Xeroderma pigmentosum C is involved in Epstein–Barr virus DNA replication

Chih-Chung Lu,t Yi-Chun Chen,t Jiin-Tarng Wang, Pei-Wen Yang and Mei-Ru Chen

Correspondence
Mei-Ru Chen
mrc@ntu.edu.tw

Graduate Institute and Department of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan 100, PR China

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Cells of mismatch and base-excision repair machineries have been shown to be involved in Epstein–Barr Virus (EBV) lytic DNA replication. We report here that nucleotide-excision repair (NER) may also play an important role in EBV lytic DNA replication. Firstly, the EBV BGLF4 kinase interacts with xeroderma pigmentosum C (XPC), the critical DNA damage-recognition factor of NER, in yeast and in vitro, as demonstrated by yeast two-hybrid and glutathione S-transferase pull-down assays. Simultaneously, XPC was shown, by indirect immunofluorescence and co-immunoprecipitation assays, to interact and colocalize with BGLF4 in EBV-positive NA cells undergoing lytic viral replication. In addition, the efficiency of EBV DNA replication was reduced about 30–40% by an XPC small interfering RNA. Expression of BGLF4 enhances cellular DNA-repair activity in p53-defective H1299/bcl2 cells in a host-cell reactivation assay. This enhancement was not observed in the XPC-mutant cell line XP4PA-SV unless complemented by ectopic XPC, suggesting that BGLF4 may stimulate DNA repair in an XPC-dependent manner. Overall, we suggest that the interaction of BGLF4 and XPC may be involved in DNA replication and repair and thereby enhance the efficiency of viral DNA replication.

INTRODUCTION

Genomic DNA damage may be induced by various environmental agents, such as UV light, ionizing radiation and genotoxic chemicals, or the products of cellular metabolism (Hoeijmakers, 2001). To maintain genome integrity, mammalian cells have evolved various processes to monitor and repair damaged DNA. DNA damage may trigger cell-cycle-checkpoint activation, transcriptional activation, repair mechanisms, damage tolerance or even apoptosis, if the damage is severe (Friedberg, 2001). The repair mechanisms include recombination repair, mismatch repair (MMR), base-excision repair (BER) and nucleotide-excision repair (NER). Double-stranded breaks in DNA are usually recognized by the MRN (Mre11–Rad50–NBS1) complex and the activated ataxia telangiectasia mutated/related (ATM/ATR) pathway. The binding of the MRN complex activates cellular damage machinery through ATM and ATR (Shiloh, 2003). The post-replicative MMR system plays important roles in removing base–base and small insertion–deletion mismatches that arise during DNA replication (Kunkel & Erie, 2005). BER is the major process for removing a damaged or inappropriate base. Hydrolysis of the N-glycosidic bond creates an abasic site in the DNA, allowing AP endonuclease 1 to generate a nick, for polymerase to fill in the correct nucleotide through short-patch BER or via long-patch BER, which depends on replication proteins (Krokan et al., 2002). NER is used widely to remove many types of DNA lesions, such as DNA damage caused by exposure to UV light and bulky DNA induced by chemicals (Friedberg, 2001).

The basic steps of NER include damage recognition, dual incision and release of a short damaged region (24–32 nt), repair synthesis to fill the gap, and ligation (Friedberg, 2001). Two subpathways are distinguishable in the repair synthesis and ligation process: transcription-coupled NER (TC-NER), which removes damaged DNA located on the transcribed strand, and global genomic NER (GG-NER), which repairs lesions throughout genomic DNA. For GG-NER, xeroderma pigmentosum C (XPC) binds tightly to hHR23B to perform the damage recognition. The binding of the XPC–hHR23B complex triggers the binding of XPA, TFIIH, XPG, RPA and ERCC1–XPF. The ERCC1–XPF complex then serves as a duplex/single-stranded DNA endonuclease to cut the damaged strand at the 5’ junction, whilst XPG cuts the damaged strand at the 3’ junction to release the short damaged fragment to initiate the subsequent repair process (Friedberg, 2001).

Under such stringent control of cellular DNA fidelity, viral DNA replication within the nucleus strongly elicits host DNA-repair systems. Viruses thus need to inactivate the DNA signalling pathway or modify host repair or replication factors to facilitate replication of their genomes.

†These authors contributed equally to this work.
recruited to viral replication compartments (Weitzman et al., 2004). ATM and BER proteins have been shown to be recruited to viral replication compartments (Weitzman et al., 2004).

Epstein–Barr virus (EBV) is a widely distributed γ-herpesvirus that infects about 90% of the population worldwide. Following primary infection, it becomes latent in the B lymphocytes of most individuals. Reactivation of EBV can be induced by chemical agents, UV light or surface IgG cross-linking (Rickinson & Kieff, 2001). Viral DNA replication initiates at oriLyt, with the viral DNA replication core machinery containing seven virus-encoded proteins: BZLF1 (an oriLyt-binding protein), BALF5 (DNA polymerase), BMRF1 (also called EA-D, a polymerase processivity factor), BALF2 (a single-stranded-DNA-binding protein), BBLF4 (helicase), BSLF1 (primase) and BBLF2/3 (helicase–primase–associated protein) (Fixman et al., 1995). During lytic replication, the cellular DNA-repair response may be stimulated because of the accumulation of abundant viral DNA. Phosphorylated ATM and MRN complex are recruited to the EBV replication compartment (Kudoh et al., 2005). Although the cellular DNA-damage response is activated by lytic virus replication, p53 signalling downstream of the ATM checkpoint signal is blocked to prevent cell-cycle arrest and apoptosis. Moreover, the levels of cyclin E/A and the hyperphosphorylated form of RB are increased during lytic infection. This S-phase-like environment may benefit EBV lytic replication (Kudoh et al., 2005). Recently, it was demonstrated that proliferation cell nuclear antigen (PCNA), the clamp loader RF-C and MMR proteins MSH-2, MSH-6, MLH1 and hPMS2 are recruited to EBV replication compartments, suggesting that host MMR might function to increase viral genome integrity (Daikoku et al., 2006). Our previous study on viral and cellular uracil–DNA glycosylase also indicated that the cellular BER machinery may supplement viral DNA replication in cell culture (Lu et al., 2007). The interplay between viral and cellular DNA replication and repair machineries may play a pivotal role in EBV replication.

BGLF4 kinase is the only Ser/Thr protein kinase of EBV and phosphorylates several viral substrates, including DNA polymerase processivity factor BMRF1, EBNA-2, EBNA-LP and BZLF1 (Asai et al., 2006; Chen et al., 2000; Kato et al., 2003; Yue et al., 2005). It also phosphorylates cellular translation elongation factor 1 delta (EF-1δ) and casein kinase IIβ at cellular cdc2-targeted sites (Kawaguchi et al., 2003). BGLF4 localizes within the viral replication compartment and is packaged into virions (Wang et al., 2005), indicating that BGLF4 may be involved in viral lytic DNA replication and virion egress. Indeed, viral lytic DNA replication was decreased by 30% and viral nucleocapsids were retained in the nuclei when the expression of BGLF4 was knocked down by small interfering RNA (siRNA) (Gershburg et al., 2007). Interestingly, BGLF4 also phosphorylates MCM4 at sites inactivating DNA helicase activity of the MCM4–6–7 complex, which might lead to the blocking of chromosomal DNA replication in virus-reactivated cells (Kudoh et al., 2006). Recently, we have also observed that expression of BGLF4 can induce premature chromosome condensation through a cdc2-independent mechanism (Lee et al., 2007).

In this study, BGLF4 protein was shown, through yeast two-hybrid screening, to interact with XPC. This interaction was confirmed by co-immunoprecipitation and immunofluorescence assay in HeLa and EBV-positive NA cells. Functionally, BGLF4 stimulates cellular repair activity in host-cell reactivation in an XPC-dependent manner. By using an siRNA approach, XPC was shown to be required for efficient viral genome replication, suggesting that the cellular NER machinery also may contribute to viral genome integrity during its replication.

**METHODS**

**Yeast two-hybrid system.** The yeast two-hybrid system protocol was adapted from that described by James et al. (1996). The bait plasmid pGBDU-BGLF4 (S300P), containing a spontaneous mutation at aa 300, was used to screen a cDNA library of the human lymphocyte MATCHMAKER (Clontech) fused to the GAL4-AD in pACT. The bait plasmid and the cDNA library were transformed sequentially into the yeast strain P69-4A (MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4a gal80α lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (James et al., 1996). The transformants were then plated on synthetic complete medium lacking adenine (Ade), uracil (Ura) and leucine (Leu). The Ade Δ/ura Δ/leu Δ colonies were purified and the cDNA clones were isolated, sequenced and subjected to a BLAST search of GenBank.

**Plasmids.** Wild-type BGLF4 (pYPW17) and catalytic-site mutant (‘kinase-dead’) BGLF4 (K102I, pYPW20) are pSG5-based constructs (Wang et al., 2005). To express glutathione S-transferase (GST) fusion protein, Bglf4HI–EcoRI fragments containing BGLF4 ORFs from pYPW1 or pYPW20 were modified to be in frame with GST and cloned into pGEXXJ (Pharmacia Biotech). The p53–expressing plasmid (pCMV-p33) and pCMV-Luc, which contains a Renilla luciferase gene driven by the cytomegalovirus (CMV) promoter, have been described previously by Liu et al. (2004). The XPC–expressing plasmid (pDNA3.1B/XPCΔ; a gift from Dr Wenyu Huang, National Cheng-Kung University, Taiwan) has been described previously by Chang et al. (2003). The Rta-expressing plasmid RTS15 (pSG5-Rta, a gift from Dr Diane Hayward, Johns Hopkins School of Medicine, Baltimore, MD, USA) was described by Ragoczy & Miller (1999). The siGFP plasmid is the pSUPER-based construct described by Chang et al. (2004). The siRNA-expressing plasmids (siXPC-1, siXPC-2 and siXPC-3) were constructed by cloning siRNA sequences, which were predicted using an online program (http://www.sirnawizard.com/sirna.php), into pSUPER via the BamHI and HindIII sites. The siRNA sequences were subjected to a BLAST search against the human genome and EST databases to ensure that no other human genes were targeted. The siXPC-1, siXPC-2 and siXPC-3 RNAs are directed against the XPC sequences 5’-GAGCCCTCTCTCACAAGGT-3’ (nt 208–226 of the cDNA), 5’-GAAGACAAAAAGAAGAATG-3’ (nt 590–608 of the cDNA) and 5’-GAGAAATGGCTCACCATT-3’ (nt 957–975 of the cDNA), respectively.

**Cell culture and induction of the viral lytic cycle.** H1299/bcl2 cells (Liu et al., 2004) had been established previously by transfecting a plasmid expressing bcl-2 into H1299 cells, a human large cell lung carcinoma cell line without the p53 gene (Mitsudomi et al., 1992).
NPC-TW01 is an NPC cell line lacking the EBV genome (Lin et al., 1993) and the NA cell line was derived from NPC-TW01 and converted with a neomycin-containing recombinant Akata EBV (Chang et al., 1999). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. XP4PA-SV cells, which carry a deletion mutation in the xpc gene (Chang et al., 2003), were obtained from Dr Wenyua Huang and maintained in DMEM supplemented with 15% fetal calf serum. For induction of the EBV lytic cycle, NA cells were transfected with the Rta-expressing plasmid RTS15.

**Antibodies.** The primary antibodies used for the immunoprecipitation assay, immunoblotting and indirect immunofluorescence included Rta mAb 467, Zta mAb 4F10 (Tsai et al., 1997), EA-D mAb 88A9 (Tsai et al., 1991), BGLF4 mAb 2224 (Wang et al., 2005), XPC mAb (Abcam), β-actin mAb (Sigma), HA mAb (Covance) and rabbit antiserum against BGLF4 (Wang et al., 2005).

**Immunoprecipitation assay and immunoblotting.** HA-XPC- and BGLF4-expressing plasmids were transfected into 293T cells by using the calcium phosphate method (Chen & Okayama, 1987). At 48 h post-transfection, cells were lysed in NP-40 lysis buffer [50 mM Tris/HCl (pH 8.0), 120 mM NaCl and 0.5% Nonidet P-40]. To detect the interaction of BGLF4 and XPC in virus-replicating cells, Rta-, BGLF4- and XPC-expressing plasmids were cotransfected into NA cells by using Lipofectamine 2000 (Invitrogen). At 36 h post-transfection, cells were lysed in NP-40 lysis buffer containing 100 mM sodium fluoride, 2 mM sodium vanadate and 1× Complete Mini Protease Inhibitor mixture (EDTA-free; Roche). Lysates (1.5 mg total protein) were precleared with protein A–Sepharose CL-4B beads (Amersham Biosciences) at 4°C for 1 h. The supernatants were incubated with anti-BGLF4 mAb 2224 (Wang et al., 2005) or anti-HA mAb at 4°C for 1.5 h. The immunocomplexes were precipitated with protein A–Sepharose beads at 4°C for 1.5 h and washed extensively with PBS. After incubation, the blot was developed with substrate for 35S incorporation and the primers and probes have been described previously (Lo et al., 2002). The EBNA1 region of the EBV genome was the detection target for real-time PCR and the primers and probes have been described previously (Lo et al., 1999). Endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA, which served as a control, was quantified by using specific primers (Chang et al., 2002) and Universal Probe Library probe 045 (Roche). The H2B4 cell line, harbouring one EBV genome per cell, was used to generate a standard curve for quantification and the EBV copy number was calculated by comparison with the standards (Chang et al., 2002). All samples were tested in duplicate and the experiments were repeated twice independently.

**Detection and quantification of EBV DNA.** For quantification of EBV DNA, real-time PCR was performed according to the manufacturer’s instructions (Applied Biosystems). Briefly, cells were lysed, digested with proteinase K and then subjected to real-time PCR analysis as described previously (Chang et al., 2002). The EBNA1 gene of the EBV genome was the detection target for real-time PCR and the primers and probes have been described previously (Lo et al., 1999). Endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA, which served as a control, was quantified by using specific primers (Chang et al., 2002) and Universal Probe Library probe 045 (Roche). The H2B4 cell line, harbouring one EBV genome per cell, was used to generate a standard curve for quantification and the EBV copy number was calculated by comparison with the standards (Chang et al., 2002). All samples were tested in duplicate and the experiments were repeated twice independently.

**Host-cell reactivation (HCR) assay.** The HCR assay protocol was adapted from that described by Liu et al. (2004). Briefly, a firefly luciferase reporter plasmid (pCMV-Luc), either damaged with 1000 or 100 J UV light m⁻² (Stratalinker UV Cross-linker 1800) as indicated or mock-treated, together with an undamaged Renilla luciferase reporter plasmid (pWP-1) as an internal control, were cotransfected with effector plasmids BGLF4, BGLF4 (K1021), p53 or XPC into H1299/bcl2 or XP4PA-SV cells. At 24 or 48 h post-transfection, the cells were harvested and subjected to a luciferase assay with a Dual-Glo assay kit (Promega) according to the manufacturer’s instructions. The activity of Renilla luciferase (from the internal control) was used to calibrate the activity of firefly luciferase (from the reporter). Repair activity was determined from two parameters, repair conversion and fold HCR. Repair conversion was calculated first by dividing normalized luciferase activity from cells transfected with UV-light-irradiated pCMV-Luc by that of non-irradiated pCMV-Luc transfectants. The repair conversion of vector transfectants was set to 1. The fold HCR was then calculated by comparing the repair conversion of effector to that of vector transfectants.
RESULTS

BGLF4 interacts with DNA repair protein XPC

To identify potential BGLF4-associated cellular and viral proteins, yeast two-hybrid screening was performed by using a ‘kinase-dead’ version of BGLF4 (S300P) as bait to avoid a possible effect of BGLF4 kinase activity on yeast growth. The screening was performed against a cDNA library derived from an EBV-transformed human peripheral blood B lymphocyte (Clontech). Among five BGLF4-interacting cDNA clones identified, one contained the coding region, aa 509–835, of XPC, which is believed to be responsible for recognizing bulky DNA lesions in GG-NER. XPC protein was found to interact with a variety of factors, including hHR23B, TFIIH and DNA (Fig. 1a). As illustrated in Fig. 1(a), aa 509–835 of XPC overlap partially with the interacting domain for hHR23B and TFIIH. The interaction of XPC (aa 509–835) with full-length (S300P) BGLF4, its N terminus (aa 1–293) and C terminus (aa 201–429) was reproduced in a yeast two-hybrid system (data not shown), suggesting that XPC may interact with either a domain within the aa 201–293 region or separate domains in the N and C termini of BGLF4. To confirm the interaction of BGLF4 with XPC in mammalian cells, a ‘kinase-dead’ version of BGLF4 (K102I) and HA–XPC (aa 509–835) were cotransfected into 293T cells for co-immunoprecipitation assays with either anti-BGLF4 or anti-HA antibody. HA–XPC (aa 509–835) was precipitated specifically from BGLF4 (K102I) and HA–XPC (aa

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**Fig. 1.** Interaction of BGLF4 kinase with cellular protein XPC. (a) The structure and functional domains of XPC. The region that shares high homology with *Saccharomyces cerevisiae* RAD4 is shown as a grey box within the open bar representing the XPC gene (940 aa). The functional domains of XPC responsible for hHR23B interaction, DNA binding and TFIIH interaction are delineated by lines (Uchida et al., 2002). The cDNA product containing aa 509–835 of XPC was shown to interact with BGLF4 in a yeast two-hybrid system. (b) 293T cells were transfected with plasmids expressing ‘kinase-dead’ BGLF4 (K102I) and HA–XPC (509–835) for 48 h. Protein lysates were immunoprecipitated (IP) from cell extracts by using an anti-BGLF4 mAb (2224) or anti-HA antibody. The immunocomplexes were separated by SDS-PAGE and subjected to Western blotting with anti-BGLF4 (upper panel) or anti-HA (lower panel) antibodies. (c) 293T cells were transfected with plasmids expressing wild-type BGLF4 and full-length XPC for 48 h. Proteins were immunoprecipitated from cell extracts by using an anti-BGLF4 mAb (2224) or anti-HA antibody. The immunocomplexes were separated by SDS-PAGE and subjected to Western blotting with anti-BGLF4 (upper panel) or anti-HA (lower panel) antibodies. (d) Samples (3 μg) of GST, GST–BGLF4 or GST–BGLF4(KD) protein were coupled to glutathione-Sepharose beads to pull down in vitro-expressed, [35S]methionine-labelled XPC in binding buffer. After extensive washing, the complexes were separated by SDS-PAGE and analysed by autoradiography (upper and middle panels are short and long exposures of the same blot). The amounts of GST or GST fusion proteins were revealed by Coomassie brilliant blue staining. Molecular mass markers are indicated.
509–835)-cotransfected 293T cells by using anti-BGLF4 antibody (Fig. 1b, lane 1). This interaction also was detected in a reciprocal co-immunoprecipitation assay performed with anti-HA antibody (Fig. 1b, lane 4). Moreover, full-length XPC also was co-immunoprecipitated from wild-type BGLF4 and XPC-cotransfected 293T cells using anti-BGLF4 antibody (Fig. 1c, lane 1). GST pull-down experiments further revealed that in vitro-expressed [\(^{35}\)S]methionine-labelled XPC protein can bind to both GST–BGLF4 and GST–BGLF4 KD (Fig. 1d). Even though the signal is not very strong, these results indicate that BGLF4 interacts with XPC both in vitro and in vivo.

### Colocalization and interaction of BGLF4 and XPC in EBV-positive NA cells

To detect intracellular colocalization of BGLF4 and XPC, HeLa cells were cotransfected with BGLF4- and XPC-expressing plasmids. Following transient transfection, BGLF4 was detected by using a specific rabbit antiserum and XPC was visualized with a mouse mAb. As shown in Fig. 2(a), colocalization of BGLF4 and XPC was observed in the nucleus by using an indirect immunofluorescence assay (IFA). BGLF4 has been shown to colocalize with the DNA polymerase processivity factor BMRF1 (EA-D) and the immediate-early transactivator Zta in the EBV lytic replication compartment during the early stages of replication (Asai et al., 2006; Wang et al., 2005). Therefore, we sought to determine whether XPC colocalizes with BGLF4 in the viral replication compartment. EBV-positive NA cells were cotransfected with plasmids expressing XPC and EBV immediate-early transactivator, Rta, which can induce EBV lytic DNA replication. Ectopic expression of Rta induced the expression of BGLF4 in NA cells, as detected by IFA (Fig. 2b). Moreover, XPC partially colocalized with BGLF4 in cells undergoing lytic DNA replication at 24, 36 and 48 h post-induction (p.i.). To confirm this observation further, the Rta- and XPC-cotransfected NA cells were treated with 0.5 % Triton X-100–mCSK buffer to solubilize DNA-unbound forms of BGLF4 and XPC proteins, and fixed (Fig. 2c). Similar colocalization patterns of BGLF4 and XPC were observed in the nuclei of detergent-treated cells, suggesting that at least a portion of XPC appears to localize to the viral replication compartment while viral genomes are synthesized.

![Fig. 2. Colocalization and interaction of BGLF4 and XPC in HeLa and EBV-replicating NA cells. (a) HeLa cells were co-transfected with BGLF4 and XPC expression plasmids for 48 h. The cells were incubated with rabbit anti-BGLF4 serum and mouse anti-XPC mAb, following by FITC-conjugated anti-rabbit IgG and rhodamine red-conjugated anti-mouse antibodies and observed by fluorescence microscopy. (b) EBV-positive NA cells were cotransfected with plasmids expressing Rta and XPC for 24, 36 and 48 h. The expression of endogenous BGLF4 (green) and XPC (red) was detected with rabbit anti-BGLF4 serum and mouse anti-XPC mAb, following by FITC-conjugated anti-mouse IgG and rhodamine red-conjugated anti-rabbit antibodies and observed by fluorescence microscopy. (c) EBV-positive NA cells were cotransfected with plasmids expressing XPC and/or Rta for 24 h. Cells were treated with 0.5 % Triton X-100–mCSK buffer, fixed with methanol and co-immunostained with antibodies to BGLF4 (red) and XPC (green). The nuclei were stained with Hoechst 33258.](image-url)
To look for further evidence of such an interaction in EBV-positive cells, we cotransfected NA cells with expression plasmids for full-length XPC, BGLF4 and Rta and immunoprecipitated BGLF4 from the derived extracts. Western blots showed that XPC was coprecipitated with BGLF4 (Fig. 3), indicating that BGLF4 interacts specifically with XPC in NA cells undergoing lytic DNA replication.

**Requirement for XPC in EBV lytic replication**

To determine whether the recruitment of XPC by BGLF4 in virus-replicating cells contributes to EBV lytic DNA replication, experiments were carried out with a pSUPER-based system to drive siRNA synthesis in Rta-transduced NA cells. Ectopic expression of Rta induced the expression of BZLF1 and BMRF1 proteins in NA cells, as detected by immunoblotting. Expression of XPC was inhibited specifically by transfection with siXPC-1 or siXPC-3 and inhibited slightly by siXPC-2, but not by the siGFP control, in Rta-induced NA cells (Fig. 4a). Amplification of EBV DNA was detected simultaneously by using quantitative real-time PCR (Fig. 4b). Expression of siXPC-1 and siXPC-3 resulted in around 30–40% inhibition of Rta-induced amplification of viral genomes at 48 h p.i. In contrast, expression of siXPC-2, siGFP and pSUPER had no effect on Rta-induced amplification of the viral genome. This observation suggests that XPC may be required for increased efficiency of EBV DNA replication.

**BGLF4 enhances DNA-repair activity in an XPC-dependent manner**

Repair of UV damage is carried out by NER. XPC is the critical factor for recognizing the DNA lesion in NER. To gain insights into the functional significance of the BGLF4–XPC interaction, we investigated the effect of BGLF4 on the DNA-repair activity of XPC in an HCR assay, which measures the cellular repair activity. In this assay, plasmids expressing BGLF4 or empty vector, the UV-damaged pCMV-Luc and untreated Renilla luciferase reporter plasmid were cotransfected into p53-null H1299/bcl2 cells. Compared with cells transfected with empty vector, cells transfected with the plasmid expressing p53 enhanced the repair of the UV-damaged reporter plasmid by 5.07-fold. Meanwhile, the same amounts of BGLF4 plasmid showed a 2.29-fold enhancement of DNA-repair activity, in a dose-dependent manner (Fig. 5a). Expression of 'kinase-dead' BGLF4 (K102I) did not enhance DNA-repair activity in H1299/bcl2 cells (Fig. 5b), suggesting that BGLF4 enhances DNA-repair activity in a kinase activity-dependent and p53-independent manner.

Further, to determine whether BGLF4 enhances DNA-repair activity through an XPC-dependent pathway, the XP4PA-SV cell line, which was derived from a XPC patient, was used in an HCR assay. Compared with the vector control, no stimulating effect on DNA repair was observed in XP4PA-SV cells in the presence of wild-type or 'kinase-dead' BGLF4 (Fig. 5c). As expected, ectopic expression of XPC in XP4PA-SV cells enhanced DNA repair in a dose-dependent manner (Fig. 5d; data not shown). In the presence of 0.015 μg XPC plasmid, the XPC-mediated DNA-repair activity was enhanced further by BGLF4 in a dose-dependent manner (Fig. 5d). Interestingly, expression of BGLF4 (K102I) also enhances DNA repair in an XPC-dependent manner in XP4PA-SV cells (Fig. 5c, d).

**DISCUSSION**

Several lines of evidence from this study indicate that the critical DNA damage-recognition factor of NER, XPC, contributes to EBV lytic replication. Firstly, XPC associates with viral lytic kinase BGLF4 in vitro and in vivo (Fig. 1). Although the interaction is not very strong, data presented here demonstrated repeatedly that BGLF4 could interact with XPC in vitro and in vivo. Because BGLF4 has also recently been demonstrated to interact with many viral and cellular factors in a yeast two-hybrid system (Calderwood et al., 2007), we speculate that BGLF4 may interact with different cellular and viral factors in cells and that this is why only a small portion of BGLF4 or protein was immunoprecipitated reciprocally. Secondly, XPC colocalizes and interacts with BGLF4 in virus-replicating NA cells (Figs 2 and 3). Thirdly, when siXPC1 and siXPC3 were used to knock down XPC, EBV lytic DNA replication was suppressed to 60–70% of the level observed for the vector.
control (Fig. 4). To our knowledge, this is the first study to demonstrate that NER contributes to EBV lytic DNA replication.

Complex interactions among viral and cellular DNA-replication and -repair systems, contributing to efficient virus replication, have been reported in several studies of herpesviruses. By using immunoprecipitation and mass spectrometry, the single-stranded DNA-binding protein ICP8 of herpes simplex virus 1 (HSV-1) has previously been shown to be associated with over 50 cellular and viral proteins. Many of the co-immunoprecipitated cellular proteins are involved in DNA replication, recombination and repair, suggesting that HSV-1 recruits them to aid its replication or that they target abnormal viral DNA structures generated during replication (Taylor & Knipe, 2004). For example, the ATM signal-transduction pathway is activated in HSV-1-infected and EBV-reactivated cells (Kudoh et al., 2005; Shirata et al., 2005). In EBV-replicating cells, Mre11-Rad50-NBS1 (MRN) complexes recognize newly synthesized viral DNA and are recruited to sites of viral replication. Phosphorylated p53 is also recruited to the replication compartment and interacts with Zta. However, p53 downstream signalling is blocked; therefore, the virus can actively promote an S-phase-like environment to facilitate its replication (Kudoh et al., 2005). Lytic infection of HSV-1 induces NBS-1 phosphorylation and recruits NBS-1 to viral precursors of replication compartments, suggesting that the activation component of ATM signalling is also beneficial for efficient virus infection (Shirata et al., 2005). Overall, various mistakes generated during viral DNA replication may rely on cellular machineries to provide repair activities.

In addition, PCNA and components of mismatch repair (MMR) factors, including MSH-2, MSH-6, MLH-1 and hPSM2, are recruited to the EBV lytic replication compartment. Their recruitment might be required for PCNA to load onto newly synthesized viral DNA (Daikoku et al., 2006). Recently, our study also indicated that cellular UNG2 and EBV BKRF3-encoded viral uracil-DNA glycosylase (UDG) activities play an important role in viral lytic DNA replication (Lu et al., 2007). UDG is the primary DNA repair enzyme of the BER pathway responsible for removal of inappropriate uracil from DNA. Moreover, cellular UDG (UNG2) can interact and colocalize with PCNA and replication protein A (RPA) in cellular replication foci (Otterlei et al., 1999). BKRF3 also is associated with BGLF4 and viral DNA polymerase (BALF5) in the viral replication compartment (Lu et al., 2007). In addition, depletion of UNG2 in Kaposi’s sarcoma-associated herpesvirus-positive cells using short hairpin RNA (shRNA) reduced the number of copies of the viral genome (Verma et al., 2006). Regarding the role of the DNA-repair system in viral DNA replication, NER is shown here for the first time, to our knowledge, to contribute to EBV lytic DNA replication. Notably, RPA associated with XPC to recognize DNA lesions in NER. PCNA has also been shown previously to be required for NER for the DNA-resynthesis step in vitro (Shivji et al., 1992). This study reveals that XPC interacts with and colocalizes with BGLF4 in EBV-positive cells undergoing viral lytic DNA replication (Figs 1, 2 and 3). Considering these observations together, we postulate that the interaction of BGLF4 and XPC can recruit cellular replication and repair proteins such PCNA and RPA to the viral replication compartment to enhance replication efficiency. This suggestion is partly supported by the recent observation that knockdown of BGLF4 using siRNA reduced viral DNA synthesis to about 70% (Gershburg et al., 2007).
Several virally encoded proteins have been shown to regulate NER activity. The hepatitis B virus oncoprotein HBx suppresses both global and transcription-coupled NER (Groisman et al., 1999; Mathonnet et al., 2004). Human T-cell leukemia virus type 1 (HTLV-1) transactivator and transforming protein Tax has also been shown to inhibit BER and NER (Kao & Marriott, 1999; Philpott & Buehring, 1999). In this study, we observed that expression of BGLF4 stimulated the DNA-repair activity of exogenous UV-damaged substrates in an HCR assay (Fig. 5a). The enhancement was not observed in XPC-defective XP4PA-SV cells, indicating that BGLF4 enhances DNA repair through an XPC-dependent pathway in vivo (Fig. 5b, c). Notably, BGLF4 could enhance DNA-repair activity in both a p53-dependent and -independent manner, and kinase activity was not required to stimulate HCR in p53-positive cells. We suspect that BGLF4 may function as a recruitment protein and may also provide some DNA damage signalling simultaneously. In NER, p53 can modulate the helicase activity of XPB and XPD, regulate the expression of XPC and serve as a chromatin-accessibility factor for NER of DNA damage (Adimoolam & Ford, 2002; Rubbi & Milner, 2003; Wang et al., 1995), whereas BGLF4 can interact with XPC and topoisomerase II (Lee et al., 2007). Such interactions might be expected to somehow enhance DNA-repair efficiency through recruitment of proteins for formation of more stable complexes. Within p53-negative cells, because BGLF4 is known to

Fig. 5. BGLF4 stimulates cellular DNA-repair activity. (a) H1299/bcl2 cells were cotransfected with either various amounts of BGLF4 or p53 expression plasmids and UV-damaged (1000 J m$^{-2}$) or undamaged pCMV-Luc reporter plasmid, together with pWP-1, a plasmid expressing Renilla luciferase, as a transfection control. Firefly and Renilla luciferase activities were detected 48 h after transfection. Fold HCR represents DNA-repair activity, calculated as described in Methods. The results were calculated from three independent experiments. (b) Various amounts of ‘kinase-dead’ BGLF4 (K102I) were transfected into H1299/bcl2 cells with the other reporters as described for (a). The results were calculated from three independent experiments. (c) XP4PA-SV cells were cotransfected with either various amounts of BGLF4 or BGLF4 (K102I) expression plasmids and UV-damaged (100 J m$^{-2}$) or undamaged pCMV-Luc reporter plasmid, together with pWP-1 as a transfection control. Firefly and Renilla luciferase activities were detected 24 h after transfection. (d) XP4PA-SV cells were cotransfected with 0.015 μg XPC expression plasmid and various amounts of BGLF4 or BGLF4 (K102I) expression plasmids and the other reporters as described in (c). The results were calculated from three independent experiments. The expression of BGLF4 in HCR assay was detected by using immunoblotting analysis. Molecular mass markers are indicated.

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phosphorylate cdk1 and cdk2 substrates, it is possible that BGLF4 may phosphorylate some unknown cellular factors participating in the DNA-repair signalling pathway, thereby enhancing the DNA-repair activity. This may also explain why, in the presence of p53, the kinase activity of BGLF4 was not required to enhance DNA-repair activity. Overall, we suggest that BGLF4 may recruit XPC to the viral replication compartment, enhance NER activity and thereby promote efficient production of viral DNA.

In summary, we demonstrated that the viral kinase BGLF4 interacts specifically and colocalizes with the critical NER factor XPC in vivo. Furthermore, BGLF4 enhances XPC-mediated DNA-repair activity through p53-dependent and -independent mechanisms. Inhibition of XPC expression results in suppression of EBV lytic DNA replication, indicating that XPC might play an important role in EBV lytic DNA replication. Our results suggest that, in addition to BER, MMR and double-stranded DNA repair, NER also contributes to EBV DNA replication, although the specific mechanisms have yet to be identified. Future insights into the mechanisms of interplay of cellular DNA repair and viral DNA replication should allow a more complete understanding of EBV lytic cycle replication control and the pathogenesis of EBV-associated malignancies.

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