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An Epstein-Barr Virus Protein Associated with Cell Growth Transformation Interacts with a Tyrosine Kinase

RICHARD LONGNECKER,[1] BRIAN DRUKER,[2] THOMAS M. ROBERTS,[2] AND ELLIOTT KIEFF[1]*

Department of Microbiology and Molecular Genetics and Medicine, Harvard Medical School and Brigham and Women's Hospital,[1] and Dana Farber Cancer Institute, Harvard Medical School,[2] Boston, Massachusetts 02115

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Epstein-Barr virus (EBV) encodes two integral membrane proteins in latently infected growth-transformed cells. One of these, LMP1, can transform rodent fibroblasts and induce markers of B-lymphocyte activation. The second, LMP2, colocalizes with LMP1 in a constitutive patch in the EBV-transformed B-lymphocyte plasma membrane. The experiments reported here demonstrate that LMP2 may biochemically interact with LMP1 and that LMP2 closely associates with and is an important substrate for a B-lymphocyte tyrosine kinase in EBV-transformed B lymphocytes or in B-lymphoma cells in which LMP2 is expressed by gene transfer. LMP2 is also serine and threonine phosphorylated. LMP2 localizes to a peripheral membrane (presumably plasma membrane) patch in transfected B-lymphoma cells and colocalizes with much of the cellular tyrosine-phosphorylated proteins. LMP2 undergoes tyrosine phosphorylation in anti-LMP2 or antiphosphotyrosine immunoprecipitates from transfected B-lymphoma cells or EBV-transformed B lymphocytes. The first 167 of the 497 amino acids of LMP2 retain full ability to associate with and act as a substrate for a tyrosine kinase. A 70-kDa phosphotyrosine cell protein associates with LMP2 in transfected cells or in EBV-transformed B lymphocytes and could be a mediator of the effects of LMP2.

Epstein-Barr virus (EBV) causes infectious mononucleosis in normal adolescents and malignant B-lymphocyte proliferation in immunocompromised patients, in marmosets, or upon transfer of infected human B lymphocytes into mice with severe combined immunodeficiency (for a review, see references 27 and 39). EBV is also etiologically associated with African Burkitt's lymphoma and nasopharyngeal cancer.

EBV-infected, growth-transformed B lymphocytes contain EBV episomes and eight virus-encoded proteins. Six are nuclear proteins, EBNA-1, EBNA-2, EBNA-3a, EBNA-3b, EBNA-3c, and EBNA-LP (for a review, see reference 27). Two are integral membrane proteins, LMP1 (20, 33) and LMP2 (17, 35, 48), with 6 and 12 hydrophobic transmembrane domains, respectively. These eight proteins are presumed to mediate latent virus infection or B-lymphocyte proliferation.

LMP1 has important effects on cell growth and probably interacts with LMP2. Following single-gene transfer into immortalized rodent fibroblasts, LMP1 causes loss of anchorage dependence or tumorigenicity (2, 57, 59). In human B-lymphoma cells, LMP1 induces activation and adhesion molecule expression (59). LMP1 partially localizes to a patch in the lymphocyte plasma membrane (32–34), where it presumably provides a constitutive activating signal with a membrane-associated cell growth regulation protein. LMP2 may complement the LMP1-mediated activation since LMP2 partially colocalizes to the LMP1 plasma membrane patch (35).

There are two LMP2s encoded by two mRNAs which have different 5′ exons followed by eight common exons (31, 50). The LMP2A predicted primary amino acid sequence (Fig. 1) includes 119 amino acids at the amino terminus, 12 hydrophobic domains of at least 16 amino acids, each of which is likely to traverse a membrane, and a 27-amino-acid carboxy-terminal domain. LMP2B initiates at a methionine 120 amino acids into LMP2A and lacks the entire amino-terminal cytoplasmic domain (Fig. 1, arrow). The membrane localization of LMP2 was confirmed by insertion into membranes after in vitro translation (50), by immunoblots of membrane fractions from EBV-transformed lymphocytes (35), and by immunofluorescence microscopic analyses (35) with LMP2-specific antibody. The model (Fig. 1) has the amino and carboxy termini in the cytoplasm. Most positively charged amino acids are in the cytoplasm, characteristic of membrane proteins.

Nothing is known about the precise biochemical functions of LMP2. Some similar integral membrane proteins interact with or affect the activity of plasma membrane-associated enzymes which may have important regulatory functions, such as G proteins or tyrosine kinases. Tyrosine phosphorylation could be particularly important in B lymphocyte activation since cross-linking of surface immunoglobulin (Ig), the normal signal for B-lymphocyte activation, induces tyrosine phosphorylation (3, 5, 6, 18). Because of the possible key regulatory role of tyrosine kinases in cell growth regulation, we investigated whether LMP2 might undergo tyrosine phosphorylation or interact with a membrane-associated tyrosine kinase.

MATERIALS AND METHODS

Cells. LCL1 and LCL2 are EBV-transformed fetal (LCL1) or adult (LCL2) lymphoblastoid cell lines (LCL) which are not permissive for virus replication. BJAB is an EBV-negative B-lymphoma cell line. All lymphoid cell lines were grown in RPMI 1640 with 10% fetal bovine serum and 4 μg of gentamicin per ml. For transfections, a total of 10^7 cells in log phase growth were suspended in 0.4 ml of RPMI 1640 medium with 10% fetal bovine serum at room temperature and placed in a Gene Pulser Cuvette (Bio-Rad Laboratories,

* Corresponding author.

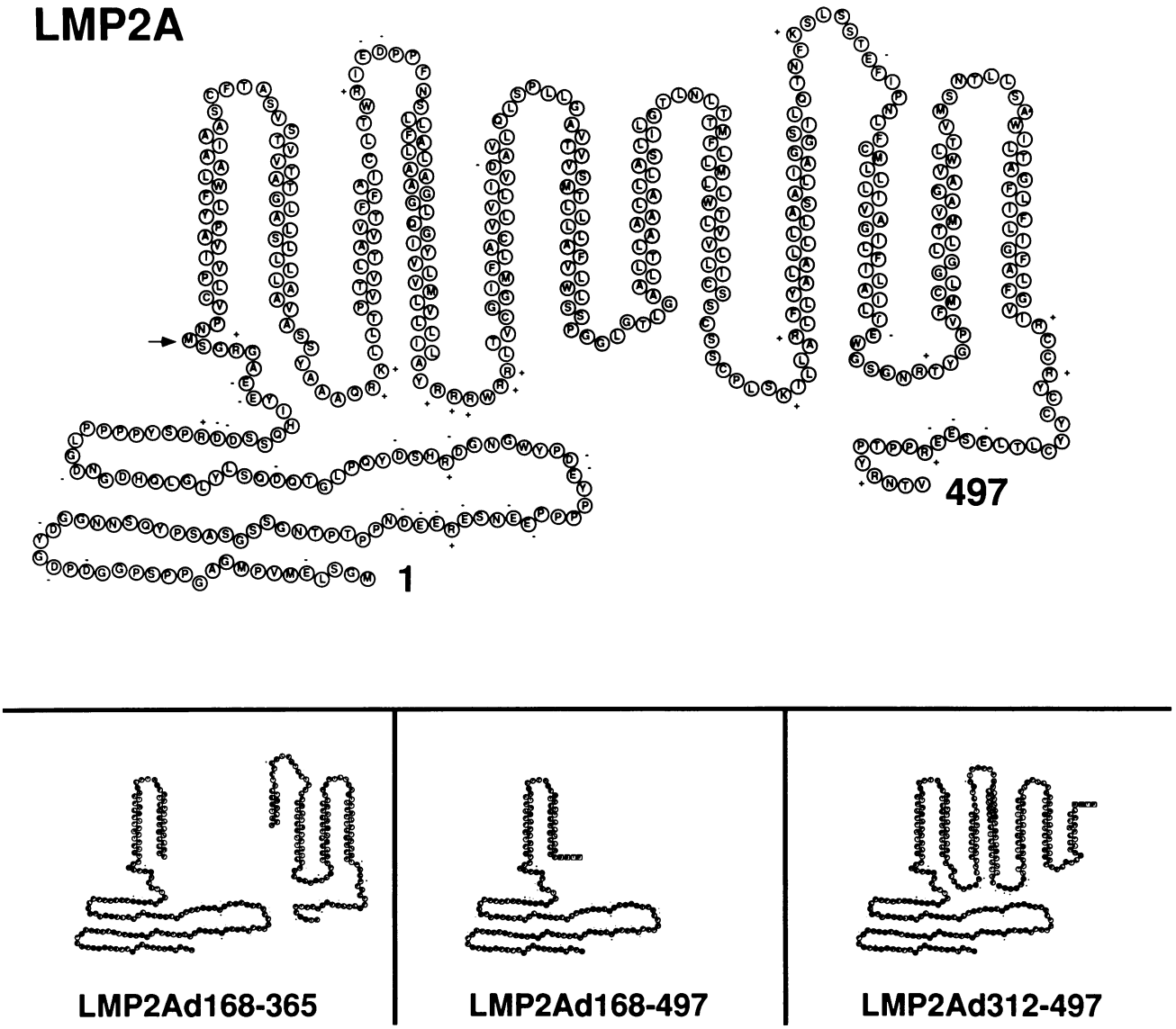

FIG. 1. Schematic of the predicted structure of LMP2A in the cell plasma membrane. An arrow indicates the translational initiation site for LMP2B. Charged amino acids are indicated with a plus or minus sign. In the structure shown, all but two positive charges are on the cytoplasmic side of the membrane (lower). The three lower panels delineate the LMP2A deletion mutants used in these studies.

Richmond, Calif.). A total of 60 μg of DNA was used in each transfection. Cells were electroporated at 200 V and 960 μF and suspended in media as described in the text.

Radiolabeling of cells. For $^{32}P_i$ labeling, cells were washed three times with phosphate-free medium and incubated in phosphate-free medium with 4 mCi of $^{32}P_i$ per ml for 4 h. For [^{35}S]methionine labeling, cells were incubated for 10 h in 95% methionine-free medium with 0.5 mCi of [^{35}S]methionine per ml.

Immunoprecipitations. Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed at 4°C for 10 min in 1% Nonidet P-40 (NP-40) lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 10 mM sodium fluoride, and 1 mM sodium orthovanadate. Insoluble material was removed by centrifugation at 4°C for 15 min at 10,000 × *g*. Cell lysates were precleared for 1 h with normal rabbit serum adsorbed to protein A-Sepharose and for 1 h with protein A-Sepharose. Precleared cell lysates were then mixed with affinity-purified rabbit serum or agarose-linked anti-phosphotyrosine (anti-P-Tyr) monoclonal antibody PY20 (ICN, Costa Mesa, Calif.) for 4 to 8 h at 4°C. Protein A-Sepharose was used to collect the antigen-antibody complexes when rabbit serum was used. The immunoprecipitates were washed four times with 1% NP-40 lysis buffer, twice with 0.5 M LiCl–0.1 M Tris (pH 7.4), and twice with 1% NP-40 lysis buffer before the immunoprecipitated proteins were removed from the immune complex by boiling in sodium dodecyl sulfate (SDS) sample buffer. For LMP2A phosphoamino acid analysis from LCL1, the cleared NP-40 cell lysate was adjusted to 1% SDS and the samples were boiled and then adjusted to 0.1% SDS prior to immunoprecipitation.

In vitro kinase assays. Protein kinase assays were per-

formed by washing immunoprecipitates twice with protein kinase buffer (50 mM Tris [pH 7.4], and 10 mM $MgCl_2$) and then adding 40 μl of kinase buffer with 10 μCi of [γ-^{32}P]ATP. After incubation for 20 min at 25°C, the assays were terminated by the addition of SDS sample buffer prior to analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

Construction of pLMP2A and LMP2A deletion mutants. pLMP2A and pLMP1 contain the *Eco*RI LMP2A and LMP1 cDNA fragment (20, 50), respectively, cloned into the *Eco*RI site of pSG5. pSG5 (Stratagene, La Jolla, Calif.) is a eukaryotic expression vector containing the simian virus 40 early promoter, intron II of the rabbit β-globin gene, and a polyadenylation signal. pLMP2Ad168–497 was constructed by removing a *Msc*I-*Sma*I fragment from pLMP2A. It is deleted from amino acid 168 to the end of LMP2A. Four additional amino acids (Gly, Gly, Ile, and His) are added to the end of this deletion mutant (Fig. 1) as a result of cloning. pLMP2Ad168–365 was constructed by removing a *Msc*I-*Nhe*I fragment from pLMP2A. It is deleted from amino acids 168 to 365 (Fig. 1). pLMP2Ad312–497 was constructed by removing a *Nhe*I-*Sma*I fragment from pLMP2A. It is deleted from amino acid 312 to the end of the LMP2A protein. Four additional amino acids (Gly, Gly, Ile, and His) are added to its end (Fig. 1) as a result of cloning.

Antibodies. Purified TrpE-LMP2A fusion protein or TrpE was purified from bacterial cells that were grown and induced (52). The insoluble proteins were recovered and electrophoresed on a 7% (TrpE-LMP2A fusion) or 10% (TrpE) preparative polyacrylamide gel. The appropriate bands were excised and electroeluted. The purified proteins (approximately 2 mg) were then coupled to 1-ml Actigel A beads (Sterogene, San Gabriel, Calif.) as described by the manufacturer. Coupled beads were then transferred to a small column and washed successively with 1 M NaCl and TBS buffer (20 mM Tris [pH 7.5], 150 mM NaCl), 3 ml of elution medium (Sterogene), and 50 ml of TBS buffer. Immune sera from the immunized rabbits were first applied to the TrpE column and subsequently to the LMP2 fusion protein column. The columns were extensively washed with TBS buffer containing 0.1% bovine serum albumin. Antibodies bound to the affinity matrix were eluted by washing the column with elution medium. The eluate was dialyzed against TBS buffer (pH 7.5)–0.1% sodium azide, divided, and frozen. The TrpE affinity-purified antiserum was subsequently used for control precipitations.

S12 is a monoclonal antibody directed against LMP1 (36). PY20, an anti-P-Tyr monoclonal antibody, and PY20 coupled to agarose were purchased from ICN.

Immunoblots. PY20 immunoblots were performed as recommended by the manufacturer (ICN). LMP1 and LMP2 immunoblots were done as previously described (33, 35).

Phosphoamino acid analysis. Phosphorylated proteins were eluted from SDS-PAGE gels (51) or transferred to Immobilon (25) and digested with 6 N HCl. For one-dimensional separations, phosphoamino acids were separated by electrophoresis on cellulose plates at pH 3.5 for 60 min at 1,000 V in glacial acetic acid-pyridine-H_2O (50:5:945, vol/vol/vol). For two-dimensional separations, phosphoamino acids were analyzed by electrophoresis on cellulose plates at pH 1.9 for 30 or 45 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 30 or 70 min at 1,000 V in glacial acetic acid-pyridine-H_2O (50:5:945, vol/vol/vol) (23). Phosphoamino acid standards (Sigma, St. Louis, Mo.) were stained with 0.25% ninhydrin in *n*-butanol.

RESULTS

Rabbit antibody specifically immunoprecipitates LMP2A. Rabbit antibody to an *Escherichia coli* TrpE-LMP2 fusion protein (35) was tested for LMP2-specific reactivity in immunoprecipitation. TrpE-specific antibody was first purified from the TrpE-LMP2 immune rabbit serum by TrpE affinity column chromatography for use as a control. LMP2-specific antibody was then purified by TrpE-LMP2 fusion protein column chromatography. Antibody specificity was evaluated by using proteins from B-lymphoma cells transfected with LMP1 (pLMP1), LMP2A (pLMP2A), or control (pSG5) expression vectors. Following transfection, cells were incubated with [^{35}S]methionine for 10 h and lysed in nonionic detergent. Immunoprecipitations with TrpE or LMP2 antibody demonstrated specific reactivity with LMP2A in cells transfected with pLMP2A but not in cells transfected with pSG5 or pLMP1 (Fig. 2). [^{35}S]methionine-labeled LMP2A was approximately 54 kDa in 6 to 18% (Fig. 2, lane 8) or 6% (lane 9) gels, as was previously noted for immunoblots of latently infected LCLs (35). A 47-kDa protein was nonspecifically precipitated with LMP2 or control rabbit serum. Other background proteins were occasionally evident in the immunoprecipitations. For example, Fig. 2, lane 5, contains other background proteins, whereas a repeat of the same precipitation in lane 12 has only the 47-kDa protein. Also, in the ^{35}S-labeled LMP2 antibody immunoprecipitate from pLMP2-transfected cells in lane 9, but not in lane 8, a discrete and a diffuse band of labeled protein is evident above the 200-kDa size marker. LMP2-specific antibody variably reacted with proteins in this size range in immunoblots of LMP2 precipitates of pLMP2-transfected cells (data not shown). Variable aggregation of LMP2 despite reduction and SDS denaturation has been previously noted (35).

LMP1 coprecipitated with LMP2 antibody from extracts of cells which were transfected with pLMP2A and pLMP1 (Fig. 2, lanes 6 and 11). LMP2 antibody did not precipitate LMP1 from cells transfected with pLMP1 only (lanes 7 and 10). The coprecipitation of LMP1 with LMP2A was confirmed by the presence, in immunoblots, of LMP1 in the LMP2 antibody immunoprecipitation from LMP1- and LMP2A-transfected cells (lane 15) and the absence of LMP1 from control immunoprecipitations (lanes 13, 14, and 16). Thus, these experiments indicate the specificity of the LMP2 antibody and also indicate that LMP1 coimmunoprecipitates with LMP2A. However, nonspecific aggregation of LMP1 based on hydrophobicity is not excluded by these experiments. Their coimmunoprecipitation and the previous colocalization of LMP1 and LMP2 in EBV-transformed LCLs are consistent with the hypothesis that LMP2 interacts with LMP1 in cell growth transformation.

LMP2A is tyrosine phosphorylated in EBV-transformed LCLs. LCL1, an early-passage EBV-transformed cord blood B-cell line, was labeled for 4 h in the presence of $^{32}P_i$. Following labeling, the cells were lysed and immunoprecipitations were done with an agarose-coupled mouse monoclonal antibody to P-Tyr, with rabbit antibody to LMP2, or with TrpE control rabbit antibody. Immunoprecipitations were done from unlabeled extracts in parallel and separated on the same SDS-PAGE gel as the labeled immunoprecipitations to allow alignment of the precipitated proteins. The resulting autoradiogram of the ^{32}P-labeled proteins is shown in Fig. 3. The LMP2 immunoprecipitate has two major labeled phosphoproteins at 54 and 135 kDa and a minor doublet at 108 kDa (Fig. 3, lane 3). The 54-kDa protein is identical in size to LMP2A. These proteins are not evident in the control rabbit

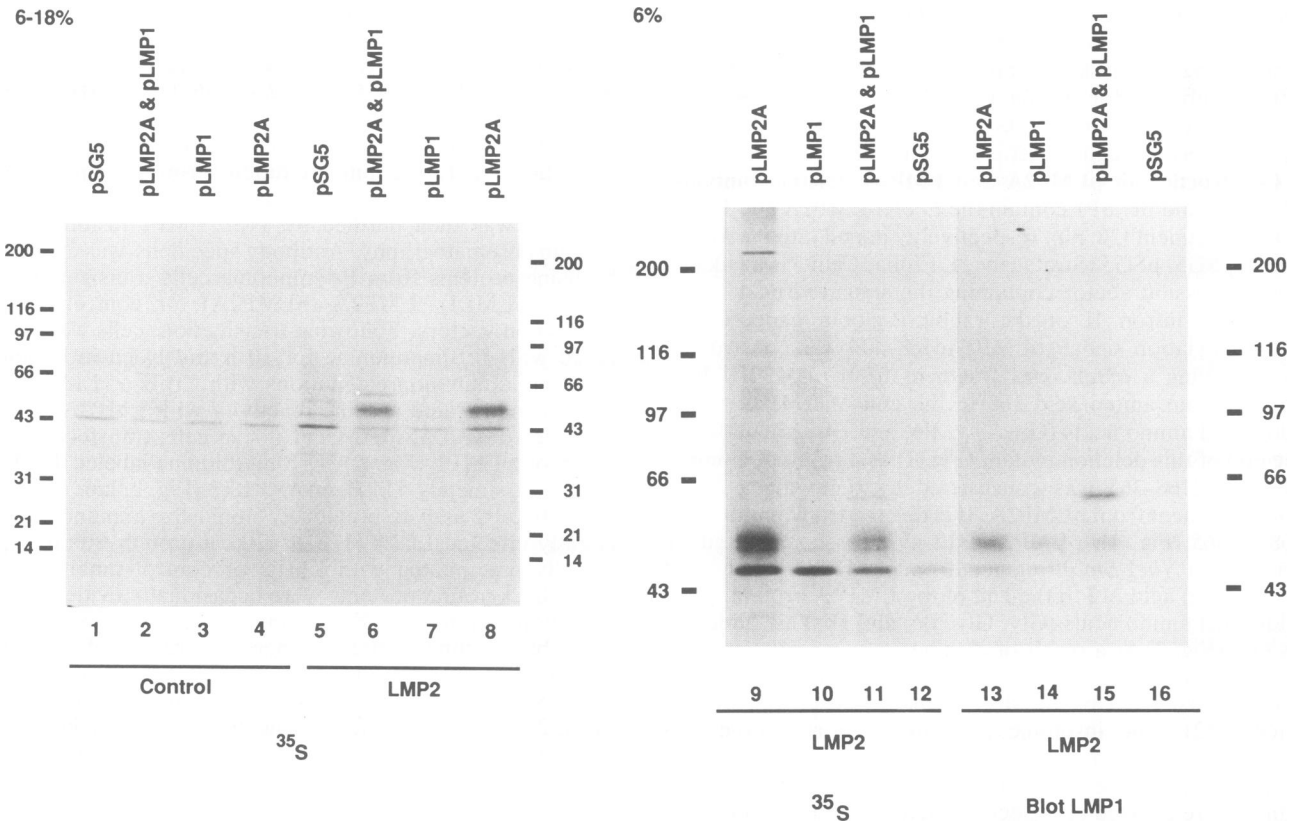

FIG. 2. Rabbit antibody to LMP2A immunoprecipitates LMP2A or LMP2A plus LMP1 from extracts of BJAB cells transfected with LMP2A or LMP2A plus LMP1 expression plasmids. BJAB cells were transfected with vector control (pSG5), pLMP1, pLMP2A, or both pLMP1 and pLMP2A. Proteins were labeled for 10 h with [^{35}S]methionine and solubilized in NP-40 lysis buffer, and immunoprecipitations were done with either control rabbit antiserum or affinity-purified antibody from rabbits immunized with LMP2 expressed as a fusion protein in *E. coli* (35). Control rabbit antiserum-immunoprecipitated (lanes 1 to 4) or LMP2 antibody-immunoprecipitated (lanes 5 to 8) labeled proteins were separated by SDS-PAGE (6 to 18% gradient gel) and transferred to nitrocellulose, and the resulting proteins were visualized by autoradiography. In another experiment, rabbit anti-LMP2 immunoprecipitates were separated by SDS-PAGE (6% polyacrylamide) (lanes 9 to 12). Following autoradiography, the nitrocellulose sheet was reacted in a immunoblot with S12, a monoclonal antibody which recognizes LMP1 (36). Reactivity with S12 was visualized with ^{125}I-labeled protein A (lanes 13 to 16). ^{35}S-labeled proteins were blocked by two sheets of film. The sizes of marker proteins are indicated in kilodaltons.

serum immunoprecipitations (lane 2), although a less abundant background 54-kDa phosphoprotein is evident in some control immunoprecipitations, including the one shown. Several other phosphoproteins are abundant in the control immunoprecipitation and in the LMP2 immunoprecipitation, e.g., the 60-kDa phosphoprotein in lanes 2 and 3. The P-Tyr-immunoprecipitated phosphoproteins included one the size of LMP2A and many others (lane 1). These data indicate that LMP2A undergoes phosphorylation. Further, either LMP2A is tyrosine phosphorylated or another P-Tyr protein is identical in size to LMP2A or LMP2A coimmunoprecipitates with a P-Tyr protein but is not itself tyrosine phosphorylated. To distinguish among these possibilities, unlabeled LMP2 or P-Tyr immunoprecipitates were probed in immunoblots. LMP2 antibody-precipitated proteins were probed with P-Tyr antibodies (lane 4), and P-Tyr antibody-precipitated proteins were probed with LMP2-specific antibodies (lane 5). The 54-kDa P-Tyr-immunoprecipitated protein reacted with LMP2 antibody (lane 5), and the principal P-Tyr-reactive protein in the LMP2 immunoprecipitate was 54 kDa, the size of LMP2A (lane 4). These data indicate that LMP2 undergoes tyrosine phosphorylation in EBV-transformed LCLs. The possibility that LMP2 specifically associates with a tyrosine phosphoprotein identical in size to LMP2A is highly unlikely but is not excluded. The 135-kDa protein and the 108-kDa doublet protein were detected by LMP2 antisera in the P-Tyr immunoprecipitate, suggesting that they are complexes of LMP2 with itself or other proteins. Similar LMP2 complexes have been variably detected in EBV-infected LCLs, in stably transfected BALB/3T3 cells, or in in vitro translations of LMP2A RNA (35, 50). The absence of the 135-kDa protein in the P-Tyr blot of the LMP2 immunoprecipitate could be due to poor transfer of that spot on the immunoblot or to variable solubilization of the 135-kDa complex in different samples loaded on the same gel.

LMP2A tyrosine phosphorylation is not dependent on other EBV genes. To exclude the unlikely possibility that the 54-kDa P-Tyr-reactive protein in the LMP2 immunoprecipitates (or in the reciprocal experiments) was a cell-encoded 54-kDa P-Tyr protein, LMP2A was expressed transiently in B-lymphoma cells. The P-Tyr proteins of pSG5- and pLMP2A-transfected cells could then be compared. The LMP2 antiserum, control antiserum, and P-Tyr antibody-immunoprecipitated proteins were resolved on 10%, 6%, or 6 to 18% SDS-PAGE gels and immunoblotted with P-Tyr

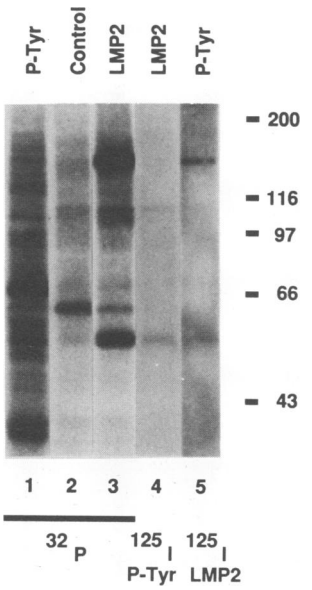

FIG. 3. Anti-P-Tyr or anti-LMP2 immunoprecipitation of $^{32}P_i$-labeled or unlabeled proteins from extracts of LCL1, an EBV growth-transformed and latently infected LCL. Cells were labeled in the presence of $^{32}P_i$ for 4 h. Anti P-Tyr (lane 1), control (lane 2), or anti-LMP2 (lane 3) immunoprecipitates were resolved by SDS-PAGE (6% polyacrylamide), transferred to nitrocellulose, and visualized by autoradiography. After exposure and development of the same autoradiogram, the appropriate lanes were repositioned for the print shown. Anti-LMP2 (lane 4) and anti-P-Tyr (lane 5) immunoprecipitates of unlabeled LCL1 cell extracts were on the same gel and were transferred to nitrocellulose. P-Tyr (lane 4) and LMP2 (lane 5) antibodies were used to identify P-Tyr proteins and LMP2 in immunoprecipitates with the reciprocal antibody.

antibody (Fig. 4). P-Tyr antibody did not react with proteins in immunoblots of the control rabbit serum immunoprecipitations (Fig. 4, lanes 1 and 2). P-Tyr antibody detected a 54-kDa protein, LMP2A, in the LMP2 immunoprecipitate of pLMP2A-transfected cells (lanes 4 and 6) but not in the LMP2 immunoprecipitate of pSG5-transfected cells (lanes 3 and 5). Most importantly, the 54-kDa P-Tyr protein, LMP2A, was evident only in the P-Tyr immunoblot of P-Tyr-immunoprecipitated pLMP2A-transfected cells (lanes 8 and 10) and not in the P-Tyr immunoblots of pSG5-transfected B-lymphoma cells (lanes 7 and 9). In the 6 to 18% gradient gel in lane 10, LMP2A is partially obscured by the 60- and 64-kDa P-Tyr proteins in P-Tyr immunoprecipitations of pSG5- or pLMP2A-transfected cells. In the 6% gel, LMP2A separates from the 60- and 64-kDa cell proteins (lane 8). Since these B-lymphoma cells do not have other EBV genes, the experiments also indicate that LMP2A tyrosine phosphorylation is not dependent on other EBV genes. Other proteins of 60 and 64 kDa were detected in the P-Tyr immunoprecipitates of both pSG5- and pLMP2-transfected cells (lanes 7 to 10) and, presumably, are abundant P-Tyr proteins of these B-lymphoma cells. A 70-kDa protein was consistently present on longer exposures of P-Tyr blots of P-Tyr immunoprecipitates from pLMP2-transfected cells but not from pSG5-transfected cells (faintly evident in lane 8, but clearly evident in lane 10). These data indicate that LMP2A induces tyrosine phosphorylation of a 70-kDa cell protein.

LMP2A and the 70-kDa proteins are labeled with $^{32}P_i$ in B-lymphoma cells transfected with a LMP2A expression vector. To verify that the 70-kDa protein and LMP2A are phosphoproteins, B-lymphoma cells were tranfected with pSG5 or pLMP2A and labeled with $^{32}P_i$ for 4 h following transfection. Duplicate cultures were not labeled. Immunoprecipitations were performed with LMP2 immune or control rabbit serum or with antibody to P-Tyr. Unlabeled proteins were immunoblotted with affinity-purified LMP2-specific antibodies. A 45-kDa background band was present in the LMP2 immunoblots of the rabbit antibody precipitations (Fig. 5, lanes 1, 2, 5, and 6). As expected, LMP2A was detected in the immunoblot of the LMP2 or P-Tyr immunoprecipitations from the BJAB cells transfected with pLMP2 (lanes 3 and 5). In parallel immunoprecipitations of ^{32}P-labeled cell proteins, P-Tyr antibody immunoprecipitated a ^{32}P-labeled 70-kDa protein and LMP2A (lane 7), whereas LMP2 antibody immunoprecipitated only ^{32}P-labeled LMP2A (lane 9). Three smaller proteins were apparent in the LMP2 antibody immunoprecipitation at 43, 37, and 34 kDa (lane 9). These are probably partially degraded LMP2, since they were variably noted in other LMP2 immunoprecipitations. Similar-sized proteins were detected in lower abundance in the LMP2 immunoblot of the LMP2 immunoprecipitation (lane 5). Labeling of the 70-kDa protein and of LMP2 was in excess of labeling of other P-Tyr proteins (compare lanes 7 and 9 with lanes 8 and 10) compared with EBV-infected LCLs (Fig. 3). These data indicate that LMP2A and a 70-kDa cell protein are major tyrosine-phosphorylated proteins in transfected B-lymphoma cells.

LMP2A associates with a protein kinase. The high LMP2A and pp70 tyrosine phosphorylation level in B-lymphoma cells transfected with the LMP2A expression vector (pLMP2A) was compatible with the hypothesis that LMP2 associates with an activated tyrosine kinase. To determine whether this putative association was stable in nonionic detergents, in vitro kinase reactions were performed with LMP2, control, or P-Tyr immunoprecipitates from pLMP2A- or pSG5-transfected BJAB cells (Fig. 6). In vitro kinase reactions with control rabbit serum immunoprecipitations resulted in little in vitro phosphorylation (Fig. 6, lanes 1 to 4). In sharp contrast, in in vitro kinase reactions with the LMP2 or P-Tyr immunoprecipitations, LMP2A was the dominant phosphoprotein (lanes 6 and 10, respectively). Several other proteins were phosphorylated in both reactions. Most notable are 108-, 70-, and 40-kDa phosphoproteins in both immunoprecipitates. The phosphoproteins which barely entered the gel (lanes 6 and 10) are probably incompletely solubilized LMP2 or LMP2 complexes. None of these phosphoproteins were evident in in vitro kinase reactions with LMP2 or P-Tyr immunoprecipitations of pSG5-transfected cells (lanes 5 and 9). Similar in vitro kinase reactions were done with immunoprecipitates from EBV growth-transformed LCLs (lanes 3, 4, 7, 8, 11, and 12). LMP2 expression in the transformed cell lines was barely detectable in immunoblots of whole-cell extracts (35) and was substantially lower than in the BJAB transfected cells (data not shown). Only LMP2A was evident in short exposures of the autoradiogram of the in vitro kinase reaction with LCL-derived LMP2 or P-Tyr immunoprecipitates (lanes 7 and 8 or 11 and 12). Longer exposures of this autoradiogram (lanes 14 and 15 or 17 and 18) revealed a 70-kDa phosphoprotein in the LMP2 and P-Tyr immunoprecipitates. A trace of the 108-kDa phosphoprotein was also evident with the LMP2 immunoprecipitate (lanes 14 and 15). Additional phosphorylated proteins were evident with the P-Tyr immunoprecipitation (lanes 17 and 18), the most notable being at 60 kDa. These results indicate that LMP2A

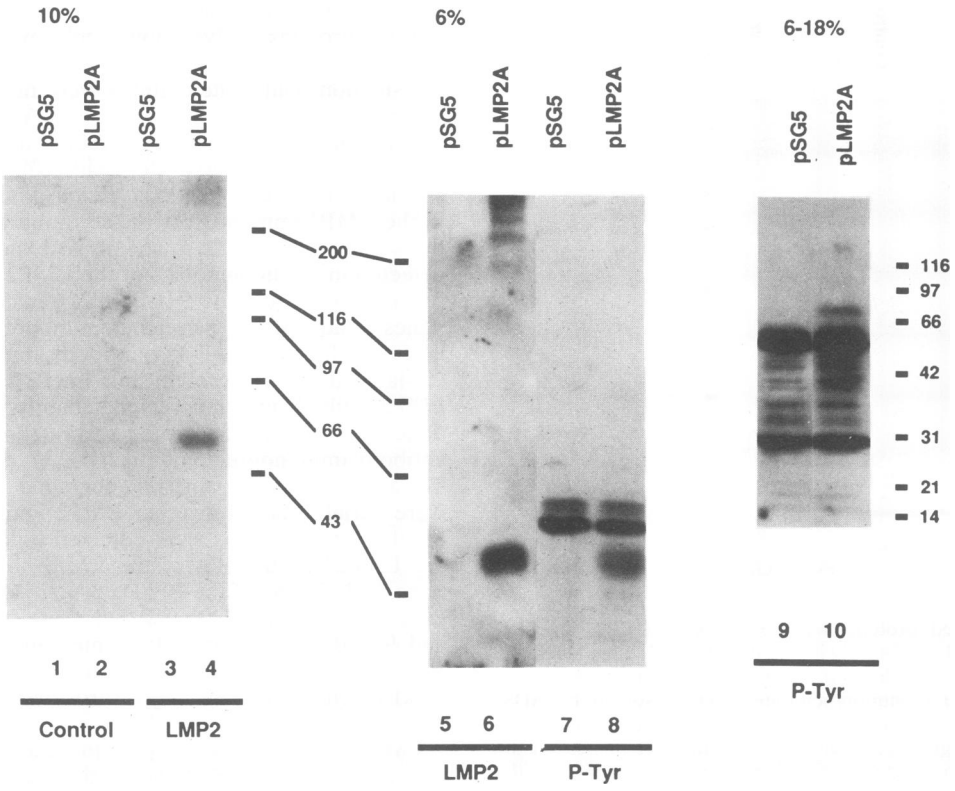

FIG. 4. Anti-P-Tyr immunoblots of immunoprecipitates from extracts of BJAB cells transfected with pLMP2A or pSG5. Immunoprecipitates were with control serum (lanes 1 and 2), LMP2-specific antibody (lanes 3 to 6), or P-Tyr-specific antibody (lanes 7 to 10). The immunoprecipitates from two different experiments were resolved by SDS-PAGE (10%, 6%, and 6 to 18% polyacrylamide).

is associated with a protein kinase, likely to be a tyrosine kinase, in both B-lymphoma cells and EBV-transformed LCLs. Further, the 70-kDa phosphoprotein might be a LMP2A-associated kinase substrate or a kinase.

LMP2A kinase association and substrate domains are within the amino terminus and first two transmembrane domains. To identify the LMP2A domains essential for kinase induction and LMP2A phosphorylation, three large deletions in the LMP2A open reading frame were made (Fig. 1) and recombined into pSG5. PLMP2A, the LMP2A deletion mutants, and pSG5 were transfected into B-lymphoma cells. Duplicate cultures were labeled with $^{32}P_i$ for 4 h posttransfection, immunoprecipitated with LMP2 antibody, and analyzed by SDS-PAGE (8% polyacrylamide) (Fig. 7, lanes 12 to 16). Unlabeled transfection products were immunoprecipitated with control, LMP2, or P-Tyr antibody and were assayed for in vitro kinase activity. The resulting labeled phosphoproteins were analyzed on SDS-PAGE (10% polyacrylamide) (lanes 1 to 11). LMP2A and each deletion mutant were phosphorylated in vivo (lanes 12 to 16). LMP2A and each deletion mutant were phosphorylated in vitro after LMP2 or P-Tyr immunoprecipitation (lanes 4 to 7 and 9 to 11, respectively). The smallest LMP2A protein, consisting of only the amino terminus and the first two transmembrane domains (167 amino acids overall), was at least as efficiently phosphorylated as LMP2A both in vitro and in vivo (lanes 6 and 7 and lanes 13 and 16). The sizes of the phosphorylated LMP2A deletion mutants were close to those expected. The 70-kDa phosphoprotein was phosphorylated to a small extent in in vitro kinase reactions with LMP2 antibody immu-

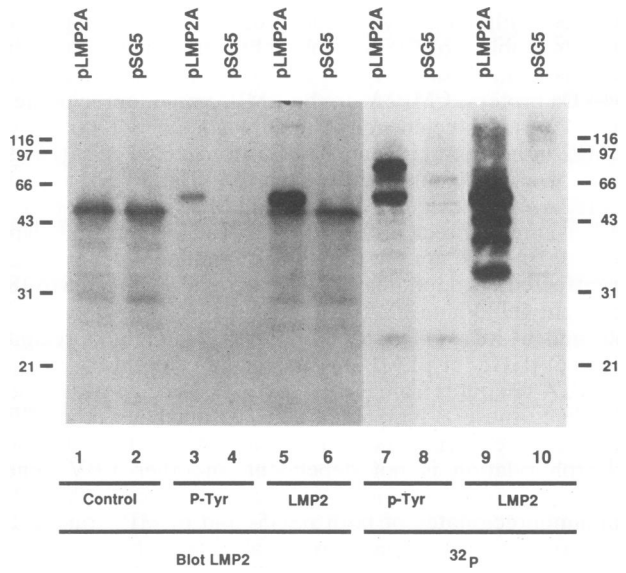

FIG. 5. LMP2 is a major P-Tyr phosphoprotein in transfected BJAB cells. Control, P-Tyr antibody, or LMP2 antibody immunoprecipitates of extracts from unlabeled (lanes 1 to 6) or ^{32}P-labeled (4-h labeling; lanes 7 to 10) pLMP2A- or pSG5-transfected BJAB cells were resolved by SDS-PAGE (10% acrylamide). ^{32}P-labeled proteins were detected by autoradiography (lanes 7 to 10). Unlabeled LMP2A in the LMP2, P-Tyr, or control immunoprecipitates was detected by immunoblot with LMP2 antibody (lanes 1 to 6).

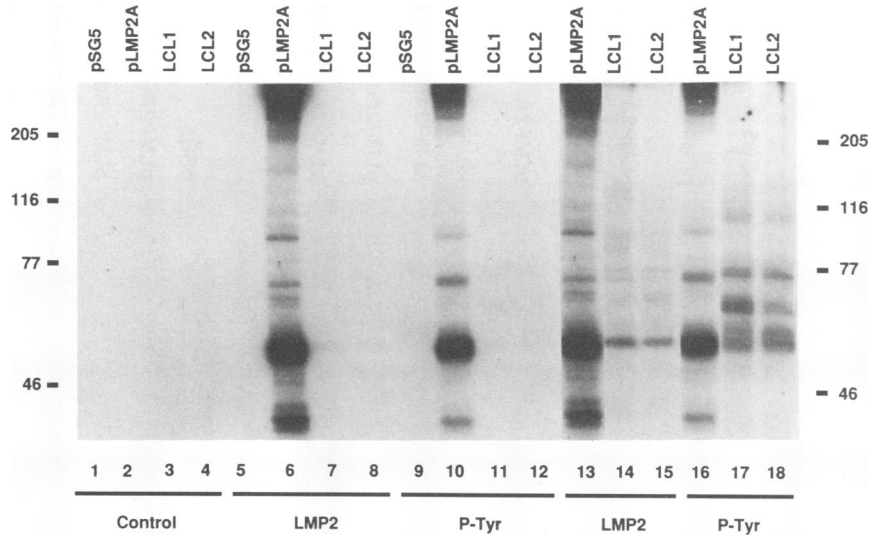

FIG. 6. LMP2A and a 70-kDa cell protein are phosphorylated in vitro in LMP2 or P-Tyr immunoprecipitates from pLMP2A-transfected cells or from EBV-transformed LCLs, LCL1 or LCL2. Labeled immune complexes were separated by SDS-PAGE (6% polyacrylamide) prior to autoradiography. Lanes 14, 15, 17, and 18 are longer exposures of lanes 7, 8, 11, and 12, respectively. Lanes 13 and 16 are duplicate exposures of lanes 6 and 10, respectively, and are included to facilitate alignment.

noprecipitates of each mutant; it was more evident with the P-Tyr immunoprecipitates (lanes 4 to 6 and 9 to 11). These results indicate that the LMP2A domain(s) which is essential for kinase interaction and substrate specificity are within the first 167 amino acids of LMP2. Further, the signal for P-Tyr phosphorylation of the 70-kDa phosphoprotein in P-Tyr immunoprecipitations must also be within the first 167 amino acids (compare Fig. 6, lane 10, and Fig. 7, lane 11).

LMP2 is phosphorylated on tyrosine in vivo and in vitro. Phosphoamino acid analysis was performed on in vivo or in vitro ^{32}P-labeled LMP2A or the 167-amino-acid LMP2A deletion mutant protein from the denatured polyacrylamide gels of immunoprecipitates from transfected B-lymphoma cells (Fig. 7). The in vitro-labeled LMP2A or the LMP2A deletion mutant proteins were phosphorylated mostly on tyrosines and to a lesser extent on serine and threonine (Fig. 8A). The in vivo-labeled LMP2A was also tyrosine phosphorylated, although most of the ^{32}P label was due to serine or threonine phosphorylation (Fig. 8B and 9). The deletion mutant was relatively less threonine phosphorylated (Fig. 8B and 9). The similarity in the P-Tyr labeling of LMP2A and the deletion mutant proteins and the absence of any LMP2A-sized labeled protein from the deletion mutant protein precipitation or of a deletion mutant-sized labeled protein from the LMP2 immunoprecipitate in the in vivo-labeled samples make it highly unlikely that the results are significantly affected by any putative comigrating protein(s) (Fig. 7 to 9). The lower relative in vivo LMP2A tyrosine to serine phosphorylation level compared with in vitro labeling could reflect differences in in vivo or in vitro tyrosine or serine kinases or phosphatase activities.

Phosphoamino acid analysis was also performed on in vivo, LCL1, ^{32}P-labeled LMP2A. Prior to immunoprecipitation, the LCL1 extract was denatured in SDS buffer to remove any phosphorylated proteins that might associate with LMP2A. The autoradiogram of the polyacrylamide gel of the immunoprecipitate was similar to that shown in Fig. 3, except that there was reduced background. The in vitro-labeled LMP2A from LCL1 was phosphorylated primarily on tyrosine and to a lesser extent on serine (Fig. 9).

P-Tyr reactivity localizes to LMP2 patches. B-lymphoma cells were transfected with pLMP2A, pLMP2Ad168–497 (Fig. 1), or pSG5. LMP2 or P-Tyr proteins were localized in the same cells by indirect immunofluorescence microscopy. LMP2 was detected with rabbit antibody and goat anti-rabbit IgG conjugated with Texas red. P-Tyr proteins were detected with anti-P-Tyr monoclonal antibody and goat anti-mouse IgG conjugated with fluorescein isothiocyanate. A Zeiss Axioskop photomicroscope equipped with epifluorescence and the appropriate barrier filters permitted visualization of intracellular fluorescein or Texas red within the same cells without spillover. As shown in Fig. 10, the LMP2-specific antibodies reacted with small discrete aggregates or with much larger patches at the periphery of pLMP2A-transfected cells. P-Tyr monoclonal antibody almost invariably reacted with LMP2 sites. No LMP2 or localized P-Tyr reactivity was detected in pSG5-transfected cells. In contrast, LMP2 antibody localized the LMP2A deletion mutant protein in pLMP2Ad168–497-transfected BJAB cells diffusely through the cytoplasm, and P-Tyr antibody reacted intensely with this diffuse antigen. These data confirm that LMP2A substantially alters protein tyrosine phosphorylation in transfected B-lymphoma cells and that the phosphorylated substrate colocalizes with LMP2. The first 167 LMP2A amino acids are sufficient to substantially alter cellular protein phosphorylation and to direct the phosphorylation to the site of mutant LMP2A localization throughout the cytoplasm. The mutant LMP2A is likely to be in cytoplasmic membranes since it has two adjacent putative transmembrane domains (Fig. 1) and since degraded products do not accumulate in cells (Fig. 7, lanes 6 and 11).

DISCUSSION

The data reported here demonstrate that LMP2A is tyrosine phosphorylated in transfected B-lymphoma cells and

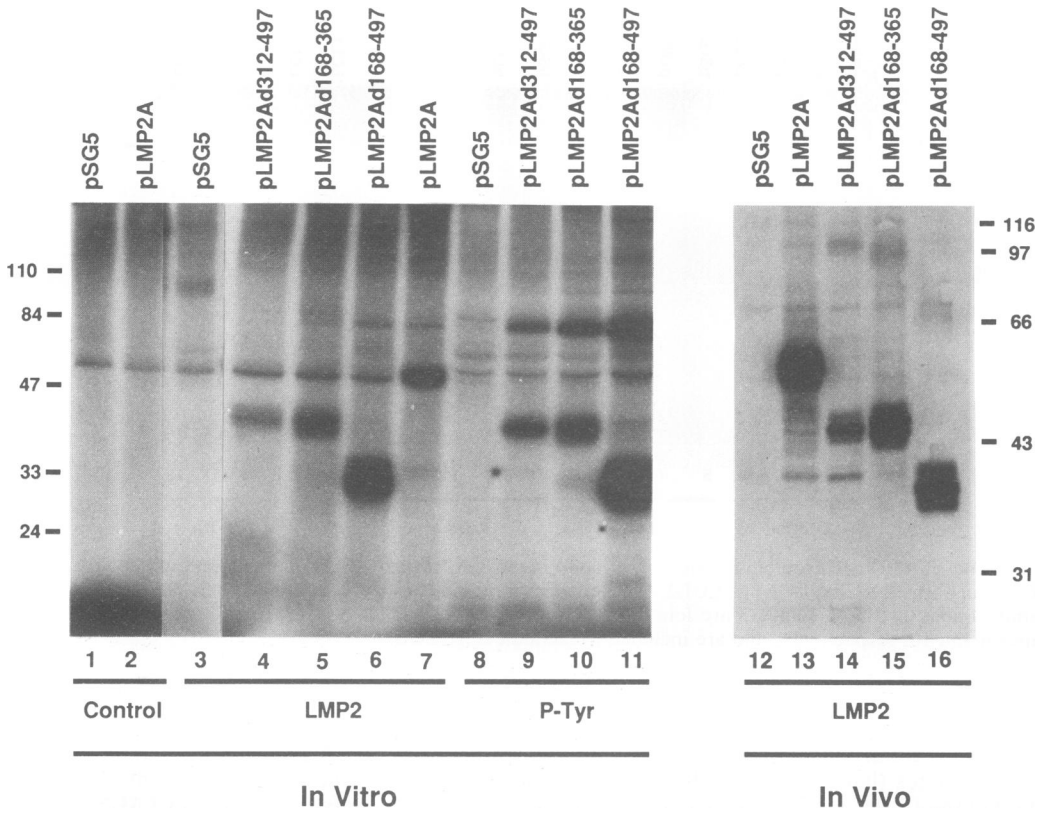

FIG. 7. Kinase association, substrate activity, and pp70 effect are within the first 167 amino acids of LMP2A. In lanes 1 to 11, in vitro kinase reactions with LMP2, P-Tyr, or control antibody immunoprecipitates from BJAB cells transfected with pSG5, pLMP2A, or LMP2A deletion mutants (described in the legend to Fig. 1) were separated by SDS-PAGE (10% polyacrylamide) and subjected to autoradiography. Lanes 11 to 16 show LMP2 antibody immunoprecipitates from in vivo $^{32}P_i$ labeling of pLMP2A or LMP2A deletion mutants transfected into a BJAB cells. Cells were transfected with pSG5, pLMP2A, or LMP2A deletion mutants; labeled with $^{32}P_i$ for 4 h posttransfection; immunoprecipitated with LMP2 antibody; separated by SDS-PAGE (8% polyacrylamide); transferred to Immobilon; and subjected to autoradiography.

in EBV-transformed B lymphocytes. In fact, LMP2A is a major tyrosine kinase substrate in both transfected B-lymphoma cells and EBV-transformed B lymphocytes. Further, tyrosine-phosphorylated LMP2A remains associated with a kinase and undergoes tyrosine phosphorylation following immunoprecipitation with P-Tyr- or LMP2-specific antibody. Moreover, LMP2A induces the phosphorylation of a 70-kDa tyrosine kinase substrate in vivo or in in vitro kinase reactions. Thus, LMP2A is not simply the end of a tyrosine kinase pathway and is likely to be actively involved in plasma membrane-mediated P-Tyr signaling.

These data are consistent with the hypothesis that LMP2A interacts with a cell tyrosine kinase or that LMP2A is a tyrosine kinase and is autophosphorylated. To evaluate the latter possibility, the LMP2A protein sequence was compared with the five consensus tyrosine kinase domains (19). The only match is with a Gly-X-Gly-X-X-Gly sequence characteristic of tyrosine kinase subdomain I, which is at amino acids 293 to 298 in LMP2A. Although most tyrosine kinases also have an X-Val after the terminal glycine, LMP2A has Ala-Ala following the terminal glycine (Fig. 1). Gly-X-Gly-X-X-Gly at 293 to 298 in LMP2A is not within the amino-terminal 167 amino acids essential for tyrosine kinase effects, as demonstrated with the LMP2Ad168–497 mutant. The first 167 LMP2A amino acids are not similar to known tyrosine kinases and are therefore not likely to have tyrosine kinase activity.

Since LMP2B lacks the amino-terminal putative cytoplasmic 119 amino acids of LMP2A and has only 47 amino acids in common with the LMP2Ad168–497 mutant, it is unlikely to have the tyrosine kinase effects of LMP2A. LMP2B and LMP2A have the Gly-X-Gly-X-X-Gly sequence. Gly-X-Gly-X-X-Gly is a domain common to ATP-binding proteins (64). The presence of this sequence in an LMP2A and LMP2B cytoplasmic reverse turn that is not essential for tyrosine kinase effects (Fig. 1) suggests that LMP2A and LMP2B may have another function requiring nucleotide binding.

Since LMP2A is not likely to be a tyrosine kinase, it probably associates with a cellular tyrosine kinase. The association of LMP2A with intracellular signal-transducing proteins is reminiscent of polyomavirus middle T (MT) antigen. MT antigen, the transforming protein of polyomavirus (53), is plasma membrane associated and has no characterized intrinsic biochemical activities. Instead, MT antigen associates with members of the *src* family of tyrosine kinases: pp60$^{c\text{-}src}$ (11, 14, 15), pp62$^{c\text{-}yes}$ (29) or pp60$^{c\text{-}fyn}$ (10, 22, 30), a phosphatidylinositol kinase (13, 26, 61, 62), and the catalytic and regulatory subunits of protein phosphatase 2A (44, 45). Each of these associated activities is necessary for MT antigen transformation. For pp60$^{c\text{-}src}$ and the phosphati-

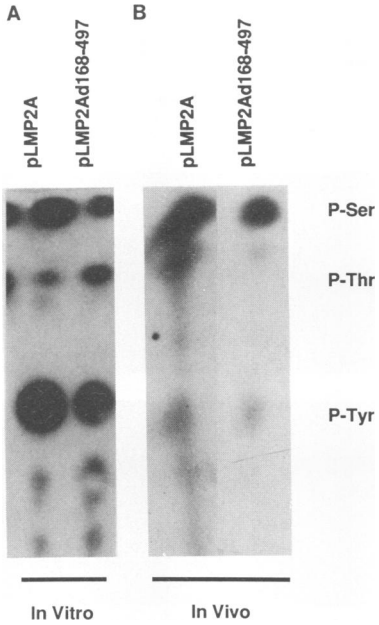

FIG. 8. One-dimensional phosphoamino acid analysis of in vivo- or in vitro-labeled LMP2A or the deletion mutant LMP2Ad167–497 proteins. LMP2A and LMP2Ad167–497 were phosphorylated in vitro after immunoprecipitation with LMP2 antibody from transfected BJAB cells. The respective labeled bands were excised from the SDS-PAGE gel (from a gel similar to that shown in Fig. 7, lanes 1 to 11), degraded, and analyzed for phosphoamino acid composition. In vivo-labeled LMP2A or LMP2Ad167–497 deletion mutant proteins were immunoprecipitated from $^{32}P_i$-labeled transfected BJAB cells. The SDS-PAGE-separated proteins were transferred to Immobilon (the actual gel is shown in Fig. 7, lanes 12 to 16), from which the single major band was excised from each lane, degraded, and analyzed. Abbreviations: P-Ser, phosphoserine; P-Thr, phosphothreonine.

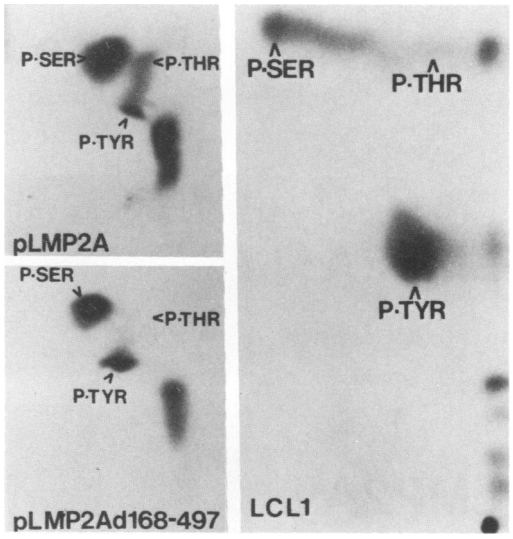

FIG. 9. Two-dimensional phosphoamino acid analysis of in vivo-labeled LMP2A or the deletion mutant LMP2Ad167–497 from transfected B-lymphoma cells or of LMP2A from in vivo-labeled LCL1. In vivo-labeled proteins were immunoprecipitated from $^{32}P_i$-labeled cells, separated by SDS-PAGE, and transferred to Immobilon, from which the single major band was excised, degraded, and analyzed. Abbreviations: P-SER, phosphoserine; P-THR, phosphothreonine. Positions were identified by using ninhydrin-stained standards.

dylinositol kinase, it has been demonstrated that association with MT antigen results in activation of their respective kinase activities (7, 8, 12, 26, 28, 46, 61, 62). Thus, LMP2 may be similar to MT antigen in its ability to associate with and alter the activity of cellular proteins which have a role in the control of cell growth.

Immunofluorescence and immunoprecipitation analyses indicate an association with LMP1 (35). LMP1 has broad effects on B-lymphocyte and rodent fibroblast growth (2, 57–59) but is not a tyrosine kinase substrate (1). It is an inducer of B-lymphocyte activation and adhesion molecules and of vimentin (4, 59). The association of these two proteins could create a macromolecular complex mediating constitutive B-lymphocyte proliferation through normal cell signal transduction pathways. The LMP2 P-Tyr- induced 70-kDa protein might be an autophosphorylated kinase or a kinase substrate which normally mediates tyrosine kinase effects. Known tyrosine kinase substrates which are probably mediators of cell growth include phospholipase C-gamma (37, 38, 56), phosphatidylinositol 3-kinase (54), *ras* GTPase-activating protein (40), and c-*raf* (41). LMP2 could interact with differentiated cell-specific components of the tyrosine kinase pathway, such as components of the membrane Ig signal transduction complex, or may interact with components common to many cell types. The LMP2 tyrosine kinase-associated proteins could have been previously identified because of their association with the Ig or the analogous T-lymphocyte–receptor complexes or could have been identified as previously characterized growth factor receptors or oncoproteins. Among lymphocyte tyrosine kinase-mediated signal transduction pathways, the T-cell receptor ligation pathways are best understood. T-cell receptor ligation upregulates membrane-associated tyrosine kinases. As many as nine tyrosine kinases are expressed in T lymphocytes (42), *lck* garnering the most attention because of its association with CD4 and CD8 (49, 55), which have important accessory roles in T-cell activation by binding to major histocompatibility class I or class II molecules and stabilizing the interaction of the latter with the T-cell receptor–CD3 complex or by playing an active role in transmembrane signaling during T-cell activation (42). Tyrosine kinase activation is necessary for phospholipase C breakdown of inositol phospholipids, releasing inositol phosphate and diacylglycerol and thereby increasing intracellular Ca^{2+} and translocating protein kinase C (60). Tyrosine phosphorylation of the zeta subunit of the T-cell receptor is essential for phospholipase C activation, interleukin-2 receptor expression, and T-cell proliferation (24, 43).

B-lymphocyte proliferation and differentiation, although less well understood, involves membrane Ig cross-linking–induced protein tyrosine phosphorylation (3, 5, 18). Membrane Ig is associated with a 38- to 40-kDa and 35- to 36-kDa membrane glycoprotein heterodimer analogous to the TCR association with CD3 (5, 9, 21, 63). Ig cross-linking leads to tyrosine phosphorylation of the Ig-associated proteins (5). A 70-kDa P-Tyr protein has also been identified in B lymphocytes following Ig cross-linking (3, 6, 18). The tyrosine kinase(s) activated by membrane Ig cross-linking is likely to be one of the *src*-like, membrane-associated, B-lymphocyte tyrosine kinases such as *lyn* (65), *blk* (16), or *hck* (47, 66). The other B-lymphocyte tyrosine kinases may mediate

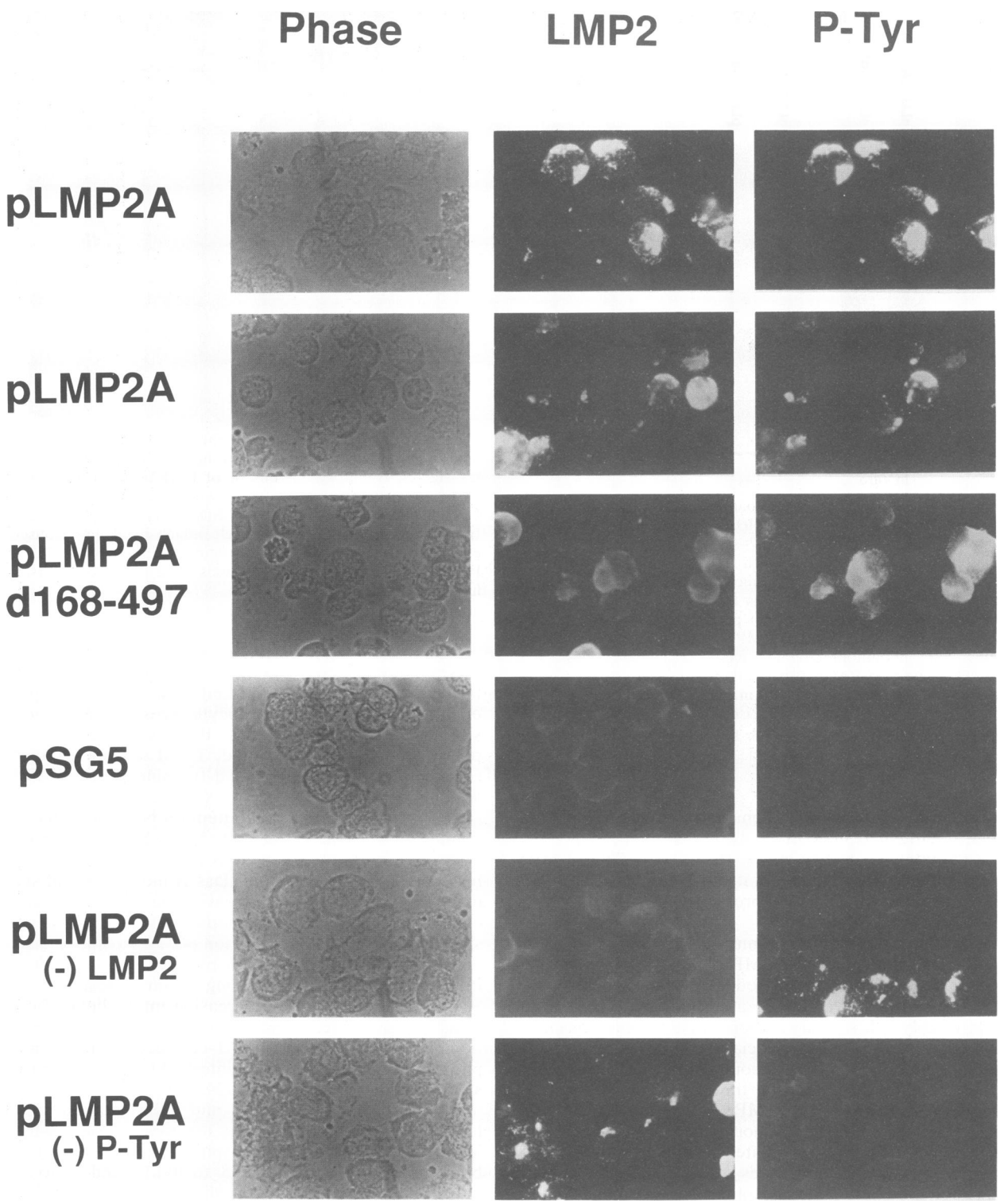

FIG. 10. LMP2- or P-Tyr-specific immunofluorescence of BJAB cells transfected with pSG5, pLMP2A, or pLMP2Ad167–497. BJAB cells were transfected with either pLMP2A, pLMP2Ad167–497, or pSG5, and slides were fixed after overnight incubation. The fixed cells were then incubated with affinity-purified rabbit LMP2-specific antiserum and mouse monoclonal antibody PY20 to P-Tyr. Slides were then incubated with Texas red-conjugated goat $F(ab')_2$ anti-rabbit Ig and fluorescein isothiocyanate-conjugated goat-$F(ab')_2$ anti-mouse Ig to visualize the primary antibodies. Photomicrographs in each row are the same field at ×1,000 with phase, Texas red-specific filter, or fluorescein isothiocyanate-specific filter (left to right). The bottom two rows are preparations in which LMP2 or P-Tyr primary antibodies were ommitted, as indicated in the figure. Note the absence of spillover in the preparations from which primary antibody was omitted.

B-lymphocyte growth factor receptor effects. Antisera specific for the various B-lymphocyte tyrosine kinases might identify the LMP2-associated tyrosine kinase. Identification of the kinase may provide an insight into the role of LMP2 in EBV-mediated cell growth transformation or into the role of the kinase in mediating B-lymphocyte growth.

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