**Epstein-Barr Virus Latent Membrane Protein 2A (LMP2A) Employs the SLP-65 Signaling Module**

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**Abstract**

In latently infected B lymphocytes, the Epstein-Barr virus (EBV) suppresses signal transduction from the antigen receptor through expression of the integral latent membrane protein 2A (LMP2A). At the same time, LMP2A triggers B cell survival by a yet uncharacterized maintenance signal that is normally provided by the antigen receptor. The molecular mechanisms are unknown as LMP2A-regulated signaling cascades have not been described so far. Using a novel mouse model we have identified the intracellular adaptor protein Src homology 2 (SH2) domain–containing leukocyte protein (SLP)-65 as a critical downstream effector of LMP2A in vivo. Biochemical analysis of the underlying signaling pathways revealed that EBV infection causes constitutive tyrosine phosphorylation of one of the two SLP-65 isoforms and complex formation between SLP-65 and the protooncoprotein CrkL (CT10 regulator of kinase like). This leads to antigen receptor-independent phosphorylation of Cbl (Casitas B lineage lymphoma) and C3G. In contrast, phospholipase C-γ2 (PLC-γ2) activation is completely blocked. Our data show that in order to establish a latent EBV infection, LMP2A selectively activates or represses SLP-65–regulated signaling pathways.

Key words: B lymphocytes • Epstein-Barr virus • antigen receptor • SLP-65 • signal transduction

**Introduction**

EBV is an oncogenic herpes virus that infects human B lymphocytes through attachment to the CD21 surface marker (for a review, see references 1 and 2). EBV is the causative agent of infectious mononucleosis and is associated with numerous malignancies including Burkitt’s lymphoma, Hodgkin’s disease, and nasopharyngeal carcinoma. Like all herpes viruses, EBV is able to persist in infected cells in a latent form. Only a few virally encoded proteins are expressed in EBV-positive peripheral B cells, such as the EBV-associated nuclear antigen (EBNA)* ¹ and the integral latent membrane protein 2A (LMP2A). Infection of B cells in vitro results in expression of additional EBNAs together with LMP1, and an LMP2A splice variant, LMP2B (3). These B cells become immortalized and are called lymphoblastoid cell lines (LCLs). EBNAs are involved in maintaining the viral genome and, together with LMP1, are required for EBV-mediated B cell transformation. It is the expression of LMP2A that regulates EBV latency by remodeling signal transduction events from the B cell antigen receptor (BCR; references 4–8).

In the absence of antigen, a yet poorly understood BCR maintenance signal drives B cells through developmental checkpoints and is indispensable for the survival of mature, peripheral B cells (9–11). Antigen engagement triggers a BCR activation signal that allows proliferation and poten-

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C-γ2; PTK, protein tyrosine kinase; pTyr, phosphotyrosine; RAG, recombination activating gene; SLP, SH2 domain-containing leukocyte protein; SH2, Src homology 2 domain; WT, wild-type.

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*Abbreviations used in this paper: BCR, B cell antigen receptor; BLNK, B cell linker protein; Btk, Bruton’s tyrosine kinase; Cbl, Casitas B lineage lymphoma; Crk, CT10 regulator of kinase; CrkL, Crk like; EBNA, EBV-associated nuclear antigen; GST, glutathione S-transferase; ITAM, immunoreceptor tyrosine-based activation motif; LCL, lymphoblastoid cell line; LMP2A, latent membrane protein 2A; PLC-γ2, phospholipase C-γ2; PTK, protein tyrosine kinase; pTyr, phosphotyrosine; RAG, recombination activating gene; SLP, SH2 domain-containing leukocyte protein; SH2, Src homology 2 domain; WT, wild-type.
tially differentiation of stimulated B cells into antibody-secreting plasma cells (12–14). Both BCR functions are dependent on the immunoreceptor tyrosine-based activation motif (ITAM) in the BCR signaling elements, Ig-α and Ig-β, and on cytoplasmic protein tyrosine kinases (PTKs) of the Src-, Syk-, and Tec families. An immediate early substrate of Syk is the Src homology 2 (SH2) domain-containing leukocyte adaptor protein (SLP)-65 (15), (also called B cell linker protein [BLNK; reference 16] or B cell adaptor containing SH2 domain [BASH; reference 17]). SLP-65 contains at least five consensus tyrosine-phosphorylation sites at the N terminus, followed by several putative binding motifs for SH3 domains and a COOH-terminal SH2 domain. Phosphorylated SLP-65 couples Syk and the Tec family member Bruton’s tyrosine kinase (Btk) to phosphorylation and hence activation of phospholipase C-γ2 (PLC-γ2; references 16 and 18–21). This triggers the mobilization of intracellular Ca²⁺ ions, which is a hallmark of the BCR activation signal (22). Mutations in BCR components or BCR effector proteins impair the development and activation of B cells in mouse and man (9, 23–26). This is also true for loss of SLP-65 expression. SLP-65 knockout mice have been generated by multiple groups (27–30) and is also true for loss of SLP-65 expression. SLP-65 knockout and activation of B cells in mouse and man (9, 23–26). This is also true for loss of SLP-65 expression. SLP-65 knockout mice have been generated by multiple groups (27–30) and is also true for loss of SLP-65 expression. SLP-65 knockout and activation of B cells in mouse and man (9, 23–26). This is also true for loss of SLP-65 expression. SLP-65 knockout and activation of B cells in mouse and man (9, 23–26).

**Materials and Methods.**

**Generation and Analysis of Transgenic Mice.** Construction and characterization of SLP-65/BLNK−/− mice (B6.129F1 background) and EμLMP2A transgenic animals have been described previously (27, 38). EμLMP2A transgenic line E (TgE) mice have been bred for over 10 generations into the C57BL/6 background. TgE males were mated with SLP-65−/− females to obtain F1 progeny. F1 progeny were further mated to obtain transgenic LMP2A SLP-65−/− mice. All animals were housed at the Northwestern University Center for Experimental Animal Resources in accordance with university animal welfare guidelines. Isolation of tail DNA and polymerase chain reaction for the presence of the LMP2A transgene, recombination activating gene (RAG)-1, and the Neomycin cassette was done as described previously (38–40). Presence of the SLP-65/BLNK gene was determined by PCR using the primers BLNK sense (5′-GGACAAA-CAGGAAGTAGTG-3′) and BLNK antisense (5′-CTGGGCA-GGGACGGTTATC-3′). Flow cytometry analysis of B cell populations was done as described previously (38–40) using antibodies purchased from BD PharMingen. Samples were run on a Becton Dickinson FACScan™ and data was analyzed using CELLQuest™ software.

**Cell Culture.** LCLs were generated by infection of peripheral IgM- and IgG-positive B cells from healthy human donors with EBV strain B95-8 and maintained, like the EBV-negative Ramos B cell line (41) in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO₂. LCLs used in this study (BS, DK, NB, and OP) are heterogeneous for surface expression of IgM, IgD, and IgG. Surface IgM expression of LCL-Bs was higher than that of Ramos B cells, while that of LCL-DK, LCL-DU, LCL-NB, and LCL-OP was reduced, and these cells became IgM-negative after a long time in culture. Stimulation of the cells with either F(ab′)₂ fragments of anti-Ig antibodies or pervanadate/H₂O₂ has been described elsewhere (42–44).

**Protein Isolation and Analysis.** After lysis of the unstimulated or stimulated B cells in 1% Nonidet P-40 buffer (44), immunoprecipitations were performed using agarose-coupled mouse monoclonal antibodies to phosphotyrosine (pTyr; 4G10; UBI), or with protein G-adsorbed mouse monoclonal antibodies to human SLP-65/BLNK (Babco), or with rabbit antibodies specific for the following BCR effector proteins: PLC-γ2 (Q20), CrKL, and C3G (Santa Cruz Biotechnology, Inc.); and Casitas B lineage.
lymphoma (Cbl; Transduction Laboratories/Becton Dickinson). Mouse monoclonal anti-CrkL (UBI) and anti-Syk (4D10; Santa Cruz Biotechnology, Inc.) were used for protein detection by immunoblot analysis. The fusion protein between the glutathione S-transferase (GST) and the tandem SH2 domains of Syk, and its application for affinity purification of phosphoprotein ligands are described in detail elsewhere (42–44).

Results

The Adaptor SLP-65/BLNK Is a Critical Downstream Effector of LMP2A. The phenotype of TgE mice demonstrated that LMP2A is capable of altering normal B cell development and enhancing survival in vivo (38). Biochemical studies in LCLs revealed that this LMP2A function is dependent on its ITAM which becomes tyrosine phosphorylated and binds Syk (7, 8, 32, 33). This suggested a role of LMP2A.

Results were obtained from another LMP2A transgenic line described in reference 27) were bred to TgE mice. Spleen and bone marrow samples isolated from WT, TgE, SLP-65+/−, and TgE SLP-65−/− animals were analyzed by flow cytometry (Fig. 1). Cells were stained with antibodies to the pan-B cell marker CD19 and IgM and relative percentages of the different B cell populations were calculated based on a lymphocyte gate. As expected, WT animals showed the normal pattern of B cell development consisting of CD19+IgM− B cells (48 ± 7%) developing into CD19+IgM+ B cells (25 ± 7%) in the bone marrow. These WT CD19+IgM+ cells colonize peripheral immune organs, such as the spleen, where most B cells display a CD19+IgM− phenotype (61 ± 7%) with variable levels of IgM surface expression. In contrast, TgE animals show the LMP2A phenotype with most developing bone marrow B cells displaying a CD19+IgM− phenotype (62 ± 9%) and a very low number of B cells being CD19+IgM+ (1 ± 1%). TgE CD19+IgM− B cells exit the bone marrow and colonize the spleen (14 ± 5%). SLP-65−/− animals display the expected phenotype of a block in B cell development from the pro-B to pre-B stage showing a small number of CD19+IgM+ B cells in the bone marrow (9 ± 1%) and the spleen (9 ± 2%). As observed previously, a small population of IgM− B cells accumulates over time in SLP-65−/− mice (27). TgE SLP-65−/− animals were indistinguishable from SLP-65−/− animals with respects to B cell development. TgE SLP-65−/− bone marrow B cells were identified as being either CD19+IgM− B cells (43 ± 5%) or CD19+IgM+ B cells (6 ± 1%). Similarly to SLP-65−/− mice, TgE SLP-65−/− animals have a small number of CD19+IgM+ B cells in the spleen (6 ± 2%). Flow cytometric analysis of B220, CD19, and CD43 expression in bone marrow samples from TgE SLP-65−/− mice and SLP-65−/− littermate controls further confirmed that both animals display an almost identical block of B cell development, i.e., at the pre-BI (B220+CD19+CD43+) to pre-BII (B220+CD19+CD43−) stage (data not shown). Similar results were obtained from another LMP2A transgenic line displaying a weaker phenotype (Tg6) when bred into the SLP-65−/− background (data not shown). These results show that LMP2A is incapable of transmitting signals to alter B cell development and survival in vivo in the absence of SLP-65. The possibility that SLP-65 could be necessary.

Figure 1. Loss of the LMP2A developmental and survival phenotype when bred into the SLP-65−/− background. (A) Mouse splenocytes (SP) and (B) bone marrow samples (BM) from WT, TgE SLP-65−/−, and TgE SLP-65−/− animals were subjected to flow cytometric analysis with at least 10,000 gated lymphocytes per animal using CD19-PE (CD19) and IgM-FITC (IgM) antibodies. Typical dot plot results of 10,000 gated lymphocytes from 5–7-wk-old animals (n = 5) are shown. The polygons represent distinct subpopulations of B cells with the average percentage of total gated lymphocytes indicated above each polygon. (A) The left polygon represents CD19+IgM− B cells typical in TgE animals; the right polygon represents CD19+IgM+ cells. (B) The left rectangle represents CD19+IgM− B cells; the right rectangle represents CD19+IgM+ B cells.
for the differentiation of an EBV target population that will be subsequently driven by LMP2A signals can be excluded, as LMP2A expression in RAG-1−/− deficient animals (TgE RAG−1−/−) releases the block of pro-B cell development (38). Thus, early pro-B cells are already susceptible to LMP2A signaling, but this developmental stage is not affected by the loss of SLP-65 (27–30).

**LMP2A Triggers Constitutive Tyrosine Phosphorylation of Syk and SLP-65.** To biochemically analyze LMP2A signaling pathways and to address the role of SLP-65 in more detail several independent LCLs were established. As shown in Fig. 2 and in line with previous reports (7, 32–35), our LCLs express the 53-kD LMP2A, which is constitutively phosphorylated on its ITAM tyrosines. The latter feature allows LMP2A to be specifically purified with a GST fusion protein encompassing the tandem SH2 domains of Syk and to be detected by anti-pTyr immunoblotting (Fig. 2 A, lanes 1–4). P53 is absent in the preparation from LMP2A-negative Ramos B cells (lanes 5 and 6). In this case, the Syk SH2 domains purify tyrosine-phosphorylated Ig-α after BCR activation of the cells. The results confirm that the previously published association between tyrosine-phosphorylated LMP2A and Syk (7, 32, 33) is mediated by the Syk SH2 domains, which possess an exquisite binding specificity for doubly phosphorylated ITAMs (42). The lack of tyrosine-phosphorylated Ig-α in stimulated LCLs (lanes 2 and 4) directly shows the LMP2A-mediated inhibition of BCR signal transduction (5–8). This is further demonstrated by anti-pTyr immunoblot analysis of LCLs (Fig. 2 B, top panel). In addition to LMP2A, a second major phosphoprotein of 70 kD (p70) can be detected (lanes 1–6). The intensity of all protein bands is independent of BCR engagement. Moreover and in marked contrast to EBV-negative Ramos B cells (lanes 7–9), BCR engagement on LCLs does not increase the complexity of the pTyr-containing protein pattern. In summary, the LCLs used in this study show the same features than those previously employed for the analysis of LMP2A function (5–8).

As reported by Miller et al. (7), the p70 phosphoprotein band of LCLs is recognized by anti-Syk antibodies (Fig. 2 B, middle panel, lanes 1–6). However, repeated preclearing with anti-Syk antibodies failed to completely remove this band from anti-pTyr immunoprecipitates (data not shown). Probing of the same material with anti-SLP-65 antibodies revealed that anti-pTyr precipitates from unstimulated and stimulated LCLs also contain SLP-65 (Fig. 2 B, bottom panel, lanes 1–6). As for Syk, detection of SLP-65 in anti-pTyr precipitates from Ramos B cells required BCR ligation (lanes 7–9). However, whereas both SL-65 isoforms, p68 and p70, are present in the preparation from activated Ramos B cells, predominantly the slower migrating form is detected in anti-pTyr precipitates from LCLs. Both isoforms are expressed by LCLs to similar amounts (Fig. 2 C). To directly test for tyrosine phosphorylation of SL-65 in LCLs, anti-SLP-65 isoforms were analyzed by anti-pTyr immunoblotting. As shown in Fig. 3, the p70 isoform of purified SL-65 from LCLs is constitutively tyrosine phosphorylated (lanes 1–4). In Ramos B cells, both SL-65 isoforms contain tyrosine-phosphorylated Ig-α.

**Figure 2.** Impaired BCR signaling but elevated levels of pTyr-containing proteins in EBV-infected B cells. Two independently obtained LCLs (lanes 1–4 in panel a and 1–6 in panel b) and EBV-negative Ramos B cells (lanes 5 and 6 in panel a and 7–9 in panel b) were left untreated or stimulated through their BCR with F(ab′)2 fragments of anti-IgM antibodies for the indicated time points (30 s or 3 min). (a) From the cleared cellular lysates, phosphoproteins were isolated by affinity purification (AP) with a GST fusion protein encompassing the tandem SH2 domains of Syk (GST–Syk[SH2]2) and detected by anti-pTyr immunoblotting. (b) Lysates were subjected to immunoprecipitation (IP) with anti-pTyr antibodies and the material was analyzed by subsequent immunoblotting with antibodies to Syk (top panel), Syk (middle panel), and SL-65 (bottom panel). (c) Cellular lysates of the different LCLs used in this study (lanes 1–4) and Ramos B cells (lane 5) were immunoblotted with anti-SLP-65 antibodies. Relative molecular masses of marker proteins are indicated on the left in kD.
forms become inducibly phosphorylated (lanes 5 and 6). Collectively, our data establish that EBV infection triggers constitutive tyrosine phosphorylation of the 70-kD form of human SLP-65.

In LCLs, the Syk/SLP-65 Complex Is Uncoupled from PLC-γ2 Activation. SLP-65 has been shown to be a specific substrate of activated Syk (16). Consistent with this, we find that Syk and SLP-65 coimmunoprecipitate after BCR ligation of Ramos B cells (Fig. 4, lanes 5 and 6). In LCLs, however, this association is stimulation independent, which explains why SLP-65 is constitutively phosphorylated in these cells (Fig. 4, lanes 1–4). A direct downstream effector protein of phosphorylated SLP-65 is PLC-γ2, which upon dual phosphorylation by Syk and Btk initiates mobilization of Ca²⁺ ions (16, 18–21). In activated Ramos B cells, PLC-γ2 is tyrosine phosphorylated and communoprecipitates with phosphorylated SLP-65 (Fig. 5, lanes 7 and 8). PLC-γ2 isolated from unstimulated or stimulated LCLs remains almost unphosphorylated (Fig. 5, top panel, lanes 1–6). In some LCLs, a weak association between PLC-γ2 and SLP-65 could be observed (Fig. 5, middle panel, lanes 4–6). The results demonstrate that EBV infection uncouples phosphorylation of Syk and SLP-65 from PLC-γ2 activation. The underlying mechanism for this phenomenon remains to be elucidated but the data provide an explanation for the described block of BCR-induced Ca²⁺ mobilization in LMP2A-expressing B cells (5). Moreover, our data suggest that in these cells, SLP-65 may be indirectly translocated to the plasma membrane by virtue of its association with phosphorylated Syk bound to the phospho-ITAM of LMP2A.

Constitutive SLP-65 Phosphorylation in LCLs Is Accompanied with a Constant Trigger of the CrkL-Cbl-C3G Pathway. The 38-kD intracellular adaptor protein CrkL consists of one SH2 and two SH3 domains and has been implicated in positive and negative regulation of lymphocyte proliferation (45–49). The communoprecipitation experiment shown in Fig. 6 A (top panel) identifies CrkL as a novel SLP-65–binding protein in vivo. The p68 and p70 isoforms of SLP-65 are detected in anti-CrkL precipitates from stimulated and weakly from unstimulated Ramos B cells (lanes 5 and 6). Also in anti-CrkL precipitates from LCLs, both SLP-65 isoforms are present but their detection is not regulated by BCR engagement (lanes 1–4). SLP-65 could not be detected in anti-Crk II precipitates (data not shown). These data show that EBV infection causes a constitutive complex formation between SLP-65 and the protooncogene product CrkL. The presence of the 68-kD isoform of SLP-65 in anti-CrkL precipitates from LCLs shows that the association is at least in part independent of SLP-65 phosphorylation and hence may involve the CrkL SH3 domain(s).

The SH2 and central SH3 domain of CrkL are known to bind the Fyn-associated ubiquitin ligase Cbl and the Rap1-specific guanine nucleotide exchange factor C3G (45–47,
50–53). Consistent with previous reports we find an increased association of CrkL with Cbl after BCR ligation of Ramos B cells (Fig. 6A, middle panel, lanes 5 and 6). The amount of Cbl that co-immunoprecipitates with CrkL from two LCLs (lanes 1–4) is almost independent of BCR stimulation in that only a slight increase of complex formation is observed in only one of the two lines (lanes 1 and 2). Similarly, CrkL and C3G immunocomplicate from LCL lysates independent of cellular stimulation (Fig. 6 A, bottom panel, lanes 1–4). After BCR ligation of Ramos B cells, a slight decrease of the anti-C3G signal was observed in some but not in all experiments (lanes 5 and 6). Fig. 6 B shows that CrkL and C3G are inducibly phosphorylated in Ramos B cells (lanes 5 and 6), but are constitutively phosphorylated in LCLs (lanes 1–4). In summary, this set of experiments revealed that LMP2A expression affects not only the phosphorylation status of the p70 SLP-65 isoform but also abolishes the requirement of BCR ligation for a phosphorylation-dependent complex formation to CrkL. The CrkL-Cbl-C3G signaling module seems to be permanently triggered in LCLs.

**Figure 6.** Association of the protooncoprotein CrkL with SLP-65. (a) Anti-CrkL immunoprecipitates (IP) were prepared from cleared cellular lysates of untreated or BCR-stimulated LCLs (lanes 1–4) and Ramos B cells (lanes 5 and 6), and analyzed by subsequent immunoblotting with antibodies to SLP-65 (top panel), Cbl (middle panel), and C3G (bottom panel). (b) Anti-pTyr immunoprecipitates from the same cells were analyzed by anti-CrkL or anti-C3G immunoblotting (top and bottom panel, respectively). Relative molecular masses of marker proteins are indicated on the left in kD.

LCLs Are Unresponsive to Pervanadate/H$_2$O$_2$ Treatment. To further compare the signaling behavior between EBV-positive and -negative B cells, LCLs and Ramos B cells were treated with the protein tyrosine phosphatase inhibitor pervanadate/H$_2$O$_2$. Subsequently, pTyr-containing proteins were purified with anti-pTyr antibodies. As reported for many cell types, pervanadate/H$_2$O$_2$ treatment of Ramos B cells changes the equilibrium between dephosphorylation and phosphorylation towards the latter and hence induces robust PTK substrate phosphorylation (Fig. 7, top panel, lanes 5 and 6). Unexpectedly, all LCLs tested in this assay were unresponsive in that no pervanadate-mediated increase of protein tyrosine phosphorylation could be observed (top panel, lanes 1–4). Probing of the filter with anti-Syk (middle panel) and anti–SLP-65 antibodies (bottom panel) showed that both signaling proteins could be purified with anti-pTyr antibodies. Hence, the data show for the first time that EBV infection renders B cells not only unresponsive to BCR activation but generally abrogates their ability to induce PTK substrate phosphorylation. Whether this is solely mediated by LMP2A remains to be elucidated.

**Figure 7.** EBV-positive B cells are unresponsive to treatment with pervanadate/H$_2$O$_2$. Two independent LCL clones (lanes 1–4) and Ramos B cells (lanes 5 and 6) were left untreated (lanes 1, 3, and 5) or incubated with 20 μM pervanadate/H$_2$O$_2$ for 3 min at 37°C (lanes 2, 4, and 6). From the cleared cellular lysates, tyrosine-phosphorylated proteins were purified by immunoprecipitation (IP) and analyzed by subsequent immunoblotting with antibodies to pTyr (top panel), Syk (middle panel), and SLP-65 (bottom panel). Relative molecular masses of marker proteins are indicated on the left in kD.
Discussion

LMP2A reorganizes BCR-regulated survival and activation in EBV-infected B cells (5–8, 32, 33, 38). Recent biochemical and genetic evidence identified the adaptor SLP-65 as a central BCR effector protein downstream of Syk (15–21, 27–30, 54). Here we have shown that loss of SLP-65 expression abrogates LMP2A-mediated signaling for B cell development and survival in vivo. Analysis of human LCLs provided insight into the molecular mechanism(s) of this phenotype. EBV infection of B cells causes constitutive tyrosine phosphorylation of human SLP-65, primarily of its p70 isoform. Other B cell signaling proteins, such as Ig-α or the SLP-65 effector protein PLC-γ2, remain unphosphorylated even after BCR ligation. Another novel finding is the association of SLP-65 with CrkL, which is regulated by the BCR in normal B cells but not in LCLs. Finally, we have demonstrated that after EBV infection, not only BCR-specific signal transduction but also pervanadate/H2O2-induced PTK substrate phosphorylation is rescinded. Hence, LMP2A expression seems to broadly inhibit the ability of cellular PTKs to respond to cellular stimulation. As a consequence, EBV infection may inhibit ligand-induced signaling from many surface receptors that transmit their activation signal through cellular PTKs. One mechanism to achieve this function might be related to the constitutive targeting of LMP2A into lipid-rafts (31). This may create a microenvironment, which prevents translocation of the activated BCR as well as of other stimulated receptors into the signaling–competent membrane compartment. Experiments are under way to test this directly.

After B cell activation, one of the earliest Syk substrates is the cytoplasmic adaptor protein SLP-65 (15, 16, 43, 55). In line with this, we found that Syk and SLP-65 communoprecipitate from lysates of BCR-stimulated Ramos B cells. The stimulation-independent association between Syk and SLP-65 observed in LCLs explains the constitutive SLP-65 phosphorylation in these cells. As described previously, efficient coupling between Syk and SLP-65 is organized by the BCR and Y→F substitutions in the Ig-α ITAM reduce SLP-65 phosphorylation (43, 55). The data presented here suggest that LMP2A can substitute this BCR function. Consistent with this, two of the eight tyrosine residues in the N terminus of LMP2A are organized as an ITAM (56), which is essential for LMP2A function in vitro and in vivo (33, 40).

Lack of SLP-65 expression leads to a partial block of B cell development in mice and to a complete absence of mature B cells in a patient suffering from severe antibody deficiency (27–30, 54). A nearly identical phenotype is caused by mutations in the btk gene, which lead to X-linked immunodeficiency (XID) in mice and X-linked agammaglobulinemia (XLA) in humans (25, 57, 58). These results suggest that SLP-65 and Btk function in a common BCR signaling pathway. In support of this, phosphorylated SLP-65 recruits both, PLC-γ (16, 18, 19) and Btk (20, 21) through their SH2 domains. These are critical events for the phosphorylation and activation of PLC-γ allowing the subsequent generation of second messenger (for reviews, see references 14 and 22). In our LCLs, phosphorylation of PLC-γ2 is not detectable, despite the prominent and constitutive phosphorylation of Syk and SLP-65. At present we can only speculate about the mechanism that is responsible for this signaling block, but it may involve an LMP2A-regulated structural organization of proteins that prevents the interaction between PLC-γ2 and the SLP-65 signaling module. Possibly, LMP2A expression excludes PLC-γ2 from entry into lipid-rafts.

In human B cells, the primary slp-65 transcript is alternatively spliced generating the 68-kD and the 70-kD SLP-65 isoforms (16). Interestingly, p68 lacks amino acids 201–225 of p70, which encompass a putative binding motif for SH3 domains. Whether p68 and p70 perform different BCR signaling functions is not known. The selective and constitutive phosphorylation of p70 in LCLs together with the phenotype of TgE SLP-65→−/− mice strongly suggest a dominant role of this SLP-65 isoform for the LMP2A-mediated B cell maintenance signal and probably also for the BCR maintenance signal. From our data, however, it is not completely clear whether the CrkL-Cbl-C3G pathway downstream of SLP-65 is involved in signaling cell survival or whether it is involved in establishing B cell anergy that renders EBV-infected B cells unresponsive to BCR ligation. Of the three known Crk isoforms (I, II, and L), particularly CrkL is known to transmit signals from lymphocyte antigen receptors (45–47). A positive signaling function of CrkL is suggested by its capacity to transform cells when mutated or overexpressed, and by the finding that CrkL is the dominant phosphoprotein in v-Abl-transformed pre-B cells (49, 59). CrkL may also participate in negative regulation of lymphocytes through its SH2-mediated binding to the ubiquitin ligase Cbl (51, 52). The constitutive phospho–Cbl/CrkL complex in LCLs could play a role in the ubiquitination-mediated degradation of signaling proteins. Indeed, three Nedd4-like ubiquitin ligases were recently found to directly associate with LMP2A (60, 61). Moreover, the structurally unusual SH2 domain of Cbl competes with PLC-γ2 for binding to phosphorylated SLP-65 (62), which could also contribute to the observed block of PLC-γ2 phosphorylation in our LCLs. In anergic T cells, which do not transcribe the Il-2 gene upon TCR activation, constitutive phosphorylation of Cbl leads to assembly of a ternary Cbl-CrkL-C3G complex that activates the Rap1 pathway independent of TCR ligation (48, 53). Overexpression of activated Rap1 in Jurkat cells recapitulated T cell anergy. The constitutive association of Cbl with CrkL and C3G in LCLs suggests that a similar pathway could operate in EBV-infected B cells to block BCR-mediated activation of the cells. The BCR can activate Rap1 via a PLC-γ-dependent pathway, which may be independent of CrkL and C3G (63).

In EBV-negative B cells, the SLP-65/CrkL complex is regulated by the BCR, indicating that phosphorylated SLP-65 is bound by the CrkL SH2 domain. Consistent with this, the four YXXP phosphorylation motifs of SLP-
65 are nearly identical to those found in known CrkL-binding proteins such as Cas (Crk-associated substrate) and Cbl. Similar amino acid sequences were selected by the CrkL SH2 domain from a phosphopeptide library (64). The finding that in LCLs, also the nonphosphorylated p68 isoform of SLP-65 coprecipitates with CrkL, suggests a function for the CrkL SH3 domain(s). At least seven PXxP consensus binding sites for SH3 domains are present in SLP-65 and a low level of SLP-65/CrkL complexes was repeatedly found in unstimulated Ramos B cells. We therefore conclude that the CrkL-derived SH2 and SH3 domains synergistically regulate the interaction with SLP-65 in resting and activated B cells.

In summary, our analysis revealed several novel aspects of LMP2A-mediated reorganization of B cell signaling elements. While a stimulation-dependent PTK substrate phosphorylation becomes generally suppressed, certain signaling pathways emanating from the SLP-65 module are selectively activated to keep EBV infected cells alive. Future studies on the assembly and function of the BCR or LMP2A signalosome will be required to better understand B lymphopoiesis and to finally interfere with it in pathological situations.

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