Influence of High Hydrostatic Pressure on Epitope Mapping of Tobacco Mosaic Virus Coat Protein

Daniel Ferreira de Lima Neto,¹ Carlos Francisco Sampaio Bonafe,² and Clarice Weis Arns¹

Abstract

In this study, we investigated the effect of high hydrostatic pressure (HHP) on tobacco mosaic virus (TMV), a model virus in immunology and one of the most studied viruses to date. Exposure to HHP significantly altered the recognition epitopes when compared to sera from mice immunized with native virus. These alterations were studied further by combining HHP with urea or low temperature and then inoculating the altered virions into Balb-C mice. The antibody titers and cross-reactivity of the resulting sera were determined by ELISA. The antigenicity of the viral particles was maintained, as assessed by using polyclonal antibodies against native virus. The antigenicity of canonical epitopes was maintained, although binding intensities varied among the treatments. The patterns of recognition determined by epitope mapping were cross checked with the prediction algorithms for the TMVcp amino acid sequence to infer which alterations had occurred. These findings suggest that different cleavage sites were exposed after the treatments and this was confirmed by epitope mapping using sera from mice immunized with virus previously exposed to HHP.

Introduction

STUDIES IN RECENT YEARS HAVE DEMONSTRATED the reversible dissociation of oligomeric proteins by hydrostatic pressure (9,51). Observations of comparable effects on multi-subunit proteins and viruses have led to the idea of using hydrostatic pressure to suppress virus infectivity, while preserving or perhaps improving the immunogenic properties. In most cases, effective immunization against viruses requires presentation of the whole virus particle to the immune system (9,50,51). This requirement, coupled to the need to eliminate infectivity, greatly limits the possibilities of preparing appropriate vaccines.

The use of hydrostatic pressure to inactivate viruses may fulfill the two requirements described above. We have previously shown that tobacco mosaic virus (TMV), a plant virus, is reversibly dissociated by pressure, and that the recovery of reassociated, but characteristically modified, virus particles decreases steeply at pressures greater than those that produce 75% dissociation (9). At higher pressures, nonspecific aggregates of capsid proteins predominate over seemingly complete reassociated capsids. The formation of imperfect virus particles after a cycle of compression and decompression has also been demonstrated for human norovirus (49) and rotavirus (41,46) by electron microscopy,

gel filtration, and spectroscopy (8,50,51,53). In this article, we describe the effects of pressure and chemical treatments on TMV by mapping the linear epitopes through spot synthesis and comparing them to results obtained with epitope prediction software. Our findings for TMV suggest the possibility of using hydrostatic pressure to prepare whole virus particles that are highly immunogenic and still retain neutralizing epitopes, in addition to unmasking other epitopes that are normally unavailable in the virus native before treatment.

Methods

ELISA

Plates with 96 wells with Maxisorp chemistry (Nunc-Immuno, Sigma) were coated overnight at 4°C with 1 µg/mL of the virus in its native conformation; after the high hydrostatic pressure treatments (triplicates for each condition), a 0.05 M carbonate/bicarbonate buffered solution (pH 9.6; 100 µL per well) was used for this step. 200 µL of a 2% (w/v) milk powder in phosphate-buffered solution (pH 7.4) was added to all wells as blocking agent; plates were then incubated for 1 h at 37°C. Sera from mice previously immunized with the TMV coat protein (native conformation and after the HHP treatments) were diluted to 1:100 in PBS

¹Laboratório de Virologia Animal, Departamentos de ¹Genética, Evolução e Bioagentes, e ²Laboratório de Termodinâmica de Proteínas, Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Cidade Universitária Zeferino Vaz, Campinas, SP, Brazil.

(pH 7.4) and 100 μ L of this solution was added to each well and incubated for 1 h at 37°C. An anti-mouse IgG conjugated with horseradish peroxidase (HRP) at 0.5 μ g/mL (Jackson Immune Research) was used as detection antibody for this system at a final volume of 100 μ L/well and incubated for 1 h at 37°C. After each step, the plates were vigorously washed with PBS for three times. Orthophenylenediamine (OPD) was used for the colorimetric readings, according to the manufacturer protocol. The enzymatic reaction was stopped with a 4 M H_2SO_4 solution. Optical densities were obtained at 495 nm on a Biotec plate reader, model ELx800, and normalized against the blanks.

TMV RNA sequencing

RNA from the reference virus strain was extracted with TRIzol[®] (Invitrogen), quantified by spectrophotometry and stored at -80°C until used. For reverse transcription, RNA (50 μ g) was converted to cDNA using Superscript III reverse transcriptase, according to the manufacturer's instructions. The reactions were done using diethyl-pyrocabonate (DEPC) water in sterile conditions. The polymerase chain reaction (PCR) mixture consisted of 5 μ L of each primer (5 pmol), 5 μ L of 10X buffer, 1 μ L of dNTPs (0.2 mM), 2 μ L of DTT, 0.5 μ L of *Taq* DNA polymerase, and DEPC water to a final volume of 50 μ L. The PCR products were visualized in 2% agarose gels stained with ethidium bromide and then analyzed in a MEGABace 1000 sequencer, according to the manufacturer's instructions. Flanking primers for the complete sequence of TMV capsid protein (TMVcp) were used. The electropherograms of the sequences were aligned using the DNASTar software package (SeqMan) and the consensus sequence was exported to BLAST and to Protean (DNASTar). The amino acid sequence was deduced from the full nucleotide sequence

using DNASTar software. Local alignment and basic protein structure properties were calculated using the CLC Main Workbench 5.5[®] (protein toolbox) and Lasergene DNASTar[®] software (EditSeq and SeqMan) packages.

Preparation of peptide arrays and epitope identification

Peptides based on the amino acid sequence determined above were synthesized by using Fmoc (9-fluorenylmethoxycarbonyl) chemistry (7,27) on PEG-derivatized cellulose membranes with an additional β -Ala anchor for the C-terminal immobilized peptides. The membranes contained overlapping pentadecapeptides spanning the complete sequence of TMVcp (residues 1–159), with an offset of three amino acid residues.

Immunogenic properties of pressure-treated TMV

The purified TMV was subjected to pressure treatment in Tris-buffered saline solution (pH 7.4), which changes very little with pressure (29). The pressure system is described in detail elsewhere (23). The temperature was maintained constant throughout the experiment via thermostatic bath. At -18°C , the temperature decrease was done at 250 MPa, pressure which prevents freezing of the sample (12). The experimental protocol consisted of subjecting a TMV preparation to 250 MPa of pressure for 1 h at 25°C for the first test group, 250 MPa for 1 h at -18°C for the second group, 250 MPa for 1 h at 25°C with 1 M urea for the third group, and 4 M urea for 1 h at 25°C as the last test group. Pressure-treated virus and control samples, along with complete Freund's adjuvant, were injected into mice. Mice injected with the pressurized virus showed no additional side effects. The neutralization of native TMV by the γ -globulin fraction obtained from immunized mice is shown in Figure 1.

FIG. 1. ELISA sensitivity of TMV isolates in serial dilution conditions. The ELISA typing test was performed on sera from immunized mice raised against the four conditions tested in this work, plus positive and negative controls (see Methods). Ratios were calculated for each condition, and normalized by subtracting the blanks for each condition. This experiment was devised to evaluate possible differences in antibody affinities for the TMV sera from HHP-derived conditions.

FIG. 2. Evaluation of the cross reactivity of the immunized mice sera against each other by ELISA assays. Native (A) TMV, or treated for 1 h at 250 MPa (B), at 250 MPa and -18°C (C), 250 MPa in urea 1 M (D), urea 4 M (E), and control (F).

Pressure-inactivated TMV elicited a similar titer of neutralizing antibodies to that seen with native TMV particles.

Serum preparation and antibody-binding assay

Balb/c female mice ($n = 5$ per experiment) obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP) were inoculated with native TMV sample plus complete Freund's adjuvant in the first inoculation and incomplete adjuvant in subsequent inoculations. The inoculations were done on Day 1, Day 15, Day 28, and Day 35, using 15, 10, 10, and 15 μg of TMV, respectively. Blood was collected 14 days after the last inoculation, pooled, and allowed to clot, after which the serum was obtained by centrifugation, inactivated by incubating for 1 h at 56°C and stored at 4°C . The protocols involving animals were approved by the institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 1717-2).

The antibody-binding assay used was described by Beutling *et al.* (7). A serum dilution of 1/100 was used in the membrane antibody-binding assays. An alkaline phosphatase-conjugated goat anti-mouse IgG (AP-IgG) (Jackson Immuno Research, Cat. No. 115-055-071) was used as secondary antibody to detect bound antibodies. The color reaction was

developed as described elsewhere (54), with an incubation time of up to 60 min. The membrane was subsequently scanned at 2400 d.p.i. with a table scanner and the software package Totallab Quant-Array Analysis was used to measure the intensity of each spot against the background intensity. The results were expressed as the mean for each spot. The membranes were regenerated using the procedure described by Beutling *et al.* (7).

An epitope-based immunoinformatics study of TMVcp

The deduced protein sequence was aligned with the protein sequence of TMVcp retrieved from the National Centre for Biotechnology Institute (NCBI) database (BLAST reference accession no. ACY41215.1). Epitope flexibility was predicted with the Karplus and Schulz flexibility scale (37) and antigenicity was predicted with the Kolaskar and Tongaonkar antigenicity scale (39). Hydrophobicity and hydrophilicity were analyzed with the Parker hydrophilicity scale. The Kyte-Doolittle hydrophobicity index (40) available from the Expasy Prot scale server was used to assess the distribution of polar and apolar residues along the protein sequence. The Kyte-Doolittle scale (window size of 7 and cutoff of 1.6) is widely used to examine the hydrophobic nature of proteins

FIG. 3. Tobacco mosaic virus coat protein epitope mapping. (A) Quantification of the spots that were recognized by the sera from mice immunized with the native conformation of the virus, prior to HHP treatments. X-axis represents the spots on the membrane. (B) Membrane used for quantification, negative control tested with antibodies against porcine parvovirus VP1 protein (not quantified), and positive control tested with antibodies against TMVcp native conformation. (C) TMVcp crystal structure (3J06.pdb) with the epitopes recognized by the test mapped on the structure using the software MOLEGRO. (D) B-cell epitope predictions (IEDB B cell epitope analysis tool) using the crystal structure from the protein data bank.

and is useful in predicting membrane-spanning domains, potential antigenic sites and regions that are likely to be exposed on the protein surface. This approach was complemented with the Hopp method for a window size of 7-mers (34,40). Ubiquitination sites were predicted as described elsewhere (22). Proteasomal cleavage sites were predicted based on proteasomal degradation experiments with β -casein (24), enolase (57), and prion proteins (55). The prediction of TAP affinity was based on a support vector machine (SVM) (22). The SVMHC (support vector MHC) method was used to predict MHC binding (8). This is an SVM-based method based on verified MHC binding peptides from the SYFPEITHI database (45). These methods have been validated elsewhere (18). Data available from the ExPASy Prot scale server were used to counter-verify the *in silico* data (28).

Combining the prediction methods

The class II MHC algorithm and the linear and conformational B cell epitope prediction algorithms were used to simulate the processing pathways for which there are *in silico* prediction algorithms, particularly with regard to the virus and host used here.

In the first step of analysis, the protein sequences were screened individually for the best binding epitopes by using the Immune Epitope Database (IEDB) algorithm. The cutoff scores were adjusted by the algorithm itself. The separate prediction methods were then combined in order to model the entire processing pathway of class I MHC antigens. Predicted peptides should have a compatible C terminus generated by the proteasome, and a relatively high affinity for TAP and for MHC molecules. The high accuracy of the final step of MHC binding prediction means that other methods can be used as filters to remove candidate peptides unlikely to be generated by proteasomal cleavage and/or transported by TAP. Peptides nine amino acids long (9-mer peptides) were extracted from the SYFPEITHI database for this mouse model (60). The data presented in this work are for 9-mer peptides (to minimize the volume of data and simplify analysis). The class I MHC alleles Kd, Dd, and Ld determine the necessary recombination for *de facto* epitope display in Balb/c mice and were used to map the potential epitopes of TMVcp. The tentative epitopes were predicted using PCM, SVM-TAP, and SVMHC methods (38,41,48,60,62).

Results

ELISA

Figure 1 shows the ELISA reactivity of the various sera screened for antibodies to TMVcp. All sera recognized TMVcp and yielded similar dilution profiles, regardless of the conditions to which the virus had been

exposed prior to inoculation. However, the antibody titers were markedly lower for TMVcp exposed to 250 MPa for 1 h at 25°C than in the other groups. Treatment with urea alone resulted in higher titers than observed with native TMV, whereas a combination of HHP and 1 M urea yielded a titer similar to that of the native protein (control).

Figure 2 shows the cross-reactivity of serum from a given group with TMV exposed to other conditions. Although the different treatments significantly altered the profile of exposed epitopes (see below), the recognition of antigenic determinants remained unchanged, despite minor nonsignificant variations among dilutions.

Mapping of TMVcp epitopes

Spot blotting with synthetic peptides was used to map the epitopes of TMVcp that interacted with the different sera; overlapping pentadecapeptides with an offset of three amino acids per spot were used. Each spot thus corresponded to a linear epitope, the sum of which allowed mapping of the whole protein. Each serum was incubated with a membrane containing bound peptides and the signal produced was quantified with the spot array module of the program Totallab Quant. Figure 3 shows the result after development with alkaline phosphatase-conjugated secondary antibodies to the native form of TMVcp compared to the negative control (serum from mice immunized with porcine parvovirus). Compared to the previously reported epitopes of native TMVcp (21), the mapping observed here revealed important differences and showed increased resolution, due to HHP treatment. By combining the results of these two studies, it is possible to obtain a global picture of the epitopes of the native TMVcp.

Mapping of TMVcp epitopes in TMV subjected to different treatments

To examine the influence of different high pressure treatments on the immunogenicity of TMV, mice were immunized with virus particles subjected to HHP, HHP plus urea 1 M, HHP plus low temperature, and 4 M urea. Figures 4–7 show the normalized intensities of the spot signals in relation to the treatments used. There was considerable variation in the epitopes recognized in TMVcp from TMV subjected to different conditions when compared with untreated (native) protein.

Influence of high pressure treatment (250 MPa) on TMVcp

Figure 4A shows the reactivity of the epitopes of TMVcp treated with HHP. Five highly reactive regions were identified: A10–A16 (residues 28–60), A20–A23 (residues

FIG. 4. Tobacco mosaic virus coat protein epitope mapping after HHP treatment. (A) Quantification of the spots that were recognized by the sera from mice immunized with the virus after to HHP treatment. X-axis represents the spots on the membrane. (B) Membrane used tested with antibodies against TMVcp after HHP treatment. (C) TMVcp crystal structure (3J06.pdb) with the epitopes recognized by the test mapped on the structure using the software MOLEGRO. (D) Differential epitopes recognized by the native-derived sera and HHP-derived sera.

58–81), B7–B4 (residues 85–108), B16–B17 (residues 121–138), and B20–B24 (residues 133–159), and two less reactive regions: A4–A7 (residues 10–33) and A25–B1 (residues 73–90). Figure 4B shows the spots used in this analysis and Figure 4C shows the PDB structure 3J06_A with the epitopes recognized in HHP-treated TMVcp, as determined using the software Molegro. Epitope mapping of HHP-treated TMVcp revealed specific epitopes not detected in native TMVcp, as shown by spots A6 (residues 16–30: SAWADPIELINLCTN), A7 (residues 19–33: ADPIELINLCTNALG), A18 (residues 52–66: VWKSPSPQVTVRF PD), A19 (residues 55–69: PSPPQVTVRFPSDF), and B10–B12 (residues 103–123: NPTTAETLDATRRVD, TAE TLDATRRVDATV, and TLDATRRVDATVAIR). On the other hand, serum raised against HHP-treated TMVcp recognized several epitopes in native TMVcp that it did not recognize in HHP-treated TMVcp, for example, spots A14–A16 (residues 40–60: QARTVVQRQFSEVWK, TVV QRQFSEVWKPSP, QRQFSEVWKPSPQV), A22–A23 (residues 64–81: FPDSDFKVYRYNAVL, SDFKVYR YNAVLDP), and A25–B1 (residues 73–90: RYNAV LDPLVTALLG, AVLDPVTALLGAFD). Figure 4D compares these results.

Influence of urea treatment on TMVcp

Figure 5A and B shows the reactivity of serum raised against TMV treated with 4 M urea. This serum recognized several epitopes exclusively in urea-treated TMVcp [i.e., spots A1–A4 (residues 1–21) and B8–B12 (residues 97–123)]. In addition, this serum recognized several epitopes exclusively in native TMVcp, as shown by spots A13 (residues 37–51: QTQQARTVVQRQFSE), A14 (residues 40–54: QARTVVQRQFSEVWK), A16 (residues 46–60: QRQFSEVWKPSPQV), and A20–A23 (residues 58–81: PQVT VRFPDSDFKVY, TVRFPDSDFKVYRYN, FPDSDFKV YRYNAVL, SDFKVYRYNAVLDP). Figure 5C shows the PDB structure 3J06_A with the epitopes recognized in urea-treated TMV, as determined using the software Molegro. Figure 5D summarizes these results.

Influence of high hydrostatic pressure and 1 M urea on TMVcp

Figure 6A and B shows the reactivity of serum raised against TMVcp exposed to a combination of HHP and 1 M urea. This serum identified specific epitopes in HHP/urea-treated TMVcp, including spots A18–A19 (residues 52–69: VWKSPSPQVTVRF PD, PSPPQVTVRFPSDF) and B3–B4 (residues 82–99: VTALLGAFDTRNR II, LLGAFDTR NR II EVE). This serum did not interact with any epitopes in native TMVcp. Figure 6C shows the PDB structure 3J06_A

with the epitopes recognized in TMV treated with HHP and urea, as determined using the software Molegro. Figure 6D summarizes these results.

Influence of high hydrostatic pressure and low temperature on TMVcp

Serum raised against TMVcp treated with a combination of HHP and low temperature (-18°C) recognized a variety of epitopes in the treated virus: spots A1–A4 (residues 1–24: MSYSITTPSQFVFLS, SITTPSQFVFLSSAW, TPSQFV FLSSAWADP, QFVFLSSAWADPIEL), A18–A19 (residues 52–69: VWKSPSPQVTVRF PD, PSPPQVTVRFPSDF), and B8–B12 (residues 97–123: EVENQANPTTAETLD, NQANPTTAETLDATR, NPTTAETLDATRRVD, TAETL DATRRVDATV, TLDATRRVDATVAIR) (Fig. 7A, B). Figure 7C shows the PDB structure 3J06_A with the epitopes recognized in TMV treated with HHP plus low temperature, as determined using the software Molegro. Figure 7D summarizes these results.

Discussion

There is extensive structural information for TMV, particularly for TMVcp. In this study, we used synthetic peptides and spot blotting to examine the reactivity of sera raised against TMVcp exposed to different conditions (HHP, HHP plus urea, and HHP plus low temperature) in order to improve our understanding of the conformational changes caused by these treatments. These results were then compared with bioinformatics epitope predictions for B and T cells in order to identify the epitopes involved. The structural location of the involved epitopes was based on the crystallographic data of TMVcp in the virion. Structural data from pressurized samples is not possible due to heterogeneity of the sample after pressure treatment (12). Multi-subunit proteins exhibit similar heterogeneity after pressure treatment, comparing to the native form (10,11,53).

Various reports have previously described the antigenic profile of native TMVcp (2,3,58) and have sought to identify the epitopes involved (5,58). In our experiments, we found no significant binding in the first 42 peptides of TMVcp, in contrast to previous results for the first 10 peptides (3,58) and for peptides 19–32 (1,59) using polyclonal sera. On the other hand, our results agreed with previous reports that identified residues 34–39 (3), 55–61 (19), and 62–68 (3) as important, highly reactive regions. We also detected strong interactions in residues 31–60, with high reactivity for residues 34–51 and 43–60. The region located between residues 58 to 81 (specifically: residues 55–61 and 62–68) was also highly reactive.

FIG. 5. Tobacco mosaic virus coat protein epitope mapping after 4 M urea treatment. **(A)** Quantification of the spots that were recognized by the sera from mice immunized with the virus after urea 4 M treatment. X-axis represents the spots on the membrane. **(B)** Membrane used tested with antibodies against TMVcp after urea treatment. **(C)** TMVcp crystal structure (3J06.pdb) with the epitopes recognized by the test mapped on the structure using the software MOLEGRO. **(D)** Differential epitopes recognized by the native-derived sera and 4 M urea-derived sera.

D

Spot	Sequence	Region	Native	Urea 4M
A1	MSYSITTPSQFVFLS	1 To 15		++++
A2	SITTPSQFVFLSSAW	4 To 18		+++
A3	TPSQFVFLSSAWADP	7 To 21		++
A14	QARTVVQRQFSEVWK	40 To 54	+	
A15	TVVQRQFSEVWKSP	43 To 57	++++	
A16	QRQFSEVWKSPPPQV	46 To 60	++++	
A20	PQVTVRFPDSDFKVY	58 To 72	+	
A21	TVRFPDSDFKVYRYN	61 To 75	+	
A22	FPDSDFKVYRYNAVL	64 To 78	+	
A23	SDFKVYRYNAVLDP	67 To 81	++	
B08	EVENQANPTTAETLD	97 To 111		+++
B09	NQANPTTAETLDATR	100 To 114		+++
B10	NPTTAETLDATRRVD	103 To 117		+++
B11	TAETLDATRRVDATV	106 To 120		+
B12	TLDATRRVDATVAIR	109 To 123		+

Sequence positions that react with polyclonal and monoclonal antibodies (MAbs) in the literature include residues 80–90, 105–112, 115–134, 134–146, and 153–156 (2,4,47). As shown here, a positive reaction was obtained for the region involving residues 85–108, with greater reactivity for residues 88–102 (spot B5). In contrast to previous work (2,6), we did not observe strong reactivity for residues 97–132, although other reports (1,4) have reported strong reactivity for this region, especially residues 121–144 (spots B16 and B17) and 133–159 (spots B20–B24). These differences may reflect the models used to investigate the antigenic profile of TMV. For example, Anderer *et al.* (4) used tryptic peptides and a fixed ELISA detection system combined with polyclonal and monoclonal antibodies to investigate this profile, whereas Al Moudallal (1) used chicken immunized sera and MAbs against TMVcp. Dekker (20) discussed the difficulties in using these methods to map a protein with MAbs or ELISA; these authors suggested that longer proteins rather than smaller fragments should be used to adequately assess antigen processing and presentation events. The SPOT technique allows this to be done and, when combined with the use of only one animal model, provides a better picture of the epitope profile, as shown here. Furthermore, the combined use of epitope prediction and an algorithm restricted to the system in which the antibodies were generated provides a more constrained strategy for fast, precise epitope mapping of a given protein.

The antigenic determinants of proteins are generally located in exposed regions that are polar and hydrophilic. Consequently, the accessibility and flexibility of these segments is higher than those of other potential epitopes located deeper in the three-dimensional structure of the molecule. As we have previously shown, Kyte-Doolittle analysis for the TMVcp sequence revealed that the regions surrounding amino acid residues 30–78, 80–120, and 130–150 have these characteristics. These regions also yielded higher intensities of antibody binding to linear epitopes, as shown for the native protein and by using prediction algorithms. Similar results were described in another report that used less accurate methods and different animal models (58).

Physical alterations can markedly affect protein function, and viruses are no exception. Modifications to capsid proteins can generate defective particles, thereby compromising the ability to self-assemble or cause successful infections (13,26). Intrinsic properties such as hydrophobicity, flexibility, and surface accessibility, secondary structure properties, exposed surfaces, and predictions based on antigenicity parameters were used here to infer epitopes based on selected algorithms and epitope mapping. In general, α -helices occur on the surface of proteins and are generally hydrophilic and accessible to the solvent. In

contrast, the protein core is essentially devoid of water molecules (45). In the case of the capsid protein VP5 of herpes simplex virus type I, the secondary structure is important for antibody binding and even small modifications may influence identification of the antigen by the immune system (52).

TMVcp has three major hydrophilic regions that span residues 30–70, 90–120, and 140–150 (21,58), all of which were also recognized in the epitope mapping of the native form of this protein. The use of mouse IgG to screen membranes containing bound linearized peptides yielded positive signals for residues 31–60, 85–108, and 133–159, in agreement with previous studies of this protein. These regions were found to be more accessible to proteasomal cleavage. These results were complemented with the predictions of TAP binding affinity. The predictions of binding to MHC class I took into consideration the results of these three combined predictive methods, as well as the mouse alleles (Dd, Ld, and Kd) that could be tested for our mouse model. The change in binding observed with this approach has been suggested to reflect structural differences that influence peptide–MHC interactions (30). In this context, *in silico* analysis revealed various possible connections for each allele tested, as shown earlier. The CBS and IEDB servers were also used to predict the MHC class II binding affinities of the full sequence. The regions with higher scores by this method were also found when mapping the native form of the protein, which suggested that the correct presentation of these peptides followed the principles well established in the literature.

The algorithm for predicting B cell epitopes reported higher binding probabilities for the region of residues 46–66, in agreement with our experimental results. Discontinuous B cell epitopes were predicted for this same region, but only in a larger fragment that extends from residues 33 to 90. There were two peaks separated by few residues (15,25,44). B cells are of particular interest because their surface receptors are formed by rearrangement of IgG-like receptors. Epitopes recognized via MHC class II and B cell receptors are of particular interest because of the biological roles of these receptors and their interplay. The results of the B cell epitope predictions described here agree with canonical TMV studies, but offer better resolution through the overlapping of linearized epitopes. However, the use of spot blotting to screen HHP-treated TMV showed that the predicted and mapped epitopes differed substantially from those previously reported (31). This discrepancy between our results and the prediction algorithms suggests different accessibility to otherwise hidden cleavage sites (see later).

Recent advances in analytical techniques for studying pressure-induced protein denaturation have facilitated the

FIG. 6. Tobacco mosaic virus coat protein epitope mapping after HHP/urea 1 M combined treatment. (A) Quantification of the spots that were recognized by the sera from mice immunized with the virus after HHP/urea 1 M combined treatment. X-axis represents the spots on the membrane. (B) Membrane used tested with antibodies against TMVcp after HHP/urea 1 M combined treatment. (C) TMVcp crystal structure (3J06.pdb) with the epitopes recognized by the test mapped on the structure using the software MOLEGRÖ. (D) Differential epitopes recognized by the native-derived sera and HHP/urea 1 M combined sera.

investigation of molecular interactions in proteins and protein complexes, and have provided new insights into the structure and function of biomolecules under conditions of elevated pressures. Moreover, as the effect of pressure on proteins is usually quite distinct when compared to that generated by extreme temperature or pH, pressure is considered to be an important variable for analyzing structure–function relationships of proteins (31). Pressure has been recognized as a potential denaturing agent for some time, although the conditions required for a given protein to denature vary considerably, depending on the structural features of the protein. Pressure-induced denaturation has been widely applied in virology. Whereas the primary structure of proteins is unchanged at pressures as high as 10 kbar, dissociation is nevertheless quite frequently observed (31,32,50,51). In general, the effect of pressure on a physicochemical process at equilibrium is governed by the volume change of the process, ΔV (31,32). Hence, the application of pressure will change the balance in favor of the state with the lowest overall volume. The magnitude of the changes in volume in the structure of a given protein will depend on specific molecular interactions (9,50,51). Because of its ability to reversibly dissociate oligomeric proteins, HHP has been increasingly used as an alternative method for viral inactivation in vaccine production. The reversible dissociation of viral capsid proteins may result in viral particles that reassociate imperfectly; such an event has been correlated with a loss of infectivity, while maintaining or even improving the immunogenicity (14,43). As shown here, we observed these changes indirectly, via linear epitope mapping of HHP-treated TMVcp. This treatment resulted in the exposure of different regions for antigen recognition and processing, and provided a detailed profile of the alterations in protein structure that were detected by the host system.

In addition to the regions more exposed to solvent that were made accessible to antigen processing, as described by Carmicle *et al.* (14) and reviewed by Neefjes *et al.* (43), the species affinity for MHC alleles was also compared with our experimental results to verify whether the predictions fell short of providing accurate data based on this design. A further aim of this study was to determine which epitopes were dependent on the three-dimensional conformation of TMVcp that previous studies had suggested was important. The three-dimensional structure of proteins generally limits the access of cleavage systems in the antigen processing machinery, thereby compromising epitope–MHC binding, as described by Thai (56).

As stated by Hubbard: “The endoproteolytic cleavage tends to occur in disordered polypeptide segments, thanks to the conformation of the segments in relation to the active sites of enzymes” (35,36), indicating that the relationship between conformation and processing is critical for antigen

presentation. In addition, immunodominance is also related to the species studied, as pointed out by Dai (16,17), who suggested that the shortening of a disordered peptide by cleavage or by structural modification may reduce its immunogenicity. In this context, the identification of regions most likely to influence the three-dimensional epitope recognition occurs in the flexible regions of the protein, as described in several earlier reports for TMVcp and revisited here with bioinformatics tools and epitope mapping via spot analysis (21).

Conformational changes in response to HHP have been extensively studied (32,33). Such changes may have occurred after the treatments used here, since distinct epitopes were recognized by sera from mice previously immunized with HHP-treated TMVcp, in contrast to the results obtained with serum raised against native TMVcp.

To test the hypothesis that protein conformation interferes with antigen processing, Dai *et al.* (16) inserted mutations at key points and used Western blot analysis to map the protein antigen recognition of these changes. These alterations were found to reduce the size of the known epitopes and also the sensitivity of the mutated sequence to proteolysis; this in turn reduced the immunogenicity of previously described epitopes. These authors proposed that the distribution of local flexibility was altered and that it influenced antigen folding, processing, and presentation. Protease-independent epitopes were restricted to regions of the protein that were flexible, and protease-dependent epitopes were constrained by structural accessibility (16,17). Many studies have evaluated the changes induced in pressurized proteins, a recent example of this being the metabolism of glucose by *Agrobacterium tumefaciens* in situ, under HHP (44). The unfolded state generated intermolecular changes that prevented reversibility of the state, especially in the interactions generated by hydrogen bonds (25).

There were distinct signals for the first pentadecapeptides after treatment with HHP plus low temperature and HHP plus 4 M urea. These epitopes have been described in the literature, but were not observed here for native TMVcp. These peptides corresponded to the amino acid sequences MSYSITPSQFVFLS, SITPSQFVFLSSAW, TPSQFVFLSSAWADP, and QFVFLSSAWADPIEL for the first four spots of TMVcp. Analysis of the surface area accessible in this region found that amino acids 3 to 15 were exposed, whereas for the remaining amino acids the results indicated internalization of the residues. The prediction algorithms for hydrophobicity and antigenicity in this region showed that this region was hydrophilic, and the initial and combined antigenic predictions for MHC I and II agreed with the possibility of presenting these epitopes. In the analysis of proteasomal cleavage, amino acids 14 and 15 were highly susceptible to proteolysis. However, based on

FIG. 7. Tobacco mosaic virus coat protein epitope mapping after HHP/low temperature combined treatment. (A) Quantification of the spots that were recognized by the sera from mice immunized with the virus after HHP/low temperature combined treatment. X-axis represents the spots on the membrane. (B) Membrane used tested with antibodies against TMVcp after HHP/low temperature combined treatment. (C) TMVcp crystal structure (3J06.pdb) with the epitopes recognized by the test mapped on the structure using the software MOLEGRO. (D) Differential epitopes recognized by the native-derived sera and HHP/low temperature combined sera.

D

Spot	Sequence	Region		Native	HHP at -18 oC
A1	MSYSITTPSQFVFLS	1	To	15	++
A2	SITTPSQFVLSSAW	4	To	18	++
A3	TPSQFVLSSAWADP	7	To	21	++
A4	QFVLSSAWADPIEL	10	To	24	++
A17	FSEVWKPSPPQVTVR	49	To	63	+
A18	VWKPSPPQVTVRFPD	52	To	66	+
A19	PSPPQVTVRFPDSDF	55	To	69	+
B08	EVENQANPTTAETLD	97	To	111	+++
B09	NQANPTTAETLDATR	100	To	114	++++
B10	NPTTAETLDATRRVD	103	To	117	++++
B11	TAETLDATRRVDATV	106	To	120	++++
B12	TLDATRRVDATVAIR	109	To	123	+++

the PDB structure and other *in silico* predictions plus the epitope mapping results, we suggest that under native conditions this region is not easily accessed by the processing system and that the treatments allowed the presentation of these epitopes to adaptive memory systems. However, TAP binding affinity data indicated a high probability of binding to the region. MHC I alleles differ in their affinities, with allele H2-Dd having a higher affinity for residues 1–5 and 16–24; the affinity of the Kd allele agrees with previous predictions and experiments, but our findings expanded the affinity to the region 16–25. MHC II predictions for this region returned the highest values for the probability affinity test.

The literature data and *in silico* analyses for this region (spots A5–A7, residues 13–33) do not match the epitopes located near an α -helix and β -sheet described in our experiments for native TMVcp. These regions are composed of hydrophobic and polar amino acids (FLSSAWADPIELINL, SAWADPIELINLCTN, ADPIELINLCTNALG) and were expected to be available on the protein surface. However, given the location between two secondary structures, the amino acid composition, and the predicted binding affinities for MHC class II, our positive results for epitope recognition in this region suggested that HHP destabilized these structures to favor the exposure and processing of these sequences into epitopes.

High binding was observed in the region of residues 28–51 (CTNALGNQFQTQQAR, ALGNQFQTQQARTVV, NQFQTQQARTVVQRQ, and QTQQARTVVQRQFSE) with antibodies against virus previously exposed to HHP, HHP plus low temperature, and HHP plus 1 M urea. Bioinformatics analysis for this region yielded higher scores for the antigenicity scale (Hopp-Woods) and for hydrophobicity (Kyte-Doolittle). However, these regions do not correspond entirely to the results for accessible surface area for the exposed residues of regions 21–37 and 57–60. Moreover, in terms of proteasomal cleavage, this region is a possible target for cleavage that was not exposed prior to HHP treatment. In agreement with this, the affinities of TAP and MHC class I for residues 20–35 and 47–58 were the highest among post-cleavage values. Although all alleles tested showed binding affinities for MHC, only allele Ld returned a higher binding affinity for the region mentioned. MHC class II predictions for residues 28 to 51 were completely within those predicted for allele IAD, suggesting the availability of this region to processing. These findings suggested that this region underwent conformational changes that allowed processing and presentation, thereby increasing the experimentally verifiable recognition discussed earlier.

A binding region specific for the responses related to HHP-treated virus was observed for residues 73–90 (RYNAVLDPVLTALLG, AVLDPLVTALLGAFD); these residues are located between two β -sheets and the beginning of an α -helix that change volume when subjected to pressure. This change in volume would expose areas for cleavage or of immunological relevance, or could alter the protein conformation, making it difficult to interact with the processes of antigen presentation. However, the predicted results for this fragment suggested a higher probability for epitope presentation. Based on this information

and on literature data showing that this region is antigenic, we suggest that the treatment failed to present this epitope because changes in the structure of TMVcp made it impossible for the immune system to process the protein.

In conclusion, the results reported here expand our knowledge of the epitope map of TMVcp in mice and also improve our understanding of the alterations induced by HHP, in contrast to immunoinformatic prediction algorithms. We found discordant regions previously considered to be well-defined epitopes, and suggest that a more careful reading of the antigen processing machinery in different animal models should be made. There was generally good agreement between algorithm-based predictions and our experimental data for the native form of the virus, but disagreement when the data for HHP-treated virus were mapped.

Epitope mapping is an effective tool for identifying the regions of a protein involved in antibody responses and, when used in conjunction with *in silico* predictions, provides a powerful set of instruments for making the best choices for vaccines based on epitope affinity and availability. Recent work has found experimental evidence to support different epitope presentation in the course of single cell infections, thus reinforcing the theory that even though immunodominant epitopes are to be selected as targets, combining the strength of several responses at the same time ultimately results in faster and more thorough clearance of the virus (15). Exposure of TMVcp to HHP, low temperature, and different concentrations of urea significantly altered the epitope profile and the recognition of linear antigens, in contrast to results reported in the literature. This information is very important, especially in cases where the regions containing the immunogenic determinants for this protein were conserved after treatment, with the unmasking of new epitopes hitherto hidden by the structure of the protein.

Future studies need to evaluate which treatments are most appropriate for different types of immune response, particularly with regard to variations in peptide synthesis. Examples of such studies include spot assays using molecules that mimic MHC class I and II interactions; the use of other immunoglobulin classes might shed light on questions related to immunodominance and VDJ recombinations. Furthermore, crystallographic studies can be done to identify changes that have occurred in the structure of a given protein. Studies based on enzymatic cleavage mediated by endogenous pathways of protein degradation in antigen presenting cells could explore whether the alterations caused by the treatments significantly impaired or favored distinct epitope profiles, as reported here for TMVcp, thereby improving the design of more effective vaccines.

Acknowledgments

The authors thank Ronald Frank and Susanne Daenicke (Helmholtz Centre for Infection Research, Braunschweig, Germany) for generous preparation of the peptide SPOT array, and Stephen Hyslop for editing the English of the manuscript. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grant

No. 2008/09835), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

Author Disclosure Statement

The authors declare that they have no competing financial interests.

References

- Al Moudallal Z, Briand JP, and Van Regenmortel MH. Monoclonal antibodies as probes of the antigenic structure of tobacco mosaic virus. *EMBO J* 1982;1:1005–1010.
- Altschuh D, Al Moudallal Z, Briand JP, *et al.* Immunochemical studies of tobacco mosaic virus.—VI. Attempts to localize viral epitopes with monoclonal antibodies. *Mol Immunol* 1985;22:329–337.
- Altschuh D, and Van Regenmortel MH. Localization of antigenic determinants of a viral protein by inhibition of enzyme-linked immunosorbent assay (ELISA) with tryptic peptides. *J Immunol Methods* 1982;50:99–108.
- Anderer FA. Preparation and properties of an artificial antigen immunologically related to tobacco mosaic virus. *Biochim Biophys Acta* 1963;71:246–248.
- Bendahmane M, Koo M, Karrer E, *et al.* Display of epitopes on the surface of tobacco mosaic virus: Impact of charge and isoelectric point of the epitope on virus-host interactions. *J Mol Biol* 1999;290:9–20.
- Benjamini E, Leung CY, and Rennick DM. Immunochemical studies on the tobacco mosaic virus protein. *Adv Exp Med Biol* 1978;98:165–179.
- Beutling U, Stading K, Stradal T, *et al.* Large-scale analysis of protein–protein interactions using cellulose-bound peptide arrays. *Adv Biochem Eng Biotechnol* 2008;110:115–152.
- Bhasin M, Lata S, and Raghava GP. TAPPred prediction of TAP-binding peptides in antigens. *Methods Mol Biol* 2007;409:381–386.
- Bispo JA, Bonafe CF, Joekes I, *et al.* Entropy and volume change of dissociation in tobacco mosaic virus probed by high pressure. *J Phys Chem B* 2012;116:14817–14828.
- Bonafe CF, Araujo JR, and Silva JL. Intermediate states of assembly in the dissociation of gastropod hemocyanin by hydrostatic pressure. *Biochemistry* 1994;33:2651–2660.
- Bonafe CF, Villas-Boas M, Suarez MC, *et al.* Reassembly of a large multisubunit protein promoted by nonprotein factors. Effects of calcium and glycerol on the association of extracellular hemoglobin. *J Biol Chem* 1991;266:13210–13216.
- Bonafe CF, Vital CM, Telles RC, *et al.* Tobacco mosaic virus disassembly by high hydrostatic pressure in combination with urea and low temperature. *Biochemistry* 1998;37:11097–11105.
- Calci KR, Meade GK, Tezloff RC, *et al.* High-pressure inactivation of hepatitis A virus within oysters. *Appl Environ Microbiol* 2005;71:339–343.
- Carmicle S, Steede NK, and Landry SJ. Antigen three-dimensional structure guides the processing and presentation of helper T-cell epitopes. *Mol Immunol* 2007;44:1159–1168.
- Croft NP, Smith SA, Wong YC, *et al.* Kinetics of antigen expression and epitope presentation during virus infection. *PLoS Pathog* 2013;9:e1003129.
- Dai G, Carmicle S, Steede NK, *et al.* Structural basis for helper T-cell and antibody epitope immunodominance in bacteriophage T4 Hsp10. Role of disordered loops. *J Biol Chem* 2002;277:161–168.
- Dai G, Steede NK, and Landry SJ. Allocation of helper T-cell epitope immunodominance according to three-dimensional structure in the human immunodeficiency virus type I envelope glycoprotein gp120. *J Biol Chem* 2001;276:41913–41920.
- Daniel S, Brusica V, Caillat-Zucman S, *et al.* Relationship between peptide selectivities of human transporters associated with antigen processing and HLA class I molecules. *J Immunol* 1998;161:617–624.
- De LMRC, and van Regenmortel MH. Immunochemical studies of tobacco mosaic virus—III. Demonstration of five antigenic regions in the protein sub-unit. *Mol Immunol* 1979;16:179–184.
- Dekker EL, Porta C, and Van Regenmortel MH. Limitations of different ELISA procedures for localizing epitopes in viral coat protein subunits. *Arch Virol* 1989;105:269–286.
- de Lima Neto DF, Stach-Machado DR, Spilki FR, Mattoso J, and Bonafe CFS. Epitope mapping of tobacco mosaic virus capsid protein: Prediction and experimental data from spot Synthesis. *Mol Biol* 2013;2:18.
- Diez-Rivero CM, Chenlo B, Zuluaga P, *et al.* Quantitative modeling of peptide binding to TAP using support vector machine. *Proteins* 2010;78:63–72.
- Duraes-Carvalho R, Souza AR, Martins LM, *et al.* Effect of high hydrostatic pressure on *Aeromonas hydrophila* AH 191 growth in milk. *J Food Sci* 2012;77:M417–M424.
- Emmerich NP, Nussbaum AK, Stevanovic S, *et al.* The human 26S and 20S proteasomes generate overlapping but different sets of peptide fragments from a model protein substrate. *J Biol Chem* 2000;275:21140–21148.
- England JL, and Haran G. Role of solvation effects in protein denaturation: From thermodynamics to single molecules and back. *Annu Rev Phys Chem* 2011;62:257–277.
- Ferreira E, Mendes YS, Silva JL, *et al.* Effects of hydrostatic pressure on the stability and thermostability of poliovirus: A new method for vaccine preservation. *Vaccine* 2009;27:5332–5337.
- Frank R, and Overwin H. SPOT synthesis. Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. *Methods Mol Biol* 1996;66:149–169.
- Garcia-Boronat M, Diez-Rivero CM, Reinherz EL, *et al.* PVS: A web server for protein sequence variability analysis tuned to facilitate conserved epitope discovery. *Nucleic Acids Res* 2008;36:W35–41.
- Gayan E, Condon S, Alvarez I, *et al.* Effect of pressure-induced changes in the ionization equilibria of buffers on inactivation of *Escherichia coli* and *Staphylococcus aureus* by high hydrostatic pressure. *Appl Environ Microbiol* 2013;79:4041–4047.
- Grey HM, Ruppert J, Vitiello A, *et al.* Class I MHC-peptide interactions: Structural requirements and functional implications. *Cancer Surv* 1995;22:37–49.
- Heremans K. High pressure effects on proteins and other biomolecules. *Annu Rev Biophys Bioeng* 1982;11:1–21.
- Heremans K, and Smeller L. Protein structure and dynamics at high pressure. *Biochim Biophys Acta* 1998;1386:353–370.
- Heremans L, and Zeyen T. Management of a cracked Acrysof intraocular lens during implantation. Case report. *Bull Soc Belge Ophtalmol* 1998;270:13–15.

34. Hopp TP, and Woods KR. Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci USA* 1981;78:3824–3828.
35. Hubbard SJ, Beynon RJ, and Thornton JM. Assessment of conformational parameters as predictors of limited proteolytic sites in native protein structures. *Protein Eng* 1998; 11:349–359.
36. Hubbard SJ, Eisenmenger F, and Thornton JM. Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. *Protein Sci* 1994;3:757–768.
37. Karplus PA, and Schulz GE. Prediction of chain flexibility in proteins. A tool for the selection of peptide antigens. *Naturwissenschaften* 1985;72:2.
38. Kim Y, Sette A, and Peters B. Applications for T-cell epitope queries and tools in the Immune Epitope Database and Analysis Resource. *J Immunol Methods* 2011;374:62–69.
39. Kolaskar AS, and Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett* 1990;276:172–174.
40. Kyte J, and Doolittle RF. A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 1982; 157:105–132.
41. Lou F, Neetoo H, Li J, *et al.* Lack of correlation between virus barosensitivity and the presence of a viral envelope during inactivation of human rotavirus, vesicular stomatitis virus, and avian metapneumovirus by high-pressure processing. *Appl Environ Microbiol* 2011;77:8538–8547.
42. Lundegaard C, Lamberth K, Harndahl M, *et al.* NetMHC-3.0: Accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. *Nucleic Acids Res* 2008;36:W509–512.
43. Neeffjes J, Jongsma ML, Paul P, *et al.* Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 2011;11:823–836.
44. Oger PM, Daniel I, and Picard A. In situ Raman and X-ray spectroscopies to monitor microbial activities under high hydrostatic pressure. *Ann NY Acad Sci* 2010;1189:113–120.
45. Pellequer JL, Westhof E, and Van Regenmortel MH. Predicting location of continuous epitopes in proteins from their primary structures. *Methods Enzymol* 1991;203:176–201.
46. Pontes L, Cordeiro Y, Giongo V, *et al.* Pressure-induced formation of inactive triple-shelled rotavirus particles is associated with changes in the spike protein Vp4. *J Mol Biol* 2001;307:1171–1179.
47. Rennick DM, Morrow PR, Benjamini E. Immunological studies with tobacco mosaic virus protein: Differential activation of B cell subpopulations. *Adv Exp Med Biol* 1982;150:201–208.
48. Roomp K, Antes I, and Lengauer T. Predicting MHC class I epitopes in large datasets. *BMC Bioinformatics* 2010;11:90.
49. Sanchez G, Aznar R, Martinez A, *et al.* Inactivation of human and murine norovirus by high-pressure processing. *Foodborne Pathog Dis* 2011;8:249–253.
50. Santos JL, Aparicio R, Joekes I, *et al.* Different urea stoichiometries between the dissociation and denaturation of tobacco mosaic virus as probed by hydrostatic pressure. *Biophys Chem* 2008;134:214–224.
51. Santos JL, Bispo JA, Landini GF, *et al.* Proton dependence of tobacco mosaic virus dissociation by pressure. *Biophys Chem* 2004;111:53–61.
52. Schlosser G, Mezo G, Kiss R, *et al.* Synthesis, solution structure analysis and antibody binding of cyclic epitope peptides from glycoprotein D of *Herpes simplex virus* type I. *Biophys Chem* 2003;106:155–171.
53. Silva JL, Villas-Boas M, Bonafe CF, *et al.* Anomalous pressure dissociation of large protein aggregates. Lack of concentration dependence and irreversibility at extreme degrees of dissociation of extracellular hemoglobin. *J Biol Chem* 1989;264:15863–15868.
54. Soutullo A, Santi MN, Perin JC, *et al.* Systematic epitope analysis of the p26 EIAV core protein. *J Mol Recognit* 2007;20:227–237.
55. Tenzer S, Stoltze L, Schonfisch B, *et al.* Quantitative analysis of prion-protein degradation by constitutive and immuno-20S proteasomes indicates differences correlated with disease susceptibility. *J Immunol* 2004;172: 1083–1091.
56. Thai R, Moine G, Desmadril M, *et al.* Antigen stability controls antigen presentation. *J Biol Chem* 2004;279: 50257–50266.
57. Toes RE, Nussbaum AK, Degermann S, *et al.* Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products. *J Exp Med* 2001;194:1–12.
58. Van Regenmortel MH. The antigenicity of tobacco mosaic virus. *Philos Trans R Soc Lond B Biol Sci* 1999;354: 559–568.
59. Van Regenmortel MH, Altschuh D, and Klug A. Influence of local structure on the location of antigenic determinants in tobacco mosaic virus protein. *Ciba Found Symp* 1986; 119:76–92.
60. Warren RL, and Holt RA. A census of predicted mutational epitopes suitable for immunologic cancer control. *Hum Immunol* 2010;71:245–254.
61. Wingfield PT, Stahl SJ, Thomsen DR, *et al.* Hexon-only binding of VP26 reflects differences between the hexon and penton conformations of VP5, the major capsid protein of herpes simplex virus. *J Virol* 1997;71:8955–8961.
62. Zhang Q, Wang P, Kim Y, *et al.* Immune epitope database analysis resource (IEDB-AR). *Nucleic Acids Res* 2008;36: W513–518.

Address correspondence to:
 Dr. Daniel Ferreira de Lima Neto
 Laboratório de Virologia Animal
 Departamento de Genética, Evolução e Bioagentes
 Instituto de Biologia
 Universidade Estadual de Campinas (UNICAMP)
 Cidade Universitária Zeferino Vaz
 Rua Monteiro Lobato, 255
 13083-862, Campinas, SP
 Brazil

E-mail: danielviro@gmail.com