Virology **286,** 91–99 (2001)
doi:10.1006/viro.2001.0965, available online at http://www.idealibrary.com on IDEAL®

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12-*O*-Tetradecanoylphorbol-13-acetate Induces Epstein–Barr Virus Reactivation via NF-κB and AP-1 as Regulated by Protein Kinase C and Mitogen-Activated Protein Kinase

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Received March 29, 2001; returned to author for revision February 26, 2001; accepted April 19, 2001

Signaling pathway components mediating Epstein–Barr virus (EBV) reactivation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were characterized in terms of induction and modification of specific transacting factors. The consequences of protein kinase C (PKC) activation by TPA in inhibiting inducible nitric oxide synthase (iNOS) mRNA expression were analyzed in the EBV-infected gastric epithelial cell line GT38. Spontaneous expression of the EBV BZLF1 gene product ZEBRA became undetectable upon long-term culturing of GT38 cells, while iNOS mRNA expression increased. In such cells the PKC inhibitors 1-(5-isoquinolinesulphonyl)-2,5-dimethylpiperazine (H7) and staurosporine inhibited TPA-induced expression of BZLF1 and BRLF1 and reversed TPA-mediated inhibition of iNOS gene expression. The mitogen-activated protein kinase inhibitor PD98059 inhibited TPA-induced BZLF1 expression. Electrophoretic mobility shift assays demonstrated that transcription factors NF-κB and AP-1 were also activated by TPA in a time-dependent manner. The TPA-induced NF-κB activation was inhibited by prior treatment of the cells with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). TPA-induced BZLF1 expression was also inhibited by the treatment with PDTC. Northern blot analyses characterized changes in levels of the c-*jun* and *jun*B expressions of the AP-1 family. These results show that TPA induces EBV reactivation via NF-κB and AP-1 and that PKC is an important mediator in regulating gene expression leading to EBV reactivation after TPA treatment of GT38 cells. © 2001 Academic Press

INTRODUCTION

Nitric oxide (NO) is a potent second messenger with diverse physiological activities, including regulation of vascular tone and neurotransmission, and killing of microorganisms and tumor cells (Nathan, 1992; Moncada and Higgs, 1993; Bredt and Snyder, 1994). The inducible form of nitric oxide synthase (iNOS) in various cell types, including macrophages, hepatocytes, and astrocytes, is expressed in response to proinflammatory cytokines and bacterial lipopolysaccharide (Stuehr and Marlettla, 1985; Togashi *et al.,* 1997; Wang *et al.,* 1998). Our previous studies showed that iNOS mRNA is constitutively expressed in Epstein–Barr virus (EBV)-infected gastric epithelial cell lines GT38 and GT39 and that NO downregulates EBV reactivation in those cells (Gao *et al.,* 1999).

EBV is a human herpesvirus that infects B cells and epithelial cells to establish a latent infection (Kieff, 1996). The latency can be disrupted by certain compounds including 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (zur Hausen *et al.,*1978; Gao *et al.,* 1999) or by cross-linking of surface immunoglobulin (Takada, 1984). These agents activate expression of the viral immediate-early protein BZLF1, which is also termed ZEBRA, EB1, Zta, or Z (Countryman and Miller, 1985; Chevallier-Greco *et al.,* 1986; Takada *et al.,* 1986). BZLF1 is a viral homolog of the cellular c-*fos* transcription factor which binds to AP-1-like sequences termed ZEBRA response elements or ZRE (Farrell *et al.,* 1989; Flemington and Speck, 1990b; Lieberman *et al.,* 1990). The BZLF1 promoter region contains several TPA-responsive binding motifs for transcription factor such as ATF1, ATF2, and c-Jun (Wang *et al.,*1997), as well as ZRE that trigger BZLF1 expression via a positive feedback mechanism (Flemington and Speck, 1990a; Daibata *et al.,*1994).

NF-κB constitutively exists as an inactive cytoplasmic heterotrimeric complex that can be activated by proper stimuli via a posttranslational phosphorylation mechanism involving several kinases (and without inducing protein synthesis) leading to the dissociation of NF-κB from cytosolic IκB. Such released NF-κB is then able to translocate into the nucleus where it binds to a specific DNA sequence to regulate expression of various genes (Adcock *et al.,* 1994; Xie *et al.,* 1994; Kim *et al.,* 1995).

It is well known that TPA is a potent protein kinase C (PKC) activator and tumor promoter (Castagna *et al.,* 1982; Nishizuka, 1984). In a previous paper, we showed that TPA induces EBV reactivation by inhibiting iNOS mRNA expression in human gastric epithelial cell lines (Gao *et al.,* 1999). However, the molecular mechanism of EBV reactivation was not defined. In the current study,

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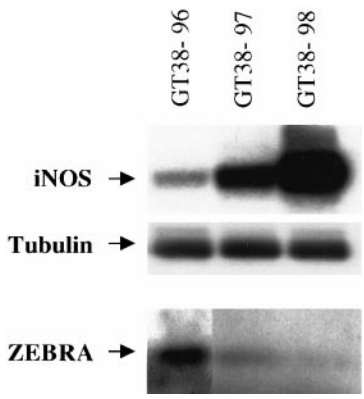

FIG. 1. Expression of iNOS mRNA and ZEBRA in GT38 cells passaged for different times after being established. GT38-96, -97, and -98 cells were passaged for 8 months, 2 years, and more than 3 years, respectively. Total RNA and protein were extracted from the confluent cultures, and RT-PCR Southern and Western blot analyses were carried out as described under Materials and Methods. Equal amounts of each sample were PCR amplified using primers specific for either iNOS or tubulin. Equal amounts of proteins were blotted onto the membrane and reacted with anti-ZEBRA monoclonal antibodies.

we have characterized in detail the intracellular signaling pathways by which TPA induces EBV reactivation and inhibits iNOS expression in human gastric epithelial cells. We have demonstrated that PKC plays an enhancing role in EBV reactivation and a suppressing role on iNOS expression.

RESULTS

iNOS gene and BZLF1 expressions in GT38 cells

The GT38 cell line was an EBV producer, expressing lytic antigens and producing infectious virus spontaneously (Takasaka *et al.,* 1998). However, we showed previously in those cells that without TPA stimulation neither BZLF1 mRNA nor the gene product ZEBRA protein was detectable and concurrently that only a low level of iNOS mRNA was constitutively expressed (Gao *et al.,* 1999). We hypothesized that the levels of iNOS expression may differ among the GT38 cell sublines depending on the cumulative duration of culture. To investigate whether endogenous NO regulates spontaneous EBV reactivation, we examined the levels of iNOS mRNA and ZEBRA by reverse transcriptase-polymerase chain reaction (RT-PCR) Southern and Western blotting, respectively, in GT38 cells cultured for 8 months after being established (GT38-96), 2 years (GT38-97), or more than 3 years (GT38-98). These "short-term," "medium-term," and "long-term" cultured sublines offer stable vignettes of varying levels of gene activation and expression for our studies. As shown in Fig. 1, the expression of ZEBRA was highest in GT38-96 cells, and it was much higher in GT38-97 cells than in GT38-98 cells, in which ZEBRA expression had almost completely disappeared. In contrast to ZEBRA, iNOS mRNA expression was highest in GT38-98 cells, and it was much higher in GT38-97 cells than in GT38-96 cells. These results suggest that constitutively produced NO may inhibit spontaneous EBV reactivation. GT38-96 cells were used for the following analyses.

Effects of PKC and mitogen-activated protein kinase (MAPK) inhibitors on EBV immediate-early gene expression and iNOS mRNA expression

The phorbol ester TPA is well known, a potent activator of PKC (Nishizuka, 1984). We reported that TPA induces EBV reactivation from latent state to lytic phase by inhibiting iNOS activation (Gao *et al.,* 1999). To analyze whether PKC and MAPK pathways are involved in the TPA-induced inhibition of iNOS gene expression and EBV reactivation, GT38 cells were treated with 1-(5-isoquinolinesulphonyl)-2,5-dimethylpiperazine (H7) or staurosporine, which are potent inhibitors of PKC, and PD98059, which is an inhibitor of MAPK, for 1 h, followed by TPA for 8 h (Fig. 2). TPA-induced expressions of BZLF1 (3.8-fold increase) and BRLF1 (3.7-fold increase) were greatly inhibited by prior treatment of the cells with either H7 (induced BZLF1 59% decrease and BRLF1 55% decrease) or staurosporine (induced BZLF1 72% decrease and BRLF1 50% decrease). PD98059 also inhibited the induction of BZLF1 by 42% and BRLF1 by 45%, although its effect appeared to be less than that of either H7 or staurosporine. On the other hand, TPA markedly inhibited iNOS mRNA expression by 93.8% and that inhibition could be reversed by prior treatment of the cells with H7, staurosporine, or PD98059 (increased 15.9-, 12.2-, and 16.5-fold, respectively, compared with TPA treatment). Taken together, these results indicated that the PKC and MAPK pathways mediate TPA-induced EBV reactivation. Furthermore, PKC and MAPK are principal negative regulators of iNOS gene expression in TPA-stimulated GT38 cells.

TPA induces NF-κB and AP-1 activation in GT38 cells

To investigate whether TPA induces NF-κB, AP-1, and SP-1 activation in GT38 cells, we performed electrophoretic mobility gel shift assays (EMSA) for these transacting factors (Fig. 3). NF-κB is composed of 50 and 65 kDa subunits (Xie *et al.,* 1994; Thanos and Maniatis, 1995; Murono *et al.,* 2000). Two bands were detected with the NF-κB probe and were NF-κB subunits in agreement with the previous papers (Fig. 3A). NF-κB binding activity rapidly increased and peaked at 0.5 h after TPA treatment, thereafter declined to a near-basal level at 2 h (Figs. 3A and 3C). AP-1 followed a similar pattern in the intensity of binding activity (Figs. 3B and 3D). TPA treatment further increased the level of activated AP-1 in the nuclear extracts. The binding activity peaked at 0.5 h and was maintained at a high level until 2 h after TPA addition. The binding activities of NF-κB and AP-1 were inhibited completely by the each competitor. As the neg-

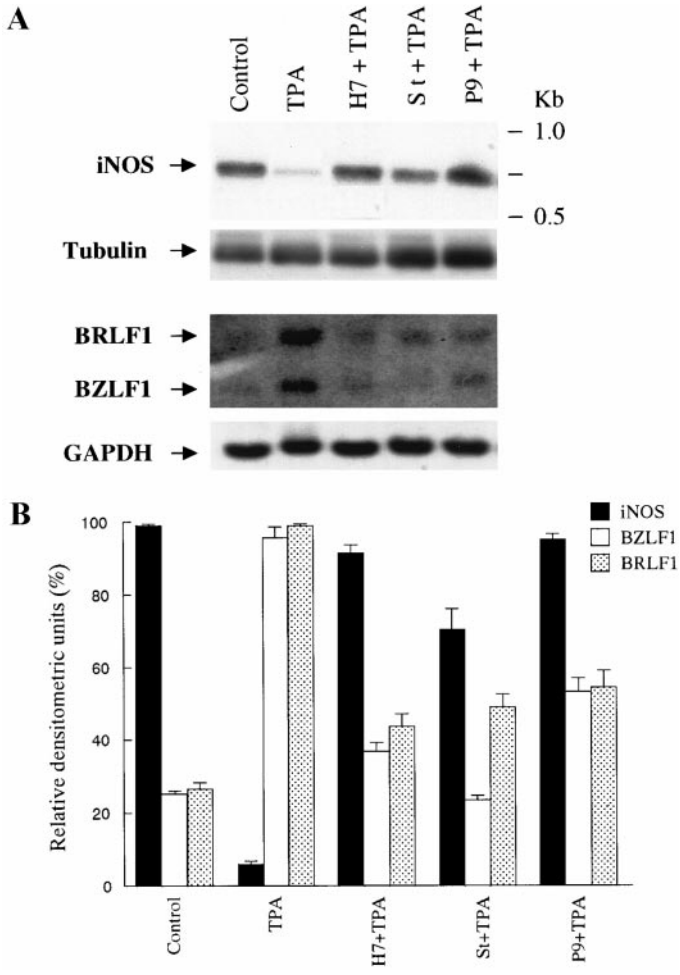

FIG. 2. The effects of PKC and MAPK inhibitors on TPA-mediated iNOS mRNA, BRLF1, and BZLF1 expression. (A) GT38 cells were pretreated with a PKC inhibitor H7 or staurosporine (St), or a MAPK inhibitor PD98059 (P9) for 1 h, and then treated with TPA (20 ng/ml) for 8 h. Total RNA was extracted, and Northern and RT-PCR Southern analyses were performed as described under Materials and Methods. (B) The autoradiographic signals were quantitated by a phosphorimager system and the relative amounts to tubulin or GAPDH signals were determined. The data represent three independent experiments, and the standard errors of the means are shown.

ative control, SP-1 binding activity was not detected (data not shown). These results showed that TPA induces NF-κB and AP-1 activation with a comparable time-dependent manner.

Pyrrolidine dithiocarbamate (PDTC) inhibits NF-κB activity and BZLF1 expression

To study further the involvement of NF-κB and AP-1 in TPA-induced BZLF1 expression, the effect of PDTC, a potent inhibitor of NF-κB activation, was examined in GT38 cells by EMSA. PDTC was added to the medium 1 h before TPA. TPA-induced NF-κB activation was clearly inhibited by PDTC treatment (Figs. 4A and 4C). In contrast, PDTC treatment did not affect TPA-induced AP-1 activation in GT38 cells (data not shown). These results differed from a previous report of experiments, indicating that PDTC can enhance the TPA-evoked activation of the AP-1 factor in HeLa cells (Menegazzi *et al.,* 1996). Furthermore, we determined expression levels of BZLF1 and BRLF1 by Northern blotting (Figs. 4B and 4D). PDTC decreased TPA-induced BZLF1 and BRLF1 expressions. These results indicated that TPA-induced EBV reactivation is mediated at least partially through NF-κB and AP-1 pathways in GT38 cells.

Effect of TPA on EBV reactivation and AP-1 family gene expressions

In EBV-containing lymphoid cell lines, EBV latency can be disrupted by TPA, whose effects are mediated by the AP-1 transcription factor c-Fos and c-Jun (Farrell *et al.,* 1989). To determine which members of the AP-1 family could be induced by TPA, the gene expressions of AP-1 constituents were examined by Northern blot analyses (Fig. 5). GT38 cells were incubated without or with TPA for intervals from 0.5 to 24 h. After TPA stimulation c-Jun was increased significantly at 0.5 h, reached a peak at 2 h, and declined to a low level at 24 h after stimulation. The junB was constitutively expressed in TPA-untreated control cells. After TPA treatment, junB was increased slightly at 0.5 h, reached a plateau at 1 h, and maintained a high level at least until 24 h. We failed to detect c-*fos* mRNA in TPA-untreated and -treated GT38 cells (data not shown). The expressions of BRLF1 and BZLF1 were induced in a time-dependent manner. These results are consistent with the view that the expressions of c-*jun* and *jun*B but not c-*fos* mediate TPA-induced EBV reactivation.

DISCUSSION

GT38 cell line has been reported as an EBV producer, expressing lytic antigens and producing infectious virus spontaneously (Takasaka *et al.,* 1998). We have also shown that neither BZLF1 mRNA nor ZEBRA was detectable without TPA stimulation and NO inhibited TPA-induced EBV reactivation (Gao *et al.,* 1999). In the present study we showed that the levels of ZEBRA and iNOS mRNA expression took a reverse order according to the term of culture of GT38 cells. These results suggested that NO is able to inhibit not only TPA-induced EBV reactivation, but also spontaneous EBV reactivation.

In this study, we have investigated the molecular mechanisms by which TPA inhibits iNOS gene expression and induces EBV reactivation in the gastric tissue-derived epithelial cell line GT38 cells. In particular, using PKC inhibitors, we first demonstrated the involvement of PKC pathway in TPA-induced EBV reactivation. This observation has led to a better insight into the signal transduction pathway associated with EBV reactivation. In our previous report, we showed that the PKC activator

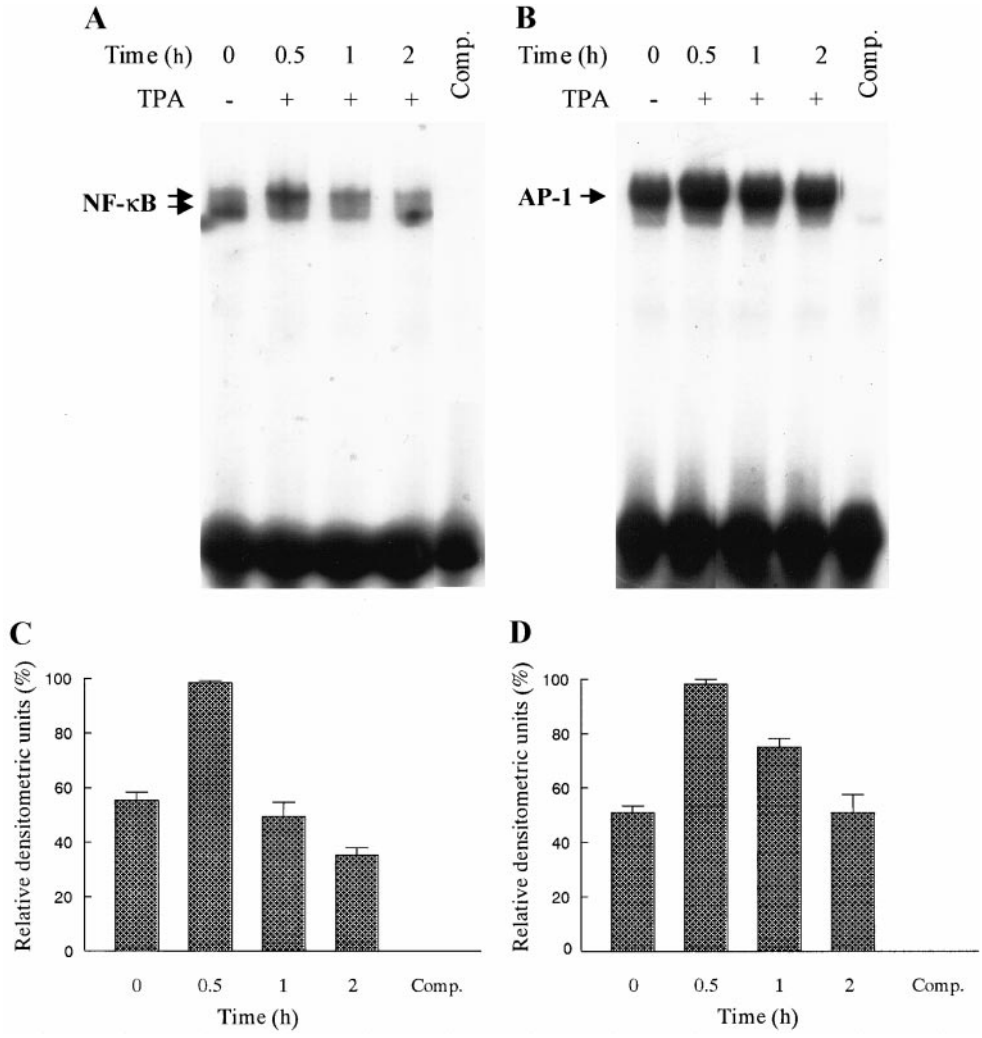

FIG. 3. Time course of NF-κB and AP-1 binding following incubation of GT38 cells with TPA. GT38 cells were incubated with or without TPA (20 ng/ml), and then nuclear extracts were prepared at the indicated times. DNA–protein binding activities of (A) NF-κB and (B) AP-1 were determined by EMSA. Competitive binding reactions (Comp.) were tested by adding a 100-fold excess of unlabeled NF-κB or AP-1 oligonucleotide for 30 min before adding the labeled probe. The relative densitometric units of NF-κB and AP-1 DNA binding activities are shown in (C) and (D), respectively. The data shown represent three independent experiments, and the standard errors of the means are shown.

TPA induced EBV immediate-early gene BZLF1 expression by inhibiting iNOS expression in GT38 cells (Gao *et al.,* 1999). In extending this observation in the present study we tested the effects of the PKC inhibitors H7 and staurosporine in those cells, finding the induction of BZLF1 expression by TPA to be abolished by those inhibitors. In contrast to that finding with BZLF1, TPA down-regulated iNOS expression was reversed by the PKC inhibitors. Taken together, these results support the view that the TPA-induced up-regulation of BZLF1 and down-regulation of iNOS expression are both mediated by a PKC-associated pathway. These results led us to postulate that potentially produced NO might down-regulate BZLF1 expression.

The most straightforward explanation for this process is that PKC primarily down-regulates iNOS gene expression, resulting in down-regulated NO production, which in turn leads to increased BZLF1 expression as a rapid response to TPA. In addition to PKC, we have reported that MAPK is also an important signal transducer for EBV reactivation in B cells (Satoh *et al.,* 1998), but its role in TPA-induced EBV reactivation has remained unclear. Thus in this study, we further tested for the role of MAPK in the TPA-induced EBV reactivation. Similar to PKC, MAPK was shown also to play an inducing role in TPA-induced EBV reactivation and iNOS gene expression (Figs. 2 and 4). Interpreting these results with those of our previous report (Gao *et al.,* 1999), we conclude that TPA-induced EBV reactivation is mediated by both PKC and MAPK pathways in which NO play a regulatory key role.

BZLF1 is a sequence-specific DNA binding protein in the bZip family of transcription factors which transactivate several early lytic-phase viral promoters via *cis-*

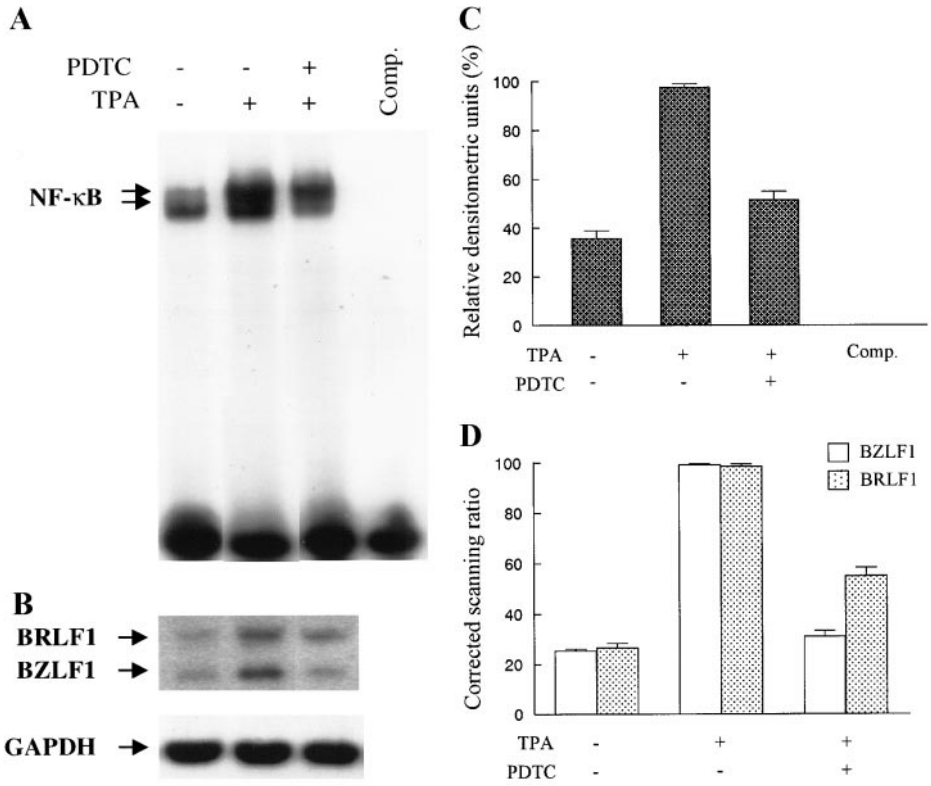

FIG. 4. PDTC inhibits TPA-induced NF-κB activation and BZLF1 expression. GT38 cells were pretreated with PDTC (100 μM) for 1 h prior to TPA (20 ng/ml) treatment. (A) Nuclear extracts were prepared at 30 min after TPA treatment, and NF-κB DNA–protein binding activity was determined by EMSA. (B) Total RNA was extracted at 8 h after TPA treatment and Northern blotting for BZLF1 and BRLF1 were carried out as described in Figs. 1 and 2, respectively. (C) Relative amounts of the NF-κB complex were determined by the densitometric analysis. (D) The autoradiographic signals of BZLF1 and BRLF1 were quantitated by a phosphorimage system and the corrected scanning ratio to the GAPDH signals is shown. The data represented were compiled from three independent experiments, and the standard errors of the means are shown.

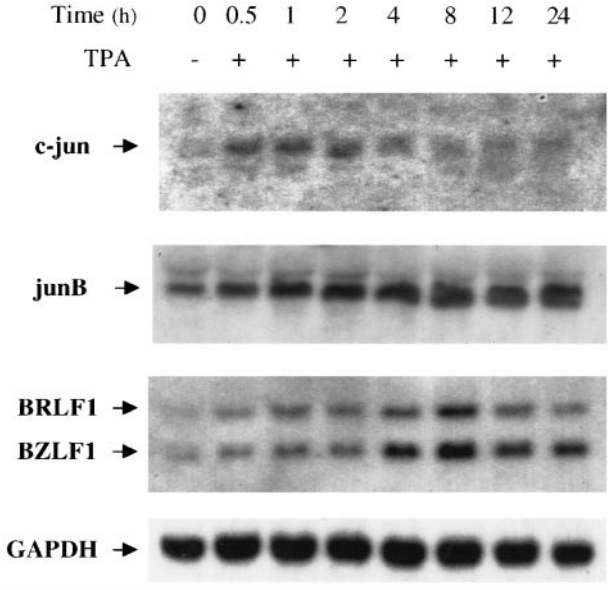

FIG. 5. The effect of TPA on BZLF1, BRLF1, c-*jun*, and *jun*B expression. GT38 cells were treated with TPA (20 ng/ml) and total RNAs were extracted at the indicated times. Northern blottings were performed as described in Fig. 1.

acting ZRE DNA binding motifs (Urier *et al.,* 1989). BZLF1 contains a carboxyl-terminal domain that mediates homodimerization through a coiled–coil interaction (Chang *et al.,* 1990; Kouzarides *et al.,* 1991) and a basic region which shares sequence homology with the DNA-binding domain of members of the AP-1 family of transcription factor (Farrell *et al.,* 1989; Urier *et al.,* 1989). Hence, BZLF1 binds to TPA responsive element (TRE) or AP-1 sequence motifs with high affinity (Farrell *et al.,* 1989; Lieberman *et al.,* 1990). Our results indicate that TPA can induce AP-1 binding activity in a time-dependent manner, suggesting the possibility that BZLF1 expression might be regulated by AP-1. This result is consistent with those by Borras and co-workers (Borras *et al.,* 1996). In addition to AP-1, treatment of GT38 cells with TPA, causing BZLF1 expression, also up-regulated NF-κB binding activity that can be reversed by addition of NF-κB inhibitor PTDC (Figs. 3 and 4). This suggests that NF-κB is also involved in the up-regulation of BZLF1 by TPA, and that, excepting the binding sequence for NF-κB on genomic DNA, the BZLF1 promoter domain might also contain binding sites for the transcription factor NF-κB. To determine finally if the TPA-induced up-regulation of BZLF1 expression is regulated by a mechanism involving NF-κB, further ex-

periments using BZLF1 promoter elements transfection assay are needed.

AP-1 transcription factor is a complex composed of proteins of the *fos* and *jun* proto-oncogene families, which need to dimerize to promote binding of the complex to the AP-1 recognition site (Hunter and Karin, 1992). Using Northern blot analysis, c-*jun* and *jun*B mRNA identified as components of AP-1-DNA neucleoprotein complexes in GT38 cells. Conceivably, regulation of the BZLF1 and BRLF1 promoters in GT38 cells may be mediated, in part, through a homodimer of c-Jun or c-Jun/JunB heterodimer. c-*jun* mRNA expression paralleled the induction of the BZLF1 and BRLF1 genes. It has been reported that TPA can induce c-*Jun* N-terminal kinase (JNK) activity (Okumura *et al.,* 1999). JNK-mediated phosphorylation is also required for activation of the c-*jun* (Kyriakis *et al.,* 1994). Our results here indicate that TPA may activate JNK, thereby inducing phosphorylation of c-Jun, which bind to the ZI and ZII elements of the BZLF1 promoter.

NF-κB and AP-1 were activated constitutively in GT38 cells (Fig. 3). TPA enhanced the NF-κB and AP-1 activities and induced EBV reactivation. What does this mean in terms of the maintaining EBV latency and reactivation from latency ? The latency in GT38 cells is type III, which expresses EBNA1, EBNA2, and LMP1 (Takasaka *et al.,* 1998; Murakami *et al.,* 2000). The observed constitutive activation of NF-κB and AP-1 may be due to the expression of LMP-1, which evokes activation of the tumor necrosis factor (TNF) receptor downstream signaling pathway (Izumi *et al.,* 1997). Cytoplasmic domain of LMP-1 contains two functional domains, namely, CTAR1 and CTAR2 (Izumi and Kieff, 1997). The ability of CTAR1 to activate NF-κB appears to be attributable to the direct interaction of TNF receptor-associated factor 2, and CTAR2-induced NF-κB is mediated through its association with TNF receptor-associated death domain (Eliopoulos *et al.,* 1999). These two functional domains of LMP1 can independently activate NF-κB (Mitchell and Sugden, 1995). In addition to NF-κB, LMP1 signals for activation of JNK pathway, a phenomenon which is mediated through CTAR2 but not CTAR1 and translates the induction of AP-1 (Kieser *et al.,* 1997; Eliopoulos and Young, 1998). The constitutive activation of these signaling molecules maintains the EBV latency. When the balance of activation signal is unbalanced by TPA, the EBV latency could be disrupted to reactivation. TPA is well known, a potent activator of PKC, and TPA induced NF-κB and AP-1 activation (Fig. 3). In the present study, we address the finding that TPA activates NF-κB and AP-1 binding activity through PKC and/or MAPK pathway, then interacts the downstream signaling pathway including *fos* and *jun* family gene expression, and reactivates BZLF1 and BRLF1 expression. However, it remains to be studied more how the signaling molecules and pathways are involved by TPA stimulation.

In conclusion, our results have for the first time demonstrated that TPA-induced EBV reactivation can occur by activating NF-κB and AP-1, and we suggest that this reactivation, marked by BZLF1 expression, is likely to be mediated at least via both PKC and MAPK pathways in the gastric tissue-derived epithelial cell line GT38.

MATERIALS AND METHODS

Cell line and reagents

GT38 cells were EBV-positive epithelial cell line derived from a human gastric tissue (Tajima *et al.,* 1998). The cells were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), in 60- or 100-mm cell culture dishes. All cells were incubated in an atmosphere of 95% air and 5% CO_2. For EBV reactivation, in some cases, cells were treated with 20 ng/ml TPA (Sigma Chemical Co., St. Louis, MO). H7, staurosporine, PD98059, and PDTC were purchased from Sigma and dissolved in dimethyl sulfoxide before adding to culture medium.

Preparation of RNA and Northern blot analysis

Total RNA from cultured cells was prepared by an ISOGEN Kit (Nippongene Inc., Tokyo, Japan) according to the manufacturer's protocol as described previously (Gao *et al.,* 1999). The RNA was dissolved in diethyl pyrocarbonate-treated water and stored at $-20°C$. All RNA samples had an OD260/OD280 ratio $>$ 1.50.

Northern blot analysis was carried out by the method described previously (Gao *et al.,* 1999). Aliquots containing 20 μg of total RNA were loaded into 1% agarose formaldehyde gels and then transferred to HybondTM-N^+ membranes (Amersham, Buckinghamshire, U.K.) by capillary transfer and UV-autocrosslinked. Membranes were prehybridized in prehybridization buffer for 6 h at 42°C, and then hybridized at 42°C for 24 h in the same solution with ^{32}P-labeled cDNA probes for BZLF1 or glyceraldehyde-3-phoshate dehydrogenase (GAPDH) (Gao *et al.,* 1999) or c-*jun* or *jun*B (Wang *et al.,* 1999). The blots were washed three times at 65°C in 1$\times$ SSPE, 0.1% SDS for 10 min. The blots were exposed to Kodak X-AR film (Eastman Kodak Co., Rochester, NY) at $-80°C$ for 1–3 days. Relative mRNA levels were quantitated with a phosphorimaging system (Molecular Imager, Nippon Bio-Rad Laboratories, Tokyo, Japan). Each Northern blot was representative of at least three separate experiments performed at different times, unless otherwise specified.

RT-PCR analysis

The PCR was performed basically as previously described (Gao *et al.,* 1999). The sequences of sense and antisense primers used for amplification of specific hu-

man iNOS cDNA are as follows: sense primer, 5′-CTGTC-CTTGGAAATTTCTGTT-3′; antisense primer, 5′-GTGATG-GCCGACCTGATGTTGC-3′, to amplify a 729-bp nucleotide product (Gao *et al.,* 1999). The oligonucleotides of the "housekeeping gene" human β-tubulin (GenBank Accession No. V00599) were designed as 5′-TGGATCTA-GAACCTGGGACCAT-3′ (sense primer) and 5′-ACCATGT-TGACTGCCAACTTGC-3′ (antisense primer) to amplify a 577-bp product (Gao *et al.,* 1999). The amplified PCR products were analyzed after electrophoresis in a 1.2% agarose gel, blot-transferred to HybondTM-N+ membrane, and UV-autocrosslinked. The membranes for iNOS or tubulin were prehybridized with prehybridization buffer and then hybridized with the labeled iNOS or tubulin probes (Gao *et al.,* 1999). The hybridized PCR product was visualized by autoradiography as for Northern blotting described above. Each illustrated RT-PCR Southern blotting is representative of at least three separate experiments performed at different times.

Western blot analysis

Western blot analyses were performed as previously described (Gao *et al.,* 1999). Protein samples (50 μg) were separated in 10% SDS–polyacrylamide gel, and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore Co., Bedford, MA), using a semidry transfer blot system. As confirmed by Coomassie brilliant blue dye, equal amounts of proteins were blotted onto the membranes, and then the membranes were incubated with anti-ZEBRA monoclonal antibodies (developed in our laboratory, 1:500) in blocking buffer. Blots were washed in blocking buffer three times and then incubated with the labeled rabbit anti-mouse IgG antibodies in the identical blocking buffer for 1 h. The blots were washed in blocking buffer three times, followed by the chemiluminescence reaction, and exposed to X-ray films.

Preparation of nuclear extract

Nuclear extracts were obtained from TPA-treated cells grown to confluence in 100-mm dishes. All extraction procedures were performed on ice with ice-cold reagents. Cells were washed with phosphate-buffered saline (PBS), harvested by scraping into 1 ml PBS, and centrifuged (12,000 rpm, 15 s). The pellet was dispersed in buffer A containing 10 mM HEPES–KOH (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. After 15 min on ice, Nonidet P-40 was added into the mixture to a final concentration of 0.6% (v/v), followed by a 10 s vortexing, and then nuclei were pelleted by centrifugation (15,000 rpm, 30 s). The nuclei pellet was dispersed in a high salt buffer (containing 20 mM HEPES–KOH (pH 7.6), 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) to solubilize DNA-binding proteins. The suspended nuclear pellet was shaken for 15 min on ice and centrifuged for 5 min at 4°C. The clear supernatant containing nuclear proteins was divided into small aliquots and were stored at −80°C.

EMSA

Double-stranded synthetic oligonucleotide probes containing the NF-κB consensus sequences (NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′) and the AP-1 consensus sequences (AP-1, 5′-CTAGTGATGAGTCAGCCG-GATC-3′) (Chen *et al.,* 1998) (Armersham Pharmacia Bioteck, Tokyo, Japan) were used in the EMSA. Forty nanograms of the double-stranded oligonucleotide were labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase (Amersham Pharmacia Bioteck) and purified through MicrospinTM G-25 columns (Amersham Pharmacia Bioteck).

Samples (10 μg) of nuclear extract and 0.1 ng (10,000 cpm) of double-stranded labeled oligonucleotide were incubated in DNA binding buffer containing 25 mM HEPES–KOH (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 7.5% glycerol, 5 mM MgCl, 2 μg BSA, and 2 μg poly(dI-dC) (Roche Molecular Biochemicals, Tokyo, Japan) at a final volume of 10 μl. The binding reaction was performed at 4°C for 30 min. Competition experiments were performed by adding a 100-fold excess of unlabeled self double-stranded oligonucleotide before the labeled probes. DNA–protein complexes were analyzed by electrophoresis on 5% polyacrylamide gels in Tris–glycine buffer (50 mM Tris, 380 mM glycine, 2mM EDTA, pH 8.2). Electrophoresis was run at 4°C at 130 V for 3 h under nondenaturing conditions. Gels were dried and exposed to X-ray films at −80°C overnight.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid of Scientific Research on Carcinogenesis in Priority Areas from the Ministry of Education, Science, Sports, and Culture of Japan; the Japan Health Science Foundation; the Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan; and the Special Coordination Funds for the Science and Technology from Science and Technology Agency. We thank Dr. Robert E. Humphreys for a critical reading of this manuscript. We also thank Dr. Haoran Wang for valuable discussions, Dr. Yukio Satoh and Dr. Yoshiko Hoshikawa for expert experimental assistance, and Sayuri Matsumoto for typing this manuscript.

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