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Short Communication

# Essential role of PKC$\delta$ in histone deacetylase inhibitor-induced Epstein–Barr virus reactivation in nasopharyngeal carcinoma cells

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Histone deactylase inhibitors (HDACi) are common chemotherapeutic agents that stimulate Epstein–Barr virus (EBV) reactivation; the detailed mechanism remains obscure. In this study, it is demonstrated that PKC$\delta$ is required for induction of the EBV lytic cycle by HDACi. Inhibition of PKC$\delta$ abrogates HDACi-mediated transcriptional activation of the Zta promoter and downstream lytic gene expression. Nuclear translocation of PKC$\delta$ is observed following HDACi stimulation and its overexpression leads to progression of the EBV lytic cycle. Our study suggests that PKC$\delta$ is a crucial mediator of EBV reactivation and provides a novel insight to study the regulation of the EBV lytic cycle.

Epstein–Barr virus (EBV) is a human gammaherpesvirus with both latent and lytic states in its life cycle (Kieff & Rickinson, 2001). EBV reactivation not only produces infectious viral progeny, but also contributes to the development of EBV-related disease (Kieff & Rickinson, 2001). Although the majority of EBV infections *in vivo* are latent, serological studies suggest that EBV reactivation may occur months or years before the clinical diagnosis of nasopharyngeal carcinoma (NPC), Hodgkin's disease and endemic Burkitt's lymphoma, serving as a risk factor for cancer development (Kieff & Rickinson, 2001). Previous studies have shown that some physiological stimuli and pharmacological agents, including transforming growth factor beta, cross-linking of surface immunoglobulin, phorbol ester, histone deacetylase inhibitors (HDACi) and several genotoxic agents, can induce EBV from latent status into the lytic cycle (Daibata *et al.*, 1994; Davies *et al.*, 1991; Feng & Kenney, 2006; Feng *et al.*, 2002; Schuster *et al.*, 1991).

The protein kinase C (PKC) family has been known for a long time to be necessary for EBV reactivation following stimulation with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) (Davies *et al.*, 1991) or anti-immunoglobulin treatment (Daibata *et al.*, 1994). The PKC family comprises 11 isozymes, which are classified into three subfamilies according to their structure and regulatory domains activated by calcium or diacylglycerol (DAG) (Newton, 1997). Conventional PKCs (PKC$\alpha$, $\beta$I, $\beta$II and $\gamma$) require calcium and DAG activators, and novel PKCs (PKC$\delta$, $\varepsilon$, $\theta$ and $\eta$) respond only to DAG and not to calcium (Newton, 1997). However, atypical PKC isoforms (PKC$\zeta$, $\iota/\lambda$ and $\mu$) are DAG-insensitive (Newton, 1997). Until now, it has not been clear which PKC members are involved in EBV reactivation. The limited information available suggests that PKCs may play an important role during different stages of various virus infections (Constantinescu *et al.*, 1991; Sieczkarski *et al.*, 2003). For example, PKC$\zeta$ or PKC$\delta$ is required for early infection or TPA-triggered lytic-cycle activation of Kaposi's sarcoma-associated herpesvirus (KSHV) (Deutsch *et al.*, 2004; Naranatt *et al.*, 2003).

HDACi, including trichostatin A (TSA), sodium butyrate (SB) and valproic acid, are common agents used to induce the EBV lytic cycle in several EBV-harbouring epithelial and B cells (Chang & Liu, 2000; Luka *et al.*, 1979). By inactivating cellular HDAC enzyme activity, HDACi can prevent histone acetyltransferase (HAT) recruitment from reducing histone hyperacetylation and chromatin accessibility, thereby facilitating transcriptional activation of their targeted genes (Marks & Dokmanovic, 2005). The molecular mechanisms by which HDACi regulate expression of their target genes remain unclear; however, some protein kinases have been reported to be required for



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HDACi-regulated gene expression, including phosphatidylinositol 3-kinase (PI3K), ATM and PKCs (Eun *et al.*, 2007; Ju & Muller, 2003; Kim *et al.*, 2003, 2007). Recent studies have shown that PKCε or PKCδ is involved in HDACi-mediated p21 or cyclin D3 gene expression (Kim *et al.*, 2003, 2007). Considering that EBV belongs to the gammaherpesviruses and its lytic cycle in response to HDACi is well-defined, it is interesting to investigate which PKC isoform is required for HDACi-induced EBV reactivation.

TPA and HDACi have been reported to induce progression of the EBV lytic cycle (Chang & Liu, 2000; Davies *et al.*, 1991). The effects of TPA or HDACi can be mediated through several pathways; however, PKC is a common signal transducer for both (Davies *et al.*, 1991; Kim *et al.*, 2007). To determine which PKC isoform is involved in HDACi-induced EBV reactivation, Gö6850 (PKCα/βI/βII/γ/ε/δ inhibitor), Gö6976 (PKCα/β/μ inhibitor) and Rottlerin (PKCδ inhibitor) were used in this study. As shown in Fig. 1(a), TSA induced immediate-early

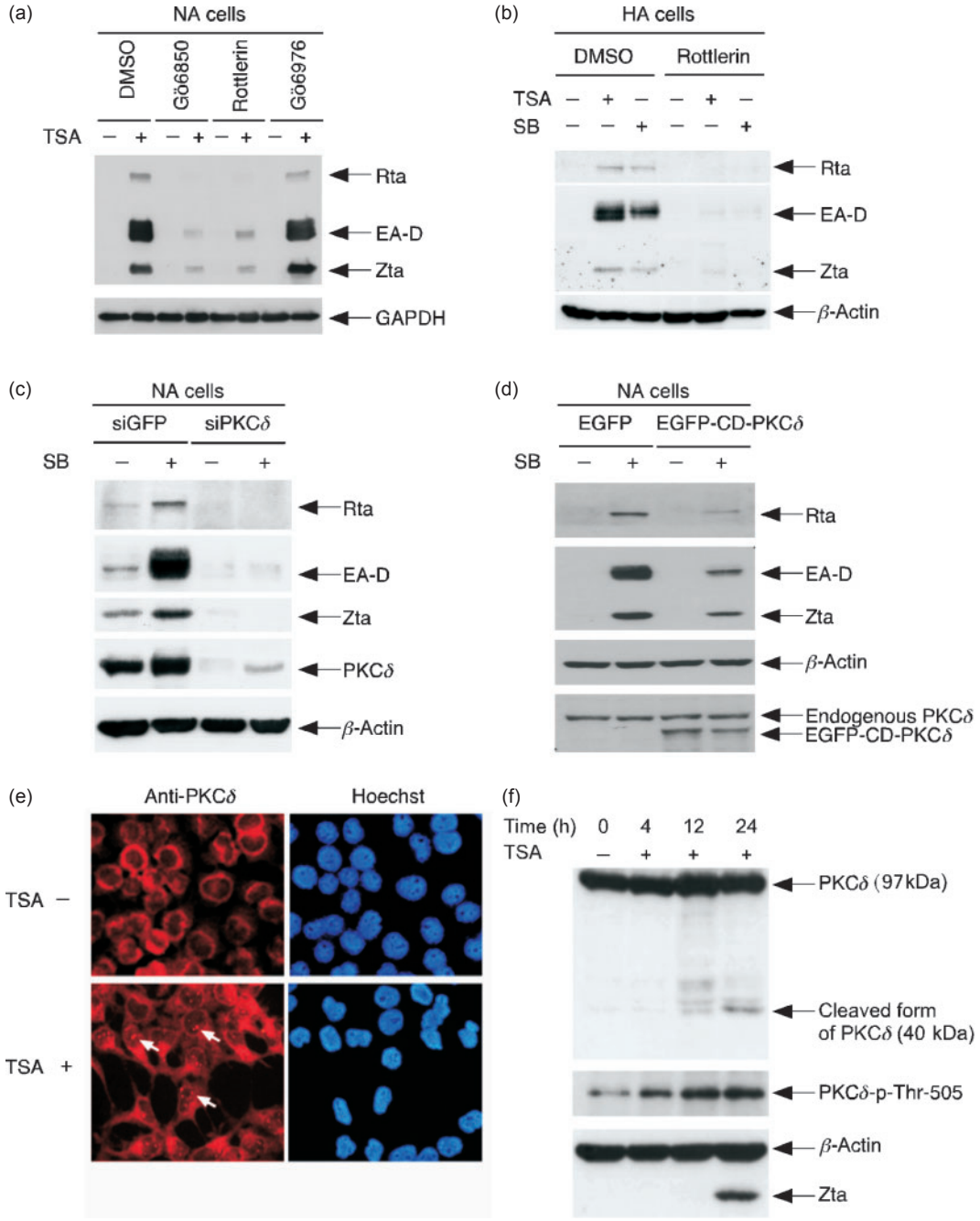

**Fig. 1.** (on previous page) PKC$\delta$ is required for TSA- or SB-induced EBV lytic-cycle progression. (a) Inhibition of TSA-triggered EBV lytic cycle by PKC inhibitors. NA cells were treated with PKC inhibitors [10 μM Gö6850, 5 μM Rottlerin or 0.4 μM Gö6976 (Calbiochem)] or with DMSO (solvent control). After 1 h, cells were treated with 1.25 μM TSA (+) or mock-treated (−) for 24 h. Proteins were detected by anti-Zta, anti-Rta, anti-EA-D and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibodies (Santa Cruz Biotechnology). (b) Suppression of TSA-triggered EBV lytic cycle by PKC$\delta$ inhibitor. HA cells were treated with Rottlerin for 1 h, and then treated with 1.25 μM TSA or 3 μM SB (+) or mock-treated (−) for 24 or 48 h. Western blotting was performed to detect protein expression as indicated. (c) Blockage of TSA-triggered EBV reactivation by PKC$\delta$ siRNA. pSUPER-derived GFP (siGFP) and PKC$\delta$ (siPKC$\delta$) siRNA-expressing plasmids were constructed as described previously (Chang *et al.*, 2004a; Storz *et al.*, 2004). NA cells were transfected once with siRNA expression plasmids and these transfected cells were reseeded every 2 days. After three rounds of transfection, cells were treated with SB (+) or mock-treated (−) for 48 h and then harvested for immunoblotting. (d) Repression of SB-induced EBV reactivation via overexpression of PKC$\delta$ dominant-negative mutants. NA cells were transfected with the EGFP-derived catalytic domain-deleted mutant of PKC$\delta$ (plasmid EGFP-CD-PKC$\delta$) or its vector control (EGFP-GFP) by using Lipofectamine 2000 reagent (Invitrogen) for 24 h, followed by treatment with SB (+) or mock treatment (−) for another 48 h. Endogenous expression of PKC$\delta$ and EGFP-CD-PKC$\delta$ was examined by using an anti-PKC$\delta$ antibody (Santa Cruz Biotechnology). (e) TSA-induced PKC$\delta$ nuclear translocation. NA cells grown on glass slides were treated with TSA or mock-treated for 8 h and then subjected to an immunofluorescence assay as described previously (Chua *et al.*, 2007). Briefly, cells were washed with PBS and fixed with acetone at −20 °C for 10 min and then incubated with anti-PKC$\delta$ antibody at 37 °C for 1 h. Cells were washed with PBS three times and then incubated with rhodamine-conjugated anti-rabbit IgG at 37 °C for 1 h, followed by another three washes with PBS. Nuclei of cells were stained with Hoechst. (f) TSA-induced PKC$\delta$ catalytic-domain cleavage and Thr-505 phosphorylation. NA cells were treated with or without TSA for 0, 4, 12 or 24 h. Cell lysates were prepared for Western blot analysis using specific antibodies against PKC$\delta$ (Santa Cruz Biotechnology) and its phosphorylation site at Thr-505 (Cell Signaling Technology). Zta expression was examined for indication of the occurrence of EBV reactivation, and $\beta$-actin was detected as an internal control.

lytic-cycle protein expression in an Akata EBV-infected NPC cell line (NA), including Zta, Rta and EA-D proteins (Chang *et al.*, 2004b). However, the TSA-induced EBV lytic protein expression could be suppressed by Gö6850 (lane 4) and Rottlerin (lane 6), but not by Gö6976 (lane 8). Furthermore, TSA- or SB-mediated EBV reactivation also could be suppressed by Rottlerin treatment of another Akata EBV-infected NPC cell line, HA (Fig. 1b) (Chang *et al.*, 2004b). These results demonstrated that PKC$\delta$ is the key mediator of TSA- and SB-induced EBV lytic-cycle progression in the EBV-positive NA and HA cell lines.

To confirm the specificity of the inhibitors and the role of PKC$\delta$ in HDACi-induced EBV reactivation, the expression of PKC$\delta$ was knocked down specifically by PKC$\delta$-targeted small interfering RNA (siPKC$\delta$) (Storz *et al.*, 2004). As shown in Fig. 1(c), EBV lytic-cycle protein expression can be induced by SB treatment in vector-control NA cells positive for green fluorescent protein-targeted siRNA (siGFP); however, the expression of SB-induced EBV lytic-cycle proteins was inhibited almost completely in the presence of siPKC$\delta$. To elucidate the working mechanisms of PKC$\delta$, a deletion mutant of the catalytic domain (from aa 299 to 654) of PKC$\delta$ (EGFP-CD-PKC$\delta$) was constructed. The expression of SB-induced EBV lytic proteins was compared in enhanced green fluorescent protein (EGFP)- and EGFP-CD-PKC$\delta$-transfected HA cells. In Fig. 1(d), SB-stimulated EBV reactivation was clearly downregulated in the presence of the PKC$\delta$ dominant-negative mutant, suggesting that CD-PKC$\delta$ could also block endogenous PKC$\delta$ activities that are required for the SB-induced EBV lytic cycle. This result also implies that HDACi may induce PKC$\delta$ activation to trigger EBV reactivation.

The activation of PKC$\delta$ can be measured by detecting several markers, including translocation to other subcellular compartments, phosphorylation at particular sites and cleavage into catalytic fragments (Shirai & Saito, 2002; Steinberg, 2004; Yamamoto *et al.*, 2006). Because PKC$\delta$ has a nuclear-localization sequence (Steinberg, 2004), we examined whether PKC$\delta$ translocated to the nucleus, which is the main site of PKC$\delta$ activation. In the absence of TSA, PKC$\delta$ was expressed mostly in the cytosol, but was also expressed at low levels in the nucleus (Fig. 1e, upper panel). However, PKC$\delta$ translocated predominantly to the nucleus 8 h after TSA treatment (Fig. 1e, bottom panel). We also found that enhancement of phosphorylation at threonine 505 (p-Thr-505) and the cleaved form of PKC$\delta$ were observed after TSA treatment (Fig. 1f). Taken together, we believe that TSA can induce PKC$\delta$ activation, which is necessary for the subsequent reactivation of EBV.

BZLF1 gene expression governs the initiation of EBV reactivation. To determine whether PKC$\delta$ affected TSA-induced BZLF1 gene expression, we investigated the expression of BZLF1 mRNA in the presence of Rottlerin in TSA-treated NA cells. Fig. 2(a) shows that TSA-induced BZLF1 gene expression was blocked by Rottlerin. To confirm whether PKC$\delta$ regulated TSA induction of BZLF1 gene expression, the promoter activity of Zp was measured in NA cells treated with Rottlerin. As shown in Fig. 2(b, c), Rottlerin suppressed TSA- and SB-induced Zp activity in NA cells (decreasing Zp activity from 5-fold to 1-fold and from 30-fold to 10-fold, respectively). Previous studies have reported that TSA stimulation usually increases expression of acetyl-histone H3 and H4 proteins, which then facilitates transcriptional activation of certain genes (Marks & Dokmanovic, 2005). We attempted to determine

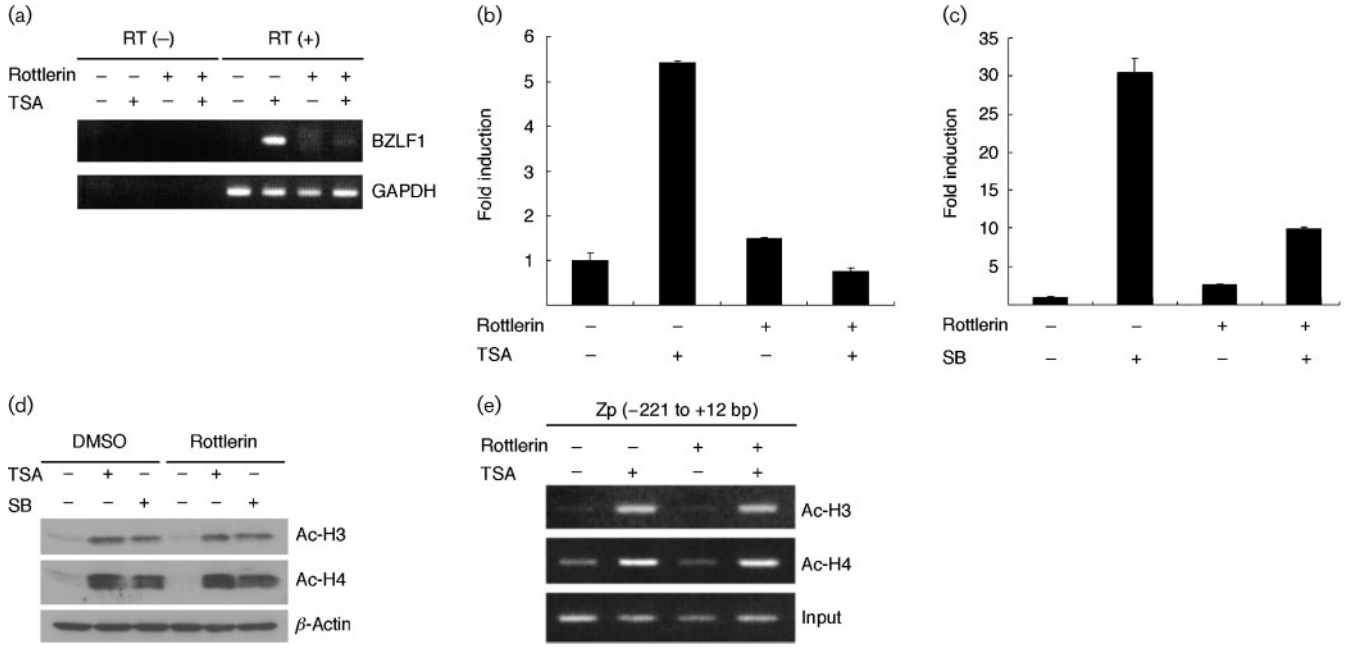


**Fig. 2.** Inhibition of PKCδ represses TSA-induced BZLF1 mRNA expression, but does not alter TSA-induced histone acetylation of Zp. (a) Expression of BZLF1 and GAPDH transcripts. NA cells were pre-treated with DMSO (–) or Rottlerin (+) for 1 h, followed by treatment with TSA (+) or mock treatment (–) for 24 h. Total RNA was extracted by TRIzol reagent (Invitrogen); mRNA expression of BZLF1 and GAPDH was analysed by RT-PCR in the presence (+) or absence (–) of reverse transcriptase (RT). PCR conditions and primers were as described previously (Chua *et al.*, 2007). (b, c) Zta promoter activities are inhibited by Rottlerin. EBV-negative NPC-TW01 cells were transfected with pGL2-basic vector (Promega) or PGL2-derived Zp reporter plasmid (pGL2-Zp-221, from −221 to +12 bp) and EGFP expression plasmid (EGFP-C1, Promega) as an internal control. After 24 h, the transfectants were pre-treated with DMSO (–) or Rottlerin (+) for 1 h, and then stimulated with TSA (+, b) or SB (+, c) or mock-stimulated (–) for another 24 or 48 h. Cell lysates were harvested and subjected to a luciferase gene reporter assay as described previously (Chang *et al.*, 2006). (d) Effect of PKCδ inhibition on global acetylation of histone H3 and H4 proteins. NA cells were pre-treated with DMSO (–) or Rottlerin (+) for 1 h and then treated with TSA or SB (+) or mock-treated (–) for 24 or 48 h. The expression of acetyl-histone H3 and H4 protein was detected by specific antibodies (Cell Signaling Technology). (e) ChIP assay to detect Zp-bound acetyl-histone H3 and H4. NA cells were pre-treated with (+) or without (–) Rottlerin for 1 h, followed by incubation with (+) or without (–) TSA for 24 h. ChIP assays were performed according to the manufacturer's protocol (Upstate Biotechnology). DNA was extracted with phenol/chloroform and precipitated with ethanol, followed by PCR analysis with Zp primers. 'Input' indicates DNA input control, as assessed by β-actin-targeted PCR analysis. PCR conditions and primers used are as described previously (Chua et al., 2007).

whether PKCδ is involved in the regulation of HDACi-induced acetyl-histone H3 and H4 protein expression, and then regulates BZLF1 gene expression further. In Fig. 2(d), it is shown that Rottlerin could not block TSA- or SB-induced acetyl-histone H3 and H4 protein expression in NA cells. To demonstrate that PKCδ did not alter the level of Zp-bound acetyl-histone H3 and H4 directly, a chromatin immunopreciptation (ChIP) assay was performed. We found no significant alteration of the level of acetyl-histone H3 and H4 proteins on Zp after Rottlerin treatment (Fig. 2e). Thus, PKCδ-mediated HDACi-induced BZLF1 gene expression is very unlikely to occur though alteration of the expression or binding of acetyl-histone H3 and H4 proteins on Zp.

We next questioned whether PKCδ alone is sufficient to induce Zp activation. In Fig. 3(a), overexpression of wild-type PKCδ could induce the activity of Zp by 5-fold without HDACi treatment. Because the N-terminal domain of PKC harbours a pseudosubstrate domain to repress its C-terminal catalytic activity (House & Kemp, 1987), it has been suggested that N-terminal deletion of PKCδ leads to its constitutive activation (Emoto *et al.*, 1995). Overexpression of the catalytic fragment of PKCδ (CF-PKCδ) could induce the activity of Zp by a further 9-fold. In contrast, expression of CD-PKCδ, with the catalytic domain of PKCδ deleted, could not induce the activity of Zp. This result implied that activation of PKCδ is necessary to upregulate Zp activity. Next, we addressed whether PKCδ alone can induce EBV lytic-cycle progression. In Fig. 3(b), overexpression of wild-type PKCδ and CF-PKCδ could induce Zta and EA-D expression without HDACi stimulation. In contrast, the CD-PKCδ mutant could not activate EBV lytic-cycle progression. It seemed that only

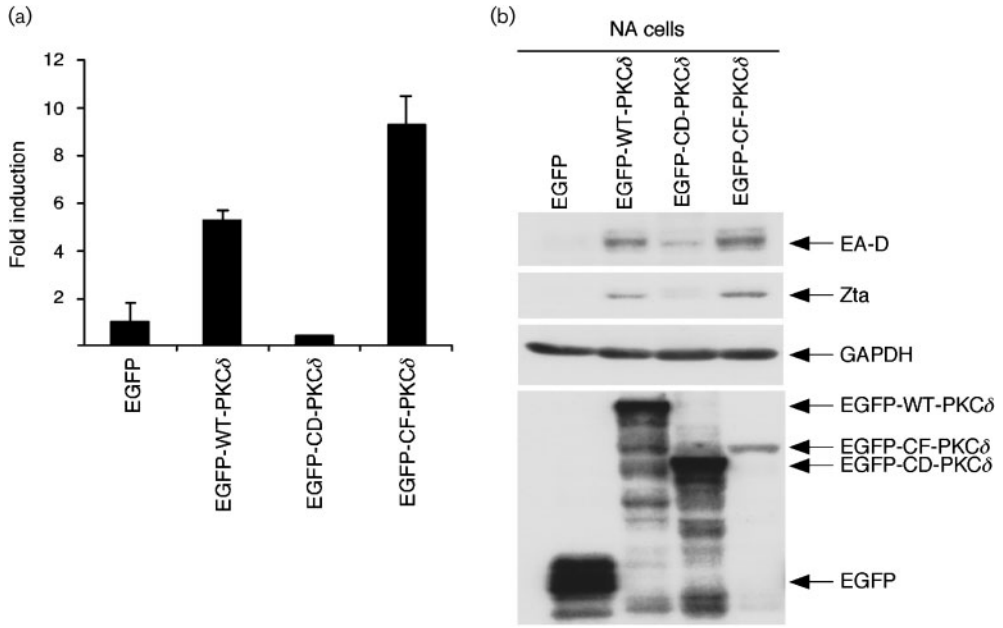


**Fig. 3.** Overexpression of PKC$\delta$ is sufficient to induce Zp activation and EBV reactivation. (a) Analysis of Zp activity in response to PKC$\delta$ overexpression. EBV-negative NPC-TW01 cells were co-transfected with pGL2-Zp-221 and GFP-derived PKC$\delta$ expression plasmids for 48 h, and then Zp activity was analysed by reporter assay. (b) Expression of EBV lytic proteins during PKC$\delta$ overexpression. NA cells were transfected with EGFP-derived wild-type PKC$\delta$ (EGFP-WT-PKC$\delta$), catalytic domain-deleted mutant of PKC$\delta$ (EGFP-CD-PKC$\delta$), EGFP-derived N-terminal-deleted mutant of PKC$\delta$ (EGFP-CF-PKC$\delta$) expression plasmid or vector control (EGFP) for 24 h. The expression of EGFP-fusion proteins was detected by an anti-GFP antibody (BD Biosciences).

CF-PKC$\delta$ expression could induce expression of Zta and EA-D sufficiently. This was consistent with the result that overexpression of CF-PKC$\delta$ enhanced the activity of Zp. Thus, these results demonstrated that activation of PKC$\delta$ is sufficient to induce EBV reactivation.

Although preferentially remaining latent within host cells, EBV has evolved some intricate mechanisms to reactivate the lytic cycle and generate viral progeny (Kieff & Rickinson, 2001). As immediate-early gene expression is the first step in initiating EBV lytic reactivation, it is plausible that immediate-early promoters must be tightly controlled to ensure successful viral reactivation. In this study, we demonstrate that PKC$\delta$ is a crucial kinase for EBV reactivation. Activation of PKC$\delta$ is sufficient to induce BZLF1 gene expression, whilst its inhibition can prevent the HDACi-triggered EBV lytic cycle. Considering that PKC$\delta$ can be activated by DNA damage or HDACi, it is worthwhile to investigate the regulation of PKC$\delta$ and its responsive effectors on immediate-early promoters in governing human gammaherpesvirus reactivation. It also raises the possibility that PKC$\delta$ may recruit particular transcription factors directly or indirectly to Zp for subsequent transcriptional activation. Several reports have indicated that an Sp1/Sp3-binding element is required for HDACi-stimulated gene expression (Choi *et al.*, 2002; Davie, 2003; Kim *et al.*, 2006, 2007; Yokota *et al.*, 2004), including KSHV ORF50 expression (Ye *et al.*, 2005). Sp1/Sp3-binding sites (ZIA, ZIC and ZID domains) are responsive elements critical for TPA-induced Zp activation (Kieff & Rickinson, 2001). It is worthwhile to investigate whether Sp1/Sp3 is required for HDACi-initiated Zp activation and to define further the roles of these transcriptional factors in PKC$\delta$-mediated Zp activation.

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