The CR4 region of EBNA2 confers viability of Epstein–Barr virus-transformed B cells by CBF1-independent signalling

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The Epstein–Barr virus (EBV) nuclear antigen 2 (EBNA2) gene product is the key regulator of the latent genes of EBV and essential for EBV-mediated transformation of human primary B cells. Viral mutants were constructed carrying a deletion of the EBNA2 conserved region 4 (CR4). Primary resting B cells infected with the ΔCR4-EBNA2 mutant virus were dramatically impaired for B cell transformation. Lymphoblastoid cell lines (LCLs) established with this mutant EBV revealed a prolonged population doubling time when cells were cultivated at low cell densities, which are not critical for wild-type-infected cells. Low-level spontaneous cell death occurred when the cells were cultivated at suboptimal cell densities. The phenotype of B cells and LCLs infected with the ΔCR4-EBNA2 mutant virus indicated that the CR4 region of EBNA2 specifically contributes to the viability of the cells rather than affecting cell division rates.

INTRODUCTION

Epstein–Barr virus nuclear antigen 2 (EBNA2) is a transactivator of viral and cellular genes, which is recruited to the promoter of target genes by sequence-specific DNA-binding proteins like CBF1 (Henkel et al., 1994; Zimber-Strobl et al., 1994). EBV infects and transforms primary B cells in culture leading to outgrowth of lymphoblastoid cell lines (LCLs). EBNA2 is absolutely required for initiation and maintenance of this process (Cohen et al., 1989; Hammerschmidt & Sugden, 1989) since it induces the G0/G1 transition (Kempkes et al., 1995; Sinclair et al., 1994). Nine conserved regions (CR1–9) within the primary structure of EBNA2 have been defined by homology of different EBV strains as well as of baboon and rhesus lymphocryptoviruses (Peng et al., 2000). The CR6 region of EBNA2 mediates binding to CBF1, which in the absence of EBNA2 functions as a transcriptional repressor by modifying the histone acetylation status of the target chromatin (Hsieh & Hayward, 1995; Hsieh et al., 1999; Kao et al., 1998). EBNA2 binding recruits the basal transcriptional machinery (Tong et al., 1995a, c) and assembles a coactivator complex by association with p300/CREB (Wang et al., 2000), p100 (Tong et al., 1995b) and the SMN protein (Barth et al., 2003), thereby mimicking the role of the activated Notch receptor (Zimber-Strobl & Strobl, 2001). If the interaction between EBNA2 and CBF1 is abolished by mutation, the resulting EBV mutant is transformation-incompetent (Yalamanchili et al., 1994).

Previously, an EBNA2 mutant virus deleted for the CR4 region had been shown to be functionally impaired but transformation-competent when tested in B cell infections (Cohen et al., 1991). LCLs, which had been generated by infection with this virus, expressed the viral latent membrane protein 1 (LMP-1) at levels similar to wild-type-transformed cells, but the growth characteristics of these cells were not described. More recently, the CR4 region of EBNA2 has been shown to bind to the Nur77/NGFI-B/TR3 protein. Nur77 is an orphan member of the steroid receptor superfamily. CR4 blocks apoptosis induced by pro-apoptotic stimuli, which specifically require Nur77 as a signal transducer (Lee et al., 2002, 2004). In summary, several lines of evidence point towards an important contribution of the CR4 region in the context of EBV growth transformation.

In this study we assess the contribution of the EBNA2 CR4 region to EBV-induced transformation of human B cells and describe the cellular phenotype of ΔCR4-EBNA2 EBV-infected and transformed B cells in detail in comparison to cells infected with recombinant EBVs with a deletion of the entire EBNA2 ORF or an EBNA2 mutant, which does not bind CBF1.

METHODS

Plasmids. The CR4 region of EBNA2 was excised by deleting an internal BamHI–BstEI fragment within the ORF of EBNA2. The
EBNA2 WW3255F mutant was kindly provided by W. Schubach. Both EBNA2 mutants were flanked by a kanamycin selection cassette, which was inserted into the Pmee site, 3’ of the EBNA2 ORF and which was derived from Cp15 (Cherepanov & Wackernagel, 1995). To generate linear fragments, which promote homologous recombination, both EBNA2 mutants were flanked with additional EBV-derived sequences. The 5’ flank corresponded to bps 35 239–36 213 and the 3’ flank corresponded to 38 017–38 341 (NCBI accession no. AJ507799). The resulting plasmid constructs were designated pKg435 (WW-EBNA2) and pKg436 (ACR4-EBNA2), and an XhoI–NotI fragment from each of these plasmids was used as a substrate for homologous recombination in pkJ46-pretransformed DH10B cells. The recombinant EBV constructs pKg447 and pKg449 were generated by homologous recombination based on pKg435 and pKg436 using the λRed system (Datsenko & Wanner, 2000). Plasmid maps for all constructs and sequence information are available on request.

**Cell lines.** The EBV-negative DG75 Burkitt’s lymphoma cell line (Ben-Bassat et al., 1977), the EBV-positive Burkitt’s lymphoma cell line Raji (Pulvertaft, 1964), the EBV-positive cell line 721 (Kavathas et al., 1980), WI38 primary human fibroblasts (ATCC) and HEK 293 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U penicillin ml−1, 100 μg streptomycin ml−1 and 4 mM glutamine at 37 °C in a 6% CO2 atmosphere. HEK 293 cells are human embryonic kidney cells transformed by the adenoviral Ela and Eib genes (Graham et al., 1977). 293/2089 and 293/2491 are HEK 293 cells transfected with recombinant EBV (2089) or EBNA2-deleted EBV mutants (2491) and have been described by Delecluse et al. (1998) and Kelly et al. (2005). 293/pKg449 and 293/pKg447 are HEK 293 cells transfected with the recombinant EBV constructs encoding ACR4-EBNA2 or WW-EBNA2 by the lipofectamine method, according to the manufacturer’s instructions. All 293 transfectants were selected for plasmid maintenance by supplementing the cell culture medium with 100 μg hygromycin B ml−1.

**PCR.** PCR amplification of viral fragments corresponding to the EBNA2 ORF was performed using genomic DNA of EBV-transformed B cells by using the forward primer 5’-AGG-GATGCCTGGACACAGGC-3’ and the reverse primer 5’-TGA-CAGAGGGTGCAAAAATGTTGG-3’. PCR conditions were 5 min at 95 °C for denaturation of the template, followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s.

**Production and quantification of viral supernatants.** HEK 293 transfectants carrying the recombinant virus plasmid were induced for virus production by cotransfection of 0.5 μg of plasmids p509, encoding BZLF1, and p2670, encoding Balf4, per one 6-well 3 ml cell culture. The supernatants of the transfectants were stored at 2–8 °C for 3 days after induction and passaged through a 0.8 μm filter. Viral titres were determined by infecting 3 × 107 Raji cells with serial dilutions of viral supernatants and measured by FACS analysis. The concentration of viral stocks was expressed as the number of Green Raji Units (GRU).

**Transformation of primary B cells by EBV.** Human primary B cells were purified from adenoids by T-cell rosetting and seeded at 1 × 10^6 cells per 100 μl culture in 96-well plates on irradiated WI38 fibroblast feeder layers. In all experiments, B cells from individual volunteers were infected in parallel with different viral preparations. For limiting dilution analysis, groups of 48 or 96 cultures were plated and infected with serial dilutions of viral supernatants. After 4–6 weeks, growing cultures were counted and the transformation efficiency was calculated as the number of GRU needed to obtain 63% growing cultures per group.

**DNA isolation and Southern blot analysis.** Genomic DNA was isolated by resuspending 1 × 10^7 cells in 3 ml lysis buffer (10 mM Tris/HCl, pH 8.0, 400 mM NaCl, 10 mM EDTA), adding 100 μl 20% SDS and 0-2 mg proteinase K ml−1 and incubation at 37 °C for >2 h. One millilitre of 5 M NaCl was added and vortexed vigorously. After 30 min incubation on ice and centrifugation at room temperature at 2500 g for 30 min, the supernatant was transferred to a fresh tube and the DNA was precipitated by adding 0-6 vols 2-propanol. The DNA was washed twice with 70% ethanol, air-dried briefly and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0). Ten micrograms of genomic DNA was digested with NcoI, separated on 0-7% agarose gels in 1 ¥ TAE, transferred to Hybond-N+ membrane (Amersham Biosciences) in 20 ¥ SSC overnight and cross-linked by baking at 80 °C for 1 h. The filters were hybridized at 68 °C overnight with 2-5 ng labelled probe ml−1 and detected as described by Engler-Blum et al. (1993).

**Western blot analysis.** For Western blot analysis, total-cell lysates were prepared by sonification in NP40 buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% NP-40, Complete Protease Inhibitor; Roche). The protein concentration was determined and 20 μg protein was separated on Laemmli 10% polyacrylamide-SDS gels. Proteins were transferred onto PVDF membrane (Immobilon P; Millipore) and detected by using the enhanced chemiluminescence system (Amersham Biosciences), according to the manufacturer’s instructions. Expression of EBNA2, LMP1 and LMP2A was detected using the monoclonal antibodies R3, LMP1G6 and TP14B7, respectively (E. Kremmer, GSF, Munich, Germany). The antibody for GAPDH was supplied by Chemicon. PARP cleavage was monitored by the mouse monoclonal antibody C2-10 (BD Pharmingen).

**Electrophoretic mobility shift assay (EMSA).** For preparation of nuclear extracts, 5 × 10^6 cells were washed in ice-cold PBS and centrifuged at 300 g and 4 °C for 10 min. The pellet was resuspended in 300 μl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1-5 mM MgCl2, 5 mM DTT and protease inhibitors) and kept on ice for 60 min. The cell suspension was transferred to a 1 ml dialysis cup, homogenized by douncing 20 times up and down with a tight pestle and transferred to a microcentrifuge tube. After centrifugation at 4 °C at 25 000 g for 10 s, 300 μl buffer A was added to the pellet and, after briefly vortexing, centrifuged again as above. Nuclei were lysed by resuspending the pellet in 300 μl buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1-5 mM MgCl2, 0-2 mM EDTA, 5 mM DTT and protease inhibitors), kept on ice for 30 min, vortexed, and centrifuged at 4 °C at 25 000 g for 20 min. The protein content of the supernatant was determined using Bio-Rad Protein Assay Kit II, according to the manufacturer’s protocol, and aliquots were stored at −80 °C.

The LMP2A oligonucleotide (Zimmer-Strobl et al., 1994) was annealed by mixing equimolar ratios of sense and antisense oligonucleotides in annealing buffer (10 mM Tris/HCl, pH 7.4, 10 mM MgCl2, 50 mM NaCl), followed by incubation at 90 °C for 10 min and cooling down to 37 °C. The annealed oligonucleotides (25 ng ml−1) were filled in with Klenow polymerase in the presence of [32P]dCTP (3000 Ci mmol−1) and unlabelled dATP, dGTP and dTTP at 37 °C for 1 h. The labelled probe was separated from unincorporated nucleotides using Nick Sephadex G50 columns (Amersham Biosciences). Two micrograms of nuclear extracts was incubated at room temperature for 30 min with 0-5–1 ng 32P-labelled oligonucleotide probe in a 20 μl reaction containing 10 mM HEPES, pH 7.9, 1 mM EDTA, 200 mM KCl, 4% Ficoll, 2 μg BSA, 2 μg poly(dI-dC), 4 mM DTT and proteinase inhibitors. Protein–DNA complexes were resolved on 4% polyacrylamide gels in 1 ¥ TBE buffer at room temperature for 3 h at 130 V. Gels were dried and exposed to X-ray film.

**MTT.** Cells were seeded at 4 × 10^5, 2 × 10^5 and 1 × 10^5 cells ml−1 in 100 μl cultures. Triplicate cultures were incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (0-5 mg ml−1) for 5 h. MTT conversion was determined in an ELISA reader at OD550-690 (Mosmann, 1983).
**RESULTS**

**Generation of recombinant EBV mutants**

Three recombinant EBV mutants were generated for this study by homologous recombination in *Escherichia coli* as described by Datsenko & Wanner (2000) and Delecluse et al. (1998) (Fig. 1a). In ΔEBNA2 EBV (p2491) the entire coding sequence of the EBNA2 gene was deleted. ΔCR4-EBNA2 EBV (pkg449) carries an EBNA2 mutant, in which a 117–146 within CR4 were deleted (Fig. 1b). WW-EBNA2 EBV (pkg447) carries two adjacent single amino acid exchanges within the CR6 region of EBNA2, which abrogates its interaction with CBF1. HEK 293 cells were stably transfected with the viral mutants and analysed by Southern blot hybridization to check the integrity of the different viral genomes (Fig. 1c). Virus stocks were generated, the viral supernatants were quantified for infectious virus units and assessed for their efficiency to transform primary human B cells in comparison to wild-type recombinant EBV based on EBV strain B95.8 (p2089) (Delecluse et al., 1998; Janz et al., 2000). In the following we will refer to recombinant B95.8 virus as wild-type EBV (EBVwt). Neither ΔEBNA2 EBV nor WW-EBNA2 EBV was able to transform primary human B cells, even when B cell cultures were infected at high virus doses. These results confirmed that EBNA2 and in particular EBNA2/CBF1 signalling is absolutely essential for B cell growth transformation (Cohen et al., 1991). In contrast, ΔCR4-EBNA2 EBV was able to transform primary B cells although at very low efficiencies (Fig. 2a, b). Whereas less than 1 GRU was sufficient to yield clonal LCLs (Fig. 2a) more than 200 GRU were necessary to establish proliferating B cell clones (Fig. 2b).

Several cultures of EBNA2 and ΔCR4-EBNA2 expressing LCLs were expanded. The presence of the CR4 deletion was confirmed by PCR amplification of the EBNA2 gene fragment from genomic DNA of the respective clones (Fig. 3a). Protein levels of EBNA2wt and ΔCR4-EBNA2 were assessed by Western blot analysis, which showed that both proteins were expressed at similar levels (Fig. 3b). Next, gel shift experiments were performed to visualize the EBNA2/CBF1 interaction of wild-type and mutant proteins. Both, EBNA2 and ΔCR4-EBNA2 bound CBF1 equally well as indicated by a prominent DNA/CBF1/ΔCR4-EBNA2 complex in gel shift experiments (Fig. 3c). These experiments showed that the EBNA2/CBF1 interaction was not affected by the CR4 deletion in our mutants. Thus, neither altered EBNA2 steady-state protein levels nor impaired CBF1 binding can account for the reduced transformation competence of the ΔCR4-EBNA2 EBV.

**Viability and cell division rate of ΔCR4-EBNA2 EBV-transformed B cells**

When ΔCR4-EBNA2 cultures expressing LCLs were expanded under routine cell culture conditions from single wells into 96-well cluster plates, cells frequently ceased proliferation and the cultures often died eventually. To gain insight into the viability of the ΔCR4-EBNA2 LCLs, MTT conversion data of triplicate cultures seeded at different densities were determined during a period of 8 days. The proliferation characteristics of the cells significantly differed in response to the culture conditions. EBVwt-transformed B cell cultures (Fig. 4a) and ΔCR4-EBNA2 EBV-transformed B cells (Fig. 4d) seeded at high density (4 × 10^5 cells ml^-1) proliferated equally well until day 3. Beyond day 3, saturation density of the cell cultures was reached and no further increase in MTT conversion was observed by EBVwt-infected cells. In contrast, ΔCR4-EBNA2 EBV-infected B cell cultures ceased to proliferate and showed a dramatic loss of viability. ΔCR4-EBNA2 EBV-infected B cell cultures plated at reduced cell concentrations (2 × 10^5 cells ml^-1) showed weak growth retardation (Fig. 4b, e). When cultures were initiated at very low cell concentrations (1 × 10^5 cells ml^-1), three out of six individual LCLs showed a very slow increase in MTT levels, whereas the remaining three clones infected with ΔCR4-EBNA2 EBV could not be expanded (Fig. 4c, f).

To characterize the ΔCR4-EBNA2 LCLs further, the proliferation rate of the cell cultures was compared to B cells infected with EBVwt (Fig. 5). Growth curves of pairs of EBNA2 LCLs and ΔCR4-EBNA2 LCLs derived from the same donor were seeded at 2 × 10^5 cells ml^-1 and viable cells, as determined by trypan blue exclusion, were counted daily over a time period of 5 days. While LCL cultures infected with EBVwt showed a population doubling time of 24 h until saturating cell concentrations were reached, mutant LCLs seeded at critical cell densities expanded extremely slowly. To differentiate between cell division rates of single cells and the proliferation rate of the cultures, we performed cell division tracking experiments using the PKH26-GL fluorescent cell linker (Fig. 6). This is a red fluorescent marker dye that stains cell membranes and has been characterized as a useful tool for *in vitro* and *in vivo* cell-tracking applications (Horan et al., 1990). To this end, cells were seeded at different cell densities, and cell division rates of LCLs infected with EBVwt or ΔCR4-EBNA2 EBV were compared. Surprisingly, both mutant and wild-type LCLs divided once per day at both cell concentrations (Fig. 6). Thus, ΔCR4-EBNA2 EBV-infected LCLs do not show altered cell division rates at the single-cell level compared to wild-type cells, and the slow population doubling time has to be due to constant and spontaneous
loss of viable cells from the culture under unfavourable conditions.

Since LMP1 and LMP2A contribute to the viability of EBV-infected B cells, we tested whether protein expression levels were altered in mutant LCLs (Fig. 7a) (Thorley-Lawson, 2001). Cells were grown under suboptimal conditions for 4 days and tested for expression of both LMP proteins. Surprisingly, both LMP expression levels increased over time, but the comparison of mutant and wild-type LCLs did not reveal obvious differences in expression levels of either protein.

The CR4 region has been shown recently to protect against apoptosis induced by stimuli, which involves the activity of Nur77 as an effector molecule. To assess the relative contribution of spontaneous apoptosis within the cell cultures, two different assays were performed. The status of PARP cleavage was determined as a parameter for apoptosis (Fig. 7a). Cells were seeded at cell densities which were critical for the viability of CR4-EBNA2 EBV-infected LCLs. In both cell lines, marginal levels of PARP cleavage could be detected, but obvious differences between wild-type and CR4-EBNA2 expressing LCLs could not be demonstrated. Subsequently, the fraction of cells with subG1 DNA content was determined by flow cytometry. CR4-EBNA2 EBV-infected LCLs showed a subtle enrichment of apoptotic cells compared to EBVwt-infected cells (Fig. 7b). Two independent experiments were performed on six pairs of LCLs derived from the same donor. On average,
the number of apoptotic cells in wild-type LCLs \( (n = 6) \) was \( 23 \pm 5.6\% \) and \( 33 \pm 10.7\% \) for experiments 1 and 2, respectively. The mean level of \( \Delta \text{CR4-EBNA2} \) EBV-infected LCLs was \( 35 \pm 14\% \) and \( 42 \pm 7\% \), respectively. \( P \)-values were 0.046 and 0.012 for experiments 1 and 2, respectively. These results indicate that spontaneous apoptosis and cell death contribute to the phenotype of \( \Delta \text{CR4-EBNA2} \) EBV-infected LCLs. Because these effects are subtle, they become evident only upon prolonged cell culture under suboptimal conditions.

**DISCUSSION**

Previously, the CR4 region has been shown to protect LCLs from specific pro-apoptotic stimuli by binding to Nur77 by using a transcomplementation system based on established conditional LCLs (Lee et al., 2004). Here we show that LCLs which are directly established from primary B cells show a moderate increase in spontaneous cell death in culture. This report characterizes the growth behaviour of \( \Delta \text{CR4-EBNA2} \)-infected LCLs extensively. Compared to EBVwt-infected cells, the mutants divided at similar rates. However, the cultures were extremely unstable and sensitive to dilution in culture to concentrations below \( 4 \times 10^5 \) cells ml\(^{-1}\), whereas
these conditions were not critical for EBVwt-transformed B cell cultures.

Deletion of EBNA2 CR4 not only reduced the growth-transforming activity of the virus 200-fold, but also significantly impaired the viability of the corresponding established LCLs. The transforming capacity of EBV is defined by limiting dilution transformation assays, which assess the frequency of proliferating cultures at 4–6 weeks after infection. The number of transforming events reflected by the number of colonies per culture cannot be directly determined after this prolonged time in suspension culture. Transformation driven by ΔCR4-EBNA2 EBV required high virus titres compared to wild-type. This result could reflect impaired transforming activities during the initial stages of transformation. Alternatively, several transformation events in parallel could be required to initiate a stable cell culture. In the light of the impaired viability of ΔCR4-EBNA2 EBV-infected LCLs upon dilution in culture we suggest that this phenotype largely accounts for the impaired transformation frequency.

The impact of the CR4 region on transformation efficiencies might be indirect via sensitization to suboptimal culture conditions. Nur77 has been shown to convert the anti-apoptotic function of bcl-2 into a pro-apoptotic activity (Lin et al., 2004). Since EBV-transformed B cells coexpress Nur77 and bcl-2, EBNA2 might be necessary to neutralize a potential pro-apoptotic bcl-2 activity in these cells.

Alternatively, Nur77 can also function as a transcriptional activator, when targeted to the nucleus, and promote cell cycle progression of lung cancer cells (Kolluri et al., 2003). However, our results do not substantiate a contribution of

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**Fig. 4.** Viability of ΔCR4-EBNA2 EBV-infected LCLs. When seeded at suboptimal densities, ΔCR4-EBNA2 EBV-infected B cell cultures are impaired in their expansion characteristics. Six individual B cell lines transformed by EBVwt (a, b, c) were compared to six ΔCR4-EBNA2 EBV-infected LCLs (d, e, f) initially seeded at 4 × 10^5 (a), 2 × 10^5 (b) and 1 × 10^5 (c) cells ml⁻¹ in triplicate. MTT assays were performed over 8 days. Results are given as the mean values of triplicates and SD is shown by error bars. Identical symbols in different graphs identify the same cell lines cultivated under different culture conditions.

**Fig. 5.** Proliferation and growth characteristics of ΔCR4-EBNA2 EBV LCLs. Pairs of established LCLs infected with either EBVwt (■, ▲) or ΔCR4-EBNA2 EBV4 (□, △) were seeded at 2 × 10^5 cells ml⁻¹ and cultured for 5 days. Viable cells, as judged by trypan blue exclusion, were counted daily. Results are presented as the mean values of triplicates and the SD is shown by error bars. ■, KG2.1 wt; ▲, KG1.3 wt; □, KG2.2 ΔCR4; △, KG1.5 ΔCR4.
EBNA2 CR4 region confers viability to EBV-infected B cells.

In summary, our results show that the CR4 region is very critical for the survival of EBV-infected B cells in culture, which is in contrast to the absolute requirement for a functional CR6 region under very stringent conditions. Previously, peptides which specifically abolish the CR6 function of EBNA2 have been demonstrated to interfere with LCL proliferation (Farrell et al., 2004). The strong contribution of the CR4 region to cell viability could suggest a potential second molecular target for antiviral therapy within the same molecule.

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Fig. 6. Cell division rate of ΔCR4-EBNA2 EBV LCLs. Established LCLs infected with either EBVwt or ΔCR4-EBNA2 EBV were stained with PKH26-GL, seeded at 1 x 10^5 cells ml⁻¹ and cultured for 5 days. The mean fluorescence intensity of viable cell populations was analysed daily by flow cytometry.

Fig. 7. Spontaneous apoptosis in ΔCR4-EBNA2 EBV LCLs. (a) Protein extracts of EBVwt- or ΔCR4-EBNA2 EBV-infected LCLs seeded at 2 x 10^5 cells ml⁻¹ and cultivated for 4 days were analysed for LMP1 and LMP2A expression and PARP cleavage by Western blot analysis. (b) The DNA content of EBVwt- (black bars) or ΔCR4-EBNA2 EBV- (white bars) infected established LCLs was analysed by flow cytometry after propidium iodide staining. The sub-G1 population was defined by FACS analysis. The results of two independent experiments are shown in the left and right panels.


