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Identifying gp85-regions involved in Epstein–Barr virus binding to B-lymphocytes

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Abstract

Epstein–Barr virus lacking glycoprotein gp85 cannot infect B-cells and epithelial cells. The gp85 belongs to the molecular complex required for virus invasion of B-lymphocyte or epithelial cells. Moreover, there is evidence that gp85 is necessary for virus attachment to epithelial cells. Thirty-six peptides from the entire gp85-sequence were tested in epithelial and lymphoblastoid cell line binding assays to identify gp85-regions involved in virus–cell interaction. Five of these peptides presented high binding activity to Raji, Ramos, P3HR-1, and HeLa cells, but not to erythrocytes; Raji-cell affinity constants were between 80 and 140 nM. Of these five peptides, 11435 (^{181}TYKRVTEKGDEHVLSLVFGK^{200}), 11436 (^{201}TKDLPDLRGPFSYPSLTSAQ^{220}), and 11438 (^{241}YFVPNLKDMFSRAVT MTAAS^{260}) bound to a 65 kDa protein on Raji-cell surface. These peptides and antibodies induced by them (recognising live EBV-infected cells) inhibited Epstein–Barr virus interaction with cord blood lymphocytes. It is thus probable that gp85-regions defined by peptides 11435, 11436, and 11438 are involved in EBV invasion of B-lymphocytes.

Keywords: EBV; gp85; Raji-cell binding peptides; HABPs

Epstein–Barr virus (EBV) is one of the most common worldwide human viruses. Most people become infected with EBV during their lives [1]; primary EBV-infection occurs via the oral route during infancy, but if primary infection is delayed until adolescence it causes infectious mononucleosis in around 50% cases [2]. EBV has also been linked to developing several malignant tumours, including nasopharyngeal carcinoma [3,4], Burkitt's lymphoma [5], and the X-associated lymphoproliferative disease, known as Duncan's syndrome [6]. EBV is also an opportunistic agent, often associated with immune compromised patients receiving allografts or with Acquired Immune Deficiency Syndrome [7–9].

EBV interaction with B-cells is a complex process involving major viral envelope glycoprotein (gp350) binding to the complement receptor type 2 molecule (CD21) [10,11] and EBV glycoprotein gp85–gp25–gp42 complex interacting with B-cell receptors [12,13]. gp42 protein binds to the HLA-DR Class-II protein β_1 domain in this interaction [14]. Both gp85 and gp25 EBV-proteins represent respective herpes simplex virus gH and gL homologues.

The gp85 is implicated in the membrane fusion process between the virus and the human lymphocytes during EBV-infection. Removing the complex containing gp85 or the anti-gp42 monoclonal antibody F-2-1 (interacting with this complex) inhibits virus ability to fuse to the cell membrane but has no effect on virus binding to B-lymphocytes [15,16]. Furthermore, EBV lacking the gp85-containing complex cannot fuse with B-cells or bind to epithelial cells, indicating that gp85 is necessary for the virus to bind itself to epithelial cells [17,18]. It has also been reported that there are two different types of gp85-containing complexes; one consisting of gp85 and gp25 and another of gp85, gp25, and gp42; these complexes are differentially used in EBV-invasion of B-lymphocytes or epithelial cells [19]. HLA Class-II in EBV-producing cells alters the trimer:dimer

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ratio; EBV from epithelial cells therefore efficiently infects B-lymphocytes whereas B-cell-derived virus tends to infect epithelial cells [20].

The purpose of this work was to identify specific EBV gp85 B-cell and epithelial cell binding sequences, which could be involved in the virus-interaction with the host cell by using peptides covering the previously reported entire gp85 protein sequence [21].

Materials and methods

Peptide synthesis. Thirty-six, 20-mer long, non-overlapping peptides, covering the total length of gp85, were produced by solid phase peptide synthesis [22]. Standard N t-Boc protected amino acids were employed (Bachem). Peptides were cleaved by the low–high HF technique [23], purified by RP-HPLC, and freeze-dried. Purity was assessed by HPLC and molecular mass was determined by MALDI-TOF mass spectroscopy. The peptide sequences are shown in Fig. 1 in one-letter code. Tyr was added to the carboxyl-terminal end of those peptides which did not contain such amino acid to enable radio-labelling.

Lymphoblastoid cell lines. The cloned Raji [24], Ramos [25], and P3HR-1 [26] lymphoblastoid cell lines, which are able to specifically bind to EBV [27–30], were cultured in RPMI 1640 (Gibco-BRL) supplemented with 10% foetal bovine serum (FBS, Hyclone), 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM Hepes (Gibco-BRL), and 1 mM sodium pyruvate. HeLa cells [31] were cultured in monolayers with RPMI 1640 medium, 10% FBS, and non-essential amino acids (Gibco-BRL) and harvested by adding PBS–EDTA, followed by spinning. All the cells were grown at 37 °C, in a 5% humidified CO_2 atmosphere. Before being used in binding assays, the cells were washed with PBS and counted in a Newbauer chamber with trypan blue and their viability was assessed.

Peptide radio-labelling. Peptides were labelled with 3.2 μl Na ^{125}I (17.2 mCi/μg) oxidised with 12.5 μl chloramine T (2.25 μg/μl), and added to 5 μl peptide (1 μg/μl) for 1 min at room temperature [32]. The reaction was stopped by adding 50 μl sodium bisulphate (2.75 μg/μl) and 50 μl isotonic PBS (pH 7.4). The radio-labelled peptide was separated from reaction products in a Sephadex G-10 column at 300 μl/min flow rate.

Cell binding assay. Increasing concentrations of radio-labelled peptide (2, 4, 8, 12 nM) were incubated with lymphoblastoid cell suspensions (10^4 cells/μl), in the absence (total binding) or presence (non-specific binding) of unlabelled peptide (1.25 μM) and incubated for 1 h at room temperature, according to previously described methodology for red blood cells [33]. After one hour, the non-bound peptide was separated from cells by spinning through dibutylphthalate–dioctylphthalate mixture ($d = 1.015$ g/ml) [34] at 5000*g* for 3 min. Cell-bound ^{125}I-labelled peptide was measured in an automatic gamma counter. The assay was carried out in triplicate under identical conditions for each radio-labelled peptide concentration and the standard deviations were always found to be below 5%.

Specific binding was calculated as being the difference between total and non-specific binding. The specific binding curve (specific bound peptide vs total added peptide) was determined for each peptide; the slope of this curve was defined as binding activity. The 15-mer gp-350 Epstein–Barr virus peptide (IHLTGEDPGFFNVEY) reported by Nemerow, which specifically binds to CR2(+) B lymphocytes and is also able to inhibit EBV-infection of peripheral blood mononuclear cells [10] was used as binding peptide control. In our assay this peptide presented a Raji-cell binding activity of 0.034; any peptide presenting Raji-cell binding activity higher than or equal to Nemerow's peptide was considered to be a high activity binding peptide (HABP). Binding assays of HABPs were performed again after Raji-cell HABP identification in a broad range of peptide concentrations (^{125}I-labelled peptide at 5–600 nM concentrations) to determine affinity constants.

HABP cell surface cross-linking and SDS–PAGE. Washed Raji or HeLa cells (10^4 cells/μl) were incubated with 100 nM ^{125}I-labelled peptide in the absence or presence of unlabelled peptide (1 μM) for 2 h at 4°C. The cells were spun at 10,000*g*, for 5 min at 4 °C. Supernatant was discarded and the pellet was washed with PBS. The pellet was incubated with 20 μM Bis (sulphosuccinimidyl suberate) (BS^3, Pierce) for 20 min at 4 °C and the reaction was stopped with 0.1 M Tris–HCl, pH 6.8. The cells were washed with PBS; cell-membranes were then

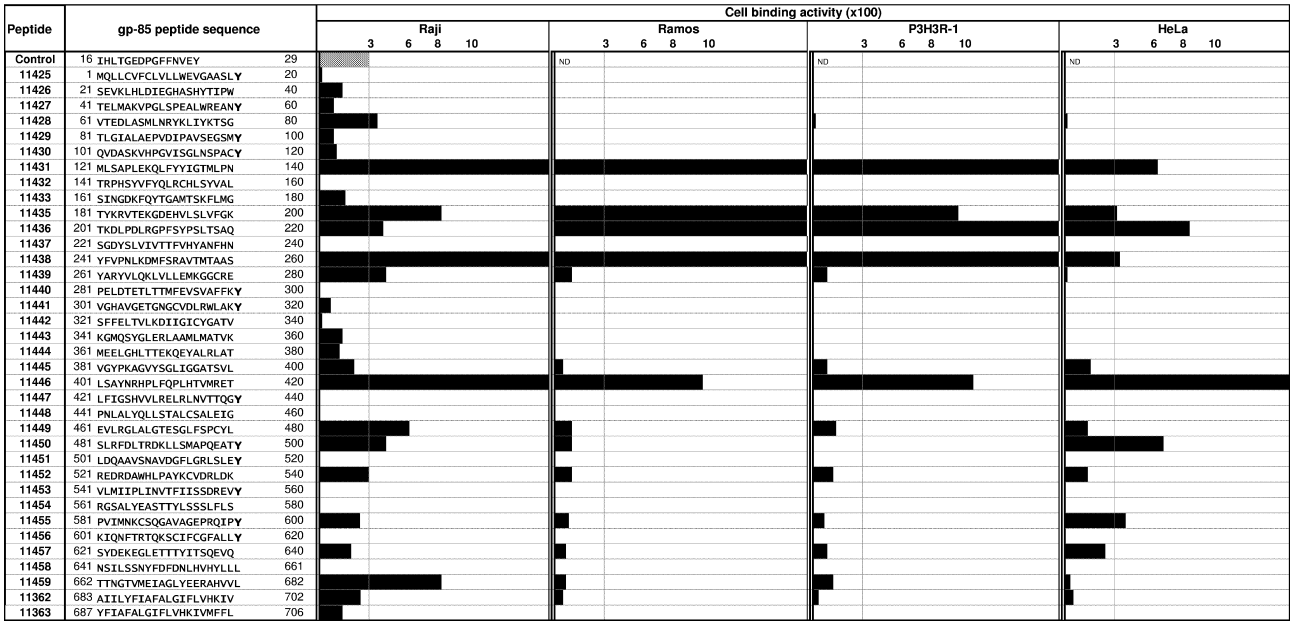

Fig. 1. gp85-peptide binding activity to Raji, Ramos, P3HR-1, and HeLa cells. The names, amino acid sequences, and position in the protein for each peptide are shown on the left. A tyrosine (shown in bold) was added in some peptides at the C-terminal to allow radio-labelling. The binding activity for each peptide to these cells is represented by the black bars. The binding activity of Nemerow's peptide is represented by the grey bar. HABPs were also tested in erythrocyte binding assays; binding activity was lower than 0.008 (data not shown).

obtained and purified as described below. These membranes were treated with Laemmli buffer, heated for 2 min at 90 °C. Solubilised proteins were separated by 12% SDS–PAGE [35], exposed for 24 h at −70 °C on Kodak film (X_OMAT). The labelled bands' molecular weight was determined with the low range molecular weight markers.

Raji-cell membrane preparation and purification. Cell membrane-containing fractions were obtained by previously reported methodology [36]; 2.5 million cells were suspended in 6 ml buffer A (0.25 M sucrose, 5 mM Tris–HCl, pH 8.0, and 1 mM $MgCl_2$) and passed through a 27 gauge syringe ten times. The mixture was centrifuged at 250*g* for 5 min at 4 °C; the supernatant was skimmed off and stored at 4 °C. The pellet was suspended in buffer A and this step was repeated twice. Supernatants were spun at 1500*g* for 10 min at 4 °C and the pellets were suspended in buffer A, pooled, and centrifuged at 16,000*g* for 30 min at 4 °C. The supernatant was discarded and buffer B (1.42 M sucrose, 5 mM Tris–HCl, pH 8.0, and 1 mM $MgCl_2$) was added to the pellet. This solution was poured into the bottom of a Falcon tube and, afterwards, 2 ml buffer B and 1 ml buffer A were added on top. The sucrose gradient was spun at 80,000*g* for 60 min at 4 °C. The ring observed in the interface was suspended in PBS and centrifuged for 60 min at 16,000*g*. The pellet was suspended in 1 ml PBS and stored at −20 °C.

Human leukocyte isolation. Cord blood was collected in EDTA-treated sterile tubes. Cord blood lymphocytes (CBLs) were separated by sedimentation on Ficoll–Hypaque gradients. The obtained lymphocytes were washed 5 times with RPMI-1640, spinning at 300*g*, for 7 min at room temperature [37]. The CBLs were counted in a Newbauer chamber and stained with trypan blue and their viability was assessed.

Epstein–Barr virus. EBV-containing supernatant used for studies of EBV and B-lymphocyte interaction was obtained from American Type Culture Collection (ATCC Catalogue No. VR-1492).

Immunisation protocol. New Zealand rabbits were subcutaneously immunised with 250 μl of each HABP solution (500 μg peptide) plus 150 μg of T-helper epitope FISEAIIHVLHSR (FIS) [38], emulsified with 250 μl of Freund's complete adjuvant, and boosted on days 20, 40, 60, and 80 with the same antigen dose in Freund's incomplete adjuvant. Blood was drawn 20 days after the second, third, and fourth doses.

ELISA and FACS analysis. Rabbit anti-peptide antibodies were tested by ELISA. In brief, micro-titre wells were coated by incubation at 37 °C with 100 μl of 10 μg/ml peptide in PBS for 1 h. Wells were then washed three times with PBS containing 0.2% Tween 20 (PBST). The wells were incubated with this buffer containing 2% powdered skimmed milk (PBSMT) for 2 h at 37 °C for blocking non-specific antibody binding. The PBSMT was then poured out and the wells were washed three times with PBST. Later on, wells were incubated with serial rabbit sera dilutions for one hour at 37 °C, washed three times with PBST, and incubated with a 1/5000 dilution of anti-rabbit peroxidase (VECTOR) at 37 °C for 1 h. They were then colour-developed after washing three times in PBST with a solution prepared by mixing equal volumes of TMB peroxidase substrate and peroxidase solution B (KPL). After incubation for 30 min, absorbancy was read at 620 nm.

FACS analysis used 500,000 cells (CBLs or B95-8) which were washed twice with PBS–BSA 0.5%, spinning at 2500 rpm for 5 min. The cells were then treated with a fixation and permeabilisation kit for flow cytometry (DAKO) according to the manufacturer's instructions. The cells were suspended in 100 μl of 0.5% BSA–PBS containing a 1/640 serum dilution of each rabbit used in immunisation experiments and incubated for 50 min at room temperature. The cells were then washed three times with PBS–BSA, spinning at 2500 rpm, for 5 min; incubated with 100 μl of 1/200 dilution of FITC-labelled goat anti-rabbit IgG F(ab)2. CBLs were additionally incubated with phycoerythrine-labelled rat anti-human-TCR for 30 min at 4 °C and finally washed twice with PBS. FACScan Becton–Dickinson equipment was used for reading cell fluorescence.

Rabbit immunoglobulin fraction isolation. Rabbit sera were four times diluted with 60 mM acetate buffer, pH 4.0; the pH was then raised to 4.5 by adding 0.1 N NaOH. Twenty-five microlitres of caprylic acid was added per 1 ml sera and stirred for 30 min. After this procedure, the samples were spun at 10,000*g* for 30 min and the supernatant was separated by filter-paper filtration. 1/10 volume of PBS 10× was added to the obtained supernatant and the pH was adjusted to 7.4 with NaOH 0.1 N. The immunoglobulin fraction was precipitated with 0.35 g/ml ammonium sulphate at 4 °C overnight. The pellet was separated by spinning at 5000*g* for 15 min at 4 °C and then suspended in PBS. The immunoglobulin solution was extensively dialysed with PBS. The protein concentration was determined by the Bradford assay and antibody activity by ELISA and immunofluorescence [39].

Epstein–Barr virus interaction with CBLs. CBLs (2×10^5) in 100 μl RPMI 1640, supplemented with 10% heat-inactivated serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.4 μg/ml cyclosporine A, and 5 mM $CaCl_2$, were incubated with 30 μl EBV-supernatant for 30 min at 37 °C in 5% CO_2 atmosphere. Then 70 μl RPMI-1640 medium was added and the samples were incubated for 16 h at 37 °C in 5% CO_2 atmosphere. After incubation, cells were washed three times with RPMI-1640 medium (FBS free). The HABP-effect on EBV-interaction with CBLs was determined by pre-incubating these cells with 8 μM of each HABP for 15 min at 37 °C immediately preceding the EBV-interaction with CBL assays. The effect of anti-peptide antibodies on EBV-interaction with CBLs was determined by incubating 30 μl isolated rabbit immunoglobulin fractions (obtained before immunisation and after four immunisations with HABPs) with 30 μl EBV-containing supernatant for 1 h at 37 °C before EBV-interaction with CBLs was performed. EBV-interaction with CBLs without HABPs and/or immunoglobulin fractions was used as positive control. CBLs, treated under the same conditions as the positive controls but without EBV-supernatant, were used as negative controls. These primers specifically amplified a 300 bp DNA fragment from EBV+ cells (Raji or B95-8), but not EBV-cells (CBLs, erythrocyte fraction or HeLa cells).

EBV-DNA identification by PCR-amplification. DNA from EBV-treated or non-treated CBLs was obtained by proteinase K digestion, followed by phenol–chloroform extraction and ethanol precipitation. This DNA was dissolved in 20μl TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA) in a final 0.5 mg/ml concentration [40]. PCR was performed in 20 μl reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM of each one of the following: deoxyribonucleotide triphosphate, 0.1–0.5 μg template DNA, 0.5 μM each primer, and 1.0 U *Taq* polymerase. Previously reported primers, that specifically amplified a 298-bp DNA-fragment, which is present 11 times EBV-genome from base 14,523 to 45,540 [41] were used to specifically amplify EBV-DNA: 5′-TTCATCACCGTCGCTGACT-3′ upstream sequence and 5′-ACCGCTTACCACCTCCTCT-3′ downstream sequence. PCR conditions consisted of 35 cycles at 95 °C (30 s), 55 °C (30 s), and 72 °C (30 s) in a Perkin–Elmer 9600 thermal cycler [41]. The PCR product was separated on 2.5% agarose gels, stained with ethidium bromide, and visualised on Molecular Imager FX (Bio-Rad).

Results

Identifying gp85 peptides that specifically bind to Raji, Ramos, P3HR-1, and HeLa cells

Initial peptide binding screening was performed with Raji cells because they bind to EBV and support virus cell invasion [42]. Peptides 11428, 11431, 11435, 11436, 11438, 11439, 11446, 11449, 11450, 11452, and 11459 (from the 36 peptides covering the entire protein) presented binding activity equal to or higher than Nemerow's peptide; they were named HABPs (Fig. 1).

The eleven HABPs mentioned above plus four Raji cell low binding activity peptides were tested in binding assays

to Ramos, P3HR-1, and HeLa cells since EBV is able to bind specifically to all these three cell lines [27–30] and also to human erythrocyte (EBV does not bind to these cells). Only HABPs 11431 (MLSAPLEKQLFYYIGTMLPN), 11435 (TYKRVTEKGDEHVLSLVFGK), 11436 (TKD LPDLRGPFSYPSLTSAQ), 11438 (YFVPNLKDMFS RAVTMTAAS), and 11446 (LSAYNRHPLFQPLHT VMRET) showed high Ramos, P3HR-1, and HeLa cell binding activity, but not human erythrocyte binding (Fig. 1). Raji HABPs 11428, 11439, 11449, 11452, and 11459 presented high binding activity only to Raji cells. Raji HABP-11450 presented high binding activity to Raji and HeLa cells. None of the low binding peptides showed high Ramos or P3HR-1 binding activity and only HABP-11455 showed high HeLa cell binding activity (Fig. 1).

Determining HABP–Raji-cell binding affinity constants

Binding affinity constant is a parameter giving information about interaction strength. Affinity constants were determined by saturation Raji-cell binding assays for the five HABPs that specifically bound to Raji, Ramos, P3HR-1, and HeLa cells. All these five HABPs (11431, 11435, 11436, 11438, and 11446) and Nemerow's peptide showed saturable Raji-cell binding (Fig. 2), allowing affinity constants and the Hill coefficients to be

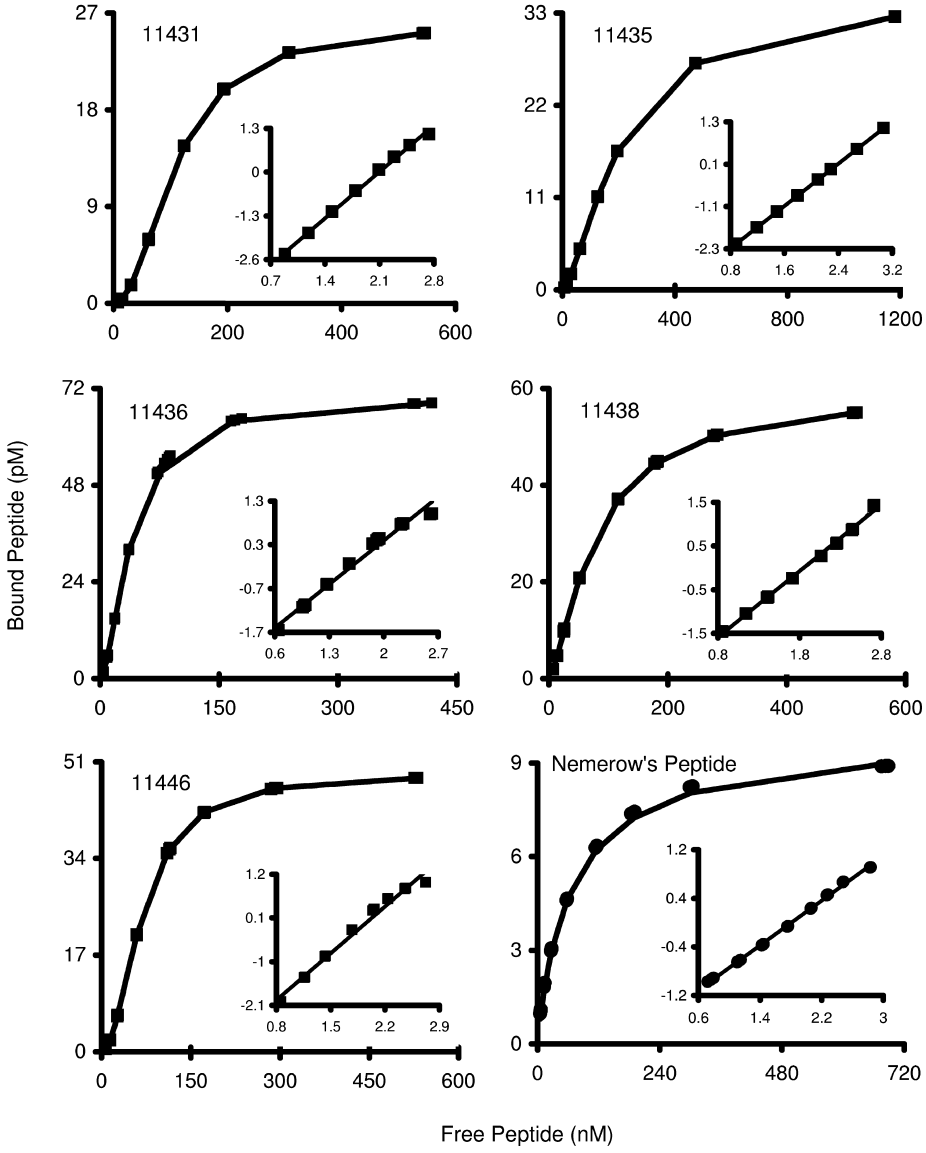

Fig. 2. Raji-cell saturation binding curves and Hill plots for HABPs 11431, 11435, 11436, 11438, 11446, and Nemerow's peptide. The *X*-axis shows non-bound peptide concentration and *Y*-axis bound peptide concentration. Inset shows the Hill plot analysis; the *X*-axis shows $\log(F)$ and in *Y*-axis $\log(B/B_{\max} - B)$; F is the non-bound peptide concentration (nM). B is the specifically bound peptide concentration and $B_{\max}$ is the maximum specifically bound peptide concentration (nM).

calculated (Fig. 2). The affinity constants (K_d) were 110, 180, 40, 80, and 70 nM for gp85 peptides 11431, 11435, 11436, 11438, and 11446 (respectively) and 68 nM for Nemerow's peptide. The number of binding sites per cell for each HABP, calculated from these data, was around 100,000; the Hill coefficients were close to 1 for all of them.

Determining the apparent molecular weight of HABP-receptors on Raji cells

Each one of these five ^{125}I-labelled HABPs was bound to intact Raji cells, in the presence or absence of the same non-labelled HABP. The surface-bound peptide was then cross-linked to Raji-cell membranes. Membrane proteins were separated by SDS–PAGE and analysed by autoradiography. All these HABPs bound specifically to a 65 kDa Raji-cell membrane surface molecule. HABPs 11431 and 11446 also bound to another two bands having lower molecular weight than 65 kDa. These HABP-bindings were specifically and completely inhibited by the same non-labelled HABP (Fig. 3).

HABPs were able to elicit antibodies recognising EBV-infected cells

HABPs 11435, 11436, and 11438 that specifically bound to one band on Raji cells were used to immunise New Zealand rabbits. Specific anti-HABP antibodies were detected by ELISA after four immunisations with these peptides, showing antibody titres between 3200 and 51,200 (Table 1). These antibodies also recognised EBV-containing supernatant by ELISA, presenting antibody titres between 3200 and 25,600. On the other hand, the EBV-containing supernatant induced antibodies recognising these HABPs with 6400 antibody titres (Table 1). No antibodies recognising EBV-containing supernatant or these HABPs were observed in the sera obtained before immunisation (pre-immune sera).

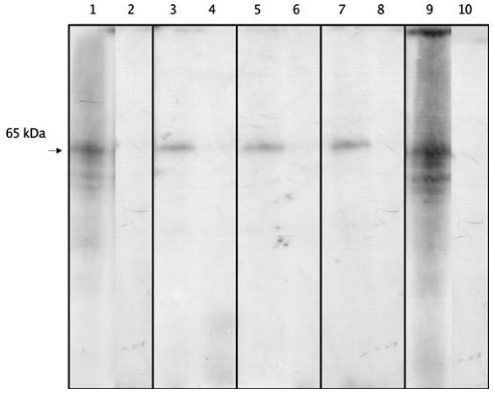

Fig. 3. Analysis of HABPs 11431, 11435, 11436, 11438, and 11446 binding to Raji-cell surface proteins by SDS–PAGE and autoradiography. Cells were incubated with ^{125}I radio-labelled peptide (100 nM) in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of unlabelled peptide (1 μM). Lanes 1 and 2 show HABP-11431; lanes 3 and 4 HABP-11435; lanes 5 and 6 HABP-11436; lanes 7 and 8 HABP-11438; and lanes 9 and 10 HABP-11446. The arrow indicates apparent molecular weight of the HABP-receptor.

Table 1
Antigen titre of HABP- or EBV-immunised rabbit sera

Immunogen	Rabbit	Antigen			
		11435	11436	11438	EBV
11435	426	>51,200			6400
	427	51,200			3200
11436	430		3200		6400
11438	432			51,200	25,600
	433			51,200	25,600
EBV	493	6400	6400	6400	51,200

All pre-immune sera shown mean O.D. of 0.050 ± 0.010. The antibody titres were calculated using the highest dilution showing O.D. higher than or equal to 0.070.

Table 2
FACS analysis of B95-8 or CBLs cells using HABP- or EBV-immunised rabbit sera[a]

Immunogen	Rabbit	B 95-8 cell		CBLs	
		Pi	PIV	Pi	PIV
11435	426	2.6	7.84	0.4	0.42
	427	1.97	8.9	0.58	0.64
11436	430	1.62	9.94	1.53	1.02
11438	432	6.27	4.42	0.2	0.4
	433	5.56	10.77	0.83	1.28
25684	129	1.56	4.26	0.53	1.61
EBV	493	4.04	31.64	1.21	3.86

[a] %Fluorescence cells treated with sera obtained before (Pi) or after four immunisations (PIV) controls: %fluorescence B95-8 or CBLs without sera and without FITC-labelled antibody 0.30 and 0.15, respectively; cells plus FITC-labelled antibody 0.40; cells plus serum 0.42.

Around 10% of B95-8 cells enter the viral lytic cycle when they are treated with TPA [43], permitting the use of these cells in identifying EBV-antigens and/or EBV-antigen-induced antibodies. Live TPA-induced B95-8 cells or CBLs were thus used for identifying antibodies induced by these HABPS that specifically recognised EBV-antigens. Flow cytometry analysis was used for determining that between 2% and 6% B95-8 cells were fluorescent when using the rabbit sera obtained before HABP-immunisation; these percentages were increased between 9% and 13% after four immunisations with these HABPs and 27% after four immunisations with EBV-containing supernatant (Table 2). B98-5 cells treated with sera obtained after four immunisations with non-relevant peptide 25,684 showed that only 4% were fluorescent cells. On the contrary, non EBV-infected CBLs presented lower than 1.7% fluorescent cells, except for those cells treated with the serum of the rabbit

immunised with EBV-containing supernatant, which presented an increase of 2% in the number of fluorescent cells (Table 2). The results obtained with HABP-11435 are shown in Fig. 4 as an example of cytometry analysis.

HABPs and the antibodies induced by HABPs specifically inhibit EBV-interaction with CBLs

The role of HABP-sequences in the interaction between EBV and CBLs was studied by incubating these cells with EBV-containing supernatant in the presence of HABPs or the antibodies induced by these HABPs. It had previously been determined that EBV-containing supernatant was able to infect CBLs not only because virus presence in these cells could be determined by PCR-amplification after 16 h but also because these cells were specifically recognised by mAb-8174 (Chemicon) which is able to specifically recognise the EBV gp350 protein. Neither HABP-11436, non-relevant peptide 25,684 nor the antibodies induced by them inhibited EBV-interaction with CBLs since EBV-DNA presence was detected in these cells after 16 h incubation (Fig. 5). On the contrary, HABPs 11435, 11438, or the antibodies induced by them, inhibited EBV interaction with CBLs since it was not possible to detect EBV-DNA in these cells after 16 h incubation with EBV-containing supernatant (Fig. 5).

Discussion

EBV binds to the target cell via high affinity interactions between virus- and cell-proteins [44]; this initiates a chain of dynamic events enabling virus invasion of the host cell. There are several EBV-proteins necessary for efficient EBV-invasion of host cells; the gp85–gp25–gp42 complex is one of them [11,17,45]. gp85 interacts with host cells mediating the fusion process and some results support the hypothesis that gp85 is necessary for virus attachment to epithelial cells. The present work has focused on the search for specific gp85-regions that could be involved in the EBV-interaction with B-cell and epithelial cells. This approach has been used for defining protein regions involved in virus and host cell interaction [36,46–50].

The target cells used for binding assays were Raji cells because they bind EBV and support virus cell invasion [42]. Eleven gp85-peptides presented Raji-cell binding activity higher than or equal to Nemerow's peptide (11428, 11431, 11435, 11436, 11438, 11439, 11446, 11449, 11450, 11452, and 11459); these were denominated Raji cell HABPs. Some of these HABPs could be binding to MHC class II molecules since it has been reported that Raji cells present different MHC class II alleles [51]. These HABPs were thus also tested in binding assays to Ramos, P3HR-1, and HeLa cells since EBV is able to bind specifically to all these three cell lines [27–30]. HABPs 11431,

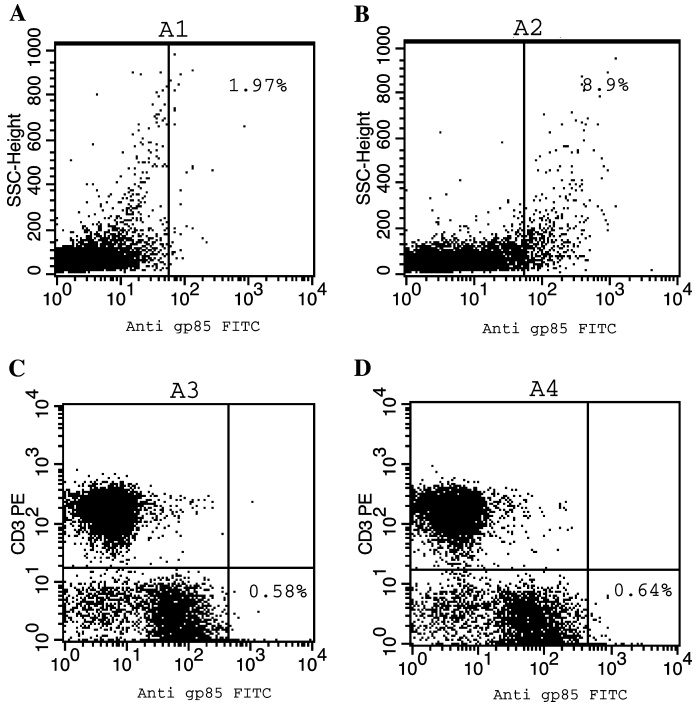

Fig. 4. Flow cytometry analysis of TPA-induced B95-8 cells or CBLs. B95-8 cells (A,B) or CBLs (C,D) were incubated with rabbit sera obtained before immunisation (A,C) or after four immunisations with HABP 11435 (B,D). The percentages of fluorescent cells are shown inside the point diagrams. (C,D) Upper cell population are CD3(+) T-cells and lower cell population are B-cells. The results obtained with sera against HABPs 11436, 11438, non-relevant peptide 25684 or EBV-supernatant are shown in Table 2.

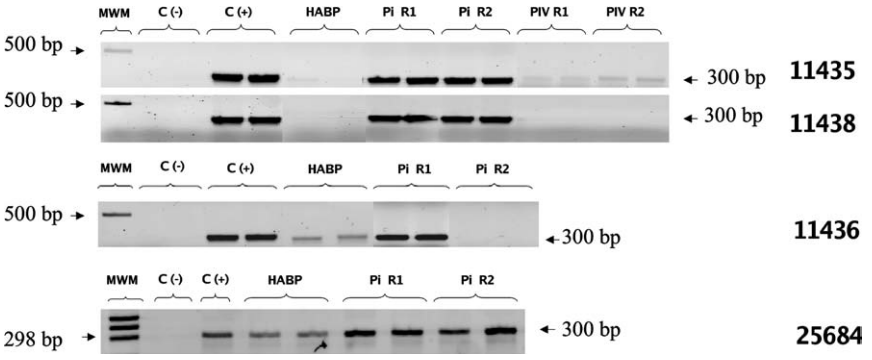

Fig. 5. PCR-amplification of EBV-DNA fragment using DNA obtained from CBLs treated with EBV-containing supernatant. CBLs treated with EBV-containing supernatant in presence of HABPs or anti-HABP antibodies were used to obtain DNA for amplifying a specific fragment, using the previously reported EBV-primers. This fragment was separated in agarose gels. In MWM: molecular weight markers; in C (+) and C (–) EBV-PCR amplification using DNA obtained from CBLs treated with or without EBV-containing supernatant, respectively (see Materials and methods). In peptide, Pi-R1, Pi-R2, PIV-R1, and PIV-R2: EBV-PCR amplification using DNA obtained from CBLs treated with EBV-containing supernatant plus HABPs, pre-immune immunoglobulin fraction or post-fourth immunoglobulin fractions, respectively; R1 and R2 represent the number of the immunised rabbit. In right appears the number of the peptide used in the rabbit immunisation.

11435, 11436, 11438, and 11446 presented high binding activity to Ramos, P3HR-1, and HeLa cells (Fig. 1), but not to erythrocytes showing cell-binding behaviour similar to EBV. It is probable that a common HABP-receptor is displayed by these cells. This receptor seems to be a 65 kDa surface protein on Raji cells and is similar for all these five HABPs (Fig. 3). This receptor is present in around 100,000 molecules per Raji cell, enough to be considered important in EBV-invasion of B-lymphocytes. The binding of these HABPs to Raji cells is strong, since the affinity constants were between 80 and 140 nM, in the same order of magnitude as Nemerow's peptide. In fact, HABP-11436 presented an affinity constant higher than Nemerow's peptide ($K_d = 68$ nM). The Hill coefficients of around one suggest that these are single peptide–Raji cell interactions.

It is probable that gp85 interacts with the specific receptors on these cells and, taking into account that HABPs 11435, 11436, and 11438 interacted with only one band on Raji surface having a similar molecular weight, they were chosen for the following experiments. HABPs 11435, 11436, and 11438 were tested in EBV-interaction with B-lymphocytes. HABPs 11435 and 11438, which specifically bound to EBV-susceptible cells, inhibited EBV-interaction with CBLs, suggesting that it is probable that these peptides are able to inhibit EBV-binding to CBLs by blocking virus binding sites. However, another effect on EBV-invasion cannot be excluded.

HABPs 11435, 11436 or 11438 elicited specific antibodies not only against themselves but also against EBV-containing supernatant, though with a lower titre (except for serum against HABP-11438) (Table 1). These results showed that these HABPs are not only immunogenic but also induced antibodies able to recognise antigens present in EBV-containing supernatant. On the other hand, EBV-containing supernatant was able to induce antibodies recognising these HABPs, suggesting that this supernatant contains an antigen able to induce anti-HABP antibodies. It is probable that this antigen could be gp85.

It is clear from the live cell immunofluorescence studies that HABPs 11435, 11436 or 11438 elicited antibodies that specifically recognised EBV-infected cells but not non-infected cells. The percentage of B95-8 cells recognised by anti HABP antibodies was similar to the percentage of TPA-treated B95-8 cells entering in the viral lytic cycle (Table 2) [43]. These results suggest that these HABPs were able to induce antibodies recognising some EBV-native antigen which is probably gp85. On the other hand, the percentage of B95-8 cells recognised by those antibodies induced by EBV-containing supernatant was higher than the percentage of TPA-treated B95-8 cells entering in the viral lytic cycle; this suggests that some EBV-antigen inducing these antibodies is expressed in the majority of B95-8 cells, not just in those cells in the viral lytic cycle.

Antibodies induced by HABPs 11435, 11436 or 11438 were tested in EBV-interaction with B-lymphocytes. The EBV-DNA fragment was found in CBLs incubated with EBV-containing supernatant plus antibodies specifically induced by non-relevant peptide 25684 (Fig. 5). This suggests that these antibodies did not have any effect on EBV-interaction with B-lymphocytes. On the contrary, no EBV-DNA was detected when antibodies induced by HABPs 11435, 11436 or 11438 were used. These results suggest a specific inhibition of EBV-interaction with B-lymphocytes by the antibodies induced by these HABPs, perhaps by inhibiting EBV-attachment to B-lymphocytes.

It is thus probable that gp85-regions defined by HABPs 11435, 11436, and 11438 are involved in EBV invasion of B-cell lymphocytes when considering that they bound with high affinity to a similar receptor on

Raji cells, also bound to Ramos, P3HR-1, and HeLa cells, inhibited EBV-interaction with CBLs, and were also able to elicit antibodies specifically inhibiting EBV-interaction with CBLs. These sequences could be used to design strategies against EBV-infection.

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