Expression of Epstein-Barr Virus BZLF1 Immediate-Early Protein Induces p53 Degradation Independent of MDM2, Leading to Repression of p53-mediated Transcription.

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Running title: p53 degradation by EBV BZLF1 protein

[3] Supported by a Research Fellowship of the Japanese Society for the Promotion of Science for Young Scientists.

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ABSTRACT

The Epstein-Barr virus (EBV) lytic program elicits ATM-dependent DNA damage response, resulting in phosphorylation of p53 at N-terminus, which prevents interaction with MDM2. Nevertheless, p53-downstream signaling is blocked. We found here that during the lytic infection p53 was actively degraded in a proteasome-dependent manner even with a reduced level of MDM2. BZLF1 protein enhanced the ubiquitination of p53 in SaOS-2 cells. The degradation of p53 was observed even in the presence of Nutlin-3, an inhibitor of p53-MDM2 interaction, and also in mouse embryo fibroblasts lacking *mdm2* gene, indicating that the BZLF1 protein-induced degradation of p53 was independent of MDM2. Furthermore, Nutlin-3 increased the level of p53 in the latent phase of EBV infection but not in the lytic phase. Although p53 level is regulated by MDM2 in the latent phase, it might be mediated by the BZLF1 protein-associated E3 ubiquitin ligase in the lytic phase for efficient viral propagation.

Keywords: p53; Epstein-Barr virus; transcription; BZLF1; lytic infection

INTRODUCTION

The Epstein-Barr virus (EBV), a human herpesvirus harboring a 170 kbp dsDNA genome, can choose between two alternative life cycles (Tsurumi, 2001). Primary EBV infection targets resting B lymphocytes, inducing their continuous proliferation. In B lymphoblastoid cell lines, the viral genome is maintained as circular plasmids forming nucleosomal structures with histones and there is no production of virus particles, this being called latent infection. When production of virus is induced, the circular genome becomes a ready template for amplification, generating thousands of copies per cell during lytic replication. The EBV lytic program is initiated by the expression of the viral immediate-early (IE) BZLF1 protein (Countryman and Miller, 1985; Rooney et al., 1989), which is a b-zip transcriptional factor which binds to AP-1-like sequences present in the promoters of early lytic genes (Farrell et al., 1989; Urier et al., 1989). Viral lytic replication occurs in discrete sites in nuclei, called replication compartments in which seven viral replication proteins, including BZLF1 protein which also acts *oriLyt* binding protein, are assembled (Takagi, Takada, and Sairenji, 1991).

Induction of the EBV lytic program elicits a cellular DNA damage response, with activation of the ataxia-telangiectasia mutated (ATM) signal transduction pathway (Kudoh et al., 2005). The DNA damage sensor, MRN complex (Mre11, Rad50, and Nbs1), and phosphorylated ATM are recruited and retained in viral replication compartments, recognizing newly synthesized viral DNAs as abnormal DNA structures. ATM DNA damage signaling activates Chk1/Chk2, leading to phosphorylation and stabilization of p53 (Kudoh et al., 2005). In general, ATM-initiated checkpoint signaling induces a p53-dependent response, featuring $p21^{WAF1/CIP1}$-mediated inactivation of Cdk2/Cyclin E (Dulic et al., 1994; el-Deiry et al., 1993;

Gu, Turck, and Morgan, 1993), leading to increase in levels of p53, $p21^{WAF1/CIP1}$, MDM2 and hypophosphorylated Rb. However, the level of the $p21^{WAF1/CIP1}$ CDK inhibitor remains unchanged and low throughout lytic infection, while the amounts of cyclin E/A and the hyperphosphorylated form of Rb increase (Kudoh et al., 2004; Kudoh et al., 2003; Kudoh et al., 2005). Thus, despite activation of the ATM checkpoint signaling, p53-downstream signaling is blocked, with rather high S-phase CDK activity associated with progression of lytic infection.

The tumor suppressor p53 plays an important role in maintaining genomic integrity (Sherr, 2004; Vogelstein, Lane, and Levine, 2000). In unstressed normal cells, p53 usually exists in latent form and at low levels due to rapid degradation through the ubiquitin-dependent proteasome pathway (Zhang and Xiong, 2001). MDM2 is a key regulator of p53 turnover by binding to p53 and promote its ubiquitination by acting as an E3 ubiquitin ligase. In response to damaged DNA, p53 is phosphorylated at Ser15 by ATM, preventing the interaction with MDM2, subsequently leading to escape from degradation by proteasome. The p53 protein level is elevated, resulting in an increase in p53-dependent transcription of its target genes, subsequently leading to cell cycle arrest or apoptosis (Bourdon et al., 2003; Oren, 2003).

To hijack the host cell system with evading host security responses and lead to an advantageous environment for viral replication, a number of viruses have a modulator(s) of p53 function. The E6 protein of the high-risk human papillomaviruses and the cellular ubiquitin-protein ligase E6AP form a complex which causes the ubiquitination and degradation of p53 (Band et al., 1993; Scheffner et al., 1990). The adenovirus E1B 55-kDa protein interacts directly with p53 and inhibits its acetylation. (Martin and Berk, 1998). E4 Orf6 binds to both p53 and E1B 55k and the trimeric complex directs the ubiquitination of p53 and thus targets it

for degradation (Querido et al., 2001).

In case of EBV, the BZLF1 immediate-early protein has been reported to be an important modulator of p53 function. Zhang *et al.* have showed that the BZLF1 protein directly binds to p53 and inhibits its ability to activate a reporter construct containing p53 binding motifs (Zhang, Gutsch, and Kenney, 1994). However, in comparison with the effects of the smaller DNA viruses, much less is known regarding the mechanism by which the protein inhibits transcriptional functions of p53. In this study, we found that the BZLF1 protein enhanced the ubiquitin-mediated degradation of p53. Since the degradation of p53 was observed even in the presence of Nutlin-3, an inhibitor of MDM2-p53 interaction, and also in mouse embryo fibroblasts lacking *mdm2* gene, the degradation of p53 was independent of MDM2 function. While p53 level is regulated by MDM2 in the latent phase, it is strongly suggested that p53 level might be downregulated by the BZLF1 protein-associated E3 ubiquitin ligase in the lytic phase. The regulation of p53 by the BZLF1 protein might serve to enhance the efficiency of lytic EBV replication.

RESULTS

Downregulation of expression levels of p53 and its target-gene product, MDM2, in the late stages of the EBV lytic infection.

We have previously demonstrated that induction of EBV lytic replication elicits ATM-dependent DNA damage signal response with phosphorylation of ATM, NBS1, Chk2, and p53 but that p53 downstream signaling is blocked (Kudoh et al., 2005). It is known that phosphorylation of p53 at Ser-15 in response to DNA damage usually correlates with both

accumulation of total p53 and the ability of p53 to transactivate downstream target genes. Lytic replication was induced in Tet-BZLF1/B95-8 cells with doxycycline (Dox) and cells were harvested at the indicated times. As shown in Fig.1 (*left panel*), despite increase in levels of p53 phosphorylated at Ser-15, levels of p53 reduced gradually with the progression of the lytic replication. Also, levels of the p53-transcriptional gene target MDM2, which is a negative regulator of p53, substantially declined as the lytic replication progressed. These results suggest that p53 is downregulated after induction of lytic infection even with a reduced level of MDM2. In contrast, levels of TBP throughout the lytic replication proved constant (Fig.1), unlike the report that the BZLF1 protein considerably reduces the level of TBP in some cell lines (Mauser et al., 2002). Progression of the lytic infection was monitored by the expression of the EBV single stranded DNA binding BALF2 early protein. Similar results were also obtained under the conditions that the lytic infection was induced by addition of chemical compounds in B95-8 cells (Fig.1; *right panel*). In Akata cell line, an EBV positive Burkitts' cell line, however, we could not observe any detectable level of p53 due to frame-shift mutation of p53 gene. Since many Burkitts' cell lines harbors mutations of p53 frequently, it should be noted that such cell lines are not suitable for the analyses.

Proteasome dependent degradation of p53 after induction of the lytic infection even with a reduced level of MDM2.

Based on the observation that steady-state levels of p53 protein are gradually decreased following the progression of EBV lytic replication (Fig. 1A), we monitored turnover of p53 in both latent and lytic phase. The half-life of p53 was shortened after induction of lytic infection (Fig. 2A). To further examine whether the decrease of p53 protein level is caused by proteasome

dependent degradation during the lytic infection, lytic replication-induced Tet-BZLF1/B95-8 cells were treated with or without MG132, 26S proteasome inhibitor. Although induction of lytic replication reduced the level of p53, the addition of MG132 increased the level of p53, including some modified forms detected as slowly migrating bands (Fig. 2B). We validated that a ladder of bands corresponded to polyubiquitinated p53 by immunoprecipitation (IP) assay with anti-Multi ubiquitin antibody (Fig. 2C). Therefore, the level of p53 was regulated by ubiquitin-mediated proteasomal degradation not only before but also after induction of lytic replication, even with a reduced level of MDM2. Usually when p53 is phosphorylated at Ser15 by ATM in response to DNA damege, the phosphorylation prevents the interaction with MDM2, leading to decrease in ubiquitination levels of p53. However, p53 was ubiquitinated to similar level to that before induction.

Association of p53 with BZLF1 protein in the lytic infection was observed especially in the presence of a proteasome inhibitor MG132.

The results described above suggest that p53 signaling pathway is blocked through the degradation of p53. EBV BZLF1 immediate early protein is a good candidate for downregulating p53, since it has been shown that BZLF1 protein interacts with p53 (Kudoh et al., 2005; Zhang, Gutsch, and Kenney, 1994). We previously reported that immunoprecipitation with anti-BZLF1 specific antibody against lysates from lytic replication-induced B95-8 cells co-precipitated both BZLF1 and p53 proteins (Kudoh et al., 2005). Inversely, immunoprecipitation using anti-p53 specific antibody was performed with lysates from lytic infection induced Tet-BZLF1/B95-8 cells in the presence and absence of a proteasome inhibitor MG132 (Fig. 3). Appreciable association of p53 with BZLF1 protein was confirmed in the

presence of MG132, but hardly in its absence under the condition. This observation suggests the possibility that p53 is a substrate for the BZLF1 protein-associated E3 ubiquitin ligase, since the association of ubiquitin ligase with its targets usually results in rapid degradation of the substrates.

Ubiquitination and proteasome dependent degradation of p53 in the presence of the BZLF1 protein in SaOS-2 cells.

To address whether the decrease of p53 protein level is caused by proteasome dependent degradation also in SaOS-2 cells, cells were co-transfected with p53 and BZLF1 expression plasmids and treated with MG132 (Fig. 4A). Treatment with MG132 resulted in restoration of the expression level of p53 in the cells where both p53 and BZLF1 protein were overexpressed, similar to the level of p53 when the cells were transfected with p53 expression vector alone (Fig. 4A). We also confirmed that the proteasomal inhibition by MG132 abolished the BZLF1 protein-mediated degradation of p53 in an MDM2 null background using *p53*- and *mdm2*-genes double knock out mouse embryo fibroblasts, 2KO cells (Montes de Oca Luna, Wagner, and Lozano, 1995) (Fig. 4B). Next, the co-transfection experiments were performed in the presence of cycloheximide. Consistently, the cycloheximide-chase assay revealed that turnover of p53 was accelerated in the presence of BZLF1 protein compared with the absence of BZLF1 protein (Fig. 4C).

We found the enhancement of p53-ubiquitination by BZLF1 protein in the presence of MG132 (Fig. 4D), implying that the BZLF1 protein causes proteasome-dependent degradation of p53 by ubiquitination.

Direct interaction between the BZLF1 protein and p53 is required for the repression of

p53-mediated transactivation and p53 degradation.

It has been previously reported that p53-mediated transactivation is repressed by expression of the BZLF1 protein in reporter gene assay (Mauser et al., 2002; Zhang, Gutsch, and Kenney, 1994). In order to examine the effect of the BZLF1 protein on p53-responsive transcription in detail, the WWP-Luc reporter plasmid, containing an authentic p21 promoter, was cotransfected with expression plasmids for p53 and BZLF1 protein into p53-null SaOS-2 cells. As shown in Fig. 5A, the p53-mediated transactivation was strongly repressed by the BZLF1 protein. In contrast, with the BZLF1 d200-227 mutant lacking p53 interaction domain (Zhang, Gutsch, and Kenney, 1994), this repression was abolished (Fig. 5A), suggesting that the physical interaction between BZLF1 protein and p53 was required for repression of p53-mediated transactivation. Western blot analysis of the lysate used in the luciferase reporter assay revealed that the levels of p53 protein were reduced with expression of the wild-type BZLF1 protein but not with the d200-227 mutant. With GFP monitoring of transfection efficiency, TBP and GAPDH expression proved constant in the presence and absence of BZLF1 protein, although it has been proposed that an indirect mechanism exists involving suppression of TBP expression by the BZLF1 protein (Mauser et al., 2002). Therefore, it appears that direct association of BZLF1 protein to p53 is required for repression of p53-specific transcription and elevated p53 degradation. It should be noted that p53 expression was not regulated by the BZLF1 protein, since the p53 protein was exogenously expressed from a human cytomegalovirus (HCMV) IE promoter that was not affected by the BZLF1 protein (Fig. 5B).

MDM2 is not required for the degradation of p53 by the BZLF1 protein.

In unstressed cells, MDM2 regulates p53 quantitatively and promote its ubiquitination by

acting as an E3 ubiquitin ligase (Zhang and Xiong, 2001). To determine whether MDM2 is required for the BZLF1 protein-mediated suppression of p53, 2KO (*p53*$^{-/-}$/*mdm2*$^{-/-}$) cells were used. As shown in Fig. 6A, the BZLF1 protein repressed the p53-mediated transactivation in a reporter assay using a PG13-Luc reporter plasmid containing p53-binding sites and allowing p53-specific transcription to be monitored. BZLF1 protein reduced the level of p53 protein in 2KO cells. On the other hand, TBP and GAPDH expression proved constant in the presence and absence of the BZLF1 protein. Furthermore, repression of p53-mediated transactivation by the BZLF1 protein correlated with the reduction of p53 was observed also in SaOS-2 cells expressing very low level of MDM2 (Capoulade et al., 2001; Chen et al., 2005b), even with Nutlin-3 that can disrupt the p53-MDM2 interaction (Vassilev et al., 2004) (Fig. 6B). Collectively, the results clearly demonstrated that MDM2 could not be involved in the BZLF1 protein-mediated p53 degradation.

Finally, to examine whether the degradation of p53 requires MDM2 in the context of the lytic infection, Tet-BZLF1/B95-8 cells were treated with Nutlin-3 in the presence or absence of doxycycline. While treatment with Nutlin-3 increased the levels of p53 just in the latent phase of infection, the compound did not change the levels of p53 in the lytic phase (Fig. 6C). Taken together, although MDM2 controls the amount of p53 protein in the latent infection, after induction of the lytic infection another E3 ligase might be involved in ubiquitin-mediated proteasomal degradation of p53.

DISCUSSION

Inhibition of p53-mediated transactivation is essential for regulating the cellular

environment advantageous for viral replication in lytic infection. This is in general agreement with a lot of reports on herpesvirus family. The molecular mechanism of inactivating p53 is controversial. For instance, in the cells infected with HCMV, pIE-86 interferes the acetylation of p53 and of histones by directly binding to the histone acetyltransferase p300/CBP and then downregulates p53-driven transcription (Hsu et al., 2004). Other reports on herpes simplex virus type 1 (HSV-1) (Wilcock and Lane, 1991) and HCMV (Fortunato and Spector, 1998) showed that the sequestration of p53 in viral replication sites as called replication compartments or centers might play a role in maintaining p53 in an inactive state. In this study, we demonstrated that expression of the EBV BZLF1 protein induced ubiquitin-mediated proteasomal degradation of p53 independent of MDM2 and resulted in reduction of the p53 protein levels, explaining the downregulation of the transcriptional function of p53 during the EBV lytic infection.

In the latent phase of EBV infection, p53 levels are regulated by MDM2 as demonstrated by Nutlin-3 treatment (Fig. 6C). One important question that arises is whether MDM2, which is clearly a major regulator of p53 stability, is required for the ubiquitin-mediated degradation of p53 by BZLF1 protein during the EBV lytic infection. We found that level of MDM2 decreased and p53 was phosphorylated at least at Ser-15 in the late stages of lytic infection (Fig. 1). Under this condition, the interaction between p53 and MDM2 is weakened because of the phosphorylation of p53 N-terminus (Shieh et al., 1997). Moreover, p53 degradation was observed even in 2KO cells lacking MDM2 in the presence of the BZLF1 protein (Fig. 6A) and also in the Nutlin-3 treated lytic infection-induced cells (Fig. 6B). Therefore, p53 are also ubiquitinated and degraded during the lytic infection, but being independent of MDM2. These findings provide us the new insight that EBV might possess another strategy to ubiquitinate the

phosphorylated p53 via the BZLF1 protein, instead of MDM2, to block the p53 downstream signaling. The identification of several new p53-E3 ligases, namely Pirh2 (Leng et al., 2003), COP1 (Dornan et al., 2004), TOPORS (Rajendra et al., 2004) and ARF-BP1 (Chen et al., 2005a), adds more complexity to the p53 ubiquitination pathway, suggesting that p53 abundance is tightly regulated by both MDM2-dependent and –independent mechanisms. It is yet uncertain exactly how those proteins are specifically regulated and under what situations they may be differentially activated. The effect of BZLF1 protein on p53 degradation might reflect the upregulation of these molecules. However, several observations do not favor this model, although direct evidence during EBV lytic infection has been lacking. First, similar to MDM2, Pirh2 is a p53 responsive gene and participates in a similar autoregulatory negative feedback loop (Leng et al., 2003). Second, as shown in Fig. 1, p53 target genes such as *mdm2* were repressed during EBV lytic replication. Third, DNA damage triggers the autodegradation of COP1 through ATM-dependent phosphorylation to permit p53 stabilization (Dornan et al., 2006). Lastly, EBV lytic replication elicits ATM-dependent DNA damage response (Kudoh et al., 2005). Therefore, it might be reasonable to predict that these E3 ligases are downregulated during EBV lytic infection. Because there is no published work regarding regulation of TOPORS and ARF-BP1, it is difficult to examine if BZLF1 protein could contribute to their expression or not.

Ubiquitination is important for the regulation of cellular processes, including signal transduction, development, apoptosis, cell cycle progression, and immune response (Hershko and Ciechanover, 1998; Liu, 2004; Pickart, 2001). In the ubiquitin-mediated proteolytic pathway, there are two distinct steps; (i) the covalent attachment of multiple ubiquitin molecules

to the substrate, and (ii) the degradation of the polyubiquitinated protein by the 26S proteasome complex (Hershko and Ciechanover, 1998). The ubiquitination of substrate requires a cascade of enzymatic reactions involving an E1 activating enzyme, an E2 conjugating enzyme, and finally an E3 ligase enzyme that covalently attaches ubiquitin to lysine residue of target protein (Scheffner, Nuber, and Huibregtse, 1995). The E3 ligase is generally thought to be a group of multisubunit complexes, which are mainly consist of the substrate-specific adaptor protein, scaffold protein, and RING finger-containing protein that interacts with E2 ligase (Bashir et al., 2004; Kamura et al., 2004; Maxwell et al., 1999; Pintard, Willems, and Peter, 2004; Wertz et al., 2004; Zheng et al., 2002). This supports the possibility that ubiquitination of p53 by BZLF1 protein requires for additional factors, since BZLF1 protein does not possess the RING finger domain. Therefore, it is likely that BZLF1 protein rather acts as an adaptor such as F-box proteins and SOCS-box proteins. Further studies are required to identify a component of BZLF1 protein-associated E3 ligase complex involved in the inhibition of p53-downstream signaling during EBV lytic infection.

However, we cannot rule out the possibility that other mechanisms are also involved in the inhibition of the p53-downstream signaling by the BZLF1 protein. Mauser and colleagues previously showed that BZLF1 protein substantially reduced the level of cellular TBP in both normal human fibroblasts and A549 cells and the inhibitory effects of the BZLF1 protein on p53 transcriptional function could be partially rescued by overexpression of TBP (Mauser et al., 2002). Although suppression of TBP expression is one of possible explanation for this observation, we could not observe any suppression of TBP expression in the transfection system in SaOS-2 cells, 2KO cells (Fig. 5A and 6A). TBP expression is stimulated with high PKC

and/or MAPK activity (Garber, Vilalta, and Johnson, 1994; Zhong, Zhang, and Johnson, 2004). The activation of PKC and/or MAPK plays an important role in transition from latent to lytic EBV infection (Gao et al., 2001; Gao, Wang, and Sairenji, 2004). Indeed, the reduction of TBP abundance was not observed also in the lytic replication-induced B95-8 cells and Tet-BZLF1/B95-8 cells (Fig. 1). Since most viral promoters contain TATA box and the binding of TBP to these promoters greatly contributes to the viral gene expressions, it is difficult to be receptive to this explanation. On the other hands, Zhang *et al.* found that p53 levels were actually increased in primary human fibloblasts, IMR-90 cells where the BZLF1 expression vector was transfected (Zhang, Gutsch, and Kenney, 1994). Also, Cayrol and Flemington reported that BZLF1 protein induced accumulation of p53 and a G1 arrest through activation of $p21^{WAF-1/CIP-1}$ in HeLa cells (Cayrol and Flemington, 1996). We confirmed this observation in HeLa cells conditionally expressing the BZLF1 protein (data not shown). In the case that BZLF1 protein is transiently overexpressed in D98/HE-R-1 cells (fusion between HeLa and EBV-positive P3HR1 cells), expression of p53 appears to be increased (Zhang, Gutsch, and Kenney, 1994). Although a cell-type-specificity will be a possible explanation for the apparent discrepancy, the expression balance between p53 and BZLF1 protein might play a key role in the fate of p53 levels in EBV-positive cells. In the late stages of infection, degradation of p53 might dominate over enhancement of p53 expression since expression level of BZLF1 protein gradually increased as shown in this study. Thus, it is likely that ubiquitin-mediated degradation of p53 by BZLF1 protein is a major mechanism of regulating p53 function during the EBV lytic infection.

The present study indicated the existence of distinct mechanisms of p53 quantitative

regulation in the latent and lytic phases of EBV infection. While the level of p53 was mainly controlled by MDM2-dependent ubiquitination in latent phase, after induction of lytic replication, MDM2 hardly interacts with p53 due to N-terminal phosphorylation of p53 (Shieh et al., 1997) resulted from the ATM-Chk2 DNA damage-signaling activation by EBV lytic program (Kudoh et al., 2005). On the basis of our findings with the BZLF1 protein, we propose a model for ubiquitination of p53 by the BZLF1 protein-associated E3 ligases, and this model may help explain how EBV prevents the cells from arresting in G1-phase or/and apoptosis in response to the activation of ATM-signal transduction pathway, and maintains the high S-phase Cdk activity that is essential for the expression of viral genes and lytic viral replication (Kudoh et al., 2004). The identification of the BZLF1 protein-associated E3 ligases is now in progress.

MATERIALS and METHODS

Cells and Reagents p53-null human osteosarcoma SaOS-2 cells, and 2KO cells, derived from mouse embryo fibroblasts lacking the *p53* and *mdm2* genes (Montes de Oca Luna, Wagner, and Lozano, 1995), were grown in DMEM supplemented with 10% fetal calf serum (FCS). 2KO cells were kindly provided by Dr. G. Lozano (M.D.Anderson Cancer Center). EBV-positive marmoset B lymphocytes B95-8 cells were cultured in RPMI medium supplemented with 10% FCS. Tet-BZLF1/B95-8 cells (Kudoh et al., 2003) were maintained in RPMI medium supplemented with 1 μg of puromycin/ml, 250 μg of hygromycin B/ml, and 10% tetracycline-free FCS. To induce lytic EBV replication, the tetracycline derivative doxycycline was added to the culture medium at a final concentration of 2 μg/ml. All cells were grown at 37 ºC in a 5% CO_2 atmosphere. For MG132 (Sigma) and Nutlin-3 (Cayman chemical)

experiments, cells were treated with MG132 (20 μM)/DMSO, and Nutlin-3 (10 μM)/DMSO for 0.5-5 h, and 6-8 h respectively, before harvesting.

Anti-p53 (FL-393) rabbit polyclonal antibody, and normal mouse IgG2a were purchased from Santa Cruz Biotechnology. Anti-Phosho-p53 (Ser15) rabbit polyclonal antibodies were obtained from Cell Signaling Technology. Mouse anti-GAPDH (Ambion), mouse anti-Multi ubiquitin (FK-2) (MBL), rabbit anti-GFP (Invitrogen), rat anti-HA (3F10) (Roche), monoclonal mouse anti-p53 (Ab-6) (Merck), mouse anti-MDM2 (Ab-3) (Merck) and mouse anti-FLAG M2 (Sigma) antibodies were also used. Horseradish-peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories. Affinity-purified anti-BZLF1, anti-BALF2, and anti-BMRF-1 protein antibodies were prepared as described previously (Daikoku et al., 2005).

Plasmids and transfection For the expression of FLAG-tagged p53, expression vectors of full-length wild type p53 (FLAG-wtp53) was constructed by inserting of p53-DNA fragment amplified by PCR into p3×FLAG-CMV-14 expression vector (Sigma). A BZLF1 mutant (BZLF1 d200-227), lacking a part of Zip domain was generated by overlapping PCR. The inserted DNA sequence of each vector was confirmed by direct DNA sequencing. The mammalian expression vector for human wild-type p53 (pcNXRS) and the BZLF1 protein expression vector (pcDNA-BZLF1) were kindly provided by Dr. T. Takahashi (Nagoya University, Japan) and Dr. K. Kuzushima (Aichi Cancer Center Research Institute, Japan), respectively. Reporter plasmids such as WWP-Luc, PG13-Luc and MG15-Luc (el-Deiry et al., 1993) were gifts from Dr. B. Vogelstein (The Johns Hopkins University Medical Institutions). WWP-Luc, PG13-Luc and MG15-Luc contain authentic p21 promoter, 13 copies of a p53-binding site, and 15 copies of a subtly mutated p53-binding site, respectively in the

upstream of reporter gene.

Cells were seeded, cultured to semiconfluence and transfected with expression plasmids using lipofection reagent (Lipofectamine™ and Plus reagent; Invitrogen) according to manufacturer's instructions.

Immunoprecipitation and Western blotting Cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.6, 120 mM NaCl, 0.1% NP40, 1 mM EDTA, 100 mM sodium fluoride, 2 mM sodium vanadate) containing a protease inhibitor cocktail (Sigma), and then sonicated. The debris was removed by centrifugation and the supernatants were applied for immunoprecipitation with specific antibodies. Complexes of antibody and antigen were collected by centrifugation and washed three times with NET-gel buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% NP40, 1 mM EDTA). The immunoprecipitates were then subjected to SDS-PAGE followed by immunoblot analyses. For in vivo ubiquitination assays, SaOS-2 cells transfected with expression plasmids of HA-ubiquitin, FLAG-p53 and BZLF1 protein by electoroporation using MicroPorator MP-100 (Digital Bio), were exposed to ionizing radiation with 20 Gy 21 hours post-transfection. Cells were treated with MG132 for 2 hours before harvesting 24 h post-transfection and then lysed in lysis buffer containing 20 μM MG132 and a protease inhibitor cocktail (Sigma) under the denaturing condition described previously (Murata and Shimotohno, 2006). FLAG-p53 derivatives were precipitated with anti-FLAG antibody and detected by immunoblot analysis. Preparation of the lysate for immunoblotting, Western blotting and detection of signals were performed as described previously (Sato et al., 2006).

Luciferase reporter assay Cells seeded in 24-well plates were transfected with 0.05 μg of the luciferase reporter plasmid (WWP-Luc, PG13-Luc or MG15-Luc), with or without 0.1 μg of

pcNXRS and 0.004, 0.02 or 0.1 μg of pcDNA-BZLF1. Simultaneously, 0.01 μg of pCMV-Rluc vector DNA was also transfected as an internal control. The amount of transfected DNA was kept constant by adding empty vector. Luciferase activity was measured with a dual luciferase assay kit (Promega) according to the manufacturer's instructions, using a luminometer (Berthoid).

ACKNOWLEDGMENTS

We thank Dr. G. Lozano, Dr. B. Vogelstein, Dr. T. Takahashi, and Dr. K. Kuzushima for invaluable materials. We also thank Yasuhiro Nishikawa for technical assistance. This work was supported by grants-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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FIGURE LEGENDS

Figure 1. Expression profiles of p53, MDM2, and TBP after induction of EBV lytic replication. Tet-BZLF1/B95-8 cells, and B95-8 cells were cultured in the presence of 2 μg/ml of doxycycline, and chemical inducers (Sodium butylate (5 mM), TPA (0.2 μg/ml) and A23187 (1 μM)), respectively, and then harvested at the indicated times. Cell lysates were prepared, and applied for immunoblot analysis with specific antibodies as indicated. IR, Tet-BZLF1/B95-8 cells were exposed to 10 Gy of γ-irradiation, harvested 2 h post-treatment, and processed similarly.

Figure 2. Induction of EBV lytic replication promotes proteasome-dependent degradation of p53. (A) Turnover of p53 is enhanced after induction of EBV lytic replication. Tet-BZLF1/B95-8 cells were cultured with (circle) or without (square) doxycycline for 48 h and treated with or without 20 μM MG132 for 3 h before harvesting. Cells were cultured in the presence of cycloheximide (CHX) for the indicated hours before harvesting. The levels of p53 and GAPDH were assessed by immunoblotting. Decay curves of p53 were generated from the band quantification after normalization against GAPDH. The data shown were obtained from at least three independent experiments and are mean ±S.E. values. (B) Proteasome-dependent degradation of p53 during EBV latent and lytic infection. Tet-BZLF1/B95-8 cells were cultured with or without of Dox for 43 h and then treated for 5 h with the proteasome inhibitor MG132 or DMSO as a control. Expression of p53 was analyzed by immunoblotting using anti-p53 antibodies with short and long exposure. $(Ub)_n$-p53 indicates the ubiquitinated form of p53. (C)

p53 ubiquitination in lytic infection. Ubiquitinated proteins were precipitated with anti-multi Ub antibodies from whole cell extracts of lytic replication-induced Tet-BZLF1/B95-8 cells treated with MG132 and probed with the anti-p53 antibody. IP, immunoprecipitation.

Figure 3. p53 is associated with BZLF1 protein in the lytic phase of EBV replication. Nuclear extracts were prepared from lytic replication-induced Tet-BZLF1/B95-8 cells with doxycycline (24 h.p.i.) in the presence of DMSO or MG132 (20 μM, 5h). Immunoprecipitation was performed with monoclonal anti-p53 or normal mouse IgG2a antibodies, followed by Western blotting using anti-BZLF1 protein, and anti-p53 antibodies. IP, immunoprecipitation.

Figure 4. BZLF1 protein enhances ubiquitination of p53 in SaOS-2 cells. (A) Expression of BZLF1 protein accelerates proteasomal degradation of p53. SaOS-2 cells were transfected with p53 and BZLF1 expression plasmids as indicated, re-seeded 8 h post-transfection and then treated with DMSO or MG132 for the indicated times before harvesting 24 h post-transfection. Cell lysates were prepared and applied for immunoblot analysis with the indicated antibodies. (B) Treatment of MG132 also restores p53 level in MDM2 null background. 2KO cells transfected with p53 and BZLF1 expression plasmids as indicated, were cultured in the presence of DMSO or MG132 for 4 h. Cell lysates were prepared and applied for immunoblot analysis with the indicated antibodies. (C) Turnover of p53 is stimulated in the presence of BZLF1 protein. SaOS-2 cells were transfected with p53 and BZLF1 expression plasmids and re-seeded 8 h post-transfection, followed by addition of cycloheximide (CHX) to the cell cultures. Cells were cultured for the indicated hours before harvesting. The cell lysates were subjected to

immunoblot analysis for p53 and GAPDH (control). (D) BZLF1 promotes polyubiquitination of p53. SaOS-2 cells were transfected with different combinations of FLAG-p53, BZLF1 protein and HA-Ub expression vectors, exposed to ionizing radiation 21 h post-transfection and treated with 20 μM MG132 for 2 h before harvesting 24 h post-transfection. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, applied to SDS-PAGE, and then immunoblotted with anti-HA antibody.

Figure 5. Repression of p53-mediated transactivation by BZLF1 protein requires the physically interaction between p53 and BZLF1 protein. (A) Repression of p53-mediated transactivation by the BZLF1 protein requires physical interaction between p53 and the BZLF1 protein. SaOS-2 cells were transfected with WWP-Luc reporter plasmids together with pCMV-Rluc, pGFP and various combinations of expression plasmids as indicated. Luciferase activity was measured 24 h post-transfection. Values are averages from three independent transfections (±S.E.) after normalization against the internal control (Renilla activity). Lysates of the luciferase samples were analyzed by Western blotting with indicated antibodies. (B) Human cytomegalovirus IE promoter was not affected by the BZLF1 protein. SaOS-2 cells were transfected with pCMV-Luc reporter plasmids together with BZLF1 protein-expression plasmids as indicated ratio. Luciferase activity was measured 24 h post-transfection and relative values were calculated with that in the absence of BZLF1 protein expression plasmid taken as 100 %. Values are averages of three independent transfections (±S.E.) Lysates of luciferase samples were analyzed by Westren blotting with anti-BZLF1 and anti-GAPDH antibodies.

Figure 6. MDM2 is not required for the BZLF1 protein-mediated downregulation of p53. (A) Repression of p53-mediated transactivation by the BZLF1 protein is independent of MDM2. 2KO (*p53*$^{-/-}$, *MDM2*$^{-/-}$) cells were transfected with PG13-Luc or MG15-Luc reporter plasmids together with pCMV-Rluc and various combinations of expression plasmids. Luciferase activity was measured 24 h post-transfection. Values are averages from three independent transfections (±S.E.) after normalization against the internal control (Renilla activity). Lysates of the luciferase samples were analyzed by Western blotting with indicated antibodies. (B) p53-specific transcription activity is repressed by expression of BZLF1 protein in a dose-dependent manner. SaOS-2 cells were transfected with PG13-Luc or MG15-Luc reporter plasmids containing a tandem array of wild-type or mutated p53-binding sites together with pCMV-Rluc as an internal control, and p53 and BZLF1 protein expression plasmids as indicated. Cells were treated with DMSO or Nutlin-3 (10 μM) for 8 h before harvesting. Luciferase activities were measured 24 h post-transfection. Values are averages from three independent transfections (±S.E.) after normalization against the internal control (Renilla activity). Lysates from the luciferase assays were applied for immunoblot analysis with anti-p53, anti-BZLF1 protein, anti-HDM2, and anti-GAPDH antibodies. (C) p53 is downregulated independent of MDM2 in the lytic infection of EBV. Tet-BZLF1/B95-8 cells were cultured in the presence (+Dox) or absence (-Dox) of doxycycline. Nutlin-3 or DMSO as a control was added to the cultures at 42 h post-induction, and cells were harvested 48 h post-induction. Expression levels of p53 were analyzed by immunoblotting with anti-p53 antibodies.

Figure 1

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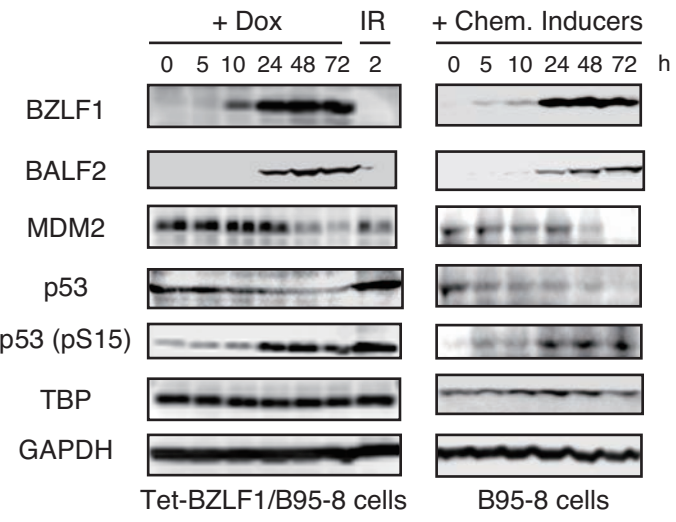

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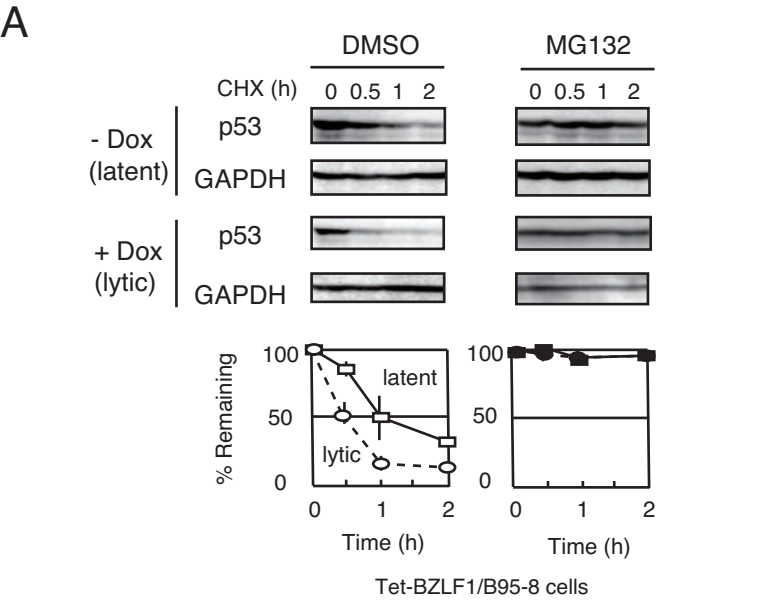

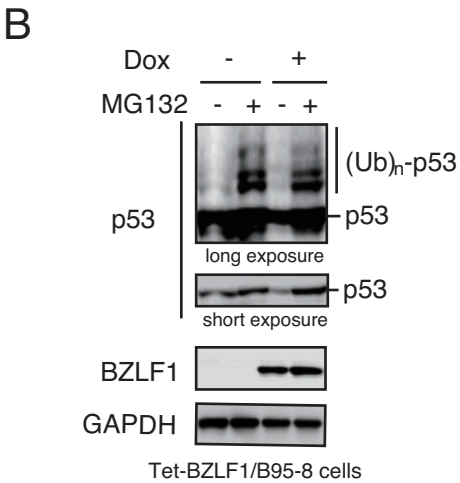

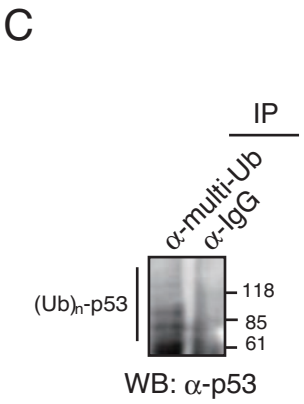

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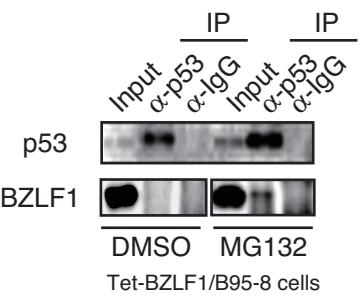

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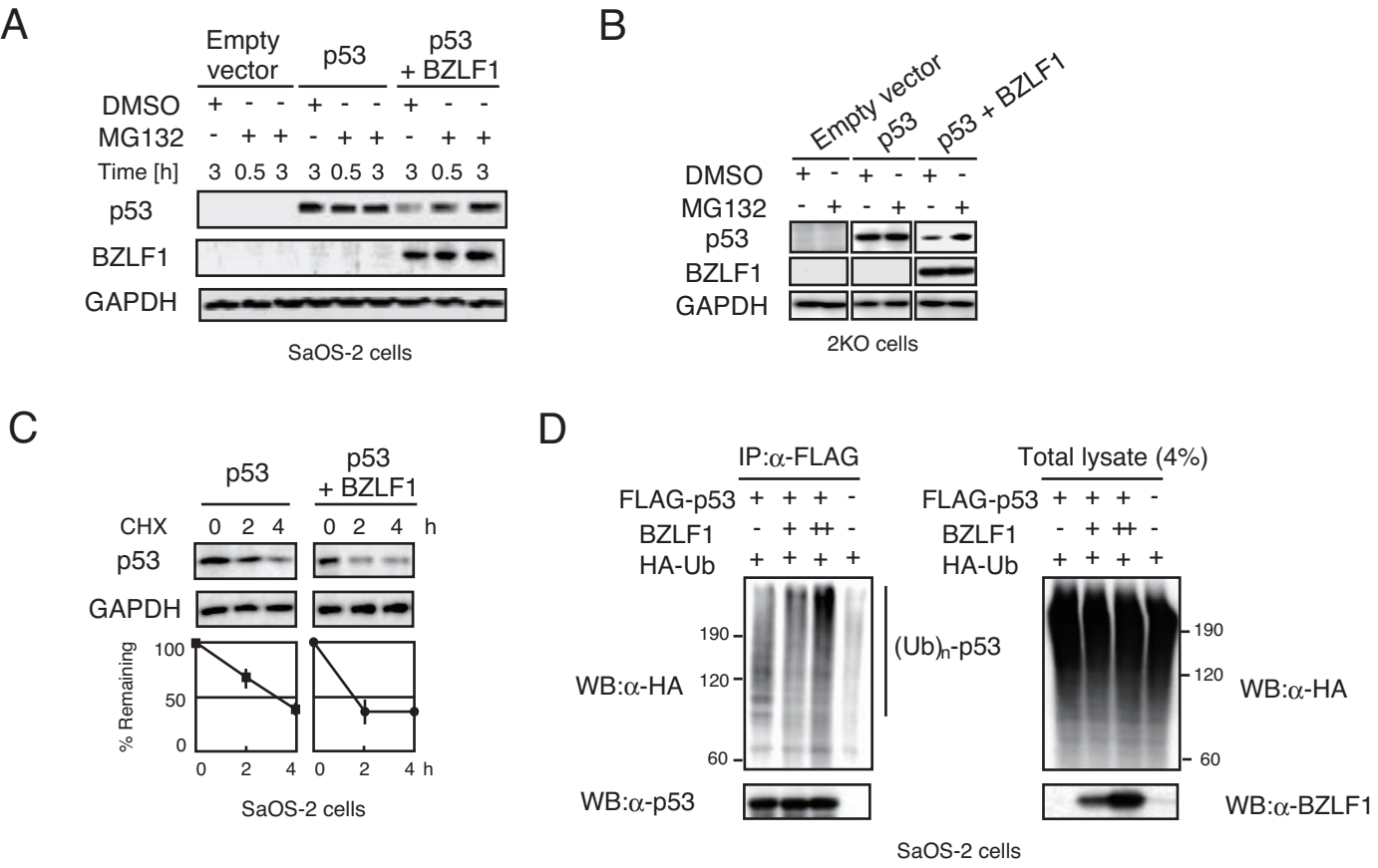

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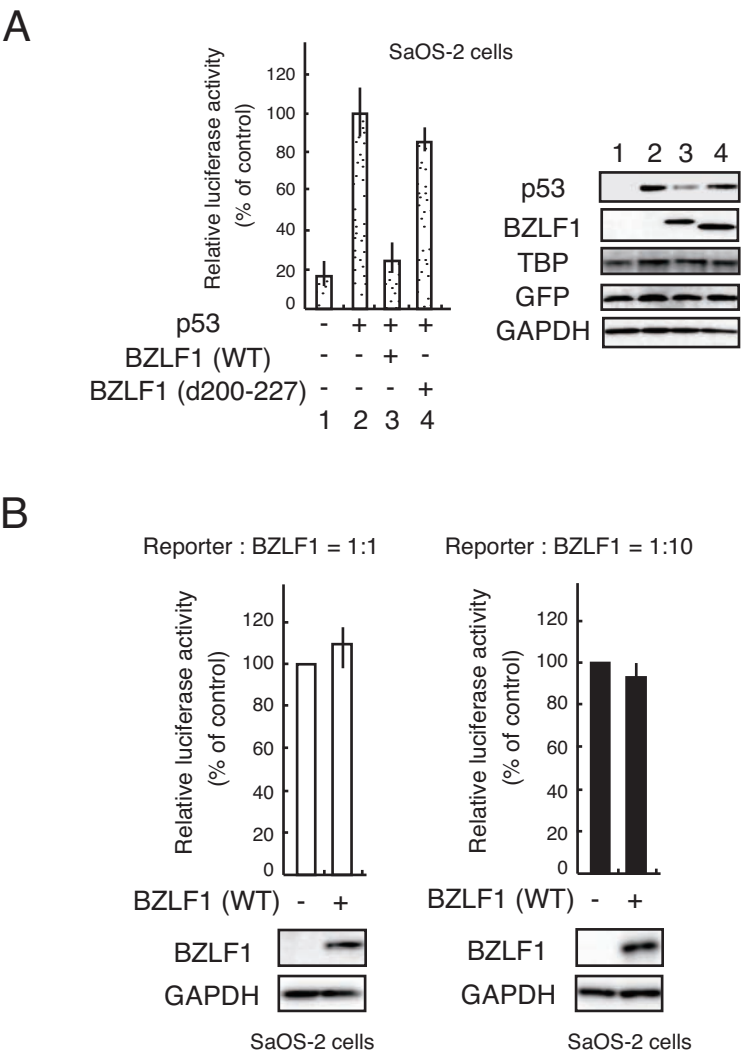

Figure 6

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