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# Analysis of the Phosphorylation Status of Epstein–Barr Virus LMP2A in Epithelial Cells

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LMP2A deletion and point mutants, with mutations in phosphotyrosine-containing protein–protein interaction motifs, were transiently expressed in 293 cells and their phosphorylation was examined in immune complex kinase assays as well as *in vivo*. *In vitro* LMP2A phosphorylation depended on tyrosine 112. *In vivo*, mutations of single tyrosines did not eliminate LMP2 phosphorylation, although mutation of the LMP2A ITAM decreased LMP2A phosphorylation. The relationship between LMP2A *in vitro* phosphorylation and that induced by cell-extracellular matrix (ECM) interactions was also investigated. While LMP2A was phosphorylated to higher levels in whole-cell extracts of stimulated cells, a difference in *in vitro* kinase assays with extracts from stimulated and unstimulated cells was not detected, indicating that the ECM-mediated regulation of LMP2A phosphorylation is lost *in vitro*. In the presence of LMP2A, several cellular proteins with molecular weights between 70 and 80 kDa were phosphorylated on tyrosine. This increase in cellular protein phosphorylation depended on the LMP2A ITAM motif and suggests that the ITAM may participate in signal-transduction events in epithelial cells. 

## INTRODUCTION

Epstein–Barr virus (EBV), a ubiquitous human herpesvirus, establishes latent infection in B cells with periodic reactivation of virus and reinfection of oropharyngeal epithelia. EBV is the causative agent of infectious mononucleosis and is associated with several human cancers, such as the lymphoid diseases Burkitt's lymphoma, posttransplant lymphoma, and Hodgkin's disease, as well as nasopharyngeal carcinoma (NPC), an epithelial malignancy (Kieff, 1996). LMP2A inhibits B cell receptor signaling through dominant negative effects on protein tyrosine kinases (Miller *et al.,* 1995, 1994a,b, 1993). In latently infected cells, LMP2A apparently acts as a decoy receptor and sequesters signaling molecules, preventing their activation in response to B cell receptor crosslinking (Burkhardt *et al.,* 1992; Longnecker *et al.,* 1991). Inhibition of these signaling events results in a block in the activation of transcription factors which would stimulate expression of EBV immediate-early genes and induce viral lytic cycle replication. *In vivo*, LMP2A provides both a development and a survival signal in B cells in the absence of normal B cell receptor signals in LMP2A-expressing transgenic mice (Caldwell *et al.*, 1998). Recent studies suggest that this signal may be a result of the specific activation of Akt by LMP2A, which has been observed both in epithelial and in B cells (Scholle *et al.,* 2000; Swart and Longnecker, 2000). In particular, the expression of LMP2A has dramatic effects on the growth of epithelial cells (Scholle *et al.,* 2000).

Tyrosine-containing protein–protein interaction motifs in the LMP2A amino terminus play critical roles in mediating these effects. Tyrosine Y112, found in the context of a YEEA sequence, closely resembles the consensus binding site for the SH2 domain of Src family kinases, YEEI (Songyang *et al.,* 1993). Binding of Lyn and Fyn to this motif leads to subsequent phosphorylation of the LMP2A immunoreceptor tyrosine-based activation motif (ITAM) at tyrosines 74 and 85. The phosphorylated ITAM motif serves as a docking site for the tandem SH2 domains of Syk, a hematopoietic cell-specific kinase (Reth, 1989; Weiss and Littman, 1994). Mutation of either of these motifs renders LMP2A nonfunctional in inhibition of B cell receptor-signal transduction (Fruehling and Longnecker, 1997; Fruehling *et al.,* 1998). Several other tyrosine-containing motifs in the LMP2A amino terminus have been suggested to constitute binding sites for SH2 domains of important cellular signaling molecules such as the docking protein Shc and the 85-kDa regulatory subunit of PI3-kinase (Miller *et al.,* 1995). The amino acid sequence surrounding tyrosine 101 has been suggested to potentially mediate binding to the SH2 domain of Csk, a negative regulator of Src. LMP2A is consistently expressed in NPC and in nude mouse passaged NPC tumors, suggesting that it is an important factor in epithelial cell infection (Brooks *et al.,* 1992; Busson *et al.,* 1992). LMP2A interacts with protein kinases in epithelial cells and its phosphorylation is induced by cell–ECM interactions (Scholle *et al.*, 1999).

To further investigate potential effects on epithelial

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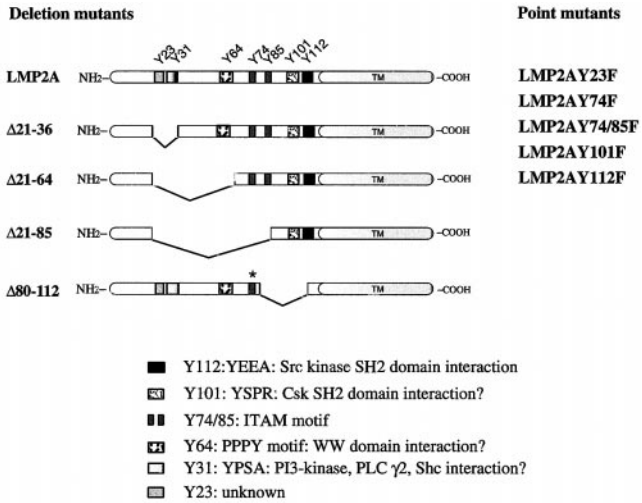


FIG. 1. LMP2A deletion and point mutants. Protein–protein interaction motifs are progressively deleted in the deletion mutants. Δ80–112 also has a point mutation at tyrosine Y74 (*). The ITAM motif is deleted in both Δ21–85 and Δ80–112. All LMP2A point mutants contain single tyrosine-to-phenylalanine substitutions with the exception of LMP2A Y74/85F, which has both tyrosines in the ITAM motif mutated.

signaling pathways that are affected by LMP2A, the individual tyrosine-containing motifs in the LMP2A N-terminus were examined for their importance for LMP2A phosphorylation in epithelial cells *in vitro* and *in vivo* using LMP2A point and deletion mutants. The results reveal differences in the protein–protein interaction motifs required for *in vitro* and *in vivo* phosphorylation. Both the LMP2A ITAM motif and the tyrosine Y112, important for LMP2A function in B lymphocytes, were also involved in LMP2A phosphorylation in epithelial cells. In addition, determination of the phosphorylation status of cellular proteins in the presence and absence of LMP2A revealed that tyrosine phosphorylation of at least three cellular proteins was increased in the presence of LMP2A and was dependent on the LMP2A ITAM motif.

## RESULTS

### LMP2A deletion and point mutants

The LMP2A amino terminus contains numerous protein–protein interaction motifs, most of which contain tyrosines, and are potential docking sites for cellular proteins when phosphorylated. A diagram of the various LMP2A deletion mutants and potential tyrosine phosphorylation sites is shown in Fig. 1. Tyrosine Y112 is in the context of a sequence YEEA, resembling the Src family kinase SH2 domain interaction motif. Tyrosine 74 and 85 constitute the LMP2A ITAM motif. ITAMs are characterized by the consensus sequence (YXXL/I)($X_{6-8}$)(YXXL/I). Protein–protein interaction motifs are progressively deleted in the deletion mutants. In addition to deletion of tyrosines 85, 101, and 112, Δ80–112 also has a point mutation in tyrosine 74, eliminating the ITAM. Δ21–85 deletes most of the tyrosines in the N-terminus, including the ITAM, but retains Y101 and Y112. Additionally, a set of point mutants was used in this study, which are single substitution mutants of individual tyrosines with the exception of Y74/85F, which contains a double mutation in the ITAM motif.

### LMP2A phosphorylation status in *in vitro* kinase assays

The LMP2A deletion and point mutants were transiently transfected into 293 cells and *in vitro* kinase assays were performed on immune complexes containing LMP2A. Wild-type LMP2A was highly phosphorylated, as were the deletion mutants Δ21–36 and Δ21–64. In contrast, the deletion mutants Δ21–85 and Δ80–112 were not phosphorylated in *in vitro* kinase assays (Fig. 2A). The ITAM motif is deleted or mutated in the latter two mutants and Δ80–112, in addition, is deleted for tyrosine Y112, the Src family kinase interaction site, suggesting an important role for these motifs in LMP2A *in vitro* phosphorylation.

In the context of the LMP2A point mutants, mutation of

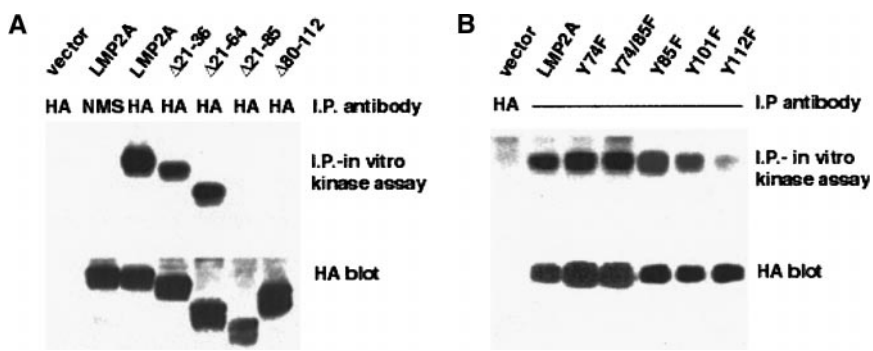


FIG. 2. LMP2A phosphorylation in *in vitro* kinase assays. LMP2A and mutants were immunoprecipitated from transiently transfected 293 cell lysates followed by immune-complex kinase assays. (A) LMP2A wt and the deletion mutants Δ21–36 and Δ21–64 are highly phosphorylated. Deletion mutants Δ21–85 and Δ80–112 are not phosphorylated. The bottom panel detects LMP2A and mutant protein expression. (B) *In vitro* phosphorylation of LMP2A point mutants. All point mutants, except Y112F, are phosphorylated compared to wild-type LMP2A. The bottom panel shows LMP2A protein levels.

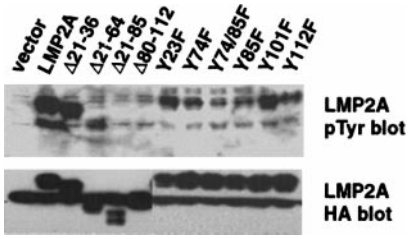


FIG. 3. Phosphorylation of LMP2A deletion and point mutants *in vivo*. Western blot analysis of 293 cell lysates, transiently transfected with wild-type or mutant LMP2A expression constructs. WT and deletion mutants Δ21–36 and Δ21–64 are phosphorylated while Δ21–85 and Δ80–112 are not. Mutation of the ITAM motif in the mutants Y74f, Y64/85F, and Y85F reduces the phosphotyrosine content. Bottom: Expression levels of LMP2A and mutants.

tyrosine Y112 inhibited *in vitro* phosphorylation, while all other LMP2A point mutants were phosphorylated to wild-type levels (Fig. 2B). These results indicate that Y112 is the primary site of phosphorylation *in vitro*. However, the deletion mutant, Δ21–85, retained Y112 and was not phosphorylated. This may indicate that phosphorylation of Y112 requires a conformation that is lost in the large deletion mutant.

### Constitutive LMP2A phosphorylation

It was of interest to investigate the phosphorylation state of the various LMP2A mutants in intact cells to determine whether *in vitro* and *in vivo* phosphorylation behaviors are consistent. Therefore LMP2A deletion and point mutants were transiently transfected into 293 cells and constitutive LMP2A tyrosine phosphorylation was examined by immunoblot and immunofluorescence. The LMP2A deletion mutants showed the same phosphorylation pattern as in the immune complex kinase assays with phosphorylation of Δ21–36 and Δ21–64 and absence of phosphorylation of Δ21–85 and Δ80–112 (Fig. 3). Although mutations in the LMP2A ITAM motif, as illustrated with the mutants Y74F, Y74/85F, and Y85F, consistently reduced the levels of LMP2A tyrosine phosphorylation, all forms of LMP2 were phosphorylated at variable levels *in vivo*.

Immunofluorescence analysis of LMP2A and selected mutants detected LMP2A predominantly in the perinuclear cytoplasm of transfected cells (Fig. 4). Using phosphotyrosine (pTyr)-specific antibodies, phosphotyrosine was readily detected in areas where LMP2A was present. The computer-generated overlay image revealed colocalization of the phosphotyrosine reactivity with LMP2A, as indicated by yellow staining. The immunofluorescence staining confirmed that the deletion mutants Δ21–85 and Δ80–112 were not phosphorylated or phosphorylated to significantly lower levels than WT LMP2A *in vivo* as little pTyr was detected even with very long exposure of pTyr staining of these mutants and as indicated by the orange color of the overlay. Although as detected by immunoblots, the Y74/85F mutant had reduced phosphotyrosine compared to the Y112F mutant (Fig. 3), phosphotyrosine was readily detected by immunostaining for both mutants and this phosphotyrosine colocalized with both mutated forms of LMP2A. The colocalization of phosphotyrosine with LMP2A Y74/85F when LMP2A itself had reduced phosphorylation may indicate colocalization of LMP2A and a phosphoprotein, possibly an LMP2A-interacting kinase.

### Differences in ECM-induced and *in vitro* LMP2A phosphorylation

Cell–ECM interactions induce tyrosine phosphorylation of LMP2A (Scholle *et al.*, 1999). To determine whether the protein kinase(s) associated with LMP2A after immunoprecipitation is activated upon cell–ECM adhesion, LMP2A was immunoprecipitated from extracts of transiently transfected cells kept in suspension for 45 min or plated on fibronectin for 45 min and *in vitro* kinase assays were performed on the immune complexes. An increase in radioactive phosphate incorporation into LMP2A in fibronectin-stimulated cells over cells kept in suspension was not detected (Fig. 5). However, determination of the total phosphotyrosine content of LMP2A by immunoblot of whole-cell extracts prior to immunoprecipitation revealed that LMP2A was phosphorylated to higher levels in fibronectin-stimulated cells in whole-cell extracts. These data indicate that the regulation of ECM-induced LMP2A phosphorylation does not occur in immunoprecipitation/*in vitro* kinase assays. These results suggest that the adhesion-enhanced kinase activity is distinct from the activity detected *in vitro*. The adhesion-enhanced kinase activity might be regulated through protein–protein interactions with the cytoskeleton or within a specific cellular localization, features that would be lost upon disruption of the cells and immunoprecipitation.

### LMP2A expression induces the phosphorylation of cellular proteins

LMP2A expression affects the phosphorylation state of several cellular proteins in B cells. Syk and the regulatory 85-kDa subunit of PI3-kinase, for example, are constitutively phosphorylated to higher levels in LMP2A-expressing B lymphocytes (Miller *et al.,* 1995). The phosphorylation state of these molecules does not change in response to B cell receptor stimulation, in contrast to non-LMP2A-expressing cells. To identify effects of LMP2A expression on cellular signaling pathways in epithelial cells, the phosphorylation state of cellular proteins in the presence or absence of LMP2A was examined in more detail. Transient transfection of LMP2A into 293 cells allowed a high level of LMP2A expression and resulted in induction of phosphorylation at variable levels of at least three cellular proteins with estimated molec-

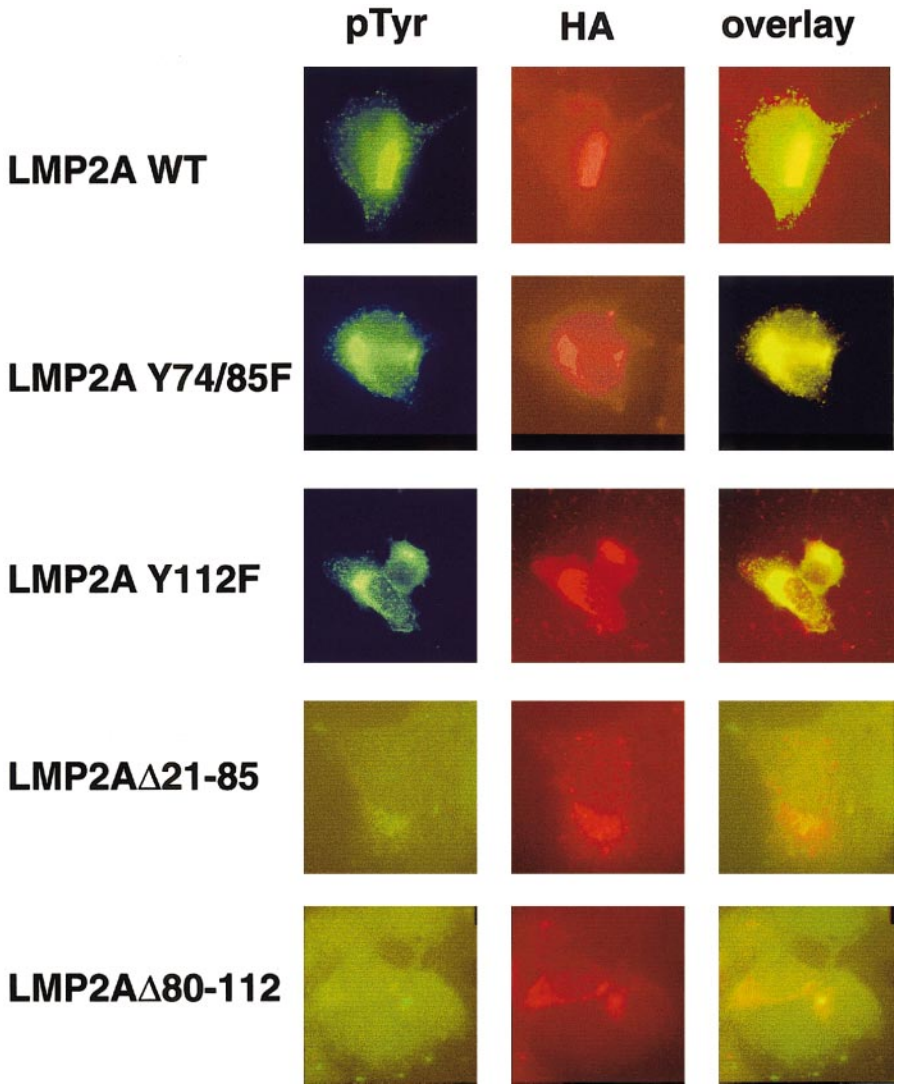


FIG. 4. Immunofluorescence analysis of the phosphorylation state of LMP2A and mutants. 293 cells were transiently transfected with LMP2A or mutant expression constructs, seeded onto coverslips, and phosphotyrosine staining (left) and HA staining (middle) was performed. The overlay (right) demonstrates colocalization of the major phosphotyrosine reactivity and LMP2A. Double mutation of the ITAM does not abolish LMP2A phosphorylation. Mutation of Y112 does not interfere with LMP2A phosphorylation *in vivo*. Deletion mutants that are not phosphorylated *in vitro* are not phosphorylated in intact cells (Δ21–85 and Δ80–112).

ular weights between 70 and 80 kDa (Fig. 6A). This induction of cellular protein phosphorylation was apparent in cells kept in suspension and could be further enhanced by fibronectin stimulation (Fig. 6B).

Although immunoblotting for HA revealed an equivalent expression of all of the LMP2A mutants, the LMP2A-deletion mutants that were not phosphorylated in epithelial cells, Δ21–85 and Δ80–112, did not induce phosphorylation of these proteins (Fig. 6C). In the context of the LMP2A point mutants, the induction of tyrosine phosphorylation of pp80 was dependent on the ITAM motif, since all three mutants in which either a single tyrosine or both tyrosines in the ITAM were mutated, Y74F, Y74/85F, and Y85F, did not cause upregulation of pp80 phosphorylation.

Analysis of the total phosphotyrosine pattern with the deletion mutants after adhesion to fibronectin confirmed that phosphorylation of pp80 was increased by adhesion (Fig. 6D). The adhesion-induced phosphorylation was variable; however, the mutants Δ21–85 and Δ80–112 did not induce phosphorylation of these proteins. These data suggest that the ITAM motif contributes to potential effects of LMP2A on epithelial cell signaling pathways, although the nature of those pathways still remains to be identified.

## DISCUSSION

The phosphorylation status of LMP2A in epithelial cells *in vitro* and *in vivo* was investigated using a combination of deletion and point mutants. Several phosphotyrosine-containing protein–protein interaction motifs

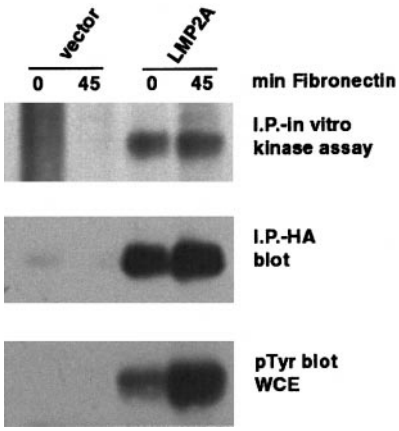


FIG. 5. LMP2A phosphorylation *in vitro* differs from cell adhesion stimulated LMP2A phosphorylation. LMP2A was immunoprecipitated from transiently transfected 293 cells, kept in suspension (0), or plated on fibronectin for 45 min (45) and *in vitro* kinase assays were performed on precipitated complexes. LMP2A did not incorporate more label in *in vitro* kinase assays with stimulated cells than unstimulated cells (top). Equal amounts of LMP2A were immunoprecipitated (middle). LMP2A in whole-cell extracts (WCE) is phosphorylated to higher levels in stimulated cells (bottom).

that had previously been shown to be important for LMP2A function in B lymphocytes were found to be involved in LMP2A phosphorylation in epithelial cells as well. *In vitro*, LMP2A phosphorylation was dependent on the presence of tyrosine 112, found in the context of a YEEA sequence, the interaction site for Src family kinases in B cells (Fruehling *et al.,* 1998). These data suggest that Y112 is important for the interaction of a protein kinase *in vitro* with the LMP2A amino terminus as both mutants that have Y112 deleted, Δ80–112 and Y112F, are not phosphorylated. The absence of phosphorylation of Δ21–85, which retains Y112, suggests that LMP2 conformation contributes to phosphorylation at this site. The strong phosphorylation *in vitro* of Y74/85F also suggests that the ITAM is not the site of phosphorylation *in vitro*. While mutation of Y112 eliminated LMP2A phosphorylation *in vitro*, the Y112 point mutant was phosphorylated to varying levels in intact cells. The requirement for this docking site therefore is less stringent *in vivo* than *in vitro*. It is possible that a direct, strong interaction with this tyrosine is required in immunoprecipitation assays to coprecipitate a protein kinase with LMP2A, whereas in intact cells colocalization of LMP2A and other kinases due to cytoskeletal interactions or a specific subcellular localization results in LMP2A phosphorylation.

Although mutation of Y112 did not significantly affect LMP2 phosphorylation *in vivo*, mutation of the ITAM consistently reduced tyrosine phosphorylation of LMP2A, indicating that the ITAM is phosphorylated in intact cells. However, it is interesting to note that none of the point mutations, including the double-ITAM mutation, completely eliminated LMP2A phosphorylation. This is in contrast to B lymphocytes where mutation of single protein–protein interaction motifs is sufficient to abrogate LMP2A phosphorylation (Fruehling *et al.,* 1998).

Immunofluorescence analysis detected the majority of LMP2A localized to the perinuclear cytoplasm, possibly associated with the endoplasmic reticulum with very little plasma membrane staining in transfected epithelial cells. This is similar to previous reports in which LMP2A was expressed in NIH 3T3 fibroblasts and detected in the same location (Longnecker and Kieff, 1990). It is possible that overexpression of LMP2A by transient transfection leads to accumulation of the protein in the ER; however, it is interesting to note that LMP2 is highly phosphorylated, suggesting that it interacts with kinases in this location.

In a previous study we demonstrated that LMP2A

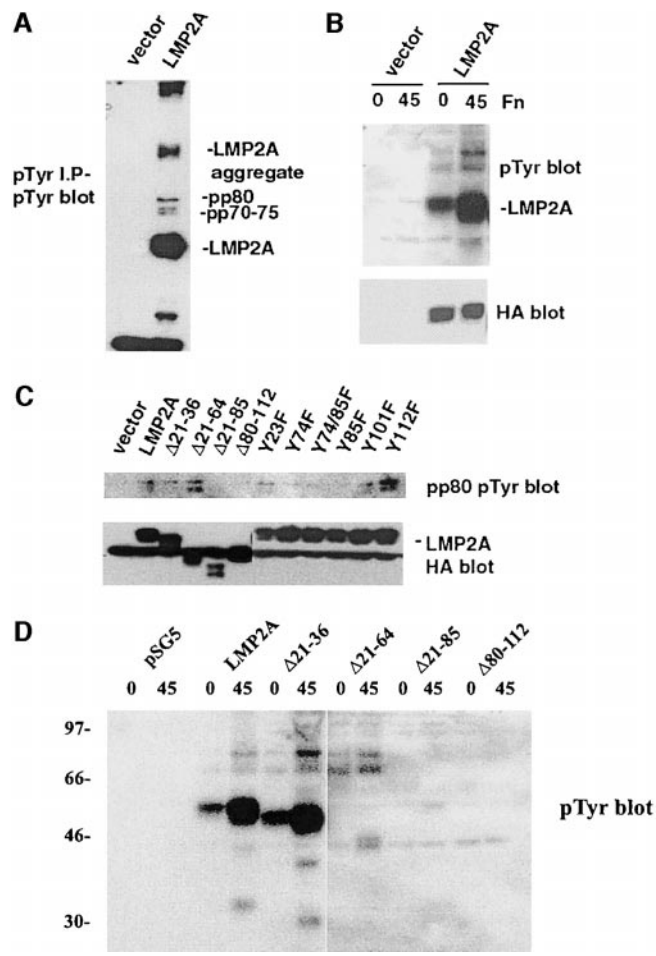


FIG. 6. LMP2A induces the phosphorylation of cellular proteins. Tyrosine-phosphorylated proteins were immunoprecipitated with antiphosphotyrosine antibodies followed by phosphotyrosine immunoblot (A). Three cellular proteins are increasingly phosphorylated in the presence of LMP2A. (B) Cell-adhesion phosphorylation of the LMP2A-induced proteins. Cells were either kept in suspension or plated on fibronectin for 45 min followed by phosphotyrosine blot. (C) Induction of cellular protein phosphorylation is ITAM-dependent. Deletion mutants Δ21–85 and Δ80–112, as well as the point mutants Y74F, Y74/85F, and Y85F, do not induce pp80 phosphorylation. (D) Adhesion-mediated phosphorylation of cellular proteins is ITAM-dependent.

phosphorylation in epithelial cells is induced by cell–ECM interactions (Scholle *et al.*, 1999). In this study, LMP2A, immunoprecipitated from lysates of cells stimulated with fibronectin, was not phosphorylated to higher levels in *in vitro* kinase assays than LMP2A immunoprecipitated from unstimulated cell extracts even though the overall phosphotyrosine content of LMP2A after fibronectin stimulation was greatly increased. These results suggest that the *in vitro* kinase activity is distinct from the kinase activity stimulated by adhesion. This may reflect a requirement for specific cofactors or subcellular localization for the adhesion activated kinase(s) to be active that are lost during lysis and immunoprecipitation. As previously reported, Csk, a negative regulator of Src kinases, phosphorylated a GST–LMP2 fusion protein *in vitro* and Csk also phosphorylated LMP2A in an adhesion-dependent manner in intact cells (Scholle *et al.*, 1999). However, fibronectin stimulation did not lead to any further increase in phosphate incorporation by Csk *in vitro*. Interestingly, Csk activity is thought to be regulated primarily by changes in its intracellular localization, depending on interactions with cytoskeletal and transport proteins, relying on its SH2 and SH3 domains (Cloutier *et al.*, 1995; Ford *et al.,* 1994; Howell and Cooper, 1994; Superti-Furga *et al.,* 1993)

Overexpression of LMP2A in 293 cells led to the induction of tyrosine phosphorylation of several cellular proteins with molecular weights between 70 and 80 kDa. The LMP2A deletion mutants that were phosphorylation defective *in vitro* and *in vivo*, Δ21–85 and Δ80–112, and the individual ITAM mutants did not induce phosphorylation of these cellular proteins, whereas mutation of Y112 alone had no effect. Therefore, the ability to induce tyrosine phosphorylation of these cellular proteins seems to depend on the presence of an intact ITAM motif. It is interesting to note that LMP2A expression in B cells leads to increased constitutive phosphorylation of the 70-kDa protein kinase Syk whose expression is restricted to cells of the hematopoietic lineage (Miller *et al.,* 1995). A homolog of Syk in epithelial cells has not been described; however, the molecular weights of two of the proteins whose phosphorylation is induced by LMP2A in epithelial cells is similar.

The function of ITAM motifs in epithelial cells is not well understood. The $\alpha_6\beta_4$ integrin is one of few epithelial cell molecules that contain an ITAM motif and an assembly of hemidesmosomes depends on the $\alpha_6\beta_4$ ITAM. While the mechanism is not understood, it has been hypothesized that the ITAM motif in this case may primarily be involved in direct assembly of structural protein–protein complexes rather than signaling functions (Mainiero *et al.,* 1995).

This study reveals important differences in the regulation of LMP2A phosphorylation in epithelial cells compared to B cells. Y112 is essential for LMP2A phosphorylation in B cells both *in vivo* and *in vitro* using B cell extracts, while a Y112F point mutant is phosphorylated in epithelial cells *in vivo*. In addition, mutation of the ITAM motif reduces but does not eliminate phosphorylation of LMP2A in epithelial cells *in vivo*. LMP2A is constitutively phosphorylated in B cells but its phosphorylation is regulated by the extracellular matrix in epithelial cells. This is only apparent *in vivo* but not *in vitro*, suggesting that specific regulatory factors are required in intact cells. These differences provide further evidence that different kinases mediate LMP2A phosphorylation in epithelial cells. Continued studies are likely to identify signaling pathways critical to epithelial cell behavior that are affected by LMP2A.

## MATERIALS AND METHODS

### Cell lines and expression constructs

Human 293 embryonic kidney cells were routinely maintained in Dulbecco's modified Eagle medium (DMEM-H) supplemented with 10% fetal bovine serum and antibiotics. pLMP2AHA, as well as the LMP2A deletion mutants pLMP2AΔ21–36, pLMP2AΔ21–64, pLMP2AΔ21–85, and pLMP2AΔ80–112, have been described previously (Fruehling *et al.,* 1996). In addition to the deletion, LMP2AΔ80–112 contains a tyrosine to phenylalanine substitution at position 74. Point-mutant expression constructs have been described previously (Fruehling and Longnecker, 1997). Tyrosines are substituted with phenylalanines. All cDNAs are tagged with a hemagglutinin epitope (HA) at the carboxyl-terminus to facilitate detection of the proteins. Transient transfections into 293 cells were performed using the calcium phosphate transfection method according to standard procedures.

### Immunoblot analysis

Whole-cell extracts were prepared by lysis of transiently transfected cells 48 h posttransfection in NP-40 lysis buffer [150 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EDTA, 1% (v/v) Nonidet-P40, 10 μg/ml aprotinin, 2 mM PMSF, 1mM sodium orthovanadate]. Lysates were incubated on ice for 15 min and clarified by centrifugation in a microcentrifuge at 13,000 rpm for 30 min at 4°C. Equal amounts of protein were separated by SDS–PAGE and transferred to Immobilon P membranes (Millipore). Immunoblotting was performed according to standard procedures. Phosphotyrosine-containing proteins were detected with the 4G10 monoclonal antibody (Upstate Biotechnology Inc.). LMP2A was detected with a polyclonal anti-HA antibody (Santa Cruz).

### Immunofluorescence analysis

Transiently transfected 293 cells were seeded onto tissue culture slides and allowed to attach for 4 h. Cells were fixed for 5 min in 1:1 methanol:acetone at −20°C

and nonspecific binding was blocked with 20% normal goat serum for 20 min. Primary antibodies were diluted in PBS and applied for 45 min. Secondary anti-mouse antibodies conjugated to FITC or anti-rabbit antibodies conjugated to Texas Red were diluted in 20% normal goat serum and applied for 30 min. Slides were mounted after extensive washing in PBS using anti-fade mounting medium (Vectashield). Tyrosine-phosphorylated proteins were detected with the PY20 antiphosphotyrosine antibody (Santa Cruz) at 1:100 dilution and LMP2A was detected with a polyclonal HA antibody at 1:50 dilution (Santa Cruz).

### Immunoprecipitations and immune complex kinase assays

After preclearing with 20 $\mu$l protein-G coupled to Sepharose, LMP2A and mutants were immunoprecipitated from 500 $\mu$g of total protein, diluted to 500 $\mu$l in lysis buffer overnight at 4°C with an anti-HA monoclonal antibody conjugated to Sepharose beads (BABCO). Immunoprecipitates were washed three times in NP-40 lysis buffer and twice in kinase buffer (20 mM HEPES, 5 mM $MnCl_2$). Kinase reactions were initiated by addition of 25 $\mu$l kinase buffer containing 10 $\mu$Ci $\gamma[^{32}P]$ATP for 15 min at room temperature. Reactions were stopped by addition of ice-cold lysis buffer, washed three times, and the products separated by SDS–PAGE after addition of 25 $\mu$l SDS sample buffer and visualized by autoradiography.

## ACKNOWLEDGMENTS

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