Brief Definitive Report

Requirement of SH2-containing Protein Tyrosine Phosphatases SHP-1 and SHP-2 for Paired Immunoglobulin-like Receptor B (PIR-B)-mediated Inhibitory Signal

By Akito Maeda,* Mari Kurosaki,* Masao Ono,‡§ Toshiyuki Takai,‡§ and Tomohiro Kurosaki*

From the *Department of Molecular Genetics, Institute for Liver Research, Kansai Medical University, Moriguchi 570, Japan; the ‡Department of Molecular Embryology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-77, Japan; and §Core Research for Evolution Science and Technology, Japan Science and Technology Corporation, Japan

Summary

Paired immunoglobulin-like receptor B (PIR-B) (p91) molecule has been proposed to function as an inhibitory receptor in B cells and myeloid lineage cells. We demonstrate here that the cytoplasmic region of PIR-B is capable of inhibiting B cell activation. Mutational analysis of five cytoplasmic tyrosines indicate that tyrosine 771 in the motif VxYxxL plays the most crucial role in mediating the inhibitory signal. PIR-B-mediated inhibition was markedly reduced in the SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2 double-deficient DT40 B cells, whereas this inhibition was unaffected in the inositol polyphosphate 5'-phosphatase SHIP-deficient cells. These data demonstrate that PIR-B can negatively regulate B cell receptor activation and that this PIR-B-mediated inhibition requires redundant functions of SHP-1 and SHP-2.

The ability of B cells to respond to antigen relies on signals transmitted through the B cell antigen receptor (BCR) complex. Activation of cytoplasmic protein tyrosine kinases is the earliest measurable biochemical response to BCR cross-linking. This initial event leads to the generation of secondary signals including R as activation, phosphatidylinositol 3-kinase activation, turnover of phosphoinositides, and calcium mobilization. Both the strength and duration of the BCR-elicited signal are important in directing biological responses of B cells such as proliferation, differentiation, and apoptosis (for reviews see references 1–4). Thus, attenuation and termination of these activation signals are also critical components for B cell response.

B cell activation is inhibited by cross-linking FcγRIIB with the BCR (5, 6). The cytoplasmic domain of FcγRIIB contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), which is necessary for the inhibitory function of the receptor (7, 8). Phosphorylation of the tyrosine in the ITIM by an activated protein tyrosine kinase(s) is critical to its inhibitory function (7). Although the phosphorylated FcγRIIB ITIM associates with the SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2, functional evidence has shown that inhibition by FcγRIIB primarily involves SHIP (11–13). In B cells, in addition to FcγRIIB, a recently cloned p91 (PIR-B) is suggested to function as an inhibitory receptor. PIR-B, a member of the immunoglobulin superfamily, is a 91-kD transmembrane glycoprotein containing four potential ITIMs in its cytoplasmic region (14, 15).

A growing family of inhibitory receptors that can interrupt the activation process have generated interest in the mechanism of inhibition and raised questions about the similarity in this mechanism used by the different receptors. To test whether PIR-B can deliver inhibitory signals in B cells, and whether both PIR-B- and FcγRIIB-mediated inhibitory responses are dependent on the same signaling molecule SHIP, we have constructed chimeric FcγRIIB–PIR-B molecules with the cytoplasmic region of PIR-B and assessed their ability to inhibit BCR signaling. We report here that SHP-1 and SHP-2, but not SHIP, are required for PIR-B-mediated inhibitory signal.

Materials and Methods

Cells, Expression Construct, and Abs. Various mutant DT 40 cells, wild-type A 20, and A 20 IIA 1.6 cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, and glutamine. FcγRIIB–PIR-B chimera and its mutants were cre-
cDNAs were subcloned into pApuro vector (16) and were electroporated into DT 40 or A20 II A.1.6 cells as previously described (17). After selecting clones in the presence of puromycin (0.5 μg/ml), cell surface expression levels of FcγRIIIB–FcγR-IIB mAb, 2.4G2 (18). Anti–chicken IgM mAb M 4, anti–SHP-Ab, and wild-type A20 cells (2 clones. After subcloning, the genomic DNA library (Clontech) was screened to obtain genomic clones. Two independent II A.1.6 transfectants expressing each FcγR-IIB–PIR-B receptor were used for calcium measurement. In the case of DT 40 cells expressing FcγR-IIB–FcγR-IIB, three different clones were used for this analysis. Calcium release from intracellular calcium store was measured in the buffer containing 1 mM EGTA. NucleaD Factor in A divated T cell L urease A says. 24 h after transfection with 20 μg of nuclear factor in activated T cell (NF-AT) (R enilla luciferase control reporter vector) luciferase reporter gene and 2 μg of pRL-CMV (Promega, Madison, WI) transfection of BCR and the FcγR-IIB–PIR-B acts at least on calcium release from intracellular calcium stores. Consistent with these results, transcriptional activation of the NF-AT luciferase reporter was inhibited by coligation of FcγR-IIB–PIR-B (Fig. 2). IIA1.6 cells expressing FcγR-IIB–FcγR-IIB mAb, 2.4G2 (Phar- mingen, San Diego, CA), and analyzed by FACScan® (Becton Dickinson, Mountain View, CA). The x and y axes for the histograms in inset boxes (Fig. 2 A, 4 A, and 5 B) indicate fluorescence intensity (4-decade-log scales) and relative cell number, respectively.

Results and Discussion

The Cytoplasmic Domain of PIR-B Is Capable of Inhibiting BCR Activation. To test whether the cytoplasmic domain of PIR-B may inhibit BCR activation, a chimeric molecule with the cytoplasmic domain of PIR-B and the extracellular domain of FcγR-IIB was constructed (Fig. 1). This molecule was transfected into the FcγR-IIB-negative mutant of the mouse A20 B cell lymphoma II A.1.6 (22) to obtain stable transfectants. Expression level of this chimeric receptor was assessed by flow cytometry analysis using anti–mouse FcγRIIIB mAb, 2.4G2 (Fig. 2 A). IIA1.6 cells expressing the FcγR-IIB–FcγR-IIB-B receptor was stimulated by BCR cross-linking alone (Fig. 2 A, solid line) or coligation of BCR and the FcγR-IIB–FcγR-IIB-B (Fig. 2 A, dashed line). Coligation of FcγR-IIB–FcγR-IIB-B to the BCR resulted in an inhibition of intracellular free calcium. Incubation with EGTA further decreased Ca2+ mobilization upon co-cross-linking of BCR with FcγR-IIB–FcγR-IIB-B, indicating that the FcγR-IIB–FcγR-IIB-B affects at least on calcium release from intracellular stores. Consistent with these results, transcriptional activation of the NF-AT luciferase reporter was inhibited by coligation of the BCR to this chimeric molecule (Fig. 2 B). Thus, the cytoplasmic domain of PIR-B can deliver a signal that inhibits BCR–mediated function in II A.1.6 B cells.
SHP-1 and SHP-2 associate with the cytoplasmic domain of PIR-B. The presence of ITIM-related sequences in the PIR-B cytoplasmic domain (Fig. 1) suggests that the PIR-B may become tyrosine phosphorylated, resulting in its association with SH2-containing signaling molecules such as SHP-1, SHP-2, and/or SHIP. Using IIA1.6 cells transfected with FcγRIIB–PIR-B, the status of tyrosine phosphorylation of FcγRIIB–PIR-B was determined by immunoprecipitation followed by Western blotting with antiphosphotyrosine mAb 4G10. As shown in Fig. 3A, the chimeric receptor was slightly tyrosine phosphorylated upon BCR cross-linking alone and this phosphorylation was markedly augmented by coligation of BCR and FcγRIIB–PIR-B. The FcγRIIB–PIR-B phosphorylation was due to tyrosine residues located in the cytoplasmic domain of PIR-B, since FcγRIIB–PIR-B(Y/F) (Fig. 1) did not undergo tyrosine phosphorylation upon coligation of BCR and FcγRIIB–PIR-B(Y/F) (data not shown). After co-cross-linking of BCR with FcγRIIB–PIR-B, the chimeric molecule was immunoprecipitated followed by Western blotting with anti-SHIP, in addition to IIA1.6 cells expressing wild-type FcγRIIB–PIR-B (lanes 1–3), wild-type A20 cells expressing FcγRIIB were stimulated similarly (lanes 4–6). FcγRIIB–PIR-B or FcγRIIB was immunoprecipitated with 2.4G2 followed by Western blotting with anti-SHIP Ab. Membranes were reprobed with anti-PKR A B Ab (bottom).

Figure 1. Schematic diagram of FcγRIIB–PIR-B chimeric molecules. FcγRIIB and PIR-B contain four ITIM-like motifs (residues 688–693, SLYASV; 717–722, ETYAQV; 769–774, VTYAQL; and 799–804, SVYATL). In addition to these tyrosines, the cytoplasmic domain of PIR-B contains one more tyrosine (residue 747). The open and shaded boxes represent portions of FcγRIIB and PIR-B, respectively.

Figure 2. Inhibition of BCR-mediated cell activation by FcγRIIB–PIR-B. (A) Calcium mobilization stimulated by BCR cross-linking (solid line) and coligation of BCR to FcγRIIB–PIR-B (dashed line) in IIA1.6 cells expressing wild-type FcγRIIB–PIR-B. Calcium release from intracellular calcium store was measured in the presence of 1 mM EGTA, shown by right panel. Histogram in the inset box indicates the expression level of FcγRIIB–PIR-B by 2.4G2 staining. Unstained cells were used as a negative control (open histogram). Stimulation of FcγRIIB–PIR-B alone by 2.4G2 did not induce calcium mobilization. (B) NF-AT activation by BCR cross-linking and the coligation of BCR to FcγRIIB–PIR-B. The results are shown as relative NF-AT activity to BCR cross-linking alone.

Figure 3. Recruitment of SHP-1 and SHP-2 to FcγRIIB–PIR-B after coligation of BCR and the chimeric receptor. (A) Tyrosine phosphorylation of FcγRIIB–PIR-B by BCR cross-linking and coligation of BCR to FcγRIIB–PIR-B. IIA1.6 cells expressing wild-type FcγRIIB–PIR-B were incubated with F(ab')2 rabbit anti-mouse IgG or with intact rabbit anti-mouse IgG, or with intact rabbit anti-mouse IgG. FcγRIIB–PIR-B was then immunoprecipitated with 2.4G2, separated by SDS-PAGE gel, transferred to membrane, and immunoblotted with antiphosphotyrosine mAb 4G10 (top). The same membrane was reprobed with anti-PKR A B Ab (bottom). (B) Immunoblotting with anti-SHP-1 and anti-SHP-2 Abs after BCR cross-linking and the coligation of BCR to FcγRIIB–PIR-B. IIA1.6 cells expressing wild-type FcγRIIB–PIR-B were stimulated with either F(ab')2 rabbit anti-mouse IgG or intact rabbit anti-mouse IgG and were lysed. FcγRIIB–PIR-B was then immunoprecipitated with 2.4G2, resolved by SDS-PAGE gel, transferred to membrane, and probed with anti-SHP-1 or anti-SHP-2 Ab. In the case of immunoblotting with anti-SHIP, in addition to IIA1.6 cells expressing wild-type FcγRIIB–PIR-B (lanes 1–3), wild-type A20 cells expressing FcγRIIB were stimulated similarly (lanes 4–6). FcγRIIB–PIR-B or FcγRIIB was immunoprecipitated with 2.4G2 followed by Western blotting with anti-SHIP Ab. Membranes were reprobed with anti-PKR A B Ab (bottom).
As a positive control, recruitment of SHIP to phosphorylated FcγRIIB molecule was clearly observed under the same experimental conditions (Fig. 3B, left, lanes 4–6).

Tyrosine 771 in the cytoplasmic domain of PIR-B is essential for inhibition. The cytoplasmic tail of PIR-B contains four copies (residues 688–693, 717–722, 769–774, and 799–804) of potential ITIMs (14, 15, 23). The inhibition assay described above allowed us to assess the structural importance of each phosphotyrosine for the inhibitory signal. Various tyrosine mutants were made (Fig. 1) and transfected into IIA1.6 cells to obtain stable transformants. IIA1.6 cells expressing comparable levels of chimeric receptors (Fig. 4A) were stimulated by BCR alone or coligation of BCR and the chimeric mutants. FcγRIIB–PIR-B almost reverted the inhibitory effect of PIR-B. This result demonstrates that phosphorylation of cytoplasmic tyrosines of PIR-B plays an important role in the inhibitory signal and that the residual inhibition may be independent of tyrosine phosphorylation on PIR-B (Fig. 4B).

SHP-1 and SHP-2 are redundantly required for PIR-B–mediated inhibition. To test directly the role of SHIP, SHP-1, and SHP-2 in the inhibitory signal delivered by PIR-B cytoplasmic tail, B cell lines deficient in SHIP, SHP-1, or SHP-2 protein expression on various gene-targeted DT40 cells. Each protein was immunoprecipitated and was detected by Western blotting analysis using anti-SHIP, anti-SHP-1, or anti-SHP-2 Ab. (B) Calcium mobilization was measured after BCR cross-linking (solid line) and coligation (dashed line) in various DT40 mutants expressing FcγRIIB–PIR-B. Surface expression levels of FcγRIIB–PIR-B are indicated in inset boxes. (C) Comparison of inhibitory effect in various mutant DT40 cells. The results are expressed as the mean from three independent clones. Error bars represent SD from the mean.

wild-type chimeric receptor, whereas the FcγRIIB–PIR-B(Y771F) mutant inhibited it to a much lesser degree than wild-type FcγRIIB–PIR-B (Fig. 4, A and B). Thus, these data indicate that both tyrosines contribute to the inhibitory effect and that tyrosine 771 plays a more critical role in inhibiting the BCR-induced calcium mobilization.

SHP-1 and SHP-2 are redundantly required for PIR-B–mediated inhibition. To test directly the role of SHIP, SHP-1, and SHP-2 in the inhibitory signal delivered by PIR-B cytoplasmic tail, B cell lines deficient in SHIP, SHP-1, or SHP-2 individually and in a combination of SHP-1 and SHP-2 were established using a chicken DT40 B cell line as a consequence of homologous recombination (Fig. 5A). A detailed analysis of BCR signal transduction in these mutants will be presented elsewhere. FcγRIIB–PIR-B chimeric molecule was transfected into DT40 wild-
type and mutant cells, and compared for its ability to mediate the inhibitory signaling response. In contrast to II 1A1.6 B cells, a more residual inhibition by FcγRIIB–PIR-B(Y/F) was observed in DT 40 cells (Fig. 5, B and C). Various genetic background DT 40 cells expressing similar levels of FcγRIIB–PIR-B were stimulated with BCR cross-linking alone (Fig. 5, solid line) or coligation of BCR and the FcγRIIB–PIR-B (dashed line). As shown in Fig. 5 B, FcγRIIB–PIR-B-mediated inhibition of calcium mobilization was unperturbed in SHIP-deficient DT 40 cells, but was substantially reduced in SHP-1/SHP-2 double-deficient cells. The reduction of the chimeric receptor-mediated inhibition was also observed by loss of either SHP-1 or SHP-2 alone. However, this reduction was less than that in double-deficient cells. These results demonstrate that redundant functions of SHP-1 and SHP-2 are required for the FcγRIIB–PIR-B-mediated inhibitory signal.

Signals from the BCR and coreceptors such as CD19, CD22, and FcγRIIB (24, 25) are integrated inside the cell, allowing the B cell to mount a response appropriate to the source of the antigen and the lymphocyte environment. In this study, we provide evidence that the cytoplasmic region of PIR-B is capable of inhibiting B cell activation by a phosphotyrosine-dependent manner in IIA1.6 and DT 40 B cells. Coligation of BCR and FcγRIIB–PIR-B induced tyrosine phosphorylation of the cytoplasmic domain of PIR-B and this phosphorylation was abrogated by the FcγRIIB–PIR-B(Y/F) mutant. Moreover, this mutant markedly reduced the inhibitory effects on BCR signaling, suggesting that the SH2-containing proteins are involved in PIR-B-mediated inhibitory signals.

The finding that the residual inhibition by FcγRIIB–PIR-B still occurs in SHP-1 and SHP-2 double-deficient DT 40 cells, implicates that, in addition to SHP-1 and SHP-2, another SH2-containing protein(s) may participate in this inhibitory signal to some extent. Nevertheless, the substantial reduction of the inhibition by loss of SHP-1 and SHP-2 clearly indicates that the redundant functions of these SH2-containing phosphatases are required for PIR-B-mediated inhibition. Supporting this conclusion, both SHP-1 and SHP-2 were recruited to the phosphorylated FcγRIIB–PIR-B. In contrast to requirement of SHIP for an inhibitory response by FcγRIIB, our biochemical and functional data demonstrate that SHIP is dispensable for the PIR-B-mediated inhibitory response.

Mutational analysis of five cytoplasmic tyrosines indicates that tyrosine 771 in the motif VTYAQL plays the most crucial role in mediating the inhibitory signal and that tyrosine 801 in the motif SVYATL also contributes. The more functional importance of tyrosine 771 may reflect the fact that the motif surrounding tyrosine 771 matches better with the consensus I/VxYxxL sequence than that of tyrosine 801 (26). Binding of SH2 domains of SHP-1 and SHP-2 to tyrosine phosphorylated PIR-B would serve to relocate SHP-1 and SHP-2 to membrane (11), where they may gain access to potential substrates. Simultaneously, these phosphatases may be converted to an open conformation by binding to phosphorylated PIR-B, thereby leading to an increase of their enzymatic activity (27).

The ligand of PIR-B is still unknown. Significant homology of PIR-B to recently isolated LIR-1 (28), together with the evidence that LIR-1 is able to bind to the MHC class I molecule, suggests that PIR-B may be a receptor for MHC class I or class I-related molecules. Thus, similar to inhibition of NK cytotoxicity by interaction between class I molecules on target cells and KIRs on NK cells (29–32), cell-cell interactions may bring PIR-B into the close proximity of antigen-bound BCR, resulting in attenuation of BCR signaling. Alternatively, interaction of PIR-B with a putative ligand may remove it from the vicinity of BCR in order to release the B cells from the negative regulatory effects. Identification of the ligand for PIR-B as well as substrates for SHP-1 and SHP-2 will further clarify biological roles of PIR-B in the immune response and its mechanism of inhibition.

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Address correspondence to Tomohiro Kurosaki, Department of Molecular Genetics, Institute for Liver Research, Kansai Medical University, Moriguchi 570, Japan. Phone: 81-6-993-9445; Fax: 81-6-994-6099; E-mail: kurosaki@mxr.meshnet.or.jp

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