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LAT Is Essential for FcεRI-Mediated Mast Cell Activation

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Summary

The linker molecule LAT is a substrate of the tyrosine kinases activated following TCR engagement of T cells. LAT is also expressed in platelets, NK, and mast cells. Although LAT-deficient mice contain normal numbers of mast cells, we found that LAT-deficient mice were resistant to IgE-mediated passive systemic anaphylaxis. LAT-deficient bone marrow–derived mast cells (BMMC) showed normal growth and development. Whereas tyrosine phosphorylation of FcεRI, Syk, and Vav was intact in LAT-deficient BMMCs following FcεRI engagement, tyrosine phosphorylation of SLP-76, PLC-γ1, and PLC-γ2 and calcium mobilization were dramatically reduced. LAT-deficient BMMCs also exhibited profound defects in activation of MAPK, degranulation, and cytokine production after FcεRI cross-linking. These results show that LAT plays a critical role in FcεRI-mediated signaling in mast cells.

Introduction

Extensive examination has revealed much about the structure and function of the mast cell high-affinity receptor for IgE, FcεRI, the receptor central to mast cell activation. As is true with many immunoreceptors, the FcεRI is comprised of multiple subunits, a ligand binding α chain, and two chains, β and γ, which function as signal transduction subunits (Blank et al., 1989; Alber et al., 1991). Both of these molecules contain an ITAM (immunoreceptor tyrosine-based activation motif), containing paired tyrosine residues (Reth, 1989). Engagement of this receptor with IgE, followed by aggregation of multiple IgE-bearing FcεRI molecules by polyvalent antigen leads to activation of the Src-family protein tyrosine kinase (PTK) Lyn, which phosphorylates the tyrosines of the β and γ ITAMs (Eiseman and Bolen, 1992; Yamashita et al., 1994). In this manner, activation creates a new surface for the Syk PTK, which binds the phosphorylated ITAM through its tandem SH2 domains. Interaction of Syk with the ITAMs also activates this enzyme. Thus, the activated FcεRI is characterized by associated and active Syk PTK (Benhamou et al., 1993).

The early events following activation of the FcεRI ultimately lead to the complex pattern of mast cell effector function. Release of granules containing histamine, serotonin, β-hexosaminidase, and mast cell–specific proteases is a prominent feature. Transcription of multiple cytokine and lymphokine genes and protein secretion also follow receptor engagement. Among the cytokines, interleukins 1–6, 9, 10, 13, 16, TNF-α, TGF-β, and GM-CSF are known to be produced in activated mast cells (Metcalfe et al., 1997). A major question in the study of FcεRI-mediated signaling is how one of the earliest events following FcεRI engagement, the activation of PTKs, leads ultimately to granule release and gene transcription (Benhamou et al., 1990). Activation of PTKs in mast cells as in other systems leads to phosphorylation and activation of PLCγ (Park et al., 1991). The resulting elevation of calcium is required for granule release and activation of such transcription factors as NF-AT. SLP-76, originally identified as a PTK substrate in T cells and required for T cell development and activation, is also expressed in mast cells (Hendricks-Taylor et al., 1997; Clements et al., 1998; Pivniouk et al., 1998; Yablonski et al., 1998). In mast cells prepared from mice genetically engineered to be deficient in SLP-76, FcεRI engagement fails to lead to β-hexosaminidase or IL-6 release in vitro. Mice lacking SLP-76 are also resistant to IgE-mediated passive anaphylaxis (Pivniouk et al., 1999).

In T cells, SLP-76 and other critical signaling molecules such as the Ras activator SOS are cytosolic proteins. They constitutively bind the small linker molecules, Grb2, and Grb2-related molecules such as Grap and Gads. These linker molecules contain two SH3 domains surrounding an SH2 domain. The SH3 domains constitutively bind SLP-76 and SOS (Buday and Downward, 1993; Egan et al., 1993; Feng et al., 1996; Liu et al., 1999). Upon TCR engagement, these small complexes of Grb2 and SLP-76 are brought to the plasma membrane by an interaction of the Grb2 SH2 domain with phosphorylated tyrosine residues of the integral membrane linker molecule LAT, which is heavily phosphorylated following TCR activation. PLCγ also docks on to LAT following TCR engagement (Buday et al., 1994; Sieh et al., 1994; Weber et al., 1998; Zhang et al., 1998). Messenger RNA encoding LAT is expressed in the mast cell line RBL-2H3 (Zhang et al., 1998). It thus became important to determine whether LAT is critical for mast cell function, as it is for T cells. To address this question, we derived long-term mast cells by IL-3 stimulation of bone marrow cells isolated from LAT-deficient mice. Our results demonstrate a profound effect on mast cell function in cells

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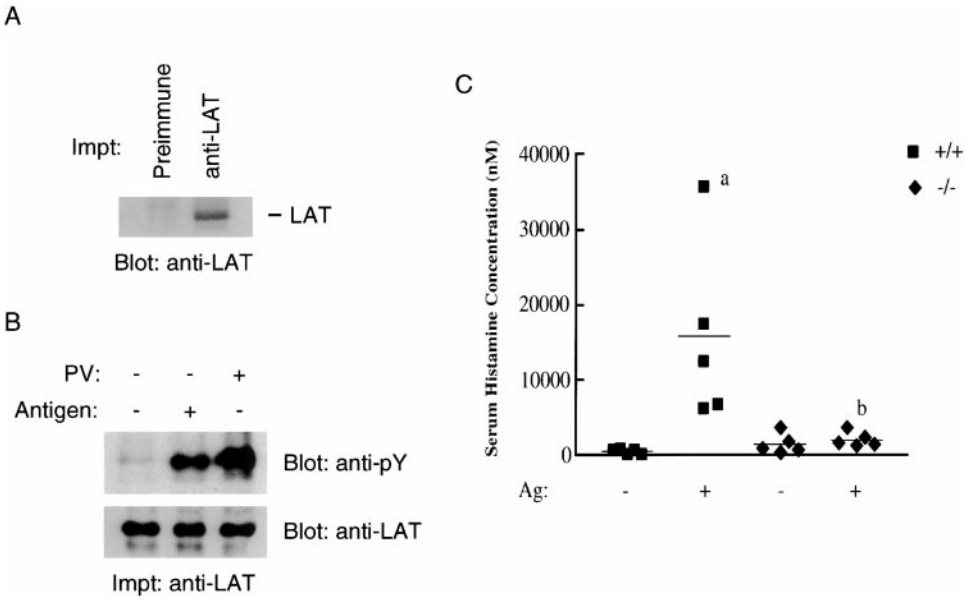

Figure 1. LAT Protein Expression and Protein Tyrosine Phosphorylation of LAT Following FcεRI Engagement in RBL-2H3 Cells and the Effect of LAT on IgE-Mediated Passive Systemic Anaphylaxis

(A) Lysates from RBL-2H3 cells (5×10^6) were immunoprecipitated with preimmune serum (control) or anti-LAT antiserum, and then immunoblotted with anti-LAT antiserum.

(B) RBL-2H3 cells (2×10^7) were sensitized in 10 ml media with anti-DNP IgE (1 μg/ml) for 12 hr and then stimulated with DNP-HSA (500 ng/ml) or pervanadate (100 μM) for 2 min. Lysates from these cells were immunoprecipitated with anti-LAT antiserum. Immunoprecipitates were fractionated by SDS-PAGE and proteins were transferred to nitrocellulose filters, which were probed with anti-phosphotyrosine antibody or anti-LAT antiserum.

(C) Mice ($n = 5$) were sensitized and challenged as described in Experimental Procedures. The data were analyzed for significance by a unpaired t test. (a) Comparison of $LAT^{+/+}$ in the presence (Ag^+) or absence of antigen (Ag^-) stimulation; $p < 0.01$. (b) Comparison of $LAT^{+/+}$ to $LAT^{-/-}$ in the presence of antigen (Ag^+); $p < 0.05$.

isolated from these mice and show that LAT is critical to normal mast cell function.

Results

To confirm that LAT protein is expressed in mast cells, we first tested RBL cells for LAT expression and determined whether LAT could be tyrosine phosphorylated following FcεRI engagement in these cells. Cells were incubated with monoclonal IgE specific for DNP, and then the FcεRI was aggregated by addition of multivalent DNP-HSA. After stimulation, postnuclear lysates were subjected to immunoprecipitation with anti-LAT antibodies. LAT protein was detected with the same antibody by Western blot analysis, and the tyrosine phosphorylation of LAT was demonstrated by blotting with anti-phosphotyrosine reagents (Figures 1A and 1B).

As a test of the role of LAT for mast cell–mediated cellular function in vivo, we compared normal and LAT-deficient mice for histamine release following engagement of mast cell receptors. This assay, a test for passive systemic IgE-dependent anaphylaxis, is dependent on in vivo mast cell degranulation and histamine release. For this assay, mice were injected with monoclonal anti-DNP IgE and were subsequently challenged with either DNP-HSA or, as a control, with PBS. After 1.5 min, the mice were euthanized and blood was obtained for the assay. Five normal mice showed a massive increase in systemic histamine, while the LAT-deficient mice and those animals not receiving antigen showed no increase in histamine levels over background (Figure 1C). These results do not reflect differences in the numbers of mast cells found in these animals. Microscopic analysis demonstrated that the number of skin mast cells detected per field were approximately equal in wild-type (18.6 ± 0), heterozygous (19.0 ± 0.8), and LAT-deficient (18.8 ± 0.4) mice.

All subsequent analyses of mast cell LAT function in this study were performed on long-term mast cell lines derived from bone marrow extracted from three populations: LAT-deficient mice (−/−), mice heterozygous for the targeted *LAT* gene (+/−) and wild-type animals (+/+). Bone marrow from these mice was cultured in the presence of 20 ng/ml IL-3. Cell growth rates for all three populations were identical regardless of whether cells were counted after 2 or 7 weeks of culture in IL-3 (data not shown). Similarly, the rates at which FcεRI-positive cells expanded in these cultures were identical in the three populations (data not shown). After 5 weeks, cells were greater than 95% FcεRI positive, as shown (Figure 2A). Cells from each of the three lines contained equivalent numbers of toluidine-positive granules (data not shown). To determine the amount of LAT protein, cells from the three lines were solubilized in detergent, and LAT protein was detected by blotting. No LAT protein was detected in mast cells from the $LAT^{-/-}$ mice. An intermediate amount was found in mast cells from the heterozygous animals (Figure 2B).

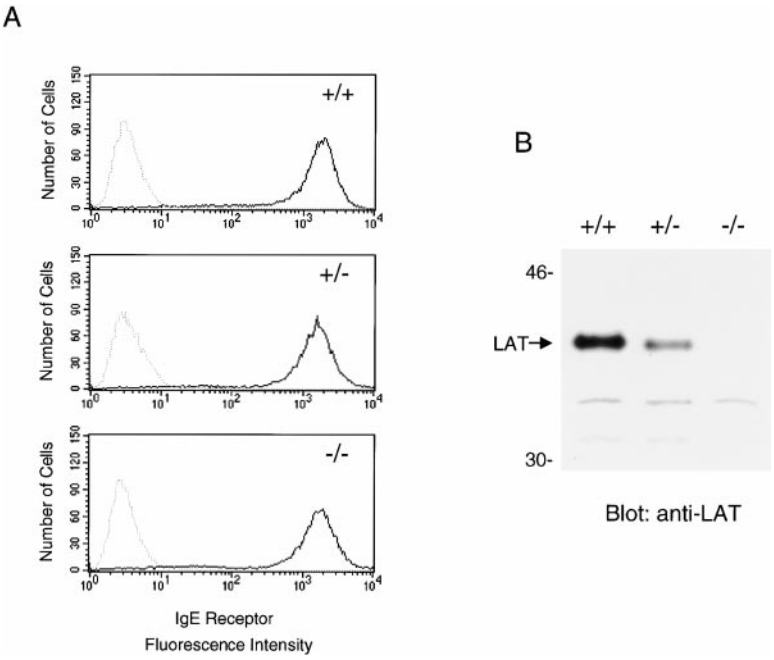

Figure 2. Surface IgE Receptor and LAT Expression Level in BMMCs from LAT$^{+/+}$, LAT$^{+/-}$, and LAT$^{-/-}$ Mice

(A) IgE receptor expression. Three populations of BMMCs (cultured for 5 weeks) were treated with anti-DNP IgE mAb, and then incubated with phycoerythrin-conjugated goat anti-mouse Ig. Control staining was with phycoerythrin-conjugated goat anti-mouse Ig alone.

(B) LAT expression level. Proteins in the lysates from three populations of BMMCs (2×10^5 cell equivalents) were separated by SDS-PAGE and LAT was detected by immunoblotting.

Cells from the mast cell lines were then stimulated through the FcεRI and intracellular phosphorylation events were evaluated. The earliest events occurring following receptor engagement are the phosphorylation of receptor subunits and associated PTKs (Paolini et al., 1991; Eiseman and Bolen, 1992). Cell lines from the three populations (+/+, +/−, and −/−) were stimulated by antigen activation. Immunoprecipitation of the β and γ chains of the receptor and analysis of the phosphotyrosine content of these subunits is a test of the function of the Src family PTK Lyn. No consistent difference in the level of phosphotyrosine or fold increase in phosphotyrosine on these subunits was observed, (Figures 3A and 3B, arrows indicate the β chains or multiple γ chain isoforms). In mast cell activation, the phosphorylation of β and γ chain ITAMs leads to recruitment and activation of the Syk PTK. The level of Syk tyrosine phosphorylation serves as an indication of activation of this enzyme. Following activation, there was no difference in the level of Syk tyrosine phosphorylation between the mast cells isolated from wild-type, heterozygous, and LAT-deficient mice (Figure 3C).

In T cells, LAT functions downstream of the PTKs activated by the TCR, and thus the above results in mast cells are not surprising. In normal FcεRI-stimulated mast cells, LAT was isolated and its tyrosine phosphorylation was detected by blotting with anti-phosphotyrosine antibodies (Figure 4A). Phosphorylation was rapid and was followed quickly by dephosphorylation. These kinetics are similar to that seen with TCR engagement (Zhang et al., 1998). LAT phosphorylation in T cells creates binding sites for signaling proteins containing SH2 domains and allows these proteins to become phosphorylated and activated at the plasma membrane. To test the effect of LAT deficiency on the global level of intracellular tyrosine phosphorylation, mast cells from wild-type and LAT$^{-/-}$ mice were activated and the phosphotyrosine content of cellular lysates was compared (Figure 4B). The loss of phosphorylated LAT, a 36–38 kDa protein, was easily detected in the lysates of (−/−) cells (open arrowheads). In addition, substrates with approximate apparent molecular weights of 76, 135, and 145 kDa (closed arrowheads) were not observed in this lysate.

Known PTK substrates were then evaluated. SLP-76, a PTK substrate in T and mast cells, is required for optimal activation of both cell types (Hendricks-Taylor et al., 1997; Yablonski et al., 1998; Pivniouk et al., 1999). In T cells, it is brought to the plasma membrane by virtue of its interaction with Gads, whose SH2 domain interacts with phosphorylated LAT (Liu et al., 1999). Mast cells from the LAT +/+, +/−, and −/− lines were activated and SLP-76 was immunoprecipitated (Figure 4C). Equivalent levels of SLP-76 protein were detected in the three populations. However, the level of tyrosine phosphorylation on SLP-76 varied with the amount of LAT protein. SLP-76 tyrosine phosphorylation was decreased in the heterozygous cells and was barely detectable in the cell lines from the LAT$^{-/-}$ mice. Vav, an activator of the Rac family of G proteins, is another substrate of PTKs activated in mast and T cells (Bustelo et al., 1992; Margolis et al., 1992; Crespo et al., 1997). It coimmunoprecipitates with LAT in T cells activated via the TCR and in mast cells after FcεRI stimulation (Arudchandran et al., 2000). This interaction is likely to be indirect and mediated, in part, by Vav binding to SLP-76 (Tuosto et al., 1996; Wu et al., 1996). In contrast to SLP-76, no significant difference in the level of Vav tyrosine phosphorylation was observed in the three cell lines (Figure 4C).

Mast cells express two isoforms of phospholipase Cγ (PLC) and both become phosphorylated on tyrosine upon receptor engagement (Barker et al., 1998). The two isoforms of PLC were isolated from LAT$^{+/+}$, LAT$^{+/-}$, and LAT$^{-/-}$ cell lines following activation. Rapid tyrosine phosphorylation of both isoforms was detected in all the cells (Figure 4D). However, the level of phosphorylation

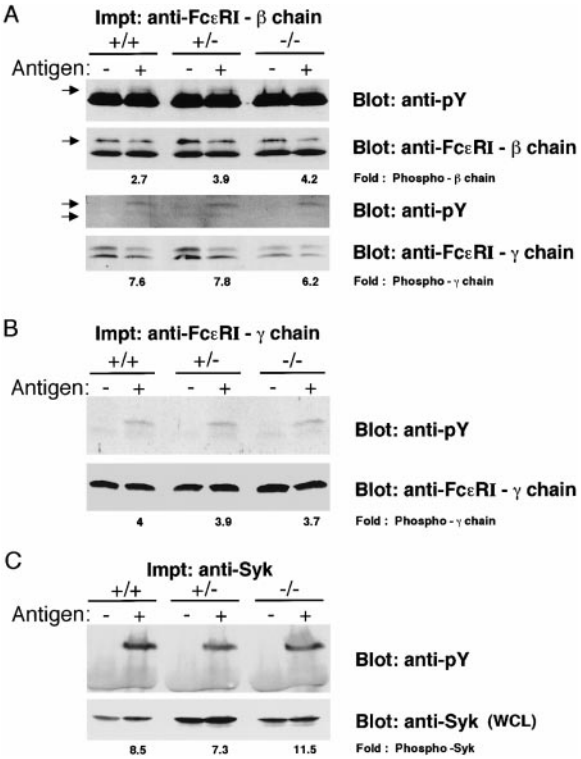

Figure 3. Protein Tyrosine Phosphorylation of Syk and FcεRI in BMMCs Following Antigen Stimulation

Three populations of BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-HSA for 1 min as described in Experimental Procedures. Lysates from these cells were immunoprecipitated with anti-FcεR-β chain (A), anti-FcεR–γ chain (B), and anti-Syk (C). Immunoprecipitated proteins were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membrane filters, and then immunoblotted with anti-phosphotyrosine antibody. After stripping, filters were reprobed with anti-FcεR–β chain and anti-FcεR–γ chain to determine protein levels. For Syk protein levels, the total lysate was immunoblotted. Arrows indicate FcεR-β chain and anti-FcεR–γ chains. Densitometric analysis of phosphorylated proteins was performed and the fold increase in intensity of the phosphoprotein from activated cells compared to nonactivated cells is shown. These densitometric values are normalized to protein levels.

varied with the level of LAT protein. Phosphorylation of the enzyme in the heterozygous cells was at an intermediate level and PLC phosphorylation in mast cells from the $LAT^{-/-}$ mice was barely detectable. Tyrosine phosphorylation of PLCγ isoforms is required for activation of the enzyme leading to cleavage of phosphoinositides and the subsequent release of diacylglycerol and inositol phosphates (Nishibe et al., 1990). Elevation of inositol phosphates leads to release of calcium from intracellular stores and calcium influx from the extracellular space. To test for activation-induced intracellular calcium elevation, the three cell lines were labeled with the calcium-sensitive dyes Fluo-3 and Fura-Red, incubated with IgE, and then activated with DNP-HSA (Figure 5A). Intracellular calcium levels were determined by calculating the ratio of fluorescence intensity of Fluo-3 and Fura-Red. Cells from the $LAT^{-/-}$ mice demonstrated a marked decrease in intracellular calcium elevation, while the heterozygous cells showed an intermediate level. Over five experiments, the mean peak calcium values in fluorescence ratio units from the three cell lines were: +/+, 101.7 ± 10.3; +/−, 72.6 ± 5.2; and −/−, 56.2 ± 4.5. The response of all three cell lines to thapsigargin was equivalent.

Receptor engagement also leads to activation of a number of serine/threonine protein kinases of the mitogen-activated protein (MAP) family. ERK activation is dependent on the Ras pathway (Hirasawa et al., 1995), which is in turn activated by SOS, a protein bound to LAT via the Grb2 linker molecule (Turner et al., 1995; Finco et al., 1998). The activation of JNK is less well characterized in hematopoietic cells. In mast cells, Vav may be in part responsible via activation of SEK1, but other pathways that involve the activation of PKC or the activation of MKK7 are also implicated in JNK activation (Teramoto et al., 1997; Kawakami et al., 1998; Song et al., 1999). Both ERK2 and JNK1 were isolated by immunoprecipitation from activated and nonactivated mast cells from $LAT^{+/+}$, $LAT^{+/-}$, and $LAT^{-/-}$ cell lines. In vitro kinase assays were performed using myelin basic protein and a c-Jun-GST chimeric protein as exogenous substrates for the ERK2 (Figure 5B) and JNK1 assays (Figure 5C), respectively. Enzyme activation was prominent in the wild-type cells, intermediate in the heterozygous, and barely detectable in the $LAT^{-/-}$ cells.

Mast cell effector function was tested first by examining antigen-induced degranulation. The mast cell lines were assayed for granule release of two mediators, β-hexosaminidase (Figure 6A) and serotonin (Figure 6B). For both assays, cells preloaded with IgE were incubated with increasing amounts of antigen. Dose-response curves demonstrated a peak of degranulation at a concentration of 10–100 ng/ml for all the cell lines and for both mediators. However, as was observed in the assays of the intracellular signaling pathways, the amount of mediator that was released depended on the amount of LAT. Activation of LAT-deficient cells resulted in only a low level of granule release, and the $LAT^{+/-}$ cells released an intermediate amount. All three cell lines released equal amounts of β-hexosaminidase in response to 1 μM thapsigargin (Figure 6C).

Cytokine RNA levels were analyzed in $LAT^{+/+}$, $LAT^{+/-}$, and $LAT^{-/-}$ cells 1 hr after FcεRI ligation using RT-PCR. Unstimulated and stimulated populations of all three cell lines contained equal amounts of D-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts, indicating appropriate normalization (Figure 7A). Five cytokines and one chemokine were analyzed, and the requirement for receptor ligation and LAT expression varied for each. For the chemokine MCP-1, transcripts were present at a high level regardless of LAT expression, and little effect of receptor ligation was observed. The generation of the other cytokines was markedly inhibited in the $LAT^{-/-}$ line. Receptor ligation in these cells failed to result in generation of IL-2 or IL-3 message, and production of IL-6, IL-10, and TNF-α mRNA was suppressed. A detailed kinetic analysis of IL-4 RNA levels was also performed (Figure 7B). The response at 30 min was similar, but over time, there was a marked decrease in the amount of IL-4 message produced in the −/− cells.

Direct analysis of cytokine protein confirmed and extended the conclusion that LAT is required for optimal

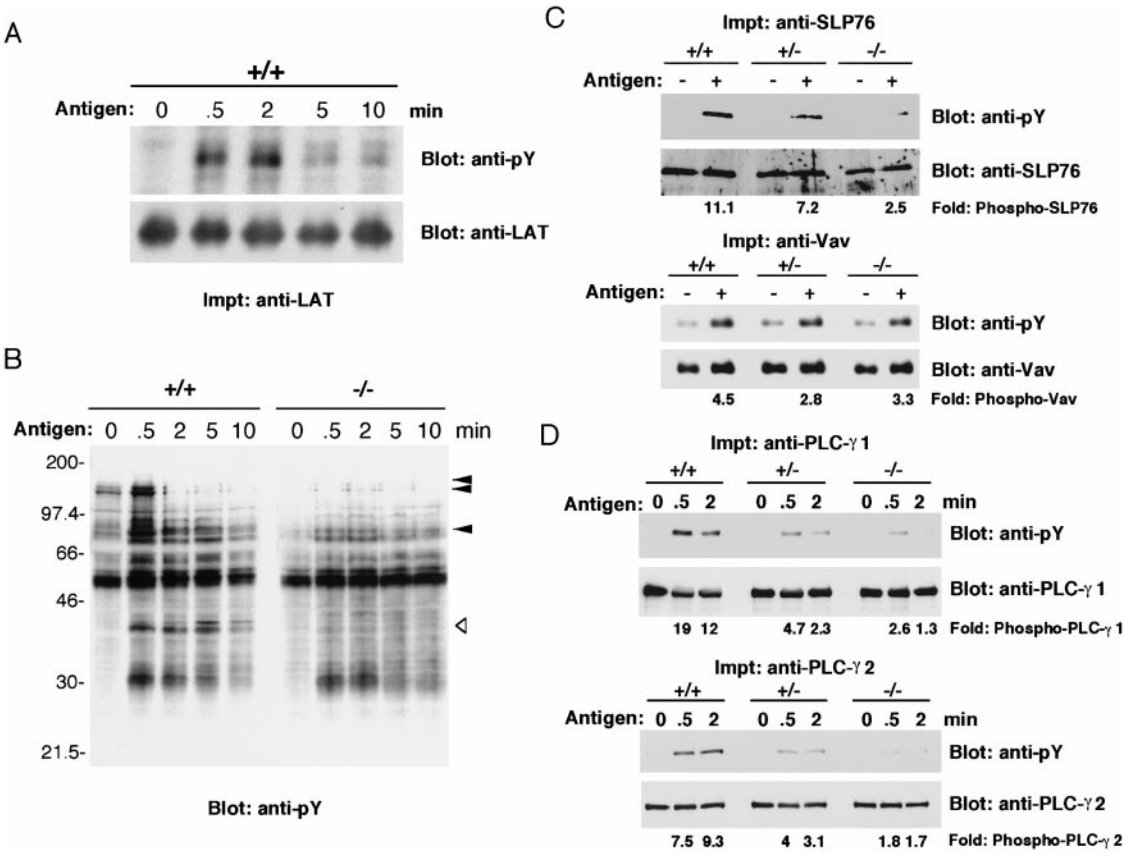

Figure 4. Protein Tyrosine Phosphorylation of LAT and Intracellular Signaling Molecules

(A) $LAT^{+/+}$ BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-HSA for the indicated time. For LAT phosphorylation, lysates from $LAT^{+/+}$ BMMCs were immunoprecipitated with anti-LAT antiserum and then immunoblotted with anti-phosphotyrosine antibody. After stripping, the filter was reprobed with anti-LAT antiserum.

(B) Total cell lysates (2.5×10^5 cell equivalents) were prepared from $LAT^{+/+}$ and $LAT^{-/-}$ BMMCs sensitized with anti-DNP IgE and then stimulated with DNP-HSA for the indicated time. Proteins were resolved on SDS-PAGE, transferred to nitrocellulose membrane filters, and then immunoblotted with anti-phosphotyrosine antibody. Molecular weight (kilodaltons) markers are shown on the left. Arrowheads are defined in the text.

(C and D) Three populations of BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-HSA for 1 min or the indicated time. Lysates from three populations of BMMCs were immunoprecipitated with anti-SLP-76 and anti-Vav (C) and anti-PLC-γ1 and anti-PLC-γ2 (D). Immunoprecipitated proteins were resolved on SDS-PAGE, transferred to nitrocellulose membrane filters, and then immunoblotted with anti-phosphotyrosine antibody. After stripping, the filters were reprobed with anti-SLP-76 and anti-Vav (C) and anti-PLC-γ1 and anti-PLC-γ2 (D) to demonstrate the amount of protein. Densitometry was used to determine the indicated relative intensity levels as in Figure 3.

cytokine production and release (Figures 7C–7F). Levels of four cytokine proteins (IL-2, IL-4, IL-6, and TNF-α) reflected the amount of LAT protein in the cell. Differences in the relative amounts of protein and RNA may reflect differences in stimulation times between the assays.

Discussion

Our data demonstrate that LAT is central to FcεRI-mediated signaling and effector function in mast cells, while not having any obvious role in mast cell development. There was no difficulty growing mast cells from the bone marrow of LAT-deficient mice. The response to IL-3 was intact, and FcεRI-bearing and functional cells were derived at the same rate from the marrow of LAT-deficient and normal mice. Mast cell FcεRI expression levels were also normal. Receptor aggregation led to the same level of proximal PTK activation as demonstrated by β and γ chain and Syk tyrosine phosphorylation. Signaling abnormalities were observed in pathways known to be downstream of PTK activation. Thus, SLP-76 and two isoforms of PLCγ demonstrated a LAT-dependent decrease in levels of phosphotyrosine following activation of the FcεRI. Phosphorylation was partially inhibited in mast cells from heterozygous mice and was further decreased in cells from $LAT^{-/-}$ mice. Decreased tyrosine phosphorylation of PLCγ correlates with a decrease in enzymatic activity, resulting in a decrease in IP3 and diacylglycerol production, which we measured indirectly as a decrease in calcium influx and MAP kinase activation. There is some low level PLC activation in the $LAT^{-/-}$ cells, and this might account for the presence of the minimal receptor-induced calcium elevation. The significance of the lack of optimal SLP-76 tyrosine phosphorylation is not as well understood. The Vav molecule, via

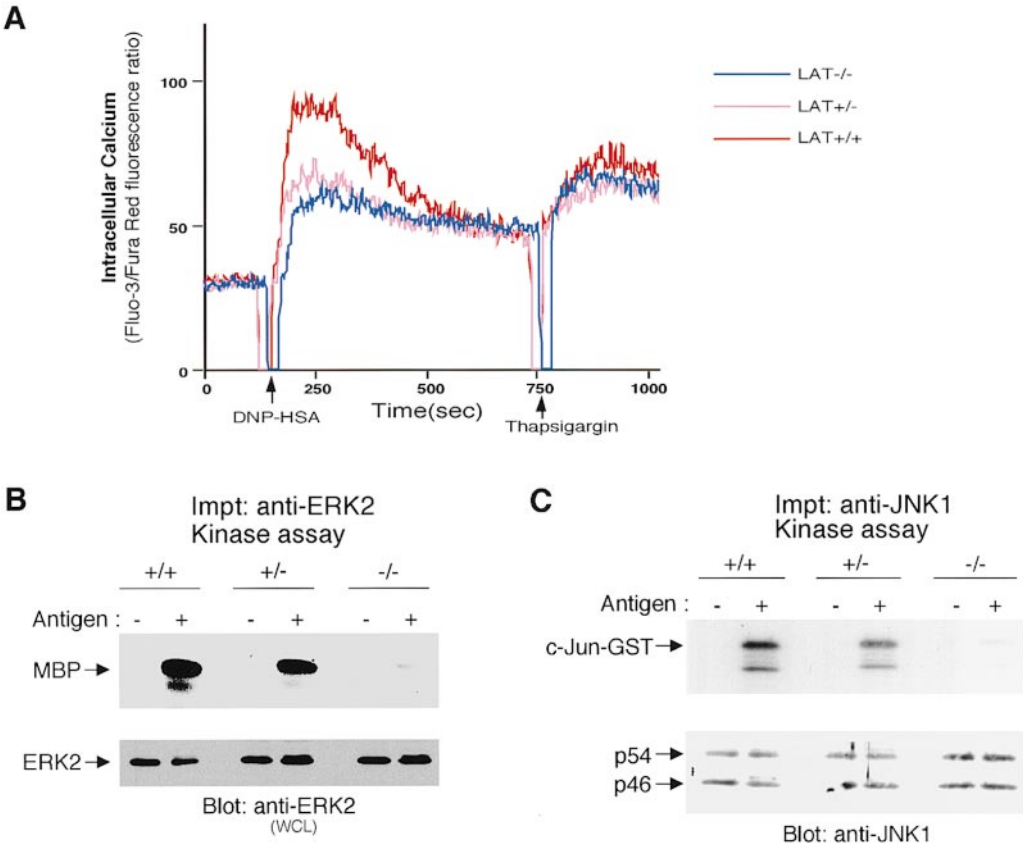

Figure 5. Ca^{2+} Mobilization and MAP Kinase Activities in $LAT^{+/+}$, $LAT^{+/-}$, and $LAT^{-/-}$ BMMCs Following Antigen Stimulation

(A) BMMCs (2×10^6/ml) were loaded with Fluo-3 and Fura Red. BMMCs were sensitized with anti-DNP IgE (1 μg/ml) and then stimulated with DNP-HSA (30 ng/ml) and thapsigargin (1 μM) at the indicated time. Fluo-3 and Fura Red fluorescence intensity after antigen stimulation was measured by flow cytometry. Intracellular Ca^{2+} concentrations were indicated by the ratio of Fluo-3/Fura Red fluorescence intensity.

(B and C) ERK and JNK activities in $LAT^{+/+}$, $LAT^{+/-}$, and $LAT^{-/-}$ BMMCs following antigen stimulation. BMMCs were sensitized with anti-DNP IgE and then stimulated with DNP-HSA. (B) ERK2 kinase activity was measured by in vitro kinase assay. The upper panel shows an autoradiograph of ^{32}P incorporated into myelin basic protein (MBP) following its phosphorylation by ERK2 immunoprecipitates from BMMCs. The lower panel shows the amount of ERK2 protein in the same cell lysates by anti-ERK2 immunoblot. (C) JNK1 kinase activity was measured by in vitro kinase assay. The upper panel shows an autradiograph of ^{32}P incorporated into c-Jun-GST following its phosphorylation by JNK1 immunoprecipitates from BMMCs. The lower panel shows the amount of JNK1 found in the in vitro kinase reaction.

its SH2 domain, has been shown to bind tyrosine-phosphorylated SLP-76 in T cells (Tuosto et al., 1996; Wu et al., 1996). Though Vav tyrosine phosphorylation appeared normal in the LAT-deficient mast cells, a failure of Vav and SLP-76 targeting to LAT in the plasma membrane could impinge on the normal Vav function of small G protein activation. This, in turn, has been demonstrated to contribute to a failure of complete JNK activation (Arudchandran et al., 2000).

Mast cell effector function was markedly impaired in LAT-deficient cells. Secretion of two mediators, serotonin and β-hexosaminidase, was partially inhibited in the heterozygous cells and markedly impaired in the $LAT^{-/-}$ cells. It is notable that a low level of release of both mediators was observed in these cells. This might also reflect the low level of PLC activation and calcium response discussed above. Production of mast cell cytokine message measured at 1 hr and cytokine protein measured at 4 hr was partially to completely inhibited in the $LAT^{-/-}$ cells. There was a good correlation between the decrease in message and protein for the various cytokines.

The genetic approach to analyzing mast cell function has proved to be quite powerful. IL-3 driven cultures of either fetal liver from mice deficient in Syk or bone marrow from SLP-76-deficient mice have also been analyzed. Receptor engagement of Syk-deficient mast cells results in an absence of PLC phosphorylation and calcium flux, Vav phosphorylation (unlike the $LAT^{-/-}$ cells), ERK activation and degranulation. The defects seem more absolute than those seen without LAT (Costello et al., 1996). SLP-76-deficient mast cells seem more like the LAT-deficient cells. There is a marked but not complete inhibition of PLC phosphorylation, calcium flux, and granule release (Pivniouk et al., 1999). These studies of Syk and SLP-76 follow extensive biochemical studies on Syk in mast cells and a few such studies focusing on SLP-76 as a PTK substrate in these cells

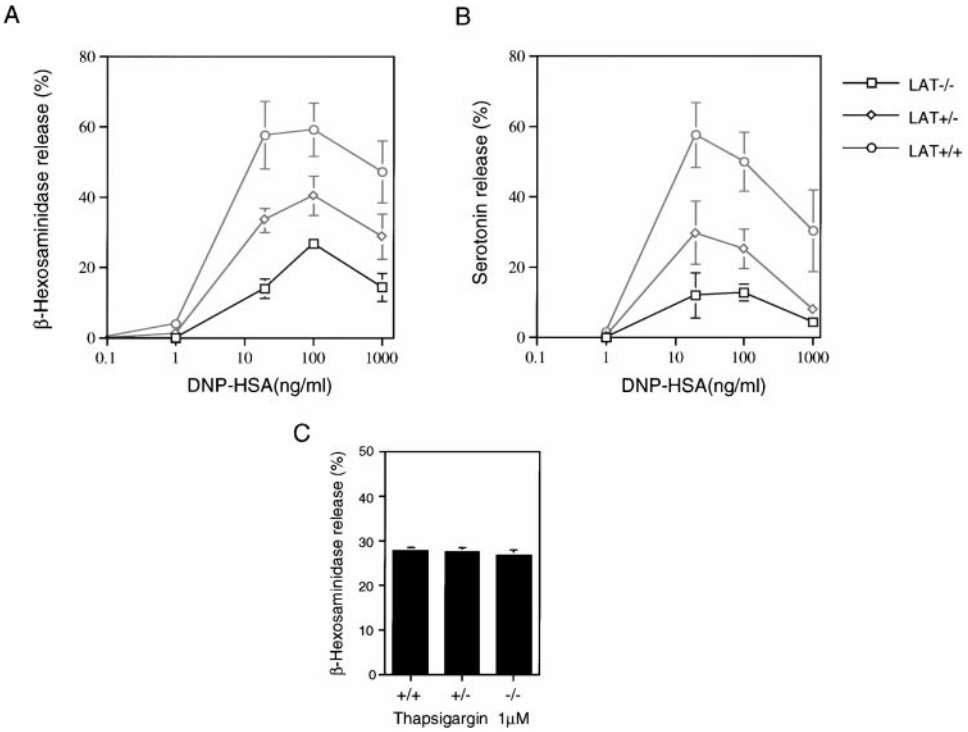

Figure 6. β-Hexosaminidase and Serotonin Release Following Antigen Stimulation of $LAT^{+/+}$, $LAT^{+/-}$, and $LAT^{-/-}$ BMMCs

The extent of degranulation was determined by measuring the release of granular enzyme β-hexosaminidase or serotonin. BMMCs were sensitized for 6 hr with anti-DNP IgE in medium (without IL-3) and then stimulated with the indicated concentration of DNP-HSA.

(A) Percent of β-hexosaminidase activity in supernatants versus total activity. Standard errors reflect four experiments.

(B) The percent release of incorporated [^{3}H]serotonin. The results are reported as net release. Standard errors reflect three experiments.

(C) Cells were incubated with thapsigargin and β-hexosaminidase release was determined. Spontaneous release ranged from 3.9% to 5.2%.

(Oliver et al., 1994; Rivera and Brugge, 1995; Zhang et al., 1996; Hendricks-Taylor et al., 1997). The role of LAT in mast cells had barely been addressed, since reagents to study this molecule did not exist until recently. From these studies and based on work in T cells, additional work on molecules that link enzymes and linker molecules to LAT are likely to be productive. In particular, it had been thought that Grb2 was the only linker bringing most effector molecules to LAT in T cells (Finco et al., 1998; Zhang et al., 1998). The more recent identification of Gads, a linker that couples SLP-76 to LAT in T cells, suggests that this molecule and other Grb2-related linkers need to be examined in mast cells (Feng et al., 1996; Bourette et al., 1998; Liu and McGlade, 1998; Qiu et al., 1998; Liu et al., 1999).

The LAT molecule is expressed in four cell types: T, mast, NK cells, and platelets (Zhang et al., 1998; Facchetti et al., 1999). Studies with cells isolated from the LAT-deficient mice indicate that the significance and function of LAT varies in the four cell types. The loss of LAT affects the T cell lineage most dramatically. There is a complete block in T cell development in mice homozygous for the LAT deletion (Zhang et al., 1999b). Interestingly, one copy of the *LAT* gene is sufficient for normal intrathymic development. The role of LAT in normal T cell function cannot be easily addressed, but two independently derived LAT-deficient variants of the Jurkat cell line show a complete block in signaling via the TCR (Finco et al., 1998; Zhang et al., 1999a). The mast cell defect defined in the current study is not as profound. As with Lyn- and Syk-deficient mice, there appears to be no defect in mast cell development (Hibbs et al., 1995; Costello et al., 1996). Mast cell activation is affected in a LAT concentration–dependent fashion. Most biochemical pathways are not absolutely blocked in the absence of LAT, though the defect is considerable. Of interest is that the only in vivo response that we tested, systemic anaphylaxis measured by serum histamine levels, is completely blocked in the absence of LAT. Platelets from the LAT-deficient mice show a block in signaling via the GPVI receptor (Pasquet et al., 1999). This molecule is associated with Syk and PLC activation and calcium release. Calcium flux in the LAT-deficient platelets does not occur in response to the appropriate peptide ligand, though other signaling pathways mediated by, for example, thrombin receptor are intact. Finally, NK cells seem to be the least affected of the four populations. NK numbers and function appear normal, at least on a population level (Zhang et al., 1999b). It should be noted that in one study, some NK clones were partially blocked by overexpression of dominant-negative LAT protein, suggesting a role for LAT in certain NK cells (Jevremovic et al., 1999). The overall impression from this ongoing work is that the four cell types rely on LAT

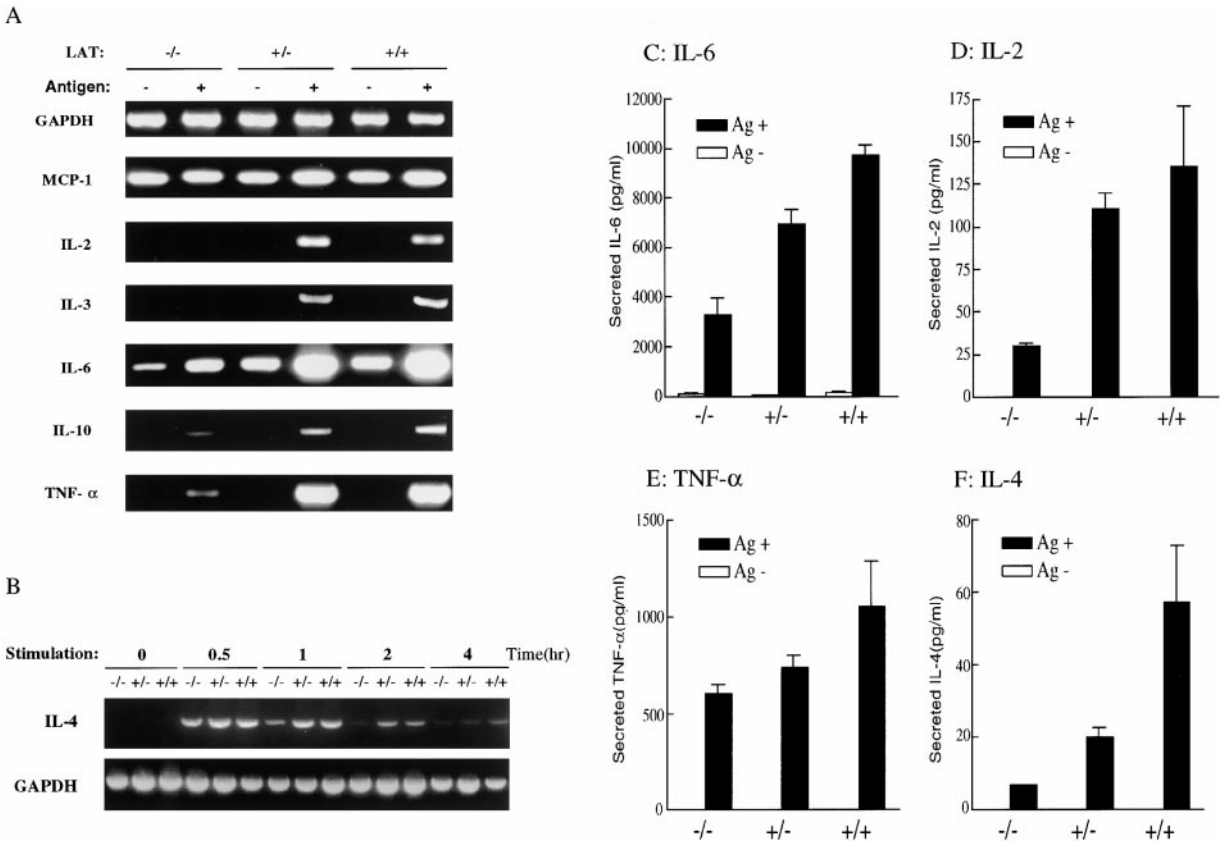

Figure 7. Expression of Cytokine mRNAs and Secretion of Cytokines Following Antigen Stimulation of LAT$^{+/+}$, LAT$^{+/-}$, and LAT$^{-/-}$ BMMCs

(A and B) BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-HSA as described in Experimental Procedures. mRNA was purified from these BMMCs and cytokine mRNAs were detected by RT-PCR using specific primers for each cytokine.

(C–F) Secretion of cytokine following antigen stimulation from LAT$^{+/+}$, LAT$^{+/-}$, and LAT$^{-/-}$ BMMCs. Cytokine levels were measured by ELISA.

as a central linker molecule to varying degrees. Where dependence on LAT is not complete, other mechanisms for bringing signaling molecules to the surface are likely to be found.

Experimental Procedures

Mice

Generation of LAT-deficient mice was reported in detail by Zhang et al. (1999b). Mice were caged in a specific pathogen-free environment in accordance with National Institutes of Health regulations. All mice were of a C57BL/6 and 129/Sv mixed background.

Skin Mast Cell Determination

Naive animals were sacrificed and an area of shaved dorsal skin was removed and fixed in 10% formalin. Slides were made and stained with toluidine blue. The number of blue staining mast cells were counted in the epidermis and dermis using three fields for each slide at 200× magnification. Cell counts did not vary significantly between animals, and thus values were averaged.

Mast Cell Isolation

Bone marrow cells taken from LAT$^{+/+}$, LAT$^{+/-}$, and LAT$^{-/-}$ mouse femurs were incubated in suspension cultures with IL-3 for 4 to 8 weeks. IgE receptor expression level was determined by flow cytometry.

Flow Cytometric Analysis of IgE Receptor Expression

Cells (1×10^6/ml) were stained for FcεRI expression with 2.5 μg/ml anti-DNP IgE (SPE-7 mAb, Sigma), followed by phycoerythrin-conjugated goat anti-mouse Ig (Southern Biotechnology) and analyzed by flow cytometry using a FACScan (Becton Dickinson) (Costello et al., 1996).

Passive Systemic Anaphylaxis

Mice were sensitized for 24 hr with 2 μg of anti-DNP IgE (Liu et al., 1980) by intravenous injection. They were subsequently challenged by intravenous injection of 500 μg of DNP-HSA (Sigma) for 1.5 min, euthanized with CO_2, and blood was immediately collected by cardiac puncture. Serum histamine concentration was determined using a competitive histamine immunoassay kit (Immunotech).

Immunoblotting and Immunoprecipitation

Cells ($2–5 \times 10^7$ in 10 ml) were preloaded for at least 4 hr with anti-DNP IgE (1 μg/ml) in medium (without IL-3). Cells were challenged in Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA) with DNP-HSA (100 ng/ml). Cell lysates for immunoblotting were prepared by direct boiling in reducing sample buffer and were separated on a 12% acrylamide gel. Prior to immunoprecipitation, cells were usually lysed in buffer containing 1% Brij, 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), PMSF, pepstatin, aprotinin, leupeptin, sodium orthovanadate, and EDTA for 30 min on ice. The detergents used for SLP-76 immunoprecipitation were 1% Triton X-100, 1% octylglucoside, and 0.1% SDS. Detergents used to immunoprecipitate FcεRI-γ chain were 0.5% Triton X-100 and 1% octylglucoside.

Antibodies used for immunoprecipitation were anti-LAT antiserum (Zhang et al., 1998), anti-Syk (a gift of U. Blank, Institut Pasteur), anti-FcεRI-β chain (Rivera et al., 1988), anti-FcεRI-γ chain (a gift of R. P. Siraganian, NIH), anti-PLC-γ1 and anti-SLP-76 (Upstate Biotechnology), anti-PLC-γ2 and anti-Vav (Santa Cruz Biotechnology). Antibodies used for immunoblotting were 4G10 and anti-Vav (Upstate Biotechnology), anti-Syk (a gift of P. Draber, Institute of Molecular Genetics, Prague), anti-FcεRI-β chain, and anti-FcεRI-γ chain (Moriya et al., 1997). Immunoprecipitations and immunoblotting were carried out by standard procedures. Reagents used for detection of antibody in immunoblots were donkey-anti-rabbit IgG-horseradish peroxidase (HRP) and sheep-anti-mouse IgG-HRP (Amersham), anti-chicken IgG-HRP (Jackson Immunoreseach Laboratories), and Protein A/Protein G-HRP (Pierce).

Measurement of Calcium Flux

Cells (2×10^6/ml) were incubated with 16 μM Fluo-3-AM and 16 μM Fura Red in Tyrode's buffer at 37°C for 45 min. Cells were preloaded with anti-DNP IgE (1μg/ml) on ice for 1 hr and then kept at room temperature for 30 min to allow cleavage of the AM esters. The cells were washed twice with Tyrode's buffer and analyzed by flow cytometry. Intracellular Ca^{2+} concentration was indicated by Fluo-3/Fura Red fluorescence intensity ratios.

MAP Kinase Assays

For ERK and JNK kinase assays, cells (3×10^6) were preloaded for 2 hr with 1 μg anti-DNP IgE in 1 ml Tyrode's buffer and stimulated with DNP-HSA (100 ng/ml) for 10 min. For ERK kinase assays, ERK2 immunoprecipitated with a rabbit polyclonal serum against ERK2 (Santa Cruz Biotechnology) was used to phosphorylate myelin basic protein (MBP) (Upstate Biotechnology) for 10 min at room temperature. ERK2 levels were detected in lysates with ERK2 antibody. For JNK assays, JNK1 immunoprecipitated with goat polyclonal serum against JNK1 (Santa Cruz Biotechnology) was used to phosphorylate the GST-Jun 5–89 fusion protein (c-Jun-GST) (Upstate Biotechnology) for 10 min at room temperature. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and phosphorylation was detected by autoradiography. To control for JNK1 levels, the membrane was subsequently immunoblotted with anti-JNK1.

Degranulation Assay

The degree of degranulation was determined by measuring the release of β-hexosaminidase and serotonin. Cells (2×10^6/ml) were preloaded for 6 hr with anti-DNP IgE (1 μg/ml) in medium (without IL-3). To measure β-hexosaminidase release, sensitized cells were stimulated with DNP-HSA or thapsigargin for 7 min in Tyrode's buffer. Samples were placed on ice and centrifuged. The enzymatic activities of β-hexosaminidase in supernatants and cell pellets solubilized with 0.5% Triton X-100 in Tyrode's buffer were measured with *p*-nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 60 min at 37°C. The reaction was stopped by the addition of 0.2 M glycine (pH 10.7). The release of the product 4-*p*-nitrophenol was detected by absorbance at 405 nm. The extent of degranulation was calculated by dividing 4-*p*-nitrophenol absorbance in the supernatant by the sum of absorbance in the supernatants and in cell pellets solubilized in detergent. To measure degranulation by release of serotonin, IgE-sensitized cells were preloaded with [^{3}H]serotonin for 6 hr. Washed cells were stimulated with DNP-HSA for 7 min in Tyrode's buffer. Samples were put on ice and centrifuged. Release of [^{3}H]serotonin to the supernatant was measured in triplicate by liquid scintillation and results are described as the percent of degranulation as above.

RT-PCR

Cells (30×10^6) from 5- to 7-week-old cultures were deprived of IL-3 for at least 6 hr to induce a resting state and were incubated with 1 μg/ml of DNP-specific IgE at 3×10^6 cells/ml. Cells were washed once and stimulated with 1 ng/ml DNP-HSA for 1 hr. Total RNA was isolated using the TRI reagent (Molecular Research Center). Cells were homogenized in 1 ml TRI reagent for 15 min at RT and the aqueous layer containing RNA was extracted with chloroform. The RNA was then precipitated with isopropanol. First strand cDNA synthesis was carried out using the Life Technologies Superscript RT-PCR kit. Three to five μg of total RNA was used in reactions primed with oligo dT to obtain cDNA. Then 1–5 μl of the synthesized c-DNA was used as the template for the cytokine mRNA amplification reactions. Primers for amplification of various cytokines were obtained from Clontech and Biosource. All cytokines were assayed with two distinct sets of primers to insure that the obtained product did not result from genomic DNA contamination.

Detection of Cytokines

For detection of IL-6, cells (5×10^6 /ml) were incubated with 1 μg/ml of anti-DNP IgE for 3 hr and stimulated for 24 hr with 30 ng/ml of DNP-HSA. Supernatant was collected and secreted IL-6 was determined using a murine specific IL-6 ELISA detection kit (Endogen). For detection of IL-2, IL-4, and TNF-α, cells (4×10^7) were incubated in 15 ml media with 1 μg/ml of anti-DNP IgE for 14 hr and subsequently stimulated for 4 hr with 30ng/ml of DNP-HSA. After stimulation, the media (15 ml) was recovered and concentrated approximately 50-fold by using Ultrafree-15 Biomax-5 (Millipore). In some experiments, cell pellets were lysed to quantitate the level of intracellular cytokines. Cytokine levels were determined by ELISA as above.

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