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LAT and NTAL Mediate Immunoglobulin E-Induced Sustained Extracellular Signal-Regulated Kinase Activation Critical for Mast Cell Survival[∇]

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Immunoglobulin E (IgE) induces mast cell survival in the absence of antigen (Ag) through the high-affinity IgE receptor, Fcε receptor I (FcεRI). Although we have shown that protein tyrosine kinase Syk and sustained extracellular signal-regulated kinase (Erk) activation are required for IgE-induced mast cell survival, how Syk couples with sustained Erk activation is still unclear. Here, we report that the transmembrane adaptors LAT and NTAL are phosphorylated slowly upon IgE stimulation and that sustained but not transient Erk activation induced by IgE was inhibited in LAT^{-/-} NTAL^{-/-} bone marrow-derived mast cells (BMMCs). IgE-induced survival requires Ras activation, and both were impaired in LAT^{-/-} NTAL^{-/-} BMMCs. Sos was preferentially required for FcεRI signals by IgE rather than IgE plus Ag. Survival impaired in LAT^{-/-} NTAL^{-/-} BMMCs was restored to levels comparable to those of the wild type by membrane-targeted Sos, which bypasses the Grb2-mediated membrane recruitment of Sos. The IgE-induced survival of BMMCs lacking Gads, an adaptor critical for the formation of the LAT–SLP-76–phospholipase Cγ (PLCγ) complex, was observed to be normal. IgE stimulation induced the membrane retention of Grb2-green fluorescent protein fusion proteins in wild-type but not LAT^{-/-} NTAL^{-/-} BMMCs. These results suggest that LAT and NTAL contribute to the maintenance of Erk activation and survival through the membrane retention of the Ras-activating complex Grb2–Sos and, further, that the LAT–Gads–SLP-76–PLCγ and LAT/NTAL–Grb2–Sos pathways are differentially required for degranulation and survival, respectively.

Immunoglobulin E (IgE) binding to its high-affinity receptor, Fcε receptor I (FcεRI), is usually considered to be a passive step called sensitization. However, recent studies have shown that IgE actively promotes several mast cell responses, such as survival, adhesion, and the promotion of *in vivo* inflammatory reactions in the absence of antigen (Ag) (3, 6, 16, 17, 26). Furthermore, high concentrations of IgE (>10 μg/ml) in the plasma of subjects with parasitic infections or atopic diseases have often been observed (18), suggesting a possible Ag-independent contribution of IgE to the exacerbation of these diseases. Recent studies have suggested that IgE may elicit weak FcεRI aggregation to induce mast cell responses, even in the absence of multivalent Ag (22, 23). IgE-induced responses in the presence and absence of Ag are known to utilize distinct signaling pathways; by manipulating the duration of extracellular signal-regulated kinase (Erk) activation, we previously showed that sustained Erk activation is critical for mast cell survival but not for degranulation (52). Although we and others have also found that IgE-induced mast cell survival requires Fc receptor γ-ITAM, Lyn, and Syk (21, 22, 24, 40), the molecular mechanism underlying the coupling of Syk with sustained Erk activation is still unclear.

Many reports have demonstrated that the duration of Erk

activation alters the quality of biological responses (29, 30, 52). In a variety of receptor systems, Erk activation is regulated by Ras through the subsequent activation of Raf and mitogen-activated protein kinase/Erk kinase (MEK). Although Ras is activated by guanine nucleotide exchange factors (GEFs) such as Sos upon growth factor stimulation (7), recent reports have suggested that another GEF, RasGRP, also activates Ras in T-cell receptor (TCR)- or B-cell receptor (BCR)-mediated signaling (10, 32). RasGRP is activated by diacylglycerol, a product of phospholipase Cγ (PLCγ)-mediated hydrolysis (11), providing a new pathway for Ras activation from Ag receptors. However, the contributions of Sos and RasGRP to FcεRI signaling through IgE in the presence and absence of Ag are still unknown.

NTAL (5), also called LAB (15), and LAT are raft-localized transmembrane adaptor molecules possessing multiple tyrosine-based activation motifs, and they are the substrates for Syk (13, 36). Although Grb2 and Gads binding motifs are conserved in both proteins, the binding site for PLCγ is present only in LAT and not in NTAL (5, 8, 14). It has been demonstrated previously that LAT is critical for mast cell activation upon IgE stimulation in the presence of Ag (38). On the other hand, NTAL has been reported to function as a negative regulator as well as a positive regulator of FcεRI signaling in mast cells upon IgE–Ag stimulation (43, 47, 57). The analysis of bone marrow-derived mast cells (BMMCs) has led to the proposal of a possible mechanism for the molecular competition between the two proteins within the lipid raft, which may

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explain how NTAL exerts a negative regulatory function in a LAT-dependent manner (47, 57).

Using BMMCs deficient in both LAT and NTAL, we showed that these two adaptors contribute to IgE-induced sustained Erk activation and survival by acting as a scaffold for the retention of the Grb2-Sos complex within the plasma membrane.

MATERIALS AND METHODS

Mice. LAT^{-/-}, NTAL^{-/-}, and LAT^{-/-} NTAL^{-/-} mice with a C57BL/6 background have been described previously (47). Syk^{-/-} mice with a BALB/c background were provided by V. L. J. Tybulewicz (24). Gads^{-/-} mice with a C57BL/6 and 129 mixed genetic background have been described previously (56). All mice were maintained under specific-pathogen-free conditions in accordance with institutional guidelines.

Abs. The mouse anti-dinitrophenol IgE antibody (Ab) H1-DNP-ε-26 was kindly provided by F.-T. Liu and T. Kawakami (La Jolla Institute, Ontario, CA) and used for survival assays. The mouse antidinitrophenol IgE Ab SPE-7 was purchased from Sigma (St. Louis, MO) and used for survival assays and biochemistry analyses. For IgE stimulation, each IgE clone was ultracentrifuged at 100,000 × g for 10 min just before use in the assays in order to exclude aggregates, as described previously (24, 33). Anti-phospho-Erk Ab was purchased from Promega (Madison, WI), anti-phospho-Syk was from Cell Signaling Technology (Beverly, MA), antiactin was obtained from Sigma, anti-phospho-PLCγ was from Biosource International (Camarillo, CA), anti-Ras was purchased from Pierce Biotechnology (Rockford, IL), anti-green fluorescent protein (anti-GFP) was obtained from Invitrogen (Carlsbad, CA), anti-GFP-horseradish peroxidase (HRP) was from Miltenyi Biotec (Bergisch, Gladbach, Germany), anti-mouse IgG-HRP and protein G-Sepharose were purchased from Amersham Biosciences (Piscataway, NJ), anti-rabbit Ig-HRP was from Zymed (San Francisco, CA), and anti-Sos Ab was obtained from BD Pharmingen (San Diego, CA). Anti-mCD63 monoclonal Ab, which has been proven to be specific to CD63 by using CD63 transfectant, was kindly provided by K. Nishida (RIKEN, Yokohama, Japan). Anti-NTAL Abs were kindly provided by V. Horejsi (Academy of Science of the Czech Republic, Prague).

Construction. To construct the Grb2-GFP fusion, the Grb2 gene was fused to that for GFP by PCR. Genes for Ras^{N17} and farnesylated Sos, which were kindly provided by K. Nishida (RIKEN, Yokohama, Japan) and A. Aronheim (Israel Institute of Technology, Haifa, Israel) (1), respectively, were cloned into the pMX-IRES-hCD8 (53) or pMX-IRES-GFP retroviral vector. Glutathione S-transferase (GST)-Raf-Ras binding domain (RBD) was kindly provided by J. L. Bos (University Medical Center Utrecht, Utrecht, The Netherlands) (9). For the construct comprising the proline-rich domains of Sos (Sos-Pro), the region of the Sos gene corresponding to amino acids 1,019 to 1,336 was amplified by PCR and subcloned into pMX-IRES-GFP (20).

Retroviral infection and BMMC induction. The preparation of BMMCs and fetal liver-derived mast cells and the retroviral infection of BMMCs were carried out as previously described (24, 52). Adherent cells were removed every 3 to 5 days. After 4 weeks, homogeneous populations of FcεRI-positive, c-kit-positive BMMCs were obtained. For the infection of differentiated BMMCs, cells were cultured with 100 ng of stem cell factor/ml and 30 ng of interleukin-3 (IL-3)/ml for 12 h and then spin infected with concentrated virus supernatant.

Western blotting. BMMCs were cultured without IL-3 for 2 h and then stimulated with IgE (SPE-7) at 37°C for the times indicated below. Cells were lysed in lysis buffer containing 1% Nonidet P-40 and subjected to Western blotting as previously described (52).

Survival assays. BMMCs (2 × 10⁵) were washed with IL-3-free medium twice and then stimulated with IgE (H1-DNP-ε-26 and SPE-7) in 400 μl of IL-3-free medium on 48-well plates. Forty-microliter samples of the cultured cells were taken daily and stained with propidium iodide, and live cells were counted with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Live-cell gating was performed on FL3 (propidium iodide)-side-scatter dot plots, which can separate out live cell populations clearly.

Real-time reverse transcription-PCR (RT-PCR). BMMCs were incubated in the absence of IL-3 for 1 h and then stimulated with IgE for 1 h. RNA was isolated by using an RNeasy minikit (QIAGEN, Valencia, CA). After the removal of genomic DNA by treatment with DNase (Wako Nippon Gene, Tokyo, Japan), randomly primed cDNA strands were generated with reverse transcriptase II (Invitrogen). By using gene-specific primers, RNA expression was quantified by real-time PCR, and values were normalized to β-actin expression.

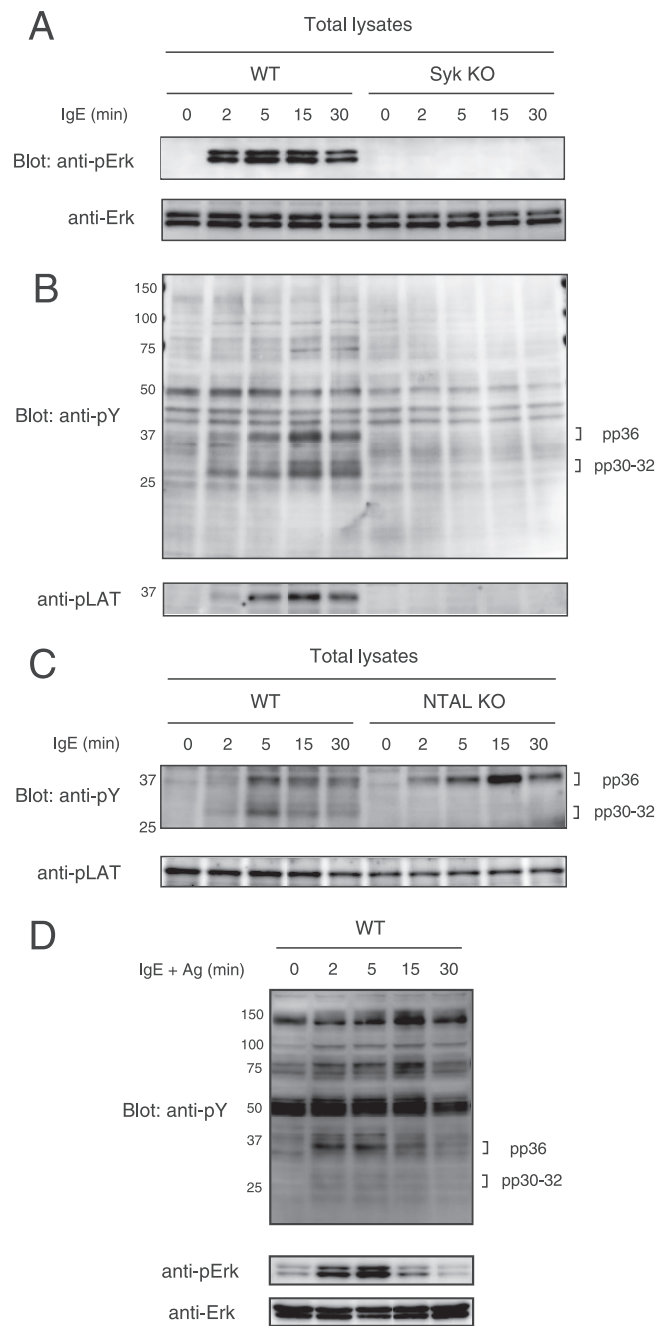


FIG. 1. LAT and NTAL are phosphorylated by IgE (without Ag) and are required for sustained Erk activation. (A and B) Syk^{+/+} (WT) and littermate Syk^{-/-} (Syk KO) fetal liver-derived mast cells were stimulated with 10 μg of IgE/ml for the indicated periods, and total cell lysates were blotted with antibodies to phospho-Erk (anti-pErk) and Erk (A) and phosphotyrosine (anti-pY) and phospho-LAT (anti-pLAT) (B). Brackets on the right indicate LAT (upper) and NTAL (lower). Numbers on the left are molecular size markers. (C) C57BL/6 (WT) and NTAL^{-/-} (NTAL KO) BMMCs were stimulated as described above. Total cell lysates were blotted with antiphosphotyrosine. (D) IgE (in the presence of Ag) induces transient phosphorylation and Erk activation. BMMCs were sensitized with 1 μg of IgE/ml for 4 h and then stimulated with 15 ng of DNP-HSA/ml. Cell lysates were blotted with antibodies to phosphotyrosine, phospho-Erk, and Erk.

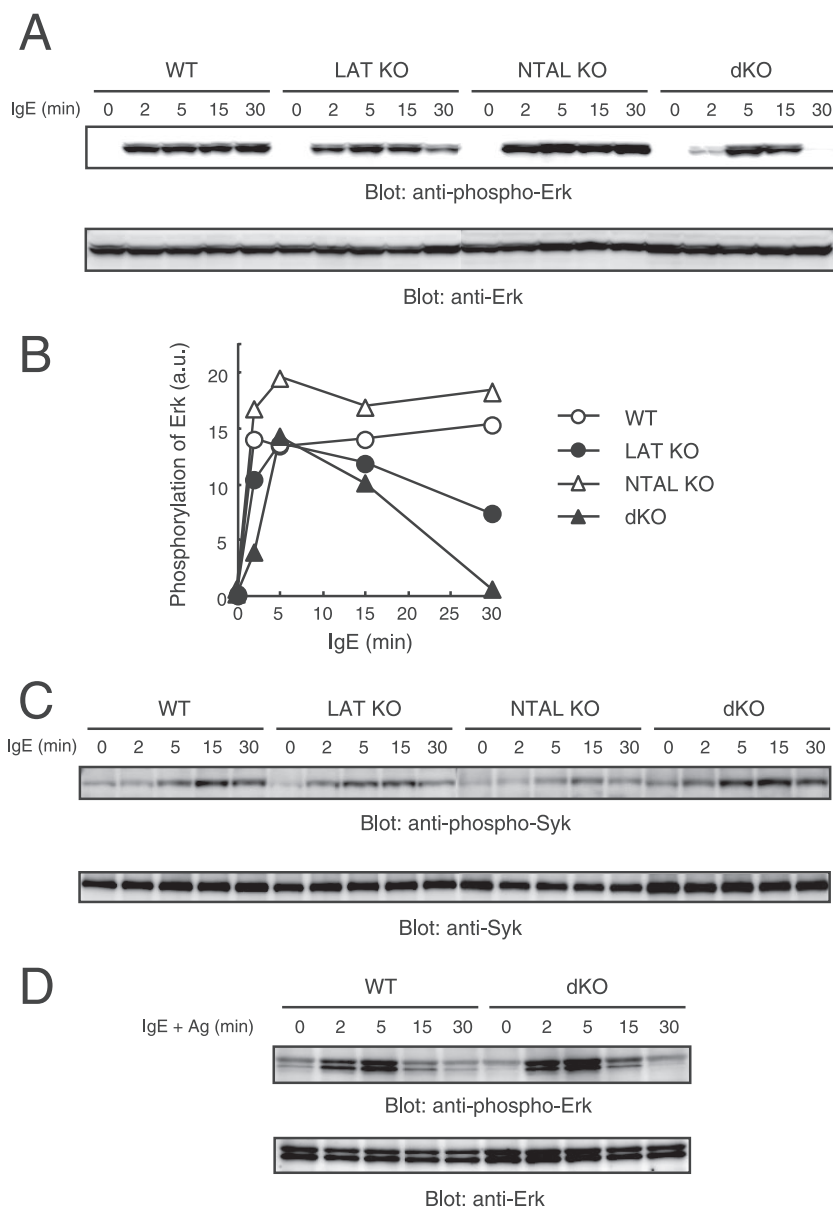


FIG. 2. LAT and NTAL are required for sustained Erk activation. (A) Activation of Erk upon IgE stimulation. C57BL/6 (WT), $LAT^{-/-}$ (LAT KO), $NTAL^{-/-}$ (NTAL KO), and $LAT^{-/-} NTAL^{-/-}$ (dKO) BMMCs were stimulated with 10 μ g of IgE/ml, and total cell lysates were immunoblotted with anti-phospho-Erk and anti-Erk. (B) Quantitative results of the analysis of phospho-Erk described in the legend to panel A. The intensity of each band shown in panel A was quantified, and the values are expressed as arbitrary units (a.u.). (C) Activation of Syk upon IgE stimulation. BMMCs were treated as described in the legend to panel A and blotted with anti-phospho-Syk and anti-Syk. (D) Activation of Erk upon Ag cross-linking. WT and dKO BMMCs were stimulated as described in the legend to Fig. 1D and blotted with anti-phospho-Erk and anti-Erk. Data are representative of findings from three independent experiments with similar results.

Gene-specific primer sequences were as follows: β -actin gene, 5'-TGG AAT CCTGTGGCATCCATGAAAC-3' (forward) and 5'-TAAAACGCAGCTCA GTAACAGTCCG-3' (reverse); IL-3 gene, 5'-ATAGGGAAGCTCCCAGA ACCTGAACTC-3' (forward) and 5'-AGACCCCTGGCAGCGCAGAGTCA TTC-3' (reverse); and histidine decarboxylase gene, 5'-AGTCTGGCGAGA AGGGAAGG-3' (forward) and 5'-TCTGGGCACTCATAGGCACA-3' (reverse).

Pulldown assay. BMMCs were lysed with lysis buffer (Ras activation kit; Pierce, Rockford, IL). Cell lysates were incubated with GST-Raf-RBD-bound glutathione-Sepharose for 1 h at 4°C. After the samples had been washed five times with lysis buffer, Sepharose-bound protein was eluted with 10 mM glutathione and analyzed by Western blotting with anti-Ras Ab.

Flow cytometric analysis of degranulation. Degranulation was analyzed by flow cytometry as described previously (31). Briefly, BMMCs were sensitized with 1 μ g of IgE (SPE-7)/ml for 4 h and then stimulated with 50 ng of dinitrophenol-conjugated human serum albumin (DNP-HSA)/ml for 30 min in Tyrode's buffer. Cells were fixed with 4% paraformaldehyde for 15 min and then stained with anti-CD63 Ab and phycoerythrin-conjugated goat anti-mouse IgG (heavy- and light-chain) Fab (Cedarlane, Ontario, Canada). The surface expression of CD63 was determined by flow cytometry.

Microscopic analysis. BMMCs were infected with pMX-Grb2/GFP-IRES-hCD8. After being sorted with anti-human CD8 (anti-hCD8), the cells were stimulated with immobilized IgE (SPE-7; 10 μ g/ml) on a glass-coated dish (Mat-Tech, Ashland, MA). After 30 min of incubation at 37°C, the cells were analyzed

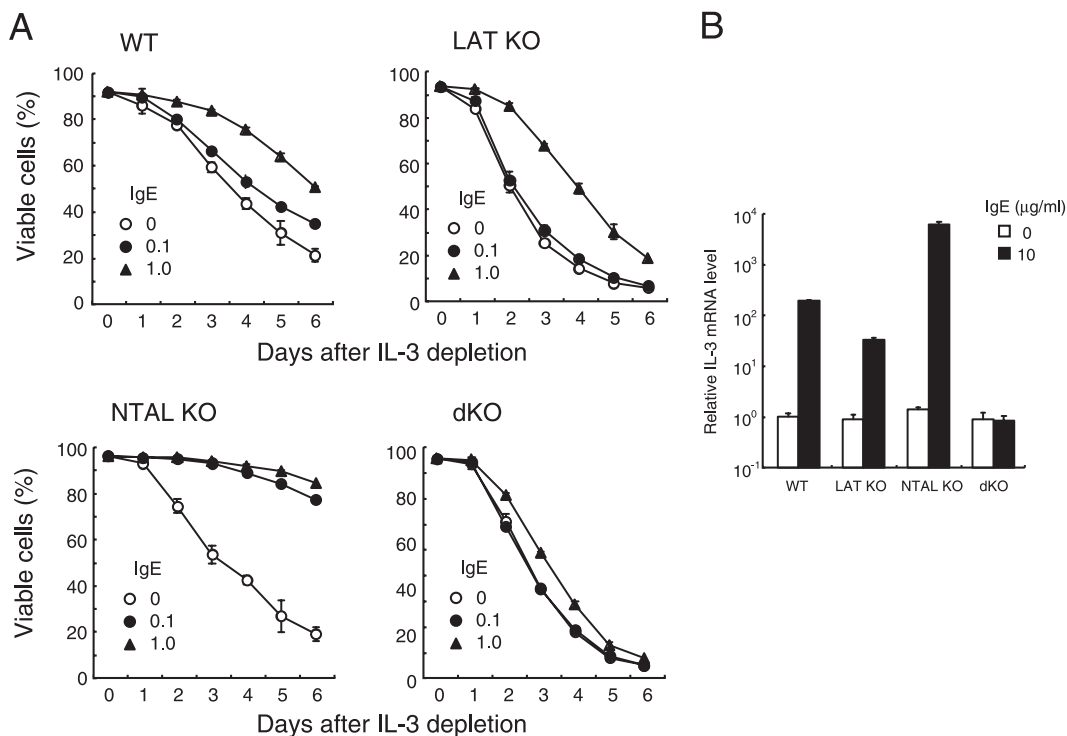


FIG. 3. LAT and NTAL are required for IgE-induced survival and IL-3 production. (A) IgE-induced survival of C57BL/6 (WT), LAT^{-/-} (LAT KO), NTAL^{-/-} (NTAL KO), and LAT^{-/-} NTAL^{-/-} (dKO) BMMCs. Cells were stimulated with 1 μg of IgE/ml in the absence of IL-3, and cell viability at the indicated periods was determined by propidium iodide staining. Data are means ± standard deviations (SD) of results from triplicate assays and are representative of findings from four independent experiments with similar results. (B) IL-3 mRNA expression. BMMCs were incubated without IL-3 for 1 h and were left unstimulated or were stimulated with IgE for 1 h. RNA was isolated, and levels of expression of IL-3 mRNA were determined by real-time RT-PCR. Levels of mRNA expression are relative to that in unstimulated WT BMMCs.

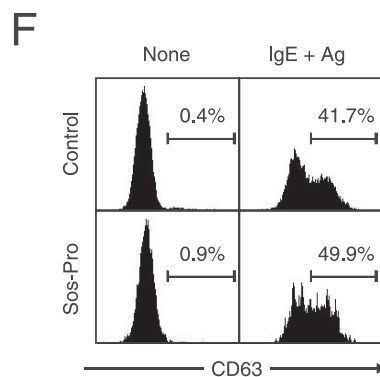
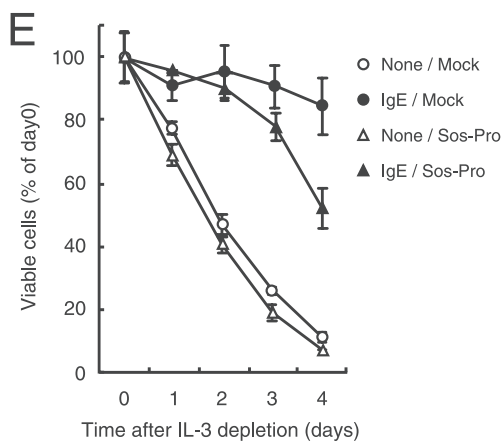
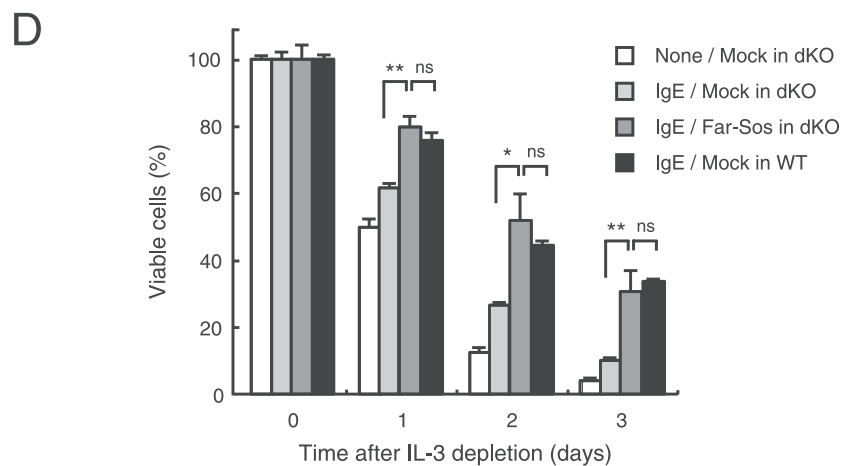
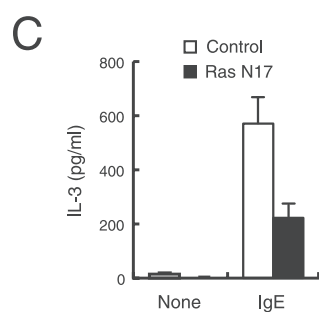
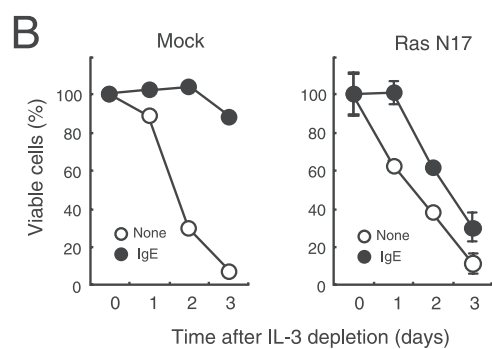
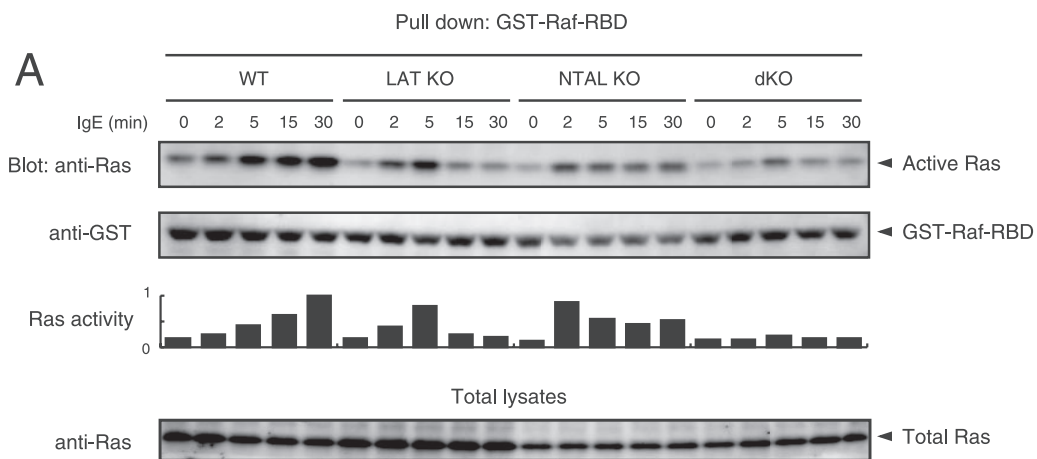
by wide-field fluorescence microscopy with an IX-81 instrument (Olympus, Tokyo, Japan). The membrane localization of Grb2-GFP was assessed by total internal reflection fluorescence (TIRF) microscopy with an IX81-ARCEVA system (Olympus, Tokyo, Japan).

RESULTS

LAT and NTAL are required for sustained Erk activation by IgE in the absence of Ag. IgE induces sustained Erk activation in mast cells in the absence of Ag (16, 52), whereas Erk activation in Syk^{-/-} mast cells was totally abolished (Fig. 1A, right lanes). To assess how Syk couples with sustained Erk activation upon IgE stimulation in the absence of Ag, we first examined the total tyrosine phosphorylation pattern induced in mast cells by IgE treatment without Ag. The phosphorylation of two major proteins, pp36 and pp30-32, was observed upon IgE treatment with slow kinetics (Fig. 1B, upper panel). These phospho-proteins were not detected in Syk^{-/-} mast cells (Fig. 1A, right lanes), suggesting that they are substrates or are located downstream of Syk. pp36 was likely to be LAT, because the anti-phospho-LAT blot exhibited similar results (Fig. 1B, lower panel), whereas pp30-32 seemed to correspond to NTAL on the basis of its gel mobility pattern (5, 15, 47) and the anti-NTAL immunoblot (data not shown). Indeed, pp30-32 was completely absent in NTAL^{-/-} BMMCs (Fig. 1C). These results are consistent with the report that LAT and NTAL are expressed in mast cells and are substrates for Syk (5). The phosphorylation of these two adaptors was also elicited, albeit

to a lesser extent in the case of NTAL, upon IgE stimulation in the presence of Ag (Fig. 1D) as reported previously (47, 57). However, the levels of phosphorylation of these adaptors rapidly declined after 5 min (Fig. 1D). Thus, LAT and NTAL are phosphorylated with slow kinetics upon IgE stimulation in the absence of Ag, in sharp contrast to the transient phosphorylation upon IgE stimulation in the presence of Ag (57).

Next, to examine the role of LAT and NTAL in IgE-induced sustained Erk activation, we stimulated BMMCs from wild-type (WT), LAT^{-/-}, NTAL^{-/-}, and LAT^{-/-} NTAL^{-/-} (double knockout [dKO]) mice with IgE in the absence of Ag. The absence of LAT and/or NTAL did not alter the level of surface expression of FcεRI in BMMCs (data not shown), consistent with results in previous reports (47, 57). We compared the kinetics of downstream signaling in these BMMCs after stimulation by IgE in the absence of Ag (Fig. 2A, B, and C) and in the presence of Ag (Fig. 2D). Although LAT plays a critical role in IgE-induced mast cell responses in the presence of Ag (38), sustained Erk activation by IgE in LAT^{-/-} BMMCs was slightly reduced but still observed. In contrast, dKO BMMCs showed severe impairment of sustained Erk activation, although transient activation was almost normal (Fig. 2A and B). A minor role for LAT and NTAL in transient Erk activation was also evident upon FcεRI stimulation by Ag cross-linking (Fig. 2D) (57). The intensity of Erk activation in NTAL^{-/-} BMMCs was augmented (Fig. 2A and B), consistent with the reports that NTAL negatively regulates mast cell responses



induced by IgE in the presence of Ag (47, 57). The level of Syk phosphorylation, as an upstream event, in dKO BMMCs was not decreased (Fig. 2C). These results indicate that IgE-induced Erk activation can be triggered but not sustained in the absence of LAT and NTAL even when FcεRI engagement and Syk activation are prolonged.

LAT and NTAL are required for IgE-induced mast cell survival and IL-3 induction. We then analyzed the contribution of LAT and NTAL to IgE-induced survival. The survival of LAT^{-/-} BMMCs was slightly impaired, but that of LAT^{-/-}NTAL^{-/-} BMMCs was almost completely inhibited (Fig. 3A), an observation that was well correlated with the degree of sustained Erk activation (Fig. 2). We have previously shown that autocrine IL-3 production plays a critical role in IgE-induced mast cell survival (24). Indeed, the induction of IL-3 mRNA in LAT^{-/-}NTAL^{-/-} BMMCs was greatly impaired (Fig. 3B). Furthermore, other events elicited by IgE stimulation in the absence of Ag, such as IL-6 production and histidine decarboxylase induction, were also blocked in LAT^{-/-}NTAL^{-/-} BMMCs (data not shown). Taken together, these results show that NTAL and LAT are crucial for IgE-mediated IL-3 induction and mast cell survival in the absence of Ag. The results were consistent when another IgE clone, SPE-7, was used (data not shown).

Ras activation through LAT and NTAL is critical for mast cell survival. We next examined how sustained Erk activation was maintained through LAT and NTAL. The activation of MEK, a kinase upstream of Erk, in LAT^{-/-}NTAL^{-/-} BMMCs was also impaired (data not shown). Since both LAT and NTAL are capable of binding Grb2, an event that potentially leads to MEK activation through Sos and Ras (5), we investigated Ras activation upon IgE stimulation in the absence of Ag. IgE binding induced sustained Ras activation in WT BMMCs, whereas the activation in LAT^{-/-}NTAL^{-/-} BMMCs was greatly diminished (Fig. 4A). To examine the role of Ras activation in IgE-induced survival, a dominant negative form of Ras (Ras^{N17}) was introduced into WT BMMCs. Ras^{N17} markedly suppressed IgE-induced BMMC survival and IL-3 production (Fig. 4B and C), although the rates of exogenous IL-3-induced survival did not differ between mock- and Ras^{N17}-transduced cells (data not shown). These results suggest that

LAT and NTAL regulate IgE-induced survival through sustained Ras activation.

We next analyzed the involvement of Sos in survival through Ras activation. If LAT and NTAL were to serve as a platform for the sustained localization of the Grb2-Sos complex within the plasma membrane, the existence of a membrane-targeted form of Sos would bypass the requirement for LAT and NTAL. Indeed, the expression of farnesylated Sos was able to abolish the defect in IgE-induced survival among LAT^{-/-}NTAL^{-/-} BMMCs and restore the survival rates to levels comparable to those of WT BMMCs (Fig. 4D). On the other hand, if Sos was required for IgE-induced survival, then Sos-Pro, which is known to block Grb2-Sos interaction (46), would suppress this event. Indeed, Sos-Pro suppressed mast cell survival induced by IgE in the absence of Ag (Fig. 4E). Collectively, sustained Ras activation by the prolonged membrane targeting of Sos through LAT and NTAL seems to be necessary and sufficient for mast cell survival.

To verify if Sos also contributes to the IgE-mediated signal in the presence of Ag, we examined the effect of Sos-Pro on degranulation induced by IgE in the presence of Ag by analyzing CD63 expression on the cell surface as a marker for degranulation (31). Surprisingly, the levels of induction of surface CD63 expression in GFP-negative (control) and GFP-positive (Sos-Pro) cells were similar (Fig. 4F). Thus, in the absence of Ag the Sos-Ras pathway is preferentially required for IgE-induced events as a downstream component of LAT and NTAL.

The Gads-mediated pathway is not essential for IgE-induced mast cell survival. In addition to Grb2, LAT and NTAL can bind Gads, an adaptor molecule that is similar to Grb2 and contains Src homology 3 (SH3)-SH2-SH3 domains. Gads is essential for linking LAT and SLP-76 (2) and is critical for TCR-mediated PLCγ1 activation and Ca²⁺ influx (56). It was recently reported that PLCγ also contributes to Ras activation through RasGRP, a diacylglycerol-dependent GEF. To examine the contribution of the Gads-mediated pathway to IgE-induced responses in the absence of Ag, we analyzed Gads^{-/-} BMMCs. PLCγ activation in Gads^{-/-} BMMCs was impaired, as expected (Fig. 5A). However, levels of sustained Erk activation, Ras activation, and mast cell survival in Gads^{-/-} BMMCs were observed to be comparable to those in WT BMMCs (Fig. 5). Thus, although LAT and NTAL

FIG. 4. NTAL and LAT mediate sustained Ras activation critical for survival induction. (A) Activation of Ras upon IgE stimulation. BMMCs were cultured in the absence of IL-3 for 2 h at 37°C, followed by stimulation with 10 μg of IgE/ml. Total cell lysates were pulled down with GST-Raf-RBD, and the amount of activated Ras was determined by immunoblotting with anti-Ras Ab (upper panel) and anti-GST to confirm the amount of GST protein used (middle panel). The ratio of the intensity of anti-Ras to that of anti-GST was calculated and expressed as Ras activity (bar chart). Total lysates were also subjected to immunoblotting with anti-Ras Ab (bottom panel). LAT KO, LAT^{-/-} BMMCs; NTAL KO, NTAL^{-/-} BMMCs. (B) Effect of dominant negative Ras on IgE-induced survival of BMMCs. Mature WT BMMCs were infected with the pMX-IRES-GFP vector alone (mock) or dominant negative Ras (Ras N17). After 2 days, cells were left unstimulated (open circles) or were stimulated with 1 μg of IgE/ml (closed circles) in the absence of IL-3 and the viability of GFP-positive BMMCs was determined by flow cytometry. For each treatment, data are expressed as the percentages of viable cells relative to the population of viable cells at day 0. Means ± SD of results from triplicate assays are shown. (C) Dominant negative Ras suppresses IgE-induced IL-3 production. WT BMMCs were infected with pMX-RasN17-IRES-hCD8. hCD8⁻ (control) and hCD8⁺ cells were sorted and stimulated with 10 μg of IgE/ml for 4 h. (D) Effect of membrane-targeted Sos on IgE-induced survival. WT and dKO BMMCs were infected with the pMX-IRES-GFP vector only (mock) or farnesylated Sos (Far-Sos), and survival upon IgE stimulation was assessed as described in the legend to panel B. Data are means ± SD of results from triplicate assays. ns, not significant; *, *P* < 0.05; **, *P* < 0.01 (Student's *t* test). (E) Effect of dominant negative Sos (Sos-Pro) on IgE-induced survival. WT BMMCs were infected with the pMX-IRES-GFP vector alone (mock) or dominant negative Sos, and survival induced by 1 μg of IgE/ml was assessed. Data are means ± SD of results from triplicate assays. (F) Effect of dominant negative Sos on degranulation by IgE (with Ag). WT BMMCs infected with pMX-Sos-Pro-IRES-GFP were left unstimulated or were stimulated with IgE and DNP-HSA. The surface expression of CD63 was determined for GFP-negative (control) and GFP-positive (Sos-Pro) populations from the same culture. Data are representative of findings from three independent experiments with similar results.

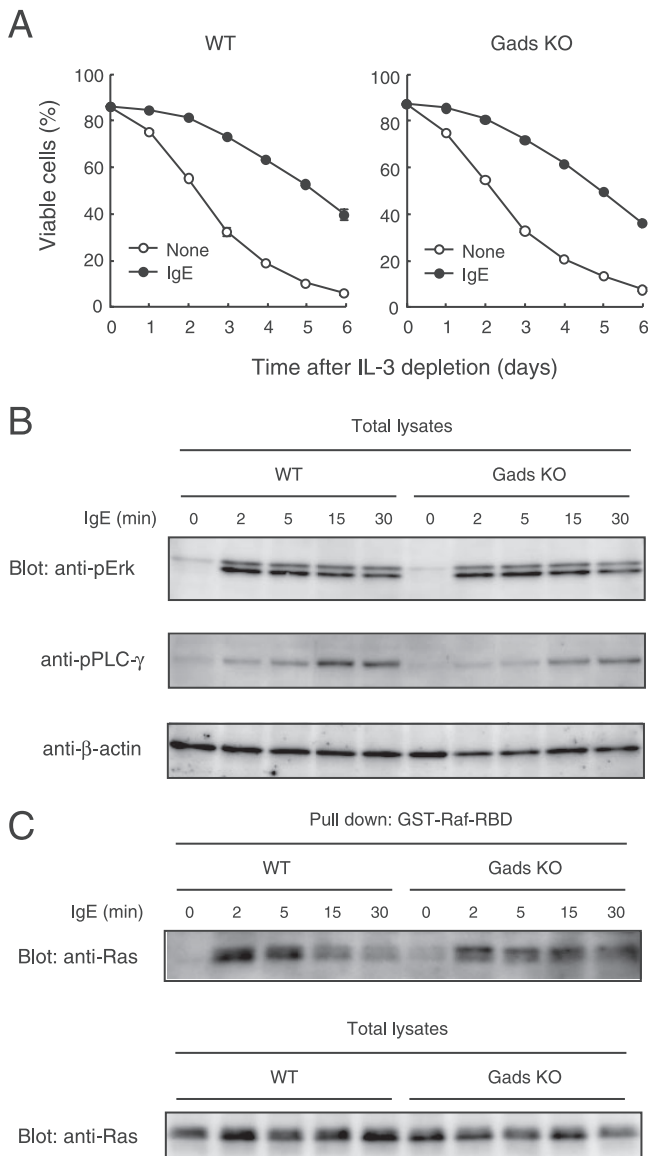


FIG. 5. The Gads-mediated PLC γ pathway is not required for sustained Erk activation and survival. (A) IgE-induced survival of Gads $^{-/-}$ BMMCs. WT and Gads $^{-/-}$ BMMCs were stimulated with 1 μ g of IgE/ml in the absence of IL-3, and cell viability was determined as described in the legend to Fig. 3A. Data are means \pm SD of results from triplicate assays and are representative of findings from three independent experiments with similar results. (B) Phosphorylation of Erk and PLC γ upon IgE stimulation. WT and Gads $^{-/-}$ (Gads KO) BMMCs were stimulated with 10 μ g of IgE/ml, and total cell lysates were immunoblotted with anti-phospho-Erk (anti-pErk) and anti-phospho-PLC γ Abs. Actin was also detected with anti-actin Ab (lower panel). (C) Activation of Ras upon IgE stimulation. WT and Gads $^{-/-}$ BMMCs were stimulated with IgE, and the amount of activated Ras was determined in a manner similar to that described in the legend to Fig. 4A.

can potentially bind both Grb2 and Gads, Gads seems to play a minor role in IgE-induced sustained Erk activation and mast cell survival in the absence of Ag. In contrast, we recently found that IgE-induced degranulation in the presence of Ag in Gads $^{-/-}$ mice is impaired (S. Yamasaki, O. Kanagawa, and T. Saito, unpublished data).

LAT and NTAL are required for membrane targeting of Grb2. Finally, to confirm the contribution of LAT and NTAL to the retention of the Grb2-Sos complex close to the inner leaflet of the plasma membrane, we visualized the intracellular trafficking of Grb2 by IgE by using Grb2-GFP fusion protein. Grb2, through its SH3 domain, is constitutively associated with Sos (35), and Grb2-GFP retained a strong affinity for endogenous Sos in mast cells (Fig. 6A). WT and LAT $^{-/-}$ NTAL $^{-/-}$ BMMCs were retrovirally transfected with Grb2-GFP, and intracellular trafficking was analyzed by fluorescence microscopy. We have previously shown that plate-coated IgE stimulation can deliver a sustained signal similar to that of soluble IgE in the absence of Ag (52). Stimulation on a plate coated with IgE induced drastic membrane localization of Grb2-GFP within 30 min (Fig. 6B). To analyze the cell surface area more precisely on an evanescent excitation field, we employed TIRF microscopy, because it enables a selective visualization of the plasma membrane (4, 44). The recruitment of Grb2-GFP to the plasma membrane was clearly observed upon IgE stimulation (Fig. 6C, upper panels). In sharp contrast, Grb2-GFP in LAT $^{-/-}$ NTAL $^{-/-}$ BMMCs did not show membrane translocation (Fig. 6C, lower panels). Importantly, equal levels of Grb2-GFP expression in WT and LAT $^{-/-}$ NTAL $^{-/-}$ BMMCs were confirmed by flow cytometry (data not shown). These results suggest that LAT and NTAL provide an essential scaffold for the IgE-induced membrane retention of the Grb2-Sos complex.

DISCUSSION

We investigated the molecular mechanism of IgE-induced sustained Erk activation and mast cell survival. Many reports have demonstrated that the duration of Erk activation alters the quality of the biological response (29, 30, 52). By manipulating the duration of Erk activation, we showed previously that sustained, not transient, Erk activation is critical for mast cell survival (52). However, how the signal duration is physiologically regulated remains to be elucidated. It has been reported previously that the duration of Erk activation is determined by multiple mechanisms, such as receptor internalization and the operation of a feedback inhibitory mechanism (34, 41). The weak cross-linking of Fc ϵ RI without internalization may induce sustained Erk activation through prolonged Fc ϵ RI ligation (23, 52, 54). Nevertheless, even when Fc ϵ RI engagement and Syk activation were prolonged, sustained Erk activation in LAT $^{-/-}$ NTAL $^{-/-}$ BMMCs was almost completely inhibited, suggesting that the two adaptors LAT and NTAL constitute some kind of timer for downstream signaling. Note that transient Erk activation was not dramatically impaired by IgE stimulation in the absence of Ag (Fig. 2A and B) and in the presence of Ag (Fig. 2D) (57) in the absence of LAT and NTAL. This result suggests that transient Erk activation via Fc ϵ RI is LAT- and NTAL-independent and that the weak and prolonged cross-linking of Fc ϵ RI may preferentially utilize LAT and NTAL for sustained Erk activation, eventually leading to mast cell survival. However, so far we cannot directly exclude the possible contribution of another pathway to sustained Erk activation, such as the activation of Rap1, B-Raf, or protein kinase C, as reported previously (19, 41, 45).

Oh-hora et al. showed the differential requirements for Ras-

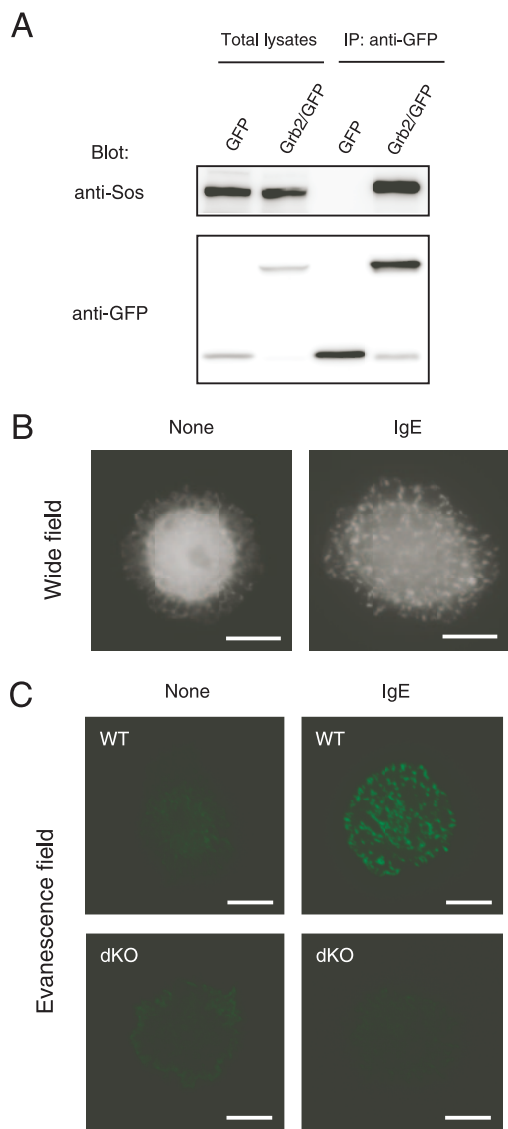


FIG. 6. LAT and NTAL are required for membrane recruitment of Grb2-GFP. (A) Constitutive association of Grb2-GFP with Sos in mast cells. RBL.2H3 cells were infected with GFP and Grb2-GFP. Total lysates and anti-GFP immunoprecipitates (IP) were blotted with anti-Sos Ab and anti-GFP-HRP Ab. (B) Fluorescence microscopy of BMMCs expressing Grb2-GFP. WT BMMCs were infected with Grb2-GFP and stimulated for 30 min on a glass-bottom dish coated with 10 μ g of IgE/ml. Then the cells were analyzed by wide-field fluorescence microscopy with an IX81-ARCEVA system. (C) TIRF microscopic images of BMMCs expressing Grb2-GFP. WT and dKO BMMCs were infected with Grb2-GFP, stimulated as described in the legend to panel B, and analyzed by TIRF microscopy with an IX81-ARCEVA system. Images representative of at least 20 independent fields are shown. Bars represent 5 μ m.

GRP and Sos in BCR- and epidermal growth factor receptor-mediated Ras activation, respectively, by using chicken B-cell lines (32). Our observation that dominant negative Sos suppresses IgE-mediated survival in the absence of Ag but not IgE-induced degranulation in the presence of Ag implies the existence of similar divergence patterns in Fc ϵ RI signaling; weak, constitutive engagement but not Ag cross-linking of

Fc ϵ RI may result in the preferential utilization of Sos rather than RasGRP for Ras activation. Although RasGRP4 is expressed in mast cells (55), the lack of Gads or LAT—important in regulating PLC γ through the formation of the LAT–Gads–SLP-76–PLC γ quaternary complex (27, 42, 51)—did not result in severe impairment of sustained Erk activation, suggesting that PLC γ -dependent RasGRP4 activation may not play a major role in the maintenance of IgE-induced signals in mast cells in the absence of Ag. Considering that LAT $^{-/-}$ mice exhibited severe impairment of IgE-induced mast cell degranulation in the presence of Ag (38, 57), NTAL appears to compensate for LAT function preferentially under stimulation via IgE without Ag. This finding may be explained by the fact that NTAL possesses binding sites for Grb2 but not for PLC γ , which is crucial for degranulation (48, 49). Consistent with this suggestion, Y136, a PLC γ binding site of LAT, has been shown to be essential for degranulation (28, 39).

Recently, accumulating evidence has suggested that the quantity and duration of signals through immune receptors determine distinct immune responses (29, 50). It is attractive to speculate that, likewise, the differential contributions of Sos and RasGRP are also operative in Ras activation during sustained Ag receptor triggering, for example, during the tonic signal of BCR (25), TCR engagement for homeostatic proliferation (12), or autonomous pre-TCR signaling (53). Indeed, RasGRP1-deficient thymocytes display no apparent defect in autonomous pre-TCR signaling, a process that is known to depend on Ras activation (10, 37).

The augmentation of mast cell function by the genetic deletion of NTAL alone has been explained by an increase in the amount of available LAT (47, 57), in light of the possible competition between the two transmembrane adaptors within the lipid raft. In line with these speculations, IgE-induced events in the absence of Ag are also upregulated in NTAL $^{-/-}$ BMMCs. As an additional possible explanation, we found that specific chemokine receptors are constitutively upregulated in NTAL $^{-/-}$ BMMCs (S. Yamasaki and T. Saito, unpublished data); this upregulation may also contribute to the augmented mast cell responses seen in NTAL-deficient single-knockout mice.

Taking these findings together, we propose herein a possible molecular mechanism by which Syk couples with sustained Erk activation upon IgE stimulation in the absence of Ag. IgE without Ag induces the delayed phosphorylation of LAT and NTAL. Phosphorylated LAT and NTAL may serve as a docking platform that enables the retention of the Grb2-Sos complex within the plasma membrane. Thus, Fc ϵ RI can differentially utilize the intracellular signaling machinery in response to different stimuli, and the raft-localized membrane adaptors LAT and NTAL are the critical transducers for timing the duration of signals from Fc ϵ RI to trigger downstream events. These results suggest that it may be possible to differentially modulate signaling events through Fc ϵ RI in order to block specific allergic reactions.

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