Inhibition of Transforming Growth Factor β Signaling and Smad-dependent Activation of Transcription by the Latent Membrane Protein 1 of Epstein-Barr Virus*

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Inhibition of transforming growth factor β (TGFβ) signaling by the Epstein-Barr virus Latent Membrane Protein 1 (LMP1) may account, at least in part, for the oncogenic activity of LMP1. We found that LMP1 is a potent inhibitor of TGFβ signaling and Smad-dependent activation of transcription in 293 epithelial cells and COS-7 fibroblasts. LMP1 strongly inhibited the uninduced and the Smad-inducible activity of the promoters of the human p21/WAF1/Cip1 gene and the mouse Smad7 gene. Inhibition of TGFβ signaling and Smad-dependent activation of transcription by LMP1 was greatly reduced by deletion of both C-terminal activating regions 1 and 2 of LMP1 as well as by overexpression of a non-degradable form of IκB. In contrast, specific inhibitors of p38 kinase or MEK kinase did not reverse the inhibitory activity of LMP1. TGFβ signaling was enhanced by overexpression of dominant negative forms of the LMP1 effectors TRAF2, NIK, and IKKβ and was abolished by overexpression of p65/RelA or a p50/p65 fusion protein. Deletion of the transactivation domain of p65 abolished its inhibitory activity. Immunoblotting and immunofluorescence microscopy indicated that suppression of TGFβ signaling and Smad transcriptional activity by LMP1 was not due to Smad degradation or cytoplasmic retention suggesting that LMP1 affects the nuclear function of Smad proteins. Our data are consistent with an essential role of NF-κB activation by LMP1 in the inhibition of TGFβ signaling and Smad-mediated transcriptional responses.

The latent membrane protein 1 (LMP1)1 of Epstein-Barr virus (EBV) is the principal transforming antigen of the virus.

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1 The abbreviations used are: LMP1, Latent Membrane Protein 1; AP1, activator protein 1; CTAR, C-terminal activating region; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; IKKβ, IκB kinase β; IKKβ-DN, dominant negative IKKβ; IκB-ND, non-degradable IκB; NF-κB, nuclear factor of κB; NIK, NF-κB-inducing kinase; PBS, phosphate-buffered saline; Smad, Sma and Mad-related protein; TES, transformation effector site; TGFβ, transforming growth factor β; TRAF, tumor necrosis factor receptor-activated factor; EBV, Epstein-Barr virus; STAT, signal transducers and activators of transcription; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; CMV, cytomegalovirus; PBS, fetal bovine serum; HRP, horseradish peroxidase; MES, 4-morpholinopentanesulfonic acid; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; wt, wild type; GFP, green fluorescence protein.
death domain protein (23, 24). NF-xB activation by LMP1 is mediated by NIK or a related MAP kinase kinase kinase and the subsequent activation of the IkB kinase (IKK) complex (25). AP1 activation by TRAF2/TESS2 is mediated through the activation of JNK and p38 (13), whereas TRAF1/TESS1 activates AP1 through p38 (14, 18). LMP1 induces multiple cellular genes involved in B lymphocyte and epithelial cell activation and survival (1). Many of these genes are induced at the transcriptional level through the activity of NF-xB and AP1.

In this report we examined the effect of LMP1 on TGFβ signaling. TGFβ is a cytokine that affects gene expression by binding to type I and type II TGFβ receptors (26, 27). Following TGFβ stimulation, the type II receptor phosphorylates the type I receptor, and the latter binds to and phosphorylates the pathway-restricted Smads 2 and 3. The phosphorylated Smads 2 or 3 proteins subsequently bind to the common partner Smad 4 and translocate to the nucleus where they affect the transcription of target genes that are involved in important biological processes such as cell growth, differentiation, development, apoptosis, and anti-inflammatory activity (26, 27). Smad proteins have been shown to mediate the transcriptional activation of various TGFβ-responsive genes such as the collagen (28), the tissue plasminogen activator inhibitor (29), the JunB protooncogene (30), and the tissue plasminogen activator (31, 32), among others.

Ebine-Barr virus interferes with the growth-suppressive pathway of TGFβ, and it has been reported that the primary effector of this antagonism in B-lymphocytes is LMP1 (33). However, a more recent report has cast doubt on the critical role of LMP1 in preventing TGFβ-mediated growth in B lymphocytes (34). In the present study we wished to determine whether the LMP1 and TGFβ signaling pathways interact at the molecular level and to identify the mechanism of interference of LMP1 in TGFβ-mediated functions in epithelial cells and fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents for cell culture (DMEM, fetal bovine serum, T cells, virus, EDTA, phosphate-buffered saline, penicillin, streptomycin, and gentamycin) were purchased from Invitrogen. O-Nitrophenyl-galactoside and the anti-myc antibody 9E10 were purchased from Sigma-Aldrich Ltd. The luciferase assay system was purchased from Promega Corp. All reagents for cell culture (DMEM, fetal bovine serum, penicillin, streptomycin) were purchased from Invitrogen.

**Plasmid Constructions**—The expression plasmids pSG5-FLAG-LMP1, pcDNA3-FLAG-LMP1-(1-231), pcDNA3-FLAG-LMP1-(188-351), pcDNA-IxN-BD, pcDNA3-FLAG-NIK, pcDNA3-NIK-DN, pcDNA3-FLAG-IKKα99 (expresses wt IKKβ), pcDNA3-FLAG-IKKj334 (expresses dominant negative IKKβ), and pcDNA3-FLAG-TRAF2 have been described previously (15, 25). Plasmid pSG5-LMP1-(1-185) has been described previously (20). The expression vector pBS-CMV-p65 was kindly offered by Dr. D. Thanos (Columbia University, New York, NY). Vectors for the constitutively active p50 subunit (pSG5-p50) and the mutant p65 subunit with a deletion of the transactivation domain (pcMV-p65ΔC) were a gift from Dr. G. Mavrothalassitis (University of Crete, Heraklion, Greece). The reporter plasmids pCAGAluc-EIB-luc and the plasmid pEGFP-Smad3 were kind gifts of Dr. Aria Moustakas (LICR-Uppsala, Sweden). The p(3XEc-L) homopolymeric promoter construct as well as the pCMV-p65/p50 vector have been described previously (35). The plasmid pBXG1-Smad3, expressing the human Smad3 protein fused at its N terminus with the DNA binding domain (amino acids 1-147) of the yeast transactivator GAL4, was constructed by cloning the Smad3 cDNA excised from plasmid pcDNA1-amp-6myc-Smad3 by EcoRI/NdeI into the corresponding sites of vector pBIX-G1, which contains the GAL4 DNA binding domain under the control of the CMV promoter. The reporter plasmid (−2.300/+8) p21-luc has been described previously (31). The reporter plasmid (−4.200/+110) Smad7-luc was a generous gift of Dr. R. Heuchel (LICR-Uppsala, Sweden).

**Cell Cultures, Transient Transfections, β-Galactosidase, and Luciferase Assays**—HEK-293, COS-7, and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% of fetal bovine serum (FBS), and penicillin-streptomycin in a 37 °C/5% CO2 incubator in a humidified atmosphere. Transient transfections were performed by the CaPO4 precipitate method (36). For transient transfections, HEK-293 and COS-7 cells were seeded at 2.5 × 10⁴ cells/well in 6-well plates or at 5 × 10⁴ cells/well in 10-cm dishes 48 h before transfection, while HepG2 were seeded at 2.5 × 10⁴ cells/well in 6-well plates and at 1 × 10⁴ cells per 10-cm dish, respectively, the day before transfection. Cells were measured with a Neubauer hemocytometer (Hauser Scientific). The total amount of DNA that was used in each transactivation experiment (6-well plates) was 6 µg. The total amount of DNA that was used in protein expression experiments (10-cm dishes) was 50 µg. Forty hours following transfection, cells were washed twice with PBS and collected in luciferase lysis buffer (Promega Corp). β-Galactosidase assays were performed as described previously (37). Luciferase assays were performed using the Luciferase assay kit from Promega Corp. according to the manufacturer’s instructions.

**Western Blotting**—For Western blotting analysis, protein extracts were resolved by 8.5% or 12.5% polyacrylamide gel electrophoresis. Electrophoresis was performed in 500 mL of 1× TBS (1 liter of 10× TBS: 30.3 g of Tris, 144.2 g of glycerol, 10 g of SDS, pH 8.3), using the Bio-Rad Protein electroblotting apparatus. Proteins on the membrane were visualized by Ponceau S staining. Nitrilcellulose membranes were washed with TBS-T (TBS + 0.05% Tween-20) for 10 min, at room temperature. Nonspecific sites were blocked by washing with TBS (1×) for 1 h at room temperature. Western blotting was performed with a 1:5000 dilution in TBS of the mouse monoclonal antibody anti-myc (9E10) or mouse monoclonal antibodies specific for the C terminus (S12) or N terminus (EBV.OT22CN) of LMP1, for 1 h at room temperature. The membranes were washed three times with TBS-T, for 10 min, at room temperature. As a secondary antibody we used goat anti-mouse horseradish peroxidase (HRP) conjugated in a 1:10,000 dilution in TBS-T, for 1 h at room temperature. After three washes of 10 min with TBS-T at room temperature, bands were visualized by enhanced chemiluminescent detection (ECL Western blotting kit, Amersham Biosciences, Inc.) using ECL-hyperfilm.

**Indirect Immunofluorescence and Confocal Microscopy**—For the indirect immunofluorescence experiments, transient COS-7 cells were plated on gelatin-coated coverslips and incubated for 16–18 h. Cells were washed three times on a slow rotating platform with PBS ++ (PBS plus 0.9 mM CaCl₂ and 0.5 mM MgCl₂) and fixed with 3% formaldehyde in PBS ++ for 5 min at room temperature. Cells were washed with PBS ++/+ three times and permeabilized with 0.5% Triton X-100 in buffer 1 (buffer 1 (1×) = 137 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 5.5 mM glucose, 4 mM NaHCO₃, 2 mM MgCl₂, 2 mM EDTA, 2 mM EGTA, 20 mM MES, pH 6.0–6.5) for 5 min at room temperature. Cells were washed three times with PBS ++/− and blocked with PBS ++/−/1.5% FBS two times. Cells were incubated with the first antibody (anti-FLAG, 1:200 dilution in PBS ++/−/1.5% FBS) for 30 min at 4 °C. Cells were washed three times with PBS ++/−/1.5% FBS and incubated with the secondary antibody (anti-mouse IgG Texas Red-conjugated, 1:50 dilution in PBS ++/−/1.5% FBS) for 30 min at 4 °C in the dark. Cells were washed three times with PBS ++/− in the dark and mounted on glass slides using mounting solution (1:1 glycerol/PBS). Cells were observed using a Leica SP confocal fluorescent microscopy.

**RESULTS**

**Inhibition of TGFβ Signaling and Smad Transcriptional Activity by the Latent Membrane Protein 1 of Epstein-Barr Virus**—To determine whether the LMP1 and the TGFβ signaling pathways exhibit cross-talk, we performed a series of transient transfection experiments in human embryonic kidney epithelial 293 cells and COS-7 fibroblasts. In these experiments, TGFβ signaling and Smad transcriptional activity was monitored by using the TGFβ-inducible reporter plasmid pCAGAluc-EIB-luc (Fig. 1E and Ref. 38). As shown in Fig. 1B, the transactivation of this promoter in 293 cells was strongly induced (17.4-fold) by the addition of 200 nM of TGFβ. However, the inducibility of this promoter was reduced to 3.6-fold by LMP1 (83% reduction). The inhibition of TGFβ signaling by LMP1 in 293 cells was also confirmed using a constitutively active type 1 TGFβ receptor (CA-ALK5) or human Smad3 and Smad4 proteins. As shown in Fig. 1B, the CA-ALK5 receptor transactivated the cDNA-(EIB-luc) promoter at 13.7-fold, and this transactivation was reduced to 1.8-fold by co-transfection.
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A Non-degradable Form of IκB Abolishes the LMP1 Inhibitory Activity on the TGFβ Signaling Pathway

To evaluate the contribution of NF-κB to the mechanism of inhibition of TGFβ of LMP1 (87% reduction). Similarly, the Smad3/Smad4 proteins transactivated the (CAGA)12-E1B promoter by 17.9-fold, and this transactivation was reduced to 4.6-fold by co-transfection of LMP1 (75% reduction).

LMP1 also suppressed TGFβ signaling in COS-7 fibroblasts. As shown in Fig. 1C, the −18-fold induction of the (CAGA)12-E1B promoter by the CA-ALK5 receptor and the Smad3 and Smad4 proteins was strongly inhibited to 2.4-fold by LMP1 (greater than 85% reduction in both cases). Furthermore, the inhibition of Smad3/Smad4-mediated transactivation of the (CAGA)12-E1B promoter by LMP1 in COS-7 cells was dose-dependent and had reached a plateau at 25 ng of the LMP1 expression vector (Fig. 1D). LMP1 had a minimal effect on the activity of the (CAGA)12-E1B promoter in both 293 and COS-7 cells (data not shown).

The findings of Fig. 1 clearly indicate that the expression of LMP1 protein in epithelial cells or in fibroblasts drastically impairs TGFβ signaling and Smad-mediated activation of transcription suggesting that the cross-talk of the two signaling pathways is antagonistic.

LMP1 Inhibits TGFβ Signaling on the p21/WAF1/Cip-1 and Smad7 Promoters—The effect of LMP1 on TGFβ signaling was investigated further by using two natural TGFβ-inducible promoters: the promoter of the human cell cycle inhibitor p21/WAF1/Cip-1 gene and the promoter of the mouse Smad7 gene. Both of these promoters were shown previously to be induced by TGFβ and Smad proteins (31, 32, 39). As shown in Fig. 2A, Smad3/Smad4 proteins transactivated the human −2300/+8 p21 promoter by 2.2-fold. LMP1 inhibited the Smad3/Smad4 transactivation of the p21 promoter by 91% and the basal activity of this promoter by 60% (Fig. 2A).

Overexpression of Smad3 and Smad4 proteins in COS-7 cells transactivated strongly (7.2-fold) the mouse −4200/+110 Smad7 promoter (Fig. 2B). However, the transactivation of the Smad7 promoter by Smads was totally abolished in the presence of LMP1. As in the case of the p21 promoter, LMP1 inhibited the Smad3/Smad4-mediated transactivation of the Smad7 promoter by 94% and the basal activity of this promoter by 50%.

Taken together, the data of Fig. 2 indicate that LMP1 is a strong inhibitor of the activity of the p21/WAF1/Cip-1 and Smad7 promoters and that the induction of these promoters by Smad proteins is abolished by LMP1.
signaling by LMP1, cells were co-transfected with the (CAGA)\textsubscript{12}-E1B-luc reporter construct along with expression vectors for CA-ALK5, LMP1, and a non-degradable form of I\(\beta\)-B (I\(\beta\)-B-ND) shown previously to block the NF-\(\kappa\)B pathway by sequestering NF-\(\kappa\)B into the cytoplasm (40). As shown in Fig. 3 (A and B), the inhibition of CA-ALK5 signaling to the (CAGA)\textsubscript{12}-E1B promoter by LMP1 was totally reversed by overexpression of the I\(\beta\)-B-ND mutant in 293 (panel A) or COS-7 (panel B) cells. The effectiveness of the I\(\beta\)-B-ND mutant in blocking NF-\(\kappa\)B activity in COS-7 cells was evaluated using the NF-\(\kappa\)B-responsive reporter construct p(3X\(\beta\)-B-L) (35). As shown in Fig. 3C, this NF-\(\kappa\)B-responsive promoter was strongly induced by LMP1 (8.6-fold), and the LMP1-mediated transactivation was totally abolished by the I\(\beta\)-B-ND mutant (0.5-fold). In contrast, a specific inhibitor of the p38 MAP kinase (SB 203580) or the MEK kinase (PD 98059) could not counteract the suppression of TGF\(\beta\) signaling by LMP1 (Fig. 3D).

The combined data of Fig. 3 (A–D) indicate that the inhibition of TGF\(\beta\) signaling by LMP1 is mediated by a signaling cascade leading to activation of the transcription factor NF-\(\kappa\)B.

**At Least One of the C-terminal Activating Regions / Transformation Effector Sites (CTAR1/TE51, CTAR2/TE52) of LMP1 Is Sufficient for Inhibition of TGF\(\beta\) Signaling**—To determine the relative contribution of each of the CTAR/TE5 domains in the mechanism of inhibition of TGF\(\beta\) signaling by LMP1, three truncated forms of LMP1 were utilized in transient transfection experiments in COS-7 cells (Fig. 4A). Mutant LMP1 (1–187) has a C-terminal truncation of the 188–386 region that contains both CTAR1/TE51 and CTAR2/TE52 domains. Mutant LMP1 (1–231) lacks the 232–386 region that contains the CTAR2/TE52 domain but retains the CTAR1/TE51 domain. Mutant LMP1 (188–351) contains an internal deletion between amino acids 188 and 351, which removes the CTAR1/TE51 domain but leaves intact the CTAR2/TE52 domain.

These truncated LMP1 forms were expressed in COS-7 cells to levels comparable with or higher than those of the wt LMP1 protein (Fig. 4B). As shown in Fig. 4C, LMP1 (1–187) was very ineffective in inhibiting CA-ALK5 receptor signaling (22% inhibition relative to 100% inhibition by the wt LMP1 protein). In contrast, both LMP1 (1–231) and LMP1 (188–351) inhibited CA-ALK5 signaling. Importantly, the inhibition of CA-ALK5 signaling by the wt and the 1–281 and 188–351 LMP1 forms was totally reversed by co-expression of the I\(\beta\)-B-ND mutant (Fig. 4C). Similar results were obtained using an LMP1 mutant (LMP1-AAA) containing a triple-amino acid substitution in the CTAR1/TE51 domain (P204A,Q206A,T208A) as well as an LMP1 mutant (Y384G) containing a replacement of threonine at position 384 by glycine in the core of CTAR2 (15–20). Both the LMP1-AAA and the LMP1-Y384G mutants inhibited the Smad3/Smad4-mediated transactivation of the (CAGA)\textsubscript{12}-E1B reporter in COS-7 cells (data not shown).

In a parallel control experiment, the effectiveness of the truncated LMP1 forms to activate NF-\(\kappa\)B in COS-7 cells was evaluated by using the p(3X\(\beta\)-B-L) reporter construct in the presence and absence of the I\(\beta\)-B-ND mutant. As shown in Fig. 4D, wt LMP1 transactivated the 3X\(\beta\)-B-L promoter by 8.7-fold. Deletion of CTAR2/TE52 (in LMP1 1–231) decreased NF-\(\kappa\)B transactivation to 4.2-fold (52% reduction) and deletion of...
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CTAR1/TES1 (in LMP1 Δ188–351) decreased the transactivation to 6.5-fold (25% reduction). Finally, deletion of both CTAR1/TES1 and CTAR2/TES2 reduced the transactivation of the 3XbB-L promoter by LMP1 to 1.9-fold (78% reduction). In all cases, co-transfection of the IxB-ND mutant abolished the transactivation of the 3XbB-L promoter by the wild type or mutant forms of LMP1 (Fig. 4D).

The combined data of Fig. 4 (A–D) indicate that at least one of the NF-κB-activating domains in the C-terminal cytoplasmic region of LMP1 (CTAR1/TES1 or CTAR2/TES2) is sufficient for induction of 3XbB-L promoter activity and inhibition of TGFβ signaling.

Inhibition of TGFβ Signaling and Smad-mediated Activation of Transcription Activity by Key Effectors of LMP1—As discussed in the introduction, NF-κB activation by the CTAR1/ TES1 and CTAR2/TES2 domains of LMP1 is likely to be mediated by the binding of TRAF2 directly or indirectly to both the CTAR1/TES1 and CTAR2/TES2 domains and the subsequent activation of the NIK or a related MAP kinase kinase kinase and the IxB kinase (IKKβ) complex. As shown in Fig. 5A, overexpression of TRAF2, NIK, and IKKβ in COS-7 cells along with Smad3/Smad4 resulted in a dramatic inhibition (92–97%) of the transactivation of the (CAGA)12-E1B promoter. In agreement with this finding, overexpression of dominant negative forms of these proteins in HepG2 cells resulted in an marked enhancement of TGFβ-mediated transactivation of the (CAGA)12-E1B promoter. The transactivation of the (CAGA)12- E1B promoter by TGFβ in the presence of TRAF-2-DN, NIK-DN, and IKKβ-DN was enhanced by 3.5-, 4-, and 1.9-fold, respectively (Fig. 5B). These findings suggest that the endogenous NF-κB activity has a negative effect on TGFβ signaling. The expression of myc-Smad3 and myc-Smad4 proteins was not affected by IKKβ, NIK, and TRAF2 as determined by Western blotting in transfected COS-7 cells (Fig. 5C).

The combined data of Figs. 1–5 strongly suggest that the inhibition of TGFβ signaling and Smad transcriptional activity by LMP1 is mediated via NF-κB activation.

Inhibition of TGFβ Signaling and Smad Transcriptional Activity by NF-κB Is Compromised by Deletion of the C-terminal Domain of p65 (Amino Acids 496–551)—To study further the mechanism of inhibition of TGFβ signaling by NF-κB, COS-7 cells were transiently transfected by the (CAGA)12-E1B-luc reporter along with the CA-ALK5 receptor and expression vectors for a constitutively active form of p50 or the p65 subunits of NF-κB. As shown in Fig. 6A, p65 but not p50 strongly inhibited TGFβ signaling in COS-7 cells. In contrast, a truncated p65 form lacking the C-terminal domain 496–551 (p65 ΔC) could not confer inhibition to the signaling pathway triggered by the ALK5 receptor (Fig. 6A). In a parallel experiment, p65 strongly inhibited the Smad3/Smad4-mediated transactivation of the (CAGA)12-E1B promoter, whereas the constitutively active p50 subunit and the p65 ΔC mutant did not. Strong inhibition of Smad-mediated transactivation of the (CAGA)12-E1B was achieved by a fusion protein in which the Rel homology domain of p65 was swapped with domains of p50 and the p65 subunits of NF-κB. As shown in Fig. 6A, p65 but not p50 strongly inhibited TGFβ signaling in COS-7 cells. In contrast, a truncated p65 form lacking the C-terminal domain 496–551 (p65 ΔC) could not confer inhibition to the signaling pathway triggered by the ALK5 receptor (Fig. 6A).

In a control experiment, the transcriptional activity of the p50, p65, p65 ΔC, and p50/p65 proteins on the 3XbB-L promoter was tested in COS-7 cells (Fig. 6C). As expected, both p65 and the p50/p65 fusion proteins strongly transactivated the 3XbB-L promoter by 43- and 37-fold, respectively, whereas the p50 subunit as well as the p65 ΔC mutant were unable to transactivate the 3XbB-L promoter.

In conclusion, the findings of Fig. 6 suggest that inhibition of TGFβ signaling and Smad function by LMP1 seems to be mediated specifically by the p65/RelA subunit of NF-κB and

**Fig. 4.** Deletion of individual CTAR/TES domains in LMP1 do not affect its potential to inhibit TGFβ signaling. A, a schematic representation of the LMP1-truncated forms used in the transfection experiments of panels C and D. Symbols are as in Fig. 1A. B, analysis of the levels of expression of LMP1 proteins in COS-7 cells by immunoblotting. COS-7 cells (5 × 10^5^ cells in 10-cm dishes) were transfected with the 5 μg of the vectors FLAG-LMP1 (wt), FLAG-LMP1-Δ188–351, and LMP1-1–187 as indicated at the top of the figures. Forty hours following transfection, cells were lysed and expression of LMP1 was monitored by SDS-PAGE and Western blotting using antibodies specific for the FLAG epitope or the N terminus (EBV.OT22CN) of LMP1 as indicated at the bottom of the figures. The position of wt and truncated LMP1 forms is shown by arrows. C, COS-7 cells were transiently transfected with the p(CAGA)12-E1B-luc reporter (500 ng) along with expression vectors for CA-ALK5 (500 ng), LMP1 (500 ng), LMP1-1–187 (500 ng), LMP1-1–231 (500 ng), LMP1-Δ188–351 (500 ng), and the IxB-ND mutant (1 μg) as indicated at the bottom of the bar graphs. The CMV-β-galactosidase plasmid (1 μg) was included in each sample for the normalization of transfection efficiency. D, COS-7 cells were transiently transfected with the p3XbB-L reporter (1 μg) along with expression vectors for LMP1 (500 ng), LMP1-1–187 (500 ng), LMP1-1–231 (500 ng), LMP1-Δ188–351 (500 ng), and the IxB-ND mutant (1 μg) as indicated at the bottom of the bar graph.

The combined data of Fig. 4 (A–D) indicate that at least one of the NF-κB-activating domains in the C-terminal cytoplasmic region of LMP1 (CTAR1/TES1 or CTAR2/TES2) is sufficient for induction of 3XbB-L promoter activity and inhibition of TGFβ signaling.
that the C-terminal region of p65/RelA (amino acids 496–551), which is part of the transactivation domain of the protein is essential for the inhibition.

Inhibition of GAL4-Smad3 Transcriptional Activity by LMP1 and p65—To investigate further the mechanism of inhibition of Smad transcriptional activity by LMP1, a GAL4-based transactivation assay was employed in COS-7 cells. In this experiment, the reporter pG3B-E1B-luc (Fig. 7A) was transactivated 7.7-fold by GAL4-Smad3 but not by GAL4 alone (Fig. 7B). The GAL4-Smad3-mediated transactivation of the GAL4 reporter was totally abolished by co-expression of either LMP1 or p65. Furthermore, the transcriptional repression posed by LMP1 on the GAL4-Smad3 could be reversed by the IκB-ND mutant shown previously to inhibit signaling of LMP1 via NF-κB (Fig. 3D). Immunofluorescence analysis showed that the GAL4-Smad3 fusion protein was localized in the nucleus either in the absence or presence of LMP1 (data not shown).

LMP1 Does Not Affect the Nuclear Localization of Smad Proteins—To determine whether LMP1 affects Smad3 transcriptional activity by altering its subcellular distribution, COS-7 cells were transiently transfected with a vector expressing Smad3 fused at the N terminus with green fluorescence protein (GFP-Smad3) along with a vector expressing FLAG-tagged LMP1 or the empty vector as a control. In the GFP-Smad3-transfected cells, the green fluorescence was detected almost exclusively in the nucleus (Fig. 8A). In the GFP-Smad3/FLAG-LMP1 co-transfected cells, GFP-Smad3 was again localized in the nucleus (panel B), whereas FLAG-LMP1 (red fluorescence) was distributed in perinuclear and other cellular membranes (panel C). Co-expression of the two proteins in the same cell was confirmed by overlaying the green and red fluorescent images (panel D). The same pattern was observed when the LMP1-(1–231) mutant was co-transfected in COS-7 cells along with GFP-Smad3 (panels E–G). These results indicate that LMP1 does not alter the subcellular distribution of Smad3 protein in the LMP1/GFP-Smad3 co-transfected COS-7 cells.

In conclusion, the data presented in Figs. 7 and 8 suggest that LMP1 does not affect the nuclear localization of Smad3 protein but rather suppresses the transcriptional activity of Smad3 in the nucleus by an as yet unknown mechanism.

**DISCUSSION**

Loss of Sensitivity to TGFβ Leads to Cell Transformation and Cancer—Transforming growth factor β (TGFβ) is a tumor suppressor cytokine that potently inhibits cell proliferation due to cell cycle arrest in the G1 phase (41). Although the mechanism by which TGFβ causes cell cycle arrest in G1 is not elucidated, several studies have suggested that this may be due to the up-regulation of cell cycle inhibitor genes such as p21/WAF1/Cip-1 and p15 (31, 32, 39, 42). Perturbation of the TGFβ/Smad signaling pathway may result in progression of tumors through resistance of the cells to the growth inhibition induced by
TGFβ. For example, loss of sensitivity to TGFβ has been observed in cells transformed by several viral oncogenes such as the T antigen of simian virus 40, the adenovirus E1A gene product, and the Latent Membrane Protein 1 (LMP1) of Epstein-Barr virus (33, 43). LMP1 protein is essential for B lymphocyte transformation associated with EBV infection (2). In a recent study, Takanashi et al. (44) showed that LMP1 transformed rodent fibroblasts such as NIH 3T3 and Rat1 in culture. Cell transformation was evidenced by loss of contact inhibition, anchorage-independent growth, and loss of TGFβ sensitivity.

In another study, Arvanitakis et al. (33) showed that LMP1 caused loss of TGFβ-mediated growth inhibition in human B lymphocytes, whereas LMP1 had no effect in the TGFβ-medi-
ated transcriptional activation of target genes such as junB. Furthermore, the EBV-transformed B cells had a normal TGFβ receptor profile and functional TGFβ receptors suggesting that the regulatory and the growth inhibitory responses of genes in B cells to TGFβ result from separate signaling cascades. However, a more recent study did not reveal a negative role of LMP1 on TGFβ-mediated growth inhibition of B lymphocytes (34). The reason for this discrepancy is unclear, but it could be due to differences in culture conditions and levels of LMP1 expression.

LMP1 is an Inhibitor of the Basal and TGFβ-inducible Activity of the Promoters of the p21/WAF1/Cip1 and Smad7 Genes—In the present study, it was shown that expression of low levels of LMP1 abolished the Smad-mediated transactivation of the homopolymeric (CAGA)_{12} reporter (Fig. 1D) suggesting that Smad function in the LMP1-transfected cells was deregulated. Most importantly, LMP1 strongly inhibited the basal as well as the TGFβ-inducible activity of the promoter of the human cell cycle inhibitor p21/WAF1/Cip-1 (p21) gene (Fig. 2). Inhibition of the constitutive p21 promoter activity by LMP1 could be explained by assuming that LMP1 affects the activity of endogenous Smad proteins, which are expressed in COS-7 cells (32). Alternatively, LMP1 could block the transactivation function of other factors such as Sp1 that bind to the p21 promoter and modulate its activity. Sp1 binds to six sites located in the region between −122 and −60 relative to the transcription initiation site of the p21 gene (45). The potential involvement of Sp1 in the LMP1-mediated repression of the p21 promoter is supported by the recent finding showing that LMP1 repressed the transcription driven by the promoter of the CD99 gene, which encodes for a protein that is involved in cell adhesion, differentiation, and apoptosis in T cells and neurons (46). The proximal promoter of the CD99 gene contains an Sp1 site, and this site appears to be indispensable for the repressive activity of LMP1 (46).

A potential mechanism by which LMP1 inhibits TGFβ signaling could be the up-regulation of the inhibitory Smad7 gene (47). Smad7 gene transcription is rapidly induced by TGFβ indicating that Smad7 is involved in the fine-tuning of the cellular response to TGFβ (48–50). In the current study, we found that Smad7 promoter activity was strongly repressed in LMP1-transfected cells. Furthermore, the Smad3/Smad4-mediated transactivation of the Smad7 promoter was totally abolished by co-transfection with LMP1 (Fig. 2B) or p65/RelA (data not shown) in agreement with previous findings (51). By suppressing Smad7 gene transcription, LMP1 could deregulate the network of TGFβ-induced genes that affect cell growth, differentiation, and apoptosis.

Critical Role of NF-κB in LMP1-mediated Inhibition of TGFβ Signaling and Smad Transcriptional Activity—The important role of NF-κB in the mechanism of inhibition of TGFβ signaling by LMP1 is supported by experiments of this study. First, we observed strongly inhibited inhibition of Smad-mediated transactivation of the (CAGA)_{12}E1B promoter by overexpression of key effectors of LMP1 signaling such as TRAF2, NIK, and IKKβ (Fig. 5A). Furthermore, dominant negative forms of the above effectors (TRAF2-DN, NIK-DN, and IKKβ-DN) induced TGFβ signaling indicating that, under conditions of TGFβ stimulation, endogenous NF-κB factor antagonizes Smad proteins. Second, overexpression of the p65/RelA subunit of NF-κB strongly inhibited both ALK-5 and Smad-mediated transactivation of the (CAGA)_{12}E1B promoter in COS-7 cells. Interactions between NF-κB and Smads have been observed previously (51–53). For example, negative interactions between p65 and Smad3 transcription factors were observed using the 3TP-lux reporter that contains Smad but not NF-κB binding sites or the activin-responsive AR3 promoter that also lacks NF-κB binding sites and requires FAST-2 for transactivation (51). Inhibition of TGFβ signaling by NF-κB correlates also with the critical role of this transcription factor in rodent fibroblast transformation by LMP1 (53).

Potential Mechanisms of Suppression of TGFβ Signaling by LMP1—Suppression of TGFβ signaling by LMP1 could be accounted for by one or more of the following putative mechanisms: (a) LMP1-mediated cytoplasmic retention or enhanced nuclear export of Smad proteins. In a previous report, it was shown that the subcellular distribution of Smad2 and Smad3 proteins could be modified by oncogenically activated Ras and this mechanism of Smad inactivation involved phosphorylation of Smad proteins in the linker region (54). In a similar manner, Smads could be modified by an LMP1-induced kinase resulting in their cytoplasmatic retention and transcriptional inactivation. Although we cannot exclude the possibility of phosphorylation of Smads by an LMP1-inducible kinase, our present data do not support the cytoplasmic retention hypothesis. Using a combination of direct and indirect immunofluorescence confocal microscopy analyses, we found that LMP1 does not affect the subcellular distribution of Smad3 protein in COS-7 cells (Fig. 8). In this experiment, the expression of FLAG-LMP1 was monitored by indirect immunofluorescence confocal microscopy using an antibody specific for the FLAG antigen fused with the LMP1 protein and a Texas Red-conjugated secondary antibody. This analysis revealed that staining of both full-length LMP1 and the truncated LMP1 form 1–231 was punctate and perinuclear (Fig. 8, C and F). Punctate staining of LMP1 in fibroblasts (clearly evident in Fig. 8F) is consistent with plasma membrane localization as reported previously (2, 55). Perinuclear localization of LMP1 has also been observed before in fibroblasts (55). This staining pattern of LMP1 is consistent with localization to the endoplasmic reticulum and is likely to be due to the ability of LMP1 to insert itself post-translationally to intracellular membranes in addition to the plasma membrane. The expression of Smad3 protein was monitored by the fluorescence emitted from the green fluorescence protein that was fused at the N terminus of Smad3 protein. GFP-Smad3 was consistently localized in the nucleus of transfected COS-7 cells even in the absence of added TGFβ possibly due to the overexpression of the transfected protein (Fig. 8A). These observations are consistent with previous work, which showed that overexpression of Smad3 and Smad4 proteins in COS-1 cells or HepG2 cells leads to exclusive nuclear localization of Smad proteins and TGFβ-independent transactivation of target promoters such as the plasminogen activator inhibitor gene promoter or the p21/WAF1/Cip1 promoter (31, 56). In the co-transfected cells, the nuclear localization of GFP-Smad3 was not affected by co-expression of the wt or the truncated 1–231 LMP1 forms (Fig. 8, D and G). Based on these observations we conclude that the dramatic inhibition of Smad3 transcriptional activity by LMP1 does not seem to result from an LMP-1-mediated inhibition of nuclear translocation of Smad3 or an enhanced export of Smad3 to the cytoplasm. (b) Enhanced degradation of Smad3 and Smad4 proteins in cells expressing LMP1. Western blotting analysis of Smad proteins expressed in COS-7 cells transfected with LMP1 or the LMP1 effectors TRAF2, NIK, and IKKβ (Fig. 5C) did not reveal any major change in the expression of Smad3 and Smad4 compared with their expression in the absence of LMP1 or its effectors. (c) Inhibition of Smad transcriptional activity in the nucleus. In the present study we have observed that LMP1 strongly repressed the transactivation of a GAL4-Smad3 fusion protein on a GAL4-dependent promoter (Fig. 7). Because the transactivation of this promoter strictly depends on DNA bind-
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ing via GAL4 and because the GAL4-Smad3 fusion protein was localized in the nucleus in the absence or in the presence of LMP1 (data not shown), it is unlikely that LMP1 represses Smad activity by interfering with its DNA binding capacity. On the other hand, direct protein-protein interactions between Smads and NF-κB family members such as p65/RelA could in principle account for the inhibition of Smad transactivation function. Such direct physical interactions between Smad3 and p52, a p50 homologue, have been recently observed resulting in the synergistic transactivation of the TGF-β-inducible JunB promoter via an NF-κB binding site (57).

Finally, squelching of co-activators such as p300 could also account for the inactivation of Smad transcriptional activity by LMP1. It has been shown previously that p300 is important for Smad function in the nucleus and interacts physically with Smads 2 and 3 (58, 59). p300 is equally important for NF-κB activity through physical interactions with the transcription domain of p65/RelA (60, 61). Thus, activation of p65/RelA by LMP1 could deplete p300 from the nuclear pool and thus prevent the Smad-mediated transactivation of promoters consisting of Smad but not of NF-κB-responsive elements.

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