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Epstein-Barr Virus Latent Membrane Protein 2A Preferentially Signals through the Src Family Kinase Lyn▽

Mark Rovedo and Richard Longnecker*

Department of Microbiology and Immunology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611

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Latent membrane protein 2A (LMP2A) is a viral protein expressed during Epstein-Barr virus (EBV) latency in EBV-infected B cells both in cell culture and in vivo. LMP2A has important roles in modulating B-cell receptor signal transduction and provides survival and developmental signals to B cells in vivo. Although Lyn has been shown to be important in mediating LMP2A signaling, it is still unclear if Lyn is used preferentially or if LMP2A associates promiscuously with other Src family kinase (SFK) members. To investigate the role of various SFKs in LMP2A signaling, we crossed LMP2A transgenic mice (TgE) with $Lyn^{-/-}$, $Fyn^{-/-}$, or $Blk^{-/-}$ mice. TgE $Lyn^{-/-}$ mice had a larger immunoglobulin M (IgM)-positive B-cell population than TgE mice, suggesting that the absence of Lyn prevents LMP2A from delivering survival and developmental signals to the B cells. Both TgE $Fyn^{-/-}$ and TgE $Blk^{-/-}$ mice have an IgM-negative population of splenic B cells, similar to the TgE mice. LMP2A was also transiently transfected into the human EBV-negative B-cell line BJAB to determine which SFK members associate with LMP2A. Lyn was detected in LMP2A immunoprecipitates, whereas Fyn was not. Both Lyn and Fyn were able to bind to an LMP2A mutant which contained a sequence shown previously to bind tightly to the SH2 domain of multiple SFK members. From these results, we conclude that LMP2A preferentially associates with and signals through Lyn compared to its association with other SFKs. This preferential association is due in part to the SH2 domain of Lyn associating with LMP2A.

Epstein-Barr virus (EBV) is a member of the gammaherpesvirus family and has been associated with the development of several human malignancies, including African Burkitt's lymphoma, Hodgkin's disease, adult T-cell leukemia, nasopharyngeal carcinoma, and some gastric cancers. EBV infects human B cells and artificially drives their development to the long-lived memory B-cell compartment (1, 2, 32, 41, 42, 85, 86, 92). EBV accomplishes this by infecting naïve B cells and using different programs of viral gene expression to manipulate the development of infected B cells. Once the infected B cells are driven to the memory B-cell compartment, the EBV gene expression switches to a nearly quiescent state. EBV transcripts have been detected in infected peripheral B cells, but few if any viral proteins are expressed, allowing the latently infected cells to avoid immune surveillance (3, 12, 13, 17, 31, 60, 72, 93). EBV then persists in these long-lived memory B cells for the lifetime of the host.

One of the EBV proteins implicated in modulating normal B-cell signaling and development is latent membrane protein 2A (LMP2A). The effects of LMP2A expression on normal B cell development have been investigated by using a transgenic-mouse model (7, 8, 36, 37, 53, 54, 67–71, 89, 90). TgE mice express LMP2A under the Eμ heavy chain promoter and intronic enhancer (8). These mice express LMP2A in B cells at the pro-B stage of development. In normal B-cell development, B cells go through a series of developmental stages in the bone marrow that are marked by gene rearrangements of the heavy chain and light chain. These gene rearrangements culminate in the expression of a mature B-cell receptor (BCR). Upon expressing a BCR, B cells migrate out into the periphery to complete their maturation. If at any stage of development the gene rearrangements are not successful, a BCR is not produced and the cell dies by apoptosis. In B cells expressing LMP2A, normal developmental checkpoints in B cells are bypassed, and these cells are able to migrate out into the periphery regardless of whether they express a BCR or not (7, 8, 53, 54). In these mice, LMP2A functions to deliver development and survival signals to B cells even in the absence of normal BCR signals (7, 8, 53, 54).

The expression of LMP2A has also been shown to modulate normal B-cell signaling in human B cells (21–23, 39, 55, 57, 58, 96). In uninfected B cells, cross-linking of the BCR by antigen results in the aggregation of the BCR into glycosphingolipid-rich microdomains (lipid rafts) of the plasma membrane (14). These lipid rafts contain an increased concentration of Src family protein tyrosine kinases (SFKs), such as Lyn, which interact with BCR immunoglobulin alpha (Igα) and Igβ subunits and mediate the phosphorylation of the BCR immunoreceptor tyrosine activation motifs (ITAMs) (40, 44, 66). The dually phosphorylated ITAM tyrosine residues become a binding site for the tyrosine kinase Syk (74). Binding of Syk to the ITAM results in its phosphorylation and activation via Lyn and activation of subsequent signaling events, including protein phosphorylation and calcium mobilization, which eventually leads to changes in gene transcription (40). LMP2A has been suggested to alter normal BCR signal transduction in B cells by recruiting cellular proteins such as Lyn and Syk, reducing the levels of Lyn and consequently downregulating tyrosine phosphorylation and calcium mobilization following BCR cross-linking (22, 23, 39, 55, 57, 58). The LMP2A N-terminal domain

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Feinberg School of Medicine, Northwestern University, Ward 6-231, 303 E. Chicago Avenue, Chicago, IL 60611. Phone: (312) 503-0467. Fax: (312) 503-1339. E-mail: r-longnecker@northwestern.edu.

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contains several protein interaction motifs similar to those found in cellular proteins (6, 22, 23, 38, 39, 46, 96). In addition to an ITAM motif, LMP2A contains a sequence based around Y112 that is very similar to motifs that associate with the SH2 domain of SFKs (23). Additionally, LMP2A contains two polyproline motifs that have been shown to recruit Nedd4 ubiquitin ligases (38, 39, 96). The recruitment of Lyn to LMP2A results in the ubiquitination and subsequent degradation of Lyn. LMP2A also delivers its own tonic signals through Lyn and Syk (7, 8, 22, 23, 53–55, 57, 58, 67, 69–71, 89, 90). The interaction with multiple cellular proteins enables LMP2A to alter normal B-cell signaling and development.

The SFKs include Src, Lyn, Fyn, Blk, Yes, Lck, Hck, Fgr, and Yrk. All SFK members are highly homologous in amino acid sequence, and many exhibit overlaps in function. The SFKs have a similar arrangement of domains which are important in facilitating protein-protein interactions and regulation of catalytic activity. One of these domains, the Src homology 2 (SH2) domain, recognizes short sequence motifs containing a phosphotyrosine residue. It has been shown previously that differences in the amino acid sequence of SH2 domains direct specificity (9, 15, 19, 61, 65, 79, 81–84). For example, while the SFK SH2 domains preferentially bind to the consensus sequence YEEI, the SH2 domain of PI3K preferentially binds to the sequence YMEM.

It has been previously shown that Lyn, Fyn, and Blk are particularly important in normal BCR signaling and development (75). Lyn is the most highly expressed SFK in B cells and has been shown to work in conjunction with other coreceptors to modulate B-cell signaling (5, 24–27, 64, 73, 76, 77, 95). This suggests that Lyn may play a particularly important role in regulating BCR signaling. Lyn has been shown to associate with LMP2A in immunoprecipitation assays (6, 23). Taken together, this led us to hypothesize that LMP2A may preferentially use Lyn to modulate normal BCR signaling. To investigate the role of various SFKs in LMP2A signaling, we crossed LMP2A transgenic mice (TgE) to various SFK knockout mice. TgE Lyn$^{-/-}$ mice had a larger IgM-positive population than TgE mice, suggesting that the absence of Lyn prevents LMP2A from delivering survival and developmental signals to the B cells. LMP2A was also transiently transfected into the human EBV-negative B-cell line BJAB to determine which SFK members associate with LMP2A. Lyn was detected in LMP2A immunoprecipitates from BJAB lysates, whereas Fyn was not; however, Lyn and Fyn were both able to bind to an LMP2A mutant which contained a sequence shown previously to bind tightly to the SH2 domain of multiple SFK members. From these results, we conclude that LMP2A preferentially associates with and signals through Lyn compared to its association with other SFKs and that this preferential association is due to the SH2 domain of Lyn associating with LMP2A.

MATERIALS AND METHODS

Cell lines, cell culture, and DNA vectors. BJAB cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1,000 U/ml penicillin, and 1,000 μg/ml streptomycin. BJAB is an EBV-negative B-lymphoma cell line (52). BJAB cell lines expressing LMP2A and LMP2A mutants were generated by infection with retrovirus stocks, using a previously described LMP2A retroviral-expression construct (pMP2). PCR-mediated mutagenesis was used to generate YEEI, YEEL, and YMEM LMP2A mutants. Y112F was described previously (23). Mutated LMP2A constructs were verified by sequencing. Retrovirus stocks were made by transient transfection of the GP2293 cell line. The resulting stocks were used to infect BJAB cells, which were then selected in 400 U/ml hygromycin.

Transfection. BJAB cells were transiently transfected by using a Bio-Rad Genepulser. Five million BJAB cells were electroporated in 0.4 ml of RPMI 1640 with 12 μg of the appropriate vector. Cells were pulsed at 210 V and 960 mF capacitance in a 0.4-cm electrode gap cuvette and then incubated in 10 ml of RPMI 1640 at 37°C for 14 h. After the 14-h incubation, cells were lysed in 1 ml of lysis buffer.

Mice. The construction and characterization of EuLMP2A transgenic mice have been previously described (8). Lyn$^{-/-}$, Fyn$^{-/-}$, and Blk$^{-/-}$ animals were graciously donated by the laboratory of A. Tarakhovsky. All animals were housed at the Northwestern University Center for Experimental Animal Resources in accordance with university animal welfare guidelines.

Fluorescence-activated cell sorter (FACS) analysis. Flow cytometry was done as previously described (7, 8). Data were recorded by using a BD LSR II FACS machine, and samples analyzed by using FACSDiva 5.0.

Antibodies. The anti-LMP2A antibody 14B7 has been described previously (21). All horseradish peroxidase-linked secondary antibodies were purchased from Amersham (Arlington Heights, IL). The mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and the anti-hemagglutinin (HA) epitope mouse monoclonal antibody (12CA5) were purchased from Abcam. Lyn, Fyn, and Blk antibodies were purchased from Santa Cruz. FACS antibodies CD19-phycoerythrin (PE) and IgM-fluorescein isothiocyanate (FITC) were purchased from BD Pharmingen.

Immunoprecipitation and immunoblots. Lysates were cleared for 1 h and then incubated with the appropriate antibodies (indicated in figure legends) overnight at 4°C. Protein G-coated beads were added and allowed to incubate for 1 h. The immunoprecipitates were then washed three times in Triton X-100 buffer, resuspended in 2× sodium dodecyl sulfate (SDS) buffer, heated to 70°C for 5 min, and separated by electrophoresis on 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels. For immunoblotting, cell lysates and immunoprecipitates were electrophoresed and transferred to Immobilon membranes. Membranes were blocked in Tris-buffered saline–Tween (TBST) containing 5% milk for 1 h at room temperature, incubated for 1 h in milk containing the appropriate primary antibodies at room temperature, washed three times in TBST, and incubated with secondary antibodies in milk for 30 min at room temperature. For detection, membranes were washed three times in TBST and detected by using ECL plus (Pierce, Rockford, IL).

RESULTS

Lyn$^{-/-}$ TgE mice have a restored IgM-positive B-cell population compared to the level of this population in TgE mice. B cells that do not go through productive gene rearrangements during development in the bone marrow do not express a BCR and are not able to move into the periphery. Instead, these cells die by apoptosis. In the TgE transgenic-mouse model, LMP2A is expressed during the pro-B stage of development and results in the bypassing of normal B-cell developmental checkpoints. In these mice, B cells that are IgM negative do not die by apoptosis but instead move out into the periphery and colonize peripheral lymphoid organs (7, 8). SFKs have been shown to play an important role in the delivery of survival and developmental signals from LMP2A to the B cell (6, 18, 23, 46, 54, 56). We hypothesized that Lyn may be a particularly important SFK in delivering developmental and survival signals from LMP2A to the B cells in TgE mice. To examine this possibility, we crossed TgE mice with Lyn$^{-/-}$, Fyn$^{-/-}$, or Blk$^{-/-}$ mice. Splenic cells were isolated from wild-type (wt), TgE, Lyn$^{-/-}$, TgE Lyn$^{-/-}$, Fyn$^{-/-}$, TgE Fyn$^{-/-}$, Blk$^{-/-}$, and TgE Blk$^{-/-}$ mice and analyzed by flow cytometry. Representative plots from several experiments are shown in Fig. 1. Cells were stained with IgM-FITC to determine IgM expression and with CD19-PE as a pan-B-cell marker. As was expected, wt mice did not have an IgM-negative population of splenic B cells, nor did Lyn$^{-/-}$, Fyn$^{-/-}$, or Blk$^{-/-}$ mice. In contrast to

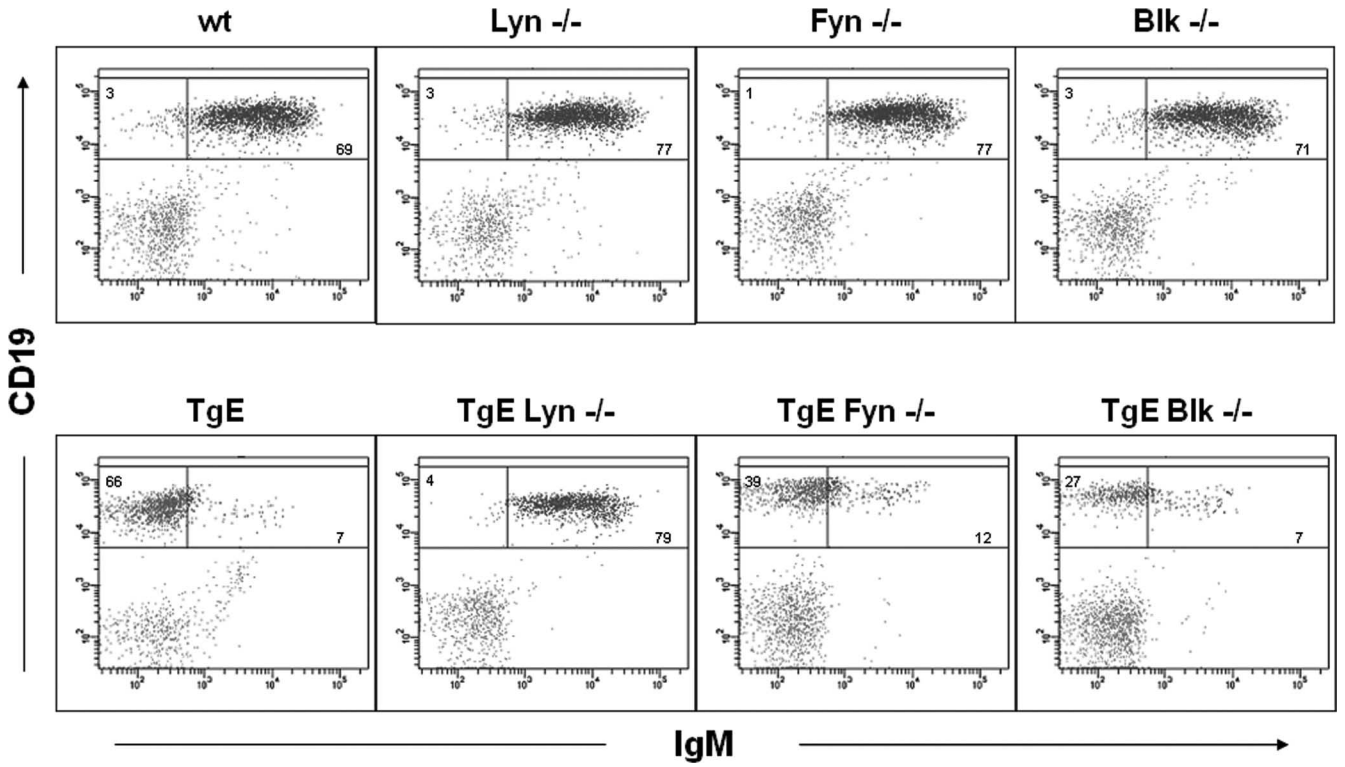

FIG. 1. Splenic B cells from Lyn$^{-/-}$ TgE mice contain an IgM-positive population. Representative flow cytometry dot plots from multiple experiments are shown. Splenic cells were harvested from 5-week-old mice. Red blood cells were lysed, and the remaining splenic cells were counted using a hemocytometer. Cells were then washed, stained with fluorescently labeled antibodies (1 million cells stained with IgM-FITC and CD19-PE), and analyzed by flow cytometry. The *x* axis represents IgM expression, and the *y* axis represents CD19 expression. IgM-positive and IgM-negative populations are marked by boxes.

this, B cells from TgE mice had a large IgM-negative population. Interestingly, TgE Lyn$^{-/-}$ mice had a normal IgM-positive population, suggesting that Lyn is important in transducing signals from LMP2A to the B cell. Both TgE Fyn$^{-/-}$ and TgE Blk$^{-/-}$ mice have an IgM-negative population of splenic B cells, similar to the TgE mice, suggesting that Fyn and Blk do not contribute to LMP2A signaling in B cells. In contrast to this, the expression of Lyn is required for the normal signaling of LMP2A in the TgE mouse model.

Data from several experiments were tabulated, and the statistical significance was determined (Fig. 2). The difference between the percentage of IgM-positive splenic B cells in TgE mice compared to the percentage in TgE Lyn$^{-/-}$ mice was statistically significant ($P < 0.005$). However, there was no statistically significant difference between TgE mice and TgE Fyn$^{-/-}$ mice or TgE mice and TgE Blk$^{-/-}$ mice. This suggests that Lyn is specifically important in transducing LMP2A signals to mouse B cells.

Expression of LMP2A results in decreased levels of Lyn, but not Fyn. It has been shown previously that the expression of LMP2A in B cells results in decreased levels of Lyn (23, 38, 39, 96). However, it has not been determined if LMP2A mediates a similar effect on other SFKs. To determine if LMP2A expression has any effect on the levels of Fyn, we ran lysates from the EBV-negative human B-cell line BJAB stably expressing LMP2A or the vector control on an SDS-PAGE protein gel and probed for Lyn, Fyn, LMP2A, and GAPDH (Fig. 3A). GAPDH was used as a protein loading control. As previously described, the expression of LMP2A in BJAB cells resulted in a decrease in the constitutive level of Lyn compared to the level with the vector control (23). However, the expression of LMP2A did not decrease the level of Fyn compared to the level with the vector control.

The effect of LMP2A expression on the levels of Lyn and

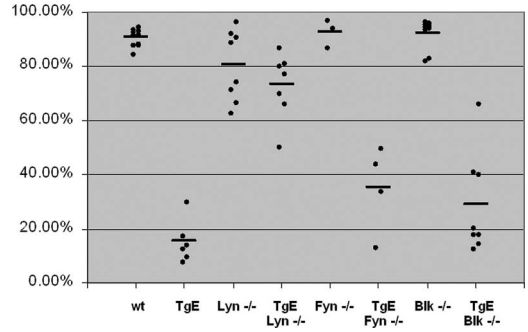

FIG. 2. Splenic B cells from Lyn$^{-/-}$ TgE mice contain an IgM-positive population that is statistically significant compared to the IgM-positive B-cell populations in TgE, Fyn$^{-/-}$ TgE, and Blk$^{-/-}$ TgE mice. Flow cytometry data tabulated from multiple experiments are shown. IgM-positive populations were calculated as the percentage of total CD19-positive cells. Dots represent individual mice. The bar in each column represents the average of the results.

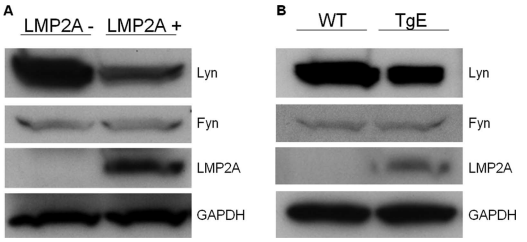

FIG. 3. Lyn levels are lower in LMP2A-expressing cells. (A) Lysates were prepared using 1 ml Triton X-100 lysis buffer and 10 million BJAB cells stably expressing LMP2A or vector control (previously described). Lysates were separated by SDS-PAGE and immunoblotted with rabbit anti-Lyn, rabbit anti-Fyn, rat anti-LMP2A, or mouse anti-GAPDH antibody. Lyn was detected at a lower level in LMP2A-positive BJAB cells than in wt BJAB cells. Similar amounts of Fyn were detected in both LMP2A-positive BJAB cells and wt BJAB cells. GAPDH served as a loading control. +, present; −, absent. (B) B cells were purified from disassociated mouse spleens by using CD19-coated magnetic beads. Cell lysates were prepared from the purified splenic B cells of TgE and wt mice. Lysates were separated by SDS-PAGE and immunoblotted with rabbit anti-Lyn, rabbit anti-Fyn, rat anti-LMP2A, or mouse anti-GAPDH antibody. Lyn was detected at a lower level in TgE mouse splenic cells than in wt mouse splenic cells. Similar amounts of Fyn were detected in both TgE and wt mouse cells. GAPDH served as a loading control.

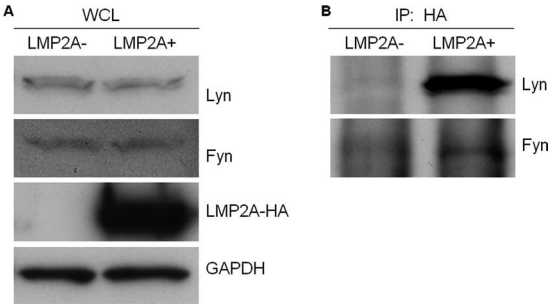

FIG. 4. LMP2A preferentially associates with Lyn. Ten million BJAB cells were electroporated with either 12 μg of an HA-tagged LMP2A-expressing plasmid or empty vector. Cells were incubated at 37°C and 5% carbon dioxide for 14 h. Lysates were prepared by using 1 ml of Triton X-100 lysis buffer per 10 million cells. (A) Whole-cell lysates (WCL) were separated by SDS-PAGE and immunoblotted with rabbit anti-Lyn, rabbit anti-Fyn, rat anti-LMP2A, or mouse anti-GAPDH antibody. (B) Lysates were immunoprecipitated (IP) with mouse anti-HA antibody, separated by SDS-PAGE, and immunoblotted with rabbit anti-Lyn or rabbit anti-Fyn antibodies. Lyn was detected in the LMP2A immunoprecipitate, but Fyn was not. +, present; −, absent.

Fyn was also investigated in the mouse model of LMP2A (Fig. 3B). B cells were purified from disassociated mouse spleens by using CD19-coated magnetic beads. Cell lysates were prepared from the purified splenic B cells of TgE and wt mice, run on an SDS-PAGE gel, and probed for the presence of Lyn, Fyn, LMP2A, and GAPDH. The expression of LMP2A resulted in a decrease in the amount of Lyn in the B cells, similar to previously observed results (36). The levels of Fyn were unchanged between the TgE and wt mice. Taken together, these data show that LMP2A expression affects the constitutive levels of Lyn, but not Fyn.

LMP2A associates with Lyn, but not with Fyn, in coimmunoprecipitations. It has been previously demonstrated that LMP2A associates with Lyn in coimmunoprecipitations (6, 23). This association is important in delivering signals via LMP2A, as well as the degradation of Lyn (23, 39, 96). Although Fyn was not degraded in the presence of LMP2A, the possibility exists that LMP2A could still be associating with both Lyn and Fyn but only facilitating the degradation of Lyn. It has been shown previously that Fyn-mediated tyrosine phosphorylation of the Nedd4 ubiquitin ligase Itch results in negative regulation of Itch ubiquitination activity (97). Additionally, the association of the tyrosine kinase Syk with LMP2A has no effect on Syk ubiquitination and degradation (22, 39). To determine if LMP2A associates with Fyn, we immunoprecipitated LMP2A and probed for the presence of Lyn and Fyn (Fig. 4). BJAB cells were electroporated with a plasmid expressing HA-tagged LMP2A and allowed to incubate for 14 h. Whole-cell lysates were run on an SDS-PAGE gel and probed for the presence of Lyn, Fyn, LMP2A, and GAPDH (Fig. 4A). Lyn levels did not decrease in the presence of LMP2A in the transiently transfected cells due to the short incubation time. LMP2A was immunoprecipitated via the C-terminal HA tag. LMP2A was immunoprecipitated by the HA tag instead of the N-terminal tail in order to prevent disruption of the SFKs' association with the N-terminal tail of LMP2A. Immunoprecipitates were run on an SDS-PAGE gel and probed for the presence of Lyn and Fyn (Fig. 4B). As previously described, Lyn coimmunoprecipitated with LMP2A. No Fyn was detected in the LMP2A immunoprecipitation. This suggests that Lyn preferentially associates with LMP2A.

Expression of YEEL mutant results in lower levels of both Lyn and Fyn. We next set out to determine which domains of Lyn were responsible for the preferential binding of Lyn to LMP2A. The SH2 domains of different proteins bind to motifs containing a phosphotyrosine and have been shown previously to have different binding specificities (9, 15, 19, 61, 65, 79, 81–84). The amino acids surrounding the phosphotyrosine residue have been shown to be essential in determining this peptide binding specificity (9, 43, 45, 49, 83). The SH2 domain of SFKs has been shown to bind particularly strongly to the sequence YEEI, which is very similar to the YEEA sequence found in LMP2A (83). Additionally, this tyrosine residue of LMP2A (Y112) has been shown to be important in the binding of Lyn to LMP2A (23). To determine if the Lyn SH2 domain interaction with the YEEA motif of LMP2A is important in imparting binding specificity, we created two LMP2A mutants. The YEEL mutant changed the alanine to lysine in the YEEA sequence of LMP2A, which is similar to a sequence in the ITAM of the Igα and Igβ subunits of the BCR that bind the SFKs Lyn, Fyn, and Blk. The YMEM mutant was created by changing the YEEA motif of LMP2A to YMEM, which is the consensus binding sequence for the SH2 domain of PI3K. Four separate cell lines were created that stably expressed wt LMP2A, the YEEL mutant, the YMEM mutant, or a control empty vector. Lysates were prepared from the four cell lines, run on an SDS-PAGE gel, and probed for the presence of Lyn, Fyn, LMP2A, and GAPDH (Fig. 5). Densometric analysis was performed to quantify the levels of Lyn and Fyn detected. As was previously described, the expression of wt LMP2A results in lower constitutive levels of Lyn, but not Fyn. Interestingly, the expression of the YEEL mutant not only resulted in sig-

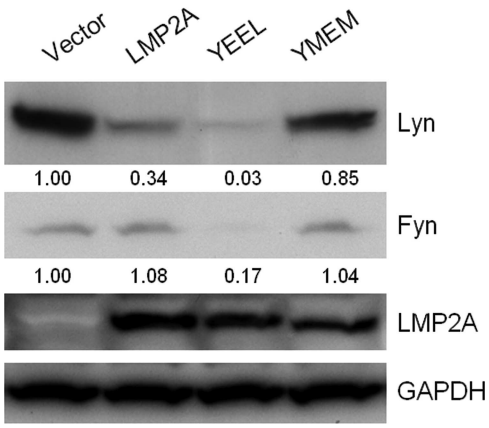

FIG. 5. Expression of the YEEL LMP2A mutant results in lower Lyn and Fyn levels. Two separate LMP2A mutants were derived by using a previously described retroviral construct (pMP2). Cells infected with the parental construct, the YEEL mutant, the YMEM mutant, or the control empty vector were selected in hygromycin to generate four different cell lines. Lysates were prepared by using 1 ml of Triton X-100 lysis buffer per 10 million cells from each of the four cell lines. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with rabbit anti-Lyn, rabbit anti-Fyn, rat anti-LMP2A, or mouse anti-GAPDH antibody. Lyn levels were lower in the LMP2A-expressing cells than in cells with vector control and lower in the YEEL mutant cell line than in LMP2A-expressing cells. There was no significant difference in Lyn levels in the YMEM cell lysates and in lysates of cells with the vector control. Fyn levels in the vector control, LMP2A-expressing, and YMEM-expressing cell lines were equivalent, but they were lower in the YEEL-expressing cell line. Densometric analysis was used to quantify protein levels normalized to the levels in the control.

nificantly reduced Lyn levels compared to the levels with wt LMP2A but also reduced the Fyn levels compared to the levels with the vector control. As was predicted, the expression of the YMEM mutant did not have any effect on Lyn or Fyn levels. Taken together, this suggests that the binding preference for Lyn may be due in part to the YEEA sequence.

YEEL and YEEI mutants associate with both Lyn and Fyn. To determine if the lower levels of Lyn and Fyn in the YEEL-expressing cells were due to increased binding, we decided to immunoprecipitate LMP2A and probe for the presence of Lyn and Fyn. Two other LMP2A mutants were used in addition to the YEEL and YMEM mutants. A YEEI mutant, which should bind strongly to the SH2 domain of SFKs, was constructed by mutating the alanine to an isoleucine in the YEEA sequence of LMP2A. The Y112F mutant has been described previously and has tyrosine 112 of LMP2A changed to a phenylalanine (23). Cells were electroporated with plasmids expressing wt LMP2A, YEEI, YEEL, YMEM, Y112F, or a control vector. Whole-cell lysates were run on an SDS-PAGE gel and probed for the presence of Lyn, Fyn, LMP2A, and GAPDH (Fig. 6A). LMP2A immunoprecipitates were run on an SDS-PAGE gel and probed for the presence of Lyn and Fyn (Fig. 6B). As previously described, Lyn, but not Fyn, coimmunoprecipitated with wt LMP2A. However, both Lyn and Fyn were detected in the immunoprecipitates of YEEL and YEEI mutants. This suggests that the YEEA motif found in LMP2A preferentially associates with the SH2 domain of Lyn, but not Fyn. LMP2A was able to bind to both Lyn and Fyn by changing

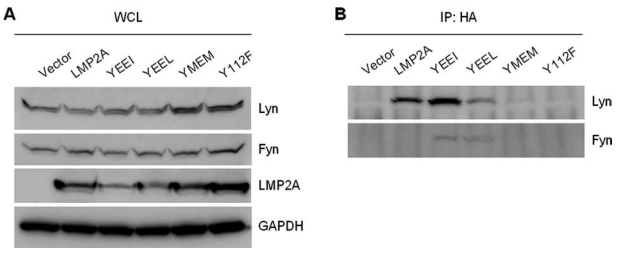

FIG. 6. YEEL and YEEI mutants associate with both Lyn and Fyn. Ten million BJAB cells were electroporated with 12 μg of an HA-tagged LMP2A-expressing plasmid, one of three HA-tagged mutant LMP2A-expressing plasmids (YEEI, YEEL, or YMEM), or empty vector. Cells were incubated at 37°C and 5% carbon dioxide for 14 h. Lysates were prepared by using 1 ml of Triton X-100 lysis buffer per 10 million cells. (A) Whole-cell lysates were separated by SDS-PAGE and immunoblotted with rabbit anti-Lyn, rabbit anti-Fyn, rat anti-LMP2A, or mouse anti-GAPDH antibody. (B) Lysates were immunoprecipitated with mouse anti-HA antibody, separated by SDS-PAGE, and immunoblotted with rabbit anti-Lyn or rabbit anti-Fyn antibody.

the YEEA sequence of LMP2A to motifs that have been demonstrated to bind to multiple SFKs, namely, YEEI and YEEL.

DISCUSSION

SFK members play similar but distinct roles in B-cell signaling and development. This is evidenced by the differences in phenotypes between various SFK knockout mice. While $Lyn^{-/-}$ mice have a slightly reduced number of splenic B cells compared to the level in their wt counterparts, $Fyn^{-/-}$ mice, as well as $Blk^{-/-}$ mice, have normal numbers of splenic B cells (35, 63, 80, 91). Cross-linking of the BCR normally results in the phosphorylation of a number of downstream proteins. B cells isolated from $Lyn^{-/-}$ mice have reduced tyrosine phosphorylation upon BCR cross-linking, whereas Fyn and Blk knockouts appear to have no discernible defect (35, 91). B cells isolated from $Lyn^{-/-}$ mouse spleens had higher proliferation than B cells from wt mice when cross-linked with $F(ab')_2$ (35). In contrast to this, $Blk^{-/-}$ B cells did not proliferate more (91). The results of studies of mice expressing constitutively active forms of different SFKs have also shed light on the unique roles each may play. Mice expressing a constitutively active form of Blk (Blk Y495F) exhibited increased responsiveness to interleukin-7 and supported maturation beyond the pro-B-cell stage even in the absence of a pre-BCR (94). In contrast to this, mice expressing a constitutively active form of Lyn (Lyn Y508F) were not more responsive to interleukin-7, although like the mice expressing Blk Y495F, they did exhibit a small subset of IgM-negative B cells in the spleen (29).

The differences in SFK roles in B cells may be accounted for by their ability to associate with and phosphorylate different targets. In particular, the SH2 domains of Blk, Lyn, and Fyn have been show to bind to and phosphorylate distinct sets of proteins (4, 47). The SH2 domain is found on a variety of cellular proteins and is an important mediator of protein-protein interactions. The SH2 domain recognizes short sequence motifs containing a phosphotyrosine residue. The amino acids surrounding the phosphotyrosine are important in directing SH2 binding specificity (83, 84).

Although there are considerable differences in the targets of

the various SFK members, Lyn, Fyn, and Blk also demonstrate overlap in function. This is evidenced by the severe B-cell developmental block in a triple-knockout mouse (75). While the knockout of any one SFK does not significantly affect early B-cell development, a triple knockout of all three results in a block between the pro-B- to pre-B-cell transition. This demonstrates the fact that Lyn, Fyn, and Blk can all partially compensate for the loss of each other in B-cell development; however, the loss of all three cannot be overcome.

Our data have demonstrated that Lyn is required for the LMP2A phenotype in mice, whereas Fyn and Blk are not required. It is interesting to speculate as to why LMP2A would preferentially associate with and signal through Lyn. Lyn may be important in transducing survival and developmental signals at particular stages of B-cell development. Lyn has been shown to play a role in the development and survival of mature B cells (11, 20, 30, 51, 63). LMP2A may signal through Lyn in B cells infected by EBV to drive differentiation to the memory B-cell compartment. In fact, knocking out Lyn has been shown to result in changes in gene transcription for a number of genes, including those which control BCR signaling, proliferation, transcription, inflammation, and cytoskeletal organization (59). Particularly interesting are data from microarrays and Lyn knockout mice which suggest that Lyn may control the formation and proliferation of germinal centers via germinal-center-associated DNA primase that cannot be compensated for by other SFKs (59). The initial infection of B cells by EBV results in viral gene expression, including LMP2A expression, which has been suggested to activate B cells and induce them to migrate into follicles to form germinal centers (1, 2, 32, 41, 42, 86, 92). LMP2A may transduce signals through Lyn that are important in the formation of these germinal centers. In addition, B cells from $Lyn^{-/-}$ mice showed increased developmental arrest and deletion when exposed to self antigen (16, 20). LMP2A may sequester and prevent normal signaling through Lyn to prevent the deletion of EBV-infected cells.

The important role that Lyn plays in B-cell signaling may also be a reason that Lyn is specifically targeted by LMP2A. In addition to the role that Lyn plays in BCR signal transduction, Lyn also plays both positive and negative regulatory roles. It has been shown that Lyn plays an important role in the ability of CD19 to amplify B-cell signal transduction (10, 25–28, 95). Lyn also plays a role in the negative regulation of B-cell signaling. Lyn has been shown to phosphorylate an inhibitory residue on Syk (34). In cells that do not express Lyn, the phosphorylation of Y317 of Syk is suppressed, which leads to elevated production of inositol 1,4,5-trisphosphate and an amplified calcium flux. Lyn and Syk may be recruited by LMP2A to facilitate the phosphorylation of the inhibitory residue on Syk, thus inhibiting normal BCR signaling. Lyn is also involved in regulating the phosphorylation of CD22 and FcγRIIB, which facilitates the recruitment of the SHP-1 and SHIP phosphatases and inhibits B-cell signal transduction (62). The ability to modulate Lyn-specific signaling at particular stages of the EBV life cycle without significantly altering the signaling of other SFKs may be important in the establishment of infection or persistence of EBV. Alternatively, Lyn may be used preferentially by LMP2A to prevent the development of human malignancies in the host. The dysregulation of SFK members other than Lyn by LMP2A may lead to the formation of B-cell tumors or other human malignancies. For example, mice expressing the constitutively active version of Blk develop B-cell tumors, whereas mice expressing the constitutively active version of Lyn do not (48).

Another possibility as to why it may be important for LMP2A to preferentially associate with Lyn at particular stages of B-cell development is that the association of LMP2A with multiple SFKs and their subsequent degradation may have adverse effects on B-cell survival and development. In TgE mice, the levels of Lyn are lower than those found in wt mice. However, the levels of other SFKs, such as Fyn, are not affected by LMP2A expression. Interestingly, while TgE $Lyn^{-/-}$ mice had numbers of total B cells that were comparable to those in wt mice, TgE $Fyn^{-/-}$ and TgE $Blk^{-/-}$ mice had lower numbers of B cells (Fig. 1, data not shown). This suggests that in the presence of LMP2A, SFK members not recruited and degraded by LMP2A may be important in compensating for the lower levels of Lyn. The degradation of multiple SFK members may negatively impact B-cell survival or development during EBV infection. By preferentially associating with Lyn at stages of B-cell maturation and development where other SFKs may be important, LMP2A is able to transduce signals to the B cell without impacting the function of the other SFKs.

Our coimmunoprecipitation studies have demonstrated that Lyn preferentially binds to the motif surrounding Y112 of LMP2A compared to the binding of other SFKs, such as Fyn. Likewise, the results of our experiments utilizing TgE mice crossed with Lyn, Fyn, and Blk knockout mice have shown that this preferential association of Lyn with LMP2A has a role in delivering LMP2A signals to B cells. Although we have demonstrated the importance of Lyn in LMP2A signaling in mice, we cannot rule out the possibility that preferential binding of Lyn to LMP2A is not essential at other stages of maturation and development in B cells. However, we believe that the murine model provides strong evidence that Lyn plays a particularly important role in LMP2A signaling. Unfortunately, to our knowledge there is not an appropriate human B-cell model that replicates the effect of LMP2A on human B-cell development and survival in vivo. Additional studies will be needed to understand the role that the preferential binding of Lyn to LMP2A plays in the EBV life cycle.

It is likely that there are other domains of Lyn besides SH2 that contribute to the specificity of binding to LMP2A. One possibility is the unique domain, which is thought to confer binding specificity due to its divergence in amino acid sequence among the SFK members. It was shown previously that the unique domain of Lck is important in its association with the T-cell coreceptors CD4 and CD8 alpha (78). Likewise, it was shown that the Yes unique region cannot substitute for the Src unique domain (33, 88).

The SH3 domain is another possible domain that could contribute to the specificity of binding to LMP2A. The SH3 domain recognizes proline-rich sequences bearing the PXXP motif. Using chimeras of the Src and Yes SFKs, it was shown that the SH3 domain of Yes could not substitute for the SH3 domain of Src (87). Although the target protein of Src was phosphorylated just as well by Src as it was by the Yes SH3 Src chimera, the Yes SH3 Src chimera did not bind the target protein nearly as well as wt Src (87). Although LMP2A contains two such SH3 binding sequences (PXXP), there is a

considerable amount of variation in the structure and binding modes of the SH3 domain, and the binding of SH3 domains is moderately promiscuous (50). Although it may play a role in Lyn binding to LMP2A, it is unlikely that it contributes to the specificity.

Although the SH2 is a significant contributor to the preferential binding of Lyn to LMP2A, there are likely other domains of Lyn that are important in this interaction, possibly in the initial recruitment of Lyn to LMP2A. The contribution of other domains will need to be studied in further detail. The fact that Lyn is used preferentially by EBV to deliver signals to B cells via LMP2A identifies it as a potential drug target. The inhibition of multiple SFK members would likely have very severe side effects. However, the inhibition of a single SFK may have less severe side effects due to the redundancy in function exhibited by many of the family members.

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