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Epstein-Barr Virus Latent Membrane Protein 1 Blocks p53-Mediated Apoptosis through the Induction of the A20 Gene

KATHERINE L. FRIES, WILLIAM E. MILLER, AND NANCY RAAB-TRAUB*

Department of Microbiology and Immunology and the Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295

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Two Epstein-Barr virus (EBV)-associated malignancies, nasopharyngeal carcinoma and posttransplant lymphoma, rarely have mutations in the p53 tumor suppressor gene, suggesting that a viral protein interferes with p53 function. The EBV oncogene, LMP1, induces expression of the cellular antiapoptotic genes *bcl-2* and A20 and could in this way interfere with p53-mediated apoptosis. Two derivatives of the p53-null epithelial cell line H1299 were prepared, one of which (H1299-p53) stably expressed a temperature-sensitive (ts) p53 protein, and another (H1299-p53+LMP1) which stably expressed both ts-p53 and latent membrane protein 1 (LMP1). At the permissive temperature, the p53 protein in the H1299-p53 cell line transcriptionally activated two of its target genes, the cyclin-dependent kinase inhibitor p21 and the mdm2 gene product, in an LMP1-independent manner. Upon serum withdrawal at the permissive temperature, p53-mediated apoptosis was induced in 50 to 60% of the cells. In the H1299-p53 cell line which stably expressed LMP1, however, only 20 to 25% of the cells underwent apoptosis. While stable expression of LMP1 did not affect levels of bcl-2 family members in these cells, it did induce expression of A20. Stable expression of A20 in the H1299-p53 cell line inhibited p53-mediated apoptosis equivalent to inhibition by LMP1. The induction of A20 may underlie the ability of LMP1 to protect EBV-infected epithelial cells from p53-mediated apoptosis.

Epstein-Barr virus (EBV) is an important factor in the development of both lymphoid and epithelial malignancies, including Burkitt's lymphoma (BL), posttransplant lymphoma (PTL), and nasopharyngeal carcinoma (NPC) (14, 22, 28, 39, 60, 61, 85). EBV gene expression is distinct in BL compared with NPC and PTL. In BL, only the EBV nuclear antigen 1 (EBNA1) is expressed while NPC tumors express EBNA1, two latent membrane proteins (LMP1 and LMP2), and transcripts from the *Bam*HI A region of the EBV genome (6, 19, 23, 24, 31, 65, 66, 84). PTL gene expression resembles that of EBV-infected lymphocytes in vitro with expression of EBNA1, -2, -3A, -3B, -3C and -LP genes, as well as the two LMPs (25, 74, 83).

LMP1 is essential for transformation of lymphocytes in vitro and has transforming properties in rodent fibroblasts (3, 40, 53, 76). Expression of the LMP1 protein induces many cellular changes in lymphocytes including activation of the NF-κB transcription factor (27, 42, 64) and induction of cellular genes including the B-cell activation antigen CD23; adhesion molecules such as LFA-1, LFA-3, and ICAM-1; and the antiapoptotic genes A20 and *bcl-2* (30, 42, 77). In epithelial cells, LMP1 is also able to activate NF-κB and induce expression of A20 and, unlike in lymphocytes, is able to induce expression of the epidermal growth factor receptor (51, 59).

The tumor suppressor gene, p53, is frequently mutated in many human malignancies, leading to loss or inactivation of p53 function (32, 55). The p53 protein can also be inactivated by viral oncogenes including simian virus 40 T antigen (19, 20, 36, 50), adenovirus E1B 55K (19, 81), and human papillomavirus E6 (50, 68, 78). Previous studies have identified differences in the frequency of p53 mutations in malignancies associated with EBV. In NPC and PTL, p53 is rarely mutated, although it is frequently mutated in BL (16, 17, 21, 44, 71). The absence of p53 mutations in NPC and PTL suggests that a viral protein may interfere with p53 function, eliminating a selection for mutational inactivation.

One function of p53 is to block the progression of the cell cycle in response to DNA damage, causing arrest in G_1 (37, 38, 41). This involves p53-dependent upregulation of the p21 protein (18), an inhibitor of the kinase activity of cyclin-dependent kinase complexes required for G_1/S transition (26, 29, 79). It has been previously shown that the expression of LMP1 during EBV infection does not affect the ability of p53 to block cell cycle progression in G_1, as many studies identifying induction of the growth arrest and DNA damage genes were performed in EBV-immortalized B cells which express LMP1 (7, 38).

A second function of p53 is to mediate apoptosis (69, 82). Various stimuli have been found to induce p53-mediated apoptosis, including DNA-damaging agents such as ionizing radiation, growth factor, or serum withdrawal, and viral oncogenes such as adenovirus E1A and human papillomavirus E7 (10–12, 33, 43, 45–47). In lymphocytes, LMP1 may inhibit p53-mediated apoptosis through the induction of bcl-2 (30); however, bcl-2 levels do not appear to be altered by LMP1 in NPC tumors (1, 48). Expression of bcl-2 has been detected both in normal nasopharyngeal epithelia and in primary and metastatic NPC tumors (48), indicating that the induction of bcl-2 does not play an important role in the regulation of apoptosis in EBV-infected epithelial cells. The effect of LMP1 in epithelial cells on the expression of other bcl-2 family members such as bcl-x_L, a protein that functions similarly to bcl-2 to protect cells from apoptosis (5), and bax, a protein that promotes apoptosis (57), has not previously been determined.

A20, which was originally identified as a tumor necrosis factor (TNF)-inducible gene product (15), can protect breast cancer cells and fibroblasts from TNF-mediated apoptosis (58). CD40 cross-linking can also induce A20, rendering B cells

* Corresponding author. Phone: (919) 966-1701. Fax: (919) 966-3015. Electronic mail address: nrt@med.unc.edu.

resistant to apoptosis induced by serum withdrawal (67). A20 is also induced by LMP1 in both lymphocytes and epithelial cells, through activation of the NF-κB transcription factor (42, 51), suggesting that A20 could possibly protect epithelial cells from apoptosis.

The studies presented here were designed to determine if p53-mediated apoptosis is affected by the expression of LMP1 in epithelial cells. The H1299 p53-null human lung carcinoma cell line was modified to stably express a temperature-sensitive (ts) p53 protein (52). Apoptosis was efficiently induced in a p53-dependent manner in 50 to 60% of the H1299-p53 cells upon serum withdrawal. Although expression of LMP1 in the H1299-p53 cell line did not affect the ability of p53 to induce expression of p21 and mdm2, the H1299-p53+LMP1 cell line was significantly protected from the effects of serum withdrawal, with only 20 to 25% of the cells undergoing p53-mediated apoptosis. In these cells, the levels of bcl-2 family members were not affected while expression of A20 was significantly increased. Stable expression of A20 in the H1299-p53 cell line also inhibited p53-mediated apoptosis equivalent to inhibition by LMP1. These data indicate that in epithelial cells, LMP1 can specifically block p53-mediated apoptosis through induction of the A20 antiapoptotic gene.

MATERIALS AND METHODS

Cell lines. The H1299 human non-small-cell lung carcinoma cell line, which contains a homozygous deletion of the p53 gene (52), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. Stable clones expressing the mouse *ts*-p53 protein were selected in 600 μg of G418 per ml (Gibco) 48 h after transfection with the pLTRcGval135neo expression vector (12, 49). This vector contains the mouse *ts*-p53 gene under control of the Harvey sarcoma virus long terminal repeat promoter and was modified by addition of a neomycin resistance gene in the *Sal*I site.

Additional H1299-p53 cell lines were derived by stably expressing the LMP1 cDNA in the pMEP4 expression vector (Invitrogen), containing the EBV origin of replication, oriP; the EBV EBNA1 protein; and the hygromycin resistance gene. The A20 cDNA was cloned into the pcDNA3 expression vector (Invitrogen) modified to contain a hygromycin resistance gene, with the neomycin resistance gene removed. Stable H1299-p53+LMP1 and H1299-p53+A20 pools and clones were selected with 200 μg of Hygromycin B per ml (Boehringer Mannheim) 48 h after transfection. The addition of 5 μM $CdCl_2$ for 24 h was used to induce high-level LMP1 expression from the metallothionein promoter (75).

Immunoblot analysis. Protein extracts were prepared by direct lysis into sodium dodecyl sulfate (SDS) sample buffer (51). Equal loading of protein was determined by staining SDS-polyacrylamide gels with Coomassie brilliant blue (Sigma). Equivalent samples were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a supported nitrocellulose filter (Schleicher and Schuell) with a Hoefer semidry transfer apparatus. Filters were stained with Ponceau S (Sigma) to verify equal transfer and blocked overnight in Tris-buffered saline with 0.1% Tween 20 and 5% nonfat dried milk (BLOTTO). The p53, p21, and mdm2 proteins were detected with the p53 Ab-3, p21 Ab-1, and the mdm2 Ab-1 monoclonal antibodies, respectively (Oncogene Science), at a 1:100 dilution. Immunoblot analysis of p21 and mdm2 included protein lysate from the MCF7 human breast carcinoma cell line containing wild-type p53 (8) and constitutive expression of p21 (70) as a positive control. The C33A human cervical carcinoma cell line with a missense mutation in codon 273 of the p53 gene (72) was used as a negative control. The bcl-2 protein was detected with the 124 monoclonal antibody (DAKO) at a 1:70 dilution, and bcl-x_L and bax were detected with rabbit peptide antisera S18 and N20 (Santa Cruz) at a 1:2,000 dilution. Protein lysates from the BJAB and K562 cell lines were included in the immunoblot analysis of bcl-x_L and bax (respectively) as positive controls. The LMP1 protein was detected as described elsewhere (51), using the S12 monoclonal antibody at a 1:10 dilution. The A20 protein was detected with an A20-specific monoclonal antibody at a 1:500 dilution (35). Reactivity was detected using a horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham) at a 1:2,000 dilution with enhanced chemiluminescence according to the manufacturer's specifications (Amersham).

Apoptosis assay. Cells were seeded in chamber slides (Nunc) and incubated either at the restrictive temperature (39°C) or at the permissive temperature (32°C), under the conditions described. The cells were incubated for 120 h and fixed, and the apoptotic cells were detected by Tdt-mediated dUTP nick end labeling (TUNEL), using the In Situ Cell Death Kit (Boehringer Mannheim). The cells were counterstained using antifade mounting medium containing propidium iodide (Oncor) and viewed by fluorescent microscopy. A minimum of 100 cells was counted, the values representing an average of at least three experiments ± standard error.

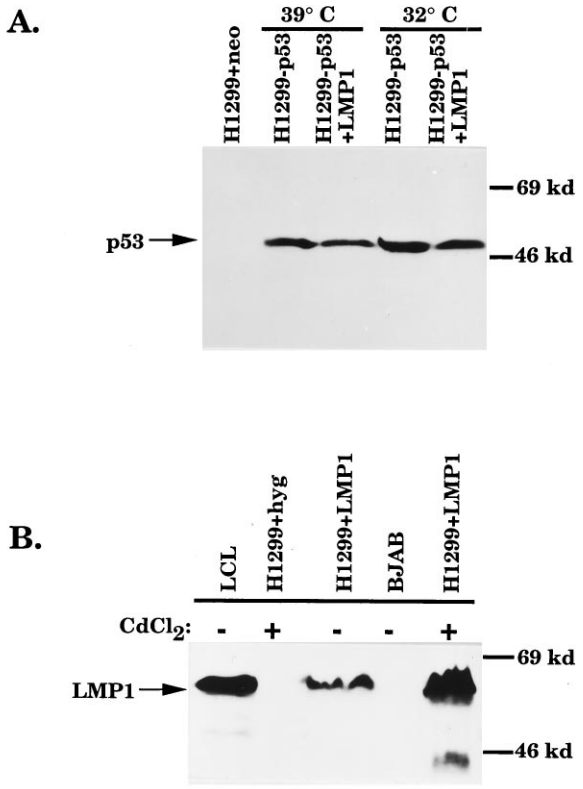

FIG. 1. Stable expression of the ts-p53 protein and the EBV LMP1 protein in the H1299 cell line. (A) Immunoblot analysis of stable H1299 clones expressing either the neo control vector, the ts-p53 protein, or both the ts-p53 protein and LMP1, at both 39 and 32°C. (B) Detection of the LMP1 protein in the stable H1299-p53 clones, which contain the LMP1 cDNA under control of the $CdCl_2$-inducible metallothionein promoter, or the hyg control vector at 39°C. LMP1 expression was induced in the H1299-p53+LMP1 cell line by addition of 5 μM $CdCl_2$ for 24 h. LCL, an EBV-positive human B-cell line; BJAB, an EBV-negative human B-cell line; and H1299-hyg were used as positive and negative controls for LMP1 detection. The positions of p53, LMP1, and the molecular mass markers are indicated.

Growth curves. The H1299-p53 and H1299-p53+LMP1 cell lines were seeded in 12-well tissue culture plates and allowed to adhere. The cells were incubated at 39 or 32°C, in the presence or absence of serum. Cells were trypsinized at the indicated times, and the number of viable cells per well was determined by trypan blue exclusion.

RESULTS

Stable expression of ts-p53 in the H1299 lung carcinoma cell line. To determine the effect of the EBV LMP1 protein on p53 function in epithelial cells, an H1299 cell line stably expressing the ts-p53 protein was created. The H1299 human lung carcinoma cell line contains a homozygous deletion of the p53 gene (52), enabling analysis of p53 function without interference from endogenous p53 protein. The ts-p53 protein is in the wild-type conformation at 32°C and is in the mutant conformation at 39°C (49). Stably transfected clones were screened for p53 expression by immunoblotting (Fig. 1A). One selected clone was transfected with an expression vector containing the LMP1 cDNA under control of the inducible metallothionein promoter. Stable LMP1-expressing pools and clones were then selected, and a clone expressing LMP1 and containing p53 protein levels equivalent to the original H1299-p53 cell line was used for further studies. The levels of the p53 protein in

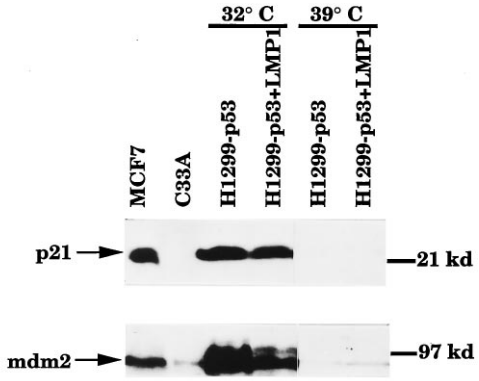

FIG. 2. Induction of p21 and mdm2 by wild-type p53. The levels of the two proteins in the H1299-p53 cell lines, with and without the LMP1 protein, were determined by immunoblot analysis. The cell lines were grown in the presence of $CdCl_2$ for 24 h at either 32 or 39°C. MCF7 is a breast carcinoma cell line containing wild-type p53, and C33A is a cervical carcinoma cell line with a mutant p53 protein. The positions of p21, mdm2, and molecular mass markers are indicated.

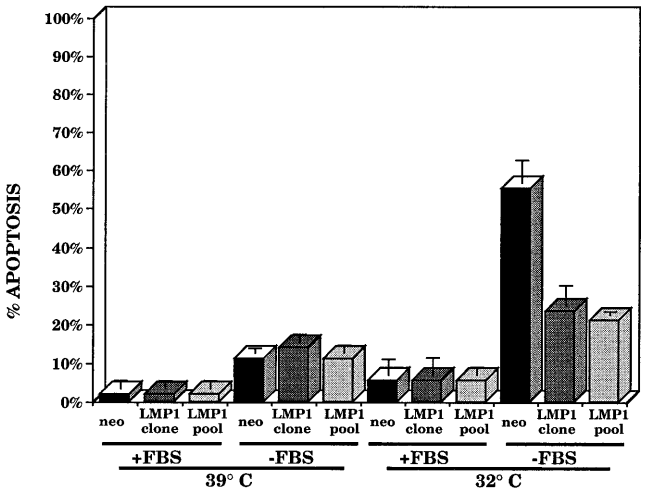

FIG. 3. Effect of LMP1 on p53-mediated apoptosis. The percentage of apoptotic cells was determined in the derivatives of the H1299-p53 cell line. The cell lines were a representative stable clone (LMP1 clone), a stable pool of cells that express LMP1 (LMP1 pool), or a control vector (neo). The three cell lines were grown in the presence or absence of fetal bovine serum (FBS) at 32 or 39°C in media containing 5 μM $CdCl_2$. The cells were fixed after 120 h, TUNEL staining was performed, and the percentage of apoptotic cells was determined by fluorescent microscopy.

the H1299-p53 and H1299-p53+LMP1 cell lines at 39 and 32°C were determined to be equivalent by immunoblot analysis (Fig. 1A). LMP1 expression was readily detectable in this cell line, and expression was increased with addition of $CdCl_2$ (Fig. 1B).

Effect of LMP1 expression on p53-dependent induction of p21 and mdm2. To verify that the p53 protein is in the wild-type conformation at 32°C, p53 induction of the p21 cell cycle inhibitor and the mdm2 gene product was determined. The p21 protein, which is induced by p53, inhibits cyclin-Cdk complex formation, resulting in G_1 arrest (18, 29, 79, 80). The p53 protein also induces expression of mdm2, which negatively regulates the expression of p53, providing a negative regulatory loop for p53 (4). At 32°C, in the wild-type conformation, p53 induced expression of p21 in both the H1299-p53 and LMP1-expressing cell lines to equal levels (Fig. 2). Expression of the mdm2 protein was also induced when the H1299-p53 cell lines were incubated at 32°C, in the presence or absence of the LMP1 protein. The cell lines MCF7, which has wild-type p53 (8), and C33A, which has mutant p53 (72), were included as positive and negative controls, respectively. The induction of p21 and mdm2 upon temperature shift to 32°C demonstrated that the ts-p53 protein functions at the permissive temperature and that expression of LMP1 does not affect the ability of p53 to transcriptionally activate its target genes.

Effect of LMP1 expression on p53-mediated apoptosis. To determine if LMP1 affects the ability of p53 to mediate apoptosis, the levels of apoptotic cells in the H1299-p53 cell line as well as stable pools and individual clones of the H1299-p53+LMP1 cell line were examined. Evidence of apoptosis was detected in less than 5% of H1299-p53 and H1299-p53+LMP1 cell lines at 39°C, as determined by TUNEL staining, in the presence of fetal bovine serum (Fig. 3). Following serum withdrawal, apoptosis was detected in approximately 10 to 15% of the cells at 39°C. This p53-independent apoptosis was not affected by LMP1. At 32°C, with p53 in the wild-type conformation, apoptosis was detected in only a few percent cells in the presence of serum. However, following serum withdrawal at 32°C apoptosis was detected in approximately 50 to 60% of the H1299-p53 cells by TUNEL staining. In the presence of LMP1, apoptosis was significantly inhibited and was detected in only 20 to 25% of the cells following serum withdrawal. This protection was evident in both the pool of LMP1-expressing cells and the selected clone (Fig. 3). These data revealed that LMP1 provides significant protection from p53-dependent apoptosis, but does not affect p53-independent apoptosis.

Growth of the H1299-p53 and H1299-p53+LMP1 cell lines in the presence of mutant and wild-type p53. To confirm that LMP1 did not affect p53-mediated growth arrest in epithelial cells, the growth characteristics of the H1299-p53 and the H1299-p53+LMP1 cell lines were determined in the presence or absence of serum at both 39 and 32°C (Fig. 4). Growth curves were performed by counting duplicate wells every 24 h for 5 days. In the presence of serum, the H1299-p53 cell line continued to proliferate at 39°C but was growth arrested at 32°C (Fig. 4A). In the absence of serum, the number of H1299-p53 cells remained constant at 39°C but decreased in cell number at 32°C (Fig. 4B). This confirmed the induction of cell death by serum withdrawal that was detected in the apoptosis assay. Similarly, the H1299-p53+LMP1 cell line demonstrated significant proliferation at 39°C with growth arrest at 32°C (Fig. 4C). However, in the absence of serum the H1299-p53+LMP1 cell line continued to proliferate at 39°C, confirming a previous study (51). At 32°C, the cells again were growth arrested; however, in contrast to the H1299-p53 cells, there was no detectable decrease in cell numbers, confirming the protection from cell death detected in the apoptosis assay (Fig. 4D). These data indicate that in epithelial cells LMP1 does not affect p53-mediated growth arrest but does inhibit p53-mediated apoptosis.

Effects of LMP1 on cellular genes that affect apoptosis. To further examine the mechanism by which LMP1 protects epithelial cells from p53-mediated apoptosis, protein levels of bcl-2 family members that either protect from apoptosis (bcl-2 and bcl-x_L) or promote apoptosis (bax) were determined. The levels of the 27-kDa bcl-2 protein, the 30-kDa bcl-x_L protein, and the 21-kDa bax protein were not affected by LMP1 in the H1299-p53 cell line, as determined by Western blot (immunoblot), regardless of the conformation of the p53 protein (Fig. 5). BJAB and K562 protein lysates were included as positive

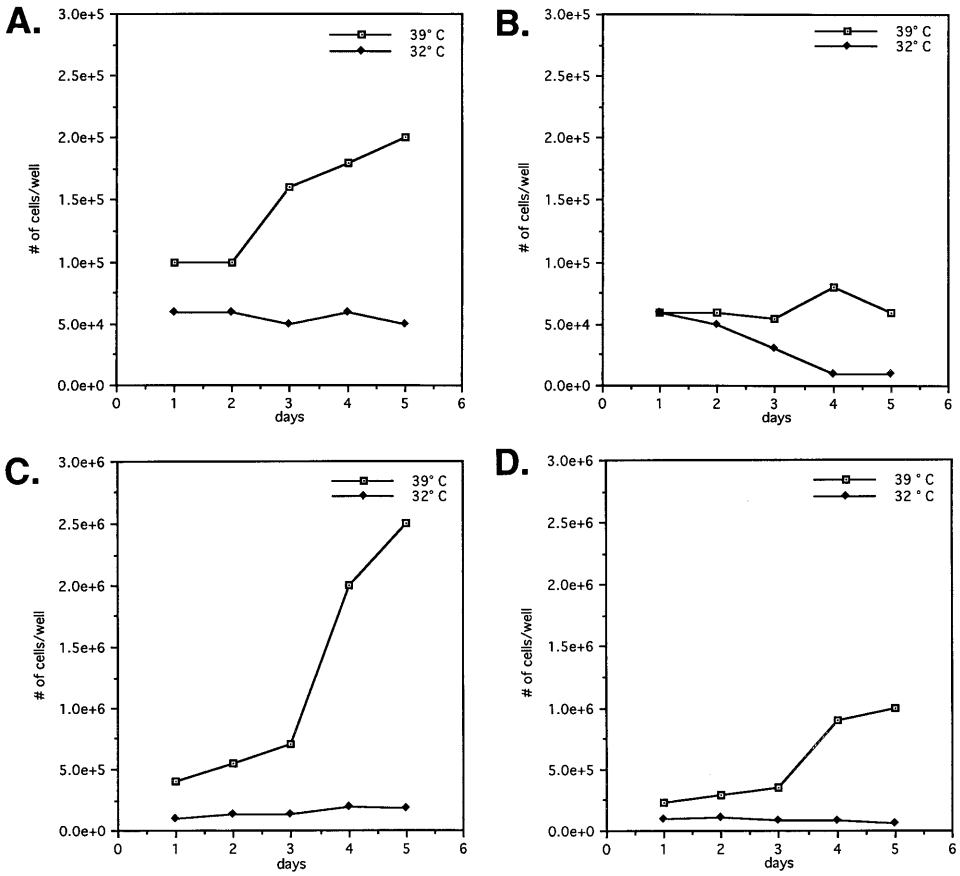

FIG. 4. Growth curves of the H1299-p53 or H1299-p53+LMP1 cell line at either 32 or 39°C, in the presence or absence of serum. The cells were seeded in 12-well plates and trypsinized at the times indicated, and viable cells were counted using trypan blue exclusion ($e + x = 10^x$, e.g., $1.0e + 6 = 1 \times 10^6$). (A) Growth of the H1299-p53 cell line in the presence of serum. (B) Growth of the H1299-p53 cell line in the absence of serum. (C) Growth of the H1299-p53+LMP1 cell line with serum. (D) Growth of the H1299-p53+LMP1 cell line without serum.

controls for immunoblot analysis of bcl-x_L and bax, respectively.

In contrast, LMP1 expression significantly affected expression of the antiapoptotic A20 gene. While only a trace amount of A20 protein was detected by immunoblot analysis in the H1299-p53 cell line, significant levels were detected in the H1299-p53+LMP1 cell line (Fig. 6). Additional higher-molecular-weight proteins were detected nonspecifically in both cell lines by the A20 monoclonal antibody. Northern (RNA) blot analysis also indicated an increase in the levels of A20 mRNA in the H1299-p53+LMP1 cell line over that of the H1299-p53 cell line (data not shown). The induction of A20 by LMP1 could therefore be an important factor in the protection of epithelial cells from p53-mediated apoptosis.

Effect of A20 on p53-mediated apoptosis. To determine if A20 expression was sufficient to protect epithelial cells from p53-mediated apoptosis, the A20 gene was expressed in the H1299-p53 cell line under control of the cytomegalovirus immediate early promoter. Stable pools and individual clones were selected with hygromycin, and immunoblot analysis with an A20-specific monoclonal antibody was used to determine the level of A20 protein expression. While only a trace of A20 protein was detected in the H1299-p53 cell line, a significant amount of A20 was easily detectable in the H1299-p53 A20 pool and in two A20 clones (Fig. 7).

The H1299-p53 cell line, the H1299-p53+LMP1 cell line, and a clone that stably expressed the A20 cDNA were assayed for apoptosis following serum withdrawal (Fig. 8). At 39°C, in the absence of serum, apoptosis was detected in 10 to 20% of the cells in both the A20- and LMP1-expressing H1299-p53 clones. This revealed that neither LMP1 nor A20 affected the p53-independent apoptosis pathway. At 32°C, in the absence of serum, apoptosis was detected in 50 to 60% of the H1299-p53 cells but was only detected in 10 to 15% of the H1299-

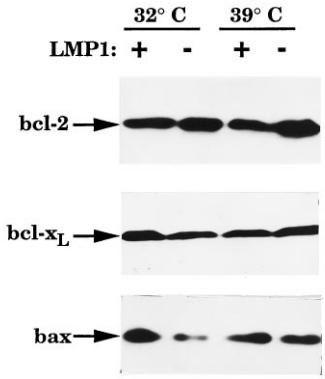

FIG. 5. Effects of LMP1 on bcl-2, bcl-x_L, and bax expression. The H1299-p53 and H1299-p53+LMP1 cell lines were grown at the permissive and restrictive temperatures in the presence of $CdCl_2$ to induce LMP1 expression. Immunoblot analysis was performed with equal amounts of protein. The positions of the 27-kDa bcl-2 protein, the 30-kDa bcl-x_L protein, and the 21-kDa bax protein are indicated.

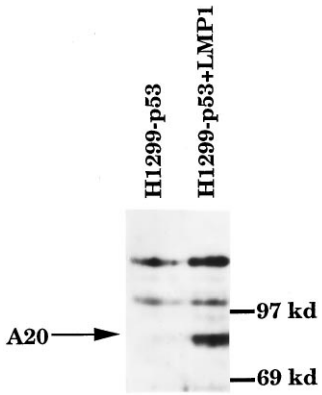

FIG. 6. Induction of A20 by LMP1 in the H1299-p53 cell line. The amount of A20 protein was determined by immunoblot analysis in the H1299-p53 and H1299-p53+LMP1 cell line, with the addition of $CdCl_2$. The position of the 87-kDa A20 protein as well as of the molecular mass markers is indicated.

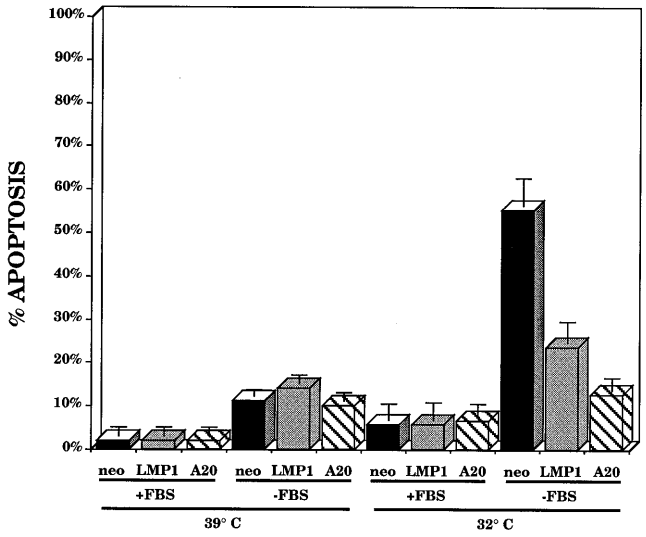

FIG. 8. Effect of LMP1 and A20 on p53-mediated apoptosis. The H1299-p53 cell line (neo), the stable LMP1-expressing clone under control of the metallothionein promoter (LMP1), and the stable A20-expressing clone under the control of the cytomegalovirus promoter (A20) were grown at either 39 or 32°C in the presence or absence of fetal bovine serum, in media containing 5 μM $CdCl_2$. After incubation at 32°C for 120 h, the cells were fixed, stained by the TUNEL assay, and viewed by fluorescent microscopy to determine the percentage of apoptotic cells.

p53+A20 cells. The protection of epithelial cells from p53-mediated apoptosis by stable expression of A20 suggests that induction of the A20 protein by LMP1 is sufficient for this protection which is independent of the effects of LMP1 on expression of other genes.

DISCUSSION

The ability of various tumor virus gene products to bind and inactivate p53, thereby blocking its function in mediating growth arrest or apoptosis, has defined a role for both p53 and viral oncogenes in the cell transformation pathway. The absence of mutations in the p53 tumor suppressor gene in both primary NPC biopsies and PTL suggested that an EBV viral gene product may interfere with some function of p53, eliminating a selection for p53 mutations (16, 17). LMP1 is expressed in both of these EBV-associated malignancies in contrast to BL, which lacks expression of LMP1 and frequently contains mutations in the p53 gene. As LMP1 activates expression of several genes implicated in blocking apoptosis, it was important to determine the effects of LMP1 on p53 function in epithelial cells.

Experiments that identified a defect in the p53-dependent G_1 arrest in lymphocytes from Ataxia-Telangiectasia patients revealed a normal induction of p53 protein and cell cycle arrest following ionizing radiation in EBV-immortalized lymphocytes from normal patients (7, 38). This revealed that EBV does not affect the ability of p53 to arrest lymphocytes in the G_1 phase of the cell cycle in response to DNA damage. Another study using EBV-infected lymphocytes indicated that treatment with the DNA-damaging agent cisplatin resulted in apoptosis promoted by an EBV-induced increase in p53 expression (2). The data presented in this study indicate that as in lymphocytes, LMP1 does not affect p53-mediated growth arrest in epithelial cells. However, following serum withdrawal, LMP1 significantly affected the ability of p53 to mediate apoptosis.

The arrest of cells in G_1 mediated by p53 requires the induction of the CDK inhibitor, p21, as embryonic fibroblasts from p21-null mice are deficient in the ability to arrest in G_1 (13). Thymocytes from these animals are able to undergo p53-mediated apoptosis, however, indicating that p21 is not required for this process. The ability of LMP1 to interfere with only p53-mediated apoptosis and not with growth arrest provides further evidence that these two functions of p53, arresting cellular growth and mediating apoptosis, are separate pathways.

Protection of lymphocytes from apoptosis by EBV has been attributed to the induction of bcl-2 by LMP1 (30), and a recent study suggested that LMP1 could protect lymphocytes from p53-mediated apoptosis possibly by upregulating bcl-2 expression (56). However, in that study, while all of the LMP1-expressing clones contained equivalent levels of bcl-2, one clone was not equally protected from apoptosis (56). This suggests that induction of bcl-2 expression is not sufficient for protection from p53-mediated apoptosis. The data presented in this study reveal that the levels of bcl-x_L and bax are also not affected by LMP1, suggesting that the effect of LMP1 on apoptosis is not due to effects on the expression of these two bcl-2 family members.

The antiapoptotic A20 gene is induced by LMP1 in both lymphocytes (42) and epithelial cells (51), and in this study, A20 was induced by LMP1 in the H1299-p53 cell line. A20 has been shown to protect many cell types from TNF alpha-medi-

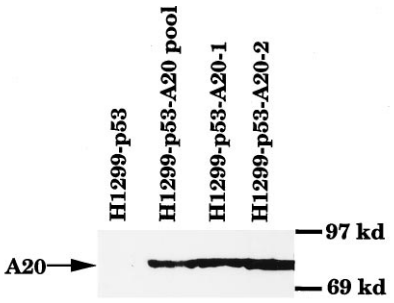

FIG. 7. Stable expression of the A20 protein in the H1299-p53 cell line. Immunoblot analysis was performed on the parental H1299-p53 cell line, a stable A20 pool, and two different clones stably expressing the A20 cDNA under control of the cytomegalovirus promoter, at 39°C. The position of A20 as well as of the molecular mass markers is shown.

ated apoptosis (58) and also protects lymphocytes from apoptosis induced by serum withdrawal (67). The data presented here are the first to show that expression of the A20 protein in epithelial cells specifically protects against p53-mediated apoptosis induced by serum withdrawal but not against p53-independent apoptosis. This study also reveals that of the many effects of LMP1 on cellular gene expression, the ability of LMP1 to induce expression of A20 is sufficient to protect epithelial cells from p53-mediated apoptosis. This ability is likely to underlie the striking absence of p53 mutations in primary NPC tumors. Of the few NPC samples with mutations in p53, most were obtained from metastases arising after radiation or chemotherapy (17, 44, 71). This radiation or chemotherapy could provide a selection for further inactivation of p53 function.

A recent study has shown that LMP1 directly interacts with TRAF3 (54), a signaling molecule that also interacts with members of the TNF alpha superfamily of receptors including CD40 and the type 2 TNF receptor (p75) (9, 34, 54, 62). This family of membrane receptors lacks intrinsic kinase activity but may transmit signals through interactions with various members of the TRAF family (62, 63, 73). The interaction of LMP1 with TRAF3 suggests that this viral oncogene may act as an activated receptor, as it patches in the membrane of infected cells similar to ligand-activated TNF receptors (54). Activation of TNF receptors typically results in two downstream actions: activation of the transcription factor NF-κB and induction of cell death (73). The abilities of LMP1 to engage the TRAF signaling pathway and to induce expression of A20, a molecule shown in this study to inhibit p53-mediated apoptosis, are important molecular properties of LMP1 that enable activation of transcription factors important in the induction of cellular activation genes without the induction of the cell death pathway.

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