Paired Immunoglobulin-like Receptor (PIR)-A Is Involved in Activating Mast Cells through Its Association with Fc Receptor γ Chain

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Summary

Paired immunoglobulin-like receptor (PIR)-A and PIR-B possess similar ectodomains with six immunoglobulin-like loops, but have distinct transmembrane and cytoplasmic domains. PIR-B bears immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences in its cytoplasmic domain that recruit Src homology (SH)2 domain-containing tyrosine phosphatases SHP-1 and SHP-2, leading to inhibition of B and mast cell activation. In contrast, the PIR-A protein has a charged Arg residue in its transmembrane region and a short cytoplasmic domain that lacks ITIM sequences. Here we show that Fc receptor γ chain, containing an immunoreceptor tyrosine-based activation motif (ITAM), associates with PIR-A. Cross-linking of this PIR-A complex results in mast cell activation such as calcium mobilization in an ITAM-dependent manner. Thus, our data provide evidence for the existence of two opposite signaling pathways upon PIR aggregation. PIR-A induces the stimulatory signal by using ITAM in the associated γ chain, whereas PIR-B mediates the inhibitory signal through its ITIMs.

Key words: activation signal • Fc receptor γ chain • immunoreceptor tyrosine-based activation motif • mast cell • paired immunoglobulin-like receptor A

Cross-linking of immune receptors such as BCR, TCR, or FcR on a variety of cells leads to their activation through the sequential activation of protein tyrosine kinases (PTKs) (1-3). Several features have emerged that are common to these activating receptors. They are all oligomeric complexes in which ligand binding and signal transduction are compartmentalized into distinct receptor subunits. Hence, these receptors comprise one or more immunoreceptor tyrosine-based activation motif (ITAM)-containing subunits. When the ligand-binding subunit(s) of the receptor is engaged, the cytoplasmic ITAMs are tyrosine phosphorylated by src-family PTKs. This leads to the recruitment of syk-family PTKs, which trigger a cascade of intracellular phosphorylations that result in cellular activation.

Balancing these activation responses are the inhibitory receptors and their associated signaling molecules, which are responsible for setting threshold levels for activation signals as well as terminating activation responses. These inhibitory receptors also share several common features. They have one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains, tyrosine phosphorylation of which leads to the recruitment of signaling molecules capable of inhibiting cell activation (4-7). Indeed, NK inhibitory receptors recruit tyrosine phosphatase SHP-1 to inhibit NK cell activation (8-10). Certain isoforms of NK receptors that lack ITIM sequences have been proposed to function as activation receptors rather than inhibitory receptors (11). A conspicuous feature of these noninhibitory NK receptors is the presence of a basic amino acid in the transmembrane domain, which may allow their association with signal-transducing proteins such as DAP12, as demonstrated recently for killer-activating receptors (12, 13).

The paired Ig-like receptors (PIRs) recently identified on B cells and myeloid lineage cells include PIR-A molecule, which has a short cytoplasmic domain, and PIR-B molecule, which bears four potential ITIMs in its cytoplasmic domain (14, 15). In contrast to the unique transmembrane and cytoplasmic domains between PIR-A and PIR-B, extracellular regions of these molecules are very homologous, suggesting that both molecules bind the putative common ligand. We and others have recently demonstrated that PIR-B functions as an inhibitory receptor in B...
Materials and Methods

Cells. Expression Constructs, and Abs. Chicken DT 40 cells and mouse A20 IIA1.6 cells (FcγRII-negative A20 derivative) were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, glutamine, and 50 μM 2-ME. RBL-2H3 cells (provided by N. Yamamoto and T. Yasuda, Bayer Yakuhin, Ltd., Kyoto, Japan) and 293 T cells were cultured in MEM and DMEM, respectively, instead of RPMI 1640. FcγRIII–PIR-A chimera, mutated Fcγ chain (18), and hemagglutinin (HA)-tagged PIR-A were created by the PCR method and subcloned into the pAPuro (19), pAneo (20), and pApuro (19) vectors, respectively. FcγRIII–PIR-A chimera contains the extracellular domain of human FcγRIIIα- and the transmembrane and cytoplasmic regions of PIR-A (15). Resulting constructs were confirmed by DNA sequencing. These expression constructs were transfected into DT40, A20 IIA1.6, and RBL-2H3 cells by electroporation, and selected in the presence of puromycin (0.5 μg/ml) or G418 (2 mg/ml). Cell lysates and phosphatase inhibitors were added to both digitonin and NP-40 buffer as previously described (20). Immunoprecipitates were separated by SDS-PAGE gel, transferred to nitrocellulose membrane and detected by appropriate Abs and ECL system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation and Western Blotting Analysis. To detect association of γ chain with FcγRIII–PIR-A, cells were solubilized in 1% digitonin buffer (1% digitonin, 0.12% Triton X-100, 150 mM NaCl, and 20 mM triethanolamine, pH 7.8). Cell lysates were sequentially incubated with mAb 3G8 or anti-HA mAb, and then with anti-mouse IgG or streptavidin in RBL-2H3 or A20 IIA1.6 cells, respectively. Surface expression levels of FcγRIII–PIR-A were checked by flow cytometric analysis using anti-human FcγRIII mAb, 3G8 (22), and expression level of Fcγ chain was analyzed by Western blotting using anti-γ chain Ab (23). The calcium phosphate precipitation method was used for transfection into 293 T cells. Biotin-tagged 3G8 mAb, streptavidin, antiphosphotyrosine mAb (4G10), F(ab′)2 rabbit anti-mouse IgG, and anti-HA epitope mAb were purchased from PharMingen (San Diego, CA), Vector Labs. (Burlingame, CA), Upstate Biotechnology, Inc. (Lake Placid, NY), Chemicon International, Inc. (Temecula, CA), and Boehringer Mannheim (Tokyo, Japan), respectively.

Northern Analysis. Total RNA was prepared from A20 IIA1.6 and RBL-2H3 cells using the guanidinium thiocyanate method. Total RNA (20 μg) was separated in a 1.2% formaldehyde gel, transferred to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech), and probed with 32P-labeled Fcγ chain cDNA.
Ca2+ measurements. Cells (5 × 10^6) were suspended in PBS containing 20 mM Hapes (pH 7.2), 5 mM glucose, 0.025% BSA, and 1 mM CaCl₂, and loaded with 3 μM Fura-2/AM at 37°C for 45 min. Cells were washed twice and adjusted to 10⁶ cells/ml. Continuous monitoring of fluorescence from the cell suspension was performed using Hitachi F-2000 fluorescence spectrophotometer (Hitachi Limited, Tokyo, Japan) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as previously described (24). FcRIII–PIR-A on RBL-2H3 and DT40 cells was stimulated with anti-FcRIII mAb (3G8, 10 μg/ml) and was subsequently cross-linked with F(ab')₂ anti-mouse IgG (25 μg/ml). Biotin-tagged 3G8 (10 μg/ml) and streptavidin (25 μg/ml) were used for stimulation of FcRIII–PIR-A on A20 IIIA1.6 cells.

**Results and Discussion**

PIR-A is capable of activating mast cells. Structural features of PIR-A have prompted us to test whether PIR-A is able to transmit activation signals. For this purpose, a chimeric molecule with the transmembrane and cytoplasmic domains of PIR-A and the extracellular domain of human FcγRIII was constructed (FcγRIII–PIR-A), and transfected into mouse A20 IIIA1.6 B cells and rat RBL-2H3 basophilic leukemia cells in order to obtain transformants. Expression level of this chimeric receptor was assessed by flow cytometric analysis using anti-human FcγRIII mAb, 3G8 (Fig. 1A, insets). As shown in Fig. 1A, stimulation of FcγRIII–PIR-A chimeric receptor on RBL-2H3 cells with 3G8 and subsequent cross-linking resulted in a transient rise in 

Figure 3. Expression of FcγRIII–PIR-A and γ in DT40 transformants. (A) FACS analysis of DT40 cells by using FITC-conjugated 3G8. Parental DT40 cells were used as a negative control. (B) Expression of γ by Western blot analysis using anti-γ Ab. Transformant cells were directly dissolved in SDS-sample buffer.

Figure 4. The FcγRIII–PIR-A complex transmits activation signals in the presence of γ. (A) Calcium mobilization by cross-linking of FcγRIII–PIR-A. Transfomants shown in Fig. 3 were stimulated by 3G8 (left arrows) and F(ab')₂ anti-mouse IgG (right arrows). (B) Tyrosine phosphorylation of γ by cross-linking of FcγRIII–PIR-A. Transfomant cells were stimulated with 3G8 (3 min), and then F(ab')₂ anti-mouse IgG (3 min). Cells were dissolved by NP-40 buffer, immunoprecipitated by anti-γ Ab, separated by 15% SDS-PAGE gel, and blotted with anti-γ chain Ab, demonstrating the association of γ chain with FcγRIII–PIR-A in RBL-2H3 cells (Fig. 1C). This association was also shown by transfection experiments using 293 T cells. FcγRIII–PIR-A by itself can be expressed on the cell surface of 293 T cells to some extent, whereas cotransfection of γ with FcγRIII–PIR-A enhanced cell surface expression of this chimeric receptor (Fig. 2A), suggesting that γ chain is involved in transport of FcγRIII–PIR-A to cell surface in 293 T cells. Moreover, the interaction of γ chain with FcγRIII–PIR-A was reconstituted in 293 T cells, as demonstrated by immuno precipitation experiments using digitonin buffer (Fig. 3).

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Fcγ Chain Is Involved in Transmitting Activation Signals through FcγRIII–PIR-A Complex by ITAM-dependent Mechanism. To show that the association of γ chain with FcγRIII–PIR-A is critical for transmitting activation signals, we used the DT 40 B cell system, since this cell line does not express γ chain like A20 cells. After obtaining DT 40 transformant expressing FcγRIII–PIR-A (Fig. 3 A), we transfected wild-type γ chain or mutated γ chain into this DT 40 transformant using another drug selection marker to isolate DT 40 cells expressing both FcγRIII–PIR-A and γ chain. Mutation of the γ chain (Tyr65 and Tyr76 to Phe) was designed to test whether the activation signal is dependent on the ITAM sequence located in the cytoplasmic domain of γ chain. Expression level of γ chain was assessed by Western blotting analysis with anti–γ chain Ab, and clones expressing comparable levels of wild-type γ and mutated γ were selected (Fig. 3 B) and analyzed. Although FcγRIII–PIR-A alone was expressed on the cell surface of DT 40 B cells, introduction of either wild-type γ chain or mutated γ chain enhanced the level of cell surface expression of the chimeric receptor about fivefold, as demonstrated by flow cytometric analysis (Fig. 3 A). These observations strengthen our previous conclusion that γ chain contributes to cell surface expression of FcγRIII–PIR-A. However, this γ chain function does not require phosphorylation of tyrosine residues in its ITAM sequence.

Stimulation of FcγRIII–PIR-A complex with wild-type γ chain evoked calcium mobilization upon receptor engagement, whereas this [Ca^{2+}] increase could not be detected in DT 40 cells expressing FcγRIII–PIR-A alone nor in the receptor with mutated γ chain (Fig. 4 A), indicating that ITAM of γ chain is essential for transmitting the activation signal. This conclusion was supported further by the observation that γ chain, but not mutated γ chain, is tyrosine phosphorylated by FcγRIII–PIR-A stimulation (Fig. 4 B). Taken together, our results demonstrate that FcγRIII–PIR-A is complexed with Fcγ chain, the presence of which is essential for transmitting activation signals.

Coexistence of both activation and inhibitory pathways upon receptor aggregation allows immune cells to generate graded responses under different ligand conditions. Assuming that PIR-A and PIR-B recognize the same ligand, the data presented here together with previous observations (16, 17), support the model that cell surface receptors with same or related ligand-binding specificity transmit both activation and inhibitory signals through ITAM and ITIM sequences, respectively (26). Ruminant of this type of regulation is Fc receptors; FcγRIIIA transmits the activation signal, whereas FcγRIIB inhibits the ITAM-induced activation signal despite the same ligand specificity between these two receptors (25).

Fcγ chain is physically and functionally associated with FcγRIII–PIR-A. Since the γ chain has a very short extracellular domain (18), this association is presumably mediated by transmembrane–transmembrane interactions. Supporting this notion, the transmembrane region of another γ chain–interacting receptor, FcεR, is very similar to that of PIR-A in that positively charged Arg residue is located in the same position, although their extracellular and cytoplasmic domains are divergent (27). Given the evidence that HA-tagged PIR-A is also associated with γ chain (Fig. 2 B), our data strongly suggest that native PIR-A associates with Fcγ chain in mast cells.

Functional interaction was demonstrated by requirement of γ chain in FcγRIII–PIR-A-induced calcium mobilization. More importantly, ITAM of the γ chain was tyrosine phosphorylated upon receptor aggregation (Fig. 4 B), indicating that the PIR-A complex transmits the activation signal through an ITAM-dependent mechanism. Thus, PIR-A uses src- and syk-family PTKs to transmit the positive signal, in a similar manner to the situation with other ITAM-bearing receptors such as TCR, BCR, or FcγRs, whereas PIR-B mediates the negative signal through recruitment of SHP-1 and SHP-2 to phosphorylated ITIMs in its cytoplasmic domain.

Both PIR-A and PIR-B appear to be expressed in B cells and myeloid lineage cells, as assessed by reverse transcriptase PCR (15). The RNA content of PIR-A and PIR-B may not necessarily reflect the cell surface expression of each molecule, because associated molecules such as γ chain may enhance the transport of PIR-A to cell surface, as seen in this study. Since the relative expression level of PIR-A and PIR-B on cell surface is one of the critical determinants for stimulatory or inhibitory responses, associated chains such as γ participate in PIR functions by not only conferring the stimulatory capability on PIR-A but also promoting its cell surface expression.
Note added in proof. The results that PIR-A is able to transmit a stimulatory signal have been obtained independently (Yamashita, Y., M. Ono, and T. Takai. J. Immunol. In press).

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