Epstein-Barr Virus Latent Membrane Protein 1 (LMP1) Activates the Phosphatidylinositol 3-Kinase/Akt Pathway to Promote Cell Survival and Induce Actin Filament Remodeling*

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The Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) is an integral membrane protein that functions as a constitutively activated member of the tumor necrosis factor receptor family. Whereas LMP1 has been shown to activate the NF-κB and mitogen-activated protein kinase pathways, these effects alone are unable to account for the profound oncogenic properties of LMP1. Here we show that LMP1 can activate phosphatidylinositol 3-kinase (PI3K), a lipid kinase responsible for activating a diverse range of cellular processes in response to extracellular stimuli. LMP1 was found to stimulate PI3K activity inducing phosphorylation and subsequent activation of Akt, a downstream target of PI3K responsible for promoting cell survival. Treatment of LMP1-expressing cells with the PI3K inhibitor LY294002 resulted in decreased cell survival. The tumor necrosis factor receptor-associated factor-binding domain of LMP1 was found to be responsible for PI3K activation. The ability of LMP1 to induce actin stress-fiber formation, a Rho GTPase-mediated phenomenon, was also dependent on PI3K activation. These data implicate PI3K activation in many of the LMP1-induced phenotypic effects associated with transformation and suggests that this pathway contributes both to the oncogenicity of this molecule and its role in the establishment of persistent EBV infection.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus associated with the development of both lymphoid and epithelial tumors. As a common virus infection, EBV appears to have evolved to exploit the process of B cell development to persist as a lifelong asymptomatic infection. However, the virus can contribute to oncogenesis as evidenced by its frequent detection in certain tumors, namely Burkitt's lymphoma, post-transplant B cell lymphomas, Hodgkin's disease, and nasopharyngeal carcinoma (NPC), and by its unique ability to efficiently transform resting B cells in vitro into permanently growing lymphoblastoid cell lines (1). These transforming effects are associated with the restricted expression of EBV genes such that only a subset of so-called latent virus proteins are expressed in virus-infected tumors and in lymphoblastoid cell lines. The full complement of eight latent genes comprising six nuclear antigens (EBNAs) and two membrane proteins (LMP1 and LMP2) are expressed only in post-transplant B cell tumors and in lymphoblastoid cell lines whereas different forms of latency are manifest in Burkitt’s lymphoma (EBNA1 only) and in Hodgkin’s disease and NPC (EBNA1, LMP1, and LMP2). These distinct forms of EBV latency appear to be a vestige of the pattern of latent gene expression used by the virus during the establishment of persistent infection within the B cell pool (2). Key to the ability of EBV to efficiently colonize memory B cells is the expression of LMP1 and LMP2 both of which provide essential survival signals. The aberrant adoption of these forms of latency can contribute to transformation as evidenced by expression of LMP1 and LMP2 in NPC and Hodgkin’s disease.

LMP1 is the major transforming protein of EBV behaving as a classical oncogene in rodent fibroblast transformation assays and being essential for EBV-induced B cell transformation in vitro (3, 4). LMP1 has pleiotropic effects when expressed in cells resulting in induction of cell surface adhesion molecules and activation antigens (5), up-regulation of anti-apoptotic proteins (Bcl-2, A20) (6, 7), and stimulation of cytokine production (interleukin-6 and interleukin-8) (8, 9). Recent studies have demonstrated that LMP1 functions as a constitutively activated member of the tumor necrosis factor receptor (TNFR) superfamily activating a number of signaling pathways in a ligand-independent manner (10, 11). Functionally, LMP1 resembles CD40, a member of the TNFR, and can partially substitute for CD40 in vivo providing both growth and differentiation responses in B cells (12).

The LMP1 protein is an integral membrane protein of 63 kDa and can be subdivided into three domains: (a) a NH2-terminal cytoplasmic tail (amino acids 1–23) that tethers and orientates the LMP1 protein to the plasma membrane, (b) six hydrophobic transmembrane loops that are involved in self-aggregation and oligomerization (amino acids 24–186), and (c) a long COOH-terminal cytoplasmic region (amino acids 187–386) that possesses most of the signaling activity of the molecule. Two distinct functional domains referred to as COOH-terminal activation regions 1 and 2 (CTAR1 and CTAR2) have been identified on the basis of their ability to activate the NF-κB transcription factor pathway (13). This effect contributes to the many phenotypic consequences of LMP1 expression including the induction of various anti-apoptotic and cytokine genes. LMP1 is also able to engage the mitogen-activated pro-
tein kinase cascade resulting in activation of extracellularly regulated protein kinase, c-Jun NH₂-terminal kinase, and p38 and to stimulate the JAK/signal transducers and activators of transcription pathway (9, 14–17). Many of these effects result from the ability of TNF-associated factors (TRAFs) to interact either directly with CTAR1 or indirectly via the death domain protein TRADD to CTAR2 (1). The binding of TRAFs to the multimerized cytoplasmic tails of LMP1 provides a platform for the assembly and activation of upstream signaling molecules including the NIK and Tpl-2 mitogen-activated protein kinase kinase kinases (18, 19). The precise mechanisms responsible for signal initiation from these multiprotein complexes remain unknown. The region between CTAR1 and CTAR2 (so-called CTAR3) has been suggested to be responsible for the JAK/signal transducers and activators of transcription pathway although other data refute this finding and deletion of this region has no effect of the efficiency of B cell transformation (17, 20).

The expression of LMP1 in NPC is associated with increased metastatic spread, an effect that is also reflected in the ability of LMP1 to induce increased cell motility and invasive growth when expressed in epithelial cells in vitro (21–25). Thus it appears that the transforming ability of LMP1 may be regulated by novel signaling pathways that, in addition to the NF-κB and mitogen-activated protein kinase cascades, may account for the ability of this molecule to influence cell motility and to provide a profound survival advantage. One pathway that fulfills these criteria is that mediated by phosphatidylinositol 3-kinase (PI3K), which via generation of specific phospholipids, activates a diverse range of cellular processes including cell growth, motility, adhesion, and survival (26).

The PI3K family of enzymes is activated by a wide range of extracellular growth and mitogenic stimuli including ligands of the TNFR family such as TNF-α and CD40, thus suggesting that LMP1 may also target this pathway (27, 28). PI3K-generated phospholipids are responsible for activating the Akt (PKB) kinase thereby promoting cell survival and for regulating the Rho family of small GTPases resulting in effects on the actin cytoskeleton and on cell signaling. Here we demonstrate that LMP1 can activate the PI3K/Akt pathway and that this effect is responsible for LMP1-induced actin polymerization, morphological transformation, and also contributes to cell survival. Furthermore, we show that the region of LMP1 previously identified as being essential for EBV-induced B cell transformation, CTAR1, is also responsible for PI3K activation.

MATERIALS AND METHODS

Cell Lines and the Establishment of Derivatives—293RcRLMP1 cells carrying an ecdysone-regulatable LMP1 gene have been described previously (9). 293RcRLMP1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin with 400 µg/ml G418. LMP1 expression was induced in 293RcLMP1 cells after the addition of 10 µM muristerone A (Invitrogen). HeLa cell medium and the cells incubated for an additional 24 h. Cells were microinjected with pSG5-based expression vectors containing wild-type LMP1 (pSG5-LMP1), or mutant LMP1 proteins carrying a defective CTAR1 domain (pSG5-LMP1AAA), defective CTAR2 domain (pSG5-LMP1AAA2786stop), or defective CTAR1 and CTAR2 domains (pSG5-LMP1AAA2786stop, or defective CTAR1 and CTAR2 domains (pSG5-LMP1AAA2786stop). These mutants have been described previously (9). For certain experiments, cells were co-microinjected with pSG5-based expression vectors containing wild-type LMP1 (pSG5-LMP1) and expression vectors carrying dominant negative forms of Cdc42 (pRK5-Myc-N17Cdc42), Rac (pRK5-Myc-N17Rac), or Rho (pRK5-Myc-N17Rho) (kind gifts from Professor Alan Hall, Ludwig Institute for Cancer Research, London, UK). Expression vectors containing a constitutively active PI3K (p110CAAX) or a dominant-negative p85 were kind gifts from Dr. Julian Downward, ICRF, London, UK. p110CAAX encodes the catalytic subunit of tyrosine kinase linked to a CAAX membrane targeting motif of Ras. Dominant-negative p85 encodes a wild-type p85 regulatory subunit deleted for the amino-terminal Src homology 2 domain.

To evaluate PI3K activation in microinjected cells, pSG5-based LMP1 expression vectors were co-microinjected with an expression vector carrying a green fluorescent protein (GFP)-tagged GRP1 PH domain fusion protein (29). Between 3 and 5 h after microinjection, cells were fixed in 4% paraformaldehyde and visualized under UV fluorescence for evidence of membrane targeting of the GFP tag. In each experiment, at least 200 cells were microinjected; each experiment was performed at least three times.

FIG. 1. The p85 regulatory subunit of PI3K associates with LMP1 in vivo. A, LMP1 immunoprecipitated from HeLa LMP1 clones carrying the CS1–4 antibody is detected with the LMP1-specific monoclonal antibody, S12. B, probing of the same immune complexes with a polyclonal antiserum to p85 demonstrated the presence of p85 in LMP1 immune complexes. C, p85 immunoprecipitated with the U13 monoclonal antibody confirmed reduced expression of p85 in LMP1 expressing cells. D, reciprocal immunoprecipitations confirmed the association of LMP1 with p85 in p85 immunoprecipitates.

bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. 5 × 10⁷–10⁸ cells were seeded onto each well of a Teflon-coated microscope slide (Hendley-Exess) in 100 µl of medium. After overnight attachment, the medium was replaced with serum-free medium and the cells incubated for an additional 24 h. Cells were microinjected with pSG5-based expression vectors containing wild-type LMP1 (pSG5-LMP1), or mutant LMP1 proteins carrying a defective CTAR1 domain (pSG5-LMP1AAA), defective CTAR2 domain (pSG5-LMP1AAA2786stop), or defective CTAR1 and CTAR2 domains (pSG5-LMP1AAA2786stop). These mutants have been described previously (9). For certain experiments, cells were co-microinjected with pSG5-based expression vectors containing wild-type LMP1 (pSG5-LMP1) and expression vectors carrying dominant negative forms of Cdc42 (pRK5-Myc-N17Cdc42), Rac (pRK5-Myc-N17Rac), or Rho (pRK5-Myc-N17Rho) (kind gifts from Professor Alan Hall, Ludwig Institute for Cancer Research, London, UK). Expression vectors containing a constitutively active PI3K (p110CAAX) or a dominant-negative p85 were kind gifts from Dr. Julian Downward, ICRF, London, UK. p110CAAX encodes the catalytic subunit of tyrosine kinase linked to a CAAX membrane targeting motif of Ras. Dominant-negative p85 encodes a wild-type p85 regulatory subunit deleted for the amino-terminal Src homology 2 domain.

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Immunostaining of Microinjected Cells for LMP1 and Actin—Between 3 and 5 h after microinjection, cells were fixed and permeabilized with 0.5% Triton X-100. Cells were immunostained for LMP1 expression using the LMP1-specific monoclonal antibody OTT22C (a kind gift from Dr. J. Middledorp, Netherlands) and Oregon Green-conjugated goat anti-mouse IgG (Molecular Probes). F-actin was visualized using a 1/400 dilution of TRITC-labeled phalloidin (Sigma).

Immunoprecipitations and Western Blotting—Subconfluent cultures were lysed in an Nonidet P-40-based lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 0.1 mM sodium orthovanadate, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). 400 μg of cleared total cell lysate was immunoprecipitated with either 15 μl of anti-p85 (Sero tec) or 25 μl of anti-LMP1 (C51-4). Samples were incubated at 4 °C for 2 h, and immune complexes were washed with a further 2-h incubation with protein A-agarose (Sigma). After 4 washes in cold lysis buffer, the beads were resuspended in 50 μl of gel sample buffer and boiled for 5 min. Immunoprecipitated proteins were separated on 7.5% polyacrylamide gels and proteins transferred to nitrocellulose. After blocking, membranes were probed with a monoclonal antibody to p105 (S12), or a rabbit polyclonal antibody to p85 (Upstate Biotechnology). Bound antibodies were detected using anti-mouse horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham Biosciences).

Akt Pathway—PI3K activity in p85 immunoprecipitates was analyzed in vitro by lipid kinase assay using phosphatidylinositol as a substrate (*, average of duplicate determinations). B, total cell lysates from the same panel of clones were analyzed for evidence of Akt phosphorylation after immunoblotting with a rabbit polyclonal antibody specific for the Ser-473 phosphorylated form of Akt. Akt kinase activity was assessed in the presence or absence of 20 μM LY294002, a selective inhibitor of PI3K.

Akt Kinase Assays—PI3K activity in p85 immunoprecipitated overnight with immobilized mouse anti-Akt IgG. After washing in two changes of lysis buffer and three changes of kinase assay buffer, the beads were resuspended in kinase assay buffer containing 1 μg of GSK3 peptide substrate and 10 μM ATP. After incubation at 30 °C for 30 min, the reaction was stopped with the addition of 20 μl of gel sample buffer. Supernatants were resolved by SDS-PAGE and proteins transferred to nitrocellulose. After blocking, membranes were probed with a phospho-GSK3 specific antibody (Cell Signaling Technology). Bound antibody was detected using anti-mouse horseradish peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham Biosciences).
Cell Viability and Apoptosis Assays

Cell viability was measured using the AlamarBlue™ Assay (Serotec). Briefly, cells were plated out at 5 × 10^3 cells/well in triplicate on a 96-well plate and left overnight. Cell viability was analyzed at 72 h post-treatment with or without 20 μM LY294002. AlamarBlue™ was added aseptically at 10% of the culture volume, and the cells were incubated for 4 h at 37°C. Fluorescence was measured with excitation wavelength at 560 nm and emission wavelength at 590 nm. For detection of apoptosis, cells were stained with acridine orange. Vector control or LMP1-expressing clones were seeded into each well of a 6-well plate at a concentration of 5 × 10^5 cells per well. 24 h later, medium was replaced with serum-free medium containing 20 μM LY294002 or Me2SO vehicle control. Cells were cultured for an additional 72 h after which time they were recovered as single cell suspensions. Cells were examined for evidence of apoptosis after incubation of cell suspensions with 5 μg/ml acridine orange followed by microscopic analysis.

RESULTS

LMP1 Interacts with p85, the Regulatory Subunit of PI3K—

The recent demonstration that a number of TNFR family members such as CD40, TNFR1, and TRANCE-R engage the PI3K/Akt signaling pathway prompted us to investigate whether LMP1 could also activate this pathway. For both CD40 and the TRANCE receptor, the regulatory subunit of PI3K, p85, is recruited to the activated receptor complex after receptor ligation (31, 32). Given that LMP1 behaves as a constitutively activated TNF receptor, it was reasoned that PI3K would associate with LMP1 in a constitutive manner. Thus immunoprecipitation experiments were performed in HeLa cells stably expressing LMP1 to determine whether PI3K formed part of the LMP1 signaling complex (Fig. 1). LMP1 was efficiently immunoprecipitated from HeLa cells (Fig. 1A) and these immunoprecipitates were found to contain a significant proportion of p85 when probed with a specific p85 antibody (Fig. 1B). The reciprocal analysis using p85 immunoprecipitates confirmed the interaction with LMP1 (Fig. 1D) and demonstrated a diminished ability to immunoprecipitate p85 in LMP1-expressing cells (Fig. 1C). This may result from the LMP1-p85 interaction reducing the efficiency of p85 antibody binding as total cellular levels of p85 were unaltered in LMP1-expressing cells (data not shown).

LMP1 Expression Results in Constitutive Activation of the PI3K/Akt Pathway—

To directly examine the ability of LMP1 to activate PI3K, the basal level of PI3K activity was measured in three independent HeLa clones stably expressing LMP1. These cells were serum-starved for 24 h and the ability of immunoprecipitated PI3K (p85 immunoprecipitates) to phosphorylate PI, assayed by lipid kinase assay. As shown in Fig. 2A, the basal levels of PI3K activity were up to 2-fold greater in LMP1-expressing cells compared with two vector control transfectants. The elevated PI3K activity in LMP1-expressing cells correlated with activation of Akt as determined using a phosphospecific antibody that recognizes Akt phosphorylated on serine 473 (Fig. 2B). To confirm that LMP1 expression resulted in activation of Akt, in vitro kinase assays were performed using Akt immunoprecipitates and a GSK3 peptide as substrate. These assays demonstrated significantly higher levels of

![Graph A](image)

**Fig. 3.** PI3K activity and Akt phosphorylation coincide temporally with the induction of LMP1 in 293 cells. 293 RcR cells carrying an ecdysone-regulatable LMP1 gene were serum-starved for 24 h prior to incubation with 10 μM Muristerone A to induce expression of LMP1. At selected time points, cell lysates were prepared and assayed for LMP1 expression, after immunoblotting with the CS1-4 pool of antibodies specific for LMP1 (A), PI3K activity by in vitro lipid kinase assay (B), p85 phosphorylation, after immunoprecipitation of p85 and subsequent bloting with a phosphotyrosine-specific antibody (C), and phosphorylation of Akt after immunoblotting with a rabbit polyclonal antibody specific for the serine 473-phosphorylated form of Akt (D).
basal Akt activity in LMP1-expressing HeLa cells compared with vector controls and this activity was inhibited by the LY290042 compound confirming the dependence of this effect on PI3K (Fig. 2C). Identical results were obtained using A431 cells stably expressing LMP1 (data not shown).

Transient Induction of LMP1 Expression Results in Activation of the PI3K/Akt Pathway—The constitutively active nature of LMP1 can obscure the impact of this protein on cell signaling pathways. To overcome this problem, an ecdysone-inducible system was generated to provide regulatable expression of LMP1. In this system, binding of the steroid hormone Muristerone A to a hybrid ecdysone-modified retinoic acid receptor allows the subsequent activation of an ecdysone-responsive target gene. HEK 293 cells carrying the pVgRXR plasmid (293EcR), which encodes the receptor subunits, and pIND-LMP1, which contains the LMP1 cDNA under the control of ecdysone-responsive elements, were generated. A tightly regulatable clone was identified that demonstrated induction of LMP1 expression after 6 h of Muristerone A treatment with steadily increasing LMP1 levels up to 48 h post-treatment and no LMP1 expression in cells not treated with the hormone (Fig. 3A). This induction of LMP1 expression correlated temporally with increased PI3K activity which at 6–48 h post-treatment was 2–3-fold higher than that observed in non-LMP1 expressing cells (Fig. 3B). Consistent with this increase in PI3K activity, induction of LMP1 resulted in phosphorylation of p85, which was evident as early as 3 h after induction and remained elevated at 12 h (Fig. 3C). Likewise, Akt activity as determined by phosphorylation at serine 473 was induced after 3 h and increased steadily over 24 h in concert with PI3K activity (Fig. 3D). To rule out the possibility that hormone treatment of 293 cells resulted in PI3K/Akt activation, parental 293 RXR cells lacking pIND LMP1 were serum-starved prior to treatment with 10 μM LY294002 and, 60 min after cross-linking, assayed for Akt phosphorylation by Western blotting using a rabbit polyclonal antibody specific for the Ser473-phosphorylated form of Akt.

Membrane Oligomerization of LMP1 Is Required for PI3K Activation—To determine whether membrane oligomerization of LMP1 is an essential requirement for PI3K activation, we used an expression system in which a chimeric RatCD2-LMP1 chimera can be induced to signal after the addition of antibodies to CD2 and subsequent cross-linking with anti-mouse IgG (9). As the chimeric molecule lacks the amino terminus and transmembrane spanning regions of LMP1, this approach also allowed us to assess the contribution of the cytosolic COOH terminus to LMP1-mediated Akt activation. Treatment of Rat-1 cells stably expressing the Rat CD2:LMP1 chimera with the anti-CD2 mAb (OX34) and cross-linking anti-mouse immunoglobulin G induced Akt phosphorylation within 30 min and this continued to increase for up to 24 h (Fig. 4A). Consistent with the increase in Akt activity, an antibody specific for the phosphorylated form of GSK-3

![Fig. 4. LMP1-induced activation of PI3K/Akt requires the cytosolic COOH terminus of LMP1.](image-url)
(p-GSK3) demonstrated phosphorylation of this Akt substrate after CD2 engagement (Fig. 4B) and this effect was further confirmed by in vitro kinase assays (Fig. 4C). Pretreatment of Rat CD2LMP1-expressing cells with LY294002 significantly reduced Akt phosphorylation induced by CD2 engagement (Fig. 4D). To rule out the possibility that antibody treatment alone of cells could contribute to PI3K/Akt activation, a neomycin-resistant Rat-1 cell clone, Rat-1 neo clone 2, was incubated in the presence of the Rat CD2 monoclonal antibody and subsequent cross-linking with anti-mouse IgG. Unlike the Rat CD2LMP1-expressing cells, treatment of the Rat-1 neo cells with anti-CD2 and anti-mouse IgG failed to induce either PI3K activity or Akt phosphorylation (data not shown). These data demonstrate that LMP1 oligomerization is required for activation of the PI3K/Akt pathway and that this activation is mediated by the LMP1 cytoplasmic domain.

**The CTAR1 Domain of LMP1 Is Responsible for PI3K Activation**—To identify the domain within the LMP1 cytoplasmic tail that is responsible for PI3K/Akt activation, we made use of an expression vector, pH-GRP1-GFP, comprising GFP fused to the PH domain of the GRP1 protein. The PH domain of GRP1 is a specific receptor for 3′-phosphorylated phosphoinositide lipids (phosphatidylinositol 1,4,5-trisphosphate (PIP3) and phosphatidylinositol 3,4-bisphosphate (PI3-4P2)) generated as a consequence of PI3K activity and, as a GFP fusion, can be used to monitor the PI3K activity at the cellular level (29).

Swiss 3T3 cells co-microinjected with pSG5 and pH-GRP1-GFP showed a diffuse cytoplasmic distribution (Fig. 5A) whereas cells co-microinjected with a constitutively activated form of the catalytic subunit of PI3K, p110CAAX, showed clear plasma membrane association resulting from translocation of the GFP-tagged fusion protein to sites of PI3K activity at the plasma membrane (Fig. 5B). Co-microinjection of wild-type LMP1 resulted in translocation of GRP1-GFP to the plasma membrane confirming that the expression of wild-type LMP1 resulted in 3′-phosphoinositide generation in cell membranes (Fig. 5C). To identify the domain of LMP1 required for PI3K activation, co-microinjection experiments were performed with LMP1 mutants defective for the CTAR1 and CTAR2 effector domains. Co-expression of the CTAR1 mutant pSG5LMP1AAA did not result in GRP1-GFP translocation to the cell membrane implicating CTAR1 as the domain of LMP1 responsible for this response (Fig. 5D). This was confirmed with the CTAR2 mutant, pSG5LMP1Stop, where co-expression resulted in translocation of the GFP-tagged GRP1 fusion protein to the plasma membrane (Fig. 5E).

**FIG. 5.** LMP1-mediated PI3K activation is mediated through the CTAR1 domain of LMP1. To assay PI3K activity in vivo, quiescent 3T3 fibroblasts were co-microinjected with a GRP1-PH-GFP fusion protein and pSG5 (A), p110CAAX (B), wild-type LMP1 (C), LMP1AAA (D), or LMP1Stop (E). 3–5 h after microinjection, cells were fixed in 4% paraformaldehyde, and evidence of PI3K activity (3′-phosphoinositide generation) as demonstrated by membrane targeting of the GFP- PH-GRP1 fusion protein was established for each protein. A representative photomicrograph is shown for each plasmid. In each case, between 200 and 250 cells were microinjected per experiment. Bar represents 30 μm.

**FIG. 6.** LMP1-mediated stress-fiber formation is mediated through the small Rho GTPases. To investigate the role of the small Rho GTases in LMP1-mediated actin stress-fiber formation, quiescent Swiss 3T3 fibroblasts were co-microinjected with LMP1 and empty vector (A and B) or dominant negative forms of Rho (C and D), Rac (E and F), and Cdc42 (G and H). LMP1 expression (A, C, E, and G) was visualized after fixing and staining with the CS1–4 pool of antibodies and Oregon Green-conjugated anti-mouse IgG, whereas actin stress-fiber formation was visualized after incubation with rhodamine-conjugated phalloidin (B, D, F, and H). A representative photomicrograph is shown for each plasmid. In each case, between 200 and 250 cells were microinjected per experiment. Bar represents 30 μm.
membrane (Fig. 5E). The CTAR1/CTAR2 double mutant pSG5LMP1AAA/378Stop failed to induce GRP1-GFP translocation consistent with the role of CTAR1 in this effect (data not shown).

**LMP1-induced Actin Polymerization Requires PI3K Activity and Is Regulated by the Small Rho GTPases**—In addition to activating downstream targets such as Akt, 3-phosphoinositide-dependent protein kinase 1 (PDK1), and protein kinase C, PI3K also plays a key role in regulating actin cytoskeletal organization through activation of the small Rho GTPases. The lipid products generated by PI3K regulate the activity of a number of guanine nucleotide exchange factors for the small Rho GTPases. The recent demonstration that LMP1 induces filopodia and actin stress-fiber formation (33) prompted us to examine whether the induction of PI3K by LMP1 resulted in actin polymerization. In agreement with our previous findings, microinjection of LMP1 resulted in robust actin stress-fiber formation (Fig. 6, A and B). Co-microinjection of LMP1 with dominant-negative Rho (N17Rho), completely inhibited actin stress-fiber formation (Fig. 6, C and D), demonstrating the requirement for Rho in this response. Similarly, co-microinjection of dominant negative Cdc42 (N17Cdc42) almost completely abrogated LMP1-induced actin stress-fiber formation (Fig. 6, E and F) whereas dominant-negative Rac (N17Rac) partially blocked this effect (Fig. 6, G and H).

To establish a role for PI3K in LMP1-mediated actin stress-fiber formation, two approaches were employed. First, LMP1 was microinjected into cells that were then treated with the LY294002 compound. Compared with cells microinjected with LMP1 alone (see Fig. 6A), cells treated with LY294002 showed very little stress-fiber formation (Fig. 7B) although there was clear expression of the LMP1 protein (Fig. 7A). In other experiments LMP1 was co-microinjected with a dominant-negative form of p85 (#H9004Np85) that is deleted for the amino-terminal Src homology 2 domain and thus competes for binding of the catalytic p110 subunit. Co-microinjection of LMP1 with #H9004Np85 resulted in complete inhibition of actin stress-fiber formation (Fig. 7, C and D). Consistent with this observation, co-microinjection of LMP1 with N17Ras also inhibited LMP1-mediated actin stress-fiber formation (Fig. 7, E and F) presumably via the ability of N17Ras to sequester p85-p110 complexes rendering them unavailable for recruitment to activated receptors. Unlike the wild-type LMP1 protein (Fig. 8, A and B), microinjection of the CTAR1-deletion mutant pSG5LMP1AAA failed to induce actin stress-fiber formation (Fig. 8, C and D), whereas microinjection of the CTAR2 mutant, pSG5LMP1378Stop, resulted in robust stress-fiber formation (Fig. 8, E and F). This result is consistent with the ability of CTAR1 to stimulate PI3K activity thus substantiating the role of PI3K in LMP1-induced stress-fiber formation. Microinjection of the CTAR1/CTAR2
double mutant pSG5LMP1 AAA/378Stop failed to induce actin stress-fiber formation (Fig. 8, G and H) demonstrating that the amino terminus, transmembrane spanning regions, and the repeat region between CTAR1 and CTAR2 do not contribute to this effect.

Role of LMP1-induced PI3K Activity in Morphological Transformation and Cell Survival—Previous work demonstrating that PI3K activation is responsible for the morphological transformation of Rat-1 fibroblasts (34) prompted us to examine whether PI3K activation plays a role in the morphological transformation of Rat-1 fibroblasts by LMP1. Vector control and LMP1-expressing Rat-1 cells were cultured in the presence or absence of 20 μM LY294002 for 18–24 h, and the morphology of the cells subsequently examined by phase microscopy. Whereas treatment of vector control cells with LY294002 was associated with slight growth inhibition (Fig. 9, A and B), it was not associated with an alteration in cell morphology. In marked contrast, treatment of LMP1-expressing cells with LY294002 resulted in a profound alteration in cell morphology (Fig. 9 D). Whereas control untreated LMP1 expressing cells cultured showed evidence of reduced contact inhibition and cell-rounding (Fig. 9C), cells cultured in the presence of 20 μM LY294002 showed a clear-cut reversal of the transformed phenotype, with cells re-acquiring a flatter morphology (Fig. 9D).

To investigate the contribution of PI3K activation to cell survival, vector control and LMP1-expressing Rat-1 cells were cultured in the presence or absence of LY294002 under serum-free conditions. Whereas vector control clones showed only minimal evidence of cell death after treatment (Fig. 10, A and C), prolonged treatment of LMP1-expressing cells with 20 μM LY294002 resulted in a profound decrease in cell viability (Fig. 10, B and D). To investigate this phenomenon further, a panel of vector control and LMP1-expressing clones were treated with increasing concentrations of LY294002, and the effects on cell survival after serum withdrawal were analyzed in a cell viability assay. Increasing concentrations of LY294002 resulted in a 20–50% decrease in cell number in vector control clones, attributable to the effects of PI3K inhibition on cell proliferation (Fig. 10E). In contrast, Rat-1 cells expressing LMP1 were clearly more sensitive to PI3K inhibition, with cell numbers being reduced by 85–90%. To confirm that the cytotoxicity observed after LY294002 treatment was because of apoptosis, suspensions of control and LY294002-treated cells were suspended in a solution containing 5 μg/ml acridine or-

![Fig. 8. LMP1-mediated actin stress-fiber formation maps to the CTAR1 domain of LMP1.](image)

To determine the domain of LMP1 required for LMP1-mediated actin stress-fiber formation, quiescent Swiss 3T3 fibroblasts were co-microinjected with LMP1 (A and B) and a panel of LMP1 mutants defective for CTAR1 (C and D), CTAR1 (E and F), or both CTAR1 and CTAR2 (G and H). LMP1 expression (A, C, E, and G) was visualized after fixing and staining with the CS1–4 pool of antibodies and Oregon Green-conjugated anti-mouse IgG, whereas actin stress-fiber formation was visualized after incubation with rhodamine-conjugated phalloidin (B, D, F, and H). A representative photomicrograph is shown for each plasmid. In each case, between 200 and 250 cells were microinjected per experiment. Bar represents 30 μm.

![Fig. 9. LMP1-induced transformation of Rat-1 fibroblasts requires PI3K activity.](image)

Vector control (A and B) or LMP1-expressing (C and D) clones were incubated in the presence (A and C) or absence (B and D) of the PI3K inhibitor LY294002 for 48 h, and cell morphology recorded by phase-contrast microscopy. Magnification ×400.
LMP1 Activates the PI3K/Akt Pathway

We have shown that LMP1 can activate the PI3K/Akt pathway and that this is responsible for some of the phenotypic effects of LMP1 associated with cell transformation. This result is not surprising given the similarities between LMP1 signaling and that elicited from the TNF receptor family, members of which can activate PI3K. Thus, TNF-α has been shown to activate PI3K and Akt and this effect was reported to be required for activation of NF-κB (35, 36). Inhibition of PI3K was found to potentiate TNF-α-induced apoptosis, an effect reminiscent of that observed in this study when LMP1-expressing cells were treated with the LY294002 inhibitor. LMP1 most closely resembles an activated CD40 in its phenotypic effects and LMP1 can partially correct the B cell development defect in CD40-deficient mice (12). It has thus been suggested that this ability to mimic CD40 is required for EBV to efficiently colonize the B cell pool and establish persistence in the healthy host (2). Previous studies have demonstrated that CD40 ligation can activate PI3K and Akt in B cells and that this effect is important for both cell proliferation and survival (27, 32). Mice deficient in the p85 subunit of PI3K are severely impaired in B cell development with reduced proliferative responses to CD40 ligation (37, 38). Taken together these data highlight the role of the PI3K pathway in B cell growth and suggest that the ability of LMP1 to activate this pathway contributes to EBV persistence in B cells.

An understanding of the signaling capacity of LMP1 is key to defining its role in EBV-induced oncogenesis and in identifying pathways that may be of general significance in the transformation process. As the only EBV-encoded protein with the characteristics of a classical oncogene, the signaling pathways engaged by this molecule are clearly crucial in effecting B cell transformation and in inducing a plethora of phenotypic effects relevant to cellular transformation. Whereas previous work has focused on the contribution of the NF-κB and mitogen-activated protein kinase pathways to LMP1-induced effects, we now demonstrate that the PI3K/Akt pathway is also activated by LMP1 and that this may account for some of the more profound oncogenic properties of the protein. This observation supports our previous work suggesting that certain aspects of LMP1 behavior in epithelial cells resemble those induced by an activated ras gene that also functions via activation of PI3K (39). Using co-immunoprecipitation experiments we have demonstrated that LMP1 associates with the p85 regulatory subunit of PI3K, thus showing that PI3K is recruited to, and forms part of, an LMP1 signaling complex. That this association results in PI3K activation is evidenced by increased basal lipid kinase activity in cells stably expressing LMP1 and by the ability of inducible LMP1 expression to also stimulate PI3K activity. Although PI3K associates with the LMP1 signaling complex, the exact nature of this interaction is currently unknown. The finding that a dominant-negative form of p85, deleted for the amino-terminal Src homology domain, can partially correct the B cell development defect in CD40– mice suggests that the LMP1-p85 association may be mediated through a novel interaction(s). Use of the chimeric CD2-LMP1-(192–386) molecule demonstrated that the cytosolic carboxyl terminus of LMP1 and not the amino terminus and transmembrane spanning regions was responsible for PI3K and Akt activation. To further define the LMP1 domains responsible for PI3K activation, a GFP-tagged GRP1 fusion protein that has high affinity and specificity for phosphatidylinositol 3,4,5-triphosphate was used in microinjection studies (29). This approach showed that the CTAR1 domain of LMP1 is responsible for mediating PI3K activation. This finding identifies the TRAF binding region of LMP1 as the mediator of PI3K activation and suggests that PI3K recruitment to LMP1 is mediated through a TRAF molecule or an as yet unidentified adaptor protein.

The ability of LMP1 to activate PI3K helps to explain a
number of the phenotypic consequences of its expression in different cellular environments. PI3K plays a key role in regulating actin cytoskeletal organization and cell shape remodeling by regulating the activity of the small Rho GTPases (40, 41). We recently reported that LMP1 induces filopodial extensions associated with lamellopodia and stress fibers in Swiss 3T3 cells (33). In this report we show that LMP1 can induce robust actin stress-fiber formation that is dependent on the small Rho GTPases and on PI3K. Through the use of a panel of LMP1 mutants, we have identified CTAR1 as the region of LMP1 essential for actin stress-fiber formation and this correlates with the ability of CTAR1 to mediate PI3K activation. In our earlier study (33) we demonstrated that Cdc42-induced filopodia formation was mediated through the transmembrane spanning regions of LMP1 whereas here, Rho-mediated stress-fiber formation requires CTAR1-generated signals. Although, Cdc42-induced filopodia formation was not investigated in this current study, the possibility that LMP1 may activate both Cdc42 and Rac/Rho independently through distinct mechanisms clearly warrants further examination. An important consequence of these effects on actin filament remodeling and cell shape change is the ability of LMP1 to induce morphological transformation in Rat-1 cells. Interesting, this phenotype could be reversed by the LY294002 PI3K inhibitor suggesting that PI3K-generated signals are an important component in LMP1-mediated cell transformation. Future studies will elucidate the precise mechanisms involved in this process and the relative contributions of the small Rho GTPases. The significance of these observations for the function of LMP1 in EBV-infected epithelial cells and B cells is worthy of consideration. Previous studies have demonstrated that LMP1 expression in NPC is associated with more advanced tumors and with increased metastatic spread (22, 24, 25). These observations concur with in vitro data showing that LMP1 induces morphological transformation as well as a more motile and invasive phenotype in Madin-Darby canine kidney cells (23).2 Interestingly these effects of LMP1 were mapped to the CTAR1 region that we have now shown to be responsible for PI3K activation. The activation and migration of B cells also requires small Rho GTPase-dependent cytoskeletal changes that are crucial for the formation of germinal centers (42, 43). Thus the ability of LMP1 to induce actin reorganization is likely to contribute to the establishment of EBV persistence in the memory B cell pool.

The role of Akt in cellular growth transformation is now clearly established (44). Akt has emerged as a critical signaling molecule that regulates a variety of cellular processes including cell growth, proliferation, and apoptosis. Although Akt is implicated in the regulation of various metabolic events, it is its role in regulating aspects of cell survival that has received most attention. Akt targets and inactivates a number of pro-apoptotic molecules associated with the induction of apoptosis (45). These include the pro-apoptotic Bcl-2 family member, Bad, caspase 9, and GSK3 among others (46). The ability of Akt to modulate these key effector molecules explains how cytokines and growth factors are able to promote cell survival in response to growth factor or serum withdrawal. The recent finding that Akt is able to induce NF-κB activation by directly phosphorylating and activating IκB kinase suggests that in addition to directly inactivating pro-apoptotic effector molecules, Akt may contribute to the suppression of apoptosis by activating NF-κB. Although this phenomenon appears to be cell type-specific, it points to the ability of Akt to provide an additional level of protection from apoptotic stimuli through the activation of NF-κB. Our findings that PI3K inhibition results in robust apoptosis in LMP1-expressing cells implies that LMP1-generated signals activate signal transduction pathways that generate pro-apoptotic signals and that the PI3K/Akt pathway serves to counteract this effect. The recent demonstration that PI3K negatively regulates the activity of c-Jun NH2-terminal kinase and signal transducers and activators of transcription (47) points to a role for these factors in LMP1-associated cytotoxicity. It is possible that PI3K/Akt plays a role in LMP1-mediated NF-κB activation and that the apoptosis observed in LY294002-treated cells is a result of NF-κB down-regulation. However, our preliminary work indicates that LY294002 treatment does not affect the ability of LMP1 to induce NF-κB in both Rat-1 fibroblasts or in HeLa cells stably expressing LMP1 (data not shown). These data suggest that in addition to the ability of LMP1 to up-regulate Bcl-2 in B cells (6), Akt may provide another anti-apoptotic pathway activated by LMP1 that contributes to the survival of EBV-infected cells. This

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2 C. W. Dawson, G. Tramountanis, A. G. Eliopoulos, and L. S. Young, unpublished observations.
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possibility is supported by recent work demonstrating that PI3K plays a role in both the survival and proliferation of EBV-transformed B cells (48). These studies also identify PI3K as an attractive target for the development of novel approaches for the treatment of EBV-associated tumors.

Given the profound effects of activating PI3K on cell survival and proliferation, it is not surprising that a diverse range of viruses have evolved to target this pathway for the efficient promotion of virus infection and replication. This is particularly evident for tumor viruses where oncoproteins such as LMP1 as an attractive target for the development of novel approaches for the treatment of EBV-associated tumors.

Indeed, PI3K activity was first discovered particularly evident for tumor viruses where oncoproteins such as LMP1 as an attractive target for the development of novel approaches for the treatment of EBV-associated tumors.

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