Toward Effective HIV Vaccination

INDUCTION OF BINARY EPITOPE REACTIVE ANTIBODIES WITH BROAD HIV NEUTRALIZING ACTIVITY*

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We describe murine monoclonal antibodies (mAbs) raised by immunization with an electrophilic gp120 analog (E-gp120) expressing the rare ability to neutralize genetically heterologous human immunodeficiency virus (HIV) strains. Unlike gp120, E-gp120 formed covalent oligomers. The reactivity of gp120 and E-gp120 with mAbs to reference neutralizing epitopes was markedly different, indicating their divergent structures. Epitope mapping with synthetic peptides and electrophilic peptide analogs indicated binary recognition of two distinct gp120 regions by anti-E-gp120 mAbs, the 421–433 and 288–306 peptide regions. Univalent Fab and single chain Fv fragments expressed the ability to recognize both peptides. X-ray crystallography of an anti-E-gp120 Fab fragment revealed two neighboring cavities, the typical antigen-binding cavity formed by the complementarity determining regions (CDRs) and another cavity dominated by antibody heavy chain variable (VH) domain framework (FR) residues. Substitution of the FR cavity VH Lys-19 residue by an Ala residue resulted in attenuated binding of the 421–433 region peptide probe. The CDRs and VH FR replacement/silent mutation ratios exceeded the ratio for a random mutation process, suggesting adaptive development of both putative binding sites. All mAbs studied were derived from VH1 family genes, suggesting biased recruitment of the V gene germ line repertoire by E-gp120. The conserved 421–433 region of gp120 is essential for HIV binding to host CD4 receptors. This region is recognized weakly by the FR of antibodies produced without exposure to HIV, but it usually fails to induce adaptive synthesis of neutralizing antibodies. We present models accounting for improved CD4-binding site recognition and broad HIV neutralizing activity of the mAbs, long sought goals in HIV vaccine development.

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The atomic coordinates and structure factors (codes 3CLE and 3CLF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/). The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) GU240574, GU240573, GU240580, GU240579, GU240578, GU240577, GU240576, GU240575, GU240582, and GU240581.

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Induction of neutralizing antibodies (Abs)2 via adaptive immune processes is the cornerstone of vaccination against microbial antigens. The antigen-binding site is mostly formed by the complementarity determining regions (CDRs) of the light and heavy chain variable domains (V\(_{\text{L}}\) and V\(_{\text{H}}\) domains). Vaccine-induced adaptive Ab responses entail sequence diversification of Ab V domains expressed within the B cell receptor (BCR) complex, selective noncovalent antigen binding to the high affinity BCR mutants, and proliferation of the mutant B cell clones. No HIV vaccine is available. The surface of HIV is studded with noncovalently associated oligomers of gp120 complexed to gp41. HIV infection and experimental HIV vaccination attempts induce robust Ab responses to the immunodominant epitopes of gp120, which are structurally divergent in various HIV strains responsible for infection in different parts of the world. Abs to such epitopes express strain-specific neutralization (1, 2), i.e. they neutralize the HIV strain from which the immunogen was isolated but not strains genetically heterologous to the immunogen.

The gp120 site responsible for binding host CD4 receptors (CD4BS) is structurally more conserved. Precise conformational details of the CD4BS expressed on the HIV surface are not available, but crystallography suggests a large, discontinuous determinant composed of regions distant from each other in the linear protein sequence (3, 4). The 421–433 peptide region is essential for CD4 binding by gp120, suggested by contacts in the crystallized complex and loss of CD4 binding function by site-directed mutagenesis in this region (5, 6). The 421–433 region is a member of a small group of microbial polypeptide sites recognized selectively by Abs produced by the immune system without prior infection by the microbe (preimmune Abs) (7–9). Such sites are designated B cell “superantigens” (SAgs) because of their selective and widespread recognition by the comparatively conserved framework regions (FRs)

2 The abbreviations used are: Ab, antibody; BCR, B cell receptor; BSA, bovine serum albumin; Bt, biotinamidohexanoyl; CD4BS, CD4-binding site; CDR, complementarity determining region; EGF, epidermal growth factor; Fab, fragment antigen binding; Fv, fragment variable; FR, framework region; HIV, human immunodeficiency virus; mAb, monoclonal antibody; R/S ratio, replacement/silent mutation ratio; SAg, superantigen; sCD4, soluble CD4, scFv, single chain Fv; VH, antibody heavy chain variable domain; VH, antibody light chain variable domain; VIP, vasoactive intestinal peptide; CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PDB, Protein Data Bank; ELISA, enzyme-linked immunosorbent assay; E-, electrophilic.
of Ab V domains (10, 11). Noncovalent SAg binding by preimmune Abs, however, is characterized by low-to-moderate binding strength (12). Most gp120-binding preimmune Abs from humans without infection display poor or no HIV neutralizing activity (13). Patients with the autoimmune disease lupus and no HIV infection produce increased amounts of Abs to the 421–433 CD4BS region (14). A single chain Fv (scFv; V\textsubscript{L}, and V\textsubscript{H} domains linked by a flexible peptide) from the lupus Ab repertoire that binds the 421–433 region reversibly neutralizes genetically diverse strains of HIV (15). Following completion of the noncovalent binding step, certain Abs can hydrolyze polypeptides via nucleophilic attack on carbonyl groups (16–21). The proteolytic reaction imparts improved antigen inactivation potency to Abs (22). We reported the neutralization of HIV by secretory IgA from humans without infection, an Ab class distinguished by the ability to catalyze the hydrolysis of gp120 selectively because of initial noncovalent recognition of the 421–433 CD4BS region (13).

The conserved character of the CD4BS in genetically diverse HIV strains renders it suitable as a vaccine target. The CD4BS, however, is poorly immunogenic. Traditional immunization methods do not stimulate the adaptive synthesis of neutralizing Abs to the 421–433 region or other CD4BS epitopes. Neutralizing Abs that bind the CD4BS are found in the blood of a subset of patients after years of HIV infection, but the target epitope is not identified, and Ab response is weak (23, 24). Certain monoclonal Abs (mAbs) that bind the CD4BS expressed by purified monomer gp120 do not neutralize HIV appreciably or display limited ability to neutralize genetically diverse HIV strains (25, 26). The CD4BS is a flexible structure expressed in differing conformational states by monomer gp120 and the native gp120 oligomers of the virus (27–30). Moreover, the process of binding CD4 may induce movements within the CD4BS (31). Reproducing the native CD4BS conformation in experimental vaccine candidates has been difficult. A CD4BS mimetic of the epitope recognized by a well known anti-CD4BS neutralizing mAb (clone b12) did not induce the synthesis of neutralizing Abs (32). Polyclonal Abs raised by immunization with synthetic peptides spanning the 421–433 CD4BS region neutralized laboratory-adapted, coreceptor CXCR4-dependent HIV strains inconsistently (33–35). Neutralization of coreceptor CCR5-dependent strains responsible for initiating most HIV infections was not studied. Importantly, small synthetic peptides are often more flexible than the corresponding native protein segments. Inducing a traditional adaptive immune response in which the Ab CDRs develop binding specificity for the peptide immunogen therefore does not ensure recognition of the native 421–433 CD4BS region (35, 36). From mutagenesis and sequence identity studies, the gp120-binding site of preimmune Abs, in contrast, is composed mainly of the V\textsubscript{H} domain FR1 and FR3 (10, 11, 37). As certain preimmune Abs express HIV neutralizing activity attributable to recognition of the 421–433 region (13), the FR-dominated site must recognize the native state of this CD4BS epitope expressed on the viral surface.

There is, however, substantial difficulty in amplifying and improving the subset of preimmune Abs with HIV neutralizing activity for vaccination against the virus; SAg binding to Ab FRs fails to stimulate adaptive B cell differentiation and synthesis of specific IgG class Abs (38, 39). Indeed, the binding at the FRs may even lead to premature death of the B cells (12, 40). The SAg character of the 421–433 CD4BS epitope is therefore predicted to render it hypoimmunogenic with respect to the adaptive synthesis of neutralizing Abs following infection or traditional vaccination procedures.

We reported previously the induction of nucleophilic Abs by covalent immunization with full-length gp120 and a gp120 V3 peptide containing strongly electrophilic phosphate esters (41–43). The electrophile reacts covalently with BCRs (44), resulting in adaptively strengthened nucleophilic reactivity coordinated with specific noncovalent recognition of gp120. The Abs obtained by covalent immunization formed very stable immune complexes with HIV resulting from pairing of Ab nucleophiles with the naturally occurring electrophilic groups of gp120 (e.g. the backbone and side chain carbonyls, see Refs. 42, 43). A minority of the Abs proceeded to catalyze the hydrolysis of gp120, aided by water attack on the covalent acyl-Ab complex (41). Here we report the neutralization of HIV strains heterologous to the full-length electrophilic gp120 immunogen (E-gp120) by mAbs with binary CD4BS and V3 loop recognition capability. We also present models that explain synthesis of the mAbs in response to immunization with E-gp120.

**MATERIALS AND METHODS**

**Immunogens and Proteins—**Mice were immunized repeatedly with two electrophilic gp120 preparations, E-gp120 1a and 1b (41). E-gp120 1a contains phosphate groups attached to surface Lys side chain amines via a suberic acid linker (41). E-gp120 1b has an identical structure except that it contains the longer linker S-(3-carboxypropyl)-2,5-dioxopyrrolidin-3-yl)-Cys-Glu-Tris; see Fig. 1A. We have reported that Abs raised by immunization with gp120 bind E-gp120 1a, and Abs raised by immunization with E-gp120 1a bind gp120 specifically (41). Both immunogens were prepared from purified gp120 of HIV strain MN (subtype B). E-gp120 1b was prepared by reacting 0.92 mg of gp120 for 2 h with N-(y-maleimidobutyryloxy)succinimide ester (0.99 mg; Sigma) in 10 mM phosphate (EMD Chem). Deprotection was with trifluoroacetic acid.

E-gp120 1b was prepared by reacting 0.92 mg of gp120 for 2 h with e.g. 7% dimethyl sulfoxide, followed by removal of excess acylating agent (Econopack 10DG; Bio-Rad), reaction for 3 h with 0.76 mg of the phosphate linker reagent in the same buffer, i.e. Cys-Glu(diphenyl amino(4-amidinophenyl)methane phosphonate)-Tris, and recovery of E-gp120 by gel filtration (Biospin 6, Bio-Rad). The phosphate linker reagent was prepared by stepwise coupling of Tris (Trizma (Tris base), Sigma) to the following: (a) N-tert-butoxycarbonyl-Glu(O-tert-butyl) using the N-hydroxysuccinimide ester (EMD Chem, Gibbstown, NJ); (b) N-tert-butoxycarbonyl-Cys(trityl) using the N-hydroxysuccinimide ester (Chem-Impex, Wood Dale, IL), and (c) diphenyl amino(4-amidinophenyl)methanephosphonate using benzotriazol-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate (EMD Chem). Deprotection was with trifluoroacetic acid, yielding the phosphate linker reagent (observed m/z from electrospray ionization mass spectrometry, 717.4; calculated (M + H\textsuperscript{+} for C\textsubscript{32}H\textsubscript{41}N\textsubscript{6}O\textsubscript{9}PS, 717.2). The molar phosphate/gp120 stoichiometry values for E-gp120 1a and 1b determined as in Ref. 41 were, respectively, 28 and 29. Soluble
CD4 (scCD4, extracellular 4-domain version) was from Protein Sciences (Meriden, CT). Bovine serum albumin (BSA) and ovalbumin were from Sigma.

**Peptide and Electrophilic Probes**—Synthetic 15-mer gp120 fragment peptides (MN strain) were from the National Institutes of Health AIDS Research and Reference Reagent Program. Synthesis of the non-peptide phosphate E-hapten 2 has been described previously (45). The electrophilic peptide analog corresponding to gp120 residues 288–306 with an N-terminal biotin (E-288–306 3; Bt-LNESVQINCTRPNYNKRKR; where Bt is biotinamidohexanoyl; C’ is S-acetamidomethyl-Cys; phosphonate groups at Lys-303 and Lys-305 side chains) was prepared by acylation of the peptide with the N-hydroxysuccinimide ester of the suberyl phosphonate unit as described in Ref. 43 (observed m/z from electrospray ionization mass spectrometry, 1299.1, 974.5, and 779.5; calculated (M + 3H)3+, (M + 4H)4+, and (M + 5H)5+ for C179H266N63O38P2S2, 1299.6, 975.0, and 780.2). The biotinylated peptide corresponding to gp120 residues 292–306 (292–306 4; Bt-VQINCTRPNYNKRKR) was from EZBiolab (Westfield, IN) (observed m/z 1115.5, 744.0, and 558.2; calculated (M + 2H)2+, (M + 3H)3+, and (M + 4H)4+ for C95H161N33O25S2, 1115.6, 744.1, and 558.3). Preparation of biotinylated and BSA-conjugated electrophilic peptide analog of subtype B gp120 consensus residues 416–433 (E-421–433 6a and 6b) was described previously (46). Biotinylated and BSA-conjugated E-416–433 6a and 6b contain gp120 residues 416–433 with phosphonate groups at Lys-421 and Lys-432 side chains (LPSRIKQINMWQVGEKA; a Bt residue was placed at the N terminus for detection of mAb binding, and Cys-418 was replaced with a Ser residue to preclude S–S bond formation). Synthesis and purification of E-416–433 6a were as described for E-288–306 3 (observed m/z, 1164.8; calculated (M + 3H)3+ for C162H244N60O38PS22, 1164.4). E-416–433 containing Cys at the N terminus (observed m/z, 1627.2, 1085.4, and 814.3; calculated (M + 2H)2+, (M + 3H)3+, and (M + 4H)4+ for C152H232N33O25PS22, 1627.8, 1085.5, and 814.4) was conjugated to BSA using N-(γ-maleimidobutyryloxy)succinimide ester, yielding 6b (6.1 mol of peptide/mol of BSA). The control Sh-E-421–433 probe 7 has an identical structure except for the shuffled 421–433-amino acid sequence (IWN- QVQEIKMG-amino(4-amidinophenyl)methanephosphonic acid diphenyl ester). Probe 7 was prepared in the same manner as E-421–433 (observed m/z, 1025.1; calculated (M + 2H)2+ for C94H139N22O22PS22, 1025.0). The control Sh416–433 8 with the shuffled 416–433 amino acid sequence (GQKSWEIPAKNRLIMVQ) was from Sigma (observed m/z from matrix-assisted laser desorption-time of flight mass spectrometry, 2451.8; calculated (M + H)+ for C111H184N83O29P3S2, 2451.3). Preparation of the control E-VIP peptide 9 containing phosphonate groups on Lys-20 side chain was described (47). Human epidermal growth factor (EGF) produced in a yeast expression system was from Austral Biologicals (San Ramon, CA). E-EGF was prepared in the same manner as described for E-gp120 (1.8 phosphonate/EGF).

**mAbs**—IgG class mAbs that bind E-gp120 1a and 1b covalently were identified, respectively, by screening three sets of splenocyte hybridomas, two from 1a-immunized mice (640 wells) and one 1b-immunized mouse (318 wells) by a denatur-
sequenced in both directions and yielded identical sequences. Germ line gene and family assignments were as in Ref. 48. Somatic hypermutation replacement (R)/silent (S) ratios were computed for CDRs (CDRs 1 and 2) and FRs 1–3 and did not include mutations attributable to V(D)J junctional diversification. Average R/S ratios expected for a random mutational process occurring in the CDRs and FRs are adjusted for codon usage and region length (49). Junctional nucleotide deletions and insertions were analyzed with IMGT® (the international ImMunoGeneTics information system) (87). To count junctional amino acid deviations from the germ line sequence, the assigned V(D)J germ line genes were aligned end-to-end; the minimum number of nucleotides required to maintain the reading frame downstream from the junctions was deleted in silico, and the sequences of the resultant junctions were compared with the anti-E-gp120 junctions.

Crystal Structures—Amino acids in the 292–306 and 421–433 interpeptide region in the gp120 crystal structure (PDB 2B4C; see ref. 4) were identified as residues within 8 Å of the plane formed by Arg-298 Ca, Lys-421 Ca, and Gln-422 Ca. Percent solvent exposure was computed as follows: $100 \times$ (solvent-accessible surface area in the crystal structure)/(solvent-accessible surface area in unfolded state), with unfolded state accessible area calculated using surface-accessible area of each amino acid (R) in the tripeptide Gly-R-Gly (where R indicates any amino acid) (50). Screening of Fab crystallization conditions was with Hampton crystal screening kits at room temperature by vapor diffusion in hanging drops. High quality Fab crystals were grown using the following: (a) 2 μl of 4 mg of Fab/ml in 10 mM Tris, pH 7.5, mixed with 2 μl of the reservoir solution (16% w/v polyethylene glycol 6000, 0.1 M HEPES, pH 7.4); or (b) 1.5 μl of 5.5 mg of Fab/ml mixed with 1 μl of the reservoir solution (8% w/v polyethylene glycol 6000, 0.1 M Tris-HCl, pH 8). Rod-shaped crystals belonging to space group P212121, with one Fab molecule/asymmetric unit were obtained under both conditions (unit cell parameters, respectively, a = 52.29 Å, b = 62.92 Å, c = 135.17 Å, and a = 52.18 Å, b = 63.13 Å, c = 135.41 Å). The crystals were cryo-protected in 25% glycerol and flash-frozen in liquid nitrogen. Diffraction data for crystals a and b, respectively, were collected at 100 K at the Advanced Light Source, beamline 4.2.2, Berkeley, CA, or the Advanced Photon Source, beamline 22ID. Using crystal a, diffraction data were solved by molecular replacement and refined to the crystallographic R factor of 0.225 using 95% of the data between resolution limits 10 and 2.5 Å. The free R factor was 0.272 for the remaining 5% of randomly excluded reflections. The final model consisted of 430 amino acid residues and 70 water molecules. Average B factor was 31.4 Å$^2$, and deviations from standard covalent bond lengths and angles were 0.005 Å and 1.22°, respectively. Ramachandran plot showed 89.3% of amino acid residues in the most favored regions. The Fab model from diffraction data a and b was built using the atomic coordinates of Fab a as the starting point and refined within resolution limits 50-2 Å. Fab model b included 434 amino acid residues and 269 water molecules. The final R factor was 0.220, and the free R factor was 0.265. Average B factor was 29.6 Å$^2$, and deviations from standard bond lengths and angles were 0.006 Å and 1.37°, respectively. Ramachandran plot showed 89% residues in the most favored regions. The atomic coordinates and structure factors were deposited in the Protein Data Bank with accession numbers 3CLE (a) and 3CLF (b). Cavities were identified using the Binding Site tool of Discovery Studio version 1.7, Accelrys (amino acids within 8 Å of the surface of an object fitted into the cavity). Solvent-accessible surface areas were obtained using a probe radius of 1.40 Å.

Binding Assays—Murine mAb binding to biotinylated gp120 (MN strain; 40 ng/well) immobilized on streptavidin-coated plates was determined by ELISA in the presence or absence of synthetic gp120 fragments (corresponding to the gp120 MN strain sequence, National Institutes of Health AIDS Research and Reference Reagent Program; catalog numbers 6215–6336). ELISAs using various immobilized peptide and protein probes coated on the wells were conducted similarly using conditions that were standardized to yield specific binding (A$_{490}$ > 0.3 after subtracting binding of an irrelevant mAbs in control wells): BSA-E-421–433 5b, 400 ng of peptide equivalents/well; BSA-E-416–433 6b, 70 ng of peptide equivalents/well; Bt-292–306 4, 400 ng/well. Binding of scFv YZ23 to BSA-E-416–433 6b (140 ng/well) or Bt-292–306 4 (400 ng/well) and of scFv JL427 to gp120 or E-gp120 1a (40 ng/well) was detected, respectively, using peroxidase-conjugated anti-polyhistidine Ab (1:1,000, Sigma) and anti-c-Myc Ab (51). Bound Fab was detected with peroxidase-conjugated anti-mouse Fab Ab (1:500, Sigma). The ability of mAb YZ23 to inhibit binding of human mAbs b12, 2G12, 17b, 48d, and 447-52D was tested with gp120 immobilized using Ab to gp120 epitope 497–511 (Cliniqa; 1 μg/well) and peroxidase-conjugated goat anti-human IgG (1:1,000, Sigma). mAb binding of intact HIV virions (strain MN) was measured by capture of immune complexes using immobilized protein G (1 μg/well) and p24 measurement (43). mAb binding of Bt-E-peptide probes was also measured by denaturing SDS-electrophoresis using boiled reaction mixtures (52). mAb-peptide adduct bands were quantified by densitometry and expressed in arbitrary volume units or biotin/mAb ratio (mol/mol) using biotinylated BSA as standard (7 biotins/BSA; Sigma).

HIV Neutralization—Infection of phytohemagglutinin-stimulated peripheral blood mononuclear cell cultures by clinical HIV isolates treated for 1 h with increasing mAb concentrations was measured in four independent culture wells by enzyme immunoassay of the capsid protein p24 in the laboratories of Dr. Carl Hanson (15) and Dr. David Montefiori (53). Neutralization was computed as percent decrease of p24 concentrations in mAb-containing wells compared with infection occurring in phosphate-buffered saline. Inter-assay variability of neutralization was determined in 26 repeat assays using the clade C virus ZA009 and mAb YZ23. The geometric mean and 95% confidence intervals of the IC$_{50}$ values were, respectively, 7.5 and 4.7–11.6 μg/ml.

RESULTS

Immunogen Properties—The electrophilic analogs of gp120 (E-gp120 1a and 1b; Fig. 1A) hold the potential of inducing an immune response different from gp120 by virtue of these structural modifications: (a) the presence of electrophilic phosphate groups placed at Lys side chains, and (b) formation of cova-
lent aggregates. The precursor gp120 devoid of electrophilic phosphonate groups were linked to a Lys side chain with a suberic acid group (E-gp120 1a) or a longer linker containing hydrophilic groups (E-gp120 1b). E-hapten 2 is a small molecule amidinophosphonate. Also shown are silver-stained reducing SDS-electrophoresis gel lanes of precursor gp120 devoid of phosphonate groups (lane 1) and E-gp120 incubated at 37 °C for 0 (lane 2), 3 (lane 3), and 6 h (lane 4). B, gp120 and E-gp120 inhibition of HIV binding by mAbs to reference neutralizing epitopes (mAb clones b12, 447-52D, and 2G12). The mAbs were incubated for 20 h with intact HIV virions (strain MN, 1.6 × 10^5 TCID_{50}/ml; mAb clone b12, 45 μg/ml; clone 447-52D, 0.8 μg/ml; clone 2G12, 7.5 μg/ml) in the presence or absence of gp120, E-gp120 1a, EGF, and E-EGF (0.5 μM). mAb-HIV complexes were captured using immobilized protein G, and viral p24 was measured by ELISA. Plotted are values of residual HIV binding (%; mean ± S.D. of 4–6 replicates) defined as 100 × (A_{490} in the presence of competitor)/A_{490} in the absence of competitor). All values are corrected for nonspecific binding observed in diluent instead of the mAbs. mAb binding (A_{490}) in the absence of competitors is as follows: clone b12, 0.176 ± 0.018; clone 447-52D, 0.291 ± 0.085; and clone 2G12, 0.246 ± 0.046. C, binding of immobilized gp120 and E-gp120 1a to mAbs b12 and 268 DIV determined by ELISA (mAb clone b12, 10 μg/ml; clone 268 DIV, 2 μg/ml). D, binding of immobilized gp120 and E-gp120 by anti-421–433 scFv JL427 determined by ELISA. E-gp120 1a binding was measured in the absence or presence of excess E-hapten 2 (100 μM) to eliminate covalent binding effects.

The alterations in antigenic structures were probed by comparing the ability of E-gp120 and gp120 to compete with intact HIV virions for binding to previously described reference mAbs. E-gp120 1a and gp120 inhibited HIV binding by the CD4BS-directed mAb b12 equivalently, but E-gp120 1a was recognized less efficiently by mAb 447-52D directed to the apex of the V3 loop (residues 310–313; Fig. 1B). The irrelevant EGF and E-EGF probes did not influence mAb-HIV binding appreciably, indicating the specificity of E-gp120 and gp120 inhibition of mAb-HIV binding. Similarly, E-gp120 1a and monomer gp120 binding by mAb b12 was near-equivalent in a direct binding assay format, whereas E-gp120 1a was bound minimally by mAb 268-DIV to the V3 loop residues 308–313 (Fig. 1C). Recombinant scFv fragments from patients with the autoimmune disease lupus that bind the CD4BS 421–433 epitope have been described (15, 37). scFv JL427 specific for the 421–433 epitope displayed superior binding to E-gp120 1a compared with monomer gp120 (Fig. 1D). After the noncovalent
**Heterologous HIV neutralization by mAbs to E-gp120.** A, aligned sequences of the immunodominant 307–325 region epitope (HXB2 numbering) of the gp120 immunogen (strain MN) and clinical HIV isolates tested for mAb neutralization. , identities; #, unidentified residues. B, neutralization of heterologous CCR5-dependent subtype C strain ZA009 with anti-E-gp120 mAbs. Shown are IC50 values for seven neutralizing mAbs obtained from assays conducted at increasing mAb concentrations. The following 10 mAbs did not neutralize the virus: YZ20, YZ24, SK-T01, SK-T02, SK-T03, SK-T04, 2F2, 7H3, 1F4, and 5E11 (IC50 > 20 μg/ml). C, representative strain ZA009 neutralization data at varying mAb YZ23, 3A5, and SK-T03 concentrations. Values are means of four cultures ± S.D.

binding step is complete, covalent binding of Ab nucleophiles by E-gp120 phosphonates results in increased immune complex accumulation (41). To distinguish between noncovalent versus covalent binding as the cause of improved scFv binding, the assay was repeated in the presence of excess hapten electrophile (E-hapten 2). This reagent saturates Ab nucleophiles without compromising noncovalent Ab-antigen binding (43). The scFv maintained its superior reactivity with E-gp120 compared with gp120 in the presence of E-hapten 2, ruling out nucleophile-electrophile pairing as a factor (Fig. 1D) and suggesting improved noncovalent binding at the 421–433 epitope as the reason for the superior reactivity.

**HIV-neutralizing mAbs—** Immunization with E-gp120 1a was reported previously to induce the synthesis of nucleophilic Abs that bind gp120 irreversibly (42) and hydrolyze gp120 slowly (41). Immunization with gp120 devoid of the electrophilic group induces strain-specific neutralizing Abs but not Abs that neutralize genetically heterologous HIV strains (2, 55, 56). The electrophilic immunogens were prepared from recombinant gp120 with a sequence corresponding to subtype B, strain MN gp120 sequence. To minimize detection of strain-specific neutralizing activity, the heterologous subtype C strain ZA009 was tested. The V domains of strain MN gp120 and ZA009 gp120 are highly divergent, illustrated by the immunodominant V3 epitope composed of residues 307–325 (Fig. 2A). Of 17 anti-E-gp120 1a and 1b mAbs tested, 7 neutralized HIV ZA009 at IC50 < 20 μg/ml (50% inhibitory concentration; 5/11 anti-1a mAbs, 45%; 2/6 anti-1b mAbs, 33%; Fig. 2B). Therefore, there is no evidence that the longer hydrophilic linker of E-gp120 1b favors development of mAbs with superior HIV recognition capability (for clarity, we do not discriminate between the mAbs raised to E-gp120 1a and 1b in the studies described below; see under “Materials and Methods” for identity of individual anti-1a and anti-1b mAbs). Ten mAbs did not meet the criterion of HIV neutralization with IC50 < 20 μg/ml. Fig. 2C is an example of HIV neutralizing activity as a function of mAb concentration.

mAb YZ23 was reported to neutralize three coreceptor CCR5-dependent HIV strains previously (42). Here we tested neutralization of 11 CCR5-dependent subtype A–C HIV strains by this mAb. All strains tested were neutralized in a concentration-dependent manner by the mAb (Table 1). The potency (IC50) with which mAb YZ23 neutralized the 11 strains was uncorrelated with the extent of sequence divergence between the V domains of the E-gp120 immunogen (strain MN) and gp120 expressed by these strains (% mismatches for the individual gp120 V1, V2, V3, V4, and V5 domains; % mismatches, 27 ± 9, 57 ± 11, 55 ± 8, 54 ± 9, and 48 ± 13, respectively; mean ± S.D.). An anti-E-gp120 mAb 1b, clone 6B11, also neutralized four genetically divergent strains drawn from different HIV subtypes (Table 1). It is therefore difficult to attribute the neutralizing activity to mAb recognition of a nonconserved epitope.

Endotoxin (lipopolysaccharide) induces chemokine release from monocytes that can bind chemokine coreceptors and inhibit HIV infection (57–59), and endotoxin contamination may cause artifactual HIV neutralization. According to the Limulus amebocyte lysate test, the endotoxin concentration of the mAb YZ23 stock solution (0.4 mg of IgG/ml) used in Table 1 was 1.14 ± 0.56 endotoxin units/ml. At the mAb concentration yielding 50% neutralization of the 11 HIV strains, the final endotoxin concentration was 0.001–0.097 endotoxin units/ml. Endotoxin does not inhibit HIV infection of peripheral blood
mononuclear cells or macrophages at these concentrations. Moreover, further reduction of the endotoxin concentration in a stock mAb YZ23 solution by ion exchange chromatography (from 0.38 to 0.06 endotoxin units/ml) did not influence the neutralizing activity (supplemental Fig. S1). Therefore, there is no evidence that endotoxin is the cause of HIV neutralization.

**Binary Peptide Reactivity**—The reactivity of anti-E-gp120 mAb clones YZ23 and YZ18 with synthetic gp120 peptides and certain electrophilic analogs of the peptides was studied. mAb binding to immobilized gp120 or E-gp120 was measured in the absence or presence of 122 synthetic gp120 peptides (15-mer overlapping peptides corresponding to residues 27–512 of strain MN gp120; 50 μg/ml). Four peptides displayed concentration-dependent inhibition of mAb-gp120 binding, the peptides corresponding to residues 292–306, 296–310, 421–435, and 421–435 (data for peptides 292–306 and 421–435 shown in Fig. 3, A and B). Peptides 292–306 and 296–310 were equipotent inhibitors, suggesting the overlapping region 296–306 as a mAb recognition site. Similarly, peptides 417–431 and 421–435 inhibited the binding with near-equal potency, suggesting residues 421–431 as the second recognition site. There is no evident sequence similarity between residues 296–306 and peptide 421–431, arguing against binding of the two peptides at a common site of the mAbs. The binding assays were conducted in the presence of excess albumin (1%, w/v), and other 15-mer synthetic gp120 peptides did not inhibit mAb YZ23 or mAb YZ18 binding to gp120 (e.g. peptides 203–212 or 142–154 in Fig. 3, A and B). gp120 binding by another anti-E-gp120 mAb, the non-neutralizing clone SK-T03, was inhibited competitively by peptide 463–477 but not peptides 292–306, 296–310, 417–431, or 421–435 (42). The data suggest selective recognition of the 296–306 and 421–431 peptides by mAbs YZ23 and YZ18.

The binary reactivity was confirmed by measuring the immune complexes in ELISA and electrophoresis tests using peptide 292–306 4 and the electrophilic analogs of peptides 288–306 (E-288–306 3), 421–433 (E-421–433 5a and 5b), and 416–433 (E-416–433 6a and 6b) containing phosphonate diester groups (Fig. 4; see supplemental Fig. S2A for comparative binding of the alternate E-421–433 and E-416–433 probes). E-288–306 3 and E-421–433/E-416–433 (5a, 5b, 6a, and 6b) contain peptide regions that bind mAbs by traditional noncovalent forces. In addition, they contain the phosphonate electrophile that forms covalent complexes with mAb nucleophilic residues, permitting electrophoretic estimation of specific immune complexation (52). Binding of mAb YZ23 to immobilized peptide 292–306 4 and E-421–433 5b was observed in ELISA tests, whereas the control anti-E-gp120 mAb SK-T03 displayed little or no binding to either peptide antigen (Fig. 4B). mAb binding to the immobilized peptides was inhibited by the corresponding soluble peptides (supplemental Fig. S2B). These observations indicate the saturable and selective character of mAb-peptide binding.

IgG class mAbs are bivalent, possessing two equivalent antigen-binding sites, each composed of a V L and V H domain (Fv region). To determine whether the binary peptide reactivity is a property of the individual antigen-binding sites, we studied the univalent Fab and recombinant scFv fragments containing the V L and V H domains of IgG YZ23. The Fab fragment displayed binding to peptide 292–306 4 as well as E-421–433 5b, whereas Fab from the control anti-E-gp120 mAb was devoid of binding reactivity (Fig. 4C). Similarly, the recombinant scFv YZ23 fragment displayed binding to peptide 292–306 4 as well as E-416–433 6b (Fig. 4D). It may be concluded that the binary peptide reactivity is a property of the individual Fv region of the mAbs.

mAb YZ23 formed covalent adducts with biotinylated E-288–306 3 and E-421–433 5a identified by denaturing electrophoresis and staining with streptavidin-peroxidase (Fig. 5A). Under reducing conditions, the E-421–433 5a adduct migrated on the gels as a band with mass predicted for the mAb heavy chain subunit adduct (50-kDa subunit; mass of 5a, 2 kDa). The light chain subunit was labeled with this peptide at very low levels indistinguishable from nonspecific labeling with the iso-

| Subtype | Strain  | IC 50 μg/ml | mAb YZ23 |
|---------|---------|-------------|-----------|
| A       | 92RW008 | 0.22        |           |
| B       | PAV0    | 2.52        |           |
|         | SF162   | 0.33        |           |
|         | 92BRR021| 14.59       |           |
|         | QH0692  | 19.88       |           |
| C       | 98BRR004| 1.40        |           |
|         | Du172   | 2.21        |           |
|         | Du422   | 3.51        |           |
|         | 97TZA009| 7.5         |           |
|         | 98TZ017 | 1.13        |           |

TABLE 1
Neutralization of genetically diverse HIV strains by mAbs YZ23 and 6B11

mAb concentrations at which 50% neutralization occurred (IC 50) are reported. Data are from plots of mAb concentration versus neutralization fitted to the equation, % neutralization = 100/(1 + 10^-log IC 50). Each mAb concentration was tested using four independent culture replicates. Outliers were eliminated by the ROUT method (coefficient Q, 90%; GraphPad Prism). Peripheral blood mononuclear cells were used as the host cells. All HIV strains were coreceptor CCR5-dependent.

FIGURE 3. Competitive inhibition of mAb YZ23 binding to immobilized gp120 A and mAb YZ18 binding to immobilized E-gp120 1a 8 by synthetic 421–435 and 292–306 peptides. Binding was determined by ELISA. mAbs are 5 μg/ml. Plotted are % values of binding (mean ± S.D. of duplicates) in the absence (A pop for YZ23, 0.768 ± 0.035; YZ18, 0.836 ± 0.028) or presence of increasing competitor peptide concentrations. Values are corrected for nonspecific binding observed in diluent instead of the mAbs. A noninhibitory control peptide is shown in each panel.
type-matched control mAb light chain (expected mass, 25 kDa). E-288–306 labeled both mAb subunits (50-kDa heavy chain and 25-kDa light chain). mAb adducts with the E-288–306 and E-421–433 probes were formed more rapidly than with control irrelevant peptide probes containing the electrophilic phosphonate group but no specific peptide epitope that can bind noncovalently to antibodies (E-VIP and the shuffled sequence Sh-E-421–433). The rates of binding by the mAb were in agreement with the one-site exponential binding model (Fig. 5B; % binding = 100(1 − e−kt), where k is the first-order rate constant, and t is reaction time). Fifty % saturation times for E-288–306 and E-421–433 were 0.72 and 3.2 h, respectively. Following near-saturation of the E-288–306-binding site, the mAb still retained the ability to bind an equimolar amount of E-421–433 (Fig. 5C). It may be concluded that two different mAb sites are capable of simultaneously binding the two peptide probes.

Of 17 mAbs studied for neutralizing activity, 8 displayed the ability to bind both E-421–433 and E-288–306 in electrophoresis tests (Fig. 6A). Six of the 8 binary peptide-reactive mAbs neutralized HIV strain ZA009. Six of the 7 neutralizing mAbs in Fig. 2B displayed binary peptide reactivity (clones YZ18, YZ21, YZ22, YZ23, 3A5, and 6B11; the neutralizing mAb clone YZ19 did not meet the binding activity threshold stated in Fig. 6A legend; neutralizing mAbs are shown as red symbols in Fig. 6A). The association between HIV neutralizing activity and the binary peptide reactivity was statistically significant (Fig. 6B; p < 0.015, two-sided Fisher’s exact test). The ability to bind E-421–433 was alone a sufficient predictor of HIV neutralizing activity, but E-288–306 binding alone was not (Fig. 6C). Six of 8 E-421–433-binding mAbs neutralized the virus (p < 0.015). In contrast, only 7 of 14 E-288–306-binding mAbs displayed neutralizing activity (p = 0.228), and only 1 of 6 mAbs with E-288–306 binding activity and no E-421–433 binding activity displayed neutralization. Three mAbs did not bind either peptide probe, suggesting that they recognize epitopes not involving residues 288–306 or 421–433. It may be concluded that the binary and 421–433 region binding activities...
are expressed preferentially by the mAb subset with heterologous HIV neutralizing activity.

Epitope Properties—Residues 421–433 located in the C4 gp120 domain are highly conserved in genetically diverse HIV strains (supplemental Table S2). Residues 288–306 in the V3 domain stem also express only limited sequence variability. The 421–433 peptide region is essential for HIV binding to CD4. We used the E-416–433 peptide probe 6b to study the relationship between the mAb and CD4-binding sites expressed on the full-length protein (Fig. 7A). Soluble CD4 (sCD4, extracellular 4-domain version) but not an irrelevant control E-VIP probe adducts were detected as in A. Band intensities quantified by densitometry are shown in duplicate. Biotin quantification was by comparison with a standard curve constructed using biotinylated BSA.

C ternary complexation of mAb YZ23 with E-288–306 and E-421–433 binding by mAb YZ23. mAb YZ23 (150 μg/ml) reacted with E-288–306 3 or E-421–433 5a (10 μM) for varying lengths of time was subjected to SDS-electrophoresis and the mAb-peptide adducts were detected as in A. Band intensities quantified by densitometry were shown in duplicate. Biotin quantification was by comparison with a standard curve constructed using biotinylated BSA.

mAb Sequences—Immune processes that can contribute to adaptive selection of E-gp120-recognizing Abs are as follows: somatic sequence diversification of the V(D)J junctions, mutations upstream of the junctions (the somatic hypermutation process), and preferential usage of individual germ line V gene products with innate gp120 binding activity. All five anti-E-gp120 mAbs sequenced contained abundant junctional diversification (Table 2). V domain amino acid replacements attributable to somatic hypermutation were low to moderate. However, three mAbs contained at least one V domain with a CDR R/S value of less than 0.5, suggesting antigen-driven selection of CDR mutations. Antigen-driven selection of FR mutations does not occur under normal circumstances, as these regions do not participate in binding conventional antigens. The R/S ratios for the CDRs of the five mAbs exceeded the random mutation ratio, suggesting antigen-driven selection of CDR mutations. Only two mAbs contained V1 +3 domain FRs with R/S ratios exceeding the predicted random mutation ratio (mAbs YZ21, YZ22, and 3A5), and the overall V1 +3 FR R/S ratio for the five mAbs also exceeded the predicted value (Table 2). The number of replacement mutations in the anti-E-gp120 V1 +3 FRs was greater than the number of silent mutations (p = 0.029; two-sided Student’s unpaired t test; see Fig. 8). There was no significant difference in the number of replacement and silent mutations in the V1 +3 FRs of the anti-E-gp120 mAbs or the VL and VH FRs of a panel of reference mAbs directed to conventional microbial antigens. We and other groups have observed previously that the 421–433 gp120 epitope is recognized weakly by preimmune Abs at a site dominated by V1 +3 domain FRs (10, 11, 37, 71). The anti-E-gp120 mAb sequence information is consistent with improved 421–433 region recognition because of adaptive mutations occurring in the V1 +3 FRs. As there was no evidence that the 288–306 gp120 region is recognized by preimmune Abs, emergence of this binding activity following E-gp120 immunization likely derives from adaptive structural changes occurring in the CDRs by junctional diversification and somatic hypermutation processes.

All five anti-E-gp120 mAbs sequenced were derived from the V1 +3 family germ line genes (Table 2). Approximately 45–49% of expressed murine Abs belong to the V1 +3 family (72, 73). The probability that all five mAbs utilize V1 +3 germ line genes by
two-sided Fisher’s exact test. Red symbols denote mAbs that neutralized subtype C strain 97ZA009 (IC50 < 20 μg/ml); black symbols, non-neutralizing mAbs. Numbers in parentheses correspond to the number of mAbs in the quadrant. A, association of binary peptide binding with HIV neutralizing activity. The frequency of the binary peptide binding activity in groups of mAbs without or with strain ZA009 neutralizing activity was compared. p value from two-sided Fisher’s exact test. B, frequency of binary E-288–306 binding by individual mAbs with adduct densities at least 4-fold greater than the background mean band intensity of adducts formed by control E-peptide probes were considered positive for binding activity (background intensity was the average value of E-VIP units (2926 ± 2926 AU); cyan quadrant, binary peptide binding mAbs; yellow quadrant, monoreactive E-288–306 binding mAbs. Red symbols denote mAbs that neutralized subtype C strain 97ZA009 (IC50 < 20 μg/ml); black symbols, non-neutralizing mAbs. Numbers in parentheses correspond to the number of mAbs in the quadrant. B, association of binary peptide binding with HIV neutralizing activity. The frequency of the binary peptide binding activity in groups of mAbs without or with strain ZA009 neutralizing activity was compared. p value from two-sided Fisher’s exact test. C, association of mAb neutralizing activity with the individual E-421–433 Sa and E-288–306 b binding activities. The frequencies of the neutralizing activity in groups of mAbs without or with the individual peptide binding activities were compared. p value from two-sided Fisher’s exact test.

Preferential VH gene utilization therefore must be considered as a factor influencing E-gp120 recognition. No information is available about preferential gp120 binding by preimmune murine Abs derived from various VH family genes. Human VH family Abs produced without exposure to HIV are reported to bind gp120 preferentially (7). Seventeen VH1 FR residues were suggested previously to contribute to the gp120 recognition by sequence comparison of preimmune human Abs without and with gp120 binding activity (10). Of these 16 residues, 9–11 are present in the murine anti-E-gp120 VH1 FRs (identities + conservative substitutions; supplemental Table S3).

Binding Site Analysis—The crystal structure of the Fab fragment prepared from mAb YZ23 was solved by the molecular replacement method. Two crystals obtained in different solvents yielded virtually identical structures (see “Materials and Methods”; structure a, PDB code 3CLE, and structure b, PDB code 3CLF; root mean square deviation of α-carbons for the two structures, 0.44 Å; resolution, 2.5 and 2.0 Å, respectively; R factor, 0.225 and 0.22, respectively). Details of the Fab a structure follow. A T-shaped canonical antigen-combining site cavity composed mainly of CDR residues was evident (24 amino acids; number of residues contributed by CDRH1, CDRH2, CDRH3, FRH1, FRH3, CDRL1, and CDRL3, 4, 3, 7, 1, 2, 1, and 6 residues, respectively, designated the CDR cavity; Fig. 9A and supplemental Table S4). Five CDR cavity residues are derived from somatic sequence diversification processes (VH Ser-92; VH Gly-95, Arg-96, Ser-98, and Gly-100). The cavity surface area is 968 Å2, a value comparable with the antigen-binding cavity formed by the CDRs of other antibodies (600–900 Å2; 74–76). The CDR cavity is flanked by a shallow and irregular L-shaped cavity composed of 21 VH domain residues (designated FR cavity; surface area 1081 Å2; Fig. 9B). This cavity is dominated by VH1 FRs (3 FRH1 and 12 FRH3 residues; supplemental Table S4). The centroid-to-centroid distance between the CDR cavity and FR cavity is 21 Å. Seven residues in the FR cavity were previously suggested to contribute to binding of gp120 by preimmune Abs (supplemental Table S4). One of the amino acids located in the FR cavity of scFv YZ23, V1H residue Lys-19, was replaced by an Ala residue by site-directed mutagenesis. The K19A mutant displayed reduced binding of the E-416–433 6b probe compared with the wild type scFv (Fig. 10A). The mutant and wild type scFv displayed near-equivalent binding of the 292–306 4 probe (Fig. 10B), suggesting that the mutation did not result in a globally disruptive conformation change of the scFv. The mutation data verify that the FR cavity is the 421–433 region-binding site.
addition, an interpeptide space composed of 12 residues with a surface area of 946 Å² separates the 296–306 and 421–431 regions (residues 326–329, 418–420, 436–439, and 443; shown in purple, Fig. 9C). Moreover, the combined surface area of the two peptide regions recognized by mAb YZ23 (1783 Å²; 296–306 region, 837 Å²; 421–431 region, 946 Å²) exceeds the typical surface areas of individual peptide epitopes recognized by antibodies (600–900 Å²; Refs. 74–76).

Specific covalent binding of the E-peptide phosphonate probes (Fig. 5) requires a nucleophilic site located close to the noncovalent mAb-binding site. Examples of such nucleophilic sites are diads of amino acids containing hydroxyl side chains hydrogen-bonded to a general base (side chains of His, Lys, Arg, Tyr, Glu, and Asp residues; backbone carbonyl groups; Ref. 42 and references cited therein). Examination of the Fab YZ23 crystal structure revealed several Ser, Thr, and Tyr residues in the CDR cavity and FR cavity within proximity of a general base. Example diads with proton donor-acceptor distance <4.2 Å are (distances in parentheses) as follows: CDR cavity, V₃ Ser-92 O- H Ser-30 NH₂ (3.1 Å); CDR cavity, V₄ Thr-70 O- H Thr-128 NH₂ (2.9 Å); and FR cavity, VH Thr-70 O- H Thr-70 carbonyl O (3.4 Å). The location of candidate nucleophilic diads within the CDR and FR cavities indicates the structural feasibility of covalent mAb binding to the electrophilic gp120 peptide probes in conjunction with their noncovalent recognition. The initial noncovalent mAb-antigen binding step may induce conformational adjustments that alter the nucleophilic reactivity by changing the proton acceptor-donor distance, and the structural study of mAb-peptide probe complexes is required to identify the nucleophiles definitively.

**DISCUSSION**

The 421–433 CD4BS region is an excellent vaccine target as it is largely conserved in genetically diverse HIV strains, and binding to CD4 is an obligatory step in HIV entry into host cells. Site-directed mutagenesis and crystallography studies indicate the crucial role of this region in maintaining the integrity of the CD4BS (3–6) (supplemental Table S2). However, experimental vaccination efforts have failed to document the synthesis of neutralizing Abs to the 421–433 region (77–79). Our studies indicate that a substantial proportion of mAbs (7/17) induced by covalent immunization neutralized HIV strains genetically heterologous to the E-gp120 immunogen. The neutralizing...
activity was associated with binary mAb recognition of the 421–433 region and another gp120 peptide region. mAb binding to the 421–433 region probe (E-416–433) was inhibited by the extracellular fragment of CD4, and the E-416–433 probe was itself bound to sCD4 specifically (supplemental Fig. S3). These observations indicate overlapping mAb and CD4-binding sites expressed by gp120.

We reported the ability of anti-E-gp120 mAbs to form unusually stable complexes with HIV (42) and to hydrolyze monomer gp120 slowly (41). Unless viral gp120 is hydrolyzed substantially more rapidly than monomer gp120, proteolysis cannot account for efficient HIV neutralization observed in the present study. Irreversible gp120 binding by the mAbs due to nucleophile-electrophile covalent binding can render HIV non-infectious for prolonged durations (42, 43). However, the mAbs will recognize genetically diverse HIV strains only if they bind a conserved gp120 region. Moreover, recognition of a gp120 region essential for viral entry is preferred, as irreversible binding at a distant epitope may not hinder binding to host cell receptors. An anti-E-gp120 mAb directed to the 463–477 gp120 region, for instance, formed very stable complexes with HIV gp120 without neutralizing the virus (clone SK-T03, see Ref. 42). The epitope specificity of the mAbs therefore is an important factor determining their functional effects. IgG molecules contain two Fv regions, each capable of binding one traditional antigen molecule. Like the parent IgG, univalent Fab YY23 and scFv YY23 displayed the binary property of binding the 288–306 and 421–433 region probes. The subsites that bind the two probes are located within a single Fv region. The mAb saturated with the 288–306 peptide still displayed stoichiometric binding of the 421–433 peptide, indicating simultaneous occupancy of the two binding subsites. mAb binding to full-length gp120 was inhibited by synthetic peptides corresponding to gp120 regions 288–306 and 421–433. mAbs that recognized the 421–433 region probe dominated the neutralizing mAb subset (6/7 neutralizing mAbs). In view of the importance of this region in HIV binding to host cells, binding at the 421–433 epitope alone may be sufficient to inactivate the virus. Several anti-E-gp120 mAbs that recognized the 288–306 region probe but not the 421–433 region probe failed to neutralize HIV (7/14 mAbs). Therefore, binding of the 288–306 region alone may not be sufficient to attain virus neutralization.

E-gp120 and monomer gp120 are surrogates for the native gp120 oligomers on the viral surface. Monomer gp120 does not induce Abs with the anti-E-gp120 mAb epitope properties. Introduction of phosphonate groups on Lys side chains likely perturbs the conformation of E-gp120 monomers. Further changes in antigenic structure can occur due to the observed covalent oligomerization of E-gp120. Like enzymes and Abs, gp120 contains a naturally occurring nucleophilic site reactive with electrophilic phosphonates (54). The self-association reaction can be attributed to an intermolecular reaction between the electrophilic phosphate and the naturally occurring nucleophilic site. Of 17 anti-E-gp120-binding mAbs examined here, 14 displayed specific binding of the 288–306 or 421–433 peptide regions, suggesting that these are the immunodominant regions of E-gp120. A structural alteration involving the

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3 Even the most efficient proteolytic mAb identified (clone YZ20; $K_m$ and $k_{cat}$ 2 μM and 0.003 min$^{-1}$ respectively) will hydrolyze only 2.4% of the viral gp120 at 20 μg/ml mAb in 1 h (41).

4 Simultaneous engagement of the 288–306 and 421–433 regions expressed by gp120 devoid of the phosphonate is feasible if these regions are topographically compatible with the two mAb-binding subsites. Alternatively, inhibition of mAb-gp120 binding by both synthetic peptide probes may result from a negative cooperativity effect, whereby occupancy of one mAb subsite reduces binding at the second subsite.
421–433 CD4BS region is suggested by the superior reactivity of a neutralizing anti-421–433 region scFv with E-gp120 compared with gp120. This suggests superior conformational mimicry of the native 421–433 CD4BS region or removal of a steric hindrance effect. Two mAbs directed to the V3 loop (residues 308–313 and 310–313) were less reactive with E-gp120 compared with gp120, suggesting the feasibility of a structural perturbation involving the neighboring 296–306 region recognized by an anti-E-gp120 mAb. E-gp120 and gp120 were bound equivalently by mAbs to a carbohydrate-dependent epitope and a CD4BS epitope not involving the 421–433 region, indicating the absence of a global change in protein conformation. These observations suggest selective structural perturbations of the 421–433 and 296–306 E-gp120 regions that explain in part its ability to induce mAbs reactive with these regions.

Noncovalent binding of traditional antigens to Ab CDRs expressed within the BCR complex activates signal-transducing proteins (Igα, Igβ, CD19, CD22, and Lyn) by an allosteric mechanism, thereby driving the initial recruitment of individual B cell clones and their adaptive differentiation into Ab-secreting plasma cells (80). The 421–433 CD4BS region, however, is a B cell SAg epitope for which preimmune human BCRs and secreted Abs produced without exposure to gp120 already express a binding site encoded by VH germ line genes without a requirement for adaptive sequence diversification (7, 10, 11, 13, 71). Preimmune Abs from mice possess a similar capability (71). Binding of SAg epitopes to the FR binding is thought to induce early B cell stimulation followed by premature cell death (12, 40, 81). Indeed, it may be hypothesized that HIV has developed a CD4BS with the ability to bind Ab FRs weakly as a means to preclude adaptive synthesis of neutralizing Abs. Our studies provide the first evidence that the impediment can be bypassed. The 421–433 region binding mAbs invariably displayed binary peptide recognition, i.e., binding of the second peptide region (residues 288–306; eight mAbs). The converse was not true. This relationship suggests the hypothesis that acquisition of 288–306 binding activity may be a requirement for improved recognition of the 421–433 region.

Information about the mechanisms of antibody synthesis was gleaned from their V domain sequences. All of the anti-E-gp120 mAbs belonged to the V_{H}1 family, suggesting selective recruitment of the preimmune B cell subset expressing this germ line gene family. A nonrandom use of V_{H} germ line genes could occur due to preferential noncovalent or covalent E-gp120 recognition. No information is available about E-gp120 recognition by preimmune murine BCRs belonging to various V_{H} families. However, certain human Ab data are relevant. Noncovalent recognition of the gp120 SAg site was originally discovered as a property of preimmune human V_{H}3 family Abs (7). As might be expected from the extensive sequence similarity between various V gene families, preimmune human Abs that use other V_{H} family genes also recognize the 421–433 region (71), and even rare V_{L} domains display this property (48). Importantly, covalent E-gp120 binding to BCR nucleophic sites may be an independent factor influencing V gene usage. The phosphonate electrophile binds Ab μ and κ/λ subunits expressed by preimmune BCRs covalently (44), and different Abs express varying levels of nucleophilic reactivity (52). The energy liberated from covalent binding substantially exceeds the binding energy of noncovalent reactions (e.g., ΔG for a noncovalent binding by an Ab with K_{D} of 1 nM at 25 °C, 51.4 kJ/mol compared with a value of 599 kJ/mol for the strength of a P-O bond; Ref. 82). It is conceivable that the excess covalent binding energy is used to induce a BCR conformational transition that activates cellular proliferation and favorable differentiation outcomes differing from the classical pathways driven by noncovalent antigen binding.

The structure of Abs changes adaptively by antigen-driven selection of BCR mutants generated by the V_{H}/V_{L} domain CDR3 junctional diversification early in B cell development, followed by selection of replacement mutations occurring over the entire V domain length (the “somatic hypermutation” process; see Ref. 83). Substantial junctional sequence diversification of the anti-E-gp120 V_{H} and V_{L} domains was observed, suggesting a robust contribution of this process in development of the Ab response to E-gp120. Amino acid replacements due to somatic hypermutation were less abundant. Nonetheless, the R/S mutation ratios for the V domain CDRs of three of five mAbs and the overall R/S ratio for the CDRs of all five mAbs exceeded the ratio predicted for a nonrandom process, a characteristic of an antigen-driven Ab response (49). An E-gp120-driven selection of CDR mutants is consistent with the expression of a mAb epitope specificity not found in the preimmune B cell repertoire, i.e., 288–306 region recognition. Similarly, the R/S mutation ratios for the V_{H} domain FRs of the anti-E-gp120 mAbs also suggested an antigen-driven selection of mutations. Recognition of the 421–433 region by preimmune Abs is dominated by contacts at V_{H} domain FRs (10, 11, 37). This suggests adaptive improvement of the FR-dominated 421–433 region-binding site of preimmune Abs, as opposed to de novo generation of this epitope specificity.

Consistent with the binary recognition model, two distinct cavities were visible on the surface of an anti-E-gp120 Fab by crystallography, the traditional cavity formed by the V_{H}/V_{L} domain CDRs and a second cavity composed mainly of V_{H} FR1 and FR3 residues. The second cavity contains seven residues previously suggested to be important for recognition of gp120 by preimmune Abs (supplemental Table S4), supporting the argument that the 421–433 region binds at the V_{H} FRs. Replacement of V_{H} Lys-19, a residue located in the FR cavity, by an Ala residue resulted in reduced E-416–433 binding by scFv YZZ2. This confirms recognition of the 421–433 region peptide probe at the FR cavity. Recognition of the 288–306 region may be assumed to occur at the classical antigen-binding site, the CDR cavity, as there is no evidence that this region is recognized by preimmune Abs. Inspection of the published gp120 crystal structure supports paired recognition of the 288–306 and 421–433 regions, respectively, at the CDRs and V_{L} FR cavities (Ref. 4; gp120 bound to CD4 and a Fab fragment). The 288–306 and 421–433 regions are spatially separated entities with combined surface area exceeding the surface areas of the individual mAb cavities. Importantly, however, the fine struc-

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5 To our knowledge, no mAb probe is available for the 296–306 region.
Covalent HIV Vaccination

Vaccine goal: Induction of neutralizing anti-CD4BS Abs

1. Escape by binary epitope binding
2. Escape by covalent binding

FIGURE 11. Possible mechanisms for E-gp120-induced neutralizing mAbs synthesis. The SAg character of the 421–433 region limits B cell responses and production of neutralizing Abs directed to the CD4-binding site (bottom). Acquisition of binding specificity for the 296–306 region by the CDRs is hypothesized to generate a stimulatory signal that overcomes any down-regulatory signal due to SAg binding at BCR FRs (pathway 1). Moreover, covalent E-gp120 engagement at the FRs of the BCR is a highly energetic process that may induce productive B cell signaling instead of anergy to the 421–433 region (pathway 2). The phospho-

tates of the 421–431 and 296–306 regions in various forms of gp120 used in this study may be divergent (for mAb binding, gp120 devoid of phosphonate electrophiles and E-gp120; for induction of mAb synthesis, E-gp120; for mAb neutralization, intact virions). Additional caveats for any assertions that the two regions are fully separated spatially in the biologically relevant conformation of gp120 are as follows. (a) Certain gp120 regions undergo local conformational transitions upon CD4 binding (31, 64, 65). (b) The CD4BS conformation in native gp120 oligomers and the monomers may not be identical (27–30). (c) Deletion of certain gp120 regions necessary to reduce conformational flexibility and obtain crystalizable gp120 complexes could introduce structural deviations.

The paired recognition model is supported by evidence for antigen-driven selection at two distinct regions of the anti-E-gp120 mAbs, the CDRs and V\textsubscript{H} FRs; the existence of two spatially separated cavities in the mAbs; and formation of stoichiometric ternary mAb complexes by synthetic peptide probes of the 288–306 and 421–433 regions. Fig. 11 presents our view of how covalent immunization might bypass the down-regulatory effect of the noncovalent 421–433 region binding at the FRs. Discrete signal transduction pathways direct B cells toward productive or abortive differentiation outcomes (84). The V\textsubscript{H} FR site for binding the 421–433 region is available in the pre-immune repertoire. However, adaptive induction of Abs that express this site by immunization with gp120 is a disfavored event. According to the paired recognition model, the 288–306 and 421–433 peptide regions expressed by the E-gp120 immunogen (but not by gp120) bind simultaneously to the CDR and FR cavities, respectively. The up-regulatory cellular signal generated by CDR binding is sufficiently strong to override the down-regulatory signal from the 421–433 region binding at the FRs, permitting immunogen-driven selection of Abs with binary peptide binding activity. The validity of this prediction depends only on simultaneous CDR and FR engagement, regardless of whether the 421–433 and 288–306 regions are distinct epitopes or a single conformational epitope.

Left unexplained by the model, however, is the observation of antigen-driven selection of FR mutants. Such mutants cannot be selected if FR binding only stimulates down-regulatory signaling. Therefore, we must leave open the alternative that the covalent reaction between E-gp120 and BCRs drives synthesis of the neutralizing Abs. The covalent reaction is exceptionally energetic compared with conventional noncovalent binding (see above). We propose that the excess covalent energy can be transduced to stimulate a productive cellular activation pathway despite 421–433 binding at the FRs, explaining induction of the neutralizing Abs by immunization with E-gp120. This model implies the following: (a) the anti-E-gp120 mAbs acquire their binary peptide reactivity fortuitously, made possible because the two peptide regions of E-gp120 are correctly located to bind the CDRs and V\textsubscript{H} FRs simultaneously; and (b) gp120 devoid of phosphonate groups may present the two peptide regions in a form capable of simultaneous CDR and FR binding. gp120 will not, however, induce Abs with the binary peptide reactivity, as it does not bind BCRs covalently, the driving force for favorable B cell differentiation.

Development of an HIV vaccine has been stymied by the poor immunogenicity of the conserved, functionally important envelope regions. Our findings indicate that covalent immunization induces the synthesis of neutralizing mAbs to a conserved gp120 segment essential for viral infection, the 421–433 CD4BS region. Synthesis of the neutralizing mAbs in response to E-gp120 appears to entail contributions from innate and adaptive immune processes that facilitate improved recognition of the 421–433 region in concert with the 288–306 region. It remains to be determined whether covalent immunization with E-gp120 or future generation electrophilic peptides can induce a neutralizing Ab response that is sufficiently robust and focused at the CD4BS to prevent and treat HIV infection. Nonetheless, the ability of the mAbs to neutralize diverse HIV strains raises hope for effective vaccination against HIV.

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