Leupaxin Negatively Regulates B Cell Receptor Signaling*

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The role of the paxillin superfamily of adaptor proteins in B cell antigen receptor (BCR) signaling has not been studied previously. We show here that leupaxin (LPXN), a member of this family, was tyrosine-phosphorylated and recruited to the plasma membrane of human BJAB lymphoma cells upon BCR stimulation and that it interacted with Lyn (a critical Src family tyrosine kinase in BCR signaling) in a BCR-induced manner. LPXN contains four leucine-rich sequences termed LD motifs, and serial truncation and specific domain deletion of LPXN indicated that its LD3 domain is involved in the binding of Lyn. Of a total of 11 tyrosine sites in LPXN, we mutated Tyr22, Tyr72, Tyr198, and Tyr257 to phenylalanine and demonstrated that LPXN was phosphorylated by Lyn only at Tyr72 and that this tyrosine site is proximal to the LD3 domain. The overexpression of LPXN in mouse A20 B lymphoma cells led to the suppression of BCR-induced activation of JNK, p38 MAPK, and, to a lesser extent, Akt, but not ERK and NFκB, suggesting that LPXN can selectively repress BCR signaling. We further show that LPXN suppressed the secretion of interleukin-2 by BCR-activated A20 B cells and that this inhibition was abrogated in the Y72F LPXN mutant, indicating that the phosphorylation of Tyr72 is critical for the biological function of LPXN. Thus, LPXN plays an inhibitory role in BCR signaling and B cell function.

Engagement of the B cell antigen receptor (BCR) on B cells by antigen triggers first the activation of the Src family kinase Lyn (1–4), which is known to phosphorylate the immunoreceptor tyrosine-based activation motifs within the cytoplasmic domains of the Ig-α and Ig-β subunits that are part of the BCR complex (5). The phosphorylation of the immunoreceptor tyrosine-based activation motif then leads to the activation of the tyrosine kinase Syk (6, 7), which leads in turn to the phosphorylation of various downstream proteins such as BLNK, phospholipase Cγ2, and Btk (8). As a consequence of the activation of these signaling proteins, numerous second messengers and intermediate signal-transducing proteins are activated, and together, they lead to the activation of several key transcription factors that regulate new gene expression in B lymphocytes and that drive unique B cell physiological responses such as proliferation, cytokine secretion, and differentiation either to memory B or antibody-producing plasma cells (9).

Because BCR signaling can lead to the activation of B lymphocytes, there exist several mechanisms to down-regulate or modulate BCR signaling to prevent the overt or inappropriate activation of B cells. Several phosphatases such as the membrane-bound CD45 and intracellular SHP-1 and SHIP-1 are known to dephosphorylate and hence deactivate key signal transduction molecules in the BCR signaling pathway (10). Recent studies also revealed that, in addition to its well-established role in BCR signal initiation, Lyn can play a negative role in down-modulating BCR signaling (11, 12). Indeed, despite showing defects in B cell development, Lyn-deficient mice are also susceptible to autoimmune diseases, and Lyn-deficient B cells are hyper-responsive to BCR ligation (13–15).

Another class of signal transduction molecules known as the adaptor proteins has also been shown to play critical roles in lymphocyte signal transduction. These proteins do not have enzymatic activities but mediate protein-protein and protein-lipid interactions to provide spatiotemporal modulation of BCR signaling (16). Some of these adaptors are positive regulators of signal transduction, and they facilitate the assembly of activating signaling complexes. For example, BLNK has been widely established as an adaptor protein that couples Syk and Btk to activate phospholipase Cγ2 upon BCR ligation, and this subsequently triggers downstream calcium fluxes and inositol 1,4,5-trisphosphate production (17). On the other hand, other adaptors such as the Csk-binding protein and the Dok family members Dok-1, 2, and 3 are known to play a negative role in immunoreceptor signaling (18). Most of these inhibitory adaptors recruit additional inhibitory effectors to the vicinity of positive regulator of signaling to shut down the signal transduction processes, e.g. Csk-binding protein is known to recruit Csk, which inhibits the activation of the Src family tyrosine kinases (19), whereas Dok-3 is known to recruit SHIP, which dephosphorylates activated signaling molecules (20, 21).

Given that certain adaptor proteins such as BLNK (22), Dok-1 (23), and Dok-3 (24) have been demonstrated to play key roles in the regulation of BCR signaling, it is conceivable that other adaptor proteins that have not been studied in the context of immunoreceptor signaling may also play a critical role in BCR signal transduction. The paxillin family of adaptor proteins could be one such example. Paxillin and its related family members Hic-5, leupaxin, and PaxB have not been demon-

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2 The abbreviations used are: BCR, B cell antigen receptor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; IL-2, interleukin-2; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; LPXN, leupaxin; BSA; bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.
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Stratified to play a role in BCR signaling so far. Paxillin is a focal adhesion adaptor protein that plays an important role in growth factor- and integrin-mediated signaling pathways (25, 26). Despite its ability to bind Pyk2 and PTP-PEST, which are molecules known to play a role in BCR signaling, a previous report had indicated that paxillin is not tyrosine-phosphorylated in activated B cells (27). Thus, the role of paxillin in BCR signaling remains to be confirmed. Another family member (Hic-5) was reported to be largely absent in lymphocytes (28), hence minimizing the possibility of its participation in BCR signaling.

On the other hand, leupaxin, which is most homologous to paxillin and detectable as a 45-kDa protein, is preferentially expressed in hematopoietic cells, including B cells (29). Similar to the other paxillin superfamily members, leupaxin contains multiple N-terminal Leu- and Asp-rich sequences (LD domains) and LIM domains (26, 30). Both LD and LIM domains had been shown to be important for protein-protein interactions, and in addition, LIM domains have also been shown to play a role in the focal adhesion targeting of paxillin superfamily members (31). Recent works also established a role for leupaxin in the function of osteoclasts (32) and in the migration of prostate cancer cells (33). Leupaxin is known to interact with Pyk2 (29); Src (34); PEST domain tyrosine phosphatase (PEP) (35); and PTP-PEST, pp125FAK, and the ADP-ribosylation factor (ARF) GTPase-activating protein p95Kri (32); and some of these proteins are known to be expressed in B cells.

In this study, we examined the possible role of leupaxin in BCR signaling. We found that leupaxin was phosphorylated upon BCR engagement in human BJAB cells. We show that leupaxin bound Lyn via its LD3 domain and that Lyn phosphorylated Tyr72 of leupaxin. In addition, we demonstrate that leupaxin inhibited the JNK and p38 MAPK signaling pathways downstream of BCR signaling and suppressed the production of interleukin-2 (IL-2) by activated mouse A20 B cells and that some of these proteins are known to be expressed in B cells.

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**Experimental Procedures**

**Plasmid Construction**—The cDNAs encoding Lyn, paxillin, and Hic-5 were cloned from a murine spleen cDNA library by PCR. FLAG-tagged wild-type leupaxin was generated from the cDNA encoding wild-type leupaxin (provided by Dr. A. Gupta, University of Maryland, Baltimore, MD) (34). FLAG-tagged leupaxin deletion mutants (LD1, LD1–2, LD1–3, LD1–4, and LD3) and tyrosine-to-phenylalanine mutants (Y22F, Y72F, Y198F, and Y257F) were generated by PCR. All wild-type and mutated cDNAs were verified by DNA sequencing (data not shown).

**Cells and Transfections**—HEK293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and penicillin/streptomycin and transiently transfected using Effectene® transfection reagent (Qiagen Inc.). BIAB and A20 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, 2 mM l-glutamine, and penicillin/streptomycin. For transfection of A20 B cells, 1 × 10⁷ cells were mixed with 20 μg of plasmid DNA in 500 μl of RPMI 1640 medium and electroporated in a 0.4-cm cuvette at 950 microfarads and 300 V using a Gene Pulser (Bio-Rad). Transfection efficiency was assessed with the pEGFP-N2 vector at 36 h post-transfection by flow cytometry and was determined to be between 30 and 40%.

**Isolation of Subcellular Fractions**—BJAB cells were lysed on ice for 20 min in hypotonic buffer containing 15 mM Tris-HCl (pH 7.5), 5 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.2 mM Na₃VO₄, and protease inhibitor mixture (Roche Applied Science). Cell lysates were homogenized through a 26-gauge needle and centrifuged at 500 × g. The supernatant was transferred to polycarbonate tubes and ultracentrifuged at 20,800 × g for 1 h at 4 °C. The supernatant containing the cytosol fraction was recovered, and the pellet containing the plasma membrane fraction was solubilized in 150 mM NaCl, 15 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1% Triton X-100, 0.2 mM Na₃VO₄, and protease inhibitors.

**Antibodies**—F(ab’)2 fragments of goat anti-mouse IgG and goat anti-human IgM were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Monoclonal antibodies against human leupaxin (283C and 315G) were obtained from Dr. A. Gupta (32) and ICOS Corp. (Bothell, WA). The following commercial antibodies were also used: anti-phospho Akt (Ser473/Thr308), anti-Akt-1, anti-ERK2, anti-IkBα, anti-JNK1, anti-Lyn, anti-phospho-ERK, anti-p38, and anti-β-tubulin (Santa Cruz Biotechnology, Inc.); anti-phospho-stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185) and anti-phospho-p38 (Thr180/Tyr182) (Cell Signaling Technology); anti-FLAG polyclonal and anti-hemagglutinin (HA) monoclonal (Sigma); horseradish peroxidase-coupled anti-phosphotyrosine (4G10; Upstate Biotechnology); and Alexa 488-conjugated goat anti-mouse and Alexa 488-conjugated chicken anti-rabbit (Molecular Probes).

**Cell Stimulation, Western Blotting, and Immunoprecipitations**—Cells were resuspended in RPMI 1640 medium at 2 × 10⁶ cells/200 μl and serum-starved at 37 °C for 1 h prior to stimulation with anti-Ig antibodies. BJAB cells were stimulated with 10 μg/ml anti-human IgM F(ab’)2 fragment, and A20 cells with 15 μg/ml anti-mouse IgG F(ab’)2 fragment. After stimulation, cells were lysed on ice for 10 min in lysis buffer containing 1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.2 mM Na₃VO₄, and protease inhibitor mixture and sonicated. Cell homogenates were centrifuged at 13,000 rpm for 15 min at 4 °C, and supernatants were recovered for protein quantification using a BCA protein assay kit (Pierce). Proteins were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride immunoblot membranes (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature and incubated separately with various antibodies recognizing the different molecules studied. Protein bands were visualized using horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence ECL detection system (Amersham Biosciences). For immunoprecipitations, cell lysates were precleared with protein A/G Plus-agarose (Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C. For immunoprecipitation of endogenous proteins, anti-leupaxin (LPXN) monoclonal antibody 315G or anti-Lyn antibody
was coupled overnight to protein A/G Plus-agarose at 4 °C and washed twice with lysis buffer before overnight incubation with precleared cell lysates at 4 °C. For other immunoprecipitations, agarose beads were covalently coupled with anti-phosphotyrosine or anti-FLAG antibody and washed twice with lysis buffer before incubation with precleared cell lysates. Beads were then pelleted and washed three times with lysis buffer before boiling in loading buffer (1% SDS, 1% β-mercaptoethanol, 15% glycerol, and 0.01% bromphenol blue) for 5 min. The released proteins were resolved on SDS-polyacrylamide gels.

Confocal Microscopy—BCR-stimulated BJAB cells were washed twice with cold 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and fixed for 20 min on ice with 4% paraformaldehyde in PBS. After permeabilization at room temperature for 10 min with 0.2% saponin and 0.03 M sucrose in 1% BSA-containing PBS, cells were washed twice before being deposited onto slides. Cells were blocked with 5% normal goat serum in 1% BSA-containing PBS at room temperature for 1 h before overnight incubation with primary antibodies at 4 °C. The slides were washed three times with 1% BSA in PBS and incubated at room temperature for 1 h with Alexa 546-conjugated goat anti-mouse or Alexa 488-conjugated chicken anti-rabbit antibody to reveal the respective primary antibodies. Slides were washed three times with 1% BSA in PBS, mounted, and viewed under a Radiance 2000 confocal laser scanning microscope (Bio-Rad).

Measurement of BCR-triggered IL-2 Production—10^5 transfected A20 cells in 200 μl of culture medium were stimulated for 24 h at 37 °C in 96-well plates in the presence or absence of 10 μg/ml anti-mouse IgG F(ab’)2 fragment. The resulting production of IL-2 was measured by enzyme-linked immunosorbent assay (ELISA) using a mouse IL-2 ELISA kit (BD Biosciences) according to the manufacturer’s protocol. All cytokine secretion assays were performed in triplicate and repeated three times. To measure BCR-induced activation of the IL-2 promoter, A20 cells (10 × 10^6) were transfected with an IL-2 promoter-luciferase plasmid as described previously (36). Briefly, cells were electroporated with 15 μg of IL-2 promoter-luciferase plasmid together with 10 μg of the indicated plasmids and 1.5 μg of pRLE-TK (Renilla) plasmid (to standardize for transfection efficiency). After 40 h, 2 × 10^6 cells were stimulated for 6 h with 10 μg of IgG F(ab’)2 fragment. Cells were harvested, and cell pellets were solubilized in passive lysis buffer (Promega Corp.) and incubated on a Spiramix roller mixer for 15 min at room temperature. Cell lysate (90 μl) was assayed for both firefly and Renilla luciferase activities using the Dual-Luciferase reporter assay system (Promega Corp.) and the relative light units were measured in a TD-20/20 single tube luminometer (Turner Biosystems, Sunnyvale, CA). Luciferase activity was calculated as increments (n-fold) in IgG F(ab’)2 fragment-induced activity over basal activity obtained with unstimulated cells.

RESULTS

Leupaxin Is Activated upon BCR Engagement in Human BJAB B Cells—It was shown previously that LPXN is preferentially expressed in hematopoietic cells (29). However, the role of LPXN in BCR signaling is not known. We first observed that LPXN is highly expressed in human BJAB B lymphoma cells (Fig. 1A), suggesting that it might have a role in some aspects of B cell physiology. It is known from various studies that the engagement of BCR on B cells with anti-IgM antibodies or anti-idiotype leads to the tyrosine phosphorylation and hence activation of several downstream signaling proteins such as the tyrosine kinase Btk and the adaptor protein BLNK (37–39). LPXN is known to contain 11 tyrosine residues. Therefore, to determine whether LPXN is involved in BCR signaling, we examined whether LPXN is tyrosine-phosphorylated upon the engagement of BCR on BJAB cells. As shown in Fig. 1A, treatment of BJAB cells with 10 μg/ml anti-human IgM F(ab’)2 fragment led to the tyrosine phosphorylation of LPXN as shown by immunoprecipitation and immunoblotting with anti-phosphotyrosine antibody 4G10. The phosphorylation of LPXN occurred as early as 1 min after BCR stimulation of BJAB cells and appeared to peak at the 5- and 15-min time points before returning to the basal level at the 30-min time point. Conversely, immunoprecipitating total cellular phosphotyrosine proteins from BCR-stimulated BJAB cells with antibody 4G10 followed by immunoblotting with anti-LPXN antibody also revealed a similar pattern in which LPXN was highly activated between 5 and 15 min, as LPXN was maximally immunoprecipitated at these time points (Fig. 1B). As a comparison, the tyrosine kinase Lyn (a known component of the BCR signal transduction system) was also shown to be tyrosine-phospho-
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rlylated in BCR-activated BJAB cells. However, in contrast to LPXN, the phosphorylation of Lyn seemed to be more prolonged and was extended to 30 min after BCR ligation (Fig. 1B). This might indicate that the activation of LPXN and hence its involvement in BCR signaling could be more transient compared with Lyn. The tyrosine phosphorylation of LPXN also indicated that it could be a target of a protein-tyrosine kinase that was activated downstream of the BCR signaling pathway.

Besides the tyrosine phosphorylation of LPXN, we also observed the recruitment of LPXN to the plasma membrane of B cells upon BCR ligation. Several proteins in the BCR signaling pathway, e.g. the adaptor protein BLNK and the tyrosine kinase Btk, are known to locate to the plasma membrane and especially to the lipid raft fraction of B cells following BCR activation (17). Our results also indicated that LPXN was enriched in the membrane fraction beginning at 5 min after anti-human IgM F(ab')2 fragment treatment of BJAB cells (Fig. 1C). LPXN could still be found in the membrane fractions 15 min after BCR ligation and was subsequently sequestered back to the cytoplasm beginning 30 min after activation. This is consistent with the tyrosine phosphorylation profile of LPXN as shown in Fig. 1 (A and B). Lyn, which is known to be constitutively present in the membrane fraction of B cells (40, 41), was used as a loading control for the membrane fractions, whereas β-tubulin was used as a control for the cytoplasmic fractions (Fig. 1C). Thus, taken together, the data indicate that LPXN is tyrosine-phosphorylated and recruited to the plasma membrane following BCR cross-linking in B cells, suggesting that LPXN could play a role in BCR signaling.

Leupaxin Interacts with Lyn during BCR Signaling—Previous studies indicated that members of the paxillin superfamily of adaptors can interact with members of the Src family of tyrosine kinases, e.g. paxillin was shown to bind Src either directly or via Pyk2 (31), Hic-5 can bind Fyn (42), and LPXN can interact with Src in osteoclasts (34). Because we demonstrated that LPXN was tyrosine-phosphorylated upon BCR cross-linking and it is known that Lyn is the predominant Src family tyrosine kinase found in B cells (43), we investigated whether LPXN can physically interact with Lyn.

To accomplish this, FLAG-tagged paxillin superfamily members (LPXN, Hic-5, and paxillin) were coexpressed with HA-tagged Lyn in HEK293T cells, and whole cell lysates from the transfectedants were subjected to immunoprecipitation with anti-FLAG antibody-agarose beads. The immunoprecipitates were subsequently immunoblotted with anti-Lyn antibody. As shown in Fig. 2A, all three members of the paxillin superfamily interacted with Lyn, with Hic-5 co-immunoprecipitating a larger amount of Lyn, followed by LPXN and finally paxillin. Used as a negative control, the FLAG tag alone did not show any nonspecific interaction with Lyn.

As LPXN bound Lyn in overexpression studies in HEK293T cells, we next examined whether endogenous interaction of LPXN and Lyn can occur in B cells upon BCR activation. BJAB cells were treated with 10 μg/ml anti-human IgM F(ab')2 fragment for various time points, and cell lysates were immunoprecipitated with anti-LPXN antibody and immunoblotted with anti-Lyn antibody. As shown in Fig. 2B, the interaction between LPXN and Lyn was detected as early as 1 min and seemed to peak between 5 and 15 min after BCR ligation in BJAB cells. The binding of LPXN to Lyn appeared to occur in response to BCR cross-linking, as LPXN and Lyn could no longer be co-immunoprecipitated at the 30-min time point. We were able to show that an equivalent amount of LPXN (used as a control) was immunoprecipitated at all time points examined. Thus, Lyn binding to LPXN appears to be induced by BCR signaling.

To visualize the endogenous interaction of LPXN and Lyn, we performed immunofluorescence studies in BJAB cells. LPXN (which stained red) was found to be evenly distributed in the cytoplasm of unstimulated BJAB cells (Fig. 2C, upper panels). Upon ligation of BCR on BJAB cells, LPXN was recruited to the plasma membrane at the 5-min time point and remained there until after 15 min. However, by 30 min, LPXN was sequestered back to the cytoplasm (Fig. 2C, upper panels). This observation was consistent with our membrane fractionation data shown in Fig. 1C. On the other hand, Lyn (which stained green) (Fig. 2C, middle panels) was constitutively present in the membrane fractions, as has been reported in a previous study (17). Interestingly, the merging of the two panels revealed a pattern of BCR-induced co-localization of LPXN and Lyn upon cell activation (Fig. 2C, lower panels, yellow). The co-localization of LPXN and Lyn was clearly visible in the plasma membrane of BJAB cells at the 5-min time point following BCR ligation and was slowly diminished from the 15-min time point onward as LPXN was slowly recruited back to the cytoplasm. By the 30-min time point, the co-localization of LPXN and Lyn was minimal as LPXN was sequestered mostly away from the membrane and predominantly found localized in the cytoplasm. Taken together, the data in Fig. 2 support the finding that LPXN interacts with Lyn and that the interaction likely occurs in the plasma membrane of B cells and is induced upon BCR activation.

Leupaxin Interacts with Lyn through Its LD3 Domain—Because LPXN is a multidomain adaptor protein containing four leucine-rich LD motifs and four LIM domains, we were interested in determining the specific domain within LPXN that is responsible for mediating the interaction with Lyn. It has been shown previously that the LD2 domain of paxillin can bind several proteins, including kinases such as Src, focal adhesion kinase, and Pyk2 (25). The interaction of paxillin with Src has been shown to be either direct (near the proline-rich N terminus) or indirect (via focal adhesion kinase or Pyk2 near the LD2 domain) (31). More relevant to our study, a recent report also indicated that the LD2 domain of LPXN can interact with Src (34). Thus, we examined the specific LD domain(s) of LPXN that interact with Lyn.

To determine which LD domain(s) of LPXN are involved in binding Lyn, we constructed a series of FLAG-tagged truncation and deletion mutants of LPXN as shown in Fig. 3A. The FLAG-tagged ΔLD1, ΔLD1–2, ΔLD1–3, ΔLD1–4, and ΔLD3 deletion mutants of LPXN were overexpressed in HEK293T cells together with HA-tagged Lyn. Western blot analyses (Fig. 3B) indicated that all FLAG-tagged LPXN deletion mutants and HA-tagged Lyn proteins were expressed in the transfected cells. The various FLAG-tagged LPXN mutants were thus immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Lyn antibody. As shown in Fig. 3B, wild-type LPXN
suggested that the potential binding domain of LPXN for Lyn could be the LD3 domain. To further confirm that the LD3 domain of LPXN binds Lyn, we specifically generated the ΔLD3 mutant, in which only the LD3 domain of LPXN was deleted. Indeed, as shown in Fig. 3B (lane 7), deleting the LD3 domain of LPXN abolished the interaction of LPXN and Lyn, as the two proteins could no longer be co-immunoprecipitated. We therefore concluded that the LD3 domain of LPXN is the domain responsible for interaction with Lyn.

Leupaxin Is Phosphorylated at Tyrosine 72 by Lyn—LPXN contains 11 potential tyrosine phosphorylation sites and has been shown to be a substrate of tyrosine kinases (29). Because LPXN was able to interact with Lyn (Figs. 2 and 3), we examined whether LPXN can be a substrate of and be phosphorylated by Lyn. FLAG-tagged Lyn was hyperphosphorylated and constitutively active when transfected into HEK293T cells (Fig. 4A, upper panel, lane 2). When FLAG-tagged LPXN was coexpressed with FLAG-tagged Lyn in HEK293T cells, it was tyrosine-phosphorylated by Lyn, as shown by immunoblotting of whole cell lysates with anti-phosphotyrosine antibody 4G10 (Fig. 4A, lane 3). However, without the coexpression of FLAG-tagged Lyn, LPXN was not phosphorylated (Fig. 4A, upper panel, lane 1). Interestingly, Lyn was also able to phosphorylate paxillin and Hic-5 (Fig. 4A, lanes 4 and 5), suggesting that Lyn can potentially interact with and phosphorylate all paxillin family members. As control immunoblotting with anti-FLAG antibody indicated that all transfected proteins were equivalently expressed in HEK293T cells (Fig. 4A, lower panel).

Because Lyn could bind and phosphorylate LPXN, we next examined the tyrosine residue(s) in LPXN that can be phosphorylated by Lyn. Among the 11 tyrosine residues in LPXN, 6 were identified as potential sites for phosphorylation by kinases using the NetPhos 2.0 program, which predicts serine, threonine, and tyrosine phosphorylation sites in

**FIGURE 2. Interaction of LPXN and Lyn.** A, binding of LPXN by Lyn. HEK293T cells were transiently transfected with plasmids expressing various FLAG-tagged paxillin superfamily members and HA-tagged Lyn. Anti-FLAG immunoprecipitates (IP) were subjected to immunoblotting (IB) with anti-Lyn antibody (upper panel) to examine co-immunoprecipitation and hence interaction of LPXN and Lyn. Whole cell lysates were immunoblotted with anti-FLAG or anti-HA antibody (middle and lower panels) to examine the expression of the transfected plasmids. B, interaction of endogenous LPXN and Lyn in BJAB cells. Cells were stimulated with 10 μg/ml anti-human IgM Fab’2 fragment for the indicated time points, and cell lysates were subjected to immunoprecipitation with anti-LPXN antibody and immunoblotted with anti-Lyn and anti-LPXN (used as a loading control) antibodies. C, recruitment of LPXN to the plasma membrane upon BCR activation. BJAB cells were stimulated with 10 μg/ml anti-human IgM Fab’2 fragment for various time points, cytospun onto glass slides, and stained with anti-LPXN (red) and anti-Lyn (green) antibodies. Co-localization of the two proteins (as indicated in yellow) was evident by merging the two panels.

(lane 2) and both the ΔLD1 and ΔLD1–2 LPXN deletion mutants (lanes 3 and 4) were able to bind Lyn, whereas the ΔLD1–3 and ΔLD1–4 mutants could not (lanes 5 and 6). This
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It was possible that by generating the Y72F mutant, we had disrupted the interaction between LPXN and Lyn. To test this possibility, the various LPXN mutants and Lyn were co-immunoprecipitated from the transfected cells. As shown in Fig. 4C, all four tyrosine-to-phenylalanine mutants (and Y72F, in particular) co-immunoprecipitated with Lyn, suggesting that these mutants can still bind Lyn. We therefore concluded that the lack of phosphorylation of LPXN by Lyn is not due to its inability to interact with Lyn and that the binding and phosphorylation of LPXN by Lyn are two separable events. Furthermore, as shown in the schematic map of LPXN in Fig. 4D, Tyr$^{72}$ is proximal to the LD3 motif, which we had demonstrated above to be the domain of LPXN responsible for its interaction with Lyn (Fig. 3, A and B).

Leupaxin Selectively Inhibits JNK, p38 MAPK, and Akt Signaling in Mouse A20 B Cells—We have so far shown that LPXN was phosphorylated upon BCR engagement and that Lyn, a critical kinase in BCR signaling, bound and phosphorylated LPXN. Thus, it is likely that LPXN plays a role in some aspects of BCR signaling. BCR engagement in B cells is known to activate three major signaling pathways downstream of tyrosine kinase activation, and these include the phospholipase Cγ2/protein kinase C/calci um, phosphoinositide 3-kinase/Akt, Vav/Rac, and Ras/Raf/MAPK pathways (39). The phospholipase Cγ2/protein kinase C/calci um pathway further triggers the activation of NFκB (44).

To elucidate the role of LPXN in BCR signal transduction, we overexpressed LPXN in mouse A20 B lymphoma cells (Fig. 5A) and examined the effect of LPXN overexpression on the activation of MAPKs, Akt, and NFκB upon BCR ligation (Fig. 5, B–D). First, we examined the relative level of ectopically expressed LPXN versus the endogenous protein. A20 cells were transiently transfected with either FLAG vector or FLAG-LPXN, and whole cell lysates were immunoblotted with anti-LPXN antibody. As shown in Fig. 5A (left panels), the level of endogenous LPXN in A20 B cells was rather low, and LPXN expression in A20 cells was significantly enhanced upon transfection, thus making A20 cells ideal for assessing the effect of LPXN on BCR signaling.

A20 B cells transfected with FLAG vector or FLAG-LPXN (Fig. 5A, right panel) were stimulated with 15 μg/ml goat antimouse IgG F(ab')$_2$ fragment for various times, and cell lysates were immunoblotted with specific antibodies recognizing the phosphorylated and hence activated forms of JNK1, JNK2, p38 MAPK, ERK1, and ERK2 (Fig. 5B). Our results indicate that A20 B cells overexpressing LPXN showed a decreased in the phosphorylation of JNK1, JNK2, and p38 MAPK at the 3-, 10-, and 30-min time points after BCR stimulation compared with control FLAG-vector-transfected A20 cells. However, the phosphorylation of ERK was largely unaffected upon the overexpression of LPXN, as at all three time points examined, the extent of ERK phosphorylation was comparable between FLAG- and FLAG-LPXN-transfected A20 B cells. We thus conclude that LPXN plays a negative role in the activation of JNK and p38 MAPK, but not ERK.

We next examined the effect of the overexpression of LPXN on the activation of Akt upon BCR ligation. As shown in Fig. 5C, the phosphorylation of Ser$^{473}$ and Thr$^{308}$ in Akt, which is indic-
ative of Akt activation, was slightly reduced at the 10- and 30-min time points in BCR-stimulated A20 B cells overexpressing LPXN compared with A20 cells transfected with the control FLAG vector. However, the inhibitory effect of the overexpression of LPXN on Akt activation was not as drastic as that on JNK and p38 MAPK activation. By contrast, LPXN did not appear to inhibit the activation of NFκB, as the degradation of IκBα was largely unaffected in A20 cells overexpressing LPXN (Fig. 5D). Thus, LPXN appears to play an inhibitory role in BCR signaling and seems to negatively regulate the activation of JNK, p38 MAPK, and, to a lesser extent, Akt, but not ERK and NFκB.

Leupaxin Inhibits IL-2 Production in A20 B Cells—Because LPXN appeared to inhibit the activation of JNK and p38 MAPK during BCR signaling, we were interested to determine the effect of LPXN expression on B cell function. Previous studies indicated that the overexpression of the inhibitory adaptor Dok-3 in A20 B cells also reduces JNK phosphorylation and that this leads to a decrease in the production of IL-2 in BCR-stimulated A20 cells (20, 24). Because the overexpression of LPXN also inhibited JNK activation, we investigated whether the overexpression of LPXN could affect IL-2 secretion by activated A20 cells.

A20 cells were transiently transfected with FLAG vector and increasing amounts of FLAG-LPXN and stimulated with 10 μg/ml goat anti-mouse IgG F(ab′)2 fragment at 37 °C for 24 h before culture supernatants were recovered and assayed for IL-2 production via ELISA. As shown in Fig. 6A, A20 B cells transfected with LPXN secreted less IL-2, and there was a dose-dependent reduction in IL-2 production with increasing amounts of LPXN transfected and expressed. Thus, LPXN inhibited IL-2 secretion by activated A20 cells in a dose-dependent manner. The total amount of FLAG-tagged LPXN transfected into A20 B cells was verified by immunoprecipitation with anti-FLAG antibody and immunoblotting with anti-FLAG antibody. Increasing amounts of LPXN were shown to be transfected into A20 cells (Fig. 6A).

To reaffirm our finding, we also performed an IL-2 promoter-driven luciferase reporter assay with A20 cells overexpressing LPXN. Compared with the ELISA, the IL-2 promoter-driven luciferase reporter assay provided a more sensitive way to measure the effect of LPXN overexpression on BCR signaling in A20 cells. As shown in Fig. 6B, control FLAG vector-transfected A20 cells up-regulated the production of IL-2 when stimulated via BCR as reflected by an increase in IL-2 promoter activity in the luciferase reporter assay. By contrast, A20 cells overexpressing LPXN did not show any increase in IL-2 production when measured in a similar manner. Thus, the overexpression of LPXN inhibits the production of IL-2 by activated B cells.
Inhibitory Role of Leupaxin in BCR Signaling

Tyrosine 72 of Leupaxin Is Important for Its Inhibitory Function—As shown above (Fig. 4B), Tyr72 of LPXN was the only tyrosine site phosphorylated by Lyn. Because LPXN was phosphorylated upon BCR signaling, Tyr72 could be important for the biological function of LPXN. To examine whether this tyrosine site is important for the function of LPXN, we overexpressed wild-type LPXN and various tyrosine-to-phenylalanine mutants in A20 cells and analyzed their effect on BCR-induced IL-2 production. First, we examined whether the Y72F LPXN mutant can be tyrosine-phosphorylated upon BCR stimulation. A20 cells were transiently transfected with FLAG vector or FLAG-tagged wild-type LPXN or mutant Y22F or Y72F and stimulated via BCR. The total cell lysates from transfected A20 cells were examined for the degradation of Igβ in response to BCR stimulation. The p38 blot was included as a control for the loading of whole cell lysates.

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of LPXN upon BCR stimulation in A20 cells, consistent with our earlier results in 293T cells (Fig. 4).

Next, A20 cells were transiently transfected with FLAG alone or with FLAG-tagged wild-type LPXN and mutants Y22F and Y72F (Fig. 7B) and stimulated with 10 μg/ml goat anti-mouse IgG F(ab')2 fragment at 37 °C for 24 h before culture supernatants were recovered and assayed for IL-2 production via ELISA. A20 cells transfected with wild-type LPXN showed inhibition of BCR-induced IL-2 production (Fig. 7B), consistent with our earlier results (Fig. 6A). A20 cells transfected with the Y22F LPXN mutant also showed similar repression of BCR-induced IL-2 production compared with wild-type LPXN. By contrast, the Y72F LPXN mutant showed no significant repression of IL-2 production and produced IL-2 at a level comparable with that produced by A20 cells transfected with the control FLAG vector (Fig. 7B). These findings indicate that Tyr72 of LPXN, which was shown above to be phosphorylated by Lyn (Fig. 4B), is important for LPXN-mediated repression of BCR-induced IL-2 production.

Similar results were also obtained when we measured IL-2 promoter-driven luciferase activity in A20 cells transfected with wild-type or mutant LPXN. As demonstrated in Fig. 7C, IL-2 promoter-driven luciferase activity was significantly suppressed in A20 cells transfected with wild-type and Y22F LPXN, whereas it was largely unaffected in cells transfected with the Y72F mutant. Used as a control, FLAG-transfected A20 cells also had high levels of IL-2 promoter-driven luciferase activity. Thus, this independent set of experiments further confirmed our hypothesis that Ty72 plays an important role in the function of LPXN and could be critical for mediating the inhibitory role of LPXN in repressing BCR-induced IL-2 production in A20 B cells.

**DISCUSSION**

We have demonstrated that LPXN, a member of the paxillin superfamily, was phosphorylated and recruited to the plasma membrane upon BCR ligation in human BJAB B lymphoma cells, indicating that LPXN can potentially play a role in B cell activation. Previous studies had shown that members of the paxillin family can interact with members of the Src kinase family (31, 34, 42), and indeed, we also established an interaction between LPXN and Lyn, a Src family tyrosine kinase that plays a critical role in BCR signal transduction. Although we have shown that Lyn also interacted with paxillin and another related family member, Hic-5 (Fig. 2A), these interactions might not be physiologically significant in B cells compared with the interaction between Lyn and LPXN. This is because the roles of paxillin and Hic-5 in BCR signaling remain controversial, as paxillin was not shown to be phosphorylated upon BCR ligation (27), whereas Hic-5 is largely absent in lymphocytes (28). On the other hand, LPXN is preferentially expressed in hematopoietic cells, including B cells, and we have shown here that it could be phosphorylated in B cells upon BCR activation. Indeed, we further established an interaction between endogenous LPXN and Lyn upon BCR ligation in BJAB cells. The induced nature of their interaction further strengthened our hypothesis that LPXN plays a role in BCR signaling. Interestingly, the kinetics of the interaction between LPXN and Lyn,
as well as LPXN recruitment to the plasma membrane, corresponded with LPXN phosphorylation by Lyn (Figs. 1 and 2). We further speculate that LPXN can be recruited to the plasma membrane by a yet unknown mechanism, where it would interact with plasma membrane-located Lyn and be phosphorylated by Lyn.

We further determined the specific domain of LPXN that interacts with Lyn. In previous studies, the LD2 domain of paxillin was reported to be the domain responsible for its interaction with Src (31), whereas the LD2 domain of LPXN was demonstrated to bind Src in osteoclasts (34). However, using different truncations of the LD domains of LPXN, we found LD3 to be the domain of LPXN that binds Lyn. The reason for the discrepancy between our finding and that published previously is unclear. However, a possible explanation could be the involvement of different tyrosine kinases with different LD domains. In the two previous studies, Src was bound by the LD2 domains of paxillin and LPXN. In our study, we examined the interaction between Lyn and LPXN, and perhaps, Lyn can bind only to the LD3 domain of LPXN. Thus, additional experiments may be needed to examine whether the LD3 domain of paxillin can also bind Lyn.

Using the NetPhos 2.0 phosphorylation prediction software, we identified six potential tyrosine phosphorylation sites among the 11 tyrosine residues in LPXN. We chose to mutate 4 tyrosine residues (Tyr22, Tyr72, Tyr198, and Tyr257) to assess their importance in the function of LPXN. Among these, Tyr72 was predicted to be the critical tyrosine phosphorylation site, as it corresponds to Tyr72 of paxillin, which has been shown to be important for its activation and function (31, 45). However, our results show that Tyr72 is the tyrosine phosphorylation site important for the activation of LPXN. The phosphorylation of Y72F mutant LPXN was completely abolished even in the presence of Lyn (Fig. 4B). This apparent unexpected result marks a difference between LPXN and paxillin in terms of the tyrosine phosphorylation sites critical for their biological function. This may indicate that the function of paxillin and LPXN can be very different. At this moment, we cannot rule out the possibility that the other tyrosine sites can also be phosphorylated, perhaps by tyrosine kinases other than Lyn and in different cell types and in response to different receptor signaling.

Our biochemical studies also established a role for LPXN in BCR signaling. The overexpression of LPXN in mouse A20 B lymphoma cells led to a decrease in JNK and p38 MAPK phosphorylation, whereas the activation of ERK was largely unaffected. The specific inhibition of JNK and p38 activation indicates a specific role of leupaxin in BCR signaling. The upstream kinase mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase (MEKK) is likely to be influenced by LPXN (46), and this will be addressed in future work. The possible effect of LPXN on the activity of a downstream target of JNK, AP-1, also remains to be studied (47, 48). Besides the phosphorylation of JNK and p38, the phosphorylation of Akt was also reduced in BCR-stimulated A20 B cells overexpressing LPXN. On the other hand, NFκB activation was unaffected upon overexpression of leupaxin. Thus, LPXN appears to function as an inhibitor of specific BCR signaling pathways.

Consistent with our biochemical analyses suggesting that LPXN can selectively inhibit certain BCR signaling pathways, our functional studies showed that A20 B cells overexpressing LPXN secreted much less IL-2 when activated via their BCR compared with control cells. The reduced production of IL-2 could be the result of the inhibition of JNK, p38 MAPK, and Akt signaling in A20 B cells overexpressing LPXN. Indeed, previous reports correlated IL-2 production to JNK signaling in Jurkat T cells (47, 48), and there is also reduced JNK phosphorylation and IL-2 production in A20 B cells overexpressing LPXN. Therefore, we speculate that LPXN regulates IL-2 production in B cells via regulating JNK, p38 MAPK, and Akt activities and that Tyr72 of LPXN is critical for the inhibition of BCR-induced IL-2 production (Fig. 7).

The precise mechanisms governing the negative regulatory function of LPXN in BCR signaling are still largely unknown. LPXN as a substrate of Lyn may affect the downstream signaling pathways shown previously to be initiated by Lyn. Despite the established positive role of Lyn in BCR signal initiation, Lyn-deficient mice are susceptible to autoimmune disease, and Lyn-deficient B cells are hyper-responsive to BCR ligation (13–15). Analyses of Lyn-deficient primary B cells showed increases in MAPK and Akt activation as well as enhanced calcium signaling (54). Lyn has since been described as having both a positive and a negative regulatory role in BCR signaling (11–14, 54). On the basis of these studies, we speculate that LPXN may play a role in enhancing the negative regulatory role of Lyn. A negative signaling pathway regulated by Lyn during BCR signaling involves the phosphatase SHIP-1. Our preliminary data indicated that the phosphorylation of SHIP-1 was normal in cells overexpressing LPXN (data not shown), hence ruling out the possibility of LPXN acting on the SHIP-1 negative regulatory pathway. It is also possible that LPXN may function in a novel pathway downstream of Lyn to enhance its negative regulatory role in BCR signaling. It had been shown previously that members of the paxillin superfamily interact with the phosphatase PTP-PEST (31, 33, 55). Thus, it is possible that LPXN may exert its negative regulatory role via PTP-PEST, and this remains to be determined. Alternatively, members of the paxillin superfamily (in particular, paxillin and Hic-5) have been shown to interact with Csk, which down-regulates Lyn activity in human and murine platelets (56). Again, LPXN could potentially exert its negative function via Csk. Our preliminary data suggested that LPXN could interact with Csk (data not shown), but this awaits further experimentation and confirmation. In conclusion, we have established a previously unknown involvement of LPXN, a member of the paxillin superfamily, in BCR signal transduction and demonstrated a novel inhibitory role for LPXN in BCR signaling and B cell function.

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