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Isolation of a Monoclonal Antibody That Targets the Alpha-2 Helix of gp120 and Represents the Initial Autologous Neutralizing-Antibody Response in an HIV-1 Subtype C-Infected Individual▽

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The C3-V4 region is a major target of autologous neutralizing antibodies in HIV-1 subtype C infection. We previously identified a Center for AIDS Program of Research in South Africa (CAPRISA) participant, CAP88, who developed a potent neutralizing-antibody response within 3 months of infection that targeted an epitope in the C3 region of the HIV-1 envelope (P. L. Moore et al., PLoS Pathog. 5:e1000598, 2009). Here we showed that these type-specific antibodies could be adsorbed using recombinant gp120 from the transmitted/founder virus from CAP88 but not by gp120 made from other isolates. Furthermore, this activity could be depleted using a chimeric gp120 protein that contained only the C3 region from the CAP88 viral envelope engrafted onto the unrelated CAP63 viral envelope (called 63-88C3). On the basis of this, a differential sorting of memory B cells was performed using gp120s made from 63-88C3 and CAP63 labeled with different fluorochromes as positive and negative probes, respectively. This strategy resulted in the isolation of a highly specific monoclonal antibody (MAb), called CAP88-CH06, that neutralized the CAP88 transmitted/founder virus and viruses from acute infection but was unable to neutralize CAP88 viruses isolated at 6 and 12 months postinfection. The latter viruses contained 2 amino acid changes in the alpha-2 helix of C3 that mediated escape from this MAb. One of these changes involved the introduction of an N-linked glycan at position 339 that occluded the epitope, while the other mutation (either E343K or E350K) was a charge change. Our data validate the use of differential sorting to isolate a MAb targeting a specific epitope in the envelope glycoprotein and provided insights into the mechanisms of autologous neutralization escape.

HIV-1-infected individuals develop antibodies within a few months of infection that are capable of neutralizing the infecting virus (9, 13, 23, 33). These antibodies are often highly potent and appear to be effective *in vivo* since the virus population is rapidly replaced by neutralization-resistant variants (21, 23, 33). However, these antibodies are generally type specific and have little to no cross-neutralizing activity, suggesting that they target highly variable regions of the envelope glycoprotein. Indeed, using a series of chimeric viruses, we found that antibodies directed against the V1V2, V4, V5, and, in particular, C3 and C3-V4 regions mediated the early autologous neutralization response in HIV-1 subtype C infection (19, 21). The C3 region is located in the outer domain of gp120, expanding from the C-terminal stem of the V3 loop to the V4 region, including the alpha-2 helix and the CD4 binding loop (12). The length of the C3 region is approximately 54 amino acids (HxB2 numbering, amino acids 332 to 384) and contains at least 3 N-linked glycans (12). The alpha-2 helix, which spans 18 amino acids from positions 335 to 352, has a very conserved amphipathic structure among subtype C strains, with most variation occurring at the solvent-exposed hydrophilic face (7). The higher diversity in the alpha-2 helix of subtype C viruses compared to subtype B viruses (6) supports the experimental findings that this region is commonly targeted by autologous neutralizing antibodies (21, 24).

We have previously identified a subtype C-infected individual from the Center for AIDS Program of Research in South Africa (CAPRISA) cohort (CAP88) whose initial autologous neutralizing-antibody response targeted the C3 region of gp120 (19). These antibodies first appeared at 11 weeks of infection and peaked at 26 weeks. Escape was mediated by 2 amino acid changes in the alpha-2 helix of C3, which were first detected at 15 weeks postinfection, becoming the major population after 20 weeks of infection. One of the mutations introduced an N-linked glycosylation site at position 339, and the other involved charge changes from a negatively charged glutamic acid (E) to a positively charged lysine (K) at either position 343 or 350. While the plasma antibodies from CAP88

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at these early stages of infection were essentially monospecific, the isolation of a monoclonal antibody (MAb) was desirable, as this would conclusively prove that potent autologous neutralization was effected by a single antibody specificity. Furthermore, a MAb would enable characterization of the epitope and the mechanism of escape and also allow the analysis of antigen-specific antibody genes mediating this early antibody response.

Recent methodological advances in the ability to identify neutralizing-antibody specificities have facilitated the design of suitable antigens with which to isolate antigen-specific memory B cells. The combination of antigen-specific memory-B-cell sorting and single-cell amplification of antibody-variable regions has resulted in the isolation of a new generation of HIV-1-neutralizing MAbs (25, 26). Using a peptide tetramer to sort antigen-specific memory B cells, we recently isolated a cross-neutralizing MAb, CAP206-CH12, that recognized a novel epitope in the membrane proximal external region (MPER) of gp41 (22). In another study, structural information was used to generate probes to isolate B cells expressing antibodies to the conserved CD4 binding site, which resulted in the isolation of the very broad and potent MAb VRC01 (34). Here we describe the isolation of an autologous neutralizing antibody from participant CAP88 by a differential antigen-specific memory-B-cell sorting strategy using a chimeric gp120 where the C3 region of the autologous virus was grafted into an unrelated gp120. Furthermore, characterization of the neutralization and binding properties of this antibody provided insight into the escape pathways in this individual.

MATERIALS AND METHODS

Human samples and envelope clones. The stored plasma and peripheral blood mononuclear cell (PBMC) samples analyzed in this study were obtained from participant CAP88 from the CAPRISA 002 Acute Infection Cohort. This study was reviewed and approved by the research ethics committees of the University of KwaZulu Natal and the University of the Witwatersrand in South Africa. The envelope genes were either previously cloned in our laboratory (9, 21) or obtained from the NIH AIDS Research and Reference Reagent Program or the Programme EVA Centre for AIDS Reagents, NIBSC, United Kingdom.

Cell lines. Cells of the JC53bl-13 cell line, also known as TZM-bl cells, engineered by J. Kappes and X. Wu, were obtained from the NIH AIDS Research and Reference Reagent Program. Human embryonic kidney 293T cells were obtained from George Shaw (University of Alabama, Birmingham, AL), and *N*-acetylglucosamine transferase I-deficient 293S cells were obtained from James Binley (Torrey-Pines Institute). Both cell lines were cultured in Dulbecco modified Eagle medium (DMEM; Gibco, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO). Cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA.

Generation of chimeras and mutant envelopes. Chimeric envelopes were created using an overlapping PCR strategy with the inserts and flanking regions amplified in separate reactions. After linkage, the 3-kb chimeric PCR fragments, generated using EnvAdir and EnvM primers (5), were cloned into plasmid pcDNA3.1 (directional; Invitrogen) and screened for function as previously described (8). Chimerism was confirmed by sequence analysis. Site-directed mutagenesis was performed using a Stratagene QuikChange II kit (La Jolla, CA).

Production of pseudoviruses. Envelope-pseudotyped viruses were obtained by cotransfecting the envelope plasmids with pSG3ΔEnv (33) using FuGene 6 transfection reagent (Roche, Basel, Switzerland) as previously described (9). In some cases pseudoviruses were generated either in 293T cells treated with the glycosidase inhibitor kifunensine at 23 μg/ml (100 μM) before transfection or in GnTI-deficient 293S cells.

Production of infectious envelope clones. Replicating viruses were prepared from specific envelope clones as described elsewhere (16). The vector pHIVΔ*env*BstEII*nef-hisD* was a gift from D. R. Kuritzkes. Briefly, linearized vector pHIVΔ*env*BstEII*nef-hisD* and full gp160 envelope PCR products were cotransfected into 293T cells with FuGene 6. Supernatants were harvested on day 4 and were used to infect activated CD8-depleted PBMCs via spinoculation. Viruses were harvested from the culture supernatants every second day until day 10 and tested for infectivity in JC53bl-13 cells. The 50% tissue culture infective dose in PBMCs was determined for each virus preparation as described elsewhere (18).

gp120 production and isolation. Recombinant gp120 proteins were produced in 293T cells by transfection using codon-optimized gp120 genes cloned in the expression vector in plasmid pcDNA3.1 (Invitrogen). gp120 was isolated using *Galanthus nivalis* lectin agarose matrix (Sigma) and eluted with 1 M methyl-α-D-mannopyranoside (Sigma). Remaining protein contaminants were eliminated through ion-exchange chromatography using FastFlow Q-Sepharose (GE Healthcare Life Science, Piscataway, NJ), equilibrated in phosphate-buffered saline (PBS), and reconstituted in 2 M NaCl-PBS. The purified gp120s were collected in the flowthrough, washed in PBS, and concentrated to 5 mg/ml. The purities of the final gp120 preparations were tested by running 10 μg of protein in an SDS-polyacrylamide gel. Protein preparations with purity higher than 99% were used in subsequent experiments.

Adsorption of gp120-binding antibodies in plasma. Adsorption of anti-gp120 antibodies was done as previously described (14), using gp120 covalently coupled to tosyl-activated magnetic beads. Anti-gp120 activity was measured by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (14).

Neutralization assays. Neutralization was measured as a reduction in luciferase gene expression after a single-round infection of TZM-bl cells with envelope-pseudotyped viruses (18). Titers were calculated as the inhibitor concentration (IC_{50}) or reciprocal plasma/serum dilution (ID_{50}) causing a 50% reduction of relative light units (RLU) with respect to the virus control wells (untreated virus). Neutralizing activity was also measured as a reduction in PBMC infection by measuring p24 Gag antigen synthesis as described previously (3).

Staining and sorting B-cell populations. For antigen-specific memory-B-cell sorting, CAP63 gp120 and a chimeric protein containing the C3 region of CAP88 in the CAP63 backbone (63-88C3) were labeled with Pacific Blue and Alexa Fluor 647, respectively, using fluorochrome labeling kits (Invitrogen, Carlsbad, CA). Thawed PBMCs were stained using a viability marker (Aqua vital dye; Invitrogen, Carlsbad, CA) and a combination of the following antibodies: CD3 phycoerythrin (PE)-Cy5, CD14 PE-Cy5, CD16 PE-Cy5, CD235a PE-Cy5, CD19 allophycocyanin (APC)-Cy7, CD27 PE-Cy7, CD38 APC-Cy5.5, anti- immunoglobulin M (anti-IgM) fluorescein isothiocyanate (FITC), and anti-IgD PE (BD Biosciences, Mountain View, CA, and Invitrogen, Carlsbad, CA). The titers of all antibodies were determined and used at optimal concentrations for flow cytometry. During the sort, forward- versus side-scatter gating was used to select for lymphocytes, geometric gates were used to eliminate doublet events, and viability dye gating was used to select for viable cells. Memory B cells were gated as $CD3^-$, $CD14^-$, $CD16^-$, $CD235a^-$, $CD19^+$, and IgD negative (IgD^-). gp120-stained B cells were sorted as single cells into wells of a 96-well plate, selecting those cells that were labeled by the 63-88C3 gp120 but not by the CAP63 gp120. Cells were stored in room temperature reaction buffer at −80°C until use. Flow cytometric data were acquired on a BD Aria2 fluorescence-activate cell sorter (FACS), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Isolation of Ig variable gene transcripts. The genes encoding Ig variable region gene segments of heavy chain (V_H) and light chain (V_L) were amplified by reverse transcription-PCR using a modification of the method described by Tiller and coworkers (29). Briefly, RNA from single B cells sorted in 96-well plates were reverse transcribed using Superscript III in the presence of primers specific for human IgG, IgM, IgD, IgA1, IgA2, kappa (K), and lambda (L) constant gene regions (15). The V_H, V_K, and V_L genes were then amplified from the transcribed cDNA separately by nested PCR, as described elsewhere, and analyzed on 1.2% agarose gels (15). The second-round PCR includes tag sequences at the 5′ end of each primer pair which permit assembling of the V_H and V_L genes into functional linear Ig gene expression cassettes (see below). PCR products were purified and sequenced. The variable gene segments, isotype, and potential functionality of the immunoglobulin were determined using the SoDA program (31).

Expression of recombinant antibodies from linear expression cassettes. Three linear Ig expression cassettes, each containing the cytomegalovirus promoter and human Ig leader as one fragment, were used for small-scale expression and specificity analysis (15). Fragments for the heavy and light chains comprised either the IgG1 constant region, Ig kappa constant region, or Ig lambda constant region attached to poly(A) signal sequences. These two fragments plus either the V_H, V_K, or V_L gene amplified from single B cells as described above were assembled by overlapping PCR. PCR products containing linear full-length immunoglobulin heavy- and light-chain genes were purified, and the paired Ig heavy- and light-chain products were cotransfected into 293T cells grown in

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6-well plates using Effectene transfection reagent (Qiagen, Valencia, CA). Cultures were fed 6 to 12 h later with 2 ml fresh medium containing 2% fetal calf serum (FCS) and incubated for 72 h at 37°C in a 5% CO_2 incubator. Thereafter, culture supernatants were harvested for antibody characterization.

Production of purified recombinant MAbs. The selected immunoglobulin V_H and V_L genes from CAP88-CH06 were cloned into human Ig(γ) and Ig(λ) expression vectors in pcDNA3.3 (15). Clones with inserts of the correct size were sequenced to confirm identity with the original PCR product. For production of purified antibodies of CAP88-CH06 by batch transient transfections, 293T cells in 10 to 20 T-175 flasks or a Hyperflask (Corning) grown to 80 to 90% confluence in DMEM supplemented with 10% FCS were cotransfected with plasmids expressing HIV-1-specific Ig heavy- and light-chain genes using polyethylenimine. Recombinant antibodies were purified using protein A-agarose columns (Thermo, Rockford, IL).

Antibody binding assay. Supernatants from the small-scale transfections were tested for the amount of IgG present by ELISA and for binding to HIV antigens by ELISA using standard techniques (1) and Luminex and using a standardized custom assay (30). Antibodies were tested for reactivity with a panel of HIV-1 Env and other HIV-1 proteins. Samples were serially diluted for assay, and data were analyzed using 5-parameter curve fitting; endpoint titers were calculated to be 3-fold above assay background. ELISA testing for antigens was considered positive if the optical density reading was above 0.13 unit. Affinity of recombinant MAbs was determined using a 4-parameter curve fit of ELISA titer data. Luminex assays were considered positive if the value obtained after the value for the blank bead was subtracted was greater than 20 units and greater than 10 divided by the IgG concentration in % g/ml. Transiently expressed MAbs with low IgG concentrations were retransfected and reassayed. In cases where multiple tests of the same antibody were available, the final data set contained data from the assay with the highest concentration of MAb.

MAb binding to pseudovirus gp120. Pseudovirus supernatants were lysed in 1% Triton X-100 for 1 h. The gp120 molecules in the lysate were captured onto a solid phase via adsorbed antibody D7324 (Aalto Bio Reagents, Dublin, Ireland). MAbs were bound to gp120 in 5% milk–0.05% Tween 20 in PBS. Bound antibodies were detected using a total anti-human immunoglobulin biotinylated at 1:3,000 (KPL, Gaithersburg, MD) and antibiotin antibody-horseradish peroxidase conjugated at 1:1,000 (Calbiochem, Merck, Darmstadt, Germany). The ELISA was developed with tetramethylbenzidine substrate (Thermo Fisher Scientific, Rockford, IL) and stopped with 1 M H_2SO_4. Plates were read at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA).

RESULTS

The anti-C3 neutralizing activity can be depleted by monomeric gp120. In a previous study we demonstrated that CAP88, an HIV-1 subtype C-infected woman, developed potent autologous neutralizing antibodies first detected at 11 weeks of infection (21). The targets of these early antibodies were mapped by making chimeras between the CAP88 virus and an unrelated heterologous CAP63 virus that was insensitive to CAP88 serum antibodies. Transfer of the C3 region from the early virus of CAP88 (envelope clone 88.2mo.B5 isolated 2 months postinfection) into a CAP63 backbone resulted in a virus (clone 63-88C3) that was as sensitive to the earliest autologous neutralizing response as the CAP88 parental virus, with titers peaking at 26 weeks (Fig. 1A, gray shading) (21). This indicated that the targets of the earliest autologous response were entirely within C3. As the virus escaped these anti-C3 antibodies, a new response targeting the V1V2 loop appeared at 26 weeks, becoming dominant at 54 weeks postinfection, as demonstrated by the neutralization of the V1V2 chimera (63-88V1V2) (Fig. 1A, striped shading). Analysis of the escape mutations to the first response mapped the epitope in C3 to the alpha-2 helix in the outer domain of gp120 (21).

We hypothesized that this epitope in C3 might be apparent on monomeric gp120. To prove this, we cloned, expressed, and purified gp120 from the 88.2mo.B5 virus and used this in adsorption/depletion studies. Plasma samples collected at 26 and 54 weeks postinfection were adsorbed with CAP88 and CAP63 gp120-coated beads. Blank beads were used as negative controls. Adsorbed plasma samples were tested for neutralization against the sensitive autologous virus 88.2mo.B5 (Fig. 1B and D) and for binding to CAP88 and CAP63 gp120 (Fig. 1C and E). CAP88 gp120-coated beads completely adsorbed the autologous neutralizing activity from plasma collected at 26 weeks postinfection (Fig. 1B), and this corresponded with almost total depletion of CAP88 gp120-binding antibodies (Fig. 1C). This suggested that the epitope targeted by the first autologous antibody response was wholly presented on monomeric gp120. However, adsorption of the neutralizing antibodies in plasma collected at 54 weeks postinfection was less efficient (Fig. 1D), despite the fact that most antibodies binding to monomeric CAP88 gp120 were removed (Fig. 1E). This might be due to the fact that the C3 antibody had started to wane and was being replaced by antibodies targeting an epitope in V1V2 (Fig. 1A) (21) that was presumably not adsorbed by monomeric gp120. A chimeric 63-88C3 gp120 was produced to specifically target the anti-C3 antibodies in CAP88 plasma. This protein efficiently depleted antibodies binding to the 63-88C3 chimeric and CAP63 gp120 proteins in the plasma collected at 26 weeks (Fig. 1C), which resulted in a 96% reduction of neutralizing activity against the 88.2mo.B5 clone, from a titer of 1:1,637 to one of 1:55 (Fig. 1B). However, this depletion was not as efficient as that for the full CAP88 gp120, perhaps because another specificity targeting an epitope outside C3 but within monomeric gp120 made a minor contribution to the autologous neutralization. This was also suggested by the significant levels of binding of the 63-88C3-depleted plasma to CAP88 gp120 (Fig. 1C). Nevertheless, the use of a chimeric protein where only the target of interest was present likely increased the specificity by reducing the binding of non-C3 antibodies. Indeed, the heterologous CAP63 gp120 did not adsorb any neutralizing activity in the plasma from either time point and therefore served as a suitable negative control (Fig. 1B and D). This protein, however, very efficiently depleted the CAP63 binding antibodies in this plasma, as expected (Fig. 1C and E). Little or no difference in binding to CAP88 gp120 was observed after adsorption with CAP63 gp120-coated beads (Fig. 1C and E). This might be explained by the presence of a large quantity of CAP88 type-specific binding antibodies in these plasma samples that were not adsorbed by CAP63 gp120, which generated a saturating signal in the ELISA.

Epitope-specific memory-B-cell sorting. In order to isolate the B cells that produced these anti-C3 antibodies, a differential sorting strategy using the chimeric and heterologous gp120s labeled with different fluorochromes was designed. The chimeric 63-88C3 gp120 was used to identify B cells that specifically recognized the C3 epitope, while the CAP63 gp120 was used to exclude B cells expressing antibodies that bound to regions outside the C3. Given that the anti-C3 antibody titers in plasma peaked at 26 weeks postinfection (Fig. 1A) and were effectively removed by the chimeric gp120, we proposed that PBMCs collected after this time should contain memory B cells specific for this epitope. Thus, PBMCs collected at 34 weeks postinfection were stained with a panel of antibodies as well as CAP63 gp120 conjugated to Pacific Blue and 63-88C3 gp120 conjugated to Alexa Fluor 647. Memory B cells ($CD19^+$,

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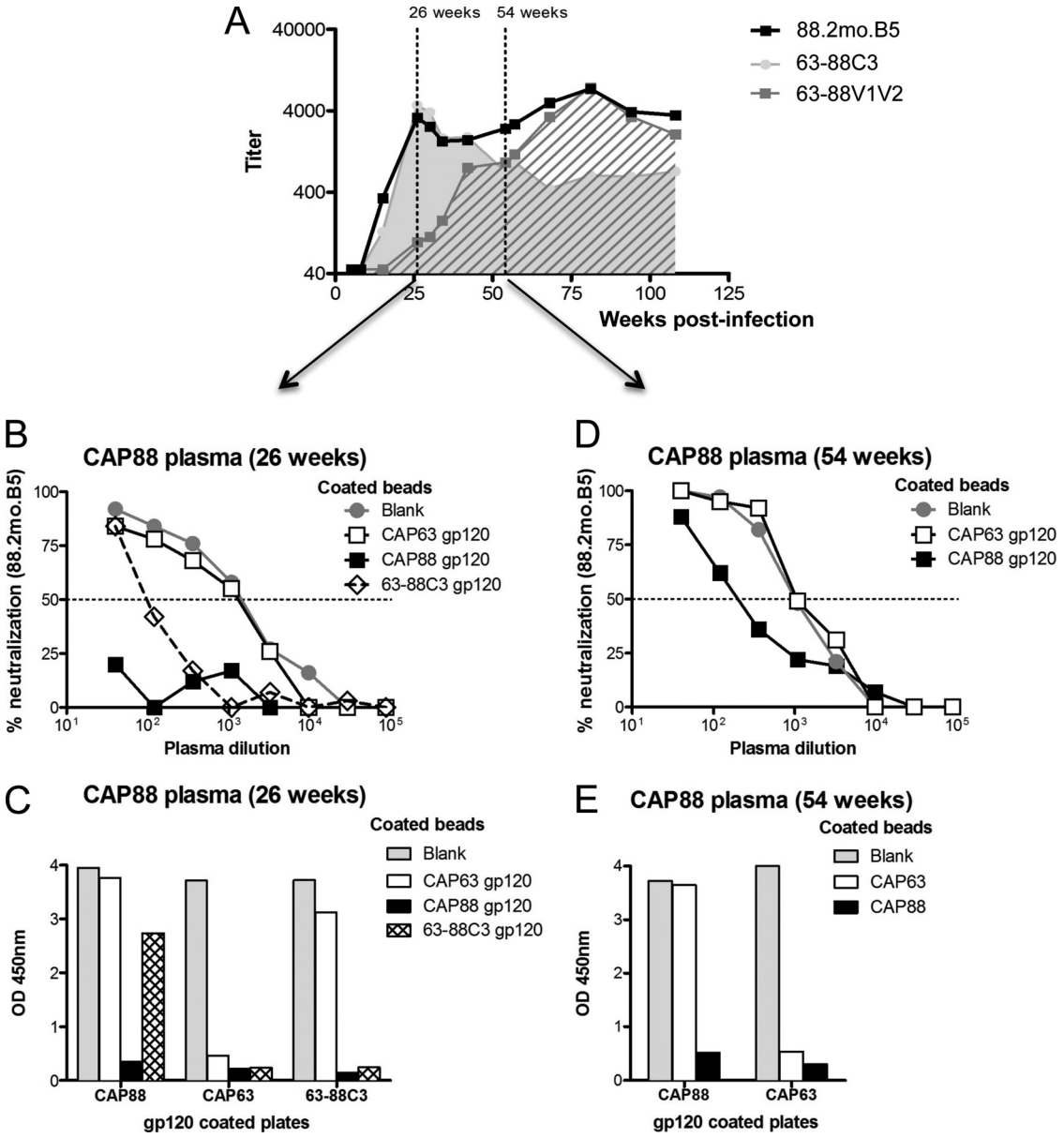


FIG. 1. Adsorption of autologous neutralizing activity by autologous monomeric gp120. (A) Graph adapted from Moore et al. (19) to show the presence of the anti-C3 and anti-V1V2 neutralizing antibodies in CAP88 plasma samples collected during the course of infection. The black line shows the response against the early CAP88 virus 88.2mo.B5, while the gray area delineates the portion attributed to anti-C3 antibodies and the gray stripped lines delineate the portion attributed to anti-V1V2 antibodies. Plasma samples collected at 26 weeks (B and C) and 54 weeks (D and E) postinfection were adsorbed with CAP88 and CAP63 gp120-coated beads as well as blank beads. The plasma sample collected at 26 weeks was also adsorbed with a chimeric CAP63 gp120 grafted with the C3 region of CAP88 (63-88C3). Absorbed plasma samples were tested for neutralization of the autologous virus 88.2mo.B5 (B and D) and for binding to all the proteins used for adsorptions (C and E). OD, optical density.

IgD^-) positively stained with 63-88C3 gp120 but negative for the heterologous CAP63 gp120 were sorted into single cells and placed into a 96-well plate for amplification of heavy-chain (V_H) and light-chain (V_L) antibody genes (Fig. 2). The frequency of epitope-specific cells acquired from this sorting was approximately 16/10,000 memory B cells. However, upon further analysis, it was noted that a group of double-positive cells was inadvertently included in the sorting gate. Of the 64 cells sorted, 16 functional heavy- and light-chain pairs were identified and expressed as full-length IgG1 recombinant antibodies in 293T cells by transient transfection using linear Ig gene expression cassettes (15). The supernatants containing the expressed antibodies were tested for binding to the 63-88C3 and CAP63 gp120s in a multiplex Luminex binding antibody assay. Seven of the 16 expressed recombinant antibodies bound 63-88C3 gp120, and 6 bound the CAP63 gp120 to various levels, possibly due to the inclusion of the double-positive population (Fig. 3A). The one MAb which failed to bind CAP63, 3469L, had the highest binding to 63-88C3 gp120, suggesting that it specifically recognized the C3 domain of CAP88. Confirmation

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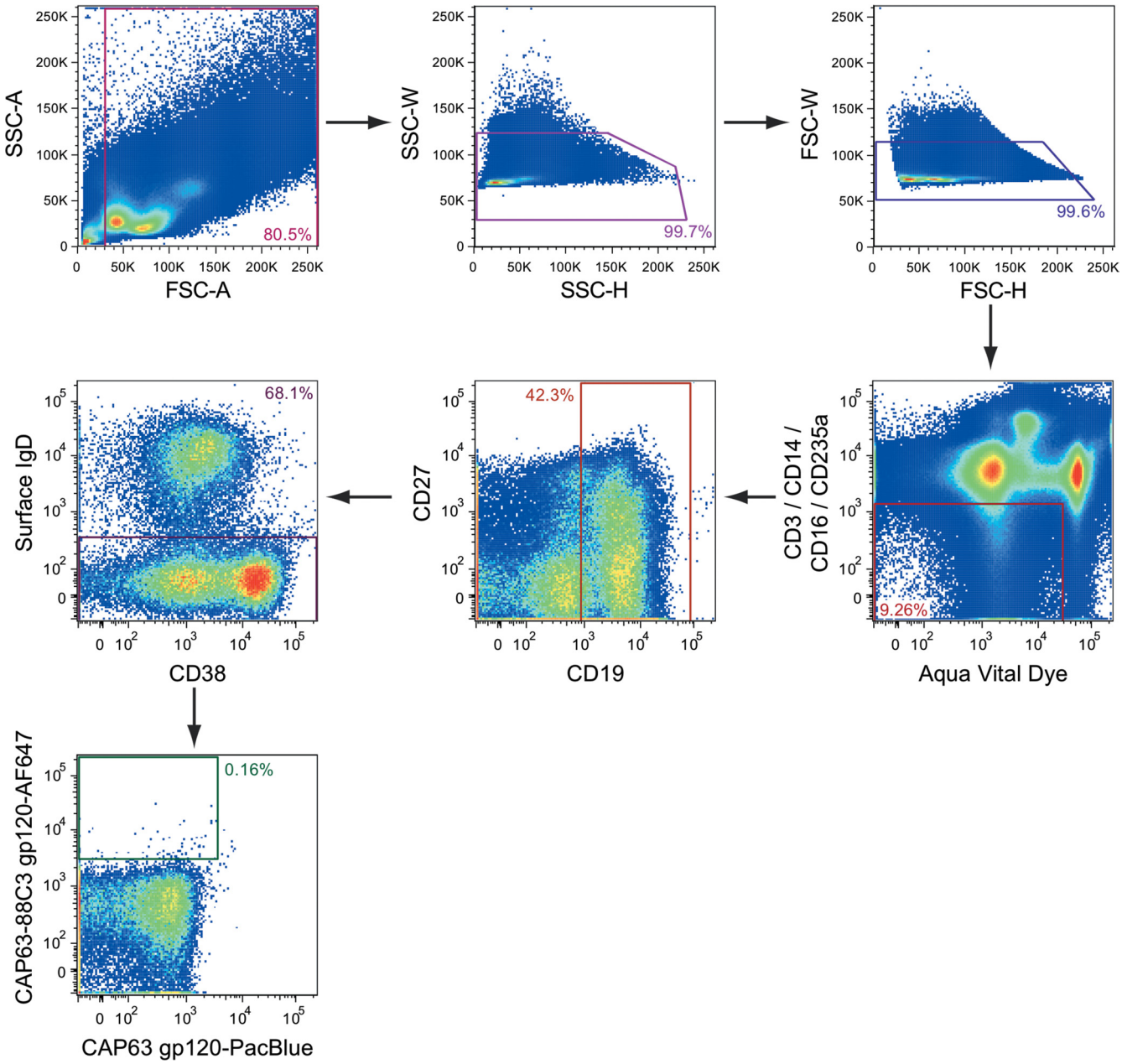


FIG. 2. Epitope-specific memory-B-cell staining. PBMCs from CAP88 at 34 weeks postinfection were stained for flow cytometric analysis and single-cell sorting. Memory B cells were selected as lymphocytes using geometric gates and live cells using a viability marker and then as CD3/CD14/CD16/CD235a$^-$, CD19$^+$, and surface IgD$^-$. Memory B cells positively stained with the chimeric protein 63-88C3-AF647 and negative for CAP63-Pacific Blue (PacBlue; square gate) were sorted individually into a 96-well plate. A population of cells that stained positive for both markers was inadvertently included in the sorted population (lower right edge of gate in the bottom panel). FSC and SSC, forward and side scatter, respectively.

of this came from the observation that the 3469L supernatant was the only one that neutralized the autologous virus 88.2mo.B5 significantly above background levels (Fig. 3B). One of the other MAbs isolated, CAP88-3468L, which bound strongly to both wild-type and chimeric proteins (Fig. 3A), was shown on further screening to recognize the V3 loop of gp120 (unpublished data). This anti-V3 MAb, however, showed no neutralization activity against the CAP88 virus.

Neutralization profile of CAP88-CH06 MAb. The variable heavy- and light-chain regions of 3469L, from now referred to as CAP88-CH06, were cloned into an IgG1-expressing vector and produced on a large scale for further assessment of neutralizing activity. In the TZM-bl pseudovirus assay, the CAP88-CH06 MAb neutralized the early-infection clone 88.2mo.B5 very potently with an IC_{50} of 13 ng/ml (Fig. 4A). A similar level of activity was noted against the autologous transmitted/

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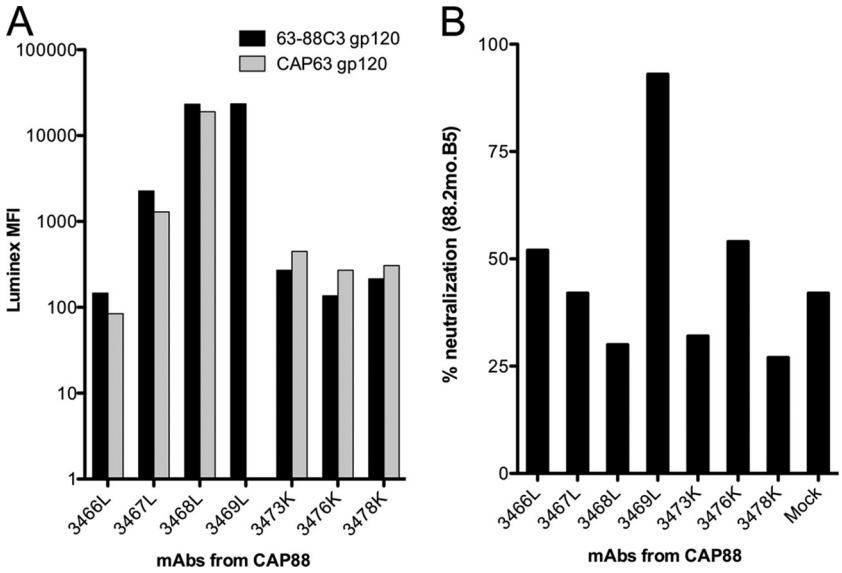


FIG. 3. Binding and neutralization of the isolated antibodies. The seven isolated V_H and V_L pairs were transiently transfected in 293T cells. Their supernatants were screened for binding to CAP63 and 63-88C3 gp120s by Luminex (A) and tested for neutralization of autologous virus 88.2mo.B5 isolated during acute infection in the TZM-bl assay (B). High background levels in the neutralization screening assay, as seen in the mock transfections, may be due to the transfection reagent or other toxins in these concentrated supernatants.

founder virus from CAP88 represented by clone 88.1mo.17, which had a gp120 amino acid sequence identical to that of 88.2mo.B5 gp120. This MAb, however, did not neutralize eight CAP88 viruses amplified from 6 and 12 months postinfection, when escape from this antibody specificity had already occurred (21) (Fig. 4A). Furthermore, no neutralizing activity was recorded at 50 μg/ml against a panel of 46 heterologous viruses, which included the subtype A ($n = 6$), B ($n = 12$), and C ($n = 12$) tier 2 panels (27) and the neutralization-sensitive strains MW965.26, SF162, and HxB2 (data not shown). It also failed to neutralize any of the 12 viruses from within the CAPRISA cohort (9) or the consensus C strain (10).

A similar pattern was observed in the PBMC neutralization assay. The MAb CAP88-CH06, was capable of neutralizing an infectious clone containing the envelope from the transmitted/founder autologous virus, 88.1mo.17, but not the escaped virus, 88.6mo.10, isolated at 6 months of infection (Fig. 4B). Interestingly, neutralization of the 88.1mo.17 virus was ~8 times more potent in the PBMC assay than in the TZM-bl pseudovirus assay, with an IC_{50} of 1.7 ng/ml, although the curves were flatter in the PBMC assay. The escaped 88.6mo.10 virus contained 2 amino acid mutations (I339N and E350K) in the alpha-2 helix of C3 which were previously shown to mediate escape from autologous plasma (21). Here we found that the CAP88-CH06 MAb was able to neutralize the escape virus obtained at 6 months in which these 2 sites were backmutated (88.6mo.10.N339I K350E), providing direct proof that this MAb targeted the predicted C3 epitope (Fig. 4C).

The CAP88-CH06 epitope is occluded by glycans. We previously showed that escape from plasma neutralization in CAP88 was mediated by the I339N mutation, which introduced an N-linked glycosylation site, in combination with either an E343K or E350K charge change in the alpha-2 helix (21). These three residues, which are separated on a linear sequence, lie adjacent to one another on the solvent-exposed side of the helix, an arrangement that likely contributed to the formation of this epitope (highlighted in red in Fig. 5).

To further explore the role of these changes in the escape from CAP88-CH06 neutralization, we introduced combinations of these 3 escape mutations into the sensitive 88.2mo.B5 virus and measured the acquisition of neutralization resistance (as opposed to measuring acquisition of sensitivity, as shown in Fig. 4C). The isoleucine (I) at position 339 in the autologous clone 88.2mo.B5 was mutated to an asparagine (N), alone or in combination with a charge change from glutamic acid (E) to lysine (K) at position 343 or 350. In addition, the isoleucine 339 was also changed to a glutamine (Q) to assess whether glycosylation *per se* or just the change from a hydrophobic to a hydrophilic residue at this position could mediate escape. The I339N substitution mediated partial resistance, with a 7-fold increase in IC_{50} from 13.2 to 102.9 ng/ml (Fig. 6A). However, the I339Q substitution had a minimal effect on neutralization resistance, emphasizing the contribution of the N-linked glycan at this position in conferring escape from this antibody. The addition of either E343K or E350K in combination with the N339 glycan resulted in complete resistance to neutralization by the CAP88-CH06 MAb, even at 10 μg/ml. Interestingly, while the E343K mutant showed a 43-fold increase in IC_{50} but remained neutralization sensitive, the introduction of the E350K mutation on its own resulted in a completely resistant variant. However, no naturally occurring sequences with only the E350K mutation were found among the 7 clones that were analyzed (21). This suggested that resistance to this MAb was mediated by the combined effects of both the charge change at position 343 or 350 and the N-linked glycan at position 339.

To further explore the role of N-linked glycosylation in CAP88-CH06 MAb neutralization, the sensitive virus 88.2mo.B5 was produced in 293T cells in the presence of kifunensine or in cells that lacked *N*-acetylglucosamine transferase I (GnTI-deficient 293S). Kifunensine inhibits the en-

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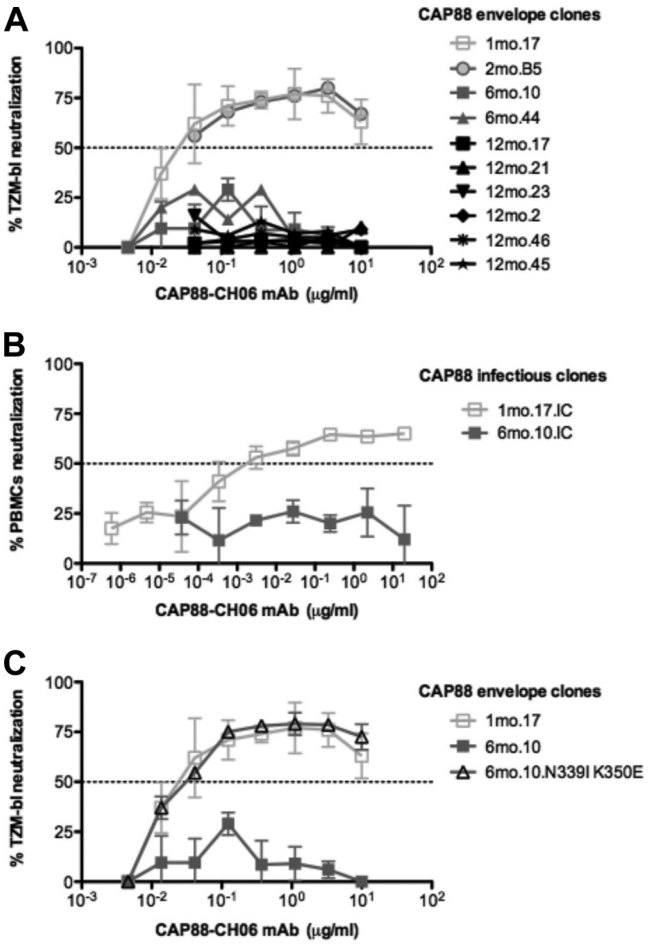


FIG. 4. Neutralization escape from CAP88-CH06 MAb. Purified CAP88-CH06 MAb was tested for neutralization of autologous CAP88 clones isolated at 1, 2, 6, and 12 months postinfection in the TZM-bl assay (A), infectious molecular clones of viruses from 1 and 6 months in a PBMC neutralization assay (B), and a clone obtained at 6 months in which the N339I and K350E changes were introduced (C). MFI, mean fluorescence index.

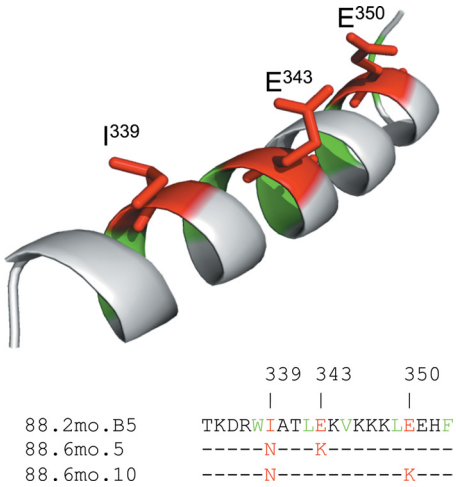


FIG. 5. Location within the alpha-2 helix of the amino acid residues involved in neutralization escape. The CAP88.2mo.B5 gp120 amino acid sequence was modeled using coordinates from the CAP210 gp120 structure in complex with secretory CD4 and 21c Fab (Protein Data Bank accession no. 3LQA) and the Modeler (version 9.8) program. A ribbon diagram of the alpha-2 helix was used to display the location of the 3 amino acids (in red) in the alpha-2 helix of CAP88 that mediated escape from the CAP88-CH06 MAb. Sequences of this region from 2 representative clones at 6 months compared to the sequence of the clone at 2 months showed that the addition of a glycan at position 339 was common, while charge changes at position 343 or 350 were mutually exclusive, occurring on equal numbers of separate genomes (21). The figure was generated with the PyMOL program (DeLano Scientific LLC, South San Francisco, CA [http://www.pymol.org]).

zyme mannosidase I, which is responsible for trimming the Man9 precursor into Man5. This is the preliminary step in the synthesis of hybrid and complex glycans, and treatment with kifunensine results in a virus rich in large high-mannose Man9 glycans (2). Conversely, viruses grown in GnTI-deficient 293S cells mainly carry Man5 glycans, due to the failure of these cells to produce hybrid or complex glycans. It has been proposed that viruses produced in 293T cells express Man5-rich glycans on their envelope glycoproteins and therefore do not substantiality differ from viruses grown in GnTI-deficient 293S cells (4). The virus produced in the presence of kifunensine was resistant to neutralization by CAP88-CH06, while the virus produced in GnTI-deficient 293S cells was more sensitive than the control virus grown in 293T without kifunensine (Fig. 6B). Altogether, these data suggested that the exposure of the CAP88-CH06 epitope is affected by the glycan moieties, which may have been exploited by the virus to escape from this antibody response.

Escape did not result in loss of binding to recombinant gp120. Having found mutations in the C3 region that totally abrogated neutralization by CAP88-CH06, we investigated whether these escape mutations resulted in the loss of binding to gp120. Monomeric gp120 proteins from the clone 88.2mo.B5 and the escape mutant 88.2mo.B5.I339N E343K were produced in 293T cells and tested for binding to the MAb CAP88-CH06. CAP63 gp120 was used as a negative control. The anti-V3 MAb CAP88-3468L, which was used as a positive control, bound all three proteins equally well, as expected (Fig. 7B). The autologous neutralizing antibody CAP88-CH06 did not bind the CAP63 gp120, confirming the isolate-specific nature of this MAb (Fig. 7A). However, CAP88-CH06 bound both CAP88 gp120s, including the one containing the two escape mutations, albeit at slightly lower levels. This indicated that neutralization escape does not necessarily preclude binding to recombinant gp120. Given this dichotomy between neutralization and binding, we captured gp120 from lysed pseudovirus supernatants grown in 293T cells and tested them for binding to the MAbs. Interestingly, the MAb CAP88-CH06 was not able to bind the gp120 from the escape mutant or the heterologous gp120, while it efficiently bound the gp120 derived from the enrollment virus (Fig. 7C), consistent with the neutralization profile and in contrast to the binding to recombinant gp120. The anti-V3 MAb was able to bind all three proteins similarly, confirming that the differences in CAP88-CH06 binding were not the result of variations in the amount of captured gp120.

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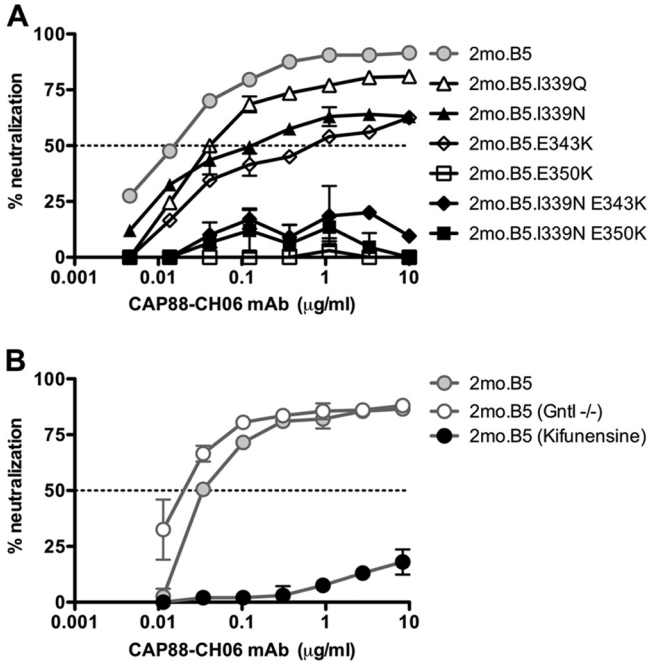


FIG. 6. Effect of N-linked glycosylation on neutralization. (A) The I339 residue in the neutralization-sensitive clone 2mo.B5 was replaced by a glutamine (Q) or asparagine (N). Charge change E343K or E350K was introduced with or without the N339 glycan. All mutants were tested for neutralization by the CAP88-CH06 MAb starting at 10 μg/ml. (B) The CAP88 clone 2mo.B5 was grown in the presence of 100 μM kifunensine or in GnTI-deficient 293S cells to determine the effect of the glycans on the neutralization by CAP88-CH06 MAb.

Sequence analysis of variable region of the MAb CAP88-CH06. The V_H and V_L sequences of CAP88-CH06 were analyzed using the SoDA (31) and JoinSolver (28) programs. The heavy chain was shown to belong to the IgA1 subclass, and the maximum-probability inferred gene recombinations were $V_H4{\sim}39{*}01$, $D_H3{\sim}3{*}01$, and $J_H4{*}02$ genes for the heavy chain and $V_\lambda3{\sim}21{*}03$ and $J_\lambda2{*}01$ genes for the lambda chain. The antibody sequences were compared to the closest reported germ line sequence and indicated that CAP88-CH06 had 21 mutations (5.9%) in the heavy-chain gene with insertions of 6 nucleotides in the CDR2 and 8 mutations (3.3%) in the light-chain gene. A high ratio of synonymous to nonsynonymous mutations was observed, suggesting that a certain level of affinity maturation from the germ line sequence took place (Fig. 8). However, it is not known if all these mutations were required to achieve the binding threshold required for neutralization.

DISCUSSION

We used epitope-specific sorting of memory B cells to isolate a monoclonal antibody that reflects the primary neutralizing response in an HIV-1 subtype C-infected individual. Mapping of this antibody specificity and its epitope was previously done using autologous plasma, and this information was used to design a suitable antigen to sort relevant B cells from stored PBMCs. This MAb, called CAP88-CH06, defined an epitope in the alpha-2 helix of the C3 region of the outer domain of the HIV-1 envelope and potently neutralized early autologous viruses. Furthermore, the isolation of this antibody allowed the precise definition of the neutralization escape mechanism that occurred in this HIV-1 subtype C-infected individual within the first 6 months of infection.

The use of a highly specific antigen combined with identification and sorting of memory B cells is an extremely powerful way to isolate antibodies of interest. Indeed, a similar strategy was used to isolate the MAbs VRC01, VRC02, and VRC03, where a mutated and unmutated resurfaced gp120 was used to specifically identify the B cells of interest (34). Here, we designed a chimeric gp120 that contained only a small fragment encompassing the target sequence to increase the specificity of sorting. Since gp120 is a complex antigen which is the target of numerous cross-binding antibodies, the heterologous gp120 employed as the chimera backbone was utilized to essentially soak up the irrelevant antibodies, as these stained with both fluorochromes. The B cells binding to the chimeric protein were therefore enriched for CAP88-C3 type-specific antibodies. Indeed, close analysis of the flow cytometric plot showed distinct populations of double-positive cells and more diffuse labeling of single-positive cells (Fig. 2). The anti-V3 MAb that was isolated in addition to the anti-C3 MAb during this procedure likely came from the single-positive-cell gate that slightly overlapped the population of double-positive cells. The proportion of single sorted cells was 0.16% (including the overlap) of memory B cells. The ability of cell sorting to identify 1 in 10,000 cells in combination with the capacity to amplify heavy- and light-chain genes from single cells makes this a highly desirable approach to fishing out MAbs of interest. While the use of a differential sorting strategy is probably less specific than sorting double-positive cells, as we did previously using a peptide tetramer to isolate an MPER-specific MAb (22), it does allow sorting of B cells where the target is part of a complex structural protein, as opposed to a peptide. However, knowledge of whether or not the antibody of interest binds the antigen and depletes the neutralizing activity is crucial before embarking on this approach. Furthermore, this method could be adapted to isolate broadly neutralizing antibodies where the epitopes on gp120 have been identified. Antibodies which recognize quaternary epitopes, present only on trimeric envelopes, such as PG9/16 (32) or even the anti-V1V2 antibody, which appears in CAP88 as the second arising specificity (21), would not be amenable to this approach at this stage.

The CAP88-CH06 MAb substantially recapitulated the activity seen in plasma, suggesting that a single antibody was responsible for the primary neutralizing response in this individual. However, since we isolated only a single antibody, we cannot be certain that other antibodies with the same specificity that either were somatic variants or used different germ line genes were also involved. Indeed, there appears to be significant redundancy in the immune system, as others have shown that both these scenarios have occurred (17, 24, 34). The CAP88-CH06 MAb was highly type specific and failed to neutralize any viruses other than the earliest autologous viral clones. It did, however, neutralize both in a cell line and in primary PBMCs, although it did so more potently in the latter assay, which may be related to glycosylation patterns and/or coreceptor density. Comparison of the CAP88-CH06 V_H and

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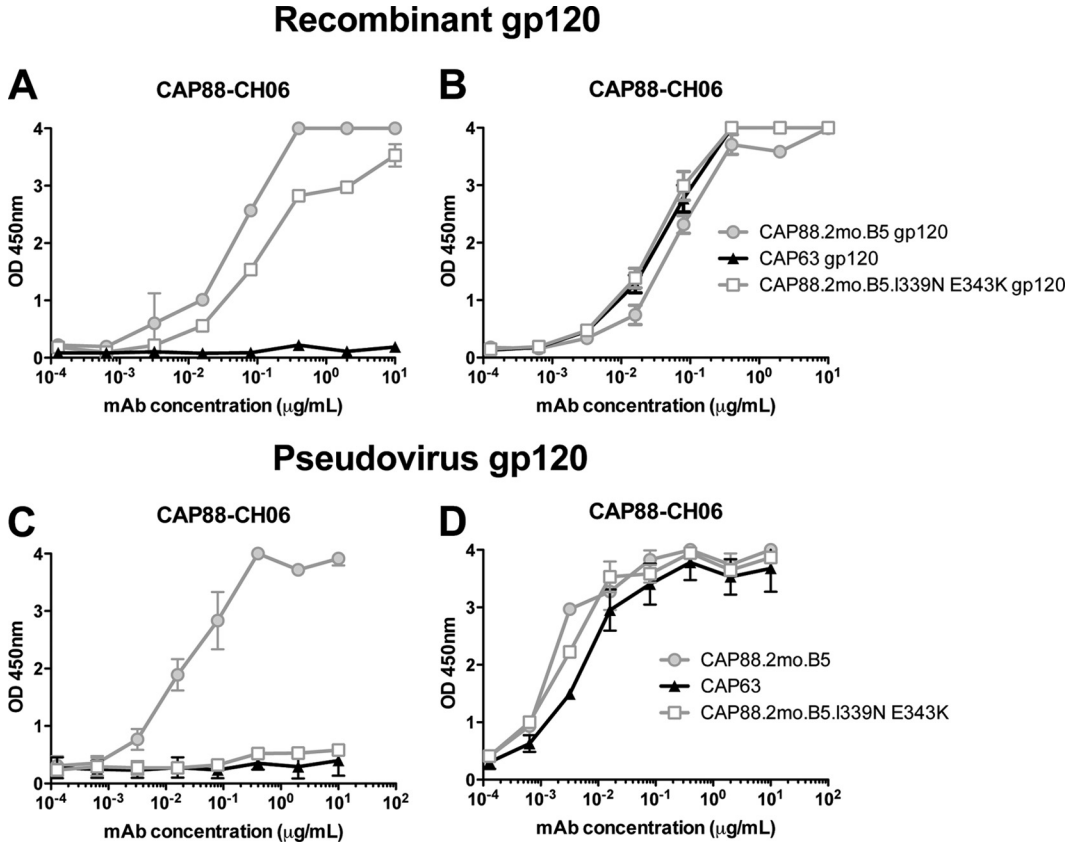


FIG. 7. CAP88 MAb binding to gp120 made from autologous and mutant viruses. Recombinant gp120s produced in 293T cells from wild-type 88.2mo.B5 and escape mutant 88.2mo.B5 containing two amino acid changes (I339, E343K) were tested in an ELISA for binding to anti-C3 CAP88-CH06 (A) and anti-V3 CAP88-3468L (B) MAbs. CAP63 gp120 was used as a control. Virion-associated gp120s were captured from lysed pseudovirus stocks produced in 293T cells via the D7324 antibody and tested for binding to anti-C3 CAP88-CH06 (C) and anti-V3 CAP88-3468L (D) MAbs.

V_L sequences to the closest germ line showed them to be 5.9% and 3.3% divergent, respectively. This is considerably lower than the divergences for existing broadly cross-neutralizing MAbs, which range from a 12% mutation rate in the V_H genes for 4E10 (11) to one of 31% for VRC01 (34). Thus, high neutralization potency was achieved without necessitating a large number of somatic mutations, which may explain in part why they arise much earlier than the highly mutated broadly cross-neutralizing antibodies. However, more early autologous neutralizing MAbs from other individuals need to be isolated and characterized to determine if this is a general feature that explains the immunodominance of such type-specific epitopes.

The observation that kifunensine treatment resulted in a virus resistant to neutralization by the MAb CAP88-CH06, while virus grown in GnTI-deficient 293S cells was more sensitive, suggested that the exposure of this epitope was dependent on the size of the "glycan fence," as first described by Binley et al (2). Indeed, escape from this MAb occurred rapidly and was mediated by the insertion of a glycosylation site, in addition to a charge change at position 343 or 350 in the alpha-2 helix. Changes in glycosylation have been shown to mediate neutralization escape (33). In this case, the N339 glycan only partially occluded the epitope, and therefore, a charge change, possibly within the binding site, was required for complete escape. It is unclear why HIV uses more than one pathway to escape pressure from such a monospecific neutralizing response. The K343E and K350E mutations were equally represented in the sequences of virus obtained at 6 and 12 months (21), suggesting that these changes were similarly favored and presumably had comparable or perhaps no fitness consequences. It is interesting that no sequences contained only the K350E change in the absence of the glycan at position 339, given that this artificially constructed virus was highly resistant to CAP88-CH06 *in vitro*. Perhaps the N339 glycan may have been performing additional functions, such as providing a compensatory mutation or mediating escape from other antibodies in the CAP88 plasma.

We observed here that mutations that rendered the virus resistant to neutralization by CAP88-CH06 MAb did not totally abrogate the binding of this antibody to recombinant gp120 but effectively precluded the recognition of pseudovirus-derived gp120. Thus, the effect of the escape mutations was apparent only in the envelope produced by the virus and not on the recombinant protein. A recent study suggested that recombinant gp120 is rich in complex-type glycans, in contrast to virion-associated gp120, which contains an oligomannose profile (Man5-9GlcNAc2) with less than 2% complex glycans (4). Given that escape from this antibody depends largely on a

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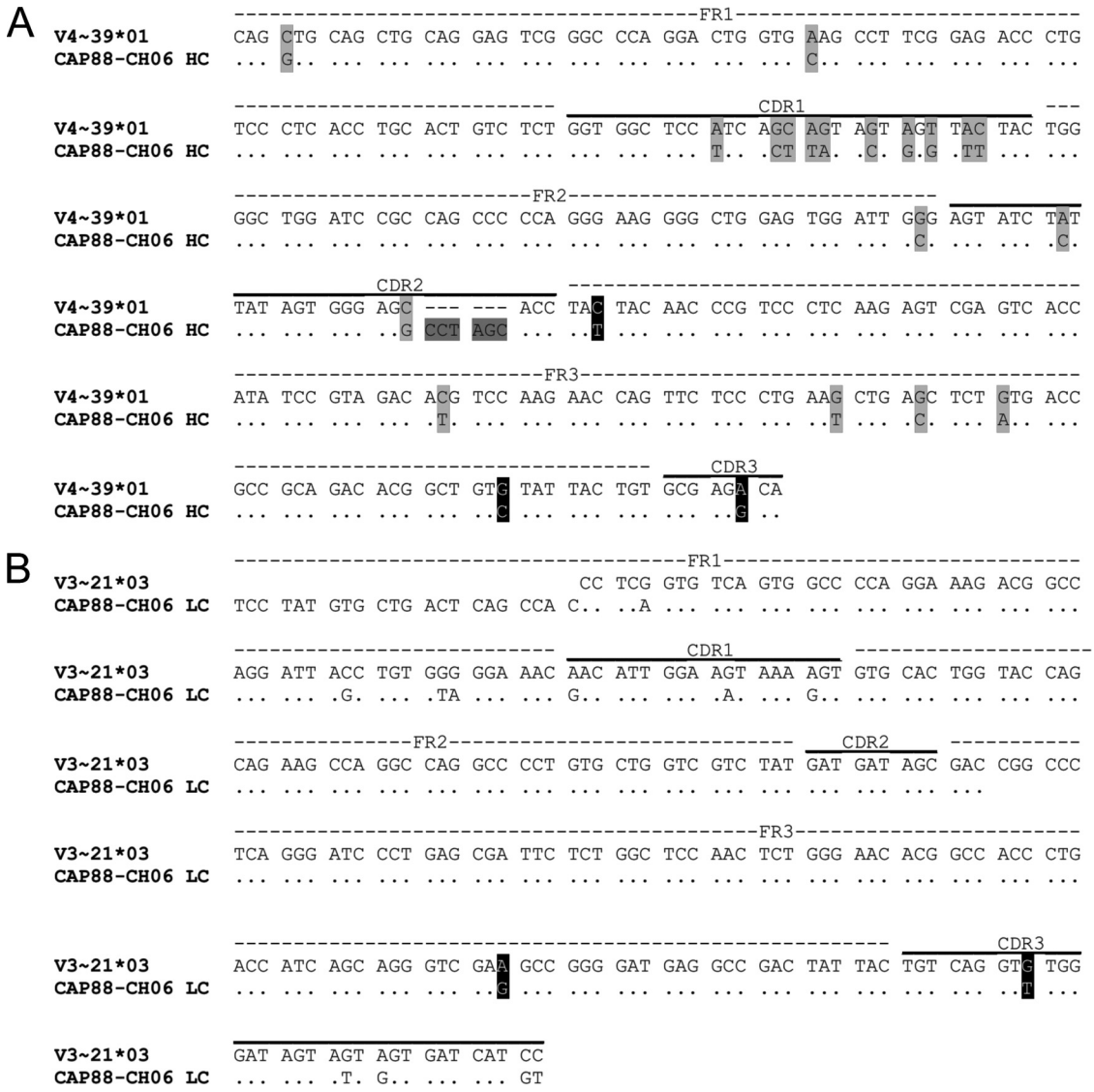


FIG. 8. Sequence analyses of the V_H and V_L genes of the CAP88-CH06 MAb. The variable region sequences of CAP88-CH06 were compared to the closest germ line sequence for both the heavy-chain (HC) (A) and light-chain (LC) (B) genes. Light gray and black highlights, nonsynonymous and synonymous changes, respectively; dark gray highlighting, a 6-nucleotide insertion in the heavy-chain complementarity-determining region 2 (CDR2). Framework regions (FR) 1, 2, and 3 and complementarity-determining regions (CDR) 1, 2, and 3 are indicated.

mannose-rich glycan, this difference may explain the paradox between these two binding experiments. This is an important observation that should be kept in mind when using recombinant gp120 either as an immunogen or a reagent.

We have previously shown that the C3 region, in particular, the alpha-2 helix, is commonly recognized by early autologous neutralizing antibodies in subtype C infections (20). Here we have isolated an antibody that represents this early specificity and that potently neutralized the autologous virus. However, escape required only two amino acid changes, attesting to the daunting plasticity faced by the immune system in dealing with HIV infection. Our findings provide clues as to why the early neutralizing responses are type specific. First, they recognize a region which is highly exposed and variable among subtype C viruses (6) as well as structurally different from subtype B envelopes (7). Second, it is possible that most antibodies against this region, like CAP88-CH06, are strongly dependent on side chain interactions for recognition and are easy to escape. The alpha-2 helix is only 18 amino acids in length and has structural restrictions, such as the need to maintain amphipathicity and a solvent-exposed face. The fact that this region is under such enormous pressure early in HIV infection suggests that it is highly immunogenic and favored by the immune system and therefore may represent an important target in vaccine design. However, finding structurally conserved motifs in this region that can be recognized by more broadly cross-neutralizing antibodies remains a significant challenge.

In summary, we successfully employed differential gp120 sorting to isolate an antigen-specific memory B cell encoding a MAb that targets a specific epitope in the envelope glycoprotein. This demonstrated that even in the absence of detailed

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structural knowledge of the target epitope, chimeric autologous proteins can be used to identify the B cells of interest. The isolated MAb CAP88-CH06 represented the primary neutralizing response and contained a low level of somatic mutations in their variable regions. Furthermore, it allowed the study of the neutralization escape from the first autologous antibody response in this HIV-1 subtype C-infected individual.

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