Development of stealth transgenes for gene therapy : evaluation of cis-acting inhibitors of antigen presentation

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Chapter 3

The Nested Open Reading Frame in the Epstein Barr Virus Nuclear Antigen 1 mRNA Encodes a Glycine, Glutamic-acid, and Glutamine-Rich Protein, Which Functions As an *In Cis*-Acting Inhibitor of Antigen Processing

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The Nested Open Reading Frame in the Epstein Barr Virus Nuclear Antigen 1 mRNA Encodes a Glycine, Glutamic-acid, and Glutamine-Rich Protein, Which Functions As an *In Cis*-Acting Inhibitor of Antigen Processing

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Abstract

The Epstein Barr Virus Nuclear Antigen-1 (EBNA-1) has a 249 amino-acids long glycine and alanine-rich repeat, which can function as an *in cis*-acting inhibitor of antigen processing. This cannot fully prevent antigenic peptide presentation since such peptides may be derived from defective ribosomal products. These DRiPs are an important source of immunogenic peptides and may be derived from translation of alternative open reading frames of the messenger. The EBNA-1 messenger contains a large nested open reading frame. Translation starting at an alternative initiator AUG downstream of the canonical initiation codon would yield a strongly acidic protein with a calculated molecular weight of 40.7 kDa. This protein would contain a 238 amino-acids long glycine, glutamine, and glutamic acid-rich repeat. We fused the nested ORF with the *Escherichia coli*-derived LacZ gene and showed β-galactosidase activity upon transfer of the fusion gene into cultured cells. Cells producing the unmodified β-galactosidase readily present the H-2L^{d}-restricted CTL epitope TPHPARIGL. In contrast, the same CTL epitope is not presented upon expression of the fusion gene. Deletion of two-thirds of the repeat does not affect its capacity to inhibit antigenic peptide generation. From these data we conclude that the glycine, glutamine, and glutamic acid-rich repeat of the nested ORF protein of EBNA-1 can act as a cis-acting inhibitor of antigen processing.

Introduction

Epstein Barr Virus (EBV) is a gamma herpes virus and infects over 90% of the human adult population. It is considered to be the classic example for immune surveillance of persistent viral infections in humans (Klein, 1994). It has three latency programs, which differ in protein expression profiles. However, the Epstein Barr virus nuclear antigen 1 (EBNA-1) is expressed in all latency programs and is therefore associated with all EBV-related malignancies.

EBNA-1 is essential for the maintenance of the viral genome as stable episomes during latency. It binds as a dimer to the viral origin of replication and ensures B-cell growth (Bochkarev et al., 1996; Shah et al., 1992; Yates et al., 1985; Rowe et al., 1992). Although EBNA-1-specific cytotoxic-T lymphocytes (CTLs) circulate in patients (Blake et al., 1997), the EBNA-1-positive cells are not recognized and killed. This has been attributed to the presence of the large glycine and alanine-rich repeat (GAr), which is not required for genome maintenance or cellular transformation (Lee et al., 1999), but has an immune evasion function. The GAr domain of EBNA-1 prevents cytotoxic T-lymphocyte (CTL)- epitope generation (Ossevoort et al., 2003) by inhibiting the proteasomal degradation (Levitskaya, et al., 1997; Levitskaya et al., 1995). The inhibition requires the interaction of at least three alanine residues of the GAr with adjacent hydrophobic binding pockets of a putative receptor

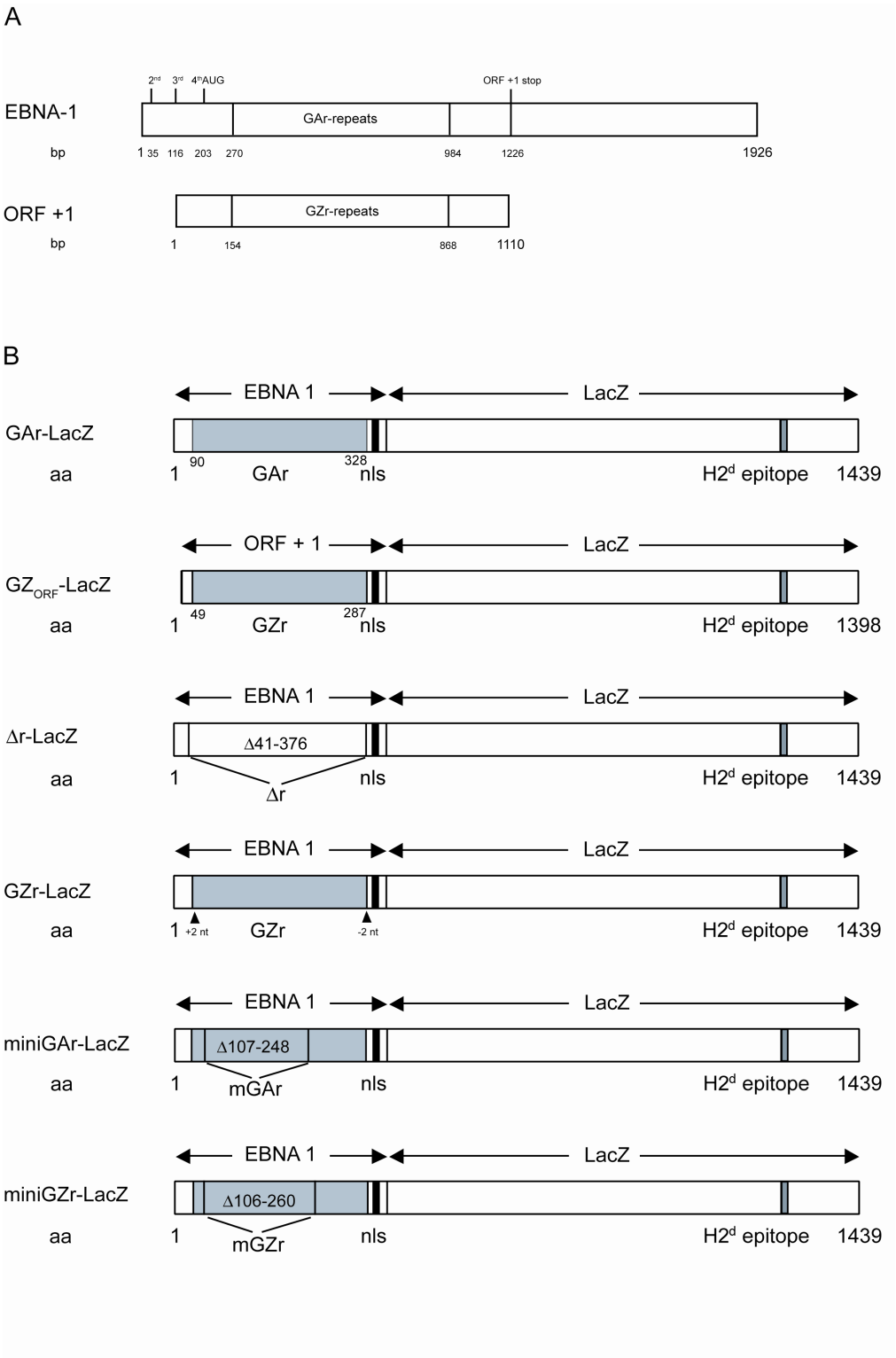
at the proteasome (Sharipo et al., 2001). Although efficient, the GAr may not be sufficient to prevent the generation and presentation of antigenic peptides altogether (Voo et al., 2004; Tellam et al., 2004; Lee et al., 2004). Especially, the GAr may not inhibit the synthesis of defective ribosomal products (DRiPs) that may generate antigen-specific antigenic peptides. These DRiPs are shown to play an important role in peptide generation (Yewdell et al., 1996; Schubert et al., 2000; Reits et al., 2000) and are actually the main source of antigenic peptides for long-lived proteins (Khan et al., 2001). Here we show that the EBNA-1 messenger RNA contains a nested open reading frame, preceded by a translation-initiation codon. The putative initiation codon adheres to the Kozak consensus sequence, in that it has an A at position -3 and a G in position +4 (Kozak, 1986; Kozak, 1997). Use of this ORF would yield a strongly acidic 40.7 kDa protein. Here we demonstrate that this protein can function as a cis-acting inhibitor of antigen presentation. These data indicate that also translation of the alternative ORF of the EBNA-1 mRNA will not result in abundant presentation of antigenic peptides and also this ORF can function as a mechanism to prevent the presentation of antigenic peptides.

Results

'In silico' analysis of the EBNA-1 mRNA sequences revealed a long open-reading frame that initiates at the third AUG codon. This start site is in open reading frame (ORF) +1 relative to the EBNA-1 ORF. Translation of the +1 ORF would yield a long and acidic protein of 370 amino acids and an estimated molecular weight of 40.7 kDa. The protein is strongly acidic and would contain 127 Glutamic-acid residues, 90 Glutamine residues and 69 Glycine residues. The polypeptide tract rich in Gly and Glx residues was named GZ- repeat (GZr) region (fig. 1). The AUG that could serve as translation initiation codon is part of the sequence 5'-ACCAUGG and is identical to the optimal Kozak consensus sequence.

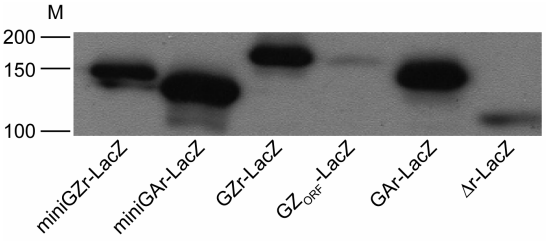
To study if the alternative open reading frame could be used, we generated a fusion of the GZr protein with the *E.coli*-derived LacZ gene. From the plasmid pGAr-LacZ, we deleted two nucleotides distal of the GAr region, merging the GZr protein ORF with the LacZ ORF. Transfection of the resulting plasmid pGZ_{ORF} into 293T cells resulted in readily detectable β-galactosidase activity. This demonstrates that under the transfection conditions translation

Figure 1 (page 95)
Schematic outline of EBNA-1 and the alternative ORF+1 proteins (A) and the fusion proteins with β-galactosidase (B). (A) The EBNA-1 coding region is depicted. The putative start codons of the alternative ORF (ORF +1) are indicated above the EBNA-1 sequence (bp = base pairs). Also indicated are the codons for the GAr in EBNA-1, and GZr in the ORF +1 protein, as well as the stop for the ORF +1 polypeptides. (B) Indicated are the Gly-Ala repeat region GAr, amino acids 90-328, the nuclear localization signal nls, aa 378-386, the entire repeat deletion aa 41-376, the deletions creating the miniGAr aa 107-248 and the miniGZr aa 106-260. The LacZ gene starts after aa 420 of the EBNA-1 ORF. In the LacZ gene the $H2^d$ epitope aa 1303-1311 is indicated. In GZ_{ORF}-LacZ the third AUG is depicted as start of the alternative ORF +1. The arrows flanking the GZr in GZr-LacZ indicate the site of the 2 bp the insertion and deletion, respectively.


A
EBNA-1
2nd
3rd
4thAUG
ORF +1 stop
GAr-repeats
bp
1 35 116 203 270 984 1226 1926
ORF +1
GZr-repeats
bp
1 154 868 1110
B
EBNA 1
LacZ
GAr-LacZ
aa
1 90 GAr 328 nls H2d epitope 1439
ORF + 1
LacZ
GZORF-LacZ
aa
1 49 GZr 287 nls H2d epitope 1398
EBNA 1
LacZ
Δr-LacZ
Δ41-376
aa
1 nls H2d epitope 1439
Δr
EBNA 1
LacZ
GZr-LacZ
aa
1 +2 nt GZr -2 nt H2d epitope 1439
EBNA 1
LacZ
miniGAr-LacZ
Δ107-248
aa
1 mGAr nls H2d epitope 1439
EBNA 1
LacZ
miniGZr-LacZ
Δ106-260
aa
1 mGZr nls H2d epitope 1439

initiation can initiate at the 3rd AUG of the EBNA1-containing mRNA's (fig. 2 and 3). This indicates that, under normal conditions, not only the GAr-containing EBNA-1 could be synthesized, but also GZr-containing polypeptides from the nested ORF. Although the GZ_{ORF}-LacZ protein is present in the transfected cells in relatively low amounts, the polypeptide may be degraded by proteasomes and yield antigenic peptides.

To facilitate studies on this protein, we enhanced its synthesis by constructing a plasmid in which the GZr region was linked to the *bona fide* EBNA-1 N terminus, allowing translation initiation to initiate at the 1st AUG. This construct allows a side-by-side comparison of GAr-LacZ and GZr-LacZ. In addition, we generated plasmids from which we deleted the GAr or GZr repeats from the LacZ fusion genes. The resulting plasmid encodes a LacZ with a small N-terminal extension consisting of 85 amino acids of the EBNA-1 ORF. In addition, we isolated some spontaneous deletion plasmids with repeat lengths considerably smaller than the 239 amino acids of the GAr and GZr. Two of these, with repeat lengths coding for 97 (in pMiniGAr-LacZ) and 82 amino acids (in pMiniGZr-LacZ), were used for further studies. All constructs are depicted in figure 1.



Protein	repeat size (aa)	Molecular weight (kD)
GAr-LacZ	239	149
miniGAr-LacZ	97	140
Δr-LacZ	none	124
GZr-LacZ	239	161
miniGZr-LacZ	82	144
GZ_{ORF}-LacZ	239	158

Figure 2

Western-blot analysis of the different LacZ fusion proteins. 293T cells and 293T cells expressing miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ genes were lysed and protein extracts were size fractionated by SDS-PAGE. The western blot was analyzed with a monoclonal mouse anti-LacZ antibody. All proteins migrate according to their expected size (indicated in the table underneath) and no smaller products are visible.

The plasmids were transferred into 293T cells and the translated proteins were analyzed by Western-blot analysis. All LacZ fusion genes yielded equivalent amount of β-galactosidase protein (fig. 2), except the GZ_{ORF}-LacZ fusion. The apparent molecular weight, as deducted from the mobility of the proteins on SDS PAGE gels, was in good agreement with the calculated molecular weight. *In-situ* staining of the transfected 293T cells for β-galactosidase activity demonstrated that the fusion proteins were biologically active (fig. 3A). Furthermore, analysis of β-galactosidase activity in protein lysates by galactolight assay further confirmed the activity of the fusion proteins. When the ratio of the activity and protein contents is plotted, it shows a clear difference between the constructs starting at the original EBNA-1 ATG and the GZ_{ORF}-LacZ. All repeat- and mini repeat containing LacZ as well as the LacZ deleted for the repeats show equivalent levels of activity (fig. 3B). Only the GZ_{ORF}-LacZ plasmid shows a significantly reduced activity level. In contrast, the GZr-LacZ, in which the GZr-LacZ fusion protein is translated from the AUG normally used by EBNA-1, has a lacZ level similar to the GAr-LacZ fusions. Since both proteins contain a GZr region of the same length, we decided to use GZr-LacZ for all further research.

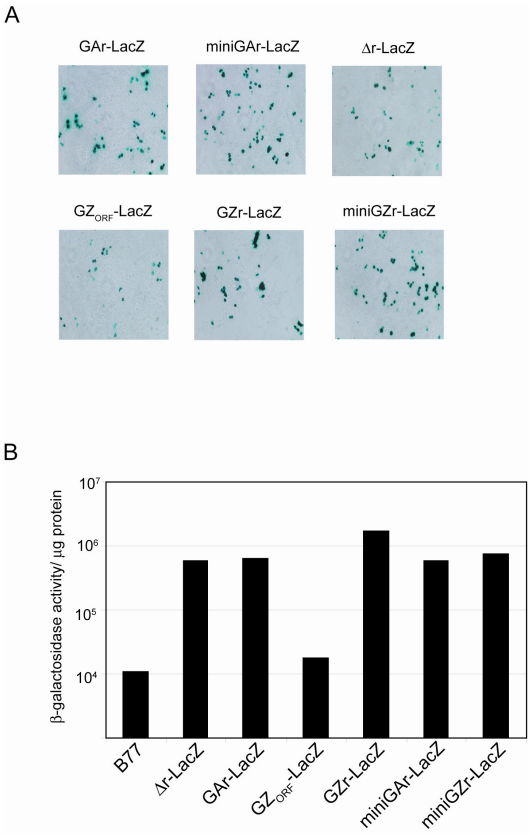


Figure 3

In-situ β-galactosidase activity assay (A) and galactolight assay (B) of the transfected 293T cells. A 293T cells were transfected with miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ-encoding plasmids. After 48 hours, the cells were fixed, and stained overnight. (B) 293T cells were transfected with miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GZ_{ORF}-LacZ, GAr-LacZ and Δr-LacZ encoding plasmids. After 48 hours, the cells were lysed and galacton was added. The β-galactosidase activity is normalized for the protein concentration in the lysate.

For further studies we generated cell lines stably expressing the fusion genes. We tested the stable cell lines by a western-blot analysis and found the proteins being present in similar amounts (fig. 4A). All constructs yielded protein of the expected size and no degradation products were apparent. These cell lines were tested for β-galactosidase activity by *in-situ* staining (fig. 4B). As a positive control served the cell line BB16, a stable B77-derived cell line expressing an unmodified LacZ gene (Hoeben et al., 1991). In contrast to the fusion constructs, which contain the EBNA-1-derived nuclear localization signal, β-galactosidase is distributed in the cytoplasm of the BB16 cells. The cell lines expressing the fusion gene and the BB16 cells all had similar levels of activity, as is evident by the *in-situ* staining. The expression levels were further confirmed by galactolight assays (fig. 4C). Taken together, our data show that all cell lines have similar amounts of β-galactosidase antigen and activity.

To measure antigenic peptide presentation, these cell lines expressing the fusion genes were tested for the presentation of LacZ-derived peptides (H_2L^d, TPHPARIGL, indicated in fig. 1) with an interferon-gamma (IFNγ) production assay using the cells expressing the fusion genes as targets. The generation of LacZ-specific CTLs was effective and the CTL recognized the positive control BB16 cells, but not the parental LacZ-negative B77 cells (fig 5A). Fusion of the full-length GAr reduced the amount of IFNγ produced clearly. Also fusion of the GZr repeat reduced INFγ production. Remarkably, the MiniGAr and MiniGZr mini repeats are more efficiently inhibiting INFγ production. When we loaded the cell lines with β-galactosidase peptide, all cell lines stimulated the antigen-specific CTLs (data not shown), demonstrating that the presence of the repeats does not frustrate the cells' capacity to present the β-galactosidase peptides. Naïve CTLs did not induce an IFNγ response.

From these data we conclude that GZ repeat that is derived from nested open reading frame in the EBNA-1 mRNA is able to inhibit presentation of linked antigens. These findings demonstrate that not only the GAr-derived sequences, but also other simple repeat sequences can inhibit antigen presentation. Further research should reveal which step in the chain of processes that leads to the presentation of antigenic peptides is inhibited.

Discussion

The Gly-Ala repeat (GAr) domain of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) prevents cytotoxic T-lymphocyte (CTL)- epitope generation (Ossevoort et al., 2003) by inhibiting the proteasomal degradation (Levitskaya et al., 1997; Levitskaya et al., 1995). However, this may not be sufficient to prevent the generation and presentation of antigenic peptides, since the GAr might not prevent formation of defective ribosomal products (DRiPs) (Voo et al., 2004; Tellam et al., 2004; Lee et al., 2004). There is a wide variety of DRiPs identified nowadays. Not only truncated or misfolded proteins, but also the products that are

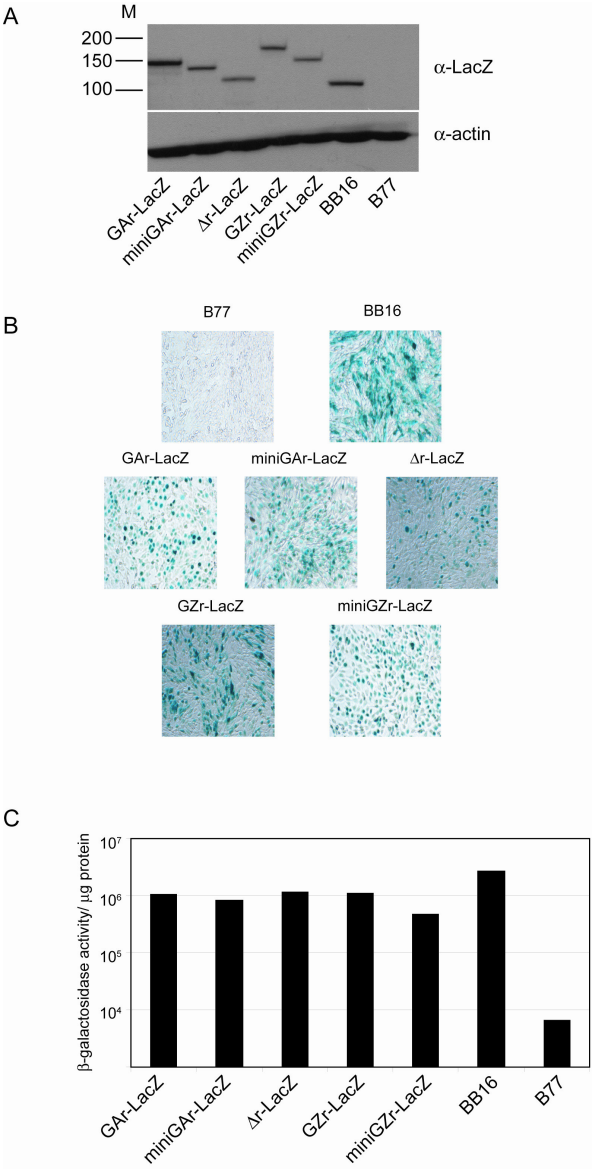


Figure 4

Characterization of the stable B77 cell lines. B77 cells were transfected with plasmids encoding miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ proteins respectively. After establishing stable cell lines, the cells expressing miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ, as well as B77 and BB16 cells (B77 cells expressing LacZ) were lysed and protein extracts were size fractionated by SDS-PAGE (A). The western blot was analyzed with a monoclonal mouse anti-LacZ antibody. All proteins migrate at the expected size, and no smaller products are visible. (B) The same cell lines, as well as B77 and BB16 were fixed stained *in-situ*. (C) Stable B77 cell lines expressing the miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ constructs, and B77 and BB16 cells were lysed and galacton was added. Plotted is the normalized β-galactosidase activity.

translated from alternative open reading frames are considered as DRiPs (Yewdell et al., 2003; Yewdell et al., 1996). Indeed several proteins are produced by translation of alternative ORFs (Fetten et al., 1991; Malarkannan et al., 1995; Shastri et al., 1995; Wang et al., 1996; Mayrand & Green, 1998; Mayrand et al., 1998; Schwab et al., 2003) and from non AUG start codons (Malarkannan et al., 1995; Shastri et al., 1995; Schwab et al., 2003). These DRiPs are shown to play an important role in peptide generation (Yewdell et al., 1996; Schubert et al., 2000; Reits et al., 2000) and are actually the main source of antigenic peptides for long-lived proteins (Khan et al., 2001).

Here we describe that polypeptides derived after translation of an alternative open reading frame of the EBNA-1 mRNA can inhibit presentation of linked antigens. This alternative ORF was observed after *in-silico* analysis of the EBNA-1 mRNA sequences and revealed a long open reading frame that initiates at ORF +1 from the original translation initiation site. It contains a repeat unit of the same length as the GAr and is rich in Gly (G) and Glx (Z) residues. Therefore, it was named GZ-rich repeat (GZr) region (fig. 1). We show that the alternative ORF is translated upon transfection of an EBNA-1 expression vector, although the steady state level of the GZr-fusion protein is lower than of the *bona fide* GAr fusion protein.

There are two possible alternative translation-initiation sites. The second AUG is followed by a single sense codon before a nonsense codon is encountered. The third and the fourth AUG codons in EBNA-1 are both in the +1 frame and would result in translation of the GZr containing polypeptide. The third AUG is has an optimal Kozak consensus sequence. It contains the A at -3 and a G at +4 and it has two Cs at positions -1 and -2. The fourth AUG contains a G residue at +4 and -3 (Kozak, 1986; Kozak, 1997). As can be seen in figures 2 and 3, the GZ_{ORF} fusion protein is present in low levels. This is not unusual for alternative ORFs (Fetten et al., 1991; Malarkannan et al., 1995; Shastri et al., 1995) and does not seem to interfere with the capacity to generate peptides and elicit a CTL response (Wang et al., 1996; Mayrand & Green, 1998; Mayrand et al., 1998; Schwab et al., 2003).

To enhance fusion protein synthesis and to make a more fair comparison between the GAr and the GZr fusion proteins, we made a GZr-LacZ fusion starting at the original AUG of EBNA-1 translation initiation. We also constructed a deleted version lacking the repeat regions and isolated comparable mini-repeats for both GAr and GZr. We could map which parts were deleted, and this mini-repeats were included in this study since it is known that short EBNA-1 derived glycine and alanine-rich sequence of only 8 amino acids are able to significantly inhibit proteasomal degradation of instable reporter protein (Sharipo, et al., 2001; Sharipo, et al., 1998; Dantuma, et al., 2000).

All these constructs were well expressed and retained their β-galactosidase activity. The stable cell lines all exhibited similar amounts of the fusion proteins and the biological activities of the proteins were equivalent (fig. 4). The cell lines were tested for peptide presentation (fig. 5).

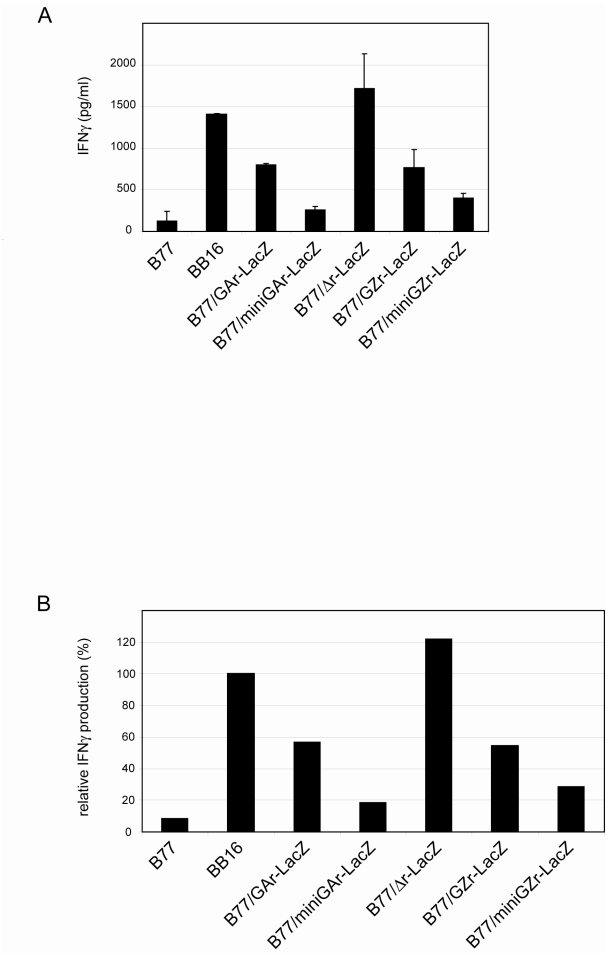


Figure 5
LacZ-specific CTLs were generated and co-cultured for 48 hours together with B77, B77 expressing miniGZr-LacZ, miniGAr-LacZ, GZr-LacZ, GAr-LacZ, and Δr-LacZ genes. Thereafter, the supernatant was collected and the amount of IFNγ was determined. (A) Absolute IFNγ production of the different cell lines. (B) Relative IFNγ production. BB16, the B77-derived cell line expressing unmodified β-galactosidase, is set to 100 percent.

Introduction of a full length repeat, both GAr and GZr, significantly decreased the IFNγ production. However, the mini-repeats blocked presentation more efficiently, as is evident by reduced IFNγ production. The mechanism for the more potent inhibition is unclear. Maybe these shorter proteins form less DRiPs then longer proteins, on the basis of size and inherent difficulties in folding or assembly (Schubert et al., 2000). Furthermore, we know that over-expression of either GAr- or GZr-containing polypeptides in B77 and MEC cells does not decrease the amount of MHC I on the cell surface (data not shown). These findings demonstrate that not only the GAr-derived sequences, but also other simple repeat sequences can inhibit antigen presentation.

The observation that also the GZr repeat blocks presentation of linked antigens is intriguing. Sharipo et al. (2001) suggested that the inhibitory effect of the GAr requires at least three alanine residues in a β-strand conformation with adjacent hydrophobic binding pockets of a putative receptor (Sharipo, et al., 2001). In the case of the GZr there are no alanine residues present and since the whole repeat is strongly acidic, it is highly unlikely that there will be hydrophobic pockets involved in the receptor part. This suggests that the GZr repeat functions in a different way. It remains to be established whether the GZr repeat protein is produced EBV infected cells and whether this protein has any physiological function.

In conclusion, we demonstrate that the GZr protein that can be translated from the nested ORF of the EBNA-1 mRNA can function as an *in cis*-acting inhibitor of antigen presentation. This ORF may function as a mechanism to prevent the presentation of antigenic peptides that are generated by translation of alternative open reading frames of the EBNA-1 messenger RNA. Further research should reveal which step in the chain of processes that leads to the presentation of antigenic peptides is inhibited. Nonetheless, the new GZ repeat may be used to stealth antigenic proteins in gene-therapy applications in which eradication of the transduced cells would frustrate the therapy.

Materials and Methods

Construction of the GAr-fusion plasmids

To obtain a fusion gene of LacZ and the EBNA-1 Gly-Ala repeats, the LacZ gene was inserted into plasmid pCMV-EBNA (Invitrogen, Groningen, The Netherlands). Firstly, plasmid pCBeb was created from plasmid pCMV-EBNA by converting the *Bst*XI site to a *Bgl*II site by insertion of a *Bgl*II adapter that was created by annealing the synthetic oligonucleotides 5'-TACGAGATCTGAAG-3' and 5'-AGATCTCGTACTTC-3' (the *Bgl*II site is underlined). Secondly, a 3072 bp *Bam*HI fragment of the retroviral vector pBag (Price et al., 1987), which carries the LacZ-coding region except the first two codons, was inserted into the *Bgl*II site of pCBeb to generate pGAr-LacZ. The presence of the intact GAr was confirmed by restriction analysis and the integrity of the complete fusion protein was confirmed by sequencing.

To derive plasmids pGZ_{ORF}-LacZ, pGZr-LacZ and pΔr-LacZ from plasmid pGAr-LacZ, the QuickChange Site-directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands) was used according to the manufacturer's protocols.

To create plasmid pGZ_{ORF}-LacZ and therefore detect translation starting at the 3rd AUG of EBNA-1, primers (sense) 5'-CAGGAGGTGGAG//CGGGGTCGAGGAGGC-3' and (antisense) 5'-GCCTCCTCGACCCCG//CTCCACCTCCTG-3' were used to delete two nucleotides (indicated with'//') at the end of the GAr-coding region of plasmid pGAr-LacZ.

This resulted in a plasmid in which the LacZ open reading frame is in frame with the GZ open reading frame. To boost the translation of the GZr containing LacZ, plasmid pGZr-LacZ was created. It was obtained by insertion of two nucleotides (insertions are underlined) near the start of the GAr coding region in plasmid pGZ_{ORF}-LacZ with the primers (sense) 5'-CACGGTGGAACA**GA**GGAGCAGGAGCAG-3' and (antisense) 5'-CTGCTCCTGCTCC**TC**TGTTCCACCGTG-3'. In this plasmid translation of the GZ open reading frame is initiated at the AUG normally used for EBNA-1.

A repeatless control was created to establish the effect of both the GAr and the GZr repeats. The codons for the repeats were deleted from plasmid pGAr-LacZ with the primers (sense) 5'-GGGGGTGATAACCATGGA|GGAGAAAAGAGGCCCAGG-3' and (antisense) 5'-CCTGGGCCTCTTTTCTCC|TCCATGGTTATCACCCCC-3', (the | mark indicates the location of the junction), yielding plasmid pΔr-LacZ. All resulting PCR products were verified by restriction analyses and complete sequence analyses for the presence of the mutations and the repeat sequences and integrity of the fusion proteins. The repeat sequences were relatively unstable in the plasmids (Ossevoort et al., 2003). We isolated spontaneously deletion plasmids with smaller repeats of both the GAr and GZr. By sequencing, the deletion was mapped and the resulting plasmids were called pMiniGAr-LacZ and pMiniGZr-LacZ.

Cell lines

The 293T and B77 (a hypoxanthine phosphoribyltransferase-negative (HPRT-) BALB/c 3T3 cell line) (Varmus et al., 1973) cell lines were cultured in DMEM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml and 0.2% glucose. P13.1 (Lammert et al., 1996) cells were cultured in IMDM (Gibco) containing 8% (vol/vol) fetal bovine serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 28 μM β-mercaptoethanol and 500 μg hygromycin B per ml.

Stable B77-derived cell lines expressing the different LacZ variants were made by plasmid transfection of cell cultures in 6-well plates with 2.7 μg LacZ construct and 0.3 μg pRSV-neo per well using the Calcium Phosphate co-precipitation technique (Graham & van der Eb, 1973). After 48 h, medium was replaced with medium containing 500 μg G-418 sulfate (Geneticin, Gibco) per ml. After elimination of the G418-sensitive cells, the cultures were maintained on medium with 200 μg G418 per ml. For isolation of clonal cell lines, highly diluted single-cell suspensions were seeded and monoclonal cell populations were isolated and expanded. The cells were evaluated for the presence of *E. coli* β-galactosidase by in-situ staining and galactolight activity assay.

β-galactosidase assays

Cell cultures were assayed for β-galactosidase activity by X-gal (5-bromo-4-chloro-3-inodyl-β-D-galactopyranoside) staining as described (Hoeben et al., 1991). Briefly, cells were

washed with ice-cold Phosphate-buffered saline containing 0.5 mM $MgCl_2$, and fixed with 5.4% formaldehyde, 0.8% glutaraldehyde in PBS at 4˚C. After washing, the cells were incubated in 5mM Potassium Ferricyanide, 5mM Potassium Ferrocyanide, 2mM $MgCl_2$ and 1 mg X-gal per ml PBS and incubated at 37˚C for 4-16 h. The β-galactosidase activity was also assayed by Galactolight assay (Applied Biosystems) according to the manufacturers instructions. In brief, cultured cells were washed twice in ice-cold PBS and lysed in lysis buffer (25mM Tris-phosphate pH 7.8, 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). Ten µl lysate was added to 100 µl of reaction buffer containing galacton and incubated at room temperature for 30 min. Hereafter, 100 µl of accelerator was added and the β-galactosidase activity was measured with an illuminometer (Lumat LB, EG&G Bertholt).

Western analysis

Cells were lysed in RIPA buffer (50 mM Tris.Cl pH=7.5, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40) and protein extracts were size-fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes and probed with mouse anti-LacZ antibodies (1/500 diluted, Roche) or mouse anti-actin antibodies (1/2000, ICN Biomedicals Inc., Aurora, OH, USA). After incubation with a peroxidase-conjugated goat-anti-mouse secondary antibody, the proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands).

Generation of b-galactosidase specific CTLs

β-Galactosidase-specific CTL clones were generated by injecting BALB/c mice intra-muscularly with $1*10^8$ PFU rAd5-nls-LacZ. Two weeks post-injection, the mice were sacrificed and splenocytes were harvested. As a control, splenocytes were harvested from naïve mice. The splenocytes were stimulated with 5000-rad irradiated The LacZ-expressing P13.1 cells for 1 week. Hereafter the effector cells were ready for use.

IFNγ ELISA

In 96-wells plates, 5000 or 2500 effectors were co-cultured with 20,000 targets/well in triplicate in the presence of 10U IL-2 per ml for 2 days at 37 ºC, at 5% CO_2. One day prior to harvesting of the supernatants, maxisorb plates (Nunc) were coated with 1 µg/ml allophycocyanin-conjugated Rat αM-IFNγ antibody (BD Biosciences) in sodium-carbonate buffer (pH=9.6) overnight at 4ºC. The coated plates were washed 4 times with PBS containing 0.05% Tween-20 and subsequently blocked with PBS containing 1% BSA and 0.05% Tween-20, for 1 hour at 37ºC. After 4 washes the plates were incubated with the supernatants for 2 h at 37ºC. A standard series starting with 10 ng IFNγ (recombinant murine IFNγ, Tebu-bio) per ml medium was diluted 1 in 2 till a final concentration of 9.7 pg per ml.

This standard series and a blank were added to the plates in duplicate. After 4 washes, 0.5 μg per ml biotinylated RatαM-IFNγ monoclonal antibody (BD Biosciences) in block solution was added as conjugate and incubated for 1 h at room temperature. Thereafter the plates were washed 4 times and subsequently streptavidin conjugated poly-Horseradish Peroxidase (Sanquin reagents, Amsterdam, The Netherlands) was added at a concentration of 133 ng per ml in block solution and incubated for 1 h at room temperature. The plates were washed again 4 times, and 4.5mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS, Sigma-Aldrich) with H_2O_2 was added to the plates, and OD was measured at 415 nm.

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