Stimulatory Function of Pairing Immunoglobulin-like Receptor-A in Mast Cell Line by Associating with Subunits Common to Fc Receptors*

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Paired Ig-like receptors (PIR) are polymorphic type I transmembrane proteins belonging to an Ig superfamily encoded by multiple isotypic genes. They are expressed on immune cells such as mast cells, macrophages, and B lymphocytes. Two subtypes of PIR have been classified according to the difference in the primary structure of the PIR transmembrane and cytoplasmic regions. These subtypes are designated as PIR-A and PIR-B. In this study, the transmembrane and cytoplasmic regions of the PIR-A subtype were shown to mediate activation signal events such as cytoplasmic calcium mobilization, protein tyrosine phosphorylations, and degranulation in rat mast cell line RBL-2H3. The association of the Fc receptor γ and β subunits with PIR-A was shown to be responsible for PIR-A function but not required for membrane expression of PIR-A on COS-7 cells. We further revealed the role of two charged amino acid residues in the transmembrane region, namely arginine and glutamic acid, in PIR-A function and its association with the above subunits. In contrast to the inhibitory nature of the PIR-B subtype, present findings reveal that PIR-A potentially acts as a stimulatory receptor in mast cells, suggesting a mechanism for regulation of mast cell function by the PIR family.

PIR-B (2, 3). mRNA expression for both subtypes has been detected in B cells, interleukin-3-induced bone marrow mast cells, and myelomonocytic lineage cells (2, 3). PIR is currently thought to be a murine receptor homologous to the human receptor ILT/LIR because of the similarity of their primary structures (3, 4), their expression patterns in immune cell types except for NK cells (5, 6), the polymorphic nature of their isotypes (4–7), and chromosomal locations (3, 8, 9). Recent studies have demonstrated inhibitory function and recognition for human major histocompatibility complex class I and virus-related major histocompatibility complex class I-like proteins by some isotypes of the ILT/LIR family, suggesting a regulatory function of ILT/LIR for immune responses in the context of major histocompatibility complex class I recognition as in the case of killer cell inhibitory receptor (6, 10). PIR-B was shown to function as an inhibitory receptor, whereas the functions of PIR-A and ligands of the entire PIR family remain unknown.

The main feature of PIR-B subtype is to harbor the conserved amino acid motifs in a cytoplasmic region denoted as immunoreceptor tyrosine-based inhibitory motif. Inhibitory function of PIR-B has been shown in splenic B cells (11), a B cell line (12), and a mast cell line (13), and the two immunoreceptor tyrosine-based inhibitory motifs of the PIR-B cytoplasmic region have been proven to affect inhibitory signaling by recruiting protein-tyrosine phosphatase, SHP-1 or SHP-2, which commonly functions as a signal transducer of immunoreceptor tyrosine-based inhibitory motif-based receptors including killer cell inhibitory receptor (14–17), Ly-49 (18), NKG2 (19), CD22 (20–23), and ILT/LIR (5, 6, 10). The inhibitory nature of PIR-B led us to postulate a role of PIR signaling in regulation of immune responses involving mast cells, B cells, and macrophages.

PIR-A is defined as a group of noninhibitory type of PIR family receptors characterized by a short cytoplasmic region that is free of any consensus amino acid sequence for activation. Instead, the transmembrane region of PIR-A harbors positively and negatively charged amino acid residues (see Fig. 7). Transmembrane-charged residues can typically be seen in stimulatory receptors mediating a variety of immune responses, such as T cell receptor, the ligand binding α chains of type I and type III Fc receptors for IgG (FcγRIa and FcγRIIa, respectively), FcγR, killer cell inhibitory receptor-2DS/3DS (alternatively called KAR), and NKR-P1 (CD161). All of these themselves have no amino acid motif for activation but associate with signaling subunits such as CD3 complex, γ and β chains (FcγR and FcγR, respectively) of type I FcR for IgE (FcεRI), and DAP12 (24–28) to generate an activation signal in response to receptor aggregation. Previous studies on T cell receptor α chain and FcεR have demonstrated the requirement of a positively charged amino acid residue in the transmem-
brane region for their function and subunit association (29, 30). The presence of charged amino acid residues in the transmembrane region of PIR-A suggests the possibility that PIR-A associates with activation subunits to deliver an activation signal into the cell. Our recent observations have suggested that one of the PIR-A isotypes, previously denoted by p91D, mediates the activation signal revealed by cytoplastic calcium mobilization and degranulation in mast cell line (13).

The present study focuses on the following two points. The first point is the mechanism of PIR-A function, and the second point is the evaluation of the role in PIR-A function of two charged amino acid residues present in the PIR-A transmembrane region. We have shown that the association of homodimeric FeRy chains and FeRβ enable PIR-A to generate an activation signal in a mast cell line and that the charged amino acid residues contribute to the subunit association and stimulatory function of PIR-A.

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—A rat cell line, RBL-2H3, was obtained from the Japanese Cancer Research Resources Bank (JCRB, Tokyo). This cell line was grown in a medium to undergo a transformation to a mast cell line. We used mouse FcR, 2.4G2, PharMingen, San Diego, CA) prepared with DEAE-cellulose column chromatography from supernatant of 100 g of spleen RNA of 129/SvJ mouse by polymerase chain reaction (PCR) using a primer pair of PAF-1 and PAR-1: PAF-1, 5'-TTCTAGCCACTCAGGCTT-3' (antisense primer) and PAR-1, 5'-GGGGCCCCACACAATGCAGAATCTC-3' (sense primer). The replaced bases are underlined. The mutation of AEQ2 was introduced into the cell. Our recent observations have suggested that one of the PIR-A isotypes, previously denoted by p91D, mediates the association of homodimeric FeRy chains and FeRβ enable PIR-A to generate an activation signal in a mast cell line and that the charged amino acid residues contribute to the subunit association and stimulatory function of PIR-A.

**DNA Constructions and Vectors**—Mouse FcRRII, FcRRIII (donated by Dr. J. Y. Ravetch, The Rockefeller University, New York, NY) and mouse FeRβ (donated by Dr. T. Kurasaki, Kansai Medical University, Osaka, Japan) in pEXV-3 vector and mouse FeRβ in pSV1 vector were used for the stable and transient expression studies. The CDNA fragment coding for transmembrane and cytoplasmic regions of PIR-A was prepared from spleen RNA of 129/SvJ mouse by polymerase chain reaction (PCR) using a primer pair of PAF-1 and PAR-1: PAF-1, 5'-GATGGGACATGGAGAATCTCATCATGATG-3' (sense primer) and PAR-1, 5'-AAGGGGCCCAATGCAGGCTTTTTCAAGGCCG-3' (antisense primer). The fragment ratio, in which the nucleotide sequence corresponding to PIR-A cDNA matched that previously reported as p91B (available from EMBL/GenBank/DBJ under accession number AF041035; Ref. 3), was digested with Apol and then ligated into the Aphi restriction site of mouse FeRRII cDNA, which locates in the transmembrane, in the sense orientation. Mutations corresponding to ARM and AEQ1 (see Fig. 1A) were generated by PCR as well using Paf-2 and Paf-3, respectively, instead of PAF-1: PAF-2, 5'-GGGCCCACCAATGGAGAATCTCATCATGATG-3' (sense primer) and PAF-3, 5'-GGGCCCACCAATGGAGAATCTCATGATG-3' (antisense primer) were used for the stable and transient expression studies. The CDNA fragment coding for transmembrane and cytoplasmic regions of PIR-A was prepared from spleen RNA of 129/SvJ mouse by polymerase chain reaction (PCR) using a primer pair of PAF-1 and PAR-1: PAF-1, 5'-TTCTAGCCACTCAGGCTT-3' (sense primer) and PAR-1, 5'-TGGAATGCTGGAGTGGC-3' (antisense primer). The replaced bases are underlined. The primer overlap each other the surrounding residue to be changed. The first round of PCR with PAF-1 plus PAR-4 and PAF-4 plus PAR-1 generated two mutant fragments that were subsequently connected by the second round of PCR with PAF-1 and PAR-1.

**Transient Assay for Membrane Expression**—20 μg of linearized DNA construct plus 1 μg of linearized pSV2-Neo vector were transfected into 5 × 10⁶ of RBL-2H3 cells by electroporation with single pulse conditions of 250 V and 975 μF (Gene Pulser II, Bio-Rad). The selection and cloning for neoycin-resistant cells were performed for 2 weeks in the presence of 100 μg/ml geneticin (Life Technologies, Inc.). For transient expression of the receptor of interest, 3 μg for a single construct or a total of 6 μg for two constructs were transfected into approximately 2 × 10⁹ COS-7 cells by a procedure with DEAE-dextran. In short, cells were incubated with DNA and DEAE-dextran (0.4 mg/ml) in serum-free Dulbecco’s modified Eagle’s medium buffered with 50 mM Tris-HCl, pH 7.4, at 37 °C for a few hours and then supplemented with 0.1 mM chloroquine (Sigma) for 3 h in serum-free Dulbecco’s modified Eagle’s medium. Cells were harvested at 48 h after transfection. Membrane expression of the receptor of interest was monitored for live cells with flow cytometric apparatus (FACSCalibur®, Becton Dickinson, San Jose, CA) by immunostaining of R-phycoerythrin-conjugated anti-FcR, biotinylated anti-FcR, anti-TNP IgG, or biotinylated anti-TNP IgE at the indicated antibody concentration for 15 min. After unbound antibodies were washed out, the receptor of interest was aggregated by 5 μg/ml of F(ab)² fragments of goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA), 5 μg/ml of streptavidin (Sigma), or 5 μg/ml of TNP-conjugated ovalbumin (Sigma) for 30 min. The percentage serotonin release (% degranulation) was calculated using the following formula: % degranulation = (cpm of supernatant)/(cpm of supernatant + cpm of cells) × 100, where cpm of cells is represented by the counts/minute in cells disrupted with 1% Nonidet P-40 plus 1% SDS solution.

**Measurement of Cytoplasmic Calcium Mobilization**—Exponentially growing 10⁶ cells in 1 ml of culture medium were labeled with 3.3 μM of fura-2 AM (Molecular Probes, Eugene, OR) for 30 min at 35 °C and then sensitized with 1 μg/ml of biotinylated aFcR or 1 μg/ml of biotinylated mouse IgE for 10 min at 25 °C. After unbound antibody was washed out, cells in 2 ml of phosphate-buffered saline supplemented with 1 mM CaCl₂ and 1 mM MgCl₂ were stimulated with 10 μg of streptavidin while agitating gently. Cytoplasmic calcium mobilization was monitored at 510 nm emission wavelength excited by 340 and 360 nm with a fluorescence spectrophotometer (Hitachi model F-4500, Hitachi Ltd., Tokyo). Calculation and calculation of calcium concentration were performed as described (36).

**Immunoprecipitation and Immunoblot Analysis**—RBL-2H3 transfected with the cDNA constructs (107) or COS-7 transfected (10⁶) were lysed in 3 or 1 ml, respectively, of digitonin-lysis buffer, pH 7.8, supplemented with 1% digitonin (Wako Pure Chemicals, Osaka, Japan), 13.6 mM triethanolamine, 150 mM NaCl, 1 mM EDTA, 10 mM iodoacetamide (Sigma), 5 μg/ml aprotinin, and 5 μg/ml leupeptin (Sigma). For immunoprecipitation of FeR by antigen stimulation, cells (10⁶) were lysed with RIPA buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 100 μg/ml genotype. For some experiments, cells were stimulated with immunocomplex made of 50 μg of anti-TNP IgG1 plus 2.5 μg of TNP-OVA/1 ml of medium before cell lysis. Cleared supernatants of cell lysates was used for immunoprecipitation with 50 μg of 2.4G2 conjugated to Sepharose 4B beads (Amersham Pharmacia Bio Tech) by 2 mg/ml bead volume. Immunoadsorbed beads were washed four times with lysis buffer, and then immunoprecipitates were denatured at 95 °C for 5 min in the presence or absence of 5% mercaptoethanol to generate reduced or nonreduced sample, respectively. Samples were separated with SDS-polyacrylamide gel electrophoresis (16.5%) and transferred onto a polyvinylidene difluoride (Millipore, Bedford, MA) membrane. For the tyrosine phosphorylation in total cellular proteins, the membrane was exposed to antiphosphotyrosine antibody, peroxidase-linked sheep anti-mouse Ig (Amersham Pharmacia Biotech), rabbit anti-phosphotyrosine antibody, peroxidase-linked donkey anti-rabbit Ig or rabbit anti-phosphotyrosine antibody, peroxidase-linked donkey anti-rabbit Ig (Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. After the primary antibody was washed out, the receptor of interest was aggregated by 5 μg/ml of aFcR or 1 μg/ml of biotinylated mouse IgE for 10 min at 25 °C. After unbound antibody was washed out, the receptor of interest was aggregated by 5 μg/ml of aFcR or 1 μg/ml of biotinylated mouse IgE for 10 min at 25 °C. After unbound antibody was washed out, the receptor of interest was aggregated by 5 μg/ml of aFcR or 1 μg/ml of biotinylated mouse IgE for 10 min at 25 °C. After unbound antibody was washed out, the receptor of interest was aggregated by 5 μg/ml of aFcR or 1 μg/ml of biotinylated mouse IgE for 10 min at 25 °C.
RESULTS

Transmembrane and Cytoplasmic Regions of PIR-A Sufficiently Function for Triggering Cellular Activation in RBL-2H3—To analyze PIR-A function in the rat mast cell line, RBL-2H3, we took advantage of the chimeric receptor consisting of the extracellular region of mouse FcγRIIB and the C-terminal portion of PIR-A encompassing the transmembrane to cytoplasmic regions, denoted by FcγRII-PIR-A (Fig. 1A). This portion is highly conserved in amino acid level among presently identified PIR-A isotypes and expresses a striking difference from the corresponding portion of PIR-B. According to the designation of PIR isotypes by Kubagawa et al. (2), the isotype of PIR-A used in this study matches PIR-A6 except for one amino acid mismatch at the second serine residue in the cytoplasmic region. The strategy of chimeric receptor enables us to perform functional analyses of PIR-A-derived signaling without the ligand or antibody to PIR, both of which are not available presently, and to analyze biochemical changes upon receptor aggregation in comparison with the established positive (mouse FcγRIII) and negative (mouse FcγRIIB) control using the same monoclonal antibody (2.4G2, denoted as αFcR in this report).

We successfully isolated stable clones of RBL-2H3 expressing the chimeric receptor, mouse FcγRIIB or FcγRIII, by cell surface immunostaining with αFcR (Fig. 1B). Immunostainings with isotype-matched control antibody or untransfected
cells assure the specificity of 2.4G2 staining for RBL-2H3 cells. The effects of receptor aggregation on cellular activation were evaluated with degranulation revealed by serotonin release (Fig. 1C), cytoplasmic calcium mobilization (Fig. 1D), and activation-induced tyrosine phosphorylation of total cellular proteins (Fig. 1E). Every mode of aggregation of FcγRII-PIR-A receptor using α-FCR induced degranulation to an extent comparable with that of FcγRIII, which is a well characterized stimulatory receptor in mast cells. Comparable induction of degranulation by intact and Fab(‘)γ fragment of α-FCR ruled out any additive stimulatory effects from recognition of α-FCR-bearing Fc portion (rat IgG2b) by unknown receptor on RBL-2H3 cells. No degranulation was detected in wild-type RBL and FcγRIIB clone in response to α-FCR stimulation, eliminating any possibility for nonspecific stimulatory effects of the reagents and methods on cellular activation. To quantify the earlier traits for cellular activation induced by the aggregation of transfected receptors, we observed the time-dependent kinetics of cytoplasmic calcium mobilization and tyrosine phosphorylation of total cellular proteins after the saturated stimulation. FcγRII-PIR-A aggregation elicited calcium mobilization in a manner essentially similar to that of FcγRIII in respect of the rapid increment comparable with that with FcεRI, slow but substantial retraction, and level of calcium concentration (Fig. 1D). FcγRII-PIR-A aggregation also induced tyrosine phosphorylation in total cellular proteins as revealed by anti-phospho-tyrosine blot (Fig. 1E). Proteins migrating around 150, 100, 70, and 30–40 kDa were extensively phosphorylated in response to FcγRII-PIR-A aggregation. This induced pattern was not grossly different from those by FcγRIII and FcεRII aggregation. These results consistently support that the PIR-A moiety corresponding to transmembrane and cytoplasmic region is sensitive to aggregation and capable of generating an activation signal in the manner similar to FcγRIII.

PIR-A Constitutively Associates with Homodimeric FcγR and an FcγRβ in RBL-2H3—PIR-A in itself is free of any known amino acid motifs for activation in its cytoplasmic region. Then the question arises as to how PIR-A generated the activation signal. The similarity of biochemical traits in activation and the conservation of charged amino acid residues in the transmembrane region (see Fig. 7) over PIR-A and FcγRIIIs led us to the hypothesis of the similar or shared receptor composition between these receptors in mast cells. To identify subunits constitutively associating with FcγRII-PIR-A, digitonin-treated cell extracts from untreated cells were subjected to immunoprecipitation with α-FCR or control antibodies. The samples in part were prepared both under reduced and nonreduced conditions. Immunoblots following the immunoprecipitations (Fig. 2) clearly revealed the reduced and nonreduced FcγR subunits at 8 kDa and mainly 16 kDa, respectively, and the reduced FcγRβ subunit at 30 kDa for FcγRII-PIR-A and FcγRII preparations. The nonreduced FcγRβ was detected at 30 kDa in the same samples (data not shown). Other signals near 16 kDa for FcγR under nonreducing conditions previously have been observed as well (26, 38), assumed to be FcγR with unknown modification. The positive reference samples of RBL-2H3 whole cell extract and the immunoprecipitation with anti-FcγR and anti-FcγRβ antibodies followed these signals as expected. No signal was detected in anti-FcγR immunoprecipitates from wild-type RBL-2H3 and FcγRIIB-expressing cells as well as a negative reference sample of COS-7 whole cell extract (Fig. 2). These results confirmed the fact shown by the recent studies in which FcγRβ associates with PIR-A (39, 40) and is also showing the new finding that FcγRβ is in the PIR-A complex, indicating that FcγRII-PIR-A receptor associates with homodimeric FcγR and single FcγRβ in RBL-2H3 to trigger the downstream events shared with FcεRI and FcγRIII in mast cells.

PIR-A Does Not Require FcγRγ or FcγRβ for Its Membrane Expression in COS-7 Cells—Because the previous study has demonstrated the necessity of FcγRγ for FcγRIIIα expression on the cell surface (41, 42), we questioned if PIR-A required FcγRγ or FcγRβ for its expression on the cell surface as well. Transient expressions of FcγRII-PIR-A, FcγRIIB, and FcγRIIIα with or without subunit are examined in COS-7 cells, in which endogenous expressions of FcγR and FcγRβ are not detected (Fig. 2). Membrane expression of FcγRII-PIR-A was detected regardless of co-expression of subunit, followed by the pattern of FcγRIIB whose expression on cell membrane is known to be independent of subunit expression (Fig. 3A). In striking contrast, membrane expression of FcγRIIIα was dependent upon co-expression of FcγR as shown previously. Utilizing the Fc binding property of extracellular regions of FcγRII-PIR-A and FcγRIII, membrane expressions of these two receptors were examined by rosetting formation with mouse IgG1-opsonized sheep red blood cells. Consistent with the data from α-FCR detection, the transfectants expressing FcγRII-PIR-A and expressing FcγRII-PIR-A plus FcγR displayed rosetting for the opsonized sheep red blood cells to a similar extent of that expressing FcγRIIIα plus FcγR (data not shown), suggesting topographically normal expression of FcγRII-PIR-A in the absence of FcγR. These results indicate that PIR-A expresses a different requirement of FcγRγ or FcγRβ for its membrane expression from FcγRIIIα in COS-7 cells. Then a question arises as to whether or not intrinsic subunits in COS-7 allowed FcγRII-PIR-A expression in place of FcγR. To answer this question in part, we attempted to detect any asso-

![Fig. 2. Association of FcγR and FcγRβ with FcγRII-PIR-A in RBL-2H3 cells.](Image 335x447 to 527x729)
brane expression of the receptor on COS-7 cells in the presence of FcR
at positions 626 and 643 (Arg626 and Glu643), respectively, in
possesses both conserved charged residues in the transmem-
portion of the transmembrane region, suggesting a role of the
secondary common feature that a negatively charged residue
lysine, methionine, or glutamine, respectively. These mutant
dna constructs and related products (proein and transfect-
tant) are denoted by AEQ1, ARM, or AEQ2, respectively (Fig.
A). All of the mutant constructs were successfully expressed
on RBL-2H3 cells as well as the prototype FcyRII-PIR-A (Fig.
B). Degranulation and calcium mobilization assay were per-
formed to examine capacity for signal transduction of mutant
receptors (Fig. 4, D and E). ARM mutation was found to totally
remove the capacity for signal transduction from FcyRII-PIR-A
receptor, and the unresponsiveness of the ARM clones could be
reconfirmed with the other clones independently isolated (Fig.
E). On the other hand, AEQ1 and AEQ2 mutation conserved
PIR-A function, although delayed calcium response was ob-
served in AEQ2 clones (Fig. 4D). These results indicate that Arg626 is necessary and Glu622 and Glu643 is not critical for
PIR-A function.

Both Arg626 and Glu643 in PIR-A Transmembrane Play an
Important Role in Subunit Association with PIR-A—We exam-
ned whether the functional alteration by single mutation could
be attributed to the difference in capacity of the mutant recep-
tor to bind to subunit. Digitonin lysates from untreated cells
were used for immunoprecipitation with the saturating amount
of aFcR antibody followed by immunoblot with anti-FcRy or
anti-FcRb antibody (Fig. 5A). ARM and AEQ2 mutation were
found to attenuate the association of both FcRy and FcRb to the
mutant receptors, although a small amount of FcRy and FcRb
was still found to be associated. By densitometric analysis, the
amount of subunits associating with ARM and AEQ2 mutant
receptors was estimated to be 8 and 23% for FcRy, respectively,
and 7 and 16% for FcRb, respectively, of that to wild-type
FcyRII-PIR-A receptor. The mutation of AEQ1 did not signifi-
cantly perturb the association of the subunits. The difference
of subunit association in quantity did not reflect the variance of
expression of subunits among the clones analyzed (Fig. 5, blots
for whole cell lysates). Decrease of subunit association by ARM
and AEQ2 mutations was supported in COS-7 cells co-express-
ing mutant receptors and FcR (Fig. 6). These results indicate that
Arg626 and Glu643 in the PIR-A transmembrane region are
respectively important for subunit association with PIR-A.

To clarify a mechanism for signal transduction by AEQ2
receptor that substantially loses the capacity for subunit asso-
ciation, we examined whether FcRy was involved in AEQ2-
derived signal transduction. By taking advantage of Ig Fc bind-
cing capacity of extracellular region of chimeric receptors used
in this study, cells were stimulated with mouse IgG1-containing
immune complex; subsequently FcRy was immunoprecipitated
and examined by tyrosine phosphorylation by anti-phos-
phorysine blot. FcRy was shown to be phosphorylated in
consequence of AEQ2 receptor aggregation as well as FcyRII-
PIR-A and AEQ1 receptor (Fig. 6A), indicating the involvement
of FcRy in AEQ2-derived signal transduction. Then cells were
stimulated with IgG1-containing immune complex as well as
above, subsequently chimeric receptors were immunoprecipitat-
ted, and FcRy and phosphorylated FcRy were respectively
detected by anti-FcRy and anti-phosphotyrosine antibodies
(Fig. 6B). The results indicate that phosphorylation of FcRy
indeed takes place in the fraction associated with AEQ2 recep-
tor but that the amount of FcRy associated with AEQ2 receptor
remains unchanged after stimulation, suggesting the mecha-
nism by which the minor fraction of AEQ2-subunit complex
sufficiently elicits signal transduction.
cell-derived inflammatory cytokines (48). These allergic manifestations can presently be attributed, at least in part, to the result of up-regulation of signals by FcεRI and/or FcγRIII in mast cells. The present findings lead to the tempting possibility that PIR-A aggregation exerts an additive effect on the signal by FcRs and, consequently, that PIR-A functions as an accelerator in developing mast cell-related pathological manifestations.

ILT/LIRs are thought to be the human homologue of PIR, and its mRNA expression in human lung mast cells has been reported by Arm et al. (9). The transmembranes of their non-inhibitory types, ILT1/LIR7, and PIR-A express strikingly conserved primary structures (Fig. 7A), suggesting that noninhibitory types ILT1/LIR7 associate with FcεRI and FcγRII. In fact, FeR association with ILT1 has been reported very recently (49). Thus, the insight from our findings may be allowed to extend to human physiology.

We have shown that PIR-A potentially acts as a stimulatory receptor, and its function relates to the association of FeRγ and FeRβ subunits in RBL-2H3 cells. Not all cell types bearing...
PIR-A express FcRβ subunits, i.e., monocytes and granulocytes. As in the case of Fc receptors (50, 51), FcRβ may not be necessary but may act as an accelerator for signal transduction. The role of FcRβ in PIR-A-derived signal transduction should be addressed by further investigation. The mRNA for PIR-A and PIR-B were also detected in mature B cells that are known to express neither FcRγ nor FcRβ. Based on current information, PIR-A cannot exert any stimulatory function, so that PIR-B has a dominant function over PIR-A in mature B cells. To further understand the mechanism of positive and negative regulations by PIR-A and PIR-B receptors, we also examined whether PIR-A requires subunits for its membrane expression. In contrast to FcγRIII, FcγRII-PIR-A did not require FcRγ for its membrane expression in COS-7 cells as well as human FcR (26, 30). Our results for PIR-A expression using COS-7 cells are similar to the results in transfected 293T cells (39) but different from the results in transfected LTK fibroblasts or splenocytes from FcRγ deficient mice (40). Because the two cell lines permissive to expression of PIR-A in the absence of FcRγ were those transformed with the gene encoding SV40 large T antigen, a high level of PIR-A translation could cause redundant accumulation of the receptor protein in these cells, resulting in membrane expression without FcRγ association. It is also possible that the FcRγ requirement for PIR-A expression might differ by cell type, although the mature cell population present in the spleen requires FcRγ for PIR-A expression (40). In this sense, the physiological requirement of FcRγ for PIR-A membrane expression still needs to be investigated using a highly sorted cell species.

The results from mutation analyses on FcγRII-PIR-A demonstrate the role of transmembrane-charged amino acids of PIR-A, Arg626 and Glu643, in subunit association and PIR-A-mediated signal transduction. Charged amino acids of the transmembrane region are commonly found in stimulatory receptors (Fig. 7). The hydrophobic nature of a helix structure is thought to be a basic requirement for membrane integration by the transmembrane region (52). Accordingly, the presence of charged amino acids is unfavorable for stable membrane expression. However, membrane expression of these stimulatory receptors is presently rationalized by association of a subunit bearing counter-charged transmembrane region to achieve hydrophobicity by neutralizing transmembrane charges. As
shown Fig. 7B, FeγR distributes two charged amino acids, aspartic acid and arginine, in the transmembrane region at seemingly parallel positions to those of PIR-A with the opposite charges. Our results indicate that both Arg626 and Glu643 of PIR-A each have an effect on the binding affinity of FeγR and Feγβ to PIR-A, supporting the existence of a mechanism for subunit assembly and specificity based on electrostatic protein interaction at a membrane site. We unexpectedly observed that the requirement of Arg626 and Glu643 for PIR-A-derived signal transduction does not parallel that for subunit association. The loss of the negative charge of Glu643 does result in a decrease of subunit association but does not affect the capacity for FeγR phosphorylation and its downstream PIR-A-mediated function. These findings brought us to assume the following two mechanisms for AEQ2-derived signal transduction. The first was that an increase in subunit association with AEQ2 receptor took place along with receptor aggregation, and the second was that efficient phosphorylation of FeγR was undertaken by the minor fraction of AEQ2 receptor where the subunit association was resistant to mutation. Stimulation of AEQ2 receptor was found to induce FeγR phosphorylation in both total and AEQ2-associated FeγR fractions to the same extent as the intact receptor, despite the fact that the amount of subunit associated with AEQ2 receptor remained much smaller than the amount of intact receptor. These findings may support the latter mechanism mentioned above and suggest the presence of a functionally competent fraction of the receptor-subunit complex in the membrane. It is important to note that our discussions were based on experiments using detergent-soluble cell fractions. Recent findings have shown the importance of detergent-insoluble fractions in signal transduction for some receptors. We did not examine whether or not AEQ2 receptor functioned in detergent-insoluble fractions. Further investigation is therefore required to understand the mechanism of receptor function and its subunit association.

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