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Non-T Cell Activation Linker Promotes Mast Cell Survival by Dampening the Recruitment of SHIP1 by Linker for Activation of T Cells

Karine Roget,*† Marie Malissen,‡§ Odile Malbec,*† Bernard Malissen,‡§¶ and Marc Daëron2*†

The linker for activation of T cells (LAT) and the non-T cell activation linker (NTAL) are two transmembrane adapters which organize IgE receptor (FcεRI) signaling complexes in mast cells. LAT positively regulates, whereas NTAL negatively regulates mast cell activation. We previously found that the four distal tyrosines of LAT can generate negative signals. We show here that two of these tyrosines provide two binding sites for SHIP1, that LAT recruits SHIP1 in vivo, and that SHIP1 recruitment is enhanced in NTAL-deficient cells. We show that NTAL negatively regulates mast cell activation by decreasing the recruitment, by LAT, of molecules involved in FcεRI-dependent positive signaling. We show that NTAL also decreases the recruitment of SHIP1 by LAT, leading to an increased phosphorylation of the antiapoptotic molecule Akt, and positively regulates mast cell survival. We finally show that the positive effect of NTAL on Akt phosphorylation and mast cell survival requires LAT. Our data thus document the mechanisms by which LAT and NTAL can generate both positive and negative signals which differentially regulate mast cell activation and survival. They also provide molecular bases for the recruitment of SHIP1 in FcεRI signaling complexes. SHIP1 is a major negative regulator of mast cell activation and, hence, of allergic reactions. The Journal of Immunology, 2008, 180: 3689–3698.

Allergies arise as consequences of the development of a local inflammatory response, initiated by the combined effects of cytokines, chemokines, lipid-derived mediators, proteolytic enzymes, and vasoactive mediators that are secreted and/or released by activated tissue mast cells. Mast cell activation is triggered by the aggregation of high-affinity IgE receptors (FcεRI) induced by the binding of a specific multivalent Ag to FcεRI-bound IgE Abs. FcεRI aggregation generates intracellular signaling complexes which build up around the intracytoplasmic domains of lipid raft-associated tyrosine-rich transmembrane adapters which function as scaffold proteins (1, 2). These include the linker for activation of T cells (LAT)3 (3) and the non-T cell activation linker (NTAL, also called LAB or LAT2) (4) which are both expressed in mast cells. Studies in mast cell-deficient mice demonstrated that LAT exerts a positive effect (5), whereas NTAL exerts a negative effect (6–8) on FcεRI signaling leading to cell activation. By contrast with the mechanisms responsible for the positive effects of LAT (9, 10), the mechanisms responsible for the negative effects of NTAL are poorly understood (6). Interestingly, both LAT and NTAL were shown to generate positive and negative signals. NTAL functions as a homolog of LAT in B cell lines where it regulates kit signaling in human mast cells (13). We found in a previous work conducted in mast cells from knockin mice, in which individual LAT tyrosines were mutated into phenylalanines (14, 15), that the four distal tyrosines of LAT could generate not only positive, but also negative, signals in mast cells. Indeed, whereas IgE-induced responses of bone marrow-derived mast cells (BMMC) from Y136F or of Y(175,195,235)F mice were markedly reduced, the responses of BMMC from Y(136,175,195,235)F mice were almost as intense as those of wild-type (WT) BMMC (16). Molecular mechanisms responsible for LAT-dependent negative signaling were not elucidated.

SHIP1 (17) is a major negative regulator of FcεRI signaling. Mast cells from SHIP1-deficient mice indeed respond more vigorously than WT mast cells to FcεRI engagement by low concentrations of IgE and/or Ag (18). Besides, inhibition of IgE-induced responses observed in excess of Ag is abrogated in SHIP1−/− mice (19). Also, SHIP1 was demonstrated to account for the negative properties of FcγRIIB, an inhibitory IgG receptor previously shown to inhibit IgE-induced mast cell activation, when coaggregated with FcεRI by immune complexes (20, 21). SHIP1 negatively regulates FcεRI signaling by two mechanisms. Through its enzymatic activity, SHIP1 removes 5-phosphate groups in the inositol ring of 3-phosphorylated inositides and phosphatidylinositides (17). Its substrates include phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) which mediates the membrane recruitment of molecules which contain a pleckstrin homology domain.

*Institut Pasteur, Département d’Immunologie, Unité d’Allergologie Moléculaire et Cellulaire, Paris, France; †Institut National de la Santé et de la Recherche Médicale (Inserm), Unité 760, Paris, France; ‡Centre d’Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France; §Inserm, Unité 631, Marseille, France; ¶Centre National de la Recherche Scientifique, Unité Mixte de Recherche 6102, Marseille, France.

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2 Address correspondence and reprint requests to Dr. Marc Daëron, Unité d’Allergologie Moléculaire et Cellulaire, Département d’Immunologie, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France. E-mail address: daeron@pasteur.fr

3 Abbreviations used in this paper: LAT, linker for activation of T cells; NTAL, non-T cell activation linker; BMMC, bone marrow-derived mast cell; WT, wild type; PI(3,4,5)P3, phosphatidylinositols (3, 4, 5)-trisphosphate; SH2, Src homology 2; PLC, phospholipase C; PDK, phosphoinositide-dependent protein kinase.

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SHIP1 can therefore prevent the PI(3,4,5)P3-dependent recruitment of critical signaling molecules (22, 23). SHIP1 can also negatively regulate the Ras pathway independently of its catalytic activity, by recruiting Dok-1 and RasGAP, when tyrosyl-phosphorylated upon membrane translocation (24, 25). As a consequence, SHIP1 is a potent inhibitor of mast cell activation when brought within signaling complexes.

The molecular bases of the recruitment of SHIP1 by FcγRIIB is well-documented. SHIP1 contains one Src homology 2 (SH2) domain which was shown to bind in vitro to the phosphorylated ITIM of FcγRIIB (26) and to mediate the in vivo recruitment of the phosphatase in signaling complexes underneath FcγRIIB-FcεRI co-aggregates. SHIP1 is constitutively associated, via C-terminal proline-rich sequences, to the C-terminal SH3 domain of cytosolic adaptors, such as Grb2 and Grap, which were demonstrated to critically stabilize the recruitment of the phosphatase by murine FcγRIIB (27). How SHIP1 is recruited by FcεRI is unclear. Although the SH2 domain of SHIP1 was reported to bind in vitro to a tyrosine residue located between the two YxxL in the ITAM of the FcγRII subunit (28), only minute amounts of SHIP1 coprecipitated with FcεRI in IgE-stimulated mast cells (29).

In this study, we show that the C-terminal end of LAT contains two tyrosine-based binding sites for the SH2 domain of SHIP1 and that, when tyrosyl-phosphorylated upon FcεRI engagement, LAT recruits SHIP1. Like that of other SH2 domain-containing molecules, the recruitment of SHIP1 by LAT was markedly enhanced in mast cells from NTAL−/− mice. As a consequence, not only LAT-dependent positive signals, but also LAT-dependent, SHIP1-mediated, negative signals were enhanced in these cells, resulting in a decreased phosphorylation of Akt and a reduced survival of IL-3-deprived mast cells.

Materials and Methods

**Mice**

LAT−/− and NTAL−/− mice (6) were backcrossed for one and seven generations, respectively, on the C57BL/6 background. LAT−/− NTAL−/− mice were obtained by crossing LAT−/− mice with NTAL−/− mice. WT littermate mice were used as controls.

**Antibodies**

The mouse IgE anti-DNP mAb 2682-I (30) was used as culture supernatant. Mouse anti-LAT and mouse anti-phospholipase C (PLC)-γ1 Abs were obtained from BD Transduction Laboratories (BD Biosciences); rabbit anti-phospho-p65 (Ser536), rabbit anti-p65, rabbit anti-phospho-Akt (Ser473), and rabbit anti-Akt were obtained from Cell Signaling; rabbit anti-LAT, mouse anti-SHPI, rabbit anti-phospho-PLC-γ1 (Tyrosine116), and HRP-conjugated goat anti-rabbit, and goat anti-mouse Ig Abs were obtained from Santa Cruz Biotechnology. The anti-phosphotyrosoine mAb 4G10 and the anti-GST mAb ND2.1 were a gift of Dr. S. Latour (Institut National de la Santé et de la Recherche Médicale (Inserm) Unité 429, Hôpital Necker, Paris, France) and Dr. J. L. Teillaud (Inserm Unité 265, Centre de Recherches Biomédicales des Cordeliers, Paris, France).

**Mast cells**

BMMC were generated from bone marrow cells as described (31). Culture reagents were obtained from Invitrogen Life Technologies.

**Cell stimulation**

BMMC, sensitized for 1 h at 37°C with 1 μg/ml IgE anti-DNP, were washed and challenged at 37°C for the indicated times with 0.1–1 μg/ml DNP-BSA or treated with 100 mM pervanadate.

**β-hexosaminidase release**

IgE anti-DNP-sensitized mast cells were challenged for 10 min at 37°C with the indicated concentrations of DNP-BSA. β-hexosaminidase released in supernatants was measured as described (16).

**Calcium mobilization**

Intracellulare-free Ca2+ concentration was determined using 5 mM fluo-3 AM (Molecular Probes/Invitrogen Life Technologies) and 0.2% pluronic F-127 (Sigma-Aldrich). Cells were stimulated with 1 μg/ml DNP-BSA, and the intracellular Ca2+ concentration ([Ca2+]i) was assessed using a FACS Calibur and the FCS Assistant 1.2.9 β software (BD Biosciences).

**Cell survival**

Cells were washed and cultured at 3 × 105 cells/ml in IL-3-free medium. At indicated days, cells were stained with annexin V-allophycocyanin (BD Biosciences). To determine viable cell counts, aliquots of stained cells were resuspended in a known volume, transferred into BD Trucount tubes (BD Biosciences) containing a known number of fluorescent beads, and analyzed by flow cytometry. A constant number of beads was acquired. Cell fluorescence was analyzed using a FACS Calibur (BD Biosciences).

**Cell proliferation**

Cells (1 × 105/ml) loaded with 1 μCFSE (Invitrogen Life Technologies) were cultured with 4% supernatant from X63-IL-3 cells. Cell division was determined by monitoring cell fluorescence using a FACS Calibur (BD Biosciences).

**Immunoprecipitation**

Five × 107 cells were lysed in lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 2 mM Na3VO4, 10 mM NaF, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF (pH 7.4). Postnuclear lysates were immunoprecipitated with protein G-Sepharose beads (Amersham Biosciences) coupled to mouse anti-LAT Ab for 2 h at 4°C.

**GST fusion proteins**

GST-LAT. The cDNA encoding the intracellular domain of LAT was amplified by PCR using cDNA generated from WT BMMC RNA. Amplified cDNA was inserted in pGEX-6P-1 (Amersham Biosciences) and trans ferrated into DH5α Escherichia coli.

**GST-SHPI.** GST-SHIP1 SH2 and GST-Grb2 SH2 fusion proteins were described previously (27). Fusion proteins were produced in DH5α following isopyrrol-1-thio-β-o-galactopyranoside induction.

**In vitro phosphorylation**

GST- and GST-LAT-coated beads were washed in kinase buffer containing 100 mM Tris, 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 2 mM DTT, and 0.25 mM Na3VO4 (pH 7.4). Beads were incubated in kinase buffer containing 100 μM ATP and 3 μl of Syk kinase (Biaffin) for 30 min at 30°C and washed in kinase buffer. One-third was subjected to SDS-PAGE and Western blotted with anti-phosphotyrosoine Abs. Two-thirds were incubated with lysates from 1 × 107 cells for 1 h at 4°C. Eluates from beads were subjected to SDS-PAGE and Western blotted as described below.

**Peptides and in vitro-binding assays**

Phosphorylated or not phosphorylated biotinylated peptides corresponding to tyrosine motifs of LAT were purchased from NeoMPS. Their sequence was: AENTIT Y VNVSPE, Y235: GEEAPD YLVLPD, Y175: Y PLVTSF, Y136: DDYPNG were: Y67: RTPAVS.
Results

LAT contains two binding sites for the SH2 domain of SHIP1

To investigate the possible interactions of SHIP1 with the four distal tyrosines of LAT which were previously observed to generate negative signals in mast cells from LAT knockin mice (16), agarose beads were coated with nonphosphorylated (Y) or phosphorylated (pY) peptides corresponding to 13-aa sequences of LAT straddling Y67, Y136, Y175, Y195, or Y235 (Fig. 1A). Y67 was included because it stands within a potential SHIP-binding consensus sequence (26). When peptide-coated beads were incubated with a BMMC lysate, SHIP1 was found to bind to pY235 and, in smaller amounts, with pY195 and pY136 peptides. As expected, PLC-yl bound to pY136 and, in smaller amounts, to pY195 peptides, whereas Grb2 bound to pY195 and, in smaller amounts, to pY235 peptides. In some experiments, minute amounts of Grb2 also bound to the pY175 peptide (data not shown). None of the molecules examined detectably bound to pY67 or to nonphosphorylated peptides (Fig. 1B).

To investigate whether the interactions of SHIP1 with pY peptides were direct or indirect, GST fusion proteins containing the SH2 domain of SHIP1 were incubated with beads coated with each of the three peptides which bound SHIP1 in cell lysates or, as a positive control, with a peptide corresponding to the FcyRIIB ITIM (26). The SH2 domain of SHIP1 bound to pY136 and to pY235, but not to pY195 peptides. Under the same conditions, the SH2 domain of Grb2 primarily bound to pY195. Smaller amounts of Grb2-SH2 bound to pY235 and even smaller amounts to pY175 peptides (Fig. 1C).

SHIP1 was previously shown to associate constitutively, via its C-terminal proline-rich region, with the C-terminal SH3 domain of Grb2 (27). To investigate whether, due to this association, the two molecules could cooperatively bind to LAT tyrosines, Y195 and pY235, pY195 and Y235, or pY195 and pY235 peptides were mixed in variable proportions, and a constant amount of the three mixtures was used to coat beads. These were used to precipitate SHIP1 and Grb2 from a BMMC lysate (Fig. 1D). SHIP1 dose-dependently bound to beads coated with the pY235 peptide, but not to beads coated with Y195 (left panel), Y235, or pY195 (middle panel) peptides. Higher amounts of SHIP1 bound to beads coated with a mixture of pY235 and pY195 peptides (right panel). Grb2 also bound to pY235 (left panel) and even more to pY195 (middle panel), but not to Y195 (left panel) or to Y235 (middle panel) peptides. Comparables amounts of Grb2 bound to the pY195 peptide, whether mixed with Y235 (middle panel) or with pY235 (right panel) peptides.

The above results altogether demonstrate that SHIP1 can bind to phosphorylated Y136 and Y235, in the intracytoplasmic domain of LAT and that binding can occur via the SH2 domain of SHIP1. They also indicate that the binding of SHIP1 to phosphorylated LAT tyrosines can be enhanced when Grb2 binds simultaneously via its SH2 domain to adjacent phosphorylated tyrosines. As PLC-yl and Grb2 also bind to pY136 and pY235, respectively, they can possibly compete with SHIP1 for the two SHIP1-binding sites in intact LAT.

LAT recruits SHIP1 upon FcεRI engagement and SHIP1 recruitment by LAT is enhanced by the deletion of NTAL

To investigate whether SHIP1 could bind to phosphorylated LAT, a GST fusion protein containing the intracytoplasmic domain of LAT (GST-LAT) (i.e., containing all potential tyrosyl-phosphorylation sites) was constructed, phosphorylated or not in vitro with the protein tyrosine kinase Syk which was previously shown to

FIGURE 1. SHIP1 binds to phosphorylated LAT tyrosines 136 and 235. A, Schematic structure of the cytoplasmic domain of murine LAT. The nine tyrosines are numbered, based on their position in the amino acid sequence. Peptides used for binding assays are shown. EC, Extracellular domain; TM, transmembrane domain; and IC, intracytoplasmic domain. B, In vitro binding of PLC-yl, SHIP1, and Grb2 to LAT peptides. Beads coated with phosphorylated or not-phosphorylated LAT peptides were incubated with BMMC lysate. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-PLC-yl, anti-SHIP1, and anti-Grb2 Abs. C, In vitro binding of SHIP1 and Grb2 SH2 domains to LAT peptides. Beads coated with phosphorylated or not phosphorylated LAT peptides were incubated with GST-SHIP1 SH2 or GST-Grb2 SH2 fusion proteins. As controls, agarose beads coated with phosphorylated or not phosphorylated FcyRIIB ITIM peptides were incubated with GST-SHIP1 SH2. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-GST Abs. D, In vitro binding of SHIP1 and Grb2 to beads coated with a mixture of LAT Y195 and LAT Y235 peptides. Phosphorylated or not phosphorylated LAT Y195 and LAT Y235 peptides were mixed in variable proportions and mixtures containing equal amounts of peptides were used to coat agarose beads which were incubated with BMMC lysate. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-SHIP1 and anti-Grb2 Abs.
phosphorylated GST-LAT (Fig. 2A). None of the three molecules bound to nonphosphorylated GST-LAT.

To investigate whether the same three molecules could be recruited by LAT, when phosphorylated upon FcεRI engagement, their coprecipitation with LAT was searched for in BMMC sensitized with IgE anti-DNP and challenged with DNP-BSA. The same cells that were either not challenged or treated with pervanadate were used as negative and positive controls, respectively. As expected, LAT was mildly phosphorylated following Ag stimulation, and much more heavily following pervanadate treatment. PLC-γ1 and, to a lower extent, SHIP1 and Grb2 coprecipitated with LAT in Ag-stimulated cells but, surprisingly, not in pervanadate-treated cells (Fig. 2B).

The deletion of NTAL was previously reported to enhance FcεRI-dependent LAT phosphorylation and, as a consequence, the recruitment of SH2 domain-containing molecules (6, 7). BMMC from NTAL−/− mice were therefore compared with BMMC from WT mice in the same experiment. As expected, LAT phosphorylation was enhanced, and higher amounts of PLC-γ1 and Grb2 coprecipitated with phosphorylated LAT in Ag-stimulated NTAL-deficient cells. The Ag-induced coprecipitation of SHIP1 with LAT was also markedly enhanced in the same cells (Fig. 2B).

Noticeably, the low molecular mass isoform of SHIP1 predominantly coprecipitated with LAT.

These results altogether indicate that SHIP1 cannot only bind in vitro to phosphorylated LAT, but also be recruited in vivo by LAT, when phosphorylated upon FcεRI engagement. Even though competition may occur between SHIP1, PLC-γ1, and Grb2, for phosphorylated LAT-binding sites, it did not prevent the three molecules from binding in vitro to a phosphorylated fusion protein containing the whole intracytoplasmic domain of LAT and from being recruited in vivo by phosphorylated LAT. Like that of phosphorylated LAT in vivo (32), and incubated with a BMMC lysate. A GST protein, treated similarly, was used as a negative control. As expected, PLC-γ1 and Grb2, but also SHIP1, bound to

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**FIGURE 2.** Tyrosyl-phosphorylated LAT recruits SHIP1. A. Pull-down assay of PLC-γ1, SHIP1, and Grb2 with in vitro-phosphorylated GST-LAT fusion proteins. GST or GST-LAT bound onto glutathione-agarose beads were subjected to an in vitro kinase assay using the Syk kinase and incubated with BMMC lysate. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-phosphotyrosine (pY), anti-PLC-γ1, anti-SHIP1, and anti-Grb2 Abs. B. Coprecipitation of PLC-γ1, SHIP1, and Grb2 with LAT. WT (left panel) and NTAL−/− (right panel) BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated, challenged with DNP-BSA, or treated with pervanadate. Cells were lysed, and LAT was immunoprecipitated. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-phosphotyrosine (pY), anti-LAT, anti-PLC-γ1, anti-SHIP1, and anti-Grb2 Abs.

**FIGURE 3.** LAT positively regulates, whereas NTAL negatively regulates, signals leading to cell activation. A. PLC-γ-1 activation. WT, NTAL−/−, and LAT−/− BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated or challenged with DNP-BSA for the indicated time and lysed. Equal amounts of proteins were fractionated by SDS-PAGE and Western blotted with anti-pPLC-γ-1 and anti-PLC-γ-1 Abs. B. NF-κB activation. WT, NTAL−/−, and LAT−/− BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated or challenged with DNP-BSA for the indicated time and lysed. Equal amounts of proteins were fractionated by SDS-PAGE and Western blotted with anti-p65-NF-κB and anti-P65-NF-κB Abs. C. Ca2+ mobilization. WT, NTAL−/−, and LAT−/− BMMC, previously sensitized with mouse mAb IgE anti-DNP, were loaded with Fluo-3 and stimulated with DNP-BSA at the indicated time (arrow), and intracellular Ca2+ concentration was measured. D. β-hexosaminidase release. WT, NTAL−/−, and LAT−/− BMMC, previously sensitized with mouse mAb IgE anti-DNP, were challenged with the indicated concentrations of DNP-BSA. β-hexosaminidase was measured in supernatant by an enzymatic assay.
PLC-γ1 and Grb2, the recruitment of SHIP1 by LAT was markedly enhanced in NTAL-deficient mast cells.

NTAL deletion enhances not only LAT-dependent positive signals which control cell activation, but also LAT-dependent negative signals which control cell survival. NTAL was described as an inhibitory molecule for FcεRI signaling (6). NTAL-dependent inhibitory signals, however, remain unknown. As previously reported, Ag-induced PLC-γ1 phosphorylation, which was markedly reduced in LAT−/− BMMC, was enhanced in NTAL−/− BMMC (Fig. 3A). When phosphorylated and activated, PLC-γ1 cleaves P(4,5)P2 into inositol Tris-phosphate and diacylglycerol. Inositol Tris-phosphate initiates the Ca²⁺ response while diacylglycerol initiates a protein kinase C-mediated pathway leading to NF-κB phosphorylation and activation (33, 34). Both the Ag-induced Ca²⁺ response (Fig. 3C) and NF-κB phosphorylation (Fig. 3B), which were reduced in LAT−/− BMMC, were enhanced in NTAL−/− BMMC. As a consequence and, as previously observed, Ag-induced release of β-hexosaminidase was decreased in LAT−/− BMMC, but enhanced in NTAL−/− BMMC (Fig. 3D). The enhancing effect of NTAL deletion on IgE-dependent mast cell biological responses may therefore primarily result from an enhancement of LAT-dependent positive signals.

If, however, LAT can recruit SHIP1 and if the deletion of NTAL enhances the recruitment of SHIP1 by LAT, we anticipated that it should decrease P(3,4,5)P3-dependent signals. The concomitant recruitment of Akt and of phosphoinositide-dependent protein

**FIGURE 4.** NTAL positively regulates, whereas LAT negatively regulates Akt activation. BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated or challenged with DNP-BSA for the indicated time and lysed. Equal amounts of proteins were fractionated by SDS-PAGE and Western blotted with anti-pAkt and anti-Akt. A, Effect of NTAL deletion in the presence of LAT. B, Effect of LAT deletion in the presence of NTAL. C, Effect of LAT deletion in the absence of NTAL. D, Effect of NTAL deletion in the absence of LAT.

**FIGURE 5.** NTAL positively regulates mast cell survival. A, Effect of LAT deletion on cell survival. B, Effect of NTAL deletion on cell survival. C, Effect of LAT and NTAL deletion on cell survival. BMMC were cultured for various periods of time in the absence of IL-3, stained with annexin V-allophycocyanin, and mixed with fluorescent beads. Daily FACS analyses were performed to evaluate the percentages of cells remaining in culture and the percentages of viable and apoptotic cells among remaining cells. Left panels, Dot plots at days 0 and 3. Gate 3 shows fluorescent beads used for cell counts. Middle panels, Percentage of annexin V-negative cells remaining in culture as a function of time (number of cells in gate 1: number of cells at the initiation of culture). Right panels, Percentage of annexin V-positive cells among cells remaining in culture (number of cells in gate 1: number of cells in gate 1 + gate 2).
kinase1 (PDK1) by PIf(3,4,5)P3 enables PDK1 to phosphorylate Akt (35). Indeed, Ag-induced Akt phosphorylation was decreased in NTAL-/- BMMC (Fig. 4A) but increased in LAT-/- BMMC (Fig. 4B). To investigate the respective roles of LAT and NTAL on the regulation of Akt phosphorylation, we compared LAT-/- NTAL-/- double-deficient BMMC with LAT-/- and with NTAL-/- single-deficient BMMC. The deletion of LAT in NTAL-deficient cells increased Ag-induced Akt phosphorylation (Fig. 4C). The deletion of NTAL in LAT-deficient cells, however, had no detectable effect on Ag-induced Akt phosphorylation (Fig. 4D). It follows that LAT can negatively regulate Akt phosphorylation in the absence of NTAL, whereas NTAL requires LAT for enhancing Akt phosphorylation.

When phosphorylated, Akt acts as an antiapoptotic molecule (36, 37). We therefore investigated the survival rates of BMMC when deprived from growth factors. We found that the viability of LAT-/- BMMC decreased similarly with time as that of WT BMMC when cultured without IL-3 (Fig. 5A). The viability of NTAL-/- BMMC, however, decreased more rapidly than that of WT BMMC, when cultured under the same conditions (Fig. 5B). Noticeably, the viability of LAT-/- NTAL-/- double-deficient BMMC was similar as that of WT BMMC (Fig. 5C). NTAL therefore enhances the survival of IL-3-deprived mast cells, and this effect requires the presence of LAT. When cultured in IL-3-containing medium, the growth (Fig. 6, A and C) and proliferation (Fig. 6, B and D) of LAT-/- or NTAL-/- BMMC were similar as those of WT BMMC.

These results altogether indicate that the deletion of NTAL amplifies not only positive but also negative LAT-dependent, SHIP1-mediated signals. LAT-dependent positive signals affect primarily mast cell activation whereas LAT-dependent negative signals affect primarily mast cell survival in the absence of IL-3. LAT-dependent negative signals are counterbalanced by the positive effects of NTAL in WT BMMC, and NTAL-dependent positive signals require LAT to increase cell survival.

Discussion
We show here that LAT contains two binding sites for the SH2 domain of SHIP1 and that, when tyrosyl-phosphorylated upon FcεRI engagement, it recruits SHIP1. LAT may thus bring SHIP1 into FcεRI signaling complexes. This phosphatase plays a prominent role in the negative regulation of IgE-induced mast cell activation. Our findings provide a molecular basis for the negative signaling properties of the four distal tyrosines of LAT that we previously unraveled in mast cells from LAT knockin mice. We also show that NTAL negatively regulates mast cell activation, but positively regulates mast cell survival, and that both effects depend on LAT. NTAL negatively regulates mast cell activation by decreasing the recruitment of molecules involved in the generation of positive signals by LAT. It positively regulates mast cell survival by decreasing the recruitment of SHIP1 by LAT, leading to an increased activation of the antiapoptotic molecule Akt.

Compelling evidence that SHIP1 negatively regulates FcεRI signaling was obtained when studying SHIP1-/- mice, in which IgE-induced anaphylactic reactions were more severe, and the mast cells of which degranulated more vigorously and secreted more cytokines than mast cells from WT mice, when sensitized with IgE Abs and challenged with Ag (38). Consistent with the previously recognized ability of SHIP1 to hydrolyze PIf(3,4,5)P3 when brought into signaling complexes (22) and to recruit Dok-1/rasGAP when phosphorylated upon FcεRI aggregation (25), IgE-induced Ca2+ responses and MAPK activation were enhanced in these cells. On the basis of these data, SHIP1 was described as “the gatekeeper of mast cell degranulation” (18). How SHIP1 is recruited into FcεRI signaling complexes is, however, unclear.

Several proteins were described to associate with SHIP1. The adapters Dok-1 and Shc bind, via their phospho tyrosine-binding domain (39), while the adapter CrkL (40) and the protein tyrosine phosphatase SHP-2 bind, via their SH2 domains (41), to tyrosyl-phosphorylated SHIP1. These molecular interactions are unlikely to contribute to the recruitment of SHIP1 into signaling complexes. SHIP1 indeed becomes phosphorylated after it has been recruited and brought close to Lyn (42). Grb2 constitutively associates with C-terminal proline-rich sequences of SHIP1 via its C-terminal SH3 domain (27). As a consequence, Grb2 may bring SHIP1 into signaling complexes, when this adapter is recruited by LAT or by another tyrosyl-phosphorylated molecule. SHIP1 was indeed recently described as forming a multimolecular complex with Dok-1,
Dok-2, and Grb2 that associated with LAT upon TCR engagement in human T cells (43). SHIP1 was also reported to interact with phosphorylated FcεRI by a yeast three hybrid assay (44) and to bind in vitro to phosphopeptides corresponding to the FcγRI and FcεRI ITAMs via its SH2 domain (45). The FcεRI ITAM contains a third tyrosine residue, in the NVYSP sequence that separates the YEEL and YSEL sequences of the canonical ITAM motif. Pull-down experiments showed that both Lyn and SHIP1 could bind in vitro to this tyrosine, and mutational analysis showed that it is involved in the negative regulation of ERK, p38, and NF-κB activation and of the secretion of IL-6, IL-13, and TNF-α induced by IgE (28). SHIP1 was less phosphorylated following FcεRI engagement in cells bearing a mutation of this tyrosine. On the basis of these data, FcεRI was proposed to bring molecules with negative properties, including SHIP1, in signaling complexes, and a faint coprecipitation of SHIP1 with FcεRI was reported in one article (29). We show here that LAT can recruit SHIP1 in mast cells.

When tyrosyl-phosphorylated upon receptor engagement, LAT provides multiple docking sites for SH2 domain-containing molecules which contribute to the FcεRI signaling complex. These include enzymes such as PLC-γ, protein tyrosine kinases of the Tec family, and the p85 subunit of PI3K, exchange factors of the Vav family and cytosolic adapters, among which are Gads, Grap, and Grb2 (10, 32, 46). Works based on mutational analysis identified the four distal tyrosines of LAT (Y136, Y175, Y195, and Y235) as being critical for the recruitment of these molecules in T cells (10, 47) and mast cells (48). We identified two binding sites for the SH2 domain of SHIP1 among these four tyrosines. These are within sequences containing Y136 and Y235. As described previously (10), the four distal tyrosines of LAT also provided two binding sites for PLC-γ1 and two binding sites for Grb2 and, not surprisingly, binding required that these sites be tyrosyl-phosphorylated. Noticeably however, each molecule bound preferentially to one of these two binding sites: SHIP1 bound to pY235 more than to pY136, PLC-γ1 bound to pY136 more than to pY195, and Grb2 bound to pY195 more than to pY235. GST fusion proteins containing the SH2 domains of SHIP1 and Grb2 bound to the same phosphorylated peptides as SHIP1 and Grb2 contained in cell lysates, and with the same hierarchy.

The three binding sites having an affinity for the SH2 domains of more than one molecule (PLC-γ1 and SHIP1 bind to pY136, Grb2 and PLC-γ1 bind to pY195, SHIP1 and Grb2 bind to pY235), SHIP1 shares its two binding sites with molecules which are likely to compete with the phosphatase for being recruited by LAT (Fig. 7A). Cooperative interactions, however, occur, which facilitate the in vivo recruitment of signaling molecules by LAT. Thus, when interacting with pY(175,195,235)-bound Gads, SLP-76 stabilizes the binding of PLC-γ1 to pY136 (10, 47, 49). We found that, when binding to pY195, Grb2 enhanced the binding of SHIP1 to pY235. This finding is reminiscent of our previous demonstration that the recruitment of SHIP1 by FcγRIIB in B cells requires the cooperative binding of SHIP1 to the phosphorylated ITIM of and Grb2 to another phosphorylated tyrosine-based motif, in the C-terminal end of mouse FcγRIIB (27). This cooperation, which can be explained by the affinity of the C-terminal SH3 domain of Grb2 for proline-rich sequences of SHIP1, is likely to compensate for competition. Indeed, all three molecules could be pulled down by a GST fusion protein containing the whole intracytoplasmic domain of LAT that was previously in vitro phosphorylated by Syk. More importantly, SHIP1, Grb2, and PLC-γ1 coprecipitated with LAT in BMMC, following FcεRI engagement by IgE and Ag. LAT was weakly phosphorylated, and small but detectable amounts of all three molecules coprecipitated with LAT under these conditions. Surprisingly, SHIP1, Grb2, and PLC-γ1 failed to detectably coprecipitate with LAT in cells treated with pervanadate, although LAT was massively phosphorylated. This suggests that, although necessary, LAT phosphorylation may not be sufficient for SH2 domain-containing molecules to be recruited. Receptors and signaling molecules which reside in distinct subcellular compartments need to be brought close to each other for being able to interact. We showed previously that, when aggregated, FcεRI becomes associated with the F-actin skeleton where a fraction of SHIP1 is constitutively associated with the actin-binding molecule Filamin-1 (50). Other conditions, which determine the intensity of LAT phosphorylation, markedly affected the recruitment of SH2 domain-containing molecules. Among these is the presence/absence of NTAL.

The deletion of NTAL indeed enhanced IgE-induced LAT phosphorylation. Although this effect has been known for some time (6), its mechanisms remains unknown. Competition between the two adapters for the kinase Syk is one possibility. If, as suggested, LAT and NTAL reside in distinct lipid microdomains, these should merge following receptor engagement (6). The dephosphorylation of LAT by SH2 domain-containing tyrosine phosphatases, possibly recruited by NTAL, is another possibility. However, neither SHP-1 nor SHP-2 was reported to coprecipitate with NTAL. Whatever the mechanism by which NTAL affects LAT phosphorylation, the recruitment of SH2 domain-containing molecules by LAT is enhanced by the deletion of NTAL (6, 7). IgE-induced activation of LAT-dependent intracellular pathways and, as a consequence, secretory responses were indeed markedly enhanced in BMMC from NTAL-deficient mice. PLC-γ1 phosphorylation and Ca2⁺ responses, which were decreased in LAT−/− cells, were increased in NTAL−/− cells, as well as β-hexosaminidase release. These effects may account for the negative regulation of FcεRI signaling that was assigned to NTAL in mast cells: rather (or more) than having a negative effect per se, NTAL can simply dampen LAT-dependent positive signals. Supporting this conclusion, the enhancing effect of NTAL deletion on IgE-induced responses was apparently not seen in LAT-deficient BMMC (6).

Noticeably, the inducible coprecipitation of SHIP1 with LAT observed upon FcεRI engagement was also markedly enhanced in BMMC from NTAL−/− mice, suggesting that LAT-dependent negative signals might also be increased in the absence of NTAL. If the recruitment of SHIP1 by LAT may, at least in part, account for these negative signals, one anticipates that PI(3,4,5)P3-dependent signaling events be enhanced in LAT-deficient cells. Akt phosphorylation, which requires that Akt and PKD1 be corecruited to the membrane by PI(3,4,5)P3 via their pleckstrin homology domain, was indeed increased in LAT−/− cells and decreased in NTAL−/− cells. Akt phosphorylation therefore appears as the first signaling event described as being negatively regulated by LAT. Noticeably, the deletion of NTAL did not affect Akt phosphorylation in the absence of LAT, indicating that the positive effect of NTAL on Akt phosphorylation requires LAT. By contrast, the deletion of LAT still increased Akt phosphorylation in the absence of NTAL, indicating that the negative effects of LAT on Akt phosphorylation do not require NTAL. Although NTAL deletion enhanced the recruitment of SHIP1 by LAT, SHIP1 phosphorylation was not detectably enhanced in whole lysates from NTAL−/− mast cells (data not shown). One possible explanation is that LAT recruits only a small proportion of the intracellular pool of SHIP1. Alternatively, SHIP1 may not be phosphorylated when recruited by LAT. The tyrosine-rich region is located in the C-terminal end which is spliced out in the low molecular mass isoforms of SHIP1. By contrast with FcγRIIB, which we previously found to predominantly recruit the high molecular mass isoform of SHIP1 (50),
LAT seems to predominantly recruit the low molecular mass isoform of SHIP1. If so, SHIP1 could hydrolyze PI(3,4,5)P3 and decrease Akt phosphorylation when recruited by LAT, but not inhibit ERK phosphorylation. SHIP1 indeed needs to be phosphorylated to recruit Dok-1/RasGAP. Supporting this possibility, the phosphorylation of ERK was marginally reduced in LAT deficient cells. Finally, our results provide a molecular mechanism to the mast cell proliferation observed in SHIP1−/− BMMC (38).

Taken together, our results support the conclusion that NTAL negatively regulates cell activation but positively regulates cell survival and that both effects of NTAL depend on LAT (Fig. 7B). The deletion of NTAL enhances both LAT-dependent positive and negative signals. As a consequence, mast cell secretory responses are enhanced because LAT-dependent positive signals that affect cell activation are dominant over LAT-dependent negative signals. NTAL deletion, however, also enhances LAT-dependent negative signals which are mediated by SHIP1 and affect primarily cell survival. Finally, our results provide a molecular mechanism to the LAT-dependent negative effects previously observed in mast cells from LAT knockin mice (16). These can indeed be explained by...
the presence of two SHIP1-binding sites in LAT (Fig. 7A). Mast cell activation results from the integration of LAT-dependent positive and negative (SHIP1-mediated) signals and of LAT-independent positive signals (mast cell responses are indeed reduced, but not abrogated in LAT−/− mast cells). LAT-independent positive signals result in part from the PI(3,4,5)P3-dependent recruitment of molecules with a pleckstrin homology domain such as PLC-γ and Btk (Fig. 7B). By removing the main PLC-γ-binding site and the secondary SHIP1-binding site, the LAT Y136F mutation abrogated most of PLC-γ-dependent positive signals and a part of SHIP1-dependent negative signals. It left intact LAT-independent positive signals, Grb2-dependent positive signals and most SHIP1-dependent negative signals. Remaining SHIP1-dependent negative signals could not affect LAT-dependent positive signals because these were virtually abrogated, but they could affect LAT-dependent positive signals. By removing all Grb2-binding sites and the main SHIP1-binding site, the LAT Y(175,195,235)F mutations abrogated Grb2-dependent positive signals and most SHIP1-dependent negative signals. Remaining SHIP1-dependent negative signals could not affect LAT-dependent positive signals because these were virtually abrogated, but they could affect LAT-dependent positive signals. By removing all Grb2-binding sites and the main SHIP1-binding site, the LAT Y(175,195,235)F mutations abrogated Grb2-dependent positive signals and most SHIP1-dependent negative signals. They left intact LAT-independent positive signals, PLC-γ-dependent positive signals, and a part of the SHIP1-dependent negative signals. The remaining SHIP1-dependent negative signals could affect PLC-γ-dependent and LAT-independent positive signals. By removing all PLC-γ, Grb2, and SHIP1-binding sites, the LAT Y(136,175,195,235)F mutations abrogated all LAT-dependent positive and negative signals. They left intact LAT-independent positive signals which were not any more negatively regulated by SHIP1. As a consequence, the secretory responses of LAT Y(136,175,195,235)F BMMC were more intense than those of LAT Y136F BMMC and than those of LAT Y(175,195,235)F BMMC (16).

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Disclosures

The authors have no financial conflict of interest.

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