Syk Activation of Phosphatidylinositol 3-Kinase/Akt Prevents HtrA2-dependent Loss of X-linked Inhibitor of Apoptosis Protein (XIAP) to Promote Survival of Epstein-Barr Virus+ (EBV+) B Cell Lymphomas*

Received for publication, April 26, 2011, and in revised form, September 1, 2011. Published, JBC Papers in Press, September 9, 2011, DOI 10.1074/jbc.M111.255125

Olivia Hatton1, Lori K. Phillips1, Maria Vaysberg1, Jordan Hurwich1, Sheri M. Kram1, and Olivia M. Martinez2

From the 4Program in Immunology and 5Department of Surgery, Division of Transplantation, Stanford University School of Medicine, Stanford, California 94305

Background: Syk activation is required for B cell survival. EBV can induce B cell lymphomas.

Results: Syk, PI3K/Akt inhibition induces apoptosis of EBV+ B cell lymphomas. Syk PI3K/Akt inhibition results in HtrA2-dependent loss of XIAP protein.

Conclusion: Syk activates PI3K/Akt to promote survival by preventing HtrA2-dependent loss of XIAP.

Significance: Syk, PI3K/Akt, and XIAP are new therapeutic targets for EBV+ B cell lymphomas.

B cell lymphoma survival requires tonic or ligand-independent signals through activation of Syk by the B cell receptor. The Epstein-Barr virus (EBV) protein latent membrane 2a (LMP2a), a mimic of the B cell receptor, provides constitutive survival signals for latently infected cells through Syk activation; however, the precise downstream mechanisms coordinating this survival response in EBV+ B cell lymphomas remain to be elucidated. Herein, we assess the mechanism of Syk survival signaling in EBV+ B cell lymphomas from post-transplant lymphoproliferative disorder (PTLD) to discover virally controlled therapeutic targets involved in lymphomagenesis and tumor progression. Using small molecule inhibition and siRNA strategies, we show that Syk inhibition reduces proliferation and induces apoptosis of PTLD-derived EBV+ B cell lines. Syk inhibition also reduces autocrine IL-10 production. Although Syk inhibition attenuates signaling through both the PI3K/Akt and Erk pathways, only PI3K/Akt inhibition causes apoptosis of PTLD-derived cell lines. Loss of the endogenous caspase inhibitor XIAP is observed after Syk or PI3K/Akt inhibition. The loss of XIAP and apoptosis that results from Syk or PI3K/Akt inhibition is reversed by inhibition of the mitochondrial protease HtrA2. Thus, Syk drives EBV+ B cell lymphoma survival through PI3K/Akt activation, which prevents the HtrA2-dependent loss of XIAP. Syk, Akt, and XIAP antagonists may present potential new therapeutic strategies for PTLD through targeting of EBV-driven survival signals.

Signals from the BCR3 are required for the survival of all benign (1–4) and most malignant (5, 6) B cells. BCR signaling is initiated by phosphorylation of the immunoreceptor tyrosine-based activation motifs of the signaling adaptors immunoglobulin (Ig) α and β by the receptor-associated Src-family protein-tyrosine kinases, most notably Lyn (7). Phosphorylated immunoreceptor tyrosine-based activation motifs recruit and activate Syk, a non-receptor-associated protein-tyrosine kinase. Syk coordinates a variety of cellular responses through direct binding with signaling intermediates, including Vav, phospholipase Cγ, PI3K, and BLNK, and the subsequent activation of downstream signaling pathways (8). Given the vital role of Syk in BCR signaling, pharmacological or genetic targeting of Syk has been pursued as a therapeutic strategy for the treatment of B cell lymphomas (9–17). Indeed, murine models of non-Hodgkin lymphomas show tumor regression or remission when Syk is targeted pharmacologically or by shRNA (11). In diffuse large B cell lymphoma (9–11), B cell lineage acute lymphoma (17), and chronic lymphocytic leukemia (12–15), pharmacological inhibition of Syk resulted in apoptosis of lymphoma cells in vitro. Moreover, Syk inhibition was efficacious in patients with relapsed/refractory B cell non-Hodgkin lymphoma and chronic lymphocytic leukemia (16). These studies have implicated the phospholipase Cγ2, PI3K/Akt, and Erk MAPK downstream signaling pathways and a number of downstream targets, including Bcl-xL, Bad, and Mcl-1, as mediators of the Syk survival signal.

The PI3K/Akt pathway is a key component of cell growth, proliferation, metabolism, and survival and is of particular interest with regard to Syk survival signaling. Akt is activated by PI3K and directly phosphorylates proteins that either inhibit the action of pro-apoptotic molecules or prevent caspase acti-
viation (18). Along these lines, Akt inhibits the pro-apoptotic molecule Bcl-2 family member Bcl-xL (21). Akt phosphorylates caspase 9 at Ser196, inhibiting its cleavage and proteolytic activity (22). Phosphorylation of FOXO transcription factors by Akt inhibits FOXO-mediated transcription of pro-apoptotic genes, including Bim and Fas ligand (23). Akt phosphorylation at Ser187 stabilizes XIAP, a member of the inhibitor of apoptosis (IAP) family, by preventing its auto-ubiquitination and degradation (24). XIAP directly binds caspases 3, 7, and 9 and inhibits their activity (25). Thus, Syk signaling may affect a variety of apoptosis-related proteins in propagation of a survival signal, although the specific mechanisms remain to be elucidated.

In addition to BCR activation, Syk can also be activated by other endogenous receptors and immunoreceptor tyrosine-based activation motif-containing viral proteins, including K1 of Kaposi sarcoma herpesvirus and LMP2a of Epstein-Barr virus (EBV) (26). LMP2a is a 54 kDa protein expressed during the latent cycle of EBV infection. LMP2a has been described as a functional mimic of the BCR, as LMP2a expression restores normal B cell development and survival in peripheral lymphoid organs of Rag2−/− mice, which lack a functional BCR (27). LMP2a has also been shown to activate many of the same signaling pathways as the BCR, including Syk, Lyn, Btk, BLNK, and PI3K/Akt, independently of Igα/Igβ (28) through self-aggregation in the membrane of latently infected cells (29). These signals, coordinated by Syk activation, function to maintain viral latency (30–33) and sustain survival (27, 34–36) in infected cells. LMP2a also blocks normal BCR triggering by sequestering the protein-tyrosine kinases Lyn and Syk (32, 33). Finally, LMP2a rescues survival of germinal center B cells with crippling mutations in the BCR (35), suggesting that LMP2a can contribute to the transformation of primary B cells (37, 38). However, a direct examination of Syk activation in EBV+ B cell lymphomagenesis, tumor survival, and proliferation has yet to be undertaken.

LMP2a expression is observed in a variety of EBV-associated malignancies, including Hodgkin lymphoma, nasopharyngeal carcinoma, AIDS-related lymphomas, and post-transplant lymphoproliferative disorder (PTLD) (39). PTLD arises in the setting of immunosuppression when the EBV-specific cytotoxic T lymphocyte response is debilitated, allowing for the proliferation and lymphomagenesis of EBV-infected B cells (40). The aims of this study are to determine whether Syk activation is critical to survival of EBV+ B cell lymphomas in PTLD and to dissect the mechanism of the Syk survival signal.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibodies to phospho-ERK (Tyr18), total ERK, and total p38 were obtained from Santa Cruz Biotechnologies. Anti-β-actin was obtained from Sigma. Anti-Syk, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-BLNK (Tyr96), and anti-BLNK were obtained from Cell Signaling Technologies. Phosphatidyethanolamine-conjugated anti-CD19, anti-X-IAP, anti-IκB, and anti-phospho-p38 (Thr180/Tyr182) were obtained from BD Biosciences. Anti-HtrA2 was obtained from R&D Systems. Whole rabbit IgG and secondary antibodies HRP-conjugated polyclonal goat-anti rabbit and HRP-conjugated polyclonal donkey anti-mouse were obtained from Jackson ImmunoResearch Laboratories. For B cell stimulation, F(ab)2 fragments of anti-human IgG and IgM were obtained from BIOSOURCE International. The small molecules inhibitors of Erk (PD98059, 20 μM; U029, 20 μM), PI3K (LY294002, 20 μM), and HtrA2 (UCF-101, 20 μM) were obtained from Calbiochem, diluted in DMSO (Sigma) to the indicated concentrations, separated into aliquots, and stored at −20°C. The active component of the Syk inhibitor fostamatinib, R406 (10 μM in DMSO), was kindly provided by Rigel Pharmaceuticals and was separated into aliquots and stored at −80°C.

**Cell Lines**—The EBV+ B cell lines derived from the blood (JB7, JC62, MF4, VB5) or lymph nodes (AB5) of patients diagnosed with PTLD were maintained as previously described (41, 42). The Jurkat human T cell line was obtained from ATCC and cultured as recommended. Cell lines were grown in a 5% CO2 humidified 37°C incubator and cultured in RPMI 1640 media (Mediatech, Inc.) supplemented with 10% heat-inactivated FBS (Serum Source International), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) unless otherwise noted.

**Purification and Stimulation of Human B Cells from Peripheral Blood**—Peripheral blood mononuclear cells were obtained from healthy donors by Ficoll density gradient centrifugation. B cells were isolated to >95% purity from peripheral blood mononuclear cells by negative selection using the MACS B cell Isolation Kit II (Miltenyi Biotec). Purity of isolation was determined by CD19 expression in B-enriched and B-depleted fractions. BCR cross-linking was initiated by the addition of F(ab)2 fragments of anti-IgM and anti-IgG (final concentration of 10 μg/ml).

**Syk Knockdown by siRNA**—Syk validated Stealth RNAs, and control medium GC oligos were obtained from Invitrogen. Syk or control oligos (200 nmol) was transfected into cells (3–5 × 106) using the Amaxa nucleofector and reagents from Lonza per the manufacturer’s instructions. Briefly, cells were harvested at 900 × g, resuspended in supplemented nucleofection reagent (Solution V) and oligo, and pulsed with program X-001. Pulsed cells were resuspended with 500 μl of prewarmed culture medium and transferred to 1 ml of pre-warmed culture medium in a 24-well plate. FITC-conjugated oligo controls indicated >95% transfection efficiency.

**Cellular Proliferation Assay**—Cells (0.25–0.5 × 106 cells/ml) were plated in triplicate in 96-well flat bottom plates in serial dilutions of small molecule inhibitors R406, PD98059, U029, or LY294002 (0–10 μM) and equivalent amounts of vehicle (DMSO; 0–1:1000) 48 h after the introduction of siRNA or after BCR cross-linking. Cells were pulsed with 0.5 μCi of [3H]thymidine (PerkinElmer Life Sciences) 18 h before collection, and plates were harvested after a total 48 h in culture at 37°C. Data were converted to a percentage of the cpm incorporated by cells grown in media alone or by cells transfected with control siRNA. IC50 values or the value at which proliferation is inhibited to 50% of media alone for each line were recorded. Cells were considered sensitive to a small molecule inhibitor if their IC50 was within the tested range (0–10 μM).

**Apoptosis Assays**—Cells (0.5–1 × 106 cells/ml) were plated in serial dilutions of small molecule inhibitors R406, PD98059, U029, LY294002, or UCF-101 (0–10 μM) or equivalent...
amounts of vehicle (DMSO; 0–1:1000) 48 h after the introduction of siRNA. For signaling inhibitor studies, both drug and media were replenished after 48 h of culture at 37 °C, and cells were cultured for an additional 48 h. As a positive control, cells were treated with 50 μM etoposide or 2 μM staurosporine for the indicated amounts of time. The percentage of apoptotic cells was determined by staining with annexin V–EGFP apoptosis detection kits (BioVision) per the manufacturer’s instructions. Data were converted to fold-increase in apoptosis by normalizing to cells grown in media alone or by cells transfected with control siRNA. Caspase enzymatic activity was quantified in stimulated lysates using caspase 3 Colorimetric Assay kits (BioVision) according to the manufacturer’s instructions. Mitochondrial damage was assessed in cells by DiOC<sub>6</sub> leakage.© 2011 The American Society for Biochemistry and Molecular Biology

RESULTS

Syk Is Constitutively Active in EBV+ PTLD-derived B Cell Lines—To assess Syk activation in EBV+ B cell lines derived from patients with PTLD, we analyzed the phosphorylation status of BLNK, which both directly binds and is exclusively phosphorylated by Syk (45, 46). BLNK was immunoprecipitated from whole cell lysates of three EBV+ PTLD-derived B cell lines (JC62, AB5, and JB7) with BLNK-specific antibody and analyzed by Western blot with antibodies to pBLNK. Consistent with the requirement of tonic BCR signaling for survival, we observed constitutive pBLNK in each of the three EBV+ PTLD-derived B cell lines (Fig. 1A, middle lanes). To ensure that this constitutive BLNK activation was indeed the result of Syk signaling, we treated the EBV+ PTLD-derived B cell lines with the small molecule R406, an ATP-competitive inhibitor of Syk (47). The constitutive activation of BLNK was completely abolished by R406 treatment (Fig. 1A, right lanes), consistent with the exclusive phosphorylation of BLNK by Syk.

Syk Promotes Proliferation of EBV+ PTLD-derived B Cell Lines—We next asked whether Syk signaling contributes to the proliferation of EBV+ PTLD-derived B cell lines. To measure the sensitivity of cellular proliferation to Syk inhibition, cell lines were treated with serial dilutions of R406 (Fig. 1B), and the IC<sub>50</sub> values were determined (Table 1). Proliferation of five of six EBV+ PTLD-derived B cell lines tested was markedly inhibited by R406 treatment, whereas the JB7 cell line was more resistant to the effects of Syk inhibition on cellular proliferation. Cells treated with equivalent amounts of the vehicle DMSO showed no effect on proliferation (supplemental Fig. 1A). Purified, anti-IgM and anti-IgG stimulated human B cells were used as a positive control for the ability of R406 to inhibit B cell proliferation (IC<sub>50</sub> value <0.156 μM) (Table 1). Conversely, R406 had no effect on the proliferation of the Jurkat T cell line (Table 1), consistent with the interpretation that R406 shows specificity for Syk and does not affect ZAP-70, a member of the Syk family of tyrosine kinases involved in T cell signaling. R406 has been reported to have minimal off-target effects on the Fli3, Jak1, Jak3, Kit, and Lck signaling pathways (8); therefore, Syk knockdown by siRNA was used to confirm Syk involvement. Syk knockdown by siRNA (Fig. 1C) significantly reduced proliferation of the JC62 and AB5 EBV+ PTLD-derived B cell lines but not the JB7 EBV+ PTLD-derived B cell line (Fig. 1D). The extent of the effect of Syk knockdown on proliferation correlated with the sensitivities of these lines to R406 treatment as determined by their IC<sub>50</sub> values. Taken together, this data suggest that constitutive Syk signaling contributes to the autonomous proliferation of EBV+ PTLD-derived B cell lines.

Syk Augments the Survival of EBV+ PTLD-derived B Cell Lines—The reduced proliferation of EBV+ PTLD-derived B cell lines measured after Syk inhibition could be due to apopto-
Syk Drives Akt- and HtrA2-dependent Survival

TABLE 1

| Cell type          | Cell line       | R406 IC<sub>50</sub> (μM) |
|--------------------|-----------------|---------------------------|
| EBV⁺, PTLD-derived B cell lines |                 |                           |
| AB5                |                 | 0.313–0.625               |
| MF4                |                 | 1.25–2.5                  |
| JB7                |                 | >10                       |
| JC62               |                 | 0.156–0.313               |
| VB5                |                 | 0.625                      |
| ZD3                |                 | 0.156                      |
| B cells            | Primary, purified cells | <0.156                    |
| T cell             | Jurkat          | >10                       |

Constitutive Syk signaling drives proliferation of EBV⁺-PTLD-derived B cell lines. Indeed, apoptosis was induced in all five R406-sensitive EBV⁺-PTLD-derived B cell lines but not the R406-resistant EBV⁺-PTLD-derived B cell line JB7 (Fig. 2, A and B). Cells treated with equivalent amounts of the vehicle DMSO showed no effect on apoptosis (supplemental Fig. 1B). Syk knockdown by siRNA (Fig. 1C) also increased apoptosis of the R406-sensitive JC62 and AB5 cell lines, albeit to different extents, but had no effect on the R406-resistant JB7 cell line (Fig. 2C). The extent of the effect of Syk knockdown on apoptosis correlated with the sensitivities of these lines to R406 treatment as determined by their IC<sub>50</sub> values. Similar to the effects on cellular proliferation, pharmacologic inhibition of Syk was more effective in inducing apoptosis of EBV⁺-PTLD-derived B cell lines than Syk siRNA. However, delivery of siRNA by nucleofection induces significant cellular apoptosis, which complicates quantitative comparison between the two methods of Syk inhibition. Taken together, these data suggest that constitutive Syk signaling is required for survival of EBV⁺-PTLD-derived B cell lines.

**Syk Signaling Results in PI3K/Akt and Erk MAPK Activation in EBV⁺-PTLD-derived B Cell Lines**—Although constitutive Syk activation is evident in all of the EBV⁺-PTLD-derived B cell lines (Fig. 1A), neither proliferation nor survival of the JB7 cell line is affected by Syk inhibition (Figs. 1B and 2, A and B). Therefore, we used the R406-resistant EBV⁺-PTLD-derived B cell line JB7 in comparison to the R406-sensitive JB7 cell line (Fig. 2C). The extent of the effect of Syk knockdown on proliferation correlated with the sensitivities of these lines to R406 treatment as determined by their IC<sub>50</sub> values. Similar to the effects on cellular proliferation, pharmacologic inhibition of Syk was more effective in inducing apoptosis of EBV⁺-PTLD-derived B cell lines than Syk siRNA. However, delivery of siRNA by nucleofection induces significant cellular apoptosis, which complicates quantitative comparison between the two methods of Syk inhibition. Taken together, these data suggest that constitutive Syk signaling is required for survival of EBV⁺-PTLD-derived B cell lines.
Akt and Erk MAPK activation are downstream of Syk signaling in EBV+ B lymphoma cell lines. Furthermore, because Akt and Erk MAPK activation were decreased in both R406-sensitive and R406-resistant EBV+ PTLD-derived B cell lines, the key anti-apoptotic Syk signal is likely to be distal to activation of these pathways.

**The Syk Survival Signal Requires PI3K/Akt Activation but Not Erk MAPK Activation**—Next we addressed whether the PI3K/Akt or Erk MAPK pathways participate in the Syk survival signal. Autocrine IL-10 secretion in EBV+ PTLD-derived B cell lines is an PI3K/Akt-, but not Erk MAPK-dependent process (44) and is required for the autonomous proliferation of PTLD lines (48). Whereas the endogenously high secretion of the autocrine growth factor IL-10 was markedly decreased after Syk inhibition in our R406-sensitive, EBV+ PTLD-derived B cell lines (Fig. 4A), the R406-resistant EBV+ PTLD-derived B cell line JB7 displayed endogenously low IL-10 secretion (Fig. 4A).

To ask whether the PI3K/Akt or Erk MAPK pathways were involved in proliferation or survival of EBV+ PTLD-derived B cells, we treated cells with pharmacologic inhibitors of the PI3K/Akt pathway (LY294002) or the Erk MAPK pathway (PD98059 and U029) and assayed both cellular proliferation and apoptosis. PI3K/Akt inhibition (Fig. 4B) reduced proliferation (Fig. 4C) and induced apoptosis (Fig. 4D) of the R406-sensitive EBV+ PTLD-derived B cell lines JC62 and AB5. In contrast, proliferation and survival of the R406-resistant EBV+ PTLD-derived B cell line JB7 was not affected by PI3K/Akt inhibition (Fig. 4, C and D). Inhibition of Erk MAPK (Fig. 4B and data not shown) resulted in no change in proliferation (Fig. 4E and data not shown) or apoptosis (Fig. 4F and data not shown) in any of the EBV+ PTLD-derived B cell lines. Taken together, these data suggest that Syk drives survival of the R406-sensitive EBV+ PTLD-derived B cell lines through the PI3K/Akt, but not the Erk MAPK, pathway.

**Syk- and PI3K/Akt-mediated Signals Prevent Loss of the Caspase Inhibitor XIAP**—Akt is known to directly phosphorylate the caspase inhibitor XIAP, thereby resulting in protein stabilization. We show that inhibition of Syk results in the complete loss of XIAP protein within 24 h in R406-sensitive, but not R406-resistant, EBV+ PTLD-derived B cell lines (Fig. 5A). However, no significant changes in XIAP transcript levels were observed after R406 treatment of EBV+ PTLD-derived B cell lines (supplemental Fig. 2). These data are consistent with regulation of XIAP at the protein rather than transcriptional level.

XIAP is known to inhibit active caspase 3 (25). Therefore, a loss in XIAP should coincide with an increase in caspase 3 activity. Consistent with the loss of XIAP, we observe increased caspase 3 activity after R406 treatment in R406-sensitive, but not R406-resistant, EBV+ PTLD-derived B cell lines (Fig. 5A). Moreover, inhibition of Akt results in loss of XIAP (Fig. 5B, first versus fifth lanes) only in the R406-sensitive lines, consistent with the known role of Akt in XIAP stabilization (24). Thus, Akt activity is required to preserve XIAP levels in EBV+ B lymphoma cells.

Akt has been described as preventing the loss of XIAP by two distinct mechanisms (24, 49), both involving regulation of XIAP at the protein level. First, phosphorylation of XIAP Ser87 by Akt has been reported to prevent autoubiquitination and subsequent degradation of XIAP, thus stabilizing the levels of protein expression (24). To address the differential loss of XIAP in R406-sensitive and R406-resistant EBV+ B lymphoma cell lines, we considered the possibility that the resistant cell line, JB7, carries a mutation that prevents XIAP autoubiquitination.
and degradation. Sequence analysis of cloned XIAP from the panel of EBV+ PTLD-derived B cell lines revealed no mutations in the XIAP ubiquitination site, its E3-ubiquitin ligase domain, or in the Akt phosphorylation site in any of the EBV+ PTLD-derived B cell lines analyzed (data not shown). Differential loss of XIAP in EBV+ PTLD-derived B cell lines is, therefore, likely not the result of a sequence mutation in XIAP.

The second mechanism involves the protease HtrA2, a homolog of the bacterial heat-inducible serine protease HtrA/DegP (50). XIAP was one of the first substrates identified for HtrA2, a small amount can be found in the cytoplasm of unstimulated cells (50). Mitochondrial damage triggers the release of HtrA2 into the cytoplasm. The variable location of HtrA2 raises the question of where XIAP and HtrA2 interact. To address this, we used DiOC₆, a dye that stains intact mitochondria, such that loss of DiOC₆ staining indicates disruption of mitochondrial inner transmembrane potential. Syk inhibition induced a loss of DiOC₆ staining in the R406-sensitive EBV+ PTLD-derived B cell lines (supplemental Fig. 3, B and C). Minimal DiOC₆ loss was observed in the R406-resistant line, JB7. Together, this demonstrates that Syk inhibition induces mitochondrial damage and is consistent with release of HtrA2 into the cytoplasm where it can interact with XIAP.

Finally, we sought to determine if blocking HtrA2 function rescued sensitive EBV+ PTLD-derived B cell lines from Syk inhibition- or PI3K/Akt inhibition-induced apoptosis. EBV+ PTLD-derived B cell lines were treated with inhibitors of Syk, PI3K/Akt, HtrA2, or a combination of inhibitors and analyzed by flow cytometry for annexin V-EGFP⁺ cells. Syk inhibition-induced apoptosis was significantly diminished when HtrA2 function was blocked in R406-sensitive cells (Fig. 5C). Similarly, PI3K/Akt inhibition-induced apoptosis was completely prevented by blocking HtrA2 function in R406-sensitive cells (Fig. 5D). Consistent with earlier results, apoptosis was not induced in the R406-resistant JB7 line (Fig. 5, C and D). Taken together
FIGURE 4. IL-10 production, cellular proliferation, and survival are PI3K/Akt-, but not Erk MAPK-dependent in EBV+ PTLD-derived B cell lines. A, PTLD lines were incubated with the indicated amounts of the Syk inhibitor R406 for 48 h. Supernatants were harvested, and an IL-10 ELISA was performed as described under “Experimental Procedures.” Data were normalized to the number of live cells, as determined by trypan blue exclusion at the time of supernatant harvest and are representative of n ≥ 3 independent experiments. B, cells were incubated with 2.5 μM Erk inhibitor PD98059, 2.5 μM PI3K inhibitor LY294002, or DMSO equivalent for 1 h. Cells were then harvested and lysed. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and Western blotting was performed for pAkt or pErk. Blots were then reprobed for total Akt, Erk, and actin as a loading control. Representative data from the AB5 PTLD line are shown. Densitometry was calculated with ImageJ and is indicated numerically below each set of blots. C and E, cells were plated in triplicate with the indicated final concentration of the PI3K inhibitor LY294002 or the Erk inhibitor PD98059 for 48 h. After pulsing with [3H]thymidine, plates were harvested, and data were converted to a percentage of the cpm incorporated by cells grown in media alone. Data are a combination of n ≥ 3 independent experiments. D and F, cells were incubated in the presence of the indicated amounts of the PI3K inhibitor LY294002 or the Erk inhibitor PD98059 for 96 h with drug and media replaced at 48 h. Cells were collected and stained for annexin V-EGFP, and the percentage of apoptotic cells was measured by flow cytometry. Data are shown as the -fold increase of apoptosis relative to media alone of n ≥ 3 independent experiments.
these data indicate that HtrA2 is involved Syk- and Akt-mediated survival of EBV+ PTLD-derived B cell lines.

**DISCUSSION**

Targeting tonic BCR signals with novel Syk inhibitors has been hotly pursued in the treatment of B cell lymphomas and has highlighted Syk activation as a critical checkpoint in B cell lymphoma survival. Syk controls a variety of cellular functions in addition to survival, however, including reactive oxygen production, cytokine release, and cellular adhesion. Understanding the precise mechanism of the Syk survival signal may highlight specific targets for therapeutic intervention in EBV+ B cell lymphomas.

The EBV+, PTLD-derived B cell lines used in this study express two potential activators of Syk, the BCR and LMP2a. Expression of LMP2a has been reported to block the ability of the BCR to activate the Src family kinases and Syk after ligation (31, 32). The EBV+ PTLD-derived B cell lines used here are similarly unable to phosphorylate BLNK after BCR ligation (data not shown). LMP2a is known to remain constitutively aggregated and phosphorylated in the membrane of infected cells, where it co-localizes with patches of anti-phosphotyrosine activity and associates with the Src family and Syk protein-tyrosine kinases (54, 55). LMP2a was more recently shown to reside in the lipid rafts and blocks BCR signaling by excluding the BCR from entering lipid rafts (33). It has been suggested that LMP2a mimics the BCR complex, competing for these protein-tyrosine kinases, and blocks the BCR from entry into lipid rafts (31, 33). Taken together these studies support the idea that unlike most B cell lymphomas that achieve Syk activation through BCR tonic signaling or antigen-dependent BCR aggregation, EBV+ B cell lymphomas activate Syk through LMP2a, the constitutively active BCR mimic encoded by EBV. However, further experiments regarding the precise mecha-
nism controlling Syk activation in EBV+ B cell lymphomas from PTLD are warranted.

Our data support a PI3K-dependent activation of Akt by Syk, but the precise molecular mechanisms of this process are unclear. Syk can directly bind the p85α regulatory subunit of PI3K (8). Syk can also phosphorylate tyrosines in LMP2a, creating docking sites for the SH2 domains of PI3K. Either mechanism may result in PI3K activation and subsequent Akt activation (56). Regardless of how Akt is activated, studies in Akt isoform-specific knock-out mice have revealed that Akt isoforms are not created equal, as each isoform has a distinct phenotype. Akt1 deficiency results in delayed pre- and postnatal growth, Akt2 deficiency results in defective glucose homeostasis, and Akt3 deficiency leads to defective brain development (57). Given the universal requirement of PI3K/Akt signaling for B cell lymphoma survival, the mechanism of Akt activation and the utilization of specific Akt isoforms may reveal further survival-specific therapeutic targets.

Although there is support for a general requirement for PI3K/Akt activation in Syk survival signaling, current data support the notion that different B cell lymphomas utilize unique PI3K/Akt anti-apoptotic targets involved in the intrinsic, or mitochondrial, pathway of apoptosis. In EBV+ B cell lymphomas, loss of the caspase inhibitor XIAP is a unique and critical outcome of Syk and PI3K/Akt survival signaling. Our studies show XIAP protein, but not transcript, levels are reduced after Syk or PI3K/Akt inhibition in the R406-sensitive, but not resistant, EBV+ PTLD-derived B cell lines. The protease HtrA2 was required for loss of XIAP and apoptosis induced by Syk and Akt inhibition. Reduction of XIAP protein levels after Syk inhibition was also observed in chronic lymphocytic leukemia (12), albeit to a markedly lower extent than in our EBV+ PTLD-derived B cell lines. In chronic lymphocytic leukemia, Syk inhibition by R406 results in the degradation of the anti-apoptotic molecule Mcl-1 (12, 15). Syk inhibition by curcumin in diffuse large B cell lymphoma results in decreased expression of the anti-apoptotic molecule Bcl-xL and loss of inhibitory phosphorylation of the pro-apoptotic molecule Bad (9). The differential use of members of the intrinsic pathway of apoptosis in malignancy is not unprecedented. Resistance of malignancies to Apo2L/TRAIL-induced apoptosis also depends on unique members of the mitochondrial pathway of apoptosis (58); for example, Bcl-2 mediates resistance in neuroblastoma and glioblastoma (59), whereas Bcl-xL mediates resistance in pancreatic adenocarcinoma (60). However, the mechanisms controlling differential utilization of unique PI3K/Akt anti-apoptotic targets in Syk inhibition-induced apoptosis of B cell lymphomas are unclear.

Common among the unique PI3K/Akt anti-apoptotic targets in B cell lymphomas is their involvement in, or requirement for, mitochondrial outer-membrane permeabilization (MOMP). MOMP results in the release of apoptogenic factors like HtrA2 from the mitochondria, also known as the “point of no return” in the intrinsic pathway of apoptosis (61). Members of the Bcl-2 family, including Bad, Bcl-xL, and Mcl-1, control MOMP in response to cellular stress, like cytokine deprivation (62). XIAP acts as an important checkpoint in this process by inhibiting caspase activity; MOMP results in the degradation or inhibition of XIAP by the release of HtrA2 or Smac/Diablo, respectively. Here we provide the first data that Syk inhibition induces MOMP, as evidenced by loss of mitochondrial DiOC6 staining in R406-sensitive EBV+ PTLD-derived B cell lines. As for the cellular stressor initiating MOMP, loss of PI3K/Akt signaling may trigger apoptosis. Intriguingly, Akt signaling alone maintains mitochondrial membrane potential and integrity (63, 64), possibly through its role in regulating Bcl-2 family members. Syk inhibition also induces growth factor withdrawal, a common cellular stressor that initiates the intrinsic pathway of apoptosis. PI3K/Akt-dependent autocrine IL-10 production (44) is required for the autonomous proliferation of EBV+ B cell lymphomas (48), but the requirement for autocrine IL-10 production for survival has yet to be directly examined. We observed loss of both PI3K/Akt signaling and high, endogenous IL-10 secretion after Syk inhibition in all our R406-sensitive EBV+ PTLD-derived B cell lines. Studies focusing exclusively on the involvement of IL-10 in PTLD should clarify a requirement for IL-10 secretion in the survival of EBV+ PTLD-derived B cell lines.

The partial rescue of Syk inhibition-induced apoptosis we observe by HtrA2 inhibition suggests Syk survival signaling is not exclusively executed through the PI3K/Akt pathway. Our data clearly show that Erk MAPK signaling is not required for proliferation or survival and that p38 MAPK and NF-κB signaling are not activated downstream of Syk. However, additional novel pathways controlling EBV+ B cell PTLD survival may be revealed by knockdown of LMP2a or Syk and subsequent proteomic analysis of signaling pathways (65). Erk MAPK activation by Syk may control other Syk-driven cellular responses, separate from apoptosis.

HtrA2 inhibition prevents both loss of XIAP and apoptosis induced by PI3K/Akt inhibition in R406-sensitive EBV+ PTLD-derived B cell lines. This strongly suggests that loss of XIAP is a crucial outcome of Syk inhibition and the primary outcome of PI3K/Akt inhibition in EBV+ B cell lymphomas. Although phosphorylation of XIAP by Akt prevents autoubiquitination and subsequent degradation (24), autoubiquitination of XIAP was recently shown as dispensable for degradation of XIAP (66). Our data suggest instead an important role for the protease HtrA2 in the loss of XIAP as well as survival of EBV+ PTLD-derived B cell lymphomas. This is consistent with findings that phosphorylation of HtrA2 by Akt attenuates its ability to degrade XIAP and induce apoptosis (49). However, a more detailed examination into the interplay between Akt, HtrA2, and XIAP is required.

Collectively, our data support a novel model of Syk survival signaling in EBV+ B cell lymphomas shown in Fig. 6. In EBV+ PTLD-derived B cell lines, Syk is activated either by the BCR or LMP2a. Syk directly activates the PI3K/Akt pathway, leading to IL-10 production. Our findings demonstrate involvement of PI3K/Akt and Erk MAPK signaling, but not NF-κB signaling, downstream of Syk activation. Whereas we demonstrate a critical requirement for PI3K/Akt signaling, we also clarify that Erk MAPK is not involved in Syk survival signaling. Finally, our studies reveal the requirement of HtrA2 in the loss of XIAP and apoptosis induced by Syk and Akt inhibition.
Survival by preventing the loss of XIAP mediated by the protease HtrA2. Activation of PI3K/Akt by Syk promotes lymphoma also induces autocrine IL-10 production. Akt has been reported to phosphor-
tutively activated by either the BCR or LMP2a. Although Syk utilizes both PI3K/Akt and Erk pathway to coordinate downstream events, Syk activation coordinates is required for EBV
PI3K/Akt and Erk pathway to coordinate downstream events, Syk activation of PI3K/Akt by Syk promotes lymphoma survival by preventing the loss of XIAP mediated by the protease HtrA2 in EBV B cell lymphomas.

Given the role of Syk in a wide and diverse set of cellular functions, Syk inhibition in murine models of PTLD will be crucial in determining the preclinical potential of this therapeutic strategy. Our studies highlight Akt, and for this first time, XIAP, as additional promising therapeutic targets for the treatment of EBV B cell lymphomas. Small molecule, peptidic, allosteric mimetic, and substrate mimetic inhibition strategies for both Akt and XIAP are currently in development and under investigation as chemotherapeutics (67, 68). These agents may constitute promising therapeutic or chemosensitizing approaches to target EBV-driven lymphomagenesis.

Acknowledgments—We thank Rigel, particularly Dr. Polly Pine, Dr. Ann Lowe, and Dr. Yasu Hitoshi, and AstraZeneca for kindly providing R406. We also thank Dr. Jeff Sharman, Amy C. Palin, Dr. Kenneth D. Gibbs, and Sadie R. Bartholomew for helpful discussions.

REFERENCES
1. Monroe, J. G. (2006) Nat. Rev. Immunol. 6, 283–294
2. Lam, K. P., Kühn, R., and Rajewsky, K. (1997) Cell 90, 1073–1083
3. Kraus, M., Alizhanov, M. B., Rajewsky, N., and Rajewsky, K. (2004) Cell 117, 787–800
4. Meffre, E., and Nussenzweig, M. C. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 11334–11339
5. Küppers, R. (2005) Nat. Rev. Cancer 5, 251–262
6. Gururajam, M., Jennings, C. D., and Bondada, S. (2006) J. Immunol. 176, 5715–5719

7. Gauld, S. B., Dal Porto, J. M., and Cambier, J. C. (2002) Science 296, 1641–1642
8. Mócsai, A., Ruland, J., and Tybulewicz, V. L. (2010) Nat. Rev. Immunol. 10, 387–402
9. Gururajam, M., Dasu, T., Shahidain, S., Jennings, C. D., Robertson, D. A., Rangnekar, V. M., and Bondada, S. (2007) J. Immunol. 178, 111–121
10. Chen, L., Monti, S., Juszczynski, P., Daley, J., Chen, W., Witzig, T. E., Habermann, T. M., Kutz, I. L., and Shipp, M. A. (2008) Blood 111, 2230–2237
11. Young, R. M., Hardy, I. R., Clarke, R. L., Lundy, N., Pine, P., Turner, B. C., Potter, T. A., and Rafaeli, Y. (2009) Blood 113, 2508–2516
12. Gobessi, S., Laurenti, L., Longo, P. G., Carsetti, L., Berno, V., Sica, S., Leone, G., and Efremov, D. G. (2009) Leukemia 23, 686–697
13. Buchner, M., Fuchs, S., Prinz, G., Pfeifer, D., Bartholomé, K., Burger, M., Chevalier, N., Vallat, L., Timmer, J., Gribben, J. G., Jumaa, H., Veelken, H., Diersc, C., and Zirlik, K. (2009) Cancer Res. 69, 5424–5432
14. Quitoga, M. P., Balakrishnan, K., Kurtova, A. V., Sivina, M., Keating, M. J., Wierda, W. G., Gandhi, V., and Burger, J. A. (2009) Blood 114, 1029–1037
15. Buchner, M., Baer, C., Prinz, G., Diersc, C, Burger, M., Zenz, T., Stilgenbauer, S., Jumaa, H., Veelken, H., and Zirlik, K. (2010) Blood 115, 4497–4506
16. Friedberg, J. W., Sharan, J., Sweetenham, J., Johnston, P. B., Vose, I. M., Lacasce, A., Schaefer-Cuttillo, J., De Vos, S., Sinha, R., Leonard, J. P., Cripe, L. D., Gregory, S. A., Sterba, M. P., Lowe, A. M., Levy, R., and Shipp, M. A. (2010) Blood 115, 2578–2585
17. Uckun, F. M., Ek, R. O., Jan, S. T., Chen, C. L., and Qazi, S. (2010) Br. J. Haematol. 149, 508–517
18. Manning, B. D., and Cantley, L. C. (2007) Cell 129, 1261–1274
19. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gohot, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
20. del Peso, L., González-García, M., Page, C., Herrera, R., and Nuñez, G. (1997) Science 278, 687–689
21. Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000) Mol. Cell 6, 41–51
22. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Science 282, 1318–1321
23. Tran, H., Brunet, A., Griffith, E. C., and Greenberg, M. E. (2003) Sci. STKE 2003, RE5
24. Dan, H. C., Sun, M., Kaneko, S., Feldman, R. I., Nicosia, S. V., Wang, H. G., Tsang, B. K., and Cheng, I. Q. (2004) J. Biol. Chem. 279, 5405–5412
25. Salvesen, G. S., and Duckett, C. S. (2002) Nat. Rev. Mol. Cell Biol. 3, 401–410
26. Fruhling, S., and Longnecker, R. (1997) Virology 235, 241–251
27. Caldwell, R. G., Wilson, J. B., Anderson, S. J., and Longnecker, R. (1998) Immunity 9, 405–411
28. Young, L. S., and Rickinson, A. B. (2004) Nat. Rev. Cancer 4, 757–768
29. Matskova, L., Ernberg, I., Pawson, T., and Winberg, G. (2001) J. Virol. 75, 10941–10949
30. Longnecker, R., and Miller, C. L. (1996) Trends Microbiol. 4, 38–42
31. Miller, C. L., Burkhartd, A. L., Lee, J. H., Stealey, B. R., Longnecker, R., Bolen, J. B., and Kieff, E. (1995) Immunity 2, 155–166
32. Miller, C. L., Lee, J. H., Kieff, E., and Longnecker, R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 772–776
33. Dykstra, M. L., Longnecker, R., and Pierce, S. K. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 57–67
34. Portis, T., and Longnecker, R. (2004) Oncogene 23, 8619–8628
35. Manco, C., and Hammerschmidt, W. (2007) Blood 110, 3715–3721
36. Merchant, M., Caldwell, R. G., and Longnecker, R. (2000) J. Virol. 74, 9115–9124
37. Scholle, F., Bendt, K. M., and Raab-Traub, N. (2000) J. Virol. 74, 10681–10689
38. Morrison, J. A., and Raab-Traub, N. (2005) J. Virol. 79, 2375–2382
39. Snow, A. L., and Martinez, O. M. (2007) Am. J. Transplant. 7, 271–277
40. Gottschalk, S., Rooney, C. M., and Heslop, H. E. (2005) Annu. Rev. Med. 56, 29–44
41. Nepomuceno, R. R., Balatoni, C. E., Natkunam, Y., Snow, A. L., Krams, S. M., and Martinez, O. M. (2003) Cancer Res. 63, 4472–4480
42. Snow, A. L., Chen, L. J., Nepomuceno, R. R., Krams, S. M., Esquivel, C. O.,
and Martínez, O. M. (2001) J. Immunol. 167, 5404–5411
43. Abramoff, M. D., Magelhaes, P. J., Ram, S. I. (2004) Biophotonics International 11, 36–42
44. Lambert, S. L., and Martínez, O. M. (2007) J. Immunol. 179, 8225–8234
45. Fu, C., Tuck, C. W., Kurosaki, T., and Chan, A. C. (1998) Immunity 9, 93–103
46. Wienands, J., Schweikert, J., Wollscheid, B., Jumaa, H., Nielsen, P. J., and Reth, M. (1998) J. Exp. Med. 188, 791–795
47. Braselmann, S., Taylor, V., Zhao, H., Wang, S., Sylvin, C., Baluom, M., Qu, K., Herlaar, E., Lau, A., Young, C., Wong, B. R., Lovell, S., Sun, T., Park, G., Aragde, A., Jurcevic, S., Pine, P., Singh, R., Grossbard, E. B., Payan, D. G., and Masuda, E. S. (2006) J. Pharmacol. Exp. Ther. 319, 998–1008
48. Beatty, P. R., Krans, S. M., and Martínez, O. M. (1997) J. Immunol. 158, 4045–4051
49. Yang, L., Sun, M., Sun, X. M., Cheng, G. Z., Nicosia, S. V., and Cheng, J. Q. (2007) J. Biol. Chem. 282, 10981–10987
50. Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001) Mol. Cell 8, 613–621
51. Srinivasula, S. M., Gupta, S., Datta, P., Zhang, Z., Hegde, R., Cheong, N., Fernandes-Alnemri, T., and Alnemri, E. S. (2003) J. Biol. Chem. 278, 31469–31472
52. Verhagen, A. M., Silke, J., Ekert, P. G., Kaufmann, H., Connolly, L. M., Day, C. L., Tikoo, A., Burke, R., Wrobel, C., Moritz, R. L., Simpson, R. I., and Vaux, D. L. (2002) J. Biol. Chem. 277, 445–454
53. Klupsch, K., and Downward, J. (2006) Cell Death Differ. 13, 2157–2159
54. Burkhardt, A. L., Bolen, J. B., Kieff, E., and Longnecker, R. (1992) J. Virol. 66, 5161–5167
55. Longnecker, R., Druker, B., Roberts, T. M., and Kieff, E. (1991) J. Virol. 65, 3681–3692
56. Portis, T., Cooper, L., Dennis, P., and Longnecker, R. (2002) Front. Biosci. 7, d414–d426
57. Dummier, B., and Hemmings, B. A. (2007) Biochem. Soc. Trans. 35, 231–235
58. Yang, A., Wilson, N. S., and Ashkenazi, A. (2010) Curr. Opin. Cell Biol. 22, 837–844
59. Fulda, S., Meyer, E., and Debatin, K. M. (2002) Oncogene 21, 2283–2294
60. Hinz, S., Trauold, A., Boenicke, L., Sandberg, C., Beckmann, S., Bayer, E., Walczak, H., Kalthoff, H., and Ungefroren, H. (2000) Oncogene 19, 5477–5486
61. Green, D. R., and Amarante-Mendes, G. P. (1998) Results Probl. Cell Differ. 24, 45–61
62. van Loo, G., Saelens, X., van Gurp, M., MacFarlane, M., Martin, S. J., and Vandenabeele, P. (2002) Cell Death Differ. 9, 1031–1042
63. Plass, D. R., and Thompson, C. B. (2005) Oncogene 24, 7435–7442
64. Robey, R. B., and Hay, N. (2006) Oncogene 25, 4683–4696
65. Chan, S. M., Ermann, J., Su, L., Fathman, C. G., and Utz, P. J. (2004) Nat. Med. 10, 1390–1396
66. Shin, H., Okada, K., Wilkinson, J. C., Solomon, K. M., Duckett, C. S., Reed, J. C., and Salvesen, G. S. (2003) Biochem. J. 373, 965–971
67. Lindsley, C. W. (2010) Curr. Top. Med. Chem. 10, 458–477
68. Schimmer, A. D., Dalili, S., Batey, R. A., and Riedl, S. J. (2006) Cell Death Differ. 13, 179–188