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ORIGINAL ARTICLE

# Decreasing the configurational entropy and the hydrophobicity of EBV-derived peptide 11389 increased its antigenicity, immunogenicity and its ability of inducing IL-6

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**Abstract** Peptide 11389 from CD21-binding region of EBV-gp350/220 protein binds to PBMCs inducing IL-6 expression and inhibiting EBV-binding to PBMCs. In addition, anti-peptide 11389 antibodies recognize EBV-infected cells and inhibit both EBV infection and IL-6 production in PBMCs. We have postulated that native structure stabilization of peptide 11389 sequence can increase its biological activity. The strategy was to modify its sequence to restrict the number of structures that peptide 11389 could acquire in solution (decreasing peptide's configurational entropy) and to weaken the non-relevant intermolecular interactions (decreasing its hydrophobicity), preserving CD21-interacting residues and structure as displayed in the native protein. Thirteen analog peptides were designed and synthesized; most of them were monomers containing an intra-chain disulfide bridge. Analog peptides 34058, 34060, 34061, 34296, 34298, 34299 and 34300 inhibited EBV invasion of PBMCs. Peptides 34059, 34060, 34295 and 34297 induced IL-6 levels in PBMCs (EC50 = 3.4, 3.3, 0.5, 0.5 μM, respectively) at higher potency than peptide 11389 (EC50 = 5.8 μM). Peptides 34057, 34059, 34060, 34301 and 34302 interacted with anti-EBV antibodies with affinities from 3 to 50 times higher than peptide 11389. Most of analog peptides were highly immunogenic and elicited antibodies that cross-react with EBV. In conclusion, we have designed peptides displaying higher biological activity than peptide 11389.

**Keywords** EBV · Peptides · Entropy · IL-6 · gp350 · Antibodies

**Abbreviations**

| | |
|---|---|
| EBV | Epstein–Barr virus |
| PBMCs | Peripheral blood mononuclear cells |
| RP-HPLC | Reverse phase-high performance liquid chromatography |
| $\Delta H$ | Enthalpy change |
| $\Delta S$ | Entropy change |
| $\Delta G$ | Gibbs-free energy change |

## Introduction

Peptide 11389 contains one of the three binding regions of the gp350/220 surface protein of Epstein–Barr virus (EBV). gp350/220 is one of the ligands used for this virus to attach and to infect host cells as has been corroborated by the gp350N-terminal structure analysis and mutagenesis studies (Szakonyi et al. 2006; Nemerow et al. 1989; Urquiza et al. 2005). The gp350/220 protein binds with high affinity to the CD21 cell receptor (KD between $1.2 \times 10^{-8}$ and $3.2 \times 10^{-9}$ M) (Tanner et al. 1987; Fingeroth et al. 1984; Nemerow et al. 1987; Moore et al. 1989) inducing several biochemical changes in host cells that involve NF kappa $\beta$ expression, the expression of

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several genes including IL-6 (D'Addario et al. 2001). EBV is highly prevalent in the human population. EBV not only infects most of human immune system cells, such as B and T lymphocytes, monocytes, NK cells and neutrophils but also epithelial cells (Kelleher et al. 1996; Trempat et al. 2002; Savard et al. 2000). This infection can induce infectious mononucleosis (Henle et al. 1968; Straus 1988), lymphoproliferative diseases and some neoplastic diseases, such as nasopharyngeal carcinoma, gastric carcinoma and Burkitt lymphoma (Pathmanathan et al. 1995; de-The et al. 1978; Purtilo et al. 1990).

Peptide 11389 blocks EBV binding to peripheral blood lymphocytes, binds to neutralizing mAb 72A1 and induces IL-6 expression in peripheral blood mononuclear cells (PBMCs) in a similar way to gp350/220 protein. On the other hand, anti-peptide 11389 antibodies are able to recognize EBV-infected cells, inhibit EBV infection of PBMCs and IL-6 expression mediated by EBV (Urquiza et al. 2005). The ability of peptide 11389 for inducing IL-6 synthesis is very important since IL-6 is a pleiotropic cytokine that prolongs lifespan of antigen-presenting cells, increases B cell maturation and antibody production and is essential for the transition of innate to acquire immune response (Jones 2005). Although increased IL-6 levels have been associated with bad prognosis in cancer (Tosato et al. 1990), it has been used to improve immune response of vaccines against tuberculosis (Okada et al. 2008), ovarian cancer in combination with anti-idiotype antibody (Reinartz et al. 2003) and DNA vaccines (Hsieh et al. 2007). It has also been tested in a clinical trial in multiple myeloma in combination with TNFα (Matsui et al. 2003; Minami et al. 2000).

The peptide 11389 sequence in the native gp350 protein is not glycosylated, displays a $\beta$-turn and is involved in binding to CD21 (Szakonyi et al. 2001). However, peptide 11389 presents a random structure in solution and tends to form aggregates. We postulate that stabilization of the $\beta$-turn structure in peptide 11389 will decrease peptide's configurational entropy (measured as the number of different structures that peptide molecules display in solution), increasing its binding affinity to the receptor as well as its biological activity. This is based on the fact that peptide's configurational entropy is substantially reduced when peptide binds to its receptor; which increases the binding entropy change. This has a direct consequence in the binding free energy change, diminishing the binding affinity of peptide and its biological activity (inhibition of EBV invasion of PBMCs, induction of IL-6 synthesis, immunogenicity and antigenicity).

Peptide design using the concept of decreasing configurational entropy to synthesize peptides with a desired biological activity has proven to be fruitful. One of these approaches is the introduction of modified amino acids with dimethyltyrosine in strategic positions of peptides to reduce the number of side-chain conformers, thus increasing the peptide biological activity. A successful example of this approach is the recent report of high affinity and ultra selective d-opioid dipeptide antagonist (Schiller et al. 1993; Balboni et al. 1997; Salvadori et al. 1997; Schiller et al. 1999; Qiu et al. 2001; Soloshonok et al. 2001a, b; Tang et al. 2000). Our approach does not involve modifications of amino acids, but strategic substitutions of amino acids in the peptide sequence to restrict the number of possible structures that peptide can acquire in solution. This was performed trying to keep not only the structure displayed in the native protein, but also, the peptide residues that could be critical in the interaction with CD21. These modifications allowed us to design peptides not only displaying higher biological activity than the native peptide but also differential activity. These new designed peptides showed different properties to modulate the immune response, block EBV virus infection or induce antibodies cross reactive with EBV that could also protect against viral infection. Further studies will show if these peptides could be used as vaccines or co-adjuvants.

In conclusion, structural substitution of amino acids made on peptide 11389 lead to the design of peptides with improved and selective biological activity, which may have important applications for antigenicity and immunogenicity studies.

## Materials and methods

### Determination of the thermodynamic parameters

Thermodynamic parameters of the extended to $\beta$-turn peptide 11389 transition was calculated based on the changes in accessible surface area (ASA) having into account that changes in heat capacity ($\Delta C_p$) and enthalpy ($\Delta H$) have been parameterized in terms of changes in polar and apolar ASA (Privalov 1989; Murphy et al. 1994):

$$\begin{aligned}\Delta C_p &= \Delta C_p, \text{ap} + \Delta C_p, \text{pol} \\ &= (0.45 \pm 0.02)\,\Delta A\text{apo} - (0.26 \pm 0.03)\,\Delta A\text{pol} \\ \Delta H &= \Delta H^* + (\Delta C_p, \text{apo} + \Delta C_p, \text{pol})\,(T - \text{TH}^*) \\ \Delta S &= \Delta S^* + \left(\Delta C_p, \text{apo} + \Delta C_p, \text{pol}\right)\,\text{Ln}\,(T/\text{Ts}^*)\end{aligned}$$

TH* is the temperature when the apolar contribution to the enthalpy is zero and based on folding data is 100 ± 6°C. $\Delta H^*$ is the polar enthalpy contribution at TH* and is equal to (35 ± 3) Δapol. Ts* is the temperature when the apolar contribution to the entropy is zero and based on folding data are 112 ± 1°C (Ysern et al. 1994).

### Peptides design

Analog peptides were designed based on the peptide 11389 sequence and the atomic coordinates of gp350/220

structure reported in the PDB (access number 2H6O) (Szakonyi et al. 2006). The amino acids exposed in the peptide 11389 but not in the native protein were identified by calculating the accessible surface area (ASA) of the protein and peptide with the program GETAREA (http://www.getarea.com), using 1.4 Å for the van der Waals radius of a water molecule. Pairs of amino acids that were less exposed to solvent and their alpha carbons were <5 Å apart in the three-dimensional structure (determined using Discover software) and were changed to Cys to allow the formation of a disulfide bridge, thus restricting the number of possible structures of peptide in solution. The hypothetical structure of these peptides was modeled using the software package Discover. In addition, the energy of these peptides was minimized to relax the structure. Then, the RMS between the analog peptide and the original peptide were determined. Analogs peptides having the lowest values of RMS as compared to the native protein structure were synthesized and their biological activity was determined. In addition, highly mobile glycines 13 and 14 were replaced by Ala to restrict the mobility; some aromatic or hydrophobic residues that are non-exposed in native protein were changed by charged amino acids to change the hydrophobicity of these regions.

## Peptide synthesis

Peptides reported in Table 1 were synthesized by solid-phase multiple peptide system (Houghten 1985; Jeener et al. 1979). MBHA resin (0.7 meq/g); t-Boc amino acid, and low–high cleavage techniques were used. Peptides were analyzed by MALDI-TOF mass spectrometry and reverse phase-high performance liquid chromatography (RP-HPLC). Peptides were reconstituted in PBS pH 7.4. The content of free cysteine (free thiol) in these peptides at 400 µM in PBS was determined using Ellman's reagent or DNTB (5,5′-dithiobis (2-nitrobenzoic acid)) according to the manufacturer's instructions based on the reaction of the thiol with DTNB to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) which is quantified by the absorbance of the anion (TNB 2-) at 412 nm.

## Immunization protocol

Each New Zealand rabbit was subcutaneously immunized with 500 µg of peptide or 50 µl of EBV (from ATCC) plus 150 µg of T-helper epitope FISEAIIHVLHSR (Prieto et al. 1995) and emulsified with 250 µl of Freund's complete adjuvant (500 µl final volume). Rabbits were later boosted on days 20, 40 and 60, with the same antigen dose in Freund's incomplete adjuvant. Blood was drawn 20 days after the second and third doses.

## Rabbit antibody isolation

Rabbit sera were diluted four times with 60 mM acetate buffer (pH 4.0); by adding NaOH 0.1 N, pH was raised to

**Table 1** Aminoacid sequence of peptide 11389 and its analogs

| Peptide | Sequence[a] | | | | | | | | | | | | | | | | | | | | | | Mass (M+1)[b] | Cys (#) | Free cys (fraction) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **11389** | | Y | V | F | Y | S | G | N | G | P | K | A | S | G | G | D | Y | C | I | Q | S | | 2,080.2 | 1 | ND |
| 34057 | – | – | – | **D** | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 2,077.2 | 1 | ND |
| 34058 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | **D** | **D** | – | – | – | – | 2,127.2 | 0 | ND |
| 34059 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | **D** | **D** | – | – | – | 2,120.3 | 0 | ND |
| 34060 | – | – | – | **D** | – | – | – | – | – | – | – | – | – | **A** | **A** | – | **R** | **K** | **R** | – | **E** | **A** | 2,177.7 | 0 | ND |
| 34061 | – | – | – | **D** | – | – | – | – | – | – | – | – | – | **A** | **A** | – | **R** | **K** | **R** | – | **E** | **E** | 2,052.8 | 0 | ND |
| 34295 | – | **C** | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | **S** | – | – | **C** | – | 2,053.8 | 2 | 0.69 |
| 34296 | – | – | **C** | – | – | – | – | – | – | – | – | – | – | – | – | – | – | **S** | – | **C** | – | – | 2,075.6 | 2 | 0.53 |
| 34297 | – | – | – | **C** | – | – | – | – | – | – | – | – | – | – | – | – | – | **S** | **C** | – | – | – | 2,043.6 | 2 | 0.25 |
| 34298 | – | – | – | – | **C** | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 2,052.5 | 2 | 0.58 |
| 34299 | – | – | – | – | – | **C** | – | – | – | – | – | – | – | – | – | – | **C** | **S** | – | – | – | – | 2,053.7 | 2 | 0.2 |
| 34300 | – | **C** | **T** | – | – | – | – | – | – | – | – | – | – | – | – | – | – | **S** | **T** | – | **C** | – | 2,043.6 | 2 | ND |
| 34301 | – | – | **T** | – | **C** | – | – | – | – | – | – | – | – | – | – | – | – | – | **T** | – | – | – | 2,041.8 | 2 | 0.22 |
| 34302 | – | – | **T** | – | – | **C** | – | – | – | – | – | – | – | – | – | – | **C** | **S** | **T** | – | – | – | 2,042.6 | 2 | ND |

*ND* not determined

[a] Bold amino acids indicate the changes made as compared to the original peptide 11389

[b] Molecular weight measured by MALDI-TOF

4.5. Then, 25 µl/ml of caprylic acid was added and stirred for 30 min. Samples were then centrifuged at 10,000×*g* for 30 min, and the supernatant was obtained. A 1/10 volume of 10× PBS was added to the supernatant and pH adjusted to 7.4 with NaOH 0.1 N. The immunoglobulin fraction was precipitated with 0.35 g/ml ammonium sulfate overnight at 4°C. The pellet was separated by spinning at 5,000×*g* for 15 min at 4°C and then suspended in PBS. The immunoglobulin solution was extensively dialyzed with PBS. Protein concentration was determined using Bradford test and antibody's activity by ELISA.

## ELISA

ELISA assays were performed to test antibodies reactivity induced by peptides to EBV coating 96-well ELISA plates with heat-inactivated EBV followed by incubation with anti-peptide serum. Testing of peptides reactivity with anti-EBV antibodies was performed coating 96-well plates with peptides (at 10 µg/ml diluted in PBS) followed by incubation with anti-EBV antibodies. Both assays were performed as follows: after coating plates with the corresponding antigen, they were incubated overnight at 4°C. Plates were then washed four times with PBS containing 0.05% Tween 20 (PBS-T). Non-specific binding sites were blocked with (200 µl) 4% non-fat dry milk in PBS-T for 2 h at 37°C. After washing as described above, rabbit serum (100 µl) at different serial dilutions was added and incubated for 1 h at 37°C. After washing, peroxidase-conjugated anti-rabbit antibody (VECTOR, Burlingame, CA) was diluted 1:5,000 in blocking buffer and was added to each well (100 µl). The plates were then incubated for 1 h at 37°C. After washing, 100 µl peroxidase substrate 3,3′,5,5′-tetramethyl benzidine (SIGMA, St. Louis, MO) was added to the plates; the reaction was stopped by adding $H_2SO_4$ 1 M and the absorbance was collected at 420 nm. The assay was performed in triplicate and considered valid only when the triplicates' coefficient of variation was lower than 10%.

Antibody titers were calculated as EC50 of dilution$^{-1}$ (median effective concentration required for antibodies to bind to 50% of the antigen) using Prism v4.0 software (nonlinear regression-curvefit).

## Human leukocyte isolation

Peripheral blood mononuclear cells (PBMCs) were collected in EDTA-treated sterile tubes. PBMCs were separated by sedimentation on Ficoll–Hypaque gradients. PBMCs thus obtained were washed five times with RPMI-1640, spinning at 300×*g* for 7 min at room temperature. Cell viability was determined by trypan blue staining (GIBCO, Carlsbad, CA).

## Epstein–Barr virus

Epstein–Barr virus-containing supernatant used for EBV and B lymphocyte interaction studies was obtained from the American-Type Culture Collection (ATCC Catalog number VR-1492).

## Effect of peptides and anti-peptide sera in IL-6 synthesis

$1 \times 10^6$ PBMCs were incubated with 20 µl of EBV-containing supernatant or peptides at different concentrations (5, 10, 15 and 20 µM) in 400 µl of RPMI-1640, supplemented with 10% heat-inactivated bovine serum and incubated for 72 h at 37°C. The supernatants were collected and IL-6 concentration was measured using BD OptEIA™ ELISA kit, following the manufacturer's instructions.

To determine the capacity of analogous peptides in inducing IL-6 production compared to EBV, the peptide concentration necessary for inducing half of the IL-6 induced by EBV was determined as EC50.

## EBV infectivity assay

Peripheral blood mononuclear cells were culture at a density of $5 \times 10^7$ cells/mL in RPMI-1640, supplemented with 10% heat-inactivated bovine serum and incubated with 27 µM of each peptide for 15 min at 37°C. Then, 4 µl of EBV-supernatant was added and incubated for 4 h at 37°C in 5% $CO_2$ atmosphere. After incubation, cells were washed three times with RPMI-1640 medium (FBS free). EBV-DNA was then detected using the PCR method described next.

## PCR amplification of EBV-DNA

DNA was obtained by phenol–chloroform extraction and ethanol precipitation. DNA was dissolved in 20 µl of TE buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA]. PCR was performed in 20 µl of reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM deoxyribonucleotide triphosphate, 0.1–0.5 µg of template DNA, each primer at 0.5 µM, and 1.0 unit of *Taq* polymerase. Previously reported primers (Chen et al. 2002) were used to specifically amplify EBV-DNA: 5′-TTCAT CACCGTCGCTGACT-3′ upstream sequence and 5′-ACC GCTTACCACCTCCTCT-3′ downstream sequence. These primers specifically amplified a 300-bp DNA fragment from EBV (+) cells (Raji or B95-8), but not EBV (−) cells (erythrocyte fraction, or HeLa cells). PCR conditions consisted of 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The amplified fragment was separated on

2% agarose gels and visualized using a Molecular Imager FX (Bio-Rad, Hercules, CA).

## Results

### Analogous peptides design

Peptide 11389 sequence displays a $\beta$-turn in native protein structure (Fig. 1) burying 753 $A^2$ of total accessible surface area (ASA) (377 $A^2$ apolar and 376 $A^2$ of polar area) mostly due to tyrosines, $^{2}$V, $^{7}$N, $^{10}$K, $^{14}$G, $^{15}$D and $^{17}$C amino acids (Fig. 2a). The thermodynamic parameters of the extended to $\beta$-turn peptide transition calculated using the changes in ASA (Fig. 2b) showed that peptide 11389 tends to be in $\beta$-turn rather than extended state ($\Delta G = -13.24$ kcal/mol). This is due to heat energy release ($\Delta H = -7.73$ kcal/mol) and increase of the entropy ($T\Delta S = 5.51$ kcal/mol). The $\beta$-turn formation diminished heat capacity of the system ($\Delta C_p = -72.2$ cal/K mol) by decreasing the apolar ASA of peptide 11389. To determine the amino acid to be changed for reducing the peptide's hydrophobicity, the difference in the ASA between peptide 11389 in solution and the same sequence in the native protein was calculated. The results showed that the first four ($^{1}$YVFY$^{4}$) and last three amino acids ($^{18}$IQS$^{20}$) are buried in the native protein and are mostly hydrophobic. These amino acids could affect the physicochemical properties and biological activity of peptide 11389 (Fig. 2b). To decrease the N- and C-terminal region's hydrophobicity and/or to induce intra-chain saline bridges, residues $^{3}$F or $^{16}$YCI$^{18}$ were changed by charged amino acids (aspartic, glutamic, lysine and/or arginine) in the design of peptides from 34057 to 34061. In addition, to diminish the configurational entropy in peptides 34060 and 34061, residues $^{13}$GG$^{14}$ and $^{20}$S were respectively changed by AA and E; $^{1}$Y was excluded and A or E was added at the C-terminal in peptide 34060 or in peptide 34061, respectively (Table 1).

On the other hand, amino acid pairs $^{20}$S-$^{1}$Y, $^{2}$V-$^{19}$Q, $^{3}$F-$^{18}$I, $^{4}$Y-$^{17}$C and $^{5}$S-$^{16}$Y were replaced by cysteines in peptides 34295 to 34302 to promote an intra-chain disulfide bridge that stabilizes the $\beta$-turn since these pairs are closer than 5 Å; aligned in the N-terminus and C-terminus antiparallel $\beta$-sheets connecting the $\beta$-turn of peptide 11389; in addition, $^{17}$C was replaced by S in most of these peptides to have no more than two cysteines per peptide molecule. Likewise, $^{18}$I that is not exposed on the native protein was additionally changed by threonine to increase the polarity of this region in peptides 34300 to 34302 (Table 1).

Peptides displayed in Table 1 were synthesized and purified to more than 90%; their molecular weight determined by mass spectrometry was similar to the expected value. The molecular weight measured for peptides 34295 to 34302 that contain two cysteines corresponded to a monomeric peptide. The free cysteines of these peptides were measured showing that the fraction of free cysteines is lower than 30% except for peptides 34295, 34296 and 34298. These data suggest that most of the peptides were monomers containing an intra-chain disulfide bridge.

The grand average of hydropathicity index (GRAVY) of linear peptide sequences was calculated using Expasy software. Increasing positive score indicates greater hydrophobicity. These values were compared with the retention time of peptides obtained by RP-HPLC analysis using C18 column (hydrophobic peptides showed higher retention time than hydrophilic peptides). According to GRAVY index calculation, 9 of 13 analog peptides would be more hydrophilic than the native peptide (Table 2) but

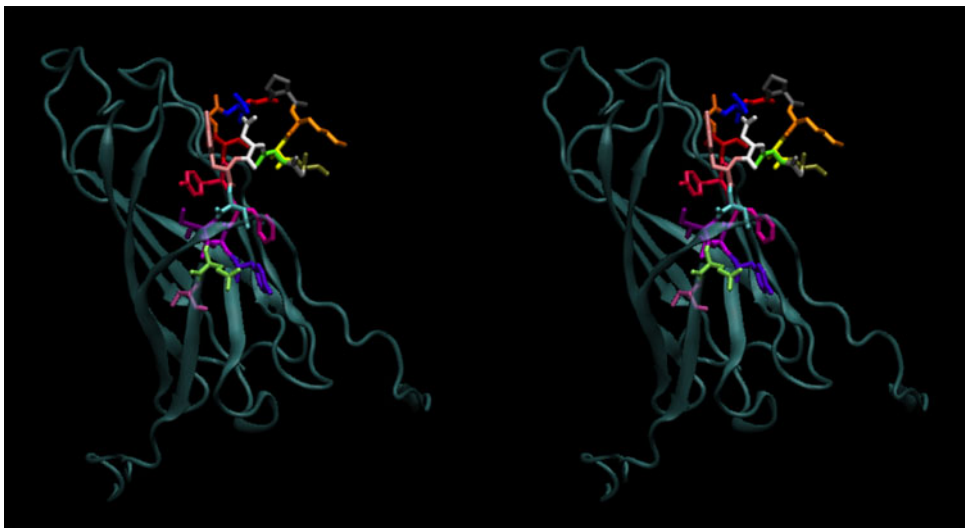

**Fig. 1** Stereoview of peptide 11389 in the gp350 protein structure. The *ribbon structure* of N-terminus region of gp350 was built using the coordinates deposit in the PDB access number 2H6O using VMD software. The configuration of this peptide is formed by two $\beta$-sheets held together with a $\beta$-turn that is mostly exposed in the native protein. Amino acids of peptide 11389 sequence are shown in stick representations. The amino acids more exposed to the solvent in this structure are $^{9}$P, $^{10}$K, $^{11}$A, $^{12}$S, $^{15}$D and $^{16}$Y

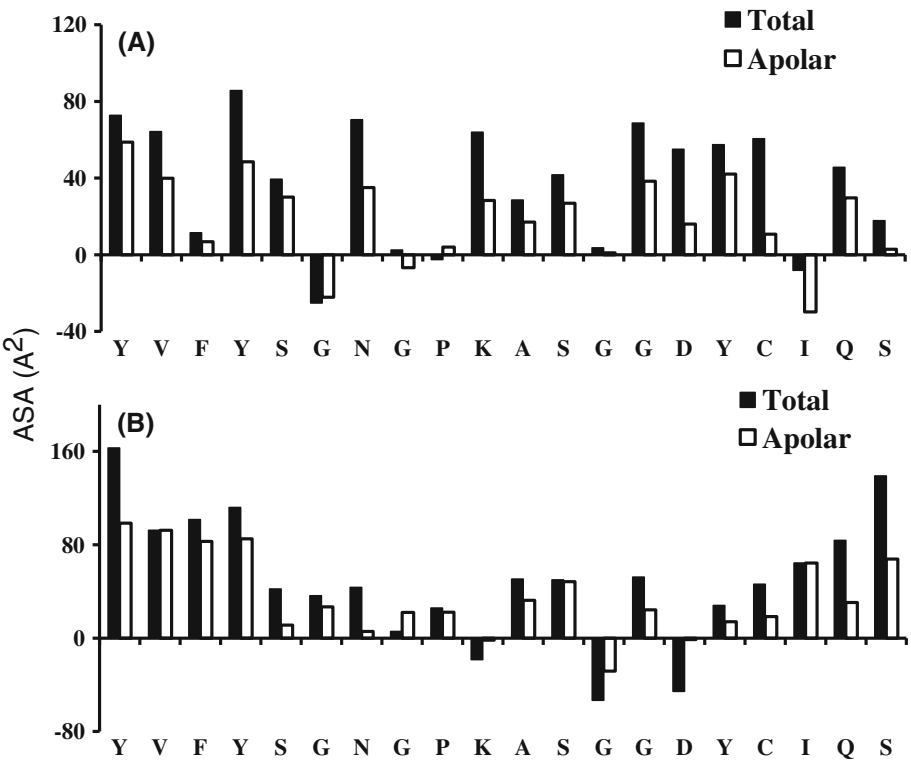


**Fig. 2** Accessible surface area of peptide 11389 in the native protein gp350 and as a monomeric peptide. **a** Differences between the accessible surface area (ASA) of peptide 11389 in a minimized lineal and folded state (as it is the native protein). **b** The amino acids exposed in the peptide 11389, but not in the native protein were calculated by the difference between the ASA of the peptide in the native protein and peptide isolated in the same structure

according to HPLC analysis all the 13 analog peptides were more hydrophilic than the native peptide 11389. This analysis indicates that most of the analog peptides were more hydrophilic than it was calculated, suggesting that part of the hydrophobic region in these peptides are buried in the folded peptide as it was expected for a $\beta$-turn.

**Table 2** Grand average of hydropathicity (GRAVY) index of analog peptides

| Peptide | GRAVY index | Retention time[a] (min) |
|---|---|---|
| 11389 | −0.405 | 22.4 |
| 34057 | −0.72 | 16.9 |
| 34058 | −0.805 | 17.81 |
| 34059 | −1.105 | 17.5 |
| 34060 | −1.41 | 13.76 |
| 34061 | −1.675 | 13.89 |
| 34295 | −0.215 | 20 |
| 34296 | −0.355 | 19.09 |
| 34297 | −0.605 | 13.38 |
| 34298 | −0.215 | 17.28 |
| 34299 | −0.215 | 18.93 |
| 34300 | −0.72 | 16.88 |
| 34301 | −0.72 | 15.23 |
| 34302 | −0.72 | 15.14 |

[a] Retention time calculated by HPLC

## Most of analogous peptides were able to inhibit EBV invasion of PBMCs

Taking into consideration that we have previously shown that peptide 11389 binds to and inhibits EBV invasion of PBMCs (Urquiza et al. 2005) by preventing the gp350-cell receptor interaction (antagonist effect), we determined whether or not peptide 11389 analogs were capable of inhibiting EBV invasion of PBMCs. These cells were incubated with either peptide 11389 or its analogs at 27 μM before EBV invasion. Then the presence of EBV-DNA in these cells was detected by PCR. When comparing band intensity of the PCR product, it was concluded that peptides 34057 and 34059 were not able of inhibiting EBV invasion. Peptides 34058, 34295, 34297, 34298, 34299, 34301 and 34302 showed a lower capacity than original peptide 11389 of inhibiting EBV invasion. Peptides 34060, 34061, 34296 and 34300 inhibited EBV invasion in a similar way than native peptide (Fig. 3).

## Some analogous peptides induced IL-6 synthesis in PBMCs with higher potency than peptide 11389

It has been reported that the interaction of either gp350 or peptide 11389 with the receptor on PBMCs induces IL-6 synthesis (Urquiza et al. 2005; D'Addario et al. 2001). It was investigated whether analog peptides were also able of

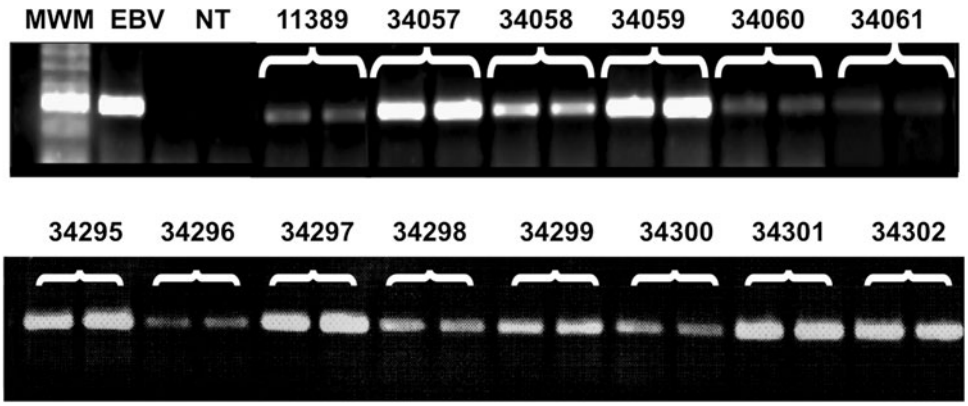


**Fig. 3** Effect of peptide 11389 and its analogous peptides in EBV invasion of host cells. PBMCs were incubated with EBV, peptide 11389 or analogous peptides at 27 μM. DNA was isolated and PCR amplification of an EBV-DNA fragment was performed. *NT* non-treatment, *MWM* molecular weight marker, *EBV* Epstein-Barr virus

inducing IL-6. Peptide 11389 or its analogs were incubated with PBMCs and IL-6 levels in the supernatant were determined. The peptide concentration necessary for inducing half of the IL-6 induced by EBV was determined (EC50). Peptide 11389 induced IL-6 in PBMCs in a dose-dependent manner with an EC50 of 5.8 μM. Interestingly, peptides 34059, 34060, 34295, 34297 (EC50 = 3.4, 3.3, 0.5 and 0.5 μM, respectively) induced IL-6 levels in PBMCs at higher potency than peptide 11389; specially, peptides 34295 and 34297 that induced the same IL-6 levels with ten times less concentration than peptide 11389. On the other hand, analog peptides 34057, 34058, 34061 and 34300 were not able of inducing IL-6 synthesis in PBMCs even using high concentrations (20 μM); peptides 34296, 34298, 34301 and 34302-induced IL-6 synthesis, but at lower potency (EC50 between 6.8 and 11.8 μM) and peptide 34299 induced IL-6 synthesis with similar potency than peptide 11389 (Table 3).

**Table 3** Induction of IL-6 synthesis by peptides and anti-EBV antibodies recognizing peptides

| Peptide | IL-6 synthesis (EC50 μM)[a] | Anti-EBV antibodies (EC50 dilution$^{-1}$)[b] |
|---|---|---|
| 11389 | 5.8 | 15 |
| 34057 | >20 | 52 |
| 34058 | >20 | 19 |
| 34059 | 3.4 | 97 |
| 34060 | 3.3 | 180 |
| 34061 | >20 | 19 |
| 34295 | 0.5 | 10 |
| 34296 | 11.8 | 9 |
| 34297 | 0.5 | 14 |
| 34298 | 11.8 | 10 |
| 34299 | 5.7 | 15 |
| 34300 | >20 | 16 |
| 34301 | 8.8 | 717 |
| 34302 | 6.8 | 63 |

[a] EC50 of peptide concentration

[b] Anti-EBV antibodies were tested for reactivity against peptides. EC50 of dilution is shown

### Peptides 34057, 34059, 34060, 34301 and 34302 showed higher reactivity with anti-EBV antibodies than peptide 11389

The cross-reactivity of anti-EBV antibodies with peptide 11389 depends mainly on the similarities not only in sequence, but also in structure between epitopes on gp350 and epitopes on peptide 11389. Antibodies elicited by EBV displayed a low reactivity with peptide 11389 despite that this region is exposed on the surface of the native protein; this could arise from higher configurational entropy of 11389 sequence in the peptide than in the native protein, generating differences in the structures displayed for peptide 11389 in solution as compared to the structure displayed by the same sequence in the native protein. The reactivity of anti-EBV antibodies with these peptides was determined to compare the structural similarities among peptides with the native protein or at least with the immuno-dominant epitopes present in these regions.

The reactivity of anti-EBV antibodies with analog peptides was compared with the reactivity with peptide 11389 (EC50 = 15 dil$^{-1}$). Peptides 34058, 34061, 34295 to 34300 and 34300 showed similar reactivity to anti-EBV antibodies than peptide 11389 (EC50 between 10 and 19), indicating that the epitope recognized by anti-EBV antibodies was not drastically modified. On the other hand, peptides 34057, 34059, 34060, 34301 and 34302 displayed a higher reactivity with anti-EBV antibodies than peptide 11389 (EC50 between 52 and 717). This suggests that these peptides displayed more similar structure to native protein region recognized by anti-EBV antibodies than peptide 11389 (Table 3).

### Analog peptides elicited antibodies with similar reactivity to EBV than antibodies induced by 11389

Peptide 11389 is immunogenic generating high titers of anti-peptide antibodies. However, these anti-peptide

antibodies display low reactivity with the intact virus. This suggests that the immune-dominant epitopes in the peptide are not exposed in native protein and/or the structure of the peptide epitopes is very different from the structure in the native protein. To overcome these flaws, analog peptides were designed to decrease the predicted hydrophobicity and to stabilize the structure. Peptide 11389 and its analogous peptides were immunized in New Zealand rabbits (two rabbits per peptide). All these monomeric peptides elicited high levels of anti-peptide antibodies with EC50 values from 1849 to 97182 except for peptides 34061 and 34297 with EC50 between 12 and 489. Despite that analog peptides induced high titers of antibodies, these displayed very low cross-reactivity with the original peptide 11389 (EC50 from 3 to 838), (except for antibodies induced in one of the rabbits immunized with peptides 34057, 34059, 34301 and 34302 and the two rabbits immunized with peptide 34298). This indicates that the epitopes in those peptides that elicited antibodies were very different from epitopes in peptide 11389. Interestingly, the majority of analog peptides induced antibodies that not only recognized the EBV-infected cells, but also showed similar reactivity with EBV (EC50 between 41.5 and 76.2) than anti-peptide 11389 antibodies (EC50 47 and 75), except for antibodies induced for one of the rabbits immunized with peptides 34057, 34058, 34060, 34299, and 34301 and the two rabbits immunized with peptides 34058 and 34300. On the other hand, one of the rabbits immunized with peptide 34061 and the two rabbits immunized with peptide 34295 showed an increase in reactivity with EBV when compared with the reactivity of anti-peptide 11389 (Table 4).

**Table 4** Anti-peptides reactivity against themselves, native peptide 11389 and EBV

| Peptide | Rabbit | Peptide's immunogenicity (EC50)[a] | Anti-peptides recognizing 11389 (EC50) | Anti-peptides recognizing EBV (EC50) |
|---|---|---|---|---|
| 11389 | 1 | 2,347 ± 398 | 2,503 ± 394 | 75 ± 9 |
| | 2 | 5,252 ± 385 | 2,278 ± 406 | 47 ± 4 |
| 34057 | 3 | 58,514 ± 9,036 | 11,689 ± 3,342 | 50 ± 3 |
| | 4 | 3,655 ± 122 | 195 ± 14 | 27 ± 2 |
| 34058 | 5 | 6,333 ± 721 | 743 ± 28 | 28 ± 3 |
| | 6 | 5,495 ± 600 | 17 ± 1 | 31 ± 2 |
| 34059 | 7 | 32,041 ± 8,238 | 1,470 ± 82 | 71 ± 5 |
| | 8 | 16,592 ± 2,247 | 34 ± 3 | 71 ± 5 |
| 34060 | 9 | 1,849 ± 547 | 24 ± 2 | 41 ± 3 |
| | 10 | 20,509 ± 4,201 | 9 ± 1 | 29 ± 2 |
| 34061 | 11 | 13 ± 3 | 3 ± 0 | 48 ± 2 |
| | 12 | 12 ± 4 | 11 ± 1 | 128 ± 16 |
| 34295 | 13 | 69,252 ± 5,072 | 214 ± 69 | 97 ± 12 |
| | 14 | 30,414 ± 7,162 | 356 ± 108 | 83 ± 21 |
| 34296 | 15 | 8,945 ± 1,169 | 367 ± 112 | 46 ± 3 |
| | 16 | 25,727 ± 3,618 | 193 ± 59 | 57 ± 4 |
| 34297 | 17 | 49 ± 6 | 496 ± 61 | 51 ± 4 |
| | 18 | 489 ± 77 | 459 ± 69 | 63 ± 4 |
| 34298 | 19 | 97,182 ± 22,099 | 3,031 ± 893 | 61 ± 4 |
| | 20 | 27,255 ± 3,494 | 2,884 ± 782 | 54 ± 4 |
| 34299 | 21 | 3,605 ± 434 | 85 ± 17 | 63 ± 5 |
| | 22 | 20,243 ± 4,743 | 838 ± 206 | 16 ± 2 |
| 34300 | 23 | 56,211 ± 8,983 | 807 ± 211 | 9 ± 2 |
| | 24 | 3778 ± 797 | 179 ± 51 | 12 ± 1 |
| 34301 | 26 | 56,211 ± 8,983 | 2,777 ± 370 | 10 ± 1 |
| | 27 | 26,137 ± 5,277 | 5 ± 0 | 76 ± 7 |
| 34302 | 28 | 29,516 ± 6,762 | 2,469 ± 382 | 38 ± 3 |
| | 23 | 56,211 ± 8,983 | 807 ± 211 | 9 ± 2 |

[a] EC50 of the dilution$^{-1}$

## Discussion

We have postulated that stabilizing the $\beta$-turn of peptide 11389 by modifying peptide's sequence can increase its biological activity. The strategies followed were both to decrease the peptide's hydrophobicity by adding charged or polar amino acids (avoiding intermolecular interactions with non-exposed regions) and to decrease peptide 11389 configurational entropy (restricting the number of structures that peptide 11389 could acquire in solution) by changing the amino acids with <5 Å of distance for cysteines to allow the formation of an intra-chain disulfide bridge. This seems to be possible having into account that the analysis of changes in ASA showed that peptide 11389 is more stable in $\beta$-turn than extended state due to entropy and enthalpy factors. This analysis also revealed that the hydrophobic regions of this peptide have to be buried by intra-chain interaction to stabilize the $\beta$-turn. However, the low solubility of peptide 11389 suggests inter-chain interactions that could involve these hydrophobic regions; this in theory could destabilize the $\beta$-turn and decrease the biological activity of this peptide.

Peptides were synthesized and mass spectra analysis and cysteine analysis indicated that peptides are monomers and most of the peptides having two cysteines seem to have intra-chain disulfide bridge. Peptides 34057 and 34059 did not show inhibition of EBV invasion of PBMC; in case of peptide 34057, the binding activity seems to be drastically affected since this peptide has also weak activity in the induction of IL-6. On the contrary, peptide 34059-induced IL-6 with higher potency than peptide 11389 suggesting that the binding to the cell receptor was not affected and in EBV invasion could be acting as agonist.

The increase in the potency for inducing IL-6, particularly for peptides 34295 and 34297 suggest that these

peptides displayed a structure more similar to native protein than peptide 11389 and interacted stronger with the cell receptor than peptide 11389. This probably was due to an entropy effect (higher structure similarity to native protein and/or lower configurational entropy than peptide 11389) more than an enthalpy effect (the generation of new contacts that increase the peptide affinity for its cell receptors) since the modifications were performed in the non-exposed regions, which do not interact with the cell receptor. On the other hand, the ability of peptides 34057, 34058 and 34300 for inducing IL-6 was drastically reduced. It is very likely that the amino acid changes made in these peptides drastically modified the peptide's structure and/or interfere with the receptor, which could be due to a steric hindrance or repulsion mediated by the new amino acids included in these peptides. The probability that the amino acids that were withdrawn from these peptides were involved in contacts with the cell receptor is very low since peptide 34057 and 34061 had the same modification than peptide 34060. Peptide 34058, similar to peptide 34059, and peptide 34300, similar to peptide 34295, displayed similar or higher potency to induce IL-6 than peptide 11389.

Most of these analog peptides displayed similar reactivity as peptide 11389 with anti-EBV, despite that peptide 11389 sequence in some cases was drastically modified indicating that the immunogenic regions and the amino acids involved in these interactions were not altered in analog peptides. Interestingly, peptides 34057, 34059, 34060, 34301 and 34302 displayed higher reactivity (EC50 from 52 to 717) with these antibodies than peptide 11389 (EC50 = 15). It could be that these peptides resemble closer the native protein structure than peptide 11389 as a consequence of the stabilization of peptide structure by decreasing the peptide configurational entropy. The formation of new interactions is not expected having into account that most of the residues that were changed in these peptides are not exposed on the native protein to interact with anti-EBV antibodies. In addition, peptides having very similar sequence (i.e. peptide 34057 and 34060 with peptide 34061; peptide 34301 with peptide 34298 and peptide 34302 with peptide 34299), displayed very different reactivity with anti-EBV antibodies (Table 2). According to these results, the amino acid residues that were changed in these peptides are not part of the main epitopes recognized by anti-EBV antibodies and it is very likely that these epitopes are located at the sequence GNGPKASGGD.

On the other hand, although the same region may mediate the activity of inhibiting EBV invasion, induction of IL-6 and anti-EBV antibody recognition, it probably involves different residues. For example, peptide 34061 inhibited EBV invasion of PBMCs, but did not induce IL-6 synthesis. Also, peptides 34295 and 34297 were more potent inducing IL-6 than peptide 11389, but they did not inhibit EBV invasion and the reactivity with the anti-EBV antibodies was very similar to peptide 11389. Interestingly, although peptide 34060 sequence was drastically modified (compared with the sequence of peptide 11389) it inhibited EBV invasion, induced IL-6 with higher potency than the original peptide and displayed higher reactivity with anti-EBV antibodies than peptide 11389.

The immune-dominant regions of peptide 11389 were modified in most of the analog peptides since the anti-peptide antibodies displayed low reactivity with peptide 11389. We were not able to significantly increase the cross reactivity of anti-analog antibodies with EBV despite their high antibody titer. This suggests that the immunogenic regions of these peptides involved some of the new residues included in this peptide and most of the residues that are not exposed in the native. The results strongly suggest that the region GNGPKASGGD is less immunogenic that the other region on these peptides (the N- and C-terminus).

In conclusion, peptides with different immunomodulatory characteristics were designed by decreasing 11389 peptide's configurational entropy and stabilizing its $\beta$-turn structure. Peptides 34057, 34059, 34060, 34295 and 34301 were better recognized by anti-EBV antibodies, and were highly immunogenic. Peptide 34297 induced high levels of IL-6 without inducing any immunogenic activity against itself. Peptide 34295 was highly immunogenic and induced high levels of IL-6. Although further studies are required, these analog peptides may be useful in modulating immune response, as vaccines against EBV as well as co-adjuvants for vaccines as previously reported for other peptides (Telusma et al. 2006; Peng et al. 2007; Goldstein 2009).

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**Conflicts of interest** The authors declare that they have no conflict of interest.

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