plays a pivotal role in regulating cells of the immune system (10).

PILRα-HA is expressed as a membrane-bound *N*- and *O*-linked glycoprotein that contains within its cytoplasmic domain three potential tyrosine phosphorylation sites. Two of these tyrosines, *e.g.* Tyr-269 and Tyr-298, are positioned in Y*XX*L consensus sequences in a manner very reminiscent of the ITIM (D/E)*XXYXX*L(X)$_{26}$Y*XX*L originally described for numerous inhibitory receptors (7–9). In the case of PILRα-HA the

A

```
PILRα MGRPLLLPLLPLLLPPAFLQPSGSTGSGPSYLYGVTQPKHLSASMGGSVEIPFSFYYPWELATAPDVRIS
PILRβ           L  Q       G                                        IV N

PILRα WRRGHFHRQSFYSTRPPSIHKDYVNRLFLNWTEGQKSGFLRISNLQKQDQSVYFCRVELDTRSSGRQQWQ
PILRβ        G                              E         R  D             R     L

PILRα SIEGTKLSITQAVTTTTQRPSSMTTTWRLSSTTTTTGLRVTQGKRRSDSWHISLETAVGVAVAVTVLGIM
PILRβ   K    T          --------    P     IA     ES GH E   L  D  IR  L  A  KTV

PILRα ILGLICLL--RWRRRKGQQRTKATTPAREPFQNTEEPYENIRNEGQNTDPKLNPKDDGIVYASLALSSST
PILRβ    L    LLW      SRAPSSDF*

PILRα SPRAPPSHRPLKSPQNETLYSVLKA*
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B

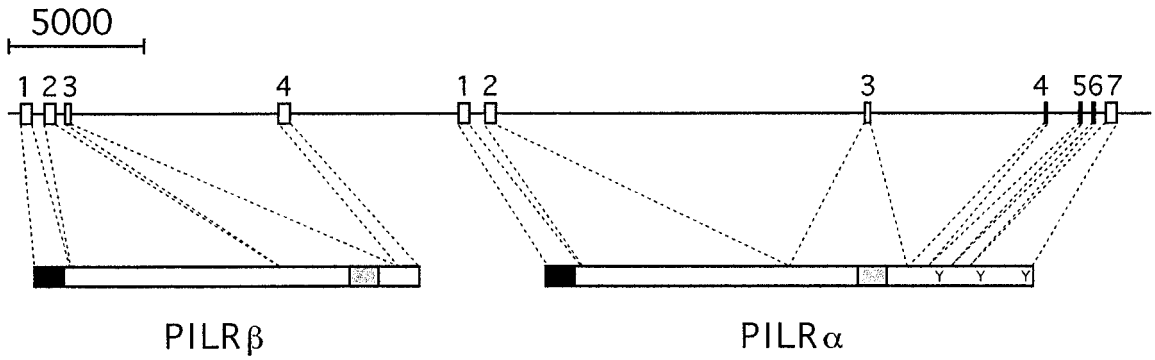

FIG. 7. **Genomic organization of the *PILRα* and *PILRβ* genes and comparison of their respective deduced amino acid sequences.** *A,* the deduced amino acid sequences of PILRα and PILRβ proteins are presented in single letter code. Only those amino acid residues that differ between the two sequences are shown in the PILRβ sequence. The *minus symbol* has been added to facilitate alignment (spacing determined by the respective nucleotide sequences). The amino-terminal signal peptide sequence is *underlined.* The transmembrane domain is displayed as a *shaded box* in both cases, and the charged lysine residue located within this region in PILRβ is highlighted in *bold. B,* schematic representation of the *PILRα* and *PILRβ* genes as well as the respective receptors. The *numbers above* the genes indicate the individual exons. The hydrophobic signal peptide (*solid*), the transmembrane region (*hatched*), and the three cytoplasmic tyrosine residues (*e.g. Y*) are indicated. The *bar* represents the scale in base pairs.

TABLE II
Intron-exon splice junction sites of the human PILRα gene

The nucleotide positions within the RG161A02 human genome clone are as follows: exon 1, 170751–171026; exon 2, 171350–171739; exon 3, 187194–187412; exon 4, 195185–195218; exon 5, 196597–196646; exon 6, 196887–196918; exon 7, 197091–197398 (polyadenylation signal at 197381). The "gt-ag" delimiting each intron is underlined.

Exo	Size	Splice donor			Splice acceptor	Intron size
	bp					*bp*
1	276	TTT CTG CAG CCT A	gtgagtacccc...	...cctcctctag	GT GGC TCC ACA GGA	323
2	390	TCC ATC ACC CAG G	gtgagtccagc...	...ctctccccag	CT GTC ACG ACC ACC	15,455
3	219	AGG AGA AGG AAA G	gtaagtgccca...	...ccccctacag	GT CAG CAG CGG ACT	7772
4	34	ACA ACC CCA GCC AG	gtgagtgctgg...	...tcccatacag	G GAA CCC TTC CAA	1378
5	50	ATC AGG AAT GAA G	gtgagtcctt...	...ttattcttag	GA CAA AAT ACA GAT	240
6	32	CTA AAT CCC AAG	gtaagcaatca...	...tctcgcccag	GAT GAC GGC ATC	172
7	308					

spacer between the two portions of the ITIM is 25 amino acids long. By using an SHP-1-D421A substrate-trapping mutant attenuated in its catalytic function but sufficiently stabilized to permit isolation of itself and the "trapped" substrate (25) indicates that tyrosine phosphorylation is required for *in vivo* association with SHP-1. Mutational analysis reveals that Tyr-269 in the motif IVYASL is the essential residue for interaction with SHP-1 although Tyr-298 in the ETLYSVL motif may also contribute marginally to the interaction. Parenthetically, both co-immunoprecipitation and surface plasmon resonance analyses indicate that the membrane-proximal tyrosine residue, *e.g.* Tyr-246, which lies outside of the ITIM consensus sequence and which does not conform to the motif thought to bind to SHP-1 SH2 domains (27) does not, in fact, contribute to the recruitment of SHP-1 by PILRα (data not shown). A parallel series of experiments indicate that PILRα-HA also interacts with the protein tyrosine phosphatase SHP-2 (data not shown) perhaps through its Tyr-298 motif which resembles the tyrosine-based motif, *e.g.* T*XYXX*(V/I), found in the human NK cell receptor 2B4 as well as in the activation molecule SLAM

TABLE III
Intron-exon splice junction sites of the human PILRβ gene

The nucleotide positions within the RG161A02 human genome clone are as follows: exon 1, ~155,321–155,672; exon 2, 155,996–156,385; exon 3, 156,643–156,843; exon 4, 164,655–165,135 (polyadenylation signal at 165,111). The "gt-ag" delimiting each intron is underlined.

Exo	Size	Splice donor		Splice acceptor		Intron size
	bp					*bp*
1	≥352	...CTG CAG CCT G	gtgagtaccca...	...cttcctctag	GT GGC TCC AC...	323
2	390	...ATC ACC CAG G	gtgagtccagc...	...ggcccagcag	CT GTC ACA AC...	257
3	201	...AGA AGG AAA G	gtaagtgccca...	...cctcctgcag	GT AGC AGG GC...	7811
4	≥481					

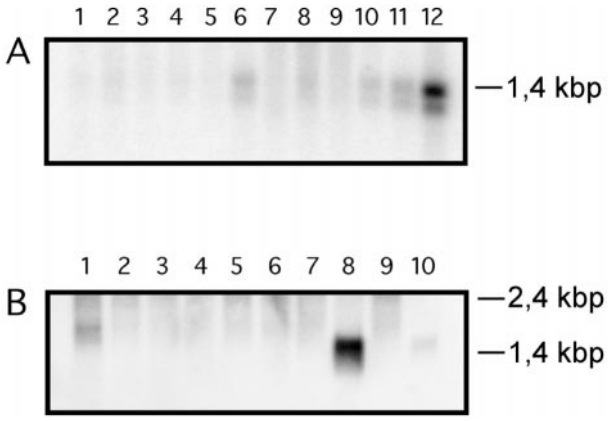

FIG. 8. **Distribution of *PILR* in selected tissues and cell lines.** Two μg of poly(A)$^+$ RNA from selected human tissues (*A*) or 10 μg of poly(A)$^+$ RNA from selected human cell lines (*B*) were subjected to Northern blot analysis with ^{32}P-labeled full-length human *PILRα* cDNA. Probing with ^{32}P-labeled β-actin cDNA indicated similar signals in the various lanes (data not shown). *A*: *lane 1,* brain; *lane 2,* heart; *lane 3,* skeletal muscle; *lane 4,* colon; *lane 5,* thymus; *lane 6,* spleen; *lane 7,* kidney; *lane 8,* liver; *lane 9,* small intestine; *lane 10,* placenta; *lane 11,* lung; *lane 12,* peripheral blood leukocyte. *B*: *lane 1,* macrophage 28SC; *lane 2,* NK92-ci; *lanes 3–5* are T-cells; *lane 3,* Jurkat; *lane 4,* KG-1; *lane 5,* K562; *lanes 6–10* are B cells; *lane 6,* Raji; *lane 7,* Daudi; *lane 8,* WIL2-NS; *lane 9*, Namalwa; and *lane 10,* 6F11.

and thought to be essential for recruitment of SHP-2 and adaptor signaling proteins such as SAP (see Ref. 28 and references therein). Our *in vivo* demonstration of an interaction between SHP-1 and PILRα is supported by our surface plasmon resonance analyses that indicate the high affinity of the amino-terminal SH2 domain of SHP-1 for the Tyr-269 phosphopeptide. A number of receptors, via their (pY)*XX*(F/P/L/Y) motif, bind the amino-terminal SH2 domain of SHP-1 (27) and, at least in the case of the erythropoietin receptor (29), the interaction with SHP-1 mediates termination of signaling initiated by the receptor itself. In contrast, immunoreceptors such as FcγRIIB (4), CD22 (30), and p58/KIR2DL3 (31, 32), which inhibit signaling initiated by other receptors, apparently do so by recruiting the carboxyl-terminal SH2 domain of SHP-1 (5, 33), an exception being pp130, the product of p91/PIR-B in activated macrophages (34), which does so by interaction with the amino-terminal SH2 domain of SHP-1. PILRα-associated SHP-1 probably exists in an active conformation since activation of SHP-1 resides in amino-terminal SH2 domain occupancy (35, 36). The length of the spacer between Tyr-269 and Tyr-298 of PILRα-HA (25 amino acids), and recognition of the amino-terminal and carboxyl-terminal SH2 domains of SHP-1 by Tyr-269 and Tyr-298, respectively, suggest the potential for tandem occupancy and, thus, maximal phosphatase activity (35, 37). Although no definitive ligand for PILRα has been identified at this time, the lack of homology, extracellular or otherwise, between PILRα and known immunoreceptors, does not necessarily preclude MHC molecules, glycoproteins on self-components, and complex carbohydrates on microbial pathogens as potential ligand candidates.

PILRα-HA is expressed in 293T cells (as well as in Cos-1 and MCF-7 cells; data not shown) as a 55-kDa species that is significantly higher than the molecular mass of 36 kDa expected from the deduced amino acid sequence. We were able to reduce the molecular mass of expressed PILRα-HA to approximately 42 kDa and thus determine that the major portion of this difference was due to the presence of *N*- and *O*-linked carbohydrate residues. An explanation for our inability to reduce completely the mass of PILRα-HA to 36 kDa may well lie in the substrate specificity of the *O*-glycosidase (*e.g.* endo-α-*N*-acetylgalactosaminidase) which only digests unsubstituted Galβ1–3GalNAc moieties while leaving all other types of *O*-glycans untouched (38). It is therefore very possible that a certain portion of *O*-linked carbohydrate residues remained bound to PILRα-HA following treatment.

One of the striking features of the ITIM-bearing family of receptors is the pairing of inhibitory receptors with complementary activating receptors. The importance of a matched receptor pair and their individual cellular regulatory roles is exemplified by the stimulation of natural killer and T cell signaling pathways upon binding of the MHC class I ligand to the killer cell activating receptor which contrasts the disrupting signal initiated by binding of the ligand to the inhibitory counterpart killer cell inhibitory receptor (39). To date, all human immunoreceptors have mapped cytogenetically to the 19q13.4 complex or leukocyte receptor cluster, or to the natural killer complex on chromosome 12. Genomic organization of *PILRα* revealed the presence of two genes, *e.g. PILRα* and *PILRβ*, with high sequence homology on chromosome 7. The ITIM-bearing gene product encoded by *PILRα* has a non-polar transmembrane domain. In contrast, the truncated protein PILRβ with the charged residue (*e.g.* lysine) in its transmembrane domain confirms that it is likely the activating counterpart of PILRα (9). Activating receptors such as the mouse PIR-A associate with FcεRI-γ (40) to deliver activation signals to macrophages. We are presently investigating the possibility that our own activating isoform PILRβ can associate with proteins bearing immunoreceptor tyrosine-based activation motifs (ITAMs) such as the Fc receptors. Control of cellular signaling pathways probably occurs through a balance between PILRα-mediated inhibition and PILRβ-mediated activation.

Based on these data and the current literature which pairs inhibitory and activating receptors such as killer cell inhibitory receptor and killer cell activating receptor, FcγRIIB and FcγRIII, and PIR-B and PIR-A, to name but a few (9, 11), we are confident in stating that PILRα and PILRβ represent a novel ITIM-bearing and non-ITIM-bearing receptor pair. Furthermore, their chromosomal localization and their lack of homology with any of the known inhibitory/activating receptors suggests that they represent a novel family of receptors. Their chromosomal localization may yet reveal itself to include other genes bearing information relevant to our understanding of the regulation of cellular signaling pathways.

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PILRα, a Novel Immunoreceptor Tyrosine-based Inhibitory Motif-bearing Protein, Recruits SHP-1 upon Tyrosine Phosphorylation and Is Paired with the Truncated Counterpart PILRβ

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