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Epstein-Barr Virus Latent Membrane Protein 2 Associates with and Is a Substrate for Mitogen-Activated Protein Kinase

CONSTANTINOS G. PANOUSIS AND DAVID T. ROWE*

Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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The latent membrane protein 2 (LMP2) of Epstein-Barr virus interferes with B-lymphocyte signal transduction through the immunoglobulin (Ig) receptor. Two isoforms of LMP2 exist and differ only in that one isoform (LMP2a) contains an N-terminal cytoplasmic domain that the other isoform does not. LMP2a is a phosphoprotein that is phosphorylated on tyrosines and serines in the cytoplasmic domain. GST1-119, a glutathione S-transferase (GST) fusion protein containing the 119 amino acids of the cytoplasmic domain, affinity precipitated serine kinase activity from BJAB cell extracts. The affinity-precipitated kinase phosphorylated LMP2a sequences, and kinase activity was increased following induction. Probing of Western immunoblots of affinity-precipitated proteins showed that the Erk1 form of mitogen-activated protein kinase (MAPK) was present. Purified MAPK phosphorylated GST fusion proteins containing the cytoplasmic domain of LMP2a and mutational analyses were used to identify S15 and S102 as the sites of in vitro phosphorylation. A polyclonal rabbit antiserum was prepared against a maltose binding protein-LMP2a cytoplasmic domain fusion protein (MBP1-119) and used to immunoprecipitate LMP2a from the in vitro-immortalized lymphoblastoid B-cell line B95-8CR. LMP2a immunoprecipitates from B95-8CR contained MAPK as a coprecipitated protein. Cross-linking surface Ig on B95-8CR cells failed to induce MAPK activity within the cells. Treatment of B95-8CR with phorbol myristate acetate (PMA) was able to bypass the Ig receptor block and activate MAPK activity. Phosphorylation of LMP2a on serine residues increased after PMA induction. The possible role for LMP2a serine phosphorylation by MAPK in the control of latency is discussed.

Epstein-Barr virus (EBV) is a gamma class human herpesvirus that infects and immortalizes B lymphocytes in vitro (25). In the host, proliferating virus-immortalized B cells are controlled by antigen-specific cytotoxic T cells which recognize virus-derived peptides (24, 37, 44, 49). Deficient T-cell responses underly the lymphoproliferative syndromes, lymphomas, and carcinomas with which EBV is associated (24, 27, 37). Normal immune surveillance is, however, apparently incapable of eliminating all virus-infected B cells from the host, and primary infections resolve into a life-long persistence, with virus being periodically detectable in throat washings and recoverable from the peripheral blood as spontaneously immortalized B lymphocytes harboring latent virus genomes (17, 25, 30, 46, 66).

In vitro, the virus-immortalized B cells express six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP), two membrane proteins (latent membrane protein 1 [LMP1] and LMP2), and two small RNAs (EBERs) (25). Genetic analyses of the virus has demonstrated roles for several of these genes (LMP1, EBNA1, -2, -3A, and -3C, and LP) in the immortalization process (19, 23, 25). One of the most important of these is LMP1, which is expressed in a number of EBV-associated lymphoproliferative diseases and malignancies and has been shown to affect a number of critical processes, including cell surface phenotype changes, NF-κB activation, and induction of antiapoptotic mechanisms (21, 43). These alterations appear to be a consequence of the ability of LMP1 to auto-cross-link in the membrane and associate with a novel tumor necrosis factor receptor-associated protein, TRAF3 (43). EBNA1, EBNA2, and EBNA3C are transcriptional activators (51, 52, 64). EBNA2, in particular, is a transcriptional activator which upregulates expression of the other EBNAs, including EBNA3C, and the LMP1 and LMP2 genes (11, 22, 67). Cellular genes CD21, CD23, and c-*fgr* are also activated in EBNA2-expressing cells (28, 65). In addition to its transcriptional activating activity, EBNA1 is an origin of replication binding protein essential for maintenance of the viral episome in proliferating cells (48). Genetic analyses suggest that the LMP2 and EBNA3B genes are not required for immortalization of B cells by virus, and their roles in the biology of EBV remain uncertain (33, 34, 35, 62).

LMP2 is made from a rightward-transcribed gene which is created upon circularization of the EBV genome (29). Two mRNAs (2.0 and 1.9 kb) produced by alternative promoter usage differ in the sequences of the first exon but share eight common 3′ exons that encode 12 membrane-spanning segments. Thus, two isoforms of the same membrane protein are made (52). The difference is that LMP2a contains an additional N-terminal cytoplasmic domain not present in the shorter LMP2b form of the protein. LMP2a is associated with Src family tyrosine kinases and is constitutively phosphorylated on tyrosines in lymphoblastoid cell lines (LCLs) and LMP2a-transfected EBV-negative Burkitt lymphoma cells (7, 32). The N-terminal domain of LMP2a contains an ARH-1 motif which is presumed to direct the interaction of LMP2a with the Syk tyrosine kinase (1, 5, 41). ARH-1 motifs are present in Fc receptors and the B- and T-cell antigen receptor-associated molecules and are used to interact with Syk family tyrosine kinases (15). When stably expressed in the EBV-negative Burkitt lymphoma cell line BJAB, LMP2a significantly inhibited calcium mobilization after cross-linking of the immunoglobulin (Ig) receptor, CD19, or major histocompatibility complex class

* Corresponding author. Mailing address: Graduate School of Public Health, Department of Infectious Diseases and Microbiology, University of Pittsburgh, 435 Parran Hall, 130 DeSoto St., Pittsburgh, PA 15261. Phone: (412) 624-1529. Fax: (412) 383-8926. E-mail: ROWE1+@PITT.EDU.

II molecules (40). In immortalized B lymphocytes, a number of signal transduction-associated molecules (Syk, phosphatidylinositol 3′-kinase, phospholipase Cα2, and Vav) were discovered to be constitutively tyrosine phosphorylated (41). Cross-linking of these cells' antigen receptors with antibody did not alter the status of the transducers and failed to induce activation of virus lytic gene expression. These effects are consistent with a role for LMP2a in latency that involves attenuation of B-cell activation. Circulating peripheral blood B cells from healthy carriers express LMP2a RNA as might be expected for a protein whose function is critical to the establishment, maintenance, and/or reactivation phases of in situ latency (47).

In addition to tyrosine phosphorylation, LMP2a expressed in immortalized lymphocytes also shows substantial phosphorylation on serine residues (32). The significance of this phosphorylation has not been explored. Neither the kinases responsible nor the sites of serine phosphorylation have been identified. Here we report that LMP2a associates with and is a substrate for mitogen-activated protein kinase (MAPK). Two sites of phosphorylation have been mapped in vitro, one of which lies within a conserved region in human and simian LMP2 proteins.

MATERIALS AND METHODS

Cell lines and reagents. BJAB is an EBV-negative Burkitt lymphoma cell line; Akata is an EBV-positive Burkitt lymphoma cell line; B95-8 is an EBV-positive monkey cell line; Akata is a Burkitt lymphoma cell line; B95-8SK is an LCL obtained by immortalization of adult peripheral B lymphocytes with B95-8 virus; B95-8CR, AG867CR, MukiraCR, MakauCR, MwikeCR, and BL72CR are in vitro-established LCLs (a gift from A. Rickinson); DT, DR, TO, SOG1, and SOG2 are spontaneous LCLs; X50-7 and IB-4 are in vitro-established cord blood lymphocyte LCLs (a gift from G. Miller); and B1.2 is an LCL (a gift from John Yates) with a disrupted LMP2a gene (part of the exon 2 has been deleted) (26). Cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (HyClone), 2 mM glutamine, 60 μg of penicillin per ml, and 200 μg of streptomycin per ml at 37°C in 5% CO_2. Anti-IgM [F(ab′)$_2$ fragment] was obtained from Accurate; phorbol myristate acetate (PMA) and protein A-Sepharose beads were purchased from Sigma; Hi-Trap affinity columns and glutathione-Sepharose beads were obtained from Pharmacia; MAPK-p44^{mpk} purified enzyme, myelin basic protein (MBP), and rabbit polyclonal anti-rat MAPK R2 (Erk1-CT) and anti-MAPK (Erk2) monoclonal antibodies were purchased from Upstate Biotechnology; rabbit polyclonal phospho-MAPK (P-MAPK)-specific antibody and rabbit polyclonal anti-p44/42 MAPK antibody were purchased from New England Biolabs. Monoclonal antiphosphotyrosine antibody PY20 was purchased from Signal Transduction Laboratories. ^{125}I-protein A, ^{125}I-goat anti-mouse, RPMI 1640 without methionine and cysteine, and RPMI 1640 without phosphate were obtained from ICN.

Construction of glutathione *S*-transferase (GST) fusion proteins. A DNA fragment encoding the N-terminal domain of LMP2a was amplified by PCR from B95-8 cDNA by using primers 1-119 (5′ TGCAAGCTTTATGGGGTC CCT 3′) and ORF3′ (5′ GGCAAGCTTAGCTTCCTCTGCC 3′). For production of truncations of the N terminus, amplification was done with primers 1-80 (5′ ACTT TAAGCTTGGGTTCC TAG 3′), 77-119 (5′ CAAAGCTTAGGAACCCAA GAT CAA 3′), and 1-61 (5′ CCAATAAG CTTACTCATAAGGCGG 3′). All of these primers have a *Hin*dIII restriction site (underlined). Point mutations were introduced by PCR by using the megaprimer method of site-directed mutagenesis (53); Ser 15 (AGC) was mutated to Ala (GCC) with the oligonucleotide 5′ ATCCCCGCCGGGGGCAGGGGGACC 3′, and Ser 102 (TCT) was mutated to Ala (GCT) with the oligonucleotide 5′ CCTCCC CCTCCCTACGC TCCACGG 3′. The PCR products were digested with *Hin*dIII and were ligated to pGEX-3X (Pharmacia). DNA sequence analysis was performed to confirm the structures of the constructs (Sequenase 2.0; U.S. Biochemical Corp.). pGEX-3X constructs were transfected into *Escherichia coli* INVα F′ (Invitrogen), and fusion proteins were prepared by inducing 1 liter of log-phase culture (A_{600} = 0.5) with 0.3 mM isopropythiogalactopyranoside for 4 h. Cells were pelleted by centrifugation, frozen and thawed once, resuspended in 10 ml of 10 mM Tris (pH 8.0)–50 mM NaCl, and lysed by sonication, and lysates were cleared by centrifugation. One milliliter of clear lysate was used to load 50 μl of glutathione-Sepharose beads (Pharmacia).

Affinity precipitations. BJAB cells (2×10^7 per sample) were collected by centrifugation and lysed with 1 ml of lysis buffer (1% Triton X-100, 137 mM NaCl, 10 mM Tris [pH 8.0], 1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM iodoacetamide, 1 μg of aprotinin, 1 μg of leupeptin). Lysates were incubated on ice for 15 min and cleared by centrifugation at 12,000 × *g* for 20 min. Clear lysates were incubated with 50 μl of GST fusion protein-bound beads overnight at 4°C. The fusion proteins absorbates were washed three times with washing buffer (1% Triton X-100, 137 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM iodoacetamide) and resuspended in 50 μl of sodium dodecyl sulfate (SDS) sample buffer.

Kinase assays. Affinity precipitates with GST fusion proteins coupled to beads were washed three times with 1 ml of kinase buffer (50 mM HEPES [pH 7.4], 30 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM Na_3VO_4) and resuspended in 20 μl of kinase buffer containing 1 μCi of [^{32}P]ATP and, where indicated, 1 U of purified p44^{mpk} (Upstate Biotechnology). The kinase reactions were performed at room temperature for 15 min, followed by washing twice with 1 ml of kinase buffer and once with washing buffer (1% Triton X-100, 10 mM Tris [pH 8.0], 137 mM NaCl, 1 mM Na_3VO_4, 1 mM iodoacetamide, 1 mM PMSF) and resuspension in 50 μl of SDS sample buffer. For the MBP-kinase assay, the beads were washed and incubated with 20 μg of MBP (Upstate Biotechnology) in 20 μl of kinase buffer in the presence of 1 μCi of [^{32}P]ATP. The reaction was terminated by the addition of 50 μl of SDS sample buffer. ^{32}P incorporation was quantitated by a PhosphorImager (Molecular Dynamics).

LMP2a-specific antibody development and purification. We used the pMAL-CRI protein fusion and expression system (New England Biolabs). The LMP2a exon 1 sequence from cloned B95-8 cDNA was prepared for insertion into the vector by PCR using the primers 5′ TGCGAATTCTATGGGGTCCCT 3′ and 5′ GGCAAGCTTAGCTTCCTCTGCC 3′, incorporating a 5′ *Eco*RI and a 3′ *Hin*dIII restriction site, and cloned downstream of the *E. coli malE* gene under the control of the inducible P_{tac} promoter. *malE* codes for MBP, permitting the fusion protein to be purified by amylose affinity column chromatography. MBP-exon 1 fusion protein was purified by amylose affinity chromatography followed by anion exchange to release residual MBP and MBP1-119 subfragments that were present in the affinity purification. The fusion protein was sent to Research Genetics, Inc., where the immunogen was emulsified with an equal volume of Freund's adjuvant and injected to rabbits; the primary immunization was followed by two boosts at weeks 2 and 6. The animals were bled at weeks 4, 8, and 10; the blood was allowed to clot, and serum was collected by centrifugation. Because of many other nonspecific interactions, we affinity purified the serum against MBP-exon 1 fusion protein. The antiserum was first passed through an amylose column loaded with MBP in order to deplete the serum of all the anti-MBP antibodies and subsequently through a Hi-Trap affinity column (Pharmacia) loaded with MBP-exon 1. The column was washed extensively, and antibodies were eluted with Tris-glycine (pH 2.5) elution buffer and dialyzed against Tris-buffered saline.

Immunoprecipitations. Cells were pelleted and lysed on ice with 50 mM Tris (pH 8.0)–137 mM NaCl–1% Triton X-100–1 mM EDTA–1 mM PMSF–5 mM NaF–0.4 mM Na_3VO_4–1% bovine serum albumin–1 mM iodoacetamide–1 μg of aprotinin–1 μg of leupeptin. Nuclei and insoluble material were removed by centrifugation. Cell lysates were incubated with antiserum for 8 h at 4°C and with 20 μl of protein-A Sepharose beads for 4 h at 4°C. Sepharose beads were washed three times with washing buffer (50 mM Tris [pH 8.0], 1% Triton X-100, 137 mM NaCl, 1 mM PMSF, 1 mM iodoacetamide) and resuspended in 50 μl of SDS sample buffer.

Metabolic labeling. For ^{32}P labeling, 10^8 cells were washed twice with phosphate-free RPMI 1640 (ICN), resuspended in 5 ml of phosphate-free RPMI 1640 containing 2% dialyzed fetal bovine serum and 1 mM glutamine, and labeled for 4 h with 5 mCi of [^{32}P]orthophosphate (ICN). Following labeling, cells were washed with 10 volumes of phosphate-buffered saline (PBS)–0.4 mM EDTA–0.4 mM Na_3VO_4 and lysed. For ^{35}S labeling, 2×10^8 cells were washed twice and incubated for 15 min in RPMI 1640 without methionine and cysteine (ICN). Cells were resuspended in 10 ml of methionine- and cysteine-free RPMI 1640 containing 2% dialyzed fetal bovine serum and 1 mM glutamine and labeled for 4 h with 2 mCi of [Tran ^{35}S] (ICN). Following labeling, cells were washed twice with 40 ml of PBS and lysed.

Phosphoamino acid analysis. Phosphoproteins were transferred onto an Immobilon-P polyvinylidene difluoride membrane and hydrolyzed for 1 h at 110°C with 6 N HCl. Labeled amino acids as well as unlabeled phosphoamino acid standards were resolved by electrophoresis in two dimensions on cellulose thin-layer chromatography plates at pH 1.9 for 90 min at 900 V in glacial acetic acid–88% formic acid–H_2O (78:25:897, vol/vol/vol) and at pH 3.5 for 45 min at 900 V in glacial acetic acid-pyridine-H_2O (50:5:945, vol/vol/vol). Phosphoserine, phosphothreonine, and phosphotyrosine were identified by staining with 1% ninhydrin.

Immunoblots. Gels to be analyzed by immunoblotting were transferred to nitrocellulose or polyvinylidene difluoride membranes and blocked for 1 h at room temperature with 5% powder milk in Tris-buffered saline or 5% bovine serum albumin in PBS–0.1% Tween 20. Blots were probed with antiserum and polyclonal serum and were developed with 1 μCi of ^{125}I-protein A (ICN) or with secondary anti-rabbit antibody conjugated to alkaline phosphatase followed by chemiluminescence (CDP-Star; New England Biolabs). Monoclonal antiserum was developed with 1 μCi of ^{125}I-goat anti-rabbit (ICN).

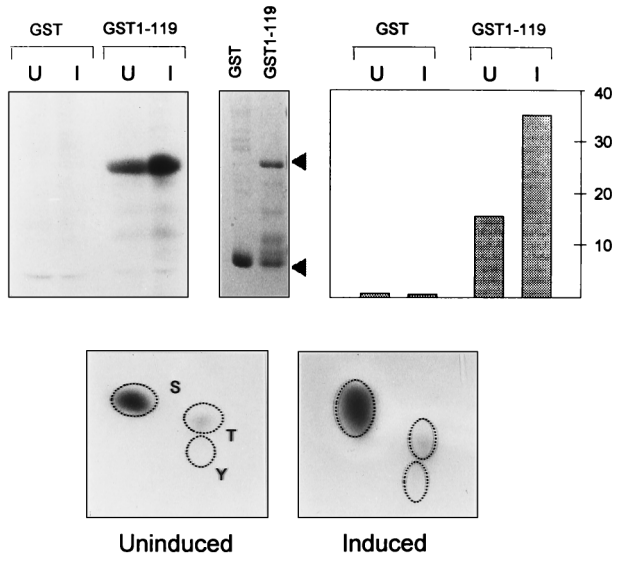

FIG. 1. Association of an inducible serine kinase activity with GST1-119. (Left) In vitro kinase assay with affinity-precipitated proteins from BJAB cell extracts uninduced (U) or induced (I) for 15 min with anti-IgM F(ab′)$_2$; (middle) Coomassie blue staining of GST and GST1-119 fusion proteins; (right) quantitation of phosphorylated proteins from the left panel, using a Molecular Dynamics PhosphorImager; (bottom) two dimensional thin-layer phosphoamino acid analyses of GST1-119 fusion protein phosphorylated in the in vitro kinase assay.

RESULTS

LMP2a exon 1 associates with a serine kinase. It has previously been shown that immunoprecipitates of LMP2a from LCLs or LMP2a expressed transiently in BJAB cells contain kinase activity that phosphorylates LMP2a on tyrosines serines and threonines (32). Subsequent studies have focused on the identity of the tyrosine kinases present and responsible for tyrosine phosphorylation on LMP2 (7, 41). Thus far, the observation that, in addition to tyrosine phosphorylation on LMP2a, the level of serine phosphorylation is also very high has not been further investigated. Using a GST fusion protein (GST1-119) that expresses the amino-terminal 119 amino acids of LMP2a (exon 1), we found that a protein kinase activity was specifically and reproducibly affinity precipitated from cell extracts of BJAB cells (Fig. 1). The kinase activity in the affinity precipitate phosphorylated GST1-119 on serine residues. In addition, an increased level of serine phosphorylation occurred in the in vitro kinase assays after induction of the BJAB cells by cross-linking the surface Ig molecules 15 min prior to preparation of the extract. We used this in vitro experimental system to explore LMP2a serine phosphorylation and identify the inducible serine kinases that were affinity precipitated by GST1-119 from cell extracts of B lymphocytes.

The inducible serine kinase is Erk1. To identify molecules associated with GST1-119, we performed affinity precipitation with ^{35}S-labeled BJAB cells. Two proteins, one of approximately 110 kDa (not shown) and a second of approximately 45 kDa, were detected (Fig. 2). The latter band appears distorted in the GST1-119 lane because of the comigration of the fusion protein. A preliminary inspection of the 13 serine residues in LMP2 exon 1 had revealed the presence of two consensus sequences around S15 (PPSP) and S102 (PYSP) for a 45-kDa signaling kinase often referred to as MAPK (4, 14). To determine whether MAPK activity might be responsible for the serine phosphorylation of GST1-119, an immunoblot of the unlabeled affinity-precipitated proteins was probed with an antiserum specific for MAPK. The autoradiogram contained a distorted band recognized by the anti-MAPK antibody in the region of 45 kDa corresponding to the ^{35}S-labeled product (Fig. 2).

The ability of Ig cross-linking to activate the MAPK cascade in BJAB cells was confirmed by probing Western blots with an antibody that recognized MAPK (both Erk1 and Erk2 isoforms) and a second antibody that specifically detected only P-MAPK. Use of the MAPK antibody showed that extracts from the uninduced and induced cells had similar amounts of MAPK (Fig. 3A). Use of the anti-P-MAPK antibody on Western blots of uninduced and induced BJAB cell extracts revealed that some activated MAPK was present in the uninduced state. Induction by Ig cross-linking led to an increase in the phosphorylation of both the Erk1 and Erk2 forms of MAPK (Fig. 3A). To differentiate which of the two isoforms was present in the GST1-119 affinity precipitate, two antisera were used, one which preferentially recognizes p44 Erk1 and another which was specific for the p42 Erk2 form of MAPK. Only the Erk1 form was detected in the affinity precipitates (Fig. 3B). In addition, the amounts of MAPK detected in the affinity precipitates from the uninduced and induced cells appeared to be approximately the same.

An increase in the amount of phosphorylation in the GST1-119 fusion protein after induction by Ig cross-linking would presumably be the result of activation of the MAPK signaling pathway. Direct detection of the induced phosphorylated form of MAPK in the affinity precipitates by high-resolution gel electrophoresis was hampered by the presence of the fusion protein. When the MAPK substrate MBP was added to the affinity precipitates, it was phosphorylated to a higher level only when the kinases were derived from induced cell extracts. Some phosphorylation of this substrate was detected in the uninduced cell extracts and also in affinity precipitates obtained by using GST alone (Fig. 3C). This result is identical to that reported for GST and GST-beta interferon receptor fusion protein affinity precipitates when these fusion proteins were used in in vitro kinase assays to detect MAPK activity (13). Only in the affinity precipitate from the induced cell extract was a higher level of phosphorylation observed. Together, these results suggest that only Erk1, not Erk2, is present in the affinity precipitate and that this kinase when

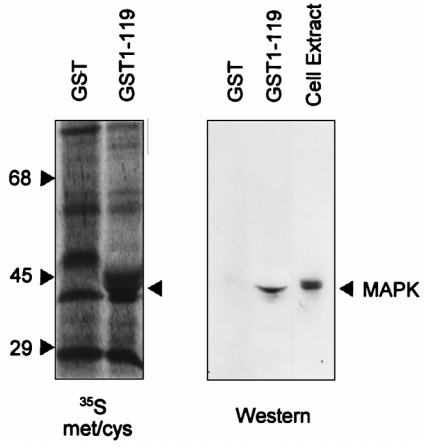

FIG. 2. Association of MAPK with GST1-119. (Right) Affinity-precipitated proteins from ^{35}S metabolically labeled BJAB cell extracts. Extracts had been precleared for 4 h with 50 μl of glutathione-Sepharose beads prior to addition of GST or GST1-119. (Left) Western blot of affinity-precipitated proteins probed with rat anti-MAPK R2 polyclonal antibody, which recognizes both Erk1 and Erk2 isoforms of MAPK. Sizes are indicated in kilodaltons.

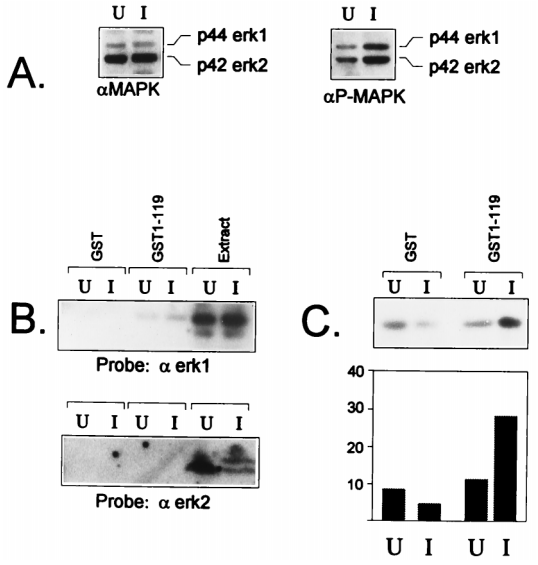

FIG. 3. The inducible serine kinase is Erk1. (A) Western blots of BJAB cell extracts from uninduced cells (U) or cells induced for 15 min with anti-IgM F(ab′)$_2$ (I) were probed with anti-MAPK (αMAPK), which detects both Erk1 and Erk2, or with anti-P-MAPK (αP-MAPK), which detects only the phosphorylated isoforms. (B) Affinity-precipitated proteins from uninduced and induced BJAB cell extracts probed with rat anti-MAPKinase R2 (Erk1-CT) polyclonal antibody (α erk1) or with anti-MAPK (Erk2) monoclonal antibody (α erk2). (C) Affinity precipitations from uninduced or induced cell extracts followed by in vitro kinase assays with exogenously added MBP. Quantitation of the phosphorylation level of MBP was measured with a PhosphorImager (Molecular Dynamics).

activated is able to phosphorylate GST1-119 on the LMP2a exon 1 sequences of the fusion protein.

Identification of MAPK sites on LMP2a. In an initial attempt to localize MAPK binding and phosphorylation within LMP2a exon 1 sequences, we made two fusion protein mutants lacking either the C-terminal region (GST1-80) or the N-terminal region (GST77-119). Each fusion protein contained only one of the two sites (S12 and S102) that were potential MAPK phosphorylation sites based on the phosphorylation consensus sequence. These fusion proteins were used in in vitro kinase assays following affinity precipitation of cellular kinases from extracts of BJAB cells which had been induced for 5 min by Ig cross-linking. Both proteins became phosphorylated (Fig. 4). Phosphoamino acid analyses of the phosphorylation of these proteins revealed that like the full-length GST1-119 fusion protein, they were both phosphorylated principally on serine residues. All three proteins showed low levels of tyrosine and threonine phosphorylation. Additional phosphorylation on tyrosines and threonines was often observed when extracts were made after very short induction times, and this presumably reflects the presence of other kinases in the affinity precipitate and the different kinetics of kinase activations following signal transduction. The full-length GST1-119 and GST77-119 showed slightly more tyrosine phosphorylation than the GST1-80. This is possibly due to the preferential phosphorylation of Y112 by Lyn and Fyn (our unpublished observations). Tyrosine 112 (YEEA) is a possible site for SH2 binding of Src family kinases (56). All three proteins were substrates for MAPK when activated MAPK was added to the fusion proteins in vitro. The in vitro kinase assay with purified MAPK showed phosphorylation only on serines (Fig. 4). GST1-80 showed a small amount of threonine phosphorylation. Although T41 and T43 (TPTPP) are surrounded by proline residues and may represent a poorly recognized potential phosphorylation motif for MAPK, threonine phosphorylation was not detected in GST1-61 or GST1-119. MAPK phosphorylation of threonine is therefore most likely to be on T79, which is exposed at the COOH end of GST1-80. In vitro MAPK binding experiments for a series of deletion mutants in the LMP2a exon 1 region revealed that neither Y74, Y85, nor the entire ARH-1 motif of LMP2a exon 1 was necessary for MAPK binding or phosphorylation (Fig. 5). Point mutations changing S15 to alanine (GST1-66A15) and S102 to alanine (GST77-119A102) were not phosphorylatable by MAPK in vitro (Fig. 5). Despite this, both proteins were still able to affinity precipitate MAPK from cell extracts. Furthermore, when the point mutant proteins were used in affinity precipitation followed by in vitro phosphorylation assays, residual serine phosphorylation was detected (not shown). This result implies that enzymes other than MAPK must be present in affinity precipitates.

LMP2a expression and MAPK activation in vivo. To examine the relationship between LMP2a and MAPK in vivo, we produced a rabbit antiserum against the MBP-LMP2a exon 1

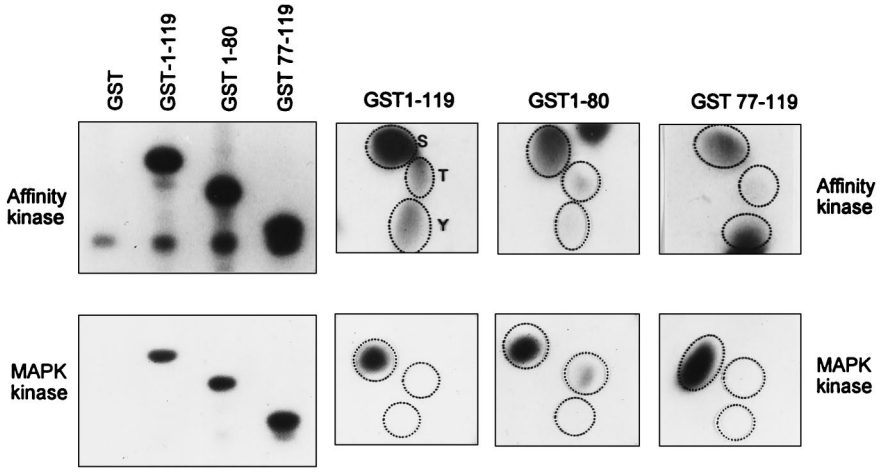

FIG. 4. At least two different serines are phosphorylated by MAPK in the LMP2a cytoplasmic domain. (Top) Affinity precipitations with GST fusion proteins from BJAB cell extracts induced for 5 min with anti-IgM F(ab′)$_2$ followed by in vitro kinase assays. Phosphoamino acid analysis was performed on the phosphorylated proteins. (Bottom) GST fusion proteins were phosphorylated in vitro by purified p44^{mpk} MAPK, and the labeled products were examined by phosphoamino acid analysis.

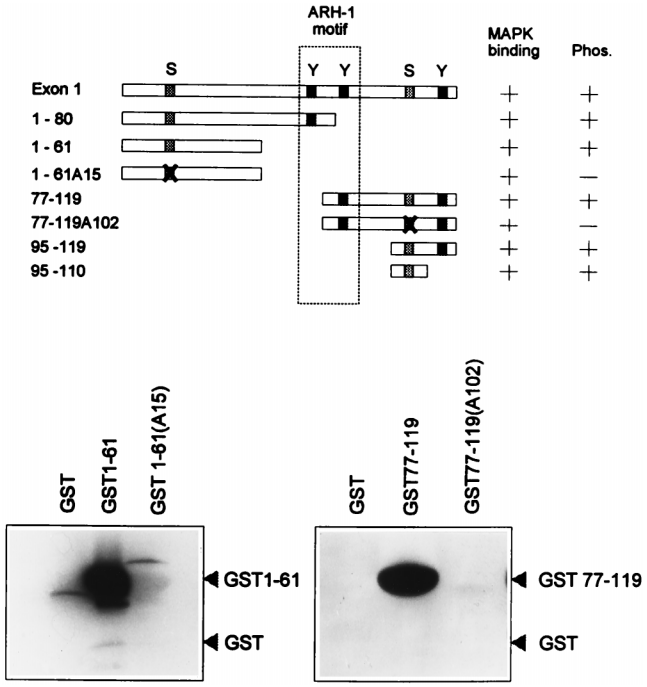

FIG. 5. Phosphorylation of S15 and S102 by MAPK. (Top) Summary of MAPK binding and phosphorylation (Phos.) for a series of different GST fusion constructs. Exon 1 refers to the full-length cytoplasmic domain (amino acids 1 to 119); the positions of S15, Y74, Y85, S102, and Y112 are indicated by grey and black boxes in the bar representing the linear sequence. The X indicates that the S residues in the fusion protein were converted to alanines. (Bottom) In vitro kinase assays with S/A point mutants that abolish phosphorylation by purified $p44^{mpk}$.

fusion protein MBP1-119. Serum from immunized rabbits was depleted against MBP and affinity purified on an MBP1-119 Sepharose column prior to use in immunoprecipitation reactions. Reactivity of the antiserum was confirmed by using GST1-119 and a BJAB cell line stably expressing a transfected LMP2a (not shown). Reactivity of the antiserum with LMP2a was tested by Western immunoblotting with a panel of LCLs. The antiserum detected a band of approximately 54 kDa from most of the LCLs tested (Fig. 6A). There was a large variation in the amount of LMP2a in the cell extracts from equal numbers of cells that did not seem correlate with the origin or handling of the cell lines in culture. BJAB (an EBV-negative control) and B1.2 (an LCL immortalized with an LMP2 deletion mutant virus) were negative for LMP2a expression (26). To confirm the status of phosphotyrosine phosphorylation in these cell lines, an immunoblot was probed with antiphosphotyrosine; however, no p54 band was detected. Several of the cell lines which showed relatively high levels of expression were examined by first immunoprecipitating the LMP2a protein from cell lysates followed by immunoblotting with the antiphosphotyrosine antiserum. Phosphorylated LMP2a was detected in every immunoprecipitate, although the level varied among the cell lines (Fig. 6B). Cell line B95-8CR, which had both a high level of expression of LMP2a and a high level of tyrosine phosphorylation, was selected for further studies.

LMP2a is a viral protein that interferes with signal transduction through the cell surface Ig receptor in B cells (41). Cross-linking of these receptors in LMP2a-positive cell lines reportedly does not lead to MAPK activation (41). To confirm these effects of LMP2a on MAPK activation through Ig receptor signaling, we analyzed the phosphorylation status of MAPK in cell lines BJAB, B95-8CR, and B1.2, which carries a B95-8 deletion mutant of the LMP2 gene and does not express a membrane-bound LMP2a protein product. Cross-linking surface Ig on B95-8CR cells did not lead to MAPK activation. No increase in P-MAPK was detected in extracts of B95-8CR cells following induction (Fig. 6C). On the other hand, both BJAB and the adult cell line B1.2 were inducible by surface Ig cross-linking. To examine serine phosphorylation of LMP2a before and after activation of the MAPK pathway, another method of inducing MAPK activation was required. When BJAB, B95-8CR, and B1.2 cells were treated with PMA, all three cell lines showed an increased level of the phosphorylated forms of both Erk1 and Erk2 5 min after induction. PMA, through direct activation of the protein kinase C pathway, circumvents the LMP2a blockade of the Ig receptor signaling and activates MAPK (Fig. 6C).

LMP2a is associated with MAPK in vivo. To examine whether LMP2a and MAPK interacted in vivo, an immunoprecipitate of LMP2a from uninduced B95-8CR cells was probed for the presence of MAPK (Fig. 7). Western immunoblotting for MAPK showed that the protein was detected as a coprecipitated protein in an immunoprecipitate of LMP2a from B95-8CR cells. Immunoprecipitation with anti-LMP2 antibodies from extracts of B95-8CR cells labeled for 4 h with

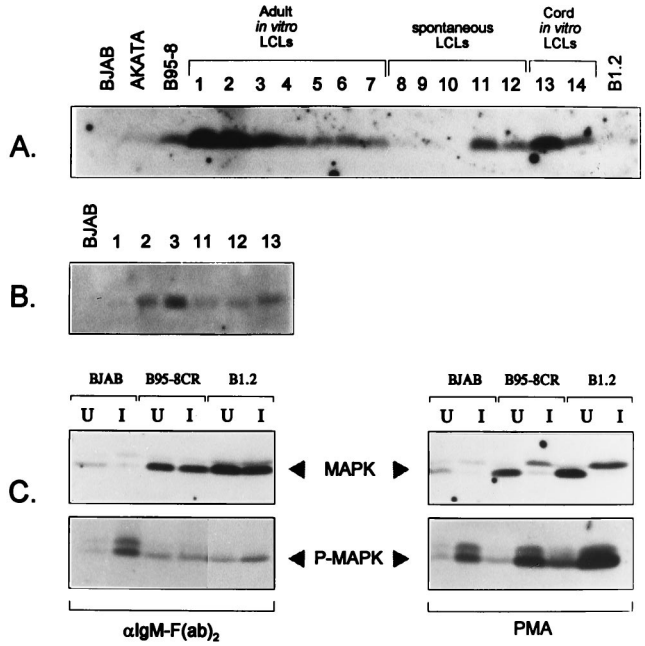

FIG. 6. (A) Western blot analysis of LMP2a expression in different cell lines. Cell extracts from 5×10^5 cells were run on a 9% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with an anti-LMP2a monospecific serum. Lanes: BJAB, an EBV-negative cell line; Akata, an EBV-positive Burkitt lymphoma cell line that has been previously reported to be negative for LMP2a expression; B95-8, a prototype marmoset cell line; 1 to 7, seven adult in vitro-established LCLs (B95-8SK, B95-8CR, AG876CR, MakauCR, MukiraCR, MwikaCR, and BL72CR, respectively); 8 to 12, five established spontaneous LCLs from the blood of latently infected donors (DT, DR, TO, SOG1, and SOG2, respectively); 13 and 14, two in vitro-established cord blood lymphocyte LCLs (X50-7 and IB-4, respectively); B1.2, which carries a B95-8 mutant virus that is not capable of expressing a membrane-bound LMP2a protein. (B) Western immunoblot of LMP2a immunoprecipitates from 5×10^7 cells probed with antiphosphotyrosine antibody PY20. (C) Western immunoblot of cell extracts from 5×10^5 cells that were either uninduced (U) or induced (I) with 20 μg of anti-IgM $F(ab')_2$ or 100 nM PMA for 5 min, probed with anti-MAPK (top) or anti-P-MAPK (bottom) antibody, and developed by chemiluminescence (CDP-Star; New England Biolabs).

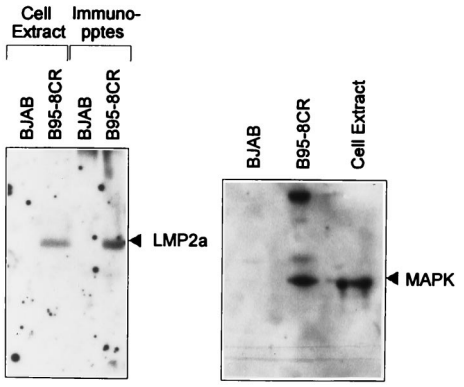

FIG. 7. Association of LMP2a with MAPK in vivo. LMP2a was immunoprecipitated from BJAB or B95-8CR cell lysates with an anti-IMP2a monospecific serum. Immunoprecipitates (Immuno-pptes) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with anti-IMP2a serum (left) or with anti-MAPK R2 (right).

$^{32}PO_4$ yielded a phosphoprotein in which label was mainly incorporated into serines. Cross-linking surface Ig did not change either the amount of label in the immunoprecipitate or the pattern of phosphoamino acids. This result is consistent with the Ig receptor signaling pathway being blocked in these cells (Fig. 8). The induction of the cell signaling produced by PMA treatment changed the level of phosphorylated LMP2a in immunoprecipitates from $^{32}PO_4$-labeled B95-8CR cells by approximately twofold. Phosphoamino acid analysis of the induced phosphorylation revealed that the protein was phosphorylated mainly on serines.

DISCUSSION

We have used primarily in vitro techniques to analyze LMP2a protein interactions and substrate specificities principally because of the overall low levels of protein expression in in vivo-immortalized cells, the inherent insolubility of LMP2a, and the difficulty associated with producing high-quality antisera capable of affinity purifying the protein from cells for metabolic labeling studies. The initial in vitro studies involved identification of cellular kinases in extracts of BJAB cells that would associate with a GST fusion protein expressing the cytoplasmic domain of LMP2a and specifically phosphorylate the fusion protein on the LMP2 sequences. Affinity-purified kinases phosphorylated LMP2a primarily on serine residues. Since serine phosphorylation of LMP2a also occurs in vivo (32), further investigation of the serine-specific kinases involved might reveal important clues to the mechanisms regulating LMP2a function. Affinity-precipitated kinase activity increased after induction of the BJAB cells by surface Ig cross-linking. This increase was seen only with the GST1-119 fusion protein, not with the GST control, and is therefore not merely due to a generalized increase in kinase activity following induction. This is demonstrated by the addition of the exogenous kinase substrate MBP, which also showed an increased phosphorylation by affinity-precipitated kinases after induction.

The presence of MAPK in affinity precipitates with GST1-119 was confirmed by immunoblotting and probing for the presence of MAPK. Another piece of evidence suggesting a specific association was the observation that only the Erk1 form of MAPK was found to be coprecipitated with the fusion protein. Proliferating BJAB cells contain some phosphorylated, activated MAPK, accounting for the kinase activity in affinity-purified precipitates from uninduced cells. The increase following induction by Ig cross-linking correlated with an increase in GST1-119 phosphorylation in the in vitro kinase assay using kinases affinity purified from induced cells.

We mapped the sites of in vitro MAPK phosphorylation on GST1-119 to S15 and S102, which are in the two putative MAPK recognition motifs. In assays using fusion proteins with these residues converted to alanines, MAPK phosphorylation

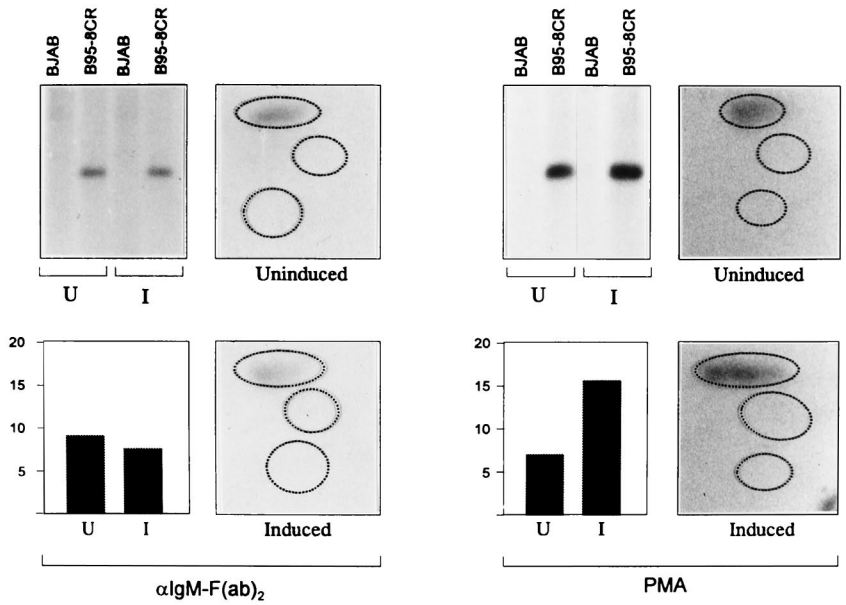

FIG. 8. LMP2a phosphorylation after induction with anti-IgM F(ab′)$_2$ or PMA. LMP2a was immunoprecipitated from 5×10^7 $^{32}PO_4$-labeled B95-8CR or control BJAB cells. Immunoprecipitates were resuspended in 0.1 volume of 1% SDS. Supernatant was brought to 0.1% SDS with lysis buffer, and LMP2a was reimmunoprecipitated. Reimmunoprecipitates were separated in an SDS–9% gel and transferred to an Immobilon membrane, and the phosphorylation of proteins was measured with a Molecular Dynamics PhosphorImager. Phosphorylated LMP2a bands excised from the membrane were examined by thin-layer phosphoamino acid analysis. Cells were induced with either 20 μg of anti-IgM F(ab′)$_2$ (left) or 100 nM PMA (right). U, uninduced; I, induced.

was abolished. Affinity precipitates with these fusions still contained some serine kinase activity capable of phosphorylating the fusion proteins. This result implies that other serine kinases are present in the affinity precipitate, and experiments are being conducted to identify the sites of additional serine phosphorylation as well as the enzymes involved. These in vitro analyses represent prerequisite to identifying functionally important serine phosphorylation sites on LMP2a and will serve as an invaluable guide for the in vivo mutational analyses to follow.

EBV efficiently induces the proliferation of peripheral blood B cells (20, 50, 55, 57). The most frequently isolated type of LCL expresses IgM, although IgG- and occasionally IgA-expressing cells may be produced by in vitro immortalization assays (10, 20). Only cells that express particular surface characteristics (i.e., have CD23 or become CD23 positive after infection) emerge as immortalized B cells spontaneously from peripheral blood or after in vitro infection with virus (2, 3, 12, 30, 50, 61, 66). Immortalized B cells in culture display a range of characteristics with respect to both virus (genome copy number and latent, early, and lytic gene expression) and cell features (surface antigen expression and Ig receptor isotype). EBV-positive Burkitt lymphoma cells are the best examples of how phenotypically diverse cell lines can arise from a common progenitor upon long-term culture (18, 50). When examining the status of LMP2a in the in vitro-immortalized or spontaneously immortalized B cells available to us, we observed that there was considerable variation in the level of LMP2a expression. Phosphorylation on tyrosine was detected by antiphosphotyrosine immunoblotting, but levels of tyrosine phosphorylation varied independently from levels of LMP2a expression. The B95-8CR line was selected for study because of its relatively high level of LMP2a expression and tyrosine phosphorylation and because signaling through Ig receptors to MAPK was blocked. The variability in LMP2a status in EBV-immortalized cell lines suggests that not all cell lines will have the same phenotype with respect to signal transduction as B95-8CR or other cell lines that have been used to study LMP2a function (32, 38, 40, 41).

An important aspect of demonstrating that a protein is a substrate for a particular kinase is showing that the phosphorylation occurs in vivo under physiological conditions. For several reasons, this has proved extremely difficult to achieve with LMP2a. Low levels of expression and low solubility have contributed to yields of metabolically $^{32}PO_4$-labeled products in immunoprecipitates that are present in levels too low for direct peptide mapping. Indeed, there are no published peptide maps even of the tyrosine phosphorylation of LMP2a, presumably for the same reasons (7, 32). Our phosphoamino acid analyses from $^{32}PO_4$-labeled cells failed to show significant incorporation of $^{32}PO_4$ into tyrosines. This may be due to lower levels of tyrosine phosphorylation than of serine phosphorylation or to lower rates of exchange and/or de novo incorporation that lead to metabolic labeling that occurs below the level of sensitivity of our assays. Evidence that MAPK phosphorylates LMP2a in vivo currently remains circumstantial. However, this conclusion is supported by three observations. First, LMP2a was phosphorylated on serines in LCLs, and the level of serine phosphorylation increased only upon MAPK activation. Second, immunoprecipitates of LMP2a coprecipitated MAPK and the isolated cytoplasmic domain affinity-precipitated MAPK from cell lysates. Third, one of the serines (S102) phosphorylated by MAPK in vitro lies within a motif that is evolutionarily highly conserved between human and simian LMP2 genes (Fig. 9) (16). In addition, this residue and its surrounding sequences are conserved among 28 isolates whose DNA sequences have been determined. Two of these isolates had mutations converting S15 to asparagine (8).

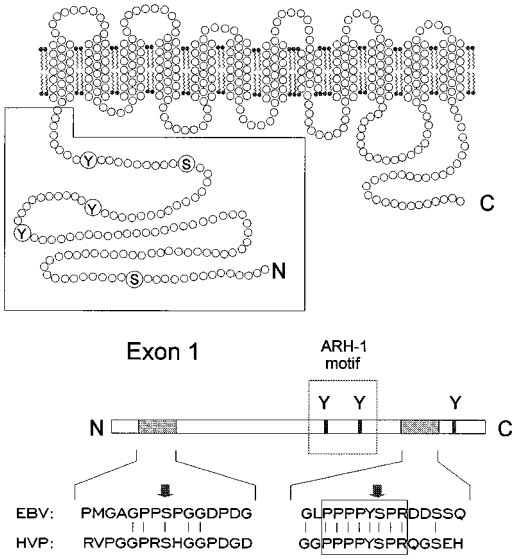

FIG. 9. Diagram showing the proposed orientation of LMP2a N- and C-terminal domains and membrane-spanning segments. Phosphorylated amino acids in the N-terminal cytoplasmic domain are indicated. Below is the linear map of the N-terminal 119 residues showing the location of the ARH-1 motif and the alignment of the sequences of EBV and herpesvirus papio (HVP) in the vicinity of S15 and S102.

EBV achieves immortalization through alterations of cellular signaling pathways. The evidence accumulated to date suggests that LMP2, although expressed in immortalized cells, is not essential to immortalization (33, 34, 39). Nevertheless, it does have cell signaling-related effects and blocks Ig receptor signaling, a phenotype that may be useful to establishing in vivo latency in B cells (41). Ig receptor signaling is integrated with signals transmitted by the multiple receptor systems of lymphocytes to achieve effects including modulation of one receptor by another, which often leads to selective desensitization (6, 9, 31, 42, 45, 54). Interposition of LMP2 into the cross talk between receptor cascades provides EBV with a powerful means of influencing outcomes and presents a model system that can provide fundamental insights into how receptor signaling cascades are regulated. In the case of EBV, intracellular signaling needs to be controlled to first establish and then maintain latency until an appropriate signal reactivates virus production. Phosphorylation is a key mechanism for regulating signal protein interactions and activities, and the implication is that LMP2a phosphorylation (on serines as well as tyrosines) has functional significance. MAPK has been shown to directly and indirectly participate in growth factor receptor regulation by phosphorylating receptors and their associated molecules (13, 31, 42, 45, 63). If MAPK activity alters LMP2 function, this effect most likely involves the S102 site. S102 lies between the ARH-1 motif and the Y112 Lyn tyrosine kinase phosphorylation site in a proline-rich conserved region (Fig. 9). Phosphorylation of S102 can be envisaged as altering LMP2 docking interactions with signal transduction kinases and/or adapter molecules. A possible role in a switching mechanism for the latent/lytic status of latently infected B cells should be considered, but further work is necessary to determine the true significance of serine phosphorylation in this region.

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