LAX is a transmembrane adaptor protein that is expressed in both T and B cells. Upon stimulation via the antigen receptors, it is tyrosine-phosphorylated and binds Grb2 and the p85 subunit of phosphatidylinositol 3-kinase. Disruption of the Lax gene causes hyperresponsiveness in T and B lymphocytes. Here, we showed that LAX was also expressed in mast cells. Upon engagement of the FcεRI, LAX was also phosphorylated and interacted with Grb2 and p85. LAX-deficient mast cells were hyperresponsive to stimulation via the FcεRI, as evidenced by enhanced degranulation, p38 MAPK, Akt, and phosphatidylinositol 3-kinase activation. This hyperresponsiveness was likely a consequence of reduced LAB expression after sensitization of mast cells with anti-dinitrophenyl IgE. In addition, FcεRI-mediated cytokine production and cell survival were also enhanced. These data suggested that LAX negatively regulates mast cell function.

The high affinity IgE receptor (FcεRI) is a multichain immune recognition receptor, which signals through the Src and Syk protein tyrosine kinases. After engagement of the FcεRI at the surface of mast cells, the Src family tyrosine kinase, Lyn, is first activated and phosphorylates the tyrosine residues within the ITAMs of the β and γ chains (1, 2). Phosphorylation of the paired tyrosines within the ITAMs leads to recruitment and further activation of Syk (3, 4). Fyn tyrosine kinase also plays an important role in mast cell function by phosphorylating Gab2, an adaptor protein that binds the p85 subunit of phosphatidylinositol 3-kinase (5, 6). These activated PTKs phosphorylate downstream signaling molecules and activate signaling cascades leading to Ras-MAPK activation, calcium mobilization, degranulation, and cytokine production (7–10). Much progress has been made in recent years to define the function of signaling proteins that contribute positively to this pathway; however, it is less clear how this pathway is negatively regulated.

Following engagement of the FcεRI on mast cells, several adaptors, such as SLP-76, Gab2, LAT, and NTAL/LAB, are phosphorylated. Previous studies show that these adaptor proteins play important roles in this pathway (6, 11–13). LAT, linker for activation of T cells, is one of the prominently phosphorylated proteins upon stimulation of mast cells from the FcεRI (12). In T cells, LAT is phosphorylated and interacts with Grb2, Gads, and PLC-γ1 upon engagement of the TCR (14–16). In LAT-deficient Jurkat cells, TCR-mediated Erk activation and calcium flux are completely defective (17, 18). Compared with the LAT-deficient Jurkat cells, Lat−/− mast cells have a less severe phenotype. Lat−/− mast cells have reduced phosphorylation of SLP-76, PLC-γ1, and PLC-γ2, and diminished calcium initiated from the FcεRI; however, they are still capable of fluxing calcium and undergo relatively normal Erk activation. Functionally, LAT deficiency leads to a dramatic reduction in mast cell degranulation and cytokine production (12). Although LAT plays a positive role, a LAT-like molecule, LAB/NTAL, functions to negatively regulate this signaling pathway and mast cell function. In Lab−/− mast cells, FcεRI-mediated Erk activation, calcium mobilization, degranulation, and cytokine production are all enhanced. Interestingly, mast cells that lack both LAT and LAB proteins have a more severe block in FcεRI-mediated signaling than Lat−/− mast cells, indicating that LAB also plays a positive role (13).

In addition to LAT and LAB/NTAL, LAX is another adaptor molecule that is expressed in lymphocytes. LAX is expressed in T cells, B cells, and monocytes; it has multiple Grb2 motifs very similar to those in LAT. However, different from LAT and LAB, LAX has no pamilatory motif and is not localized to lipid rafts (19). Upon stimulation of Jurkat T cells via the TCR, LAX is phosphorylated and interacts with Grb2, Gads, and the p85 subunit of PI3K. It is also phosphorylated upon BCR engagement. Overexpression of LAX in Jurkat cells inhibits TCR-mediated p38 MAPK and NFAT/AP1 activation. Despite these results, however, disruption of the Lax gene in mice has no impact on lymphocyte development, Lax−/− T and B cells are hyperresponsive and have a slightly enhanced calcium flux, protein tyrosine phosphorylation, and MAPK and Akt activation upon engagement of the T or B cell antigen receptors (20). Thus, our results suggest that LAX functions as a negative regulator in lymphocyte signaling.

In this report, we showed that LAX was also expressed in bone marrow-derived mast cells (BMMCs). Lax−/− BMMCs consistently grew faster in the IL-3 medium than Lax+/+ cells. They were hyperresponsive to stimulation via the FcεRI likely because of reduced expression of LAB. These data suggested that LAX negatively regulates FcεRI-mediated signaling and mast cell function.

**MATERIALS AND METHODS**

**Antibodies**—The following antibodies were used for Western blotting: polyclonal and monoclonal anti-LAT (19), monoclonal anti-LAT and LAB (13), anti-p-Tyr (4G10), the p85 subunit of PI3K, and PLC-γ1 (Upstate Biotechnology); anti-Grb2, Erk, Lyn, and Syk (Santa Cruz Biotechnology); anti-pp38, p38, pErk, pJNK, pAKT(Ser), and Akt (Cell Signaling), biotin-conjugated anti-FcεRIα, allophycocyanin-conjugated anti-c-Kit, and phycoerythrin-conjugated-Annexin V (ebioscience).

**Staining and Counting of Mast Cells**—Mast cells in the peritoneal cavity, skin, and ear were stained with toluidine blue. Peritoneal cells were spun onto microscope slides by cytospin (Cytospin 4 centrifuge,
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BMMC Proliferation and Apoptosis Assay—For proliferation, BMMCs (2–5 × 10^6/ml) were preloaded with anti-DNP IgE (0.5 μg/ml) in IMDM without IL-3 for 4 h. Cells were washed twice with IMDM and then loaded with 5 μM Fluo-4, AM (Molecular Probes) for 30 min. Cells were washed again and further incubated in IMDM for 30 min. DNP-HSA (30 ng/ml) and thapsigargin (1 μM) were used to induce calcium flux in these cells. Ca^{2+} flux was measured using a flow cytometer (FACScalibur, BD Biosciences) to monitor the fluorescence emission.

Degranulation and Cytokine Production—Degranulation of BMMCs measuring the release of β-heaminosidase and detection of cytokines by reverse transcription-PCR were performed as previously described (13). The concentrations of cytokines secreted by mast cells into the tissue culture supernatant were determined by the mouse 18-Plex cytokine assay according to the manufacturer’s protocol (Bio-Rad).

BMMC Proliferation and Apoptosis Assay—For proliferation, BMMCs (4 × 10^6 cells/per 96-well) were incubated in either medium alone or with IL-3 (0.1 or 1 ng/ml) for 36 h. [3H]Thymidine (5 μCi/ml) was added 6 h before harvesting. Incorporation of [3H]thymidine was measured by using a liquid scintillation luminescence counter (PerkinElmer Life Sciences). For apoptosis induced by IL-3 withdrawal, mast cells were washed three with phosphate-buffered saline and cultured in the IMDM without IL-3 for an additional 24, 48, and 72 h before stained with phycoerythrin-conjugated Annexin V.

Real Time PCR—Total RNAs were extracted using the TRIzol reagent (Invitrogen). cDNAs were synthesized with SuperScript reverse transcriptase (Invitrogen) using oligo(dT) as a primer. Quantitation of the Lab RNA was performed by real time PCR using SYBR Green Supermix (Bio-Rad). The following primers were used to amplify the Lab cDNA, 5'-CCTCAGAGCTATGGAAG-3' and 5'-AGCGGCAATACTCCGGACA-3'. The amount of Lab RNA was normalized by β-actin.

PI3K Determination—The PI3K level was determined using a protocol modified from procedures described previously (21). Briefly, anti-DNP IgE preloaded cells (1 × 10^7 cells/ml) were labeled with 0.2 μCi/ml [32P]orthophosphate (MP Biomedicals, carrier-free) in phosphate-free RPMI with 20 mm HEPES, pH 7.4 and 5% phosphate-free fetal bovine serum) for 1.5 h at 37°C before stimulation with DNP-HSA for 2, 5, and 10 min. The remaining steps were done as previously described (21).

RESULTS AND DISCUSSION

Expression of LAX in Mast Cells—LAX is expressed in naïve T and B cells and is up-regulated upon engagement of the antigen receptors (19). To examine whether LAX is also expressed in mast cells and further whether it functions in mast cells, we derived mast cells (BMMCs) from the bone marrow of wild type (Lax^+/+) and Lax^-/- mice. Bone marrow cells from these mice were cultured in the medium with IL-3. During the culture, Lax^-/- bone marrow cells consistently expanded faster than Lax^+/+ cells, suggesting that LAX might negatively regulate mast cell growth. To examine the expression of LAX, mast cell lysates were prepared and subjected to anti-LAX immunoprecipitation, followed by Western blotting with an anti-LAX antibody. LAX protein was clearly detected in Lax^+/+, not Lax^-/- mast cells, indicating that mast cells also express LAX (Fig. 1A).

Upon stimulation via the BCR or TCR, LAX is phosphorylated and interacts with Grb2 and p85 (19). To examine whether LAX is also phosphorylated and interacts with these proteins upon engagement of the FcεRI, mast cells were sensitized with anti-DNP IgE followed by cross-linking with DNP-HSA for 0, 2, 5, and 10 min before lysis. LAX protein was immunoprecipitated from lysates and analyzed by blotting with anti-pTyr, anti-p85, and Grb2 antibodies. As shown in Fig. 1B, LAX was weakly phosphorylated upon stimulation of Lax^+/+ mast cells. Maximal phosphorylation was 2 min after stimulation. Anti-LAX antibodies also co-precipitated a nonspecific protein with a molecular mass of ~65 kDa. Grb2 and p85 were detected in anti-LAX immunoprecipitates from stimulated Lax^+/+, not Lax^-/- mast cells. A similar amount of lysate was used in each immunoprecipitation as indicated by Western blotting the whole cell lysate with anti-Grb2 and p85 (Fig. 1B). These data indicated that as in T and B cells, LAX is also phosphorylated and interacts with Grb2 and p85 upon activation via the FcεRI.

IgE-dependent Anaphylaxis in Lax^-/- Mice—To examine whether LAX deficiency affected mast cell development, mast cells in peritoneal cavity, ear skin, and back skin of Lax^-/- and Lax^-/- mice were enumerated. Similar numbers of mast cells were observed (Table 1). To examine

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mast cell function in vivo, passive systemic IgE-dependent anaphylaxis in Lax+/+ and Lax−/− mice was performed. This assay is dependent on in vivo mast cell degranulation and histamine release after engagement of the FcεRI. Monoclonal anti-DNP IgE was first injected into mice via the tail vein. Twenty hours after injection, mice were then challenged with DNP-HSA (100 μg). 1.5 min after the challenge, blood was collected after euthanasia. Histamine concentration in the blood was measured by competitive enzyme-linked immunosorbent assay. Lax−/− mice were capable of releasing similar amounts of histamine upon challenging with DNP-HSA as Lax+/+ mice (Fig. 2A). We also performed this assay by challenging mice with different doses of DNP-HSA (20, 100, or 200 μg) after sensitization. Similar results were observed (data not shown). These data indicate that LAX deficiency did not lead to a block in mast cell function in vivo.

Enhanced Mast Cell Degranulation—We next determined whether Lax−/− mast cells had normal effector function in vitro by examining antigen-induced degranulation. After cultured in the medium with IL-3 for 3 weeks, more than 95% cells in our BMMC culture expressed FcεRI and c-Kit. FcεRI and c-Kit expression was similar on the cell surface of Lax+/+ and Lax−/− cells (Fig. 2B). To examine FcεRI-mediated degranulation, mast cells were preloaded with anti-DNP-IgE and activated by the addition of increasing amounts of DNP-HSA. The release of β-hexosaminidase from granules into the medium was assayed. As indicated in Fig. 2C, antigen-induced degranulation peaked at a concentration of 10 ng/ml for mast cells from Lax+/+ and Lax−/− mice; however, it was significantly enhanced in Lax−/− cells. These cells released similar amounts of β-hexosaminidase in response to thapsigargin, which bypasses the proximal signaling of the FcεRI (Fig. 2D).

**Table 1**
The number of mast cells in peritoneal cavity, ear, and back skin
Results are mean ± S.E. of group of 3 mice with the same age and gender.

| Mast cells      | LAX+/+ | LAX−/− |
|-----------------|--------|--------|
| Peritoneal cavity (%) | 3.03 ± 0.34 | 4.78 ± 1.01 |
| Ear skin (number/cm) | 257.4 ± 37.7 | 262.1 ± 41.4 |
| Back skin (number/cm) | 261.8 ± 38.5 | 255.4 ± 31.4 |

FceRI-mediated Signaling—To investigate whether FceRI-mediated signaling pathway was affected by the disruption of the Lax gene, we first examined the overall tyrosine phosphorylation of proteins in Lax−/− cells after the receptor engagement. As shown in Fig. 3A, there were no significant differences in tyrosine phosphorylation of proteins between Lax+/+ and Lax−/− mast cells except that there was increased phosphorylation of proteins with a molecular mass of ~32 kDa (see below). To examine FcεRI-mediated calcium flux, mast cells were sensitized with anti-DNP IgE, loaded with a calcium-sensitive dye, Fluo-4, and activated with DNP-HSA. A similar elevation of intracellular calcium after stimulation was seen in Lax+/+ and Lax−/− mast cells (Fig. 3B).

Activation via the FcεRI also leads to activation of three MAPKs, Erk, Jnk, and p38. To examine whether MAPK activation was normal in Lax−/− mast cells, lysates of activated mast cells were analyzed by Western blotting with antibodies against phosphorylated Erk, Jnk, and p38. Similar amounts of lysates loaded on each lane were indicated by reblotting with antibodies against non-phosphorylated Erk, Jnk, and p38 (Fig. 3B). Although activation of Erk and Jnk was similar in Lax+/+ and Lax−/− mast cells, activation of p38 was much stronger and sustained in Lax−/− cells (Fig. 3C). In addition to p38, Akt activation was also enhanced in Lax−/− cells. Enhanced p38 and Akt activation was not because of increased activation of PTKs after engagement of the FcεRI. Analysis of lysates by Western blotting with anti-phosphorylated forms of Lyn and Syk showed similar phosphorylation of these PTKs (Fig. 3C).

Down-regulation of LAB in Lax−/− Mast Cells—Because of decreased tyrosine phosphorylation of proteins with a molecular mass of ~32 kDa, the apparent size of LAB on SDS-PAGE in Lax−/− cells (Fig. 3A), we investigated whether LAB expression or tyrosine phosphorylation was affected in these cells. Western blot analysis showed that the amount of LAB protein was decreased dramatically in Lax−/− cells (Fig. 3C). Although it appeared that in Lax−/− cells, cross-linking with DNP-HSA led to further reduction of LAB expression. This was likely because of the fact that our anti-LAB antibodies preferentially recognized non-phosphorylated LAB.

**FIGURE 2.** The effect of LAX deficiency on IgE-mediated systemic anaphylaxis and degranulation. A, IgE-mediated systemic anaphylaxis. Mice (n = 5) were sensitized with anti-DNP IgE and challenged with 200 μg of DNP-HSA. Histamine concentration in blood was measured by enzyme-linked immunosorbent assay. B, surface IgE receptor and c-Kit expression on BMMCs from Lax+/+ and Lax−/− mice. BMMCs were stained with biotin-anti-FcεRIa (solid line) and isotype control (dashed line) followed by streptavidin-fluorescein isothiocyanate or allophycocyanin-conjugated anti-c-Kit (solid line) or isotype control (dashed line). C, FcεRI-mediated degranulation. Degranulation was determined by measuring the release of granular β-hexosaminidase after stimulation with DNP-HSA. The extent of degranulation was calculated by dividing the absorbance in the supernatant by the sum of absorbance in the supernatant and the cell pellet solubilized in 0.5% Triton X-100. D, thapsigargin-induced degranulation.
Next, we examined whether down-regulation of LAB protein was induced by sensitization with anti-DNP IgE or perhaps Lax- mast cells expressed a low amount of LAB after they were derived from bone marrow cells. Lax- and Lax+ mast cells were sensitized for 1, 6, 16, and 24 h or without sensitization before lysis. LAB expression was detected by Western blotting of the whole cell lysates with anti-LAB. As shown in Fig. 4A, while LAT expression remained the same after sensitization, LAB expression was reduced dramatically after sensitization for 16 or 24 h. The reduced expression of LAB was not at the transcriptional level because the amount of Lab RNA was slightly increased after sensitization (Fig. 4B).

Previously we showed that LAB negatively regulates mast cell function by competing with LAT for localization to lipid rafts. We next examined whether LAT localization to rafts was affected in Lax- mast cells. After sensitization of Lax- and Lax+ mast cells with anti-DNP IgE for 16 h, lipid rafts and non-raft fractions were isolated by a sucrose gradient as we did previously (13). Each fraction was analyzed by Western blotting with LAT and LAB antibodies. In Lax- mast cells, less LAB protein was detected as expected. In Lax+ mast cells, more LAT was detected in raft fractions and less LAT in non-raft fractions (Fig. 4C), indicating that LAX deficiency also affects LAT localization to rafts. Together, these data suggested that LAX likely negatively regulates FceRI-mediated signaling and mast cell function through LAB.

Enhanced Cytokine Production, Cell Survival, and PI3K Activation—Engagement of the FceRI also leads to production of multiple cytokines. Cytokine RNA levels in Lax- and Lax+ cells after stimulation for 0.5, 3, and 6 h were first analyzed by reverse transcription-PCR. Amplification of the actin transcript indicated that similar amounts of cDNAs were used. There were no significant differences in FceRI-mediated IL-3, IL-6, tumor necrosis factor-α, and tumor growth factor-β RNAs (Fig. 5A), although we observed enhanced Erk, p38, and Akt activation and granule release. However, when we quantitated the amount of cytokines secreted into the medium by these cells, the amount of IL-3, IL-6, and tumor necrosis factor-α produced by Lax- cells was slightly higher (Fig. 5B).

Because Lax- mast cells derived from bone marrow consistently grew faster than Lax+ mast cells in the medium with IL-3, we assayed mast cell proliferation in the medium with IL-3 by [3H]thymidine incorporation. Without IL-3 in the medium, mast cells from either Lax- or Lax+ mast cells failed to proliferate (Fig. 6A). With IL-3 at 0.1 ng/ml and 1 ng/ml, Lax- mast cells had more [3H]thymidine incorporated than Lax+ mast cells. The increased expansion of Lax- mast cells could be due to enhanced proliferation or decreased cell death. To distinguish these two...
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possibilities, mast cells were labeled with CFSE and were allowed to proliferate in the presence of IL-3. The CFSE intensity analyzed by FACS should be diluted after each cell division. No difference in the fluorescence intensity was seen in CFSE-labeled Lax−/− and Lax+/+ mast cells after culturing for 2–5 days (data not shown), suggesting that these cells divided at a similar rate. To examine whether these cells have any differences in cell death, we examined mast cell apoptosis induced by IL-3 withdrawal. Mast cells were washed three times with phosphate-buffered saline, and cultured for 0, 24, 48, and 72 h without IL-3 before they were stained with phycoerythrin-labeled Annexin V. Annexin V-positive cells were either dying or dead cells. As shown in Fig. 6B, the percentage of Annexin V-positive Lax−/− cells was significantly more than that of Lax+/+ cells at 24, 48, or 72 h after IL-3 withdrawal. Together, these data suggested that faster expansion of Lax−/− mast cell culture is because of reduced cell death.

Because we observed enhanced Akt and p38 activation after FcεRI engagement (Fig. 3B) and increased cell survival after IL-3 withdrawal (Fig. 6B), we asked whether LAX deficiency affects PI3K activity. PI3K plays an important role in Akt activation and cell survival. The p85 subunit of PI3K binds to LAX upon FcεRI engagement. Lax+/+ and Lax−/− mast cells were labeled with [32P]orthophosphate. The levels of FcεRI-evoked PtdIns-3,4,5-P3, a product of PI3K, were assayed by thin-layer chromatography. As shown in Fig. 6, C and D, PIP3 production after stimulation was significantly increased in Lax−/− cells compared with it in Lax+/+ cells. These data suggested that LAX negatively regulates FcεRI-mediated PI3K activation in mast cells.

Our previous studies show that overexpression of LAX in Jurkat cells inhibits p38 MAPK activation and TCR-mediated NFAT/AP-1 activation (19). A disruption of the Lax gene leads to hyperresponsiveness of T and B cells upon stimulation via the TCR or BCR. Tyrosine phosphorylation of cellular proteins, p38 MAPK and Akt activation, and cell survival are enhanced in LAX T- and B-deficient cells (20). In this study, we demonstrated that LAX was also expressed in bone marrow-derived mast cells. LAX deficiency led to enhanced mast cell degranulation, Akt, and p38 MAPK activation and cell survival. PI3K activity was also increased upon stimulation via the FcεRI. Taken together, these data suggested that LAX plays an important role in mast cell function by negatively regulating the PI3K pathway. Despite the hyperresponsiveness of LAX-deficient mast cells, we did not observe a significant enhancement of IgE-dependent systemic anaphylaxis in Lax−/− mice. It is possible that there is a compensatory mechanism in vivo to control hyperactivated mast cells. The basal IgE level in naive Lax−/− mice is higher than that in Lax+/+ mice (20). Mast cells in Lax−/− mice might bind less anti-DNP IgE after sensitization because exogenous IgE has to compete with endogenous IgE to bind the FcεRI. Even though Lax−/− mast cells were hyperresponsive in vitro, they might not be able to release more granules because of reduced receptor engagement.

The mechanism by which LAX inhibits PI3K and mast cell function is still not clear. Because tyrosine phosphorylation of LAX is very weak compared with that of LAT, it might be difficult for LAX to compete for binding

FIGURE 4. Down-regulation of LAB after anti-DNP IgE sensitization. A, Lax−/− and Lax+/+ BMMCs were sensitized with anti-DNP IgE for 0, 6, 16, and 24 h before lysis. Lysates were resolved on SDS-PAGE and blotted with anti-LAB and LAT antibodies, respectively. B, real-time PCR to detect the amount of Lab RNA. After sensitization of Lax−/− and Lax+/+ BMMCs with anti-DNP IgE for 0, 6, and 16 h, total RNAs were extracted and used for synthesis of cDNAs. The amount of Lab RNA was normalized by the β-actin RNA. C, localization of LAT and LAB in lipid rafts. After sensitization of Lax−/− and Lax+/+ BMMCs with anti-DNP IgE for 16 h, lipid rafts and non-rafts fractions were isolated by sucrose gradient. Twelve fractions were collected and analyzed by blotting with anti-LAB and LAT antibodies.

FIGURE 5. Cytokine production in Lax−/− mast cells. A, semiquantitative reverse transcription-PCR for determination of cytokine RNAs. BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-HSA for 0.5, 3, and 6 h. Relative levels of cytokine RNAs were detected by reverse transcription-PCR using specific primer pairs for each cytokine. B, cytokine production by mast cells. Cytokines secreted into tissue culture supernatants by mast cells before (0 h) and after cross-linking for 3 or 8 h were quantitated by the multiplex cytokine assay. Only IL-3, IL-6, and tumor necrosis factor-α were shown. The data shown represented the results from one of three experiments.
of Grb2 and p85 with LAT or other proteins. Even though LAX binds to Grb2 and p85 upon engagement of the FcεRI, whether binding to these two proteins contributes to its negative function remains to be determined. It is possible that LAX binds other negative regulators, such as phosphatases, to turn off FcεRI-mediated signaling. Our data showed that LAB was down-regulated in Lax−/− cells upon sensitization with anti-DNP-IgE, suggesting that regulation of mast cells by LAX might be mediated via LAB. Recent studies have shown that sensitization of mast cells with IgE not only allows binding of monomeric IgE to the FcεRI, it can also activate pathways to promote mast cell survival and activation even in the absence of antigen (22). Although we do not know how the LAX deficiency caused the down-regulation of LAB protein in mast cells, it is possible that LAX might form a complex with LAB directly or indirectly to stabilize LAB at the membrane. In the absence of LAX, sensitization of mast cells by IgE through the FcεRI could initiate signaling events to cause down-regulation of LAB from the cell surface or degradation of LAB. Because LAB is a negative regulator in the FcεRI-mediated signaling pathway, reduced LAB expression caused mast cells hyperresponsive to stimulation via the FcεRI by the IgE-antigen complex. How LAX regulates LAB expression in mast cells is very intriguing and remains to be determined in future studies.

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