Syk Tyrosine Kinase Mediates Epstein-Barr Virus Latent Membrane Protein 2A-induced Cell Migration in Epithelial Cells*

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Although spleen tyrosine kinase (Syk) is known to be important in hematopoietic cell development, the roles of Syk in epithelial cells have not been well studied. Limited data suggest that Syk plays alternate roles in carcinogenesis under different circumstances. In breast cancer, Syk has been suggested to be a tumor suppressor. In contrast, Syk is essential for murine mammary tumor virus-mediated transformation. However, the roles of Syk in tumor migration are still largely unknown. Nasopharyngeal carcinoma, an unusually highly metastatic tumor, expresses Epstein-Barr virus LMP2A (latent membrane protein 2A) in most clinical specimens. Previously, we demonstrated LMP2A triggers epithelial cell migration. LMP2A contains an immunoreceptor tyrosine-based activation motif, which is important for Syk kinase activation in B cells. In this study, we explored whether Syk is important for LMP2A-mediated epithelial cell migration. We demonstrate that LMP2A expression can activate endogenous Syk activity. The activation requires the tyrosine residues in LMP2A ITAM but not YEEA motif, which is important for Syk activation by Lyn in B cells. LMP2A interacts with Syk as demonstrated by coimmunoprecipitation and confocal microscopy. Furthermore, LMP2A-induced cell migration is inhibited by a Syk inhibitor and short interfering RNA. Tyrosines 74 and 85 in the LMP2A immunoreceptor tyrosine-based activation motif are essential for both Syk activation and LMP2A-mediated cell migration, indicating the involvement of Syk in LMP2A-triggered cell migration. The LMP2A-Syk pathway may provide suitable drug targets for treatment of nasopharyngeal carcinoma.

Spleen tyrosine kinase (Syk)4 plays pivotal roles in many important signaling pathways such as hematopoietic cell activation and differentiation (1). Structurally, Syk contains two Src homology 2 (SH2) domains and a C-terminal kinase domain joined by a linker region (2). With the distinct tandem SH2 motif, Syk can interact selectively in immune cells with proteins containing a phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) (YXX(L/I)(Xa-y)YXX(L/I)) (3). The understanding of the linkage of Syk to ITAMs comes from studies of the B cell antigen receptor (BCR). BCR activation is triggered by antigen stimulation, which results in clustering of the BCR leading to the activation of Lyn kinase through the BCR YEEA motif (4, 5). Activated Lyn phosphorylates the ITAM within the BCR, which acts as a docking site for Syk, and then triggers the autophosphorylation and the activation of Syk kinase (6). Both Syk and Lyn are important for BCR signaling (7, 8). The BCR-Lyn-Syk pathway leads to many downstream events such as the recruitment and activation of kinases, hydrolysis of phospholipids, activation of protein kinase C, and mobilization of intracellular calcium (1).

These signals stimulate B cells to proliferate and differentiate into plasma cells or memory cells (1).

Recently, additional studies in nonhematopoietic cells have shown that Syk also plays important roles in epithelial cells, hepatocytes, fibroblasts, endothelial cells, and neuronal cells (9). The role of Syk in the carcinogenesis of epithelial cells was first suggested in breast cancer, where Syk functions as a tumor suppressor gene (10). In contrast, Syk and the ITAM of murine mammary tumor virus env gene are both required for cell transformation (11). The involvement of Syk in the proliferation and migration of human umbilical vein endothelial cells implicates a potential role of Syk in the reorganization of vascular endothelial cells during angiogenesis (12, 13). Therefore, Syk might have varied roles in oncogenic transformation.

One cancer that Syk may be involved with is nasopharyngeal carcinoma (NPC). NPC is characterized by being highly metastatic and having a strong association with Epstein-Barr virus (EBV) (14, 15). EBV infects B cells and epithelial cells in vitro and in vivo (16). Among EBV gene products, LMP2A (latent membrane protein 2A) is the only EBV protein containing an ITAM and is detected in almost all NPC biopsies (17, 18). Additionally, there is a specific elevation of LMP2A antibody titers in NPC patients (19).

The roles of LMP2A in carcinogenesis are supported by the oncogenicity of LMP2A-expressing cells in nude mice (20). These tumors displayed NPC-like features, including a high rate of metastasis, and are poorly differentiated (20). In vitro, LMP2A stabilizes β-catenin to inhibit cellular differentiation (21). In addition, LMP2A induces cell migration of epithelial cells through ERK kinase (22). However, the underlying mechanism of how LMP2A transmits signals from cell membrane to induce cell motility is unclear.
In EBV-immortalized B cells, LMP2A blocks B cell activation by binding Lyn and Syk (23, 24). The binding occurs via YEEA and ITAM motifs within the LMP2A N-terminal cytoplasmic domain (25, 26). In a transgenic mouse model, LMP2A enhances the survival of immunoglobulin-negative B cells in peripheral lymphoid organ indicating that LMP2A generates a constitutive differentiation and survival signal in B cells (27). Therefore, LMP2A has dual functions in B cells (25, 27). LMP2A can substitute for normal BCR function in B cell development but can also alter normal BCR-mediated signals.

In comparison to B cells, LMP2A has been shown to possess different signals and functions in epithelial cells. The tyrosine phosphorylation status of LMP2A has been evaluated in LMP2A-expressing B cells and epithelial cells (26, 28, 29). In B cells, Lyn is essential for LMP2A phosphorylation (26). In epithelial cells, the phosphorylation of LMP2A is dependent on the stimulation of extracellular matrix and Csk kinase but not on Src kinases, including Lyn kinase (30). Accordingly, the signaling cascade of LMP2A in epithelial cells appears to be different from that in B cells.

LMP2A can induce cell migration and harbors an ITAM, the interaction motif of LMP2A with Syk in B cells (22, 25). Thus, Syk is a prime candidate for mediating LMP2A-induced cell motility in epithelial cells. In this study, we demonstrate that Syk regulates LMP2A-induced cell motility in epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human epithelial cell lines, 293 (31) and HaCaT (32) (a gift from Dr. Tzuu-Shuh Jou, National Taiwan University, Taipei, Taiwan), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Plasmid Construction**—pRc/CMV-LMP2A is an LMP2A expression plasmid driven by the cytomegalovirus promoter (22). Wild type LMP2A plasmid and plasmids with deletions in LMP2A coding sequences for 80–112, 90–211, and 80–112 (111–211) or point mutation of tyrosine codons 31 (Y31F), 74 (Y74F), 85 (Y85F), 101 (Y101F), and 112 (Y112F) to phenylalanine were described by Fruehling et al. (26). LMP2A was tagged with a hemagglutinin (HA) epitope at the C terminus. The tyrosine codon 74 was also mutated to phenylalanine in the LMP2A deletion mutant 80–112 (Δ80–112).

**Cell Transfection and LMP2A-expressing Stable Clones Establishment**—Cells were transfected using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions or by a modified calcium phosphate method, as described previously (33). Briefly, 20 μg of plasmid DNA in 360 μl of ice-cold H2O was mixed with 40 μl of 2.5 mM CaCl2 and 0.4 ml of 2×BBS (50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM NaH2PO4, pH 6.95) and incubated for 10–20 min at room temperature. The mixture was added to 50% confluent cells, which were then incubated for 18 h at 35 °C under 3% CO2. Following a media change, the transfected cells were incubated at 37 °C with 5% CO2. For transient transfections, cells were harvested 36 h post-transfection. To establish LMP2A-expressing stable clones and vector control lines, cells transfected with pRc/CMV-LMP2A or vector alone were selected in the presence of 800 μg/ml neomycin. For siRNA transfection, cells with 90% confluence were transfected with control or Syk siRNA purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Pooled siRNA duplexes for Syk or control nonspecific RNA (1 μl, 20 μM) were incubated with 3 μl of Lipofectamine 2000 (Invitrogen) in 100 μl of DMEM at room temperature for 20 min. After incubation, the RNA/liposome mixtures were added to one 12-well plate containing 500 μl of 10% fetal calf serum/DMEM. After 48 and 96 h, the transfected cells were harvested for three-dimensional collagen gel assay and Western blot analysis.

**Western Blot Analysis**—Cells were lysed in 1% Nonidet P-40 buffer (50 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium Na3VO4, 1% Nonidet P-40, 50 mM NaF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Twenty μg of extracted protein was denatured at 100 °C for 5 min and resolved on SDS-8% polyacrylamide gels. The proteins were electrotransferred onto a polyvinylidene fluoride (Immobilon-P; Millipore, Bedford, MA), and the blot was blocked in washing buffer containing 4% skim milk and then incubated with anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Inc.), anti-Syk antibody (4D10, Santa Cruz Biotechnology), anti-phospho-Syk antibody (New England Biolabs, Beverly, MA), anti-LMP2A antibody (22), anti-HA antibody (Babco, Richmond, CA), or anti-actin antibody (Sigma) for 1 h. After washing with Tris-buffered saline containing 0.05% Tween 20, the blot was incubated with peroxidase-labeled goat anti-mouse or goat anti-rabbit antibody (The Jackson Laboratory, West Grove, PA). Bands were visualized using the Renaissance kit (PerkinElmer Life Sciences).

**Immunoprecipitation**—Nonidet P-40-solubilized cell lysates (125 μg) were precleared with protein A-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C, and samples were incubated overnight with 1 μg of anti-phosphotyrosine antibody (4G10), rabbit anti-Syk antibody (N-19, Santa Cruz Biotechnology), anti-LMP2A antibody (22), mouse immunoglobulin (Dako, Glostrup, Denmark), or rabbit immunoglobulin (Dako) at 4 °C. The immune complexes were then precipitated by protein A-Sepharose at 4 °C for 2 h and washed four times with PBS or 1% Nonidet P-40 buffer. Finally, the immunoprecipitates were resolved on an SDS-8% polyacrylamide gel, and Western blot analysis was carried out as described above.

**Immunohistochemical Assay**—LMP2A monoclonal antibody was generated using a standard protocol (34). Sections of NPC biopsies, vector, and LMP2A stable clones were fixed in methanol and acetone (1:1) at −20 °C for 10 min. The slides were subsequently treated with 3% H2O2, avidin, and biotin (Zymed Laboratories Inc.) for 10 min. Between each step, the slides were washed with Tris-buffered saline, pH 7.4, three times. After blocking with 10% goat serum for 20 min, anti-LMP2A antibody (1:4) or mouse immunoglobulin (Dako) were incubated with the slides overnight at 4 °C. The slides were incubated with avidin-biotin complex (Dako), developed with diaminobenzidine (Dako), and counterstained with Mayer hematoxylin.

**In Vitro Tyrosine Kinase Assay**—Cells were lysed in 1% Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride) and subjected to immunoprecipitation as described above. The immunoprecipitates were then washed twice with washing buffer (50 mM HEPES/NaOH, pH 8.0, 10 μM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride) and subjected to immunoblotting as described above.

**Confocal Spectral Fluorescence Microscopy**—Cells were fixed to the slides with acetone at room temperature for 10 min. As described previously (35), cells were incubated with mouse anti-LMP2A monoclonal antibody (1:50), rabbit anti-Syk antibody (1:50; Santa Cruz Biotechnology), or control antibody for 1 h at 37 °C. The cell smears were then washed with PBS and incubated with 100-fold diluted fluorescein iso-
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FIGURE 1. Activation of Syk kinase in LMP2A-expressing 293 cells. Three independent LMP2A-expressing stable clones (2A-1, 2A-2, and 2A-3), LMP2A transient transfected cells (2A), and stable or transient transfected vector control cells (V) were used in the experiments. A, examination of the phosphorylation status of tyrosines 525 and 526 within the Syk activation loop. Anti-phospho-Syk antibody that recognizes phosphorylated Tyr-525/526 was used in Western blot analysis (upper panel). The immunoblot of total Syk (middle panel). Cell lysates were also probed for the expression of total Syk (middle panel), C, detection of the kinase activity of Syk. The enzymatic activity of endogenous Syk activated by LMP2A was determined by an in vitro tyrosine kinase assay. Proteins were immunoprecipitated with anti-Syk antibody. Immune complexes were incubated in kinase reaction buffer with or without ATP and subjected to Western blot analysis with anti-phospho-Syk (p-Syk, upper panel) and anti-Syk antibodies (Syk, lower panel). D, expression of LMP2A in NPC and LMP2A-3 cells. Immunohistochemical staining was performed with vector control line (vector), LMP2A-expressing stable clone (2A-3), and NPC biopsies (NPC) as described under “Experimental Procedures”. The cells were incubated with the irrelevant immunoglobulin antibody control (Ig control) or anti-LMP2A antibody (LMP2A antibody). The slides were incubated with avidin-biotin complex, developed with diaminobenzidine, and counterstained with Mayer hematoxylin. The positive diaminobenzidine staining is brown, and the hematoxylin counterstaining staining is blue. Original magnification was ×400.

RESULTS

Activation of Syk in LMP2A-expressing Epithelial Cells—To test whether Syk participates in LMP2A-triggered cell migration, the expression of endogenous Syk in epithelial cell lines was investigated. By Western blot analysis, Syk was detected in 293 and HaCaT cell lines (data not shown).

The activation of the Syk kinase requires tyrosine phosphorylation of Syk (2). To determine whether LMP2A can regulate endogenous Syk in epithelial cells, the tyrosine phosphorylation status of Syk was examined by Western blot analysis. The phosphorylation status of Syk was compared between LMP2A-expressing 293 cells and control 293 cells. The results showed that Syk was phosphorylated in LMP2A-expressing 293 cells, indicating that LMP2A can induce the activation of Syk.

Three-dimensional Collagen Gel Assay—8 × 10^5 cells were resuspended in collagen gel mixture (70 μl of 3 mg/ml rat tail collagen I, 9 μl of 10X DMEM, 2 μl of 0.2 M Hepes, pH 7.3, 5 μl of H2O2, adjusted to pH 7.4 with 1 N NaOH) (36). The mixtures were then seeded into a 96-well microtiter plate, and the gel was solidified for 30 min in a 37 °C incubator with 5% CO2. 100 μl of DMEM containing 2.5 or 1% fetal bovine serum was then added. For Syk inhibition assays, 10 μM Syk inhibitor (piceatannol; Calbiochem) was added to both the collagen gel and the culture medium. After 7–14 days of incubation, the effects on the cell spreading phenotype was observed and photographed using an inverted photomicroscope (Axiovert 10, Zeiss, Germany).

Transwell Assay—Cell motility assay was performed using the QCM—Collagen I kit, and all steps were performed according to the manufacturer’s procedure with little modification (Chemicon, Temecula, CA). Briefly, cells (3 × 10^4) in serum-free medium were seeded into the Boyden chambers having 8-μm pore size and coated with type I collagen on the underside only. For inhibition assays, 10 μM of Syk inhibitor (piceatannol) was added to both sides of the chamber. After incubating for 24 h, nonmigrating cells were removed with a flattened swab, and migrating cells on the underside of the chamber were stained, and the extent of migration was determined by counting stained cells.
Figure 2. LMP2A domains required for Syk tyrosine phosphorylation. A, LMP2A mutants used to determine regions of LMP2A important for Syk phosphorylation. LMP2A deletion mutants encompassing amino acids 21–64 (Δ21–64), 21–85 (Δ21–85), and 80–112 (Δ80–112) were used. In addition to the deletion in the LMP2A Δ80–112 mutant, tyrosine codon 74 was also mutated to phenylalanine. Specific tyrosine to phenylalanine LMP2A mutants were used in which tyrosine codon 31 (Y31F), 74 (Y74F), 85 (Y85F), 101 (Y101F), or 112 (Y112F) was mutated to phenylalanine. Mapping domains of LMP2A required for the Syk activation in 293 cells (B) and HaCaT cells (C). Vector control plasmid (V), LMP2A wild type (WT), and LMP2A mutants were transiently transfected into the cells. After 36 h, cell lysates were harvested and subjected to Western blot analysis using anti-phospho-Syk antibody against Tyr-525/526 of Syk (upper panel). The total amount of Syk was detected with anti-Syk antibody (middle panel). The expression of LMP2A was detected with anti-HA antibody (middle panel). Actin was an internal control for the quantity of protein (lower panel). D, verification of the tyrosine phosphorylation of Syk in HaCaT cells with different LMP2A transfectants. Proteins were immunoprecipitated by anti-Syk antibody (Syk), anti-phosphotyrosine antibodies (pY), or control immunoglobulin (Ig). Proteins in the immune complexes were detected with anti-phospho-Syk (Tyr-525/526) antibody (upper and lower panels). WB, Western blot.
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by immunoprecipitation and Western blotting. Anti-Syk, anti-phosphotyrosine antibodies, or immunoglobulin controls were used in immunoprecipitation experiments with LMP2A-expressing stable clones or transient transfecants (22). The immunoprecipitated proteins were then analyzed by Western blotting with anti-phosphotyrosine and anti-Syk antibodies. The results indicate that Syk was tyrosine-phosphorylated in both transient and stable LMP2A-expressing cells but not in the vector control cells (Fig. 1A).

Syk contains at least 10 tyrosine residues that are putative autophosphorylation sites (37). Among them, two tyrosine residues, amino acids 525 and 526, are essential for the activation of Syk (6, 38). To confirm that Syk is phosphorylated at these sites, Western blot analysis was performed with antibody against phosphorylated tyrosine residues 525 and 526 of Syk (Fig. 1B). The expression of LMP2A and Syk in LMP2A stable clones and transient transfecants was confirmed by Western blotting (Fig. 1B). The results clearly demonstrate that Syk is phosphorylated within the Syk activation loop in LMP2A-expressing cells and not in vector control cells (Fig. 1B). Additionally, Syk phosphorylation correlates well with the amount of LMP2A protein expressed (Fig. 1B). Actin serves as an internal control for the quantity of protein in each lane.

To confirm the activated Syk has kinase activity in LMP2A-expressing cells, an in vitro tyrosine kinase assay was performed (Fig. 1C). Syk autophosphorylation activity was detected in LMP2A-expressing cells but not in vector control cells (Fig. 1C). The kinase activity of Syk in EBV-immortalized B cell lines was also examined as a positive control in this assay (data not shown).

To gauge the level of LMP2A expression in the LMP2A stable clones in comparison to NPC tumor biopsies, immunohistochemistry was performed with NPC biopsies. A total of 68 NPC biopsies was studied in parallel with the LMP2A-3 cell line that expressed the highest levels of LMP2A (Fig. 1B). Of the 68 NPC biopsies examined, 28 cases exhibited greater staining than the LMP2A-3 cell line, whereas 36 had staining equal to or less than the LMP2A-3 cell line (representative data are shown in Fig. 1D and data not shown). No LMP2A staining was detected in vector control cells and four of the NPC biopsies (Fig. 1D and data not shown). Therefore, the expression of LMP2A and the subsequent activation of Syk would appear to be a result of physiological levels of LMP2A expression, which is similar to levels of LMP2A expression observed in NPC biopsies.

Identification of Essential Tyrosine Residues in LMP2A for Activation of Syk—LMP2A has eight tyrosine residues within its N-terminal region that could be sites for phosphorylation (39). These phosphorylated tyrosine residues are part of potential binding motifs for a variety of cellular signaling proteins (28). To evaluate the region of LMP2A that mediates Syk activity, LMP2A deletion mutant constructs (Δ21–64, Δ21–85, and Δ80–112) (Fig. 2A) and point mutants constructs (Y31F, Y74F, Y85F, Y101F, and Y112F) were transfected into cells individually. By Western blot analysis, amino acids 65–112 are essential for endogenous Syk activation (Fig. 2B). The contribution of specific LMP2A tyrosine residues for Syk phosphorylation was also tested. Tyrosine residues 74 and 85 but not 31, 101, and 112 of LMP2A were required for the tyrosine phosphorylation of Syk (Fig. 2B). Expression of LMP2A and endogenous Syk was confirmed by Western blot analysis shown in Fig. 2B. Actin was used as a loading control for each sample. These data indicated that the LMP2A Tyr-74 and Tyr-85 residues, which constitute an ITAM, are crucial for Syk phosphorylation.

A similar result was obtained when HaCaT cells were transfected with the LMP2A mutants (Fig. 2C). HaCaT is a spontaneously immortalized and non tumorigenic human epithelial cell line (32). Because the endogenous Syk content in HaCaT cells was lower than 293 cells, Syk phosphorylation was further confirmed by immunoprecipitation followed by Western blot analysis (Fig. 2D). Immunoprecipitation experiments were performed with anti-Syk, anti-phosphotyrosine antibodies, or immunoglobulin controls. Immunoprecipitations were then probed with anti-phospho-Syk antibody by Western blot. Similar to the results with 293 cells shown in Fig. 2B, the LMP2A ITAM was essential for Syk activation in HaCaT cells.

Interaction of Syk with LMP2A—To determine whether LMP2A could interact with endogenous Syk in vivo, communoprecipitation was performed with anti-Syk and anti-LMP2A antibodies. In LMP2A-expressing stable clones, LMP2A interacted with Syk (Fig. 3A). The immunoglobulin control did not immunoprecipitate LMP2A or Syk verifying the specificity of the immunoprecipitation. To further investigate the interaction of LMP2A with Syk, colocalization of Syk and LMP2A was examined by confocal microscopy. As shown in Fig. 3B, the majority of LMP2A was colocalized with Syk in three LMP2A stable clones and transient transfecants (Fig. 3B and data not shown). No signal was detected when the LMP2A antibody was used with vector control cells or control immunoglobulin with LMP2A-expressing cells (data not shown).

Mapping the Interacting Regions of LMP2A with Syk—To delineate the region of LMP2A required for interaction with Syk, the LMP2A mutants were transfected into HaCaT and 293 cells followed by immu-
noprecipitation using anti-Syk antibody, anti-LMP2A antibody, and control immunoglobulin. Immunoprecipitates were probed for Syk and LMP2A by Western blotting. The interaction of Syk with LMP2A was not apparent in cells transfected with the LMP2A Δ21–85, Δ80–112, Tyr-74, and Tyr-85 mutants, although it was readily detected with wild type LMP2A and the other LMP2A mutants in 293 cells (Fig. 4, A and B) and HaCaT cells (Fig. 4, C and D). The amount of LMP2A in each immunoprecipitation was verified by Western blotting indicating that roughly equal amounts of LMP2A or the LMP2A mutants were present in each immunoprecipitation (data not shown). This analysis indicates that the high affinity interaction of Syk with LMP2A is dependent on the ITAM present in LMP2A.

Involvement of Syk in LMP2A-mediated Cell Motility — In light of the Syk activation in LMP2A-expressing cells (Figs. 1 and 2) and previous observations indicating that LMP2A enhances cell motility (22), three-dimensional collagen gel (Fig. 5) and transwell assays (Fig. 6) were performed to investigate the role of Syk activation in LMP2A-induced cell motility. In three-dimensional collagen gel assays, cells from each of the three independent LMP2A-expressing lines extended into the collagen gel and adopted spindle-like shapes (Fig. 5, B–D). The degree of cell invasion correlated with LMP2A expression levels (Fig. 1B; Fig. 5, B–D). In contrast, vector control cells exhibited a spherical shape in the collagen gel (Fig. 5A). The invasive ability and spindle-like morphology of LMP2A-expressing cells could be blocked by the Syk inhibitor piceatannol but not by solvent control (Me2SO) (Fig. 5, E and F). Additionally, to exclude the possibility of inhibitor toxicity or nonspecific effects, we examined whether Syk siRNA could block cell migration. By three-dimensional collagen gel assay, the LMP2A-driven cell migration was inhibited in the presence of Syk siRNA but not with control siRNA (Fig. 5, G and H). The down-regulation of Syk protein expression by siRNA was confirmed by Western blot analysis (Fig. 5I). In comparison with control siRNA-transfected cells, only Syk protein but not LMP2A or actin proteins expression was suppressed by Syk siRNA (Fig. 5I).

Consistent with the collagen gel assay, the cell motility was increased in the three LMP2A stable clones in transwell assays when compared with the vector control (Fig. 6A, bars 1–4). The degree of cell migration was also proportional to the LMP2A protein expression (Fig. 1B; Fig. 6A, bars 1–4). In the presence of Syk inhibitor, the motility of LMP2A 2A-3 clone was inhibited (Fig. 6A, bar 5). Therefore, activated Syk is required for LMP2A-mediated cell migration.

To investigate the tyrosine residues in LMP2A that are essential for migration, wild type LMP2A and point mutation plasmids Y74F, Y85F, Y101F, and Y112 were transfected into HaCaT cells (Fig. 6B, bars 2 and 4–7). Protein expression of LMP2A and Syk in transfectants was con-
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FIGURE 5. The role of Syk in LMP2A-triggered cell motility. A–H, examination of LMP2A-induced cell motility in a three-dimensional collagen gel assay. LMP2A-expressing 293 cells (clones 2A-1, 2A-2, and 2A-3) and vector control cells (V) were used in the experiments. The cells were trypsinized and resuspended in a collagen gel mixture. The resuspended cells were then seeded into a 96-well microtiter plate, and 100 μl of DMEM containing fetal calf serum was added to the top of the gel. E and F, 10 μM MeSO (DMSO) or the Syk inhibitor piceatannol (inhibitor) was added to both the collagen gel and culture medium. G and H, control siRNA (si-control) or Syk siRNA (si-Syk) was transfected into the LMP2A-3 stable clone with Lipofectamine 2000. After 7–14 days of incubation, the cell spreading phenotype was observed and photographed using an inverted photomicroscope. Photomicrographs used above were 10 days post-plating. I, detection of Syk, LMP2A, and actin expression in the presence of control (con) or Syk (si-Syk) siRNA by Western blot analysis. Cell lysates were made 4 days post-transfection, and Western blot analysis was performed with anti-Syk, anti-LMP2A, and anti-actin antibodies.

DISCUSSION

Syk, an ITAM-containing protein, is essential for many cell fate determination events, such as B cell development (2). However, its role in epithelial cells is not clear. In this study, we demonstrated that Syk is regulated by a viral ITAM-containing protein LMP2A and participates in LMP2A-mediated cell migration.

We first demonstrated that endogenous Syk was activated in epithelial cells in the presence of LMP2A (Fig. 1). Tyrosine residues 74 and 85 in LMP2A were essential for Syk activation (Fig. 2). The interaction of LMP2A with Syk was demonstrated by coimmunoprecipitation assays and confocal microscopy (Fig. 3). Amino acids 65–112 of LMP2A, specifically Tyr-74 and Tyr-85 which form an ITAM, were essential for the Syk/LMP2A interaction (Fig. 4). Furthermore, activated Syk mediated LMP2A-induced cell motility (Figs. 5 and 6). In sum, our findings indicate that Syk is required for LMP2A-triggered cell migration.

The N-terminal cytoplasmic domain of LMP2A has motifs similar to those found in growth factor receptors and receptors important in immunological signal transduction. LMP2A is localized to cellular membranes and contains phosphotyrosine motifs and proline-rich motifs that function as docking sites for SH2, SH3, and WW domain containing proteins (39–41). Depending on the different cell types in which LMP2A is expressed, LMP2A may interact and activate different signal transduction pathways resulting in diverse effects. Results from our current studies suggest that the modulation of Syk activity by LMP2A has differing requirements in B cells when compared with epithelial cells. In EBV-immortalized B cells, tyrosine 112 of LMP2A is important for the interaction of LMP2A with the Lyn kinase and is also required for the phosphorylation and activation of Syk and LMP2A (26).
However, despite the expression of Lyn and other Src family kinases in epithelial cells (data not shown), Tyr-112, when mutated, had no effect on Syk activation and LMP2A-triggered cell migration (Figs. 2 and 6). In addition, in epithelial cells, Tyr-112 was not essential for LMP2A phosphorylation (data not shown) (29). Taken together, unlike its role in B cells, Lyn as well as other Src family kinases may not directly modulate LMP2A and Syk function in epithelial cells, indicating a novel interaction of Syk with LMP2A.

Then how does LMP2A interact and activate Syk in the absence of Lyn? Several possibilities exist. First, previous structural studies have indicated flexibility of the Syk SH2 domain, in contrast to the related kinase Zap-70 (3). This suggests the two SH2 domains of Syk may function as independent units and allow binding to a greater variety of signaling proteins (3). Thus, it is possible that Syk is first recruited to LMP2A via a nonconventional interaction. It has been documented that Syk can directly interact with integrins in a phosphorylation-independent manner, which implies the target proteins can interact with Syk without prerequisite activation (42, 43). Thus, the LMP2A ITAM domain might be sufficient for direct interaction and activation of Syk (Fig. 4). Alternatively, other cellular kinases have been suggested to be responsible for LMP2A phosphorylation that then allows Syk binding to the LMP2A ITAM (29). In addition, adaptor proteins in epithelial cells may also contribute to the recruitment of Syk. As well as the ITAM (Tyr-74 and Tyr-85) and Tyr-112, the LMP2A N-terminal domain contains additional tyrosine motifs and multiple proline-rich regions that may recruit specific cellular proteins (40). However, because Y31F, Y101F, and Y112F mutants of LMP2A did not block Syk activation and LMP2A-Syk interaction, it can be concluded that these tyrosines do not participate in Syk activation by LMP2A in epithelial cells (Figs. 2 and 4).

Our studies indicated that endogenous Syk is essential for LMP2A-mediated cell migration (Figs. 5 and 6). By transwell and three-dimensional collagen gel assays, the motility of LMP2A-expressing cells was blocked dramatically in the presence of a Syk inhibitor and Syk siRNA (Figs. 5 and 6). Furthermore, the mutation of Tyr-74 and Tyr-85, the LMP2A ITAM, simultaneously blocked Syk activation and cell migration (Figs. 2 and 6). These data indicated that Syk is required for LMP2A to promote cell migration in epithelial cells. Interestingly, in breast cancer, Syk has been suggested to act as a putative tumor suppressor when Syk was exogenously expressed (10). In contrast, Syk is required for murine mammary tumor virus Env protein-mediated cell transformation (11). This discrepancy may be attributable to the expression level of Syk or cell type-specific factors. Syk contains 10 tyrosine residues and is able to interact with diverse substrates and adaptor molecules (2). Thus, it is likely Syk initiates different signaling pathways in different cellular environments that may result in differing cellular phenotypic outcomes. Many tyrosine kinases, such as Btk (Bruton’s tyrosine kinase), can perform such two-edged functions (44). Btk can lead to apoptosis when exogenously expressed in HeLa cells, but it protects B cells from apoptosis (45, 46). Similarly, LMP2A can have diverse effects depending on the cell types tested. LMP2A can block BCR signaling when expressed in EBV-transformed B cells grown in cell culture (23, 47). In contrast, in primary murine B cells LMP2A does not block BCR signaling but alters normal B cell signaling and confers survival and developmental signals to BCR-negative B cells (25, 27, 48, 49). Similarly, Syk might also lead to multiple events in epithelial cells.

Previous studies have indicated that the ITAM in LMP2A is essential for the activation of the PI3K/Akt pathway and induces the nuclear translocation of the oncprotein β-catenin (21). The activation of Syk, which also requires the LMP2A ITAM, suggests the interesting possibility that LMP2A activation of Syk may also influence PI3K/Akt and β-catenin signaling directly or indirectly. Additionally, our previous studies have shown that LMP2A induces the kinase activities of ERK and c-Jun N-terminal kinase, and ERK activation contributed to LMP2A-induced cell motility (22). Other studies have demonstrated that stimulation of the Fc receptor results in the activation of ERK, and this requires the activation of Syk (50, 51). Thus, it will be interesting to determine whether LMP2A-expressing epithelial cells are stimulated through the LMP2A/Syk/PI3K-Akt/β-catenin or LMP2A/Syk/ERK pathway to propagate signals and mediate cell motility.

Cell migration is the critical step for tumor metastasis (52). The activation of Syk by LMP2A and the almost universal expression of LMP2A in NPC biopsies may indicate the contribution of LMP2A to NPC progression by enhancing cell motility. Protein-tyrosine kinases are known to play important roles in tumor development and therefore serve as logical targets for intervention (53, 54). Currently, chemotherapy is an effective treatment for primary NPC but not for metastatic NPC. Our studies suggest that targeting the LMP2A-Syk pathway may help to prevent tumor invasion and migration in NPC.

Acknowledgments—We are indebted to Tim J. Harrison of the Royal Free and University College Medical School of University College London (London, UK) for critically reviewing this manuscript. We thank Dong-Ching Fu and Yun-Wen Chen for technical assistance. We thank Yu-Chieh Yu and Tsang-Shihain Sheen from the Department of Otolaryngology, College of Medicine, National Taiwan University, for the NPC immunohistochemistry staining.

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