Peptides designed to spatially depict the Epstein-Barr virus major virion glycoprotein gp350 neutralization epitope elicit antibodies that block virus neutralizing antibody 72A1 interaction with the native gp350 molecule.

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Running head: Gp350 peptides mimic an EBV neutralizing epitope

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Abstract

Epstein-Barr virus (EBV) is the etiologic agent of infectious mononucleosis and the root cause of B-cell lymphoproliferative disease in individuals with a weakened immune system, as well as a principal cofactor in nasopharyngeal carcinoma, various lymphomas and other cancers. The EBV major virion surface glycoprotein (gp)350 is viewed as the best vaccine candidate to prevent infectious mononucleosis in healthy EBV-naive persons and EBV-related cancers in at-risk individuals. Previous epitope mapping of gp350 reveals only one dominant neutralizing epitope, which has been shown to be the target of the monoclonal antibody 72A1. Computer modeling of 72A1 antibody interaction with the gp350 amino terminus was used to identify gp350 amino acids that could form strong ionic, electrostatic or hydrogen bonds with the 72A1 antibody. Peptide DDRTTQLAQNPVYIPETYPYIKWDN (designated as peptide 2) and peptide GSAKPGNSYFASVKTEMLGNEID (designated as peptide 3) were designed to spatially represent the gp350 amino acids predicted to interact with the 72A1 antibody paratope. Peptide 2 bound to the 72A1 antibody and blocked 72A1 antibody recognition of the native gp350 molecule. Peptide 2 and peptide 3 were recognized by human IgG and shown to elicit murine antibodies that could target gp350 and block its recognition by the 72A1 antibody. This work provides a structural mapping of the interaction between the EBV neutralizing antibody 72A1 and the major virion surface protein gp350. Gp350 mimetic peptides that spatially depict the EBV neutralizing epitope would be useful as a vaccine to focus the immune system exclusively to this important virus epitope.
Importance

The production of virus-neutralizing antibodies targeting the Epstein-Barr virus (EBV) major surface glycoprotein gp350 is important for the prevention of infectious mononucleosis and EBV-related cancers. The data presented here provide the first in silico map of gp350 interaction with a virus blocking monoclonal antibody. Immunization of gp350 peptides identified by in silico mapping generated antibodies that cross-react with the EBV gp350 molecule and block recognition of the gp350 molecule by a virus neutralizing antibody.

Through its ability to focus the immune system exclusively on the gp350 sequence important for viral entry, these peptides may form the basis of an EBV vaccine candidate. This strategy would side-step the production of other irrelevant gp350 antibodies that divert the immune system from generating a protective antiviral response or that impede access to the virus blocking epitope by protective antibodies.
Introduction

Epstein-Barr virus (EBV) is the cause of infectious mononucleosis (IM) (1), and is considered a seminal contributor to the development of nasopharyngeal carcinoma and certain forms of B, NK and T cell lymphoma (2). Although EBV is ubiquitous worldwide, nearly 50% of young adults and children in developed countries are susceptible to primary EBV infection and debilitating IM (3). An important clinical consequence of primary EBV infection in immunosuppressed organ transplant patients is post-transplant lymphoproliferative disorder (PTLD) (4). Depending on the type of organ graft and on the degree of immunosuppression needed to prevent rejection, patient risk for PTLD is documented to be 10 to 76-fold higher in organ recipients who acquire primary EBV infection post-transplant compared to organ recipients who were EBV-seropositive prior to transplant (5, 6).

The EBV major virion surface glycoprotein (gp)350 is the principal target of naturally occurring neutralizing antibodies and is viewed as the best vaccine candidate to prevent IM in healthy EBV-naive young adults or to prevent PTLD in at-risk organ recipients (7, 8). The 838 amino acid ectodomain of a mature 350 kDa molecule is highly glycosylated and contains at least eight unique immunodominant B-cell epitopes (9). Experimental evidence indicates, however, that recognition of only one epitope, as represented by the monoclonal antibody 72A1 (10), effectively blocks infection by inhibiting EBV binding to the cellular receptors CD21 and CD35 (11, 12). Although an electron density map of the first 440 amino acids of the gp350 molecule localizes the 72A1 epitope to a planar structure devoid of carbohydrates (13), the amino acids within this gp350 surface structure, which interact with the 72A1 antibody and constitute the principal EBV neutralization epitope, are still unidentified.

A better elucidation of the amino acids recognized by the 72A1 monoclonal antibody should provide essential mapping points to guide the design of an EBV peptide mimetic vaccine that would focus the humoral arm of the immune system exclusively to this one...
important epitope. The present work provides the first in silico mapping, with biochemical support, of the interaction between gp350 and the virus neutralizing monoclonal antibody 72A1.
Materials and Methods

Cell culture. The EBV producing cell line P3HR1 (HTB-62, American Type Culture Collection, Manassas, VA), the anti-gp350 hybridoma 72A1 (HB-168, ATCC), the Burkitt cell line Raji (CCL-86, ATCC) and the T cell line CEM (HTB-45, ATCC) were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS and antibiotics. The Chinese hamster ovary cell line CHO-K1 (CCL-61, ATCC) was grown in Kiation’s F-12K medium supplemented with 10% FBS and antibiotics.

Peptide sequencing. The amino-terminal amino acid sequences of the 72A1 antibody heavy and light chains were determined by Edman degradation (kindly performed by Dr Claude Lazure, Institut de Recherche Clinique de Montréal, Montréal, QC) following antibody purification by protein-A affinity chromatography, resolution of the antibody heavy and light chain peptides in a denaturing 12% PAGE gel and transfer to a PVDF membrane.

Cloning of the heavy and light variable regions. Hybridoma heavy and light chain mRNA sequences were cloned as outlined in published protocols (14, 15). Briefly, total RNA was isolated from hybridoma cells using Tri reagent (Molecular Research Center, Cincinnati, OH), followed by ethanol precipitation and suspension in RNase-free water (Invitrogen Canada, Burlington, ON). cDNA strands were initially generated using the Enhanced Avian RT First Strand Kit (Sigma-Aldrich Canada, Oakville, ON) and primers encoding the conserved heavy and light chain J-sequences JHRC (5’-CTGAGGAGACGGTGACCATGGTCCCTTGGCCCC-3’) and JκRC (5’-CGTTTGATTTCCAGCTTGGTCCC-3’), respectively (14). Subsequent PCR amplification of the heavy and light chain variable region cDNA sequences was performed using AccuPrime Pfx DNA polymerase (Invitrogen Canada) in conjunction with heavy or light chain variable-region degenerate primer sets (TanTec Biosystems, Montreal, QC) (15-17). In order to differentiate authentic light chain variable region cDNA from endogenous aberrant κ-chain mRNA (18), 1.7-fold excess (175 µM) aberrant-chain-specific primer
(ACCTATTACTGTCAGCACATTA) was added to the PCR reaction mix (15). This strategy was expected to preferentially amplify the aberrant κ chain and produce a smaller PCR product enabling identification of authentic light chain variable region cDNA products. Authentic heavy chain cDNA products generated by PCR were identified by screening for the presence of aberrant heavy chain transcripts (19). Authentic heavy and light chain cDNAs were cloned into pCR-BLUNT-II TOPO vector (Invitrogen Canada, Burlington, ON) for subsequent DNA sequencing.

**Construction and expression of a human-mouse chimeric anti-gp350 IgG1 antibody.**

Human IgG1 heavy chain and human κ chain constant region cDNAs were obtained by (RT)PCR amplification of peripheral blood lymphocyte RNA using primers specific to the amino terminal segment WGQGTMVTSSAST of the human IgG heavy chain constant region (5'-TGGGGCCAAGGGACAATGGTCACCGTCTCTTCAGCCTCCACC-3'), the terminal IgG amino acids (5'-TCATTTACCCGGAGACAGGGAG-3'), the amino terminal segment GTKLEIK of the human Ig κ chain constant region (5'-GGGACCAAGCTGGAAATCAAACG-3') and the terminal Ig κ chain amino acids (5'-CTCCCTCTAACAACACTCTCCCCTG-3'). Following in-frame ligation of the 72A1 heavy and light chain variable regions to their respective human Ig constant regions, the chimeric heavy and light chain antibody cDNAs were cloned into eukaryotic expression plasmids pHook-3 and pcDNA3.1, respectively, allowing for selection of G418 and Zeocin drug resistant cells.

CHO-K1 cells were dual-transfected with chimeric light chain and heavy chain expression plasmids using Lipofectamine 2000 (Invitrogen), plated in culture medium containing 500 μg/ml G418 and 300 μg/ml Zeocin and, after three weeks of culture, individual colonies were isolated and tested for chimeric IgG1 antibody reactivity to gp350.

Gp350-expressing CEM cells, generated following transfection of the gp350 expression plasmid pCMVIE-EBMA (20), were used as a substrate to measure and confirm gp350
reactivity. Gp350 reactivity was measured by fixed cell immunofluorescence and by FACS analysis.

**EBV antibody neutralization assay.** Antibody neutralization potency was measured as the inhibition of EBV early antigen (EA) expression upon super-infection of Raji cells by P3HR1 virus (21). Mixtures of concentrated virus (2 x 10^4 EA-inducing units) and serial dilutions of purified antibody were incubated in a final volume of 0.1 ml for 60 min at 37°C prior to the addition of 5 x 10^4 Raji cells, and further incubated for 60 min at 37°C. The Raji cells were then washed in RPMI medium and cultured for 2 days. The number of Raji cells expressing EA after cell culture was measured by immunofluorescence, and relative antibody neutralization potency per µg of antibody was calculated.

**In silico identification of EBV gp350:72A1 antibody interaction.** Prediction of the gp350 domain that interacts with the 72A1 monoclonal antibody was performed by aligning 72A1 variable region protein sequences with the previously reported 3D structure for the gp350 amino terminal peptide (MMDB ID: 42235) (13). Identification of the 72A1 complementarity determining regions (CDR) was based on previously described methods [http://www.bioinf.org.uk/abs/](http://www.bioinf.org.uk/abs/) (22). The 3D structure of the 72A1 antibody variable region was generated by the Web Antibody Modeling server ([http://antibody.bath.ac.uk/index.html](http://antibody.bath.ac.uk/index.html), Dr Anthony R. Rees, Centre for Protein Analysis and Design, University of Bath, UK) and the Rosetta Antibody Modeling Server ([http://antibody.graylab.jhu.edu](http://antibody.graylab.jhu.edu)) (23). 72A1 antibody:gp350 protein docking was performed using SnugDock (24) and compared with lysozyme:gp350 docking which served as an irrelevant docking protein. The molecular modeling programs USCF Chimera 1.9 and SuperMimic were used to design mimetic peptides comprised of gp350 amino acids predicted to interact with the 72A1 antibody (25, 26).

**Peptide and whole cell ELISA.** Gp350 mimetic peptides were synthesized and HPLC purified to > 70% (Elim Biopharmaceuticals, Hayward, CA). The peptides were resuspended at
2.5 μg/ml in 0.1M carbonate buffer pH 9 prior to overnight incubation at 4°C as 100 μl aliquots in a 96-well ELISA plate (Ultident, St. Laurent, QC). Alternatively, gp350 mimetic peptides containing an additional C-terminal cysteine and coupled to maleimide-activated BSA (Sigma Conjugation Kit, Sigma-Aldrich) were suspended at 3 μg/ml in PBS, and 100 μl was added to each ELISA plate well for overnight incubation at 4°C. Peptide-coated plates were washed 4X with PBS:0.05% Tween-20, blocked with 1% BSA:PBS and incubated for a minimum of 2 h at 37°C with anti-gp350 monoclonal antibody 72A1 or the isotype-matched background control antibody (anti-LMP1 monoclonal antibody S-12) (27). Gp350 peptides coupled to BSA were also tested for human IgG reactivity using 39 sample lots of commercial intravenous immune globulin (IVIG). Bound monoclonal antibodies were detected using biotin-conjugated goat anti-mouse or anti-human IgG and horseradish peroxidase (HRP)-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) in conjunction with 3,3',5,5'-Tetramethylbenzidine substrate (TMB solution, Bioshop, Burlington ON). HRP enzyme activity was stopped with an equal volume of 2N HCl, and wells were read at 450 nm/570 nm.

Whole cell ELISA was performed using CEM cells and CEM cells expressing cell-surface gp350. The cells were washed with PBS, suspended at 1 x 10^5 cells per 40 μl which was added to each ELISA plate well. Plates were vacuum-dried and cells were fixed to the plate with cold methanol. Plates were subsequently washed with PBS and wells blocked with 1% BSA prior to a 2-h incubation with antibody. In 72A1 competition assays, plates were incubated for 2 h with gp350 mimetic peptides or mouse serum derived from peptide-immunized mice prior to addition of purified 72A1 or biotin-conjugated 72A1 antibody, respectively (28).

**Peptide immunization.** Balb/C female mice were immunized intraperitoneally with 100 μg of keyhole limpet hemocyanin (KLH) coupled with gp350 mimetic peptide (Sigma Conjugation Kit) emulsified in Sigma Adjuvant System (Sigma-Aldrich). Mice were re-injected every 21 days.
and blood was drawn 14 days after the second injection. The animal experimentation protocol was approved by our Institutional Committee for Good Animal Practice in Research.
Results

Identification and functional validation of cloned 72A1 Fab sequences. Previous epitope mapping of gp350 revealed one dominant neutralizing epitope, which was also shown to be the target of the neutralizing monoclonal antibody 72A1(13, 21, 29). Our laboratory cloned and sequenced the heavy and light chain variable region RNA sequences for this antibody, and verified by Edman protein sequencing that the cDNA sequences predicted to encode the two antibody chains matched both the mature heavy and light chain proteins. The predicted heavy and light chain variable region peptide sequences, corresponding CDRs (bold) and matching Edman amino acid sequences are shown in Figure 1.

Protein BLAST comparison of 72A1 variable region peptides with the monoclonal antibody peptides listed in the non-redundant (nr) database of the National Center for Biotechnology Information (ncbi.nlm.nih.gov) showed >98% sequence identity with several listed monoclonal antibodies for both the heavy and light chain variable regions. For the 72A1 light chain peptide, we noted a >98% identity with the anti-human tumor-associated antigen monoclonal antibody [accession # AAA39002], the anti-carcinoma-associated antigen monoclonal antibody 17-1A [accession # AAA38774], and the anti-flavivirus monoclonal antibody Fab4g2 [accession # 1UYW_L]. Analysis of the 72A1 heavy chain variable peptide showed >98% identity with that of MopC21 (30). This latter finding suggests that, in addition to forming the anti-gp350 paratope, the 72A1 heavy chain may also act as an Ig scaffold to better position 72A1 light chain interaction with the gp350 molecule (31).

In order to verify the authenticity of our 72A1 variable region cDNAs, a human-mouse chimeric antibody was constructed and tested for gp350 recognition and for virus neutralization function. As measured by fixed cell immunofluorescence, FACS analysis and culture-based EBV neutralization assay, the chimeric anti-gp350 monoclonal faithfully recognized cell-surface gp350 (Figure 2 A) and blocked P3HR1 EBV super-infection of Raji cells on par with that of the
murine version of 72A1 when tested at log10 dilutions of 0.01 to 10 µg/ml (Figure 2B). These results indicate that the 72A1 monoclonal antibody heavy and light chain variable regions were faithfully cloned.

**In silico identification of EBV gp350:72A1 antibody interaction.** Following antibody computer modeling of the 72A1 variable region peptide sequences, *in silico* identification of the gp350/220 interaction with the 72A1 antibody was performed by spatially aligning the 72A1 variable region peptide sequences with the amino-terminal portion of gp350 using the SnugDock algorithm (13). The SnugDock molecular docking program predicts the optimal alignment for close coupling of the 72A1 variable region with the gp350 molecule by identifying the lowest energy structure between two molecules, and lists the interfacing residues and types of interactions (24). SnugDock alignment indicated that the gp350 amino terminus couples to 72A1 variable region through four gp350 interfacing peptide sequences, namely 14QLTRDDP20, 144QNPVYLIPETVPYIKWDN161, 194SVKTEMLGNEIDIECIME211 and 288KASG291. Other individual amino acids on the surface of the gp350 molecule also aligned closely with the 72A1 antibody binding site and included lysine106, glutamine Q118, threonine228 and phenylalanine281. In conjunction with the weaker covalences formed between the four gp350 coupling peptides and the 72A1 variable region, individual amino acids in these four peptides were predicted to contribute strong H-bonding or to form a salt bridge. A listing of the amino acids on the surface of gp350 that interacted with the 72A1 heavy and light chain variable region, and a corresponding spatial depiction of this interaction is shown in Table 1 and depicted in Figure 3.

**Gp350 peptide mimetic blocks 72A1 binding to gp350.** In order to validate the gp350 amino acids predicted by SnugDock to couple with the 72A1 antibody variable region, two gp350 mimetic peptides were designed that were expected to faithfully position these gp350 amino acids for interaction with the 72A1 antibody paratope (Table 2). The mimetic design
GSAKPGNGSYF-A-SVKTEMLGNEID (designated as peptide 3, p3) encompassed the central area of the purported gp350 interaction domain and was comprised of domains 3 and 4 (Figure 3). The peptide was comprised of an inverted form of the gp350 sequence 282FYSGNGPKASG292 which was spatially joined via an alanine (A) to the gp350 sequence 194SVKTEMLGNEID205. The mimetic design DDRTLQL-A-QNPVYLIPETVPYIKWDN (designated as peptide 2, p2) encompassed the perimeter portion of the purported gp350 neutralizing domain and consisted of domains 1 and 2 (Figure 3). The peptide was comprised of an inverted form of the gp350 sequence 14QLTRDDP20 spatially joined with an alanine (A) to gp350 sequence 144QNPVYLIPETVPYIKWDN161. We also used the gp350 linear peptide 422SKAPESTTSTPLNTTGFDAY441 (designated as peptide 1, p1) which was previously shown to lie outside the gp350 neutralizing domain (Figure 3) and expected not to couple with the 72A1 antibody (13, 29). As shown in Figure 4A, p2 was recognized by the 72A1 antibody, whereas peptides 3 and 1 were not recognized by the 72A1 antibody. Recognition of p2 by the 72A1 antibody was shown to be specific as evidenced by the observation that the monoclonal antibody S-12, which recognizes an intracellular epitope on the EBV LMP-1 protein, failed to recognize p2 (Figure 4A).

Although p3 contained the amino acid sequence EID, which was shown by mutation studies to result in a loss of 72A1 binding to the amino terminal portion of the gp350 molecule (13), the lack of recognition by the 72A1 antibody may have been due to steric hindrance introduced during adsorption to the ELISA plate. To avoid potential ELISA plate interference, gp350 mimetic peptides were allowed to interact with 72A1 antibody in saline solution prior to subsequent antibody challenge with native gp350 protein. As shown in Figure 4B, p2 in solution was recognized by the 72A1 antibody binding site and effectively competed for gp350 recognition by the 72A1 antibody. P2 alone inhibited 72A1 recognition of gp350 by 78% compared to p1 (p=0.008). Solutions containing p2 and p1 inhibited 72A1 recognition of gp350.
by 73% compared to p1 alone (p=0.009) and solutions containing p2 and p3 inhibited 72A1 recognition of gp350 by 66% compared to p1 alone (p=0.008). Peptide 3 was not strongly recognized by the 72A1 antibody when suspended in saline, as p3 was shown to block 72A1 antibody recognition of gp350 protein by 8% (Figure 4B, peptide 3). We also noted that p2 comprised the principal elements of the 72A1 epitope, as incubation of 72A1 with an equal mixture of peptides 2 and 3 did not increase inhibition levels of gp350 recognition by 72A1 antibody (Figure 4B, 78% for p2 versus 66% for peptides 2 and 3).

In order to define whether gp350 domain 1, domain 2 or both domains of p2 were necessary for maximal epitope recognition by the 72A1 antibody, amino acids predicted by SnugDock modeling to interface with the 72A1 antibody were replaced by alanine (Table 2). Alanine was selected based on its small R-chain size and nonpolar nature. As shown in Figure 4C, alanine replacement in domain 2 abolished recognition of p2 by 72A1 antibody as compared to unaltered p2. Alanine replacement of 72A1 interfacing amino acids in p2 domain 1 resulted in a 50% reduction in recognition of p2 by the 72A1 antibody. These results indicate that amino acid sequences found in both domain 1 and domain 2 contribute to maximum epitope recognition by the 72A1 antibody.

**Gp350 mimetic peptide elicits gp350 cross-reactive antibodies and is recognized by human IgG.** In order to determine whether p2 was capable of generating antibodies that recognize native gp350 and that block recognition of gp350 by 72A1 antibody, mice were immunized with individual peptides. Following the third immunization, IgG levels of peptide reactivity were measured by ELISA. Results indicate that all mice generated antibodies to their respective peptides with average antibody concentrations (pg/ml) of 97 ± 46 SEM, 122 ± 67 and 981 ± 577 for p1, p2 and p3, respectively (Figure 5A). When these same mice sera were assayed for recognition of native gp350, results revealed a 2-log₁₀ reduction in antibody titers, with average antibody concentrations (pg/ml) of 0.015 ± 0.01 SEM, 1.3 ± 0.9 and 4.1 ± 2.4 for...
p1, p2 and p3, respectively (Figure 5B). As previously reported by Wilson and Morgan (1988), inhibition of 72A1 antibody recognition of gp350 by an ELISA-based competition assay directly correlates with the level of EBV neutralizing antibody in a given test serum (28). Competition assays to determine whether sera from peptide-immunized mice have the ability to interfere with 72A1 recognition of gp350 indicated that p2- and p3-derived antisera were capable of inhibiting 72A1 recognition of gp350 by 38% and 23%, respectively (Figure 5C). Pooled sera from both p2 and p3 immunized mice resulted in an additive reduction of 51% in 72A1 antibody recognition of gp350 (Figure 5C). These results indicate that p2, while eliciting four times less anti-gp350 antibody compared to p3 (Figure 5B), exhibited a 1.7-fold greater 72A1 antibody blocking capacity (Figure 5C).

**Gp350 mimetic peptides are recognized by human IgG.** While p2 was recognized by the murine monoclonal antibody 72A1, and both p2 and p3 were shown to generate antibodies that blocked 72A1 recognition of the gp350 epitope, it is unknown whether EBV positive human sera can recognize these gp350 mimetic peptides. The determination of human IgG reactivity against each of the three peptides could serve as a surrogate indicator of whether the gp350 mimetic peptides would be immunogenic or capable of generating an anti-gp350 antibody response. The mimetic peptides, when assayed with human EBV immune serum derived from 39 unique lots of IVIG, each representative of >1000 healthy blood donor units (32, 33), revealed that all three peptides were recognized (Figure 6). Peptide 2 exhibited the highest antibody levels at 2.16 ± 0.08 SD pg/ml. This was followed by p1 at levels of 1.83 ± 0.07 pg/ml and by p3 with antibody levels of 1.7 ± 0.06 pg/ml. These results suggest that anti-gp350 serum antibodies found in a large segment of the population recognize these gp350 mimetic peptides.
Epstein-Barr virus causes IM in approximately 500 young adults per 100,000 persons in the United States annually (3), and is associated with nearly 200,000 cases of EBV-related cancers per year worldwide (34). Although clinical symptoms of pharyngitis, lymphadenopathy, fever and severe fatigue (3) were shown to resolve in 62% of patients within 2 months, up to 10% of individuals with IM experience persistent fatigue lasting 6 months or longer (35). In addition to the human toll caused by cancer, the financial costs from treatment and spent resources, rehabilitation and indirect expenses due to lost economic output were estimated at $14 billion/year for EBV-related cancers (34, 36). Vaccines have proven to be the most cost-effective means to protect individuals and populations against infectious disease (37). Thus an EBV vaccine that protects against IM or reduces the incidence of EBV-associated cancers would constitute a substantial public health and economic benefit (38).

The EBV major virion surface glycoprotein gp350 is the principal target of naturally occurring neutralizing antibodies, and serves as a first line of defence during primary EBV infection (39). Gp350 neutralizing antibodies prevent B-cell immortalization in culture (10, 40, 41) and B-cell lymphoma in small primates (42) by blocking virus attachment to its cognate cellular receptor (11, 12). There are at least eight unique immunodominant B-cell epitopes located on the gp350 ectodomain (9); however, as revealed by experiments using the 72A1 antibody, only one highly conserved virus neutralization epitope is present on gp350 (10). The exact amino acids that comprise the core neutralization sequence for 72A1 have yet to be fully delineated (13, 21, 29). Identification of the core peptides forming the gp350 neutralization epitope for the 72A1 antibody would be extremely useful in vaccine construction, as immunization with these peptides would focus the efforts of the humoral immune system exclusively on this important virus epitope. Vaccination with the core neutralization peptide(s) or in-kind mimetics would eliminate the production of other gp350 antibodies that may divert...
the immune system from generating appropriate neutralizing antibodies or impede access to the neutralization epitope. Vaccine designs that utilize the core neutralizing epitope of gp350 would also alleviate unintended interactions with the cognate cell receptor that a larger gp350 subunit vaccine might elicit (43, 44).

SnugDock alignment indicated that the gp350 amino terminus couples to the 72A1 variable region through four interfacing peptide sequences, namely 14QLTRDDP20, 144QNPVYLIPETVPYIKWDN161, 194SVKTEMLGNEIDCIME211 and 288KASG291. These results confirm the notion that the gp350 binding site of 72A1 involves amino acids exclusive to the glycan-free region of gp350, and 72A1 binding to the domain 1 sequence QLTRDDP could mask or occlude the adjacent CD21 recognition sequence DDPGFFNVE (45, 46). Based on earlier reports, the gp350 peptide 139HHAEMQNPVYLIPETVPYIKWDN161 is known to be involved in virus neutralization (13, 21, 29) with peptide PYI involved in 72A1 antibody recognition (47). Studies by Young et al (2008), which mapped gp350 amino acids involved in CD21 binding, found that alanine substitution at tyrosine148, glutamic acid152, isoleucine157 and tryptophan159 consistently reduced gp350 binding to CD21 (46). We noted that in addition to the amino acids identified in these previous reports (grayed amino acids), the adjacent and additional amino acids QNPVYLIPETVPYIKWDN (in bold) are predicted to be involved with several H-bonds in the light chain CDR1 (-) and in the light chain framework1 leucine5 (=) (Table 1).

Szakonyi et al previously noted that double mutational changes in gp350 aspartic acid205 and glutamic acid207 resulted in a loss of gp350 recognition by 72A1 (47), while the companion studies by Young et al (2008) found that glutamic acid203, and aspartic acid residues 205 and 212 again consistently reduced gp350 binding to CD21 (46). Our SnugDock computer model agrees with and extends these earlier findings. We noted the additional involvement of amino acids (in bold) 194SVKTEMLGNEIDCIME211 and 288KASG291, some of which form H-
bonds with heavy chain CDR2 and CDR3 (=) or a salt bridge (+) with CDR3 histidine\textsubscript{104}. We observed that \textsubscript{288}K\textsubscript{ASG}\textsubscript{291} interfaced with the 72A1 heavy chain and H-bonded (=) with heavy chain CDR1 (Table 1). This observation is in agreement with an earlier report which found that the peptide \textsubscript{279}YVFYGNGPK\textsubscript{ASG}GDYCIQS\textsubscript{298} could block 72A1 binding \textit{in vitro} (29).

The design and biochemical assessment of peptides that represent these four 72A1 antibody interacting domains revealed that only p2 was recognized by 72A1 antibody and that domain 1 sequence DDRTLQL and domain 2 sequence NPVYLIPETVPYIKWDN, which comprised p2, were both required for maximum blocking of gp350 recognition by the 72A1 antibody (Figure 4C).

In order to determine whether the p2 amino acid sequence alone was capable of generating antibodies which recognize native gp350 and block recognition of gp350 by the 72A1 antibody, mice were immunized with individual peptides. Results indicate that all three peptides elicited an equal and robust antibody response, but only 1\% or less of the anti-peptide antibodies could recognize gp350 (Figure 5B) and block 72A1 antibody recognition of gp350 to a maximum of 51\% (Figure 5C). The lower level of anti-gp350 antibodies elicited by the peptide immunogens suggests that the majority of injected peptides adopted a different immunogenic conformation when coupled to KLH compared to their native conformation in the gp350 planar domain (48-50). Prior studies (51-53) suggest that transplanting the mimetic peptides onto a scaffold protein may better imitate the gp350 planar domain and aid in the generation of anti-gp350 antibodies with higher affinities compared to our current KLH terminally-coupled peptides. The 51\% inhibition of gp350 recognition by 72A1 antibody with pooled p2 and p3 antisera suggests that division of SnugDock-identified domains into two separate peptides or the omission of charged amino acids K\textsubscript{106} and Q\textsubscript{118} noted in the SnugDock model (Table 1 and Figure 3) may have been crucial to generate a more complete gp350 neutralization epitope. Future use of a scaffold protein that stabilizes the gp350
peptides into a conformation that most approximates that of the native gp350 molecule (54) or
the use of larger peptides that faithfully incorporate p2 and p3 sequences as well as K106 and
Q118, may improve generation of EBV neutralizing antibodies.

This work provides a structural understanding of EBV gp350 interaction with the EBV
neutralizing antibody 72A1. Resolution of the contacts between an antigen and its cognate
neutralizing antibody provides a launching point for gp350 vaccine designs that retain
immunologically important epitopes while eliminating potentially distracting or unnecessary
antigenic features.
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The authors declare no commercial interest in any of the products mentioned herein.
References


design of epitope-scaffolds allows induction of antibodies specific for a poorly immunogenic HIV vaccine epitope. Structure 18:1116-1126.


Table 1. Anti-gp350 variable region interaction with soluble gp350

<table>
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<th>CDR amino acid sequence</th>
<th>Predicted 72A1:gp350 amino acid bonding</th>
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<td>SER\text{\textsubscript{52}} -H- ASP\text{\textsubscript{205}}</td>
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<td>PRO\text{\textsubscript{103}} +/- SER\text{\textsubscript{194}}</td>
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<td>HIS\text{\textsubscript{104}} +/- GLU\text{\textsubscript{211}}</td>
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<td>LC-FW</td>
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<td>LEU\text{\textsubscript{5}} -H- TYR\text{\textsubscript{148}}</td>
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</table>

LC, light chain; HC, heavy chain; FW, frame-work; CDR, complementarity determining region; H, hydrogen bond; +/-, salt bridge; bold lettering represents interfacing residue. Amino acid numbering was derived from 72A1 mature heavy and light chain peptide sequences depicted in Figure 1 and the gp350 amino terminal peptide sequence depicted in Figure 3.
<table>
<thead>
<tr>
<th>Peptide ID</th>
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<tr>
<td>P1</td>
<td>419SKAPETTTSLNTTGADY439-AC”</td>
<td>Located outside the 72A1 antibody recognition domain</td>
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<tr>
<td>P2</td>
<td>19DDRTLQL14-A-145NPVYLIPETVPYIKWDN165-AC</td>
<td>Comprised of 72A1 antibody recognition of domain 1 and domain 2</td>
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<td>P2D1</td>
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<td>Measures contribution of domain 1 in 72A1 antibody recognition of peptide 2</td>
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<tr>
<td>P3</td>
<td>291GSAKPGNGSYF281-A-194SVKTEMLGNEID205-AC</td>
<td>Comprised of 72A1 antibody recognition of domain 4 and domain 3</td>
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</table>

#-AC tag was added for peptide-cysteine-3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) coupling to BSA or KLH.
Figure 1. 72A1 heavy and light chain variable region sequences. Top panels, Anti-gp350 chimeric antibody amino acid sequence with predicted CDRs (bold) and heavy (=) and light chain (-) amino acids that interact strongly with gp350. Amino-terminal peptide sequences determined by Edman sequencing (gray). The 72A1 variable region and human Ig constant region junctions are shown (†). Bottom panel, depiction of 72A1 variable region light chain (blue) and heavy chain (green). Predicted CDR (space filled) and framework amino acids in the light chain or heavy chain predicted to H-bond with gp350 are shown in yellow and purple, respectively (Table 1). Heavy chain amino acid His^{104} predicted to form a salt bridge with gp350 Glu^{211} is shown in red.

Figure 2. Chimeric anti-gp350 antibody recognizes native gp350 and blocks EBV infection of B cells. (A) Chimeric anti-gp350 antibody recognized gp350 expressed on the surface of CEM cells (CEMgp350) as measured by immunofluorescence microscopy (left panel) or by FACS analysis (right panel). (B) Log_{10} doses of chimeric anti-gp350 or of the murine 72A1 monoclonal antibody block P3HR1 virus super-infection of Raji cells.

Figure 3. Depiction of the amino acids on the surface of gp350 predicted to couple with the 72A1 variable region. Top panel, interfacing amino acids (bold) predicted to H-bond or salt bridge with the 72A1 heavy chain (=) or light chain (-) variable region in domain 1 (green), domain 2 (cyan), domain 3 (red) and domain 4 (orange) (Table 1). Peptide 1 sequence, used in Figures 4 to 6, and lying outside the gp350 neutralizing domain, is shown in blue. Bottom panel, front view (left) and side view (right) of the gp350 amino terminus (tan ribbon) with glycosylation (ball & stick) showing domain 1 to 4 peptides (space filled) predicted to interact with the 72A1 antibody.
**Figure 4.** Gp350 mimetic peptide binds to 72A1 antibody to block gp350 recognition. (A) OD$_{450}$nm values ± SE for 72A1 (closed box) or anti-LMP1 S12 (open box) binding to peptides 1 to 3 (Table 2). Plots were derived from duplicate samples and from three separate experiments. (B and C) Average percent inhibition ± SEM for 72A1 antibody recognition of gp350 in an ELISA-based competition assay by peptides 1 to 3. Plots were derived from duplicate samples and from three separate experiments. P-values < 0.01 for peptide 2 versus peptide 1 sera are indicated (*).

**Figure 5.** Gp350 peptides generate anti-gp350 antibodies that block 72A1 recognition of gp350. (A) Box plot of anti-peptide antibody concentration found in peptide immunized mice (n=4). (B) Box plot of anti-gp350 concentration found in peptide immunized mice (n=4). (C) Histogram of biotinylated-72A1 binding to gp350 protein that was pre-exposed to mouse preimmune (preimm) serum, pooled serum from peptide immunized mice (1:50 dilution), 1 µg/ml 72A1, or 0.5 mg/ml BSA. Preimmune serum recognition of peptide or gp350 is indicated (○). Average OD ± SEM was derived from three separate experiments. P-values < 0.05 for peptides 2 and 3 sera versus peptide 1 serum are indicated (*).

**Figure 6.** Human immune globulin recognized gp350 mimetic peptides. Anti-peptide antibody concentrations are depicted as a box-and-whiskers plot with sample values outside the first percentile and 99th percentile (●). Plot values were calculated from duplicate samples in three separate experiments using 39 different lots of intravenous immunoglobulin.
Mature chimeric light chain:

\[
\begin{align*}
\text{VLSQLVMTQP} & \text{KSVSTV} \text{WQKPPKLLIY} \text{GASNRTGVPDRTGS} & \text{70} \\
\text{ATDFLTISSVQAELD} & \text{GQGSPY} \text{TFFGGTKLEIKRADAAP} & \text{140} \\
\text{NFYPKDINV} & \text{SFLTGASVVCFLN} & \text{210} \\
\text{SFNRNEC}
\end{align*}
\]

Mature chimeric heavy chain:

\[
\begin{align*}
\text{DVQLVESGGGLVQPGSRKSLCAASGFTSFSGMHWVRQAPEK} & \text{GLEWVA} \text{ISSGSLHYATVGRFT} & \text{70} \\
\text{SRDNPNTLFLQMTSLRSED} & \text{WNYPHYAMYAY} \text{WQGTMVTSSASTGS} & \text{140} \\
\text{GGAALGVKDYFPEPVTVSN} & \text{GHTPAVLQSSGLYS} & \text{210} \\
\text{SNKVDKKEPKS} & \text{WYVDGEVHNAKT} & \text{350} \\
\text{QVTLPSSRDELTKQ} & \text{WQQGNFSCSV} & \text{420}
\end{align*}
\]
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**APESTTTSPTLNTTGFDAY**