Microtubule depolymerization activates the Epstein–Barr virus lytic cycle through protein kinase C pathways in nasopharyngeal carcinoma cells

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Elevated levels of antibodies against Epstein–Barr virus (EBV) and the presence of viral DNA in plasma are reliable biomarkers for the diagnosis of nasopharyngeal carcinoma (NPC) in high-prevalence areas, such as South-East Asia. The presence of these viral markers in the circulation suggests that a minimal level of virus reactivation may have occurred in an infected individual, although the underlying mechanism of reactivation remains to be elucidated. Here, we showed that treatment with nocodazole, which provokes the depolymerization of microtubules, induces the expression of two EBV lytic cycle proteins, Zta and EA-D, in EBV-positive NPC cells. This effect was independent of mitotic arrest, as viral reactivation was not abolished in cells synchronized at interphase. Notably, the induction of Zta by nocodazole was mediated by transcriptional upregulation via protein kinase C (PKC). Pre-treatment with inhibitors for PKC or its downstream signalling partners p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) abolished the nocodazole-mediated induction of Zta and EA-D. Interestingly, the effect of nocodazole, as well as colchicine and vinblastine, on lytic gene expression occurred only in NPC epithelial cells but not in cells derived from lymphocytes. These results establish a novel role of microtubule integrity in controlling the EBV life cycle through PKC and its downstream pathways, which represents a tissue-specific mechanism for controlling the life-cycle switch of EBV.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous gammaherpesvirus that infects more than 90% of the human population worldwide. EBV infections contribute to the pathogenesis of various human malignancies, such as T-cell lymphomas, B-cell lymphomas, Burkitt’s lymphoma, Hodgkin’s lymphoma, gastric carcinoma, myogenic tumours and nasopharyngeal carcinoma (NPC) (Sprangers et al., 2008; Young & Rickinson, 2004). Specifically, EBV is detected in almost all cases of NPC in high-prevalence areas such as South-East Asia (Chen et al., 1993; Han et al., 2012). Increased plasma levels of EBV DNA have been shown to correlate inversely with the prognosis and long-term survival of patients with NPC (Chan & Lo, 2002; Wang et al., 2013). Accordingly, both plasma EBV antibodies and plasma viral DNA serve as biomarkers for diagnosing and determining the prognosis of NPC (Ai et al., 2013; An et al., 2011; Chien et al., 2001; Shao et al., 2004a, b; Tedeschi et al., 2007). These studies suggest a potential role for EBV reactivation in NPC disease development and progression. It should be noted that EBV persists mainly as a latent infection with very limited expression of viral products. A switch from latent to lytic infection is required for virus replication (Tsurumi et al., 2005), it is possible that chronic and persistent virus reactivation play a role in the pathogenesis of EBV-associated diseases. An increased level of EBV DNA has been linked to the state of chronic active EBV infection (Sakamoto et al., 2012). It remains to be clarified whether chronic active EBV infection is involved in the pathogenesis of NPC.

Persistent viral reactivation has been proposed to contribute to the development of NPC via genomic instability (Ai et al., 2013; Fang et al., 2009; Tsao et al., 2012; Young &
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Nocodazole induces EBV reactivation in NA cells independently of mitosis

We first tested whether the induction of mitotic arrest could induce EBV reactivation. NA cells were treated with Taxol and nocodazole for 24 h, and the level of EBV reactivation was determined by measuring the induction of two lytic viral products, Zta and EA-D. Unexpectedly, we found that nocodazole, but not Taxol, induced Zta and EA-D expression in NA cells (Fig. 1a). As most cells treated with nocodazole were arrested during mitosis, it was not clear whether EBV reactivation was cell-cycle stage specific. Next, cells were arrested at the G1/S transition with thymidine prior to nocodazole treatment, and a similar induction of EBV reactivation was observed (Fig. 1b). We then compared the efficiency of EBV reactivation during interphase and during mitosis. Interestingly, thymidine treatment alone also induced a mild induction of Zta and EA-D. Furthermore, substantial EBV reactivation was induced in cells co-treated with nocodazole and thymidine (Fig. 1c). Because cells were arrested in interphase upon thymidine treatment, as indicated by the low level of PIK1 (Fig. 1c), these results suggested that the nocodazole-mediated EBV reactivation was independent of cell-cycle stage, although it was most apparent during interphase.

To compare the efficiency of EBV reactivation induced by conventional O-12-O-tetradecanoylphorbol-13-acetate (TPA) / sodium butyrate (TS) and nocodazole, we monitored the percentage of cells expressing Zta or EA-D and the signal intensities of these two markers in cells following treatment. The number of Zta-positive cells increased over tenfold following treatments with both TS and nocodazole (Fig. S1a, available in JGV Online). As the EA-D antibody exhibited a higher background in immunofluorescence staining, we detected only a three- and twofold increase in EA-D-positive cells following treatments with TS and nocodazole, respectively (Fig. S1b). Although nocodazole induced EBV reactivation in a similar number of cells compared with conventional TS, stronger signal intensities of Zta and EA-D were detected in individual cells in response to TS than to nocodazole (Fig. S1).

Microtubule depolymerization induces EBV reactivation

As nocodazole targets microtubule polymerization in the cell, we next investigated whether other microtubule inhibitors showed similar effects on EBV reactivation (Jordan & Wilson, 2004). Specifically, we tested the induction of Zta and EA-D in NA cells in response to colchicine and vinblastine, two other chemicals that can cause microtubule depolymerization (Fig. 2a). As expected, induction of Zta and EA-D was detected similarly to the induction observed in cells treated with nocodazole (Fig. 2b). In contrast, treatment with Taxol, which blocked the depolymerization of microtubules, failed to induce EBV reactivation. Moreover, interfering with actin polymerization using cytochalasin D had no effect on EBV reactivation (Fig. 2b). These results indicated that microtubule depolymerization, but not microtubule stabilization, triggered EBV reactivation in NA cells.

Although Zta and EA-D were induced in the absence of microtubule polymerization, it was not clear whether these inductions would interfere with the production and release of viral particles. We monitored the presence of EBV genomic DNA in the cells and cell-culture supernatants following treatments. NA cells were treated with TS or nocodazole for 72 h, and total cellular DNA and cell-culture
Fig. 1. Nocodazole induces reactivation of EBV in NA cells. (a) NA cells treated with DMSO (Ctrl), nocodazole (Noc) and Taxol for 24 h before analysis of protein expression of EA-D and Zta. Quantification results of three independent experiments are shown in the right-hand panels. (b) NA cells were synchronized by thymidine (2 mM) overnight (o/n) prior to treatment with nocodazole or Taxol as indicated. The expression of Zta, EA-D and actin in one representative experiment is shown. The right-hand panels show quantitative results of Zta and EA-D normalized to actin. (c) Induction of Zta and EA-D in NA cells synchronized by thymidine, nocodazole, Taxol or a combination. Elevated Plk1 expression is detected in mitotic-arrested cells treated with nocodazole and Taxol. All error bars represent standard deviations obtained from three independent experiments.
supernatants were examined to detect the BZLF1 gene using PCR (Chang et al., 2002). We showed that nocodazole treatment not only increased the number of EBV genome copies in the cell but also the amount of EBV genome in the culture supernatant (Fig. 3a). Similar results were obtained by treating NA cells with colchicine and vinblastine (Fig. S2). It should be noted that the proliferation of NA cells was disturbed following treatment with TS and nocodazole, and, as a result, the copy number of the actin gene was reduced in both cases after 72 h of treatment. After normalization to the actin copy number, we found that cells treated with nocodazole contained a higher cellular EBV genome content and more viral particles in the culture supernatant when compared with conventional TS treatment (Fig. 3a). These results suggested that the lytic cycle of EBV was induced in NA cells upon treatment with nocodazole.

To investigate whether the induction of Zta and EA-D by nocodazole was mediated via transcriptional regulation, we performed a reporter assay with the Zta promoter. As we found that Renilla luciferase activity was severely inhibited by sodium butyrate, TPA alone was used to treat NA cells as a positive control. As expected, treatment with TPA alone was sufficient to cause a threefold induction of Zta promoter activity (Fig. 3b). In contrast, Taxol showed no effect on the Zta promoter. A similar effect was confirmed in the parental cell line TW01 in the absence of EBV (Fig. 3c). These results indicated that EBV reactivation was mediated by regulation at the transcriptional level.

**EBV reactivation depends on PKC and the downstream p38 MAPK and JNK pathways**

To investigate which signalling pathway was involved in EBV reactivation in the presence of nocodazole, NA cells were pre-treated with various signalling pathway inhibitors 1 h prior to 24 h nocodazole treatment. It has been shown that both PKC and NFκB are critical signalling pathways involved in EBV reactivation (Gao et al., 2001; Goswami et al., 2012; Gradoville et al., 2002). Thus, we tested whether these two pathways are involved in reactivation by nocodazole. We found that inhibition of PKC with bisindolylmaleimide I and staurosporine was able to block nocodazole-mediated induction of Zta and EA-D, whereas inhibition of NFκB with BAY11-7082 had no effect (Fig. 4a). Next, we investigated the role of signalling molecules downstream of PKC, including MAPK, p38 MAPK and JNK, in nocodazole-mediated induction of Zta and EA-D. Treatment with the JNK-specific inhibitor SP600125 or the p38 MAPK-specific inhibitor SB202190 could effectively
block the induction of Zta and EA-D in combination with nocodazole treatment. Conversely, the MEK inhibitors PD98059 and U0126 had no effect on EBV reactivation (Fig. 4a). In conclusion, these results suggested that induction of EBV reactivation via disturbing microtubule polymerization depends on the activity of PKC and its downstream signalling partners p38 MAPK and JNK in NA cells (Fig. 4b).

**DISCUSSION**

This study demonstrated that microtubule deficiency could induce EBV reactivation through the PKC, p38 MAPK and JNK pathways. Interfering with microtubule polymerization, as shown by treatment with nocodazole, colchicine and vinblastine, induced the expression of two EBV lytic proteins, Zta and EA-D, in NA cells. This study therefore sheds light on a novel interaction between components of the cytoskeleton and the maintenance of EBV latency in the cell.

It has been shown that the activation of PKC and NFκB is involved in the EBV lytic cycle switch (Gao et al., 2001). NFκB was shown to be essential for EBV reactivation induced by transforming growth factor β in Burkitt’s lymphoma cells in a previous study (Oussaief et al., 2011). However, other studies showed that the level of NFκB was inversely correlated with the lytic cycle of EBV in lymphocytes (Cahir-McFarland et al., 2000; Keller et al., 2000; Li et al., 2012). In the present study, treatment with BAY11-7082 did not abolish the induction of Zta and EA-D by nocodazole (Fig. 4a). Thus, we concluded that NFκB is not relevant in the EBV lytic cycle switch induced upon microtubule defects in NPC.

The activation of PKC in response to microtubule deficiency was observed previously in endothelial and epithelial cells (Kadi et al., 2002; Tsai et al., 2012). Treatments with 0.1–50 nM Taxol were able to induce EBV lytic gene expression in an EBV-positive gastric carcinoma cell line AGS (Feng et al., 2002). In the present study, we found that Taxol could not induce the EBV lytic cycle switch in NPC NA cells, even when a higher concentration of Taxol (2 μM) was used. Thus, the occurrence of EBV reactivation in response to Taxol may be cell-type specific. PKC activity has been shown to play an important role in induction of the EBV lytic cycle, as demonstrated in several cell lines such as GT38 (a gastric tumour cell line), Raji, P3HR1 and Burkitt’s lymphoma cells (Davies et al., 1991; Goswami et al., 2012). We compared the cellular levels of Zta and EA-D induced by nocodazole and TPA/sodium n-butyrate (NaB), conventional inducers

**Fig. 3.** EBV genome expression and a reporter assay for Zta promoter activity. (a) Intracellular EBV genomes and extracellular EBV virions were measured by detecting EBV DNA. NA cells were treated with TPA/NaB (TS) or nocodazole (Noc) for 72 h. The abundance of EBV DNA in cells and culture supernatants was examined by PCR amplification of the Zta gene. The β-actin gene was used as an internal control for cell numbers. Quantification results of three independent experiments for intracellular and extracellular EBV DNA are shown (after normalization to β-actin). (b) Reporter assay for Zta promoter activity in EBV-positive NA and EBV-negative TW01 cells. Cells were transiently transfected with the Zta reporter construct for 24 h and then treated with indicated chemicals for an additional 24 h. Zta promoter activity was measured by a luciferase reporter assay. Error bars represent standard deviations obtained from three independent experiments.

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frequently used to induce an EBV lytic cycle switch. By analysing the fluorescence intensity of Zta and EA-D in individual cells, our data indicated that TPA/NaB was a stronger inducer of the EBV lytic cycle than nocodazole (Fig. S1). Notably, microtubule integrity was also affected in cells treated with TPA/NaB (Fig. 2). These results suggested that microtubule defects may also contribute to EBV reactivation triggered by TPA/NaB.

Whereas nocodazole could induce the EBV lytic cycle in NPC cells, we found that this effect was not observable in three other EBV-positive B-cell lines (P3HR1, B95.8 and Raji) (Fig. S3). In addition, nocodazole showed only a mild effect on fibroblast-derived EREV8 cells. Thus, we consider the induction of the EBV lytic cycle in response to microtubule deficiency to be a cell-type- or tissue-specific mechanism. The distinct biological differences between adherent and suspension cell types may explain why these cells respond differently to nocodazole. Adherent cells, in comparison with suspension cells, may place more demand on microtubules to maintain proper morphology and physiological activity. With regard to the observation that the disruption of actin did not activate the EBV lytic cycle in the present study (Fig. 2b), we suggest that the integrity of the cytoskeleton may play distinct roles in controlling the EBV lytic cycle among different cell types. Interestingly, actin was recently shown to play different roles in the trafficking of viral particles in epithelial and B-cells (Valencia & Hutt-Fletcher, 2012). Conversely, both EBV kinases BGLF4 and LMP1 were shown to interfere with microtubule dynamics through the induction of stathmin phosphorylation in NPC cells (Chen et al., 2010; Lin et al., 2009). As the activity of stathmin was shown to be modulated by EBV during its lytic cycle (Chen et al., 2010), the interplay between different cytoskeletal components and EBV life-cycle switches should be an interesting topic to be explored in the future.

Our study showed that microtubule deficiency can drive the EBV lytic cycle in NPC cells. This novel mechanism has several implications. First, several environmental factors have been shown to influence microtubule integrity in the cell. Smoking has been shown to correlate positively with EBV activation and is a risk factor for NPC (Xu et al., 2012). Organic constituents in cigarette smoke extract have been shown to impact microtubule polymerization (Das et al., 2009). In addition, marinated salty foods containing N-nitro compounds, such as N-methyl-N’-nitro-N-nitrosoguanidine, may also affect microtubule stability (Bouvier et al., 1991; Huang et al., 2010; Jaiswal et al., 2004). These clinical observations suggest that these environmental inducers may trigger EBV reactivation through mechanisms involving microtubule depolymerization and PKC activation. In addition, this study implies a potential novel
therapeutic approach in targeting microtubules in cancer therapy for EBV-positive tumours. In this scenario, treatment with nocodazole may induce cancer cell death via two different pathways: microtubule deficiency and activation of the lytic lytic cycle. The induction of lytic cytotoxicity has been proposed as an effective viral-targeted therapy for EBV-positive cancers (Hui et al., 2012; Wildeman et al., 2012). Considering the high prevalence of EBV in tumour cells, the induction of lytic cytotoxicity by the disruption of microtubules may serve as an effective therapeutic approach for patients with NPC.

METHODS

Cell culture and treatments. The EBV-positive cell line NA was established previously from TW01, an epithelial cell line derived from a nasopharyngeal carcinoma (Chang et al., 1999; Lin et al., 1990, 1993). All cells were grown in complete medium (high-glucose Dulbecco’s minimal essential medium with 10 % FBS and 1:100 penicillin and streptomycin) and maintained at 37 °C for 5 % CO2. The cell-culture density was 1×105 cells in a 10 cm dish, 5×104 cells in a 6 cm dish and 2×105 cells per well in a 96-well plate. The following chemicals were used with working concentrations as indicated: TPA (20 ng ml−1; Sigma), NaB (1.5 mM; Sigma), nocodazole (2 μg ml−1; Sigma), colchicine (0.1 μg ml−1; Sigma), vinblastine (2 g ml−1; Sigma), cytochalasin D (2 μM; Sigma), thymidine (2 mM; Sigma), staurosporine (50 μM; Sigma), SB202190 (20 M; Sigma), PD98059 (20 M; Sigma), U0126 (20 M; Sigma), BAY11-7082 (20 M; Sigma), paxilast (Taxol, 2 μM; Selleckchem) and bisindolylmaleimide I, hydrochloride (10 M; Merck).

Western blotting. Total cell lysates were resolved by SDS-PAGE (12 % acrylamide) followed by transfer to PVDF membranes (Millipore). Membranes were incubated with primary antibodies overnight and detected by chemiluminescence. Primary mAbs that recognize Zta (clone 4F10), EA-D (clone 88A9) and actin (Chemicon) were used as described previously (Tsi et al., 1991, 1997).

Immunofluorescence staining. Cells grown on glass coverslips were fixed with 4 % formaldehyde for 10 min followed by permeabilization with 0.1 % Triton X-100 for another 10 min. After being rinsed with PBS three times, the cells were incubated with FITC-labelled mouse anti-tubulin (Genetex) and DAPI. Fluorescence images were acquired with a Leica DMI6000 inverted microscope using a HCX PL APO ×63/NA1.4 objective and an Andor LucaR EMCCD camera. Images were acquired and analysed with Meta-morph software (Molecular Devices).

Reporter assay. Cells cultured in 96-well plates were co-transfected with the Zta reporter construct (1 g ml−1; Tsi et al., 2011) and hRLuc/TK (0.5 g ml−1; Promega) for 48 h using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase in cell lysates were detected using a Dual-Luciferase Reporter (DLR) Assay System (Promega) according to the manufacturer’s instructions.

Detection of intracellular and extracellular EBV genomic DNA. The presence of EBV viral particles in culture supernatants was monitored by detecting EBV genomic DNA using PCR as described previously (Chang et al., 2002). NA cells were treated with either TPA/NaB (TS) or thymidine/nocodazole for 72 h to induce EBV lytic reactivation. Culture supernatants were passed through a 0.45 μm filter (Millipore) and treated with DNase I (New England Biolabs) at 37 °C for 30 min. DNase I was then inactivated by adding 0.2 mM EDTA (pH 8.0) and incubated at 65 °C for 10 min. Next, the culture supernatant was treated with 0.1 mg proteinase K ml−1 at 55 °C for 3 h, and subsequently transferred to 70 °C for an additional 20 min. Two microlitres of supernatant from each group was used for detecting the EBV genome in culture supernatants. For detecting the intracellular EBV genome content, total cellular DNA was extracted with a Genomic DNA extraction kit (Viogene), and 100 ng of total genomic DNA was used for PCR amplification. The following primers were used: EBV genomic DNA-Zebra (5′-GAGTCAACATCAGGCTTG-3′ and 5′-CTGCAGACTACCTGAGG-3′) and β-actin (5′-ATCATGTTTGAACCTTCAA and CATCCTTGCTGGAATTCCA-3′).

Statistical analysis. The results are expressed as the mean±SD. All data were analysed using an unpaired t-test. The level of significance was defined as: *P<0.05; **P<0.001; ***P<0.0001.

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