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Mutational Analysis of Immunoreceptor Tyrosine-Based Inhibition Motifs of the Ig-Like Transcript 2 (CD85j) Leukocyte Receptor

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The inhibitory receptor Ig-like transcript (ILT)2 (leukocyte Ig-like receptor or CD85j) is a type I transmembrane protein expressed by different leukocyte lineages. The extracellular region of ILT2 binds HLA class I molecules, and its cytoplasmic domain displays four immunoreceptor tyrosine-based inhibition motifs. Upon tyrosine phosphorylation ILT2 recruits the Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) that is involved in negative signaling. To address the structural basis of ILT2-mediated inhibitory signaling, deletion and single tyrosine mutants were generated and transfected in the COS-7 and rat basophilic leukemia cell lines; their abilities to bind SHP-1 and to inhibit FcR-induced serotonin release in rat basophilic leukemia cells were studied. Both biochemical and functional analyses revealed tyrosines 644 (SIYATL) and 614 (VTYAQL) as the SHP-1 docking sites required for ILT2 inhibitory function. Substitution of tyrosine 562 (VTYAEL) did not alter receptor function. By contrast, mutation of tyrosine 533 (NYLAAV) interfered with ILT2 tyrosine phosphorylation and the subsequent SHP-1 recruitment, thus supporting a regulatory role for this motif. The Journal of Immunology, 2002, 168: 3351–3359.

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atural killer cells have the ability to sense the integrity of MHC expression on target cells through different inhibitory receptors specific for classical and nonclassical MHC class I molecules. Specific recognition of class I molecules represses NK cell-mediated cytotoxicity triggered by activating receptors and prevents auto-reactivity against normal cells. Human receptors fall into two different protein families: CD94/NKG2 heterodimers are homologous to C-type lectins, and killer cell Ig-like receptors (KIRs) belong to the Ig superfamily (1). The molecular basis for the functional activity of NK inhibitory receptors is the presence of immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic tails (2). Protein alignment between ITIM-bearing receptors led to the definition of ITIM as the 6-aa consensus sequence I/L/VxYxxL/V, where x represents any residue (3). Upon receptor-ligand interaction ITIMs become tyrosine phosphorylated, recruiting and activating Src homology 2 domain homolog 2 (SH2)-containing phosphatases, which mediate the inhibitory signal.

ITIMs are also displayed by other surface molecules such as FcγRIB, CD22, and CD72 in B cells; MAFA in mast cells; CDw150 (SLAM), CD33, CD31, and others (4, 5). Among them, a group of inhibitory receptors named Ig-like transcripts (ILTs) or leukocyte Ig-like receptors has been described in humans (6, 7). ILTs belong to the Ig superfamily and are encoded by genes that cluster in chromosome 19p13.4 together with KIRs. This region of chromosome 19 is known as the leukocyte receptor complex (8). In contrast to CD94/NKG2 heterodimers and KIRs, ILTs expression is not restricted to NK and T cells, and these genes have a broader distribution in different leukocyte lineages (9).

ILT2/leukocyte Ig-like receptor 1 is a 110-kDa surface glycoprotein detected on the surface of NK and T cell subsets, B cells, dendritic cells, and monocytes (10). According to a recently proposed nomenclature, ILT2 is now termed CD85j (11). It has been shown that ILT2 is expressed primarily by memory T cells (12), although some authors propose that it is present at variable levels in most T cells (13). ILT2 includes four Ig-like C2 domains in the extracellular region and a cytoplasmic tail containing four ITIM-like sequences (6). This receptor has been found to interact through its amino-terminal Ig domain with several classical and nonclassical class I molecules (10, 14–16) and with UL18, a HCMV protein homologous to HLA class I (7). Upon ligand binding, ILT2 is able to inhibit cytotoxicity in NK and T cells; moreover, ligation of ILT2 inhibited BcR-dependent Ca2+ mobilization in B cells (10) and signaling triggered through the FcγRI (CD64) in monocytes; in fact, cross-linking of ILT2 with FcγR impaired tyrosine phosphorylation of the associated γ-chain and of Syk kinase, inhibiting Ca2+ mobilization (17). Upon treatment with the phosphatase inhibitor, sodium pervanadate ILT2 becomes tyrosine phosphorylated and associates with the SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (10). SHP-1 contains two SH2 domains, and its activity is negatively controlled through the interaction of the N-terminal SH2 domain with the catalytic domain of the phosphatase (18).

Despite the fact that several reports have addressed the structural basis of SHP-1 binding to ITIMs (19, 20), a number of questions regarding the mechanisms underlying inhibitory signaling still exist. In particular, little is known about the putative relevance of the different ITIM-like sequences for ILT2 function. To address this issue we generated different mutants of the ILT2 cytoplasmic tail and transfected them in the rat basophilic leukemia (RBL) and...
COS-7 cell lines. The ability of the different mutants to assay SHP-1 and to inhibit FcεR-induced serotonin secretion in RBL cells was analyzed. Our data revealed that the ILT2 C-terminal tyrosines 614 and 644 are central sites for binding to SHP-1. Mutation of residue Y562 had no effect, whereas residue Y533 appears involved in the regulation of receptor tyrosine phosphorylation.

Materials and Methods

Cells and Abs

The RBL cell line was grown in RPMI 1640 with 10% heat-inactivated FCS. Stable transfectants were generated as previously described (21). Clones were obtained by culture under limiting dilution, selected on the basis of flow cytometry analysis, and maintained in the presence of 1 mg/ml G418. Polyclonal T cells were obtained by coculturing nonadherent PBMC with irradiated lymphoblastoid 721.221 cells. Cell populations were used at 12–14 days in culture. Anti-ILT2 HP-F1 mAb has been previously described (10). Anti-phosphotyrosine mAb 4G10 and rabbit polyclonal anti-SHP-1 were purchased from Upstate Biotechnology (Lake Placid, NY). Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Pierce (Rockford, IL). Sheep anti-mouse IgG was purchased from Sigma-Aldrich (St. Louis, MO).

Flow cytometry

Flow cytometric analysis was performed on a FACScan (BD Biosciences, San Jose, CA). Indirect immunofluorescence staining was performed as previously described (21).

DNA reagents

Briefly, ILT2 cDNA subcloned into pCDNA3 (Invitrogen, Carlsbad, CA) was subjected to site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) to generate Y535F, Y614F, and Y644F mutants. Primers used to generate the Y535F mutant were 5′-GAGAAAACCTTTTGGCTGCTG-3′ and reverse, those for Y644F mutant were 5′-GGAGTGTGACCTTCGCCCAGCTGC-3′. The sequences were confirmed by automatic sequencer. Double mutants were made by restriction subcloning and conventional molecular biology techniques. The deletion mutant ILT2Δ2′.5′′ was generated by ILT2 cDNA digestion with EcoRI (at nt 1750) and XbaI restriction enzymes and religation after treatment with T4 DNA polymerase. ILT2Δ2′′ was made by plasmid digestion with SphI at nt 1548 and ligation with blunted XbaI. Mutants were transfected into BRL cells using DOTAP liposomal reagent (Roche, Indianapolis, IN) as previously described (21). Stable transfectants were selected in 1 mg/ml G418. Clones were obtained by culture under limiting dilution conditions and were selected by flow cytometric analysis. PVS-LVL-SH1 and SHP-1ΔD149A mutants were provided by Dr. Z.-H. Xie (National Institutes of Health, Bethesda, MD).

For expression in yeast, a cDNA sequence encoding human ILT2 cytoplasmic domain was amplified by PCR and cloned in the pGAD424 vector. Wild-type and two mutated cDNA sequences encoding for the two SH2 domains of human ILT2 were subcloned in the GAL4 DNA activation domain vector pGAD424 using the EcoRI/BglII sites. The reaction was stopped by addition of 150 mM sodium orthovanadate and 10 mM H2O2 at 37°C for 10 min. After stimulation, cells were lysed in buffer containing 1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM Na3VO4, and 1 mM NaF. For receptor cross-linking, cells were resuspended at 5×10^6 cells/ml and preincubated for 15 min at 4°C with 2 μg HP-F1 Fab′1, Fab′2 sheep anti-mouse (5 μg) was added before incubating at 37°C. Cells were lysed, and immunocomplexes were isolated with protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). Lysates from pectinate-treated cells were incubated at 4°C with HP-F1 mAb, followed by protein G-Sepharose. Precipitates were washed three times with lysis buffer, resolved by 8.5% SDS-PAGE under nonreducing conditions, and transferred onto nitrocellulose membranes.

Cell hatchenization and conjugation

For cell hatchenization, 221 cells (5.10^5) cells/ml were incubated for 15 min at 37°C in PBS containing 0.5 mM trinitrobenzenesulfonic acid (Sigma-Aldrich), washed twice in 50 ml PBS, and resuspended in RPMI 1640/10% FCS medium at 8×10^6 cells/ml. Twenty-five microliters (2×10^6) of hatchenized cells were cocultured with 25 μl (2×10^6) RBL cells for 1 min at 37°C, lysed, and immunoprecipitated as previously described. To measure serotonin release stimulation, 4×10^5 hatchenized cells were cocultured with 2×10^5 [3H]serotonin-loaded RBL cells in the presence of anti-TNP IgG for 1 h in round-bottom 96-well plates in a final volume of 50 μl. The reaction was stopped by addition of 150 μl cold medium. One hundred microliters were collected from each well, and serotonin release was determined as described above. All experiments were performed in triplicate.

Results

Ligand engagement of ILT2 induces tyrosine phosphorylation

Engagement of KIR and CD94/NKG2A receptors has been reported to be sufficient for activating their tyrosine phosphorylation (21, 23–25). By contrast, it has been proposed that other inhibitory receptors require coligation with activating receptors to recruit tyrosine kinases involved in ITIM phosphorylation (26, 27). We reported that peritoneal adjuvant-induced tyrosine phosphorylation of ILT2 and recruitment of SHP-1 phosphatase (10). To determine whether receptor engagement is sufficient to promote ILT2 tyrosine phosphorylation we cross-linked Fab′2 of the ILT2-specific mAb HP-F1 on polyclonal activated T cell populations or ILT2 transfected RBL cells (RBL/ILT2) (10). As shown in Fig. 1, ILT2 engagement was sufficient to trigger tyrosine phosphorylation, which was detectable within 1–2 min of stimulation in both cell systems. To further confirm these data, we assayed the effect of ILT2 interaction with its natural ligands. RBL/ILT2 cells were treated with 10 ng/ml TNP-IgE for 1 h and then cross-linked with 20 μl TNP-specific Abs. The reaction was stopped by addition of 150 μM cold medium; subsequently, 100 μl supernatant was collected from each well, and radioactivity was measured. Serotonin release was calculated as % serotonin release = (cpm sample – cpm spontaneous release)/(cpm total – cpm spontaneous release). For peritoneal treatment, receptor cross-linking, immunoprecipitation, and Western blotting

Pervanadate treatment, receptor cross-linking, immunoprecipitation, and Western blotting

For peritoneal treatment experiments, cells were incubated in RPMI 1640 containing freshly prepared sodium pervanadate (0.1 mM sodium orthovanadate and 10 mM H2O2) at 37°C for 10 min. After stimulation, cells were lysed in buffer containing 1% (v/v) Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM Na3VO4, and 1 mM NaF. Receptor cross-linking, cells were resuspended at 5×10^6 cells/ml and preincubated for 15 min at 4°C with 2 μg HP-F1 Fab′1, Fab′2 sheep anti-mouse (5 μg) was added before incubating at 37°C. Cells were lysed, and immunocomplexes were isolated with protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). Lysates from pectinate-treated cells were incubated at 4°C with HP-F1 mAb, followed by protein G-Sepharose. Precipitates were washed three times with lysis buffer, resolved by 8.5% SDS-PAGE under nonreducing conditions, and transferred onto nitrocellulose membranes.

Three-hybrid system assay

The three-hybrid assay was conducted by transforming sequentially the yeast strain CG1945 with pBRIDGE/Fyn(s25Y,315Y-F,176R-Q) containing ILT2 and then with the vector pGAD424 containing SHP-1, wild type or mutated. Transforms were plated in synthetic dropout medium supplemented with a -Trp, -Leu dropout. Clones were tested by β-galactosidase liquid culture assay using O-nitrophenyl β-D-galactopyranoside as a substrate as previously described (22).
were incubated in the presence of 721.221 or 721.221 cells expressing either HLA-B*2705 (.221.B2705) or HLA-E (.221.AEH) (16). Interaction between ILT2+ cells and HLA+ cells was sufficient to induce receptor tyrosine phosphorylation and SHP-1 recruitment (Fig. 2B).

Next we studied whether HLA interaction with ILT2 was able to inhibit serotonin release triggered through FcεR. RBL.ILT2 cells were loaded with [3H]serotonin and incubated in the presence of either the lymphoblastoid cell line 721.221 or 721.221 cells expressing HLA-B*2705 (.221.B2705). Both cell lines were previously haptenized with TNP and incubated with anti-TNP IgE. As shown in Fig. 2A, FcεR-induced serotonin release was diminished when RBL.ILT2 cells were incubated with .221.B*2705 cells. When this assay was conducted in the presence of an anti-ILT2 mAb to block the interaction between ILT2 and HLA-B molecules, no inhibitory effect was detected (Fig. 2A). Taken together, these data support that ILT2-class I interaction is able to inhibit the signal triggered through FcεR in RBL cells, rendering this system appropriate to analyze the structural basis for ILT2 receptor function.

Tyrosines 614 and 644 are necessary for ILT2 inhibitory function

We have previously shown that coligation of ILT2 and FcεR inhibits serotonin secretion in RBL cells challenged with IgE (10). To elucidate the relative contributions of the different ITIM-like sequences in the delivery of the negative signal we first generated two ILT2 deletion mutants. Constructs encoding for a truncated version of ILT2 in which tyrosines 614 and 644 had been removed (ILT2.D2Y) as well as a receptor lacking the cytoplasmic tail (ILT2.Cyt; see Fig. 3D) were transfected into RBL cells. Fig. 3A shows surface expression of the different proteins in the RBL clones selected to perform the functional studies. To assay the ability of the receptor to inhibit serotonin secretion, ILT2 was coligated to FcεR using mouse IgE and F(ab\(^{\prime}\)) of HP-F1 mAb adsorbed onto plastic. As expected, the mutant lacking the whole cytoplasmic region (ILT2.Cyt) was unable to mediate inhibition of serotonin release (data not shown) or SHP-1 recruitment (Fig. 3C). The truncated protein in which the carboxyl-terminal tyrosines are missing (ILT2.D2Y) was phosphorylated in cells treated with pervanadate (Fig. 3B, upper panel), but it did not coprecipitate SHP-1 (Fig. 3B, lower panel) or inhibit serotonin secretion when ILT2.D2Y was cross-linked to FcεR (Fig. 3C). It is noteworthy that the lower molecular band detected in transfectants by Western blotting, which predominates in the wild-type ILT2 (ILT2wt) immunoprecipitates, was not phosphorylated after pervanadate treatment (Fig. 3B).

It has been reported that binding to both SH2 domains in SHP-1 is necessary to fully activate phosphatase activity (18). According to data previously reported for KIR, a hydrophobic residue in position -2 relative to the tyrosine in the ITIM is required to bind to SHP-1 SH2 domains (19, 20). Only tyrosine residues 562 and 614 of ILT2 fulfill this requirement. We reasoned that even if the missing Y614 in the truncated protein ILT2.D2Y could account for the lack of interaction with SHP-1, it was still possible that residue Y562 was also involved in the recruitment of SHP-1. To test this hypothesis we mutated Y562 to phenylalanine and expressed it in RBL cells (Fig. 4A). ILT2.Y562F mutant was able to inhibit serotonin release in response to IgE almost as efficiently as the wild-type receptor (Fig. 4B) and to recruit SHP-1 phosphatase upon receptor phosphorylation (Fig. 4C). According to these data, Y562 does not appear to be essential to deliver the inhibitory signal.
Thereafter we generated single mutants in which tyrosine 614, 644, or both were mutated to phenylalanine and individually transfected them into RBL cells. During the selection process of stable transfectants we did not find any clone whose ILT2 surface expression was comparable to that of RBL.ILT2wt. Therefore, we selected a new RBL.ILT2wt clone whose expression levels were similar to those of RBL.ILT2Y614F, RBL.ILT2Y644F, and RBL.ILT2.Y614, 644F cell lines. This clone, referred to as RBL.ILT2wtL, was used in the functional studies for comparative purposes (Fig. 4A). Ligation of the ILT2.Y614F single mutant on RBL cells was able to partially inhibit serotonin release in response to IgE (Fig. 4B). Accordingly, SHP-1 was recruited to the receptor upon tyrosine phosphorylation (Fig. 4C, middle panels). In contrast, the ILT2.Y644F mutant had markedly reduced its ability to inhibit degranulation (Fig. 4B), although its ability to recruit the phosphatase was not lost (Fig. 4C). The double mutant ILT2.Y614, 644F appeared inactive (Fig. 4B), further supporting that the loss of function of the ILT2.Δ2Y truncated protein is due to the lack of residues Y614 and Y644.

ILT2 tyrosines 614 and 644 are sufficient to recruit SHP-1 to the phosphorylated receptor

To circumvent the variability of ILT2 expression in different RBL clones and to estimate the relative contribution of each tyrosine to the recruitment of the phosphatase, we performed transient transfection experiments in COS-7 cells. The Src kinase Lck has been reported to catalyze tyrosine phosphorylation of KIR ITIMs (24), suggesting that members of this family of kinases would be able to phosphorylate ILT2 cytoplasmic tyrosine residues. Thus, we tested in transient transfection experiments in COS-7 cells whether either Lck or Fyn was able to phosphorylate the receptor, promoting SHP-1 recruitment. To that purpose we used a trapping mutant of SHP-1 in which D419 has been changed to A (SHP-1/D419A). This mutation within the catalytic domain still allows the phosphatase to bind to its substrate, but impairs the reaction, thus stabilizing phosphatase binding to the substrate (28). Cotransfection experiments in COS-7 cells showed that both Src kinases were able to phosphorylate the receptor and promote SHP-1 recruitment (Fig. 5), yet Fyn appeared to be slightly more efficient in several experiments performed (not shown).

In a similar set of cotransfection experiments in COS-7 cells, single mutants (ILT2.Y533F, Y562F, Y614F, and Y644F) recruited SHP-1 upon phosphorylation by Fyn (Fig. 6, A and B). The double mutant ILT2.Y533, 562F also bound SHP-1 upon tyrosine phosphorylation. By contrast, the double mutant ILT2.Y614, 644F did not coprecipitate SHP-1 when cotransfected with Fyn (Fig. 6C) even when the remaining tyrosines were phosphorylated (not shown). These results support the data obtained in RBL cells indicating that tyrosines 614 and 644 play a major role in SHP-1 recruitment and activation.

A single SH2 domain is insufficient for the recruitment of SHP-1 to the ILT2 cytoplasmic tail

To determine whether both SH2 domains of SHP-1 are involved in the interaction with the ILT2 cytoplasmic tail, mutations that eliminate phosphotyrosine binding (R30Q and R136Q) (29) were introduced into the individual SH2 domains. Wild-type SHP-1 SH2 domains or single mutants were assayed in a yeast
three-hybrid system for binding to the ILT2wt cytoplasmic tail. Fyn kinase was introduced into the yeast system to induce phosphorylation of ILT2 (22). As a result of bait and prey protein interaction, β-galactosidase gene transcription was activated, and the enzyme activity was detected. As shown in Fig. 7, wild-type SH2 domains were able to bind to ILT2, but this interaction was completely abrogated when single mutations were introduced into SHP-1 SH2 domains. Therefore, at least in the absence of the catalytic and C-terminal domains, both SH2 domains of SHP-1 appear to be required for interaction with the ILT2 cytoplasmic tail.

A single Y533F mutation was introduced in the ILT2 cytoplasmic tail, and the cDNA was transfected into RBL cells. Single clones expressing ILT2.Y533F were tested in a serotonin release assay for the ability of ILT2 to inhibit the signal mediated through FceR (Fig. 8, A and B). In none of the tested clones was the ILT2.Y533F mutant able to inhibit serotonin release stimulated by IgE (Fig. 8B shows results obtained with a representative clone). Moreover, in cells treated with sodium pervanadate, no phosphorylation of the receptor was detected by Western blotting (data not shown). In

**FIGURE 4.** Tyrosines 614 and 644 are required for delivery of the negative signal. A, Analysis by flow cytometry of ILT2 expression in ILT2wt, ILT2Y562F, ILT2Y614F, ILT2Y644F, and ILT2Y614F, Y644F transfectants. B, Inhibition of IgE-induced serotonin release in RBL.ILT2 mutants. Cells were loaded with [3H]serotonin and stimulated with purified mouse IgE (20 µg/ml) alone or in combination with HP-F1 (20 µg/ml F(ab')2) immobilized on plastic. The percentage of serotonin release was determined after 1 h at 37°C. The percentage of inhibition was calculated as compared with serotonin release triggered by IgE in the presence of F(ab')2 of HP-3B1 mAb, which was used as a control. C, Western blot analysis of tyrosine phosphorylation and SHP-1 recruitment of pervanadate-stimulated ILT2 tyrosine mutants.

**Mutation of Y533 impairs receptor phosphorylation**

A single Y533F mutation was introduced in the ILT2 cytoplasmic tail, and the cDNA was transfected into RBL cells. Single clones expressing ILT2.Y533F were tested in a serotonin release assay for the ability of ILT2 to inhibit the signal mediated through FceR (Fig. 8, A and B). In none of the tested clones was the ILT2.Y533F mutant able to inhibit serotonin release stimulated by IgE (Fig. 8B shows results obtained with a representative clone). Moreover, in cells treated with sodium pervanadate, no phosphorylation of the receptor was detected by Western blotting (data not shown). In
accordance with these data, the double mutant ILT2.Y533, 562F (Fig. 8A) was not able to inhibit degranulation of RBL cells (Fig. 8B). Recently, it has been shown that a specific sequence of events is required for efficient phosphorylation of some receptors (30, 31). Accordingly, it is possible that P-Y533 may be required to activate

**FIGURE 5.** Lck and Fyn are able to phosphorylate ILT2 in COS-7 cells. COS-7 cells were transfected with the expression plasmids indicated in the figure. Forty-eight hours post-transfection cells were collected, and ILT2 expression was assessed by flow cytometry (data not shown). ILT2 was immunoprecipitated from cell lysates. Tyrosine phosphorylation, SHP-1 recruitment, and ILT2 precipitation were analyzed by sequential immunoblotting with the respective Abs. Western blot was also performed in total lysates (WCL) to assess comparable transfection of SHP-1.

**FIGURE 6.** ILT2 tyrosines 614 and 644 are sufficient to recruit SHP-1. ILT2 mutants were coexpressed in COS-7 cells with SHP-1/D419A in the presence or the absence of Fyn tyrosine kinase. Forty-eight hours post-transfection cells were collected, and ILT2 expression was analyzed by flow cytometry (data not shown). ILT2 was immunoprecipitated from cell lysates, and immunoblotting was performed to test for SHP-1 recruitment. Western blot was also performed in crude lysates to assess comparable transfection of SHP-1.

**FIGURE 7.** The integrity of both SH2 domains is required for SHP-1 interaction with the ILT2 cytoplasmic tail. Liquid culture assay using O-nitrophenyl β-n-galactopyranoside as substrate to measure β-galactosidase activity was used to score the interaction of ILT2 with SHP-1, SHP-1_30R-Q, and SHP-1_136R-Q. For each construct at least three independent colonies were tested in the galactosidase assay.
Src kinases to further phosphorylate the cytoplasmic tail of ILT2. To test this hypothesis, we expressed either ILT2wt or the Y533F mutant in COS-7 cells in the presence of decreasing amounts of Fyn. As shown in Fig. 8C, the wild-type receptor appears more efficiently phosphorylated than the Y533F mutant. In the presence of comparable levels of Fyn, a more efficient phosphorylation of ILT2wt was observed. After densitometry and correction for the total amount of ILT2 in the immunoprecipitates, phosphorylation of wild-type protein was 6-fold that of the 533F mutant at the highest concentration of Fyn and about 12-fold at the lowest concentration (data not shown). These data support the hypothesis that tyrosine residue 533 may be involved in further activation of Src kinases (Fyn) and subsequent tyrosine phosphorylation of ILT2.

Discussion
In this study we have approached the molecular basis of ILT2 inhibitory function mediated through SHP-1 recruitment. First, we analyzed whether engagement of ILT2 was sufficient to promote receptor tyrosine phosphorylation and subsequent SHP-1 recruitment. Our results in transfected RBL cells and peripheral blood T lymphocytes show that ligation of ILT2 with a specific mAb was sufficient to initiate the events leading to receptor phosphorylation. Moreover, we observed that interaction between ILT2 expressed in the heterologous RBL cells and HLA class I-expressing cells promoted ILT2 phosphorylation (Fig. 2), strongly supporting the idea that coligation with activating receptors is not necessarily required for ILT2 function. Certainly, the possibility that tyrosine kinase activation via ITAM-bearing molecules may also promote ILT2 function is not excluded. Our data are in line with previous observations on inhibitory signaling delivery by CD94/NKG2 and KIR (21, 23–25). In contrast, some ITIM-containing receptors, such as FcγRIIB and CD72, were shown to need coligation to ITAM-containing receptors (i.e., BCR) to become tyrosine phosphorylated (26, 27). Recently, it has been reported that in ILT2-transfected Jurkat cells, cross-linking of the TCR is needed for ILT2 tyrosine phosphorylation (32). This was in contrast to our results with peripheral blood T lymphocytes, which also show that coligation with TCR is not required for ILT2 activation in T cells. The basis for the discrepancy is uncertain and probably reflects the different experimental cell systems used. In Jurkat cells Fyn activity is diminished (33). The fact that Fyn phosphorylated ILT2 more efficiently than Lck in transfected COS-7 cells (not shown) might underlie the undetectable ILT2 phosphorylation upon receptor engagement in Jurkat cells.

The cytoplasmic region of ILT2 contains four putative ITIMs, of which only two (those containing residues Y562 and Y614) fulfill the proposed ITIM consensus sequence V/I/LxYxxV/L. Pei and colleagues (29) have proposed a model for SHP-1 activation in which the amino-terminal SH2 domain of SHP-1 serves both as a regulatory domain and as a recruiting unit, whereas the C-terminal SH2 domain acts merely as a recruiting unit. According to this model, we expected that both SH2 domains would bind to residues Y562 and Y614, and we intended to determine which was the docking site for the Cterminal SH2 domain. Unexpectedly, the results from our mapping experiments in RBL and COS-7 cells indicated that SHP-1 docking sites were residues Y614 and Y644.
mutant with decreasing amounts of Fyn in COS-7 cells. We rea-
soned that if residue Y533 plays any role in amplifying Src kinase
activity, ILT2wt would be more efficiently phosphorylated by low
concentrations of Fyn than ILT2.Y533F mutant. Indeed, ILT2wt
became phosphorylated, whereas no phosphorylation of
ILT2.Y533F mutant was detected when the constructs were
co-transfected with 2 µg of a plasmid encoding for Fyn (Fig. 8C).
Interestingly, Y562 was not involved in SHP-1 recruitment and
subsequent negative signaling, but still became phosphorylated af-
after receptor engagement. We cannot rule out that other signaling
mediators may interact with this docking site. In fact, CD22, a
well-characterized inhibitory receptor that binds SHP-1, is also
able to recruit other effector molecules (i.e., Syk, phospholipase
C-γ1, and phosphatidylinositol 3-kinase) (37, 38). Further studies
will be necessary to fully understand the molecular basis of ILT2
function.

In conclusion, we have explored the structural basis of ILT2
function, showing that ILT2 negative signaling takes place through
SHP-1 recruitment to phosphoryrosine residues Y614 and Y644.
Additionally, we found that residue Y533 may play a pivotal role
in ILT2 function through a mechanism that promotes the
phosphorylation of SHP-1 docking sites.

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