Latent Membrane Protein 1 of Epstein-Barr Virus Activates the hTERT Promoter and Enhances Telomerase Activity in B Lymphocytes

Liliana Terrin, Jessica Dal Col, Enrica Rampazzo, Paola Zancai, Moreno Pedrotti, Grazia Ammirabile, Stefano Bergamin, Silvana Rizzo, Riccardo Dolcetti, and Anita De Rossi

Unit of Viral Oncology, Department of Oncology and Surgical Sciences, IOV-IRCCS, Padova, and Cancer Bioimmunotherapy Unit, Department of Medical Oncology, CRO-IRCCS, National Cancer Institute, Aviano, Italy

Received 14 February 2008/Accepted 25 July 2008

Transformation of primary B lymphocytes by Epstein-Barr virus requires the establishment of a strictly latent infection, the expression of several latent viral proteins, and sustained telomerase activity. Our previous findings indicated that induction of hTERT, the rate-limiting catalytic unit of the telomerase complex, was associated with the expression of the viral latent membrane protein 1 (LMP1). In the present study, we demonstrate that ectopic expression of LMP1 in BJAB and Ramos B cells resulted in an increase of hTERT transcripts, thus suggesting that LMP1 acts at the transcriptional level. This was confirmed by transient expression of a luciferase reporter plasmid containing the hTERT promoter cotransfected with an LMP1-expressing vector or transected into B cells in which LMP1 expression was inducible. Consistently, silencing of LMP1 by small interfering RNA resulted in a reduction of hTERT transcripts. We also provide evidence indicating that LMP1-induced hTERT activation is independently mediated by NF-κB and by mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 pathways, whereas CD40, Akt, and mTOR signaling has no involvement. Moreover, our results do not support a role for c-Myc in mediating these effects on hTERT, since ectopic expression of LMP1 did not upregulate c-Myc and silencing of this oncogene or E box mutagenesis failed to inhibit LMP1-induced hTERT activation. These findings indicate that LMP1 simultaneously modulates multiple signal transduction pathways in B cells to transactivate the hTERT promoter and enhance telomerase activity, thus confirming the pleiotropic nature of this viral oncoprotein.

Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus that establishes a life-long asymptomatic infection in immunocompetent hosts by colonizing memory B lymphocytes. EBV has a potent transforming ability, being able to efficiently induce blast transformation and uncontrolled proliferation of infected B lymphocytes in vitro. Available evidence, particularly the presence of EBV genomes and the constant expression of viral proteins, strongly supports a relevant role for EBV in the pathogenesis of a wide spectrum of human malignancies, most of which are derived from B lymphocytes (12, 44). Latently EBV-infected B cells may express a defined set of latency genes that include those encoding six nuclear antigens (EBNAs) and three latent membrane proteins (latent membrane protein 1 [LMP1], LMP2A, and LMP2B). Among the EBV latency gene products, LMP1 is considered the strongest oncoprotein, being essential for immortalization of B cells. The N terminus and the six transmembrane domains of the protein form aggregates in the cytoplasmic membrane, allowing LMP1 to act like a constitutively activated receptor (5, 15). Indeed, LMP1 shares functional properties with members of the tumor necrosis factor (TNF) receptor superfamily, particularly CD40, and induces the expression of NF-κB through activation of the TNF receptor-associated factor signaling pathway (31, 40). Consistent with this, it has been shown that LMP1 can partly restore the wild-type phenotype to mice deficient in CD40 (57). However, unlike the TNF receptor, LMP1 engages at least part of the CD40 pathway in a ligand-independent manner. In addition, LMP1 also activates other molecules affecting diverse signaling cascades, including c-Jun NH2-terminal kinase (62), p38 mitogen-activated protein kinase and extracellular signal-regulated kinases 1/2 pathways, Janus kinase (19), and phosphatidylinositol 3-kinase (9), thus behaving as a “pleiotropic” viral oncogene. The hijacking of multiple cellular signaling pathways by LMP1 likely contributes to the pathogenesis of most EBV-associated disorders through the simultaneous or sequential triggering of signals involved in the promotion of cell activation, growth, and survival.

Expression of latent EBV proteins does not suffice to fully immortalize EBV-infected B cells. In fact, only EBV-carrying B cells with sustained telomerase activity are truly immortalized, whereas telomerase-negative cells, although exhibiting a prolonged life span, eventually undergo cellular senescence and terminate their life span by shortening of telomeres (54). Telomerase, a ribonucleoprotein complex containing an internal RNA template (hTR) and a catalytic protein with a telomere-specific reverse transcriptase activity (hTERT), extends telomeres at the end of eukaryotic chromosomes, thus preventing cell senescence and death. While hTR is constitutively present in normal and tumor cells, hTERT is the rate-limiting component of the telomerase complex, and its expression correlates with telomerase activity (41). hTERT activity is repressed in somatic tissues, but both hTERT expression and telomerase activity are elevated in most human tumors (28, 35). Ectopic expression of hTERT in telomerase-negative hu-
man cells is associated with the extension of cellular life span, whereas inhibition of hTERT limits the growth of cancer cells (22). Furthermore, several pieces of data suggest that besides the maintenance of telomere length, hTERT is also involved in other cellular functions, including promotion of cell growth (6, 49) and survival (10, 39, 45), thus contributing to tumorigenesis by mechanisms independent of its ability to prevent telomere erosion (53).

We recently demonstrated that in early-passage EBV-infected B lymphocytes, activation of hTERT depends on the balance between latent and lytic EBV gene expression, with latent genes being positively associated with telomerase activation (55). We also showed that ectopic hTERT expression inhibits EBV replication and promotes the growth of primary B lymphocytes, suggesting that hTERT contributes to EBV-driven B-cell transformation by multiple pathways (55). Nevertheless, the mechanisms responsible for EBV-induced hTERT activation in B cells are still poorly elucidated. In the present study, we provide evidence indicating that, in B lymphocytes, LMP1 directly promotes the activation of telomerase by acting at the transcriptional level on the hTERT promoter. These effects are mediated by engagement of the NF-κB, MAPK, and ERK1/2 pathways.

MATERIALS AND METHODS

Cell lines. BJAB and Ramos are EBV-negative B-lymphoma cell lines, with c-Myc in germ line configuration (BJAB) or translocated (Ramos) (64). The BIABet-LMP1 and Ramos mT-LMP1 cell lines, which express LMP1 under the control of an inducible promoter, and their LMP1-negative counterparts (BIABet and Ramos mT) were used. BIABet and BIABet-LMP1 were maintained in standard medium supplemented with 1 μg/ml puromycin and 1 mg/ml Geneticin (G418; Gibco). For LMP1 induction, 1 or 10 ng/ml tetracycline (TC) was added to cell cultures, and cells were harvested after 48 h. Ramos mT and Ramos mT-LMP1 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS), 450 μg/ml hygromycin, and 0.5 mmol/liter ethylenediaminetetraacetic acid. For LMP1 induction, cells were washed with phosphate-buffered saline, 1 μmol/liter cadmium chloride was added, and cells were harvested after 24 h (20). BJAB and BJAB/LMP1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2% glutamine, 750 μg/ml of Geneticin. DG75 and DG75 (TA-LMP1) cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2% glutamine, 500 μg/ml hygromycin, and 1 mg/ml Geneticin. The addition of TC (1 μg/ml) was sufficient for LMP1 expression (55). HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. 4134 LCL cells were obtained by infecting peripheral blood from a healthy donor with the B95.8 EBV strain. These cells were used at passage 103 and were positive for hTERT and LMP1 expression, as previously reported (55). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

Northern blotting, reverse transcriptase PCR, and real-time PCR. Total cellular RNA was extracted from 1 × 106 to 5 × 106 cells by use of TRIzol reagent (Invitrogen). Contaminating genomic DNA was removed using a DNase I Amplicon grade kit (Invitrogen). The integrity of RNA was evaluated by visualizing the 18S and 28S RNAs through agarose gel electrophoresis, and the relative amount was determined by the Bio-Rad Bradford protein assay. Proteins were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Immunoblotting was performed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).
No-shift NF-κB p65 transcription factor DNA-binding activity assay. For nuclear protein extracts, cells were lysed with buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 5 µg/ml leupeptin, 1 µg/ml aprotinin, 0.5 mM PMSF) on ice for 30 min, 0.5% NP-40 was added, and the samples were vortexed for 1 min and centrifuged at 4,000 g for 20 s at 4°C. The supernatants (cytoplasmic fraction) were recovered, and the pellets (nuclear fraction) were resuspended in ice-cold extraction buffer C (20 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% [vol/vol] glycerol, 5 µg/ml leupeptin, 1 µg/ml aprotinin, 0.5 mM PMSF) and incubated on ice for 1 h. Nuclear extracts were recovered after centrifugation at 16,000 g for 20 min at 4°C. Protein concentrations were determined by the Bio-Rad Bradford protein assay. Ten micrograms of nuclear extract was used to analyze NF-κB p65 DNA-binding activity, using an EZ-Detect chemiluminescent transcription factor assay kit (Pierce Biotechnology).

RESULTS

LMP1 upregulates hTERT expression in B-lymphoma cell lines. As a first step, hTERT transcript levels and telomerase activity were analyzed in transformed B cells in which ectopic LMP1 expression was constitutive (BJAB/LMP1 cells) or inducible (Ramos cells) (Fig. 1A). BJAB cells contain a germ line nontranslocated c-Myc proto-oncogene, as usually occurs in lymphoblastoid cell lines (LCLs), and basally showed low levels of all hTERT transcripts (4.5 ± 40 hTERT-AT copies/10⁶ glyceraldehyde-3-phosphate dehydrogenase [GAPDH] copies) and the full-length hTERT transcript encoding func-
tional protein (40 ± 12 hTERT-FL copies/10^6 GAPDH copies) (Fig. 1B). Ramos cells carry a translocated c-Myc gene and showed >10-fold higher baseline levels of hTERT mRNA, consistent with the capacity of c-Myc to activate the hTERT promoter (Fig. 1B) (65). In both cellular systems, ectopic LMP1 expression was associated with induction/upregulation of the Bcl-2 protein (Fig. 1A). Constitutive LMP1 expression in BJAB cells resulted in a four- to fivefold increase in the levels of both hTERT-AT and hTERT-FL transcripts (Fig. 1B), as well as a marked enhancement of telomerase activity (Fig. 1C). A less striking increase in hTERT transcript levels and telomerase activity was also observed in Ramos cells expressing LMP1 under the control of an inducible promoter (Fig. 1B). While LMP1 mRNA was efficiently induced with a downregulation of LMP1 mRNA and protein (Fig. 2A and B). A nonsilencing, scrambled siRNA used at the same concentrations (200 and 400 nM) served as a negative control (Fig. 2A and B). Knocking down LMP1 resulted in a dose-dependent decrease in the level of hTERT transcripts (Fig. 2C). After 72 h of exposure to 400 nM anti-LMP1 siRNA, the levels of hTERT-FL mRNA were reduced >80% compared to those in control cells (Fig. 2C). Similar inhibition of hTERT mRNA was obtained when BJAB/LMP1 cells were exposed to 400 nM anti-LMP1 siRNA (not shown).

**FIG. 2.** siRNA knockdown of LMP1 decreases hTERT transcription. 4134 LCL cells were lipofected with different concentrations (200 nM and 400 nM) of anti-LMP1 siRNA (siLMP1) or control siRNA (siScramble) and analyzed after 48 and 72 h. (A) LMP1 (upper panels) and GAPDH (lower panels) mRNAs were analyzed by reverse transcription-PCR. (B) Expression of LMP1 protein was assessed by Western blotting. α-Tubulin expression was used for sample comparison. (C) hTERT-FL mRNA levels were quantified by real-time PCR with 4134 cells treated with siRNA LMP1 or control siRNA (siScramble). Means and SD (error bars) for three replicates are shown.

**SiRNA knockdown of LMP1 decreases hTERT transcription.** Silencing of LMP1 in the 4134 LCL cells by using a specific siRNA efficiently induced a downregulation of LMP1 mRNA and protein (Fig. 2A and B). A nonsilencing, scrambled siRNA used at the same concentrations (200 and 400 nM) served as a negative control (Fig. 2A and B). Knocking down LMP1 resulted in a dose-dependent decrease in the level of hTERT transcripts (Fig. 2C). After 72 h of exposure to 400 nM anti-LMP1 siRNA, the levels of hTERT-FL mRNA were reduced >80% compared to those in control cells (Fig. 2C). Similar inhibition of hTERT mRNA was obtained when BJAB/LMP1 cells were exposed to 400 nM anti-LMP1 siRNA (not shown).

**LMP1 transactivates the hTERT promoter in both epithelial and B cells.** The findings that LMP1 induced hTERT mRNA and that LMP1 silencing resulted in hTERT mRNA downregu-
lation prompted us to assess whether the hTERT promoter is regulated by LMP1. HeLa cells were cotransfected with phTERTpromoterLuc, expressing luciferase under the control of the hTERT promoter, and pcDNA3LMP1, which expresses the LMP1 protein, or the empty pcDNA3 vector as a control. As shown in Fig. 3A, the hTERT promoter was induced by ectopic LMP1 expression. We also observed that LMP1 induced hTERT promoter activity to a slightly lower level than that induced by ectopic expression of c-Myc, a known transcriptional regulator of hTERT (Fig. 3A). To verify the ability of LMP1 to induce the hTERT promoter in the B-cell background, the phTERTpromoterLuc plasmid was transfected into BJABtet-LMP1 and DG75 cells expressing LMP1 under the control of a TC-dependent promoter. In both cellular systems, LMP1 induction was associated with a strong activation of the hTERT promoter (Fig. 3B and C).

CD40 signaling is not involved in mediating the transcriptional activation of hTERT and the increase of telomerase activity induced by LMP1 in B cells. Considering that LMP1 engages at least part of the CD40 signaling pathway (31, 40, 57) and that CD40 activation may contribute to the induction of telomerase activity in B lymphocytes (25, 26, 63), we investigated whether the CD40 pathway could mediate LMP1-dependent induction of hTERT mRNA and telomerase activity in B cells. Treatment of BJAB cells (24 h) with recombinant human soluble CD40L (0.5 μg/ml), IL-4 (0.1 μg/ml), or their combination did not increase the levels of hTERT-AT and hTERT-FL transcripts (Fig. 4A), nor did it enhance telomerase activity (Fig. 4B). Activation of CD40 signaling in these cells was verified by upregulation of cell surface expression of ICAM-1/CD54 and Fas/CD95 (23; data not shown). These findings rule out the possibility that the effects induced by LMP1 on hTERT expression and telomerase activity are due to the ability of this viral protein to hijack CD40-dependent signaling.

The p42/p44 MAPK pathway contributes to LMP1-dependent induction of hTERT mRNA and telomerase activity. The LMP1 protein engages several signaling pathways that are involved in the induction of hTERT mRNA and/or telomerase activity. In particular, LMP1 is a potent stimulator of the activity of ERKs (36, 47), and it has been shown that MAPK and ERK1/2 are involved in the induction of hTERT expression and/or telomerase activity mediated by stress or growth stimuli (18, 38). Analysis of ERK1/2 activation in BJAB cells showed that LMP1 markedly enhanced the phosphorylation of ERK1/2 both in the presence and in the absence of serum, indicating that LMP1 activates the ERK1/2 pathway (Fig. 5A). Similar findings were observed in DG75 tTA-LMP1 cells (not shown). Pharmacologic inhibition of ERK1/2 by UO126 (Fig. 5B) resulted in dose- and time-dependent decreases in the levels of hTERT-AT and hTERT-FL transcripts and in telomerase activity in both BJAB and BJAB/LMP1 cells (Fig. 5C, D, and E). Notably, the inhibition was lower in BJAB/LMP1 cells than in parental BJAB cells, as a possible consequence of the higher levels of ERK1/2 activation and hTERT expression in LMP1 transfectants. These findings indicate that ERK1/2 is involved in the LMP1-dependent induction of hTERT mRNA and telomerase activity in B cells.

NF-κB but not Akt or mTOR signaling mediates LMP1-dependent induction of hTERT mRNA and telomerase activity. Previous studies have shown that LMP1 activates the phospha-
tidyinositol 3-kinase/Akt pathway (9), a cascade that regulates telomerase activity by both transcriptional and posttranscriptional mechanisms (29, 30, 68). Ectopic expression of LMP1 in BJAB cells (Fig. 6A) or in DG75 cells (not shown) did not increase the level of the phosphorylated, active form of Akt, which was instead lower than that in parental cells. Nevertheless, pharmacologic inhibition of Akt (SH5) (Fig. 6B) induced more pronounced decreases of hTERT mRNA and telomerase activity in parental BJAB cells than in LMP1 transfectants, despite the lower level of constitutive Akt activation in the latter cells (Fig. 6C and D). Pharmacologic inhibition of the mTOR kinase, a downstream target of Akt, induced only at high doses a slight decrease in the level of hTERT mRNA in both BJAB and BJAB/LMP1 cells, with no effect on telomerase activity (Fig. 6E and F). Considering that NF-κB is a downstream target of Akt (37, 43, 48, 52) and that activation of this transcription factor is crucial for LMP1-driven B-cell transformation (27, 40), we then investigated the role of NF-κB in LMP1-induced upregulation of hTERT mRNA and telomerase activity. NF-κB activation by LMP1 was demonstrated by the upregulation of the p50 and p52 subunits and the higher levels of pp65(Ser536) and pIkBα (Ser32) in BJAB/LMP1 cells than in parental BJAB cells (Fig. 7A), consistent with previous findings (2, 24). LMP1-induced NF-κB activation was also confirmed using a no-shift p65 transcription factor assay (Fig. 7B). Pharmacologic inhibition of NF-κB by BAY-11-7082 resulted in dose- and time-dependent decreases in the levels of the hTERT-AT and hTERT-FL transcripts and in telomerase activity in both BJAB and BJAB/LMP1 cells (Fig. 7C, D, and E). Similar dose- and time-dependent decreases of hTERT mRNAs and telomerase activity were also observed when NF-κB was inhibited by 6-amino-4-(4-phenoxyphenethylamino)quinazoline (not shown). Notably, the inhibition was lower in BJAB/LMP1 cells than in BJAB cells, a possible consequence of the higher levels of hTERT expression and NF-κB activation in LMP1 transfectants. NF-κB inhibition by BAY-11-7082 was confirmed by pIkBα downregulation and increased levels of the IkBα protein, as a likely consequence of its reduced degradation (Fig. 7F). These findings are consistent with a direct involvement of NF-κB in LMP1-mediated hTERT transcriptional activation. Experiments carried out with the SH5 Akt inhibitor confirmed that NF-κB is a downstream target of Akt in BJAB cells. In particular, this inhibitor decreased the phosphorylation of p65 at Ser536, downregulated the p50 and p65 proteins (not shown), and inhibited NF-κB activity, with a stronger effect in BJAB than in BJAB/LMP1 cells, where LMP1 enhances NF-κB activation (Fig. 7B). These results suggest that the downregulation of hTERT mRNA and telomerase activity observed after Akt inhibition is probably dependent on downstream effects mediated by the NF-κB transcription factor. Conversely, U0126 treatment had no effect on NF-κB activity, ruling out a possible cross talk between ERK1/2 and NF-κB in these cells (Fig. 7B).

**LMP1 does not engage c-Myc to activate hTERT.** The recent demonstration that LMP1 may upregulate hTERT expression through c-Myc activation in epithelial cells (66) prompted us to investigate the possible involvement of c-Myc in B lymphocytes. Northern blot analysis showed that ectopic expression of LMP1 in BJAB cells did not increase the level of c-Myc mRNA (not shown). We also analyzed the effects of c-Myc silencing by using a specific siRNA (Fig. 8A). A scramble siRNA was used as a negative control, whereas DG75 cells carrying a translocated c-Myc were used as a positive control (Fig. 8A). Silencing of c-Myc resulted in a strong inhibition of hTERT transcripts and telomerase activity in both DG75 and BJAB cells, while only a slight reduction of hTERT mRNA was observed in BJAB/LMP1 and DG75/LMP1 cells (Fig. 8B and C). These findings suggest that LMP1 acts as an hTERT transcriptional activator independently of c-Myc.
of c-Myc expression/activation. To verify the ability of LMP1 to induce the hTERT promoter independently of c-Myc and dependent on the NF-κB pathway, BJABtet-LMP1 cells were transfected with hTERTpromoterLucDM, containing mutations in the E box, or with a plasmid containing mutations in the NF-κB binding site. As shown in Fig. 9, disruption of the E box did not prevent LMP1-induced hTERT activation, thus confirming that LMP1 activates hTERT independently of c-Myc. In contrast, mutagenesis in the NF-κB binding site impaired LMP1-induced activation of the hTERT promoter (Fig. 9).

**DISCUSSION**

Telomerase activation is a critical step of EBV-driven cell immortalization. We recently demonstrated that activation of hTERT in early-passage EBV-infected B lymphocytes is associated with the engagement of the ERK1/2 pathway. This study further elucidates the molecular mechanism by which LMP1 induces hTERT expression, highlighting the interplay between NF-κB and c-Myc in the regulation of telomerase activity.
associated with a progressive increase in LMP1 expression and inhibition of viral lytic replication (55). The observation that LMP1 may activate telomerase activity in nasopharyngeal carcinoma cells (11, 66) prompted us to investigate the effects of LMP1 on hTERT expression and telomerase activation in the B-cell system. Here we provide evidence that ectopic expression of LMP1 in different B-cell backgrounds induces a dose-dependent increase of hTERT mRNA and telomerase activity. Consistently, inhibition of LMP1 expression by siRNA reduced the levels of hTERT transcripts, again in a dose-dependent fashion. Moreover, cotransfection experiments demonstrated that LMP1 transactivates the hTERT promoter in both carcinoma (HeLa) cells and transformed B lymphocytes (BJAB and DG75 cells). Overall, these findings indicate that LMP1 directly promotes the activation of hTERT in B cells by acting at the transcriptional level. In this respect, LMP1 mimics the effects of other proteins carried by oncogenic viruses that activate hTERT transcriptionally, such as Tax of human T-cell lymphotropic virus type 1 (51), E6 of human papillomavirus (33, 60), latency-associated nuclear antigen of Kaposi’s sarcoma-associated herpesvirus (61), and the preS2 gene product of hepatitis B virus (34). Moreover, this additional property of LMP1 reinforces the notion that this pleiotropic viral oncogene is one of the major contributors to EBV-driven B-cell

FIG. 6. Analysis of the role of Akt- and mTOR-dependent pathways. BJAB and BJAB/LMP1 cells were cultured in the absence or presence of 10% FCS (A) or treated with SH5, an inhibitor of Akt, at 20 μM (B) for 24 h. Whole-cell lysates were analyzed by Western blotting using anti-phospho-Akt (Ser473), anti-Akt, and anti-β-tubulin. BJAB and BJAB/LMP1 cells were cultured with solvent alone or with SH5 at 10 μM or 20 μM (C and D) or rapamycin, an inhibitor of mTOR, at 0.1 μM and 7 μM (E and F) and analyzed at 7 and 24 h of treatment. Levels of hTERT-AT transcripts (C and E) were quantified by real-time PCR and expressed as percentages of the hTERT-AT levels quantified in the corresponding untreated cells. (D and F) Telomerase activity was tested by TRAP assay, and levels were reported as percentages of telomerase activity quantified in the corresponding untreated cells. Values are the means and SD (error bars) for three separate experiments.
transformation. It should be considered, however, that LMP1 expression alone may not be sufficient to activate telomerase in primary B cells, since the B-cell lines we used already have baseline levels of hTERT expression and telomerase activity.

LMP1 hijacks cellular signaling pathways that are critical for B-cell growth and survival, including some cascades that are also known to regulate hTERT expression and telomerase activity. In particular, LMP1 engages at least part of the CD40

FIG. 7. LMP1 engages the NF-κB pathway to activate hTERT. (A) BJAB and BJAB/LMP1 cells were cultured in the presence or absence of 10% FCS for 24 h, and whole-cell lysates were analyzed by immunoblotting for the indicated proteins. (B) BJAB and BJAB/LMP1 cells were treated with solvent alone (NT), 20 μM SH5, or 10 μM U0126 for 24 h. Ten micrograms of nuclear protein extract was analyzed for NF-κB p65 DNA-binding activity, using an EZ-Detect chemiluminescent transcription factor assay kit from Pierce Biotechnology. Wild-type and mutant NF-κB competitor duplexes were used as signal specificity controls. Histograms are representative of three separate experiments with virtually identical results. (C and D) BJAB and BJAB/LMP1 cells were treated with BAY-11-7082 at 1 μM or 10 μM and analyzed after 24, 48, and 72 h of treatment. Levels of hTERT-AT (C) and hTERT-FL (D) transcripts were quantified by real-time PCR and expressed as percentages of the hTERT levels quantified in the corresponding untreated cells. Values are the means and SD (error bars) for three separate experiments. (E) Telomerase activity was analyzed by TRAP assay. The panel is representative of three separate experiments. TL, telomerase ladder; ITAS, internal telomerase assay standard. (F) BJAB and BJAB/LMP1 cells were treated with 3 μM BAY-11-7082 for 3 and 5 h and analyzed by Western blotting for phospho-IκBα (Ser32), IκBα, and β-tubulin expression.
signaling pathway (31, 40, 57), which may contribute to the induction of telomerase activity in B lymphocytes (25, 26, 63). Nevertheless, CD40 triggering in BJAB cells, even in combination with IL-4 costimulation, failed to reproduce LMP1-induced upregulation of \( hTERT \) expression and telomerase activity, ruling out a possible involvement of CD40 signaling in LMP1-mediated \( hTERT \) activation in this cellular system. Among the other pathways that may regulate telomerase activity, we showed that the ERK1/2 and NF-\( \kappa \)B pathways are strongly activated in BJAB cells following ectopic expression of LMP1. To our knowledge, this is the first demonstration that LMP1 activates ERK1/2 in B lymphocytes, since available data refer to experiments carried out with epithelial cells of various origins or with fibroblasts (17, 36, 47). Notably, pharmacologic inhibition of the ERK1/2 and NF-\( \kappa \)B pathways markedly decreased \( hTERT \) mRNA expression and telomerase activity in parental BJAB cells and, at lower levels, in the LMP1 transfectants. These are specific, not toxic, effects, since other inhibitors (i.e., rapamycin) failed to block the activation. These findings support a role for ERK1/2 and NF-\( \kappa \)B pathways in mediating LMP1-dependent \( hTERT \) transactivation. Pathways involving ERK1/2 activation are known to regulate telomerase activity in response to exogenous growth stimuli, even independent of proliferation (21, 38). Our results suggest that LMP1 expression in B lymphocytes may mimic the effects of growth factors by directly activating telomerase via ERK1/2, thus contributing to cell immortalization. In epithelial cells, the Ets transcription factor family, downstream of the MAPK and ERK1/2 signaling pathways, was shown to regulate telomerase activity at the transcriptional level, both directly and indirectly through the proto-oncogene c-Myc (13). Further studies are needed to assess whether Ets or other transcription factors are involved in ERK1/2-dependent activation of telomerase in B cells induced by LMP1.

The observation that NF-\( \kappa \)B is involved in mediating LMP1-dependent \( hTERT \) transactivation in B lymphocytes is consistent with findings obtained with other cellular systems supporting a role for this transcription factor in regulating telomerase (1, 50, 51). LMP1 was shown to induce telomerase activity in nasopharyngeal carcinoma cells through NF-\( \kappa \)B activation (11), an effect that was c-Myc dependent, since mutagenesis of c-Myc-responsive E box elements in the \( hTERT \) promoter inhibited \( hTERT \) transactivation induced by LMP1 (66). Our results, however, do not support a role for c-Myc in mediating the \( hTERT \) expression and telomerase activation induced by LMP1 in B lymphocytes. In these cells, in fact, ectopic expres-

FIG. 8. LMP1 does not engage c-Myc to activate \( hTERT \). BJAB, BJAB/LMP1, DG75, and DG75/LMP1 cells were lipofected with 400 nM anti-c-Myc siRNA (siMyc) or with control siRNA (siScramble) and were analyzed after 24 h. (A) c-Myc (top) and GAPDH (bottom) mRNAs were analyzed by reverse transcription-PCR. (B) \( hTERT \)-FL mRNA levels were quantified by real-time PCR. Means and SD (error bars) for three replicates are shown. (C) Telomerase activity was analyzed by TRAP assay. TL, telomerase ladder; ITAS, internal telomerase assay standard.
sion of LMP1 did not upregulate c-Myc expression, and silencing of this oncogene failed to inhibit LMP1-induced telomerase activation. Furthermore, mutagenesis in the NF-κB binding site, but not in the c-Myc binding sites, inhibited LMP1-induced activation of the hTERT promoter.

Although NF-κB may be positively regulated by ERKs (8, 59), LMP1-induced activation of telomerase via NF-κB binding site, but not in the c-Myc binding sites, inhibited LMP1-induced activation of the hTERT promoter.

Although NF-κB may be positively regulated by ERKs (8, 59), LMP1-induced activation of telomerase via NF-κB binding site, but not in the c-Myc binding sites, inhibited LMP1-induced activation of the hTERT promoter. As shown by the finding that inhibition of the MAPK/ERK pathway did not affect NF-κB activation in LMP1-expressing cells. We also investigated the possible involvement of Akt- and mTOR-dependent signaling, two pathways that may regulate telomerase (3, 29, 30, 32, 68, 69). Although we confirmed a role for Akt in regulating telomerase in B lymphocytes, LMP1 failed to activate Akt in BJAB cells, as shown by the downregulation of the phosphorylated form of Akt in LMP1-expressing cells. The slight resistance of LMP1 transfectants to Akt-dependent inhibition of hTERT expression and telomerase activity is probably due to the greater activation of NF-κB downstream of Akt. Taken together, these findings do not support a role for Akt in me-

FIG. 9. Transcriptional activation of the hTERT promoter by LMP1. (A) HeLa cells were cotransfected with vectors allowing the expression of c-Myc (pMT2Myc) or LMP1 (pcDNA-LMP1) or with control vectors (pMT2T and pcDNA3) and with a plasmid expressing luciferase under the control of the hTERT promoter containing mutations in the c-Myc binding sites (pHTERTpromoterLucDM) or NF-κB binding site (pHTERTpromoterLucNF2). (B) BJABtet-LMP1 cells, expressing LMP1 under the control of a TC-inducible promoter, were cultured without (LMP1−) or with (LMP1+) 10 ng TC for 48 h and then transfected with the pHTERTpromoterLucDM or pHTERTpromoterLucNF2 plasmid. The total amount of transfected DNA was kept constant in each experiment by adding the pBlueScript vector. A plasmid expressing the bacterial β-Gal gene was also cotransfected in each experiment as an internal control for transfection efficiency. Values were normalized for transfection efficiency by expressing them for the same amount of β-Gal counts.
diating LMP1-dependent induction of hTERT expression. Available evidence indicates that the mTOR kinase, which is downstream of Akt, may regulate hTERT expression at the transcriptional level in gynecologic tumors (68). However, mTOR inhibition by rapamycin did not affect hTERT mRNA levels or telomerase activity in B cells, ruling out a possible involvement of this kinase in LMP1-mediated hTERT activation.

Overall, the results of the present study demonstrate that the hTERT activation induced by LMP1 in B lymphocytes occurs at the transcriptional level through NF-κB- and ERK-dependent pathways. These findings also confirm the pleiotropic effects of LMP1 at the transcriptional level through NF-κB/H9260 in gynecologic tumors (68). However, downstream of Akt, Akt, heat shock protein 90, mTOR inhibition by rapamycin did not affect NF-κB-mediated anti-proliferative responses in Epstein-Barr virus-immortalized B lymphocytes (69). Therefore, the prevention and treatment of EBV-associated B-cell lymphoproliferative disorders.

ACKNOWLEDGMENTS

We thank Riccardo Dalla Favera (Columbia University, NY) for providing the BJABtet, BJABtet-LMP1, Ramos mt, and Ramos mt-LMP1 cell lines and the pMT2T-promoter and pMT2T-promoter-Luc, and pTERTpromoterLuc DM plasmids; Ethel Cesurman (Weill Cornell Medical College, NY) for providing the pcDNA-LMP1 and pcDNA, plasmids; Pierantonio Gallo for artwork; and Lisa Smith for editorial assistance. We thank Donna D’Agostino (Department of Oncology and Surgical Sciences, University of Padova) for help in constructing the pTERTpromoterLucNF2 plasmid.

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) and Programmi di Ricerca di Rilevante Interesse Nazionale (PRIN) 2005–2006. Jessica Dal Col is a fellowship recipient of AIRC.

REFERENCES


