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Functional and Structural Characterization of Human V3-Specific Monoclonal Antibody 2424 with Neutralizing Activity against HIV-1 JRFL

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ABSTRACT

The V3 region of HIV-1 gp120 is important for virus-coreceptor interaction and highly immunogenic. Although most anti-V3 antibodies neutralize only the sensitive tier 1 viruses, anti-V3 antibodies effective against the more resistant viruses exist, and a better understanding of these antibodies and their epitopes would be beneficial for the development of novel vaccine immunogens against HIV. The HIV-1 isolate JRFL with its cryptic V3 is resistant to most V3-specific monoclonal antibodies (MAbs). However, the V3 MAb 2424 achieves 100% neutralization against JRFL. 2424 is encoded by IGHV3-53 and IGLV2-28 genes, a pairing rarely used by the other V3 MAbs. 2424 also has distinct binding and neutralization profiles. Studies of 2424-mediated neutralization of JRFL produced with a mannosidase inhibitor further revealed that its neutralizing activity is unaffected by the glycan composition of the virus envelope. To understand the distinct activity of 2424, we determined the crystal structure of 2424 Fab in complex with a JRFL V3 peptide and showed that the 2424 epitope is located at the tip of the V3 crown (^{307}IHIGPGRAFYT^{319}), dominated by interactions with His^{P308}, Pro^{P313}, and Arg^{P315}. The binding mode of 2424 is similar to that of the well-characterized MAb 447-52D, although 2424 is more side chain dependent. The 2424 epitope is focused on the very apex of V3, away from nearby glycans, facilitating antibody access. This feature distinguishes the 2424 epitope from the other V3 crown epitopes and indicates that the tip of V3 is a potential site to target and incorporate into HIV vaccine immunogens.

IMPORTANCE

HIV/AIDS vaccines are crucial for controlling the HIV epidemics that continue to afflict millions of people worldwide. However, HIV vaccine development has been hampered by significant scientific challenges, one of which is the inability of HIV vaccine candidates evaluated thus far to elicit production of potent and broadly neutralizing antibodies. The V3 loop is one of the few immunogenic targets on the virus envelope glycoprotein that can induce neutralizing antibodies, but in many viruses, parts of V3 are inaccessible for antibody recognition. This study examined a V3-specific monoclonal antibody that can completely neutralize HIV-1 JRFL, a virus isolate resistant to most V3 antibodies. Our data reveal that this antibody recognizes the most distal tip of V3, which is not as occluded as other parts of V3. Hence, the epitope of 2424 is in one of the vulnerable sites on the virus that may be exploited in designing HIV vaccine immunogens.

The HIV-1 envelope glycoprotein (Env) is the only virus-encoded protein expressed on the surface of the virus and is the sole target for virus-neutralizing antibodies (Abs). On the virion surface, the HIV Env spike is a compact heterodimeric trimer made up of gp120 and gp41 subunits (1–3). The surface gp120 subunit is responsible for interacting with the host cell through binding to CD4 and the coreceptor, the chemokine receptor CCR5 or CXCR4 (4–7). On the basis of primary amino acid sequences, gp120 is divided into five conserved regions (C1 to C5), which are interspersed with five variable regions (V1 to V5) (8). The CD4-binding site and the chemokine receptor-binding site are both highly conformational and discontinuous. The chemokine receptor binding site in particular is composed of the invariant β2 and β3 strands of the V1V2 stem region, β20 and β21 strands in the conserved C4 region, and the third variable (V3) region of gp120 (3, 9). Vulnerable sites on the HIV Env have been identified based on their recognition by broadly neutralizing human monoclonal antibodies (MAbs). On gp120, these epitope sites include the CD4-binding site (10, 11), a cluster of glycans

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recognized by MAb 2G12 (12–14), and the glycan-bearing regions in V1V2 and V3 (15–17). These Abs recognize structurally complex epitopes and display an unusual VH domain exchange or an extreme level of somatic hypermutations and/or CDR3 lengths; thus, inducing such Abs by vaccination is not a simple feat.

The crown of the V3 loop, on the other hand, is highly immunogenic; antibodies to the V3 crown are induced in the vast majority of human subjects following HIV infection or after vaccination with HIV gp120 vaccines (18–23). The importance of V3 as a vaccine immunogen is further established by the fact that V3 is essential for HIV-1 infectivity (24, 25) and that antibodies binding to V3 can block the virus infection (26–31) Most V3-specific MAbs isolated from HIV-1-infected individuals are also highly cross-reactive, recognizing gp120 proteins from viruses of different HIV-1 subtypes. However, these V3-specific MAbs neutralize mainly the relatively sensitive tier 1 viruses and are ineffective against tier 2 and tier 3 isolates (2, 26, 32). The failure of anti-V3 MAbs to neutralize tier 2 and tier 3 viruses in the face of recognition of their corresponding soluble gp120 proteins indicates that V3 epitopes are present but inaccessible on the functional Env spikes on the virions (33, 34). Nonetheless, there are distinct epitopes on the V3 loop (17, 35). Many are occluded, but some may be more exposed. Immunogenic V3 epitopes that are accessible on the virus Env would be valuable new targets for HIV vaccine development.

In this study, we present the crystal structure of a V3 epitope recognized by human MAb 2424, which is distinct from the other V3 MAbs in its capacity to neutralize JRFL, a relatively resistant HIV-1 isolate. MAb 2424 was isolated from a chronically HIV-infected subject living in New York City by a cellular method in which peripheral blood mononuclear cells (PBMCs) were transformed by Epstein-Barr virus, fused with heteromyeloma cells (36, 37), and selected based on enzyme-linked immunosorbent assay (ELISA) reactivity with the V3 (consensus B)-MLV gp70 fusion protein. Unlike the other V3 MAbs, 2424 preferentially binds to V3 of subtype B viruses. Moreover, the 2424 gene usage is unusual among human anti-V3 MAbs; 2424 is IgG1 with a kappa light chain and is encoded by IGHV3-53 and IGLV2-28 genes (38). In agreement with functional studies, the crystallographic structure reveals a distinct epitope at the tip of the V3 crown that is not as influenced by glycans as the other V3 epitopes. Although it is unknown if potent 2424-like Abs can be generated by vaccination, this V3 epitope represents another important site that may be incorporated into the immunogens for vaccines designed to elicit HIV-1-neutralizing antibodies.

MATERIALS AND METHODS

Human monoclonal antibodies (MAbs) and HIV-1 Env proteins. Anti-V3 human MAbs 2424, 447-52D, 2219, 2557, 3074, and 3869 were obtained from an existing panel of HIV-specific MAbs. MAbs 2424, 447-52D, and 2219 were derived from the cells of HIV-1-infected subjects from the United States, while MAbs 2257, 3074, and 3869 were produced from Cameroonian individuals infected with CRF02_AG or other non-B subtypes (22, 38–41). All of these MAbs were generated by the cellular method as described previously (36, 37). The irrelevant human anti-parvovirus B19 MAb 1418 was used as a negative control (42). MAb b12 (provided by Dennis Burton and Carlos Barbas) and MAb 2G12 (provided by Hermann Katinger) were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, while MAb PG9 was provided by Wayne Koff (International AIDS Vaccine Initiative [IAVI]'s Neutralizing Antibody Consortium) or purchased from Polymun Scientific.

Recombinant gp120 proteins of various subtypes (produced in 293T cells) were purchased from Immune Technology Corp., except for $gp120_{JRFL}$ (produced in CHO cells by Progenics, Inc.) which was obtained from Vaccine Research and Development Branch of Division of AIDS, NIAID, NIH. Stabilized trimeric BG505-SOSIP.664 gp140 was a generous gift from John P. Moore (Weill Cornell Medical College).

Antibody binding assay. The binding of human V3 MAbs to recombinant gp120s was determined by ELISA. Briefly, 96-well ELISA plates were coated with gp120 (1.0 μg/ml in phosphate-buffered saline [PBS]) at 4°C overnight, blocked with 3% bovine serum albumin (BSA) in PBS, and reacted for 2 h at 37°C with MAbs serially diluted in PBS with 1% BSA. Bound MAbs were detected with alkaline phosphatase-conjugated goat anti-human IgG and *p*-nitrophenyl phosphate substrate.

Cell lines, plasmids, and viruses. Cells of the TZM.bl line were obtained through the NIH AIDS Research and Reference Reagent Program (contributed by J. Kappes and X. Wu). 293T/17 cell line was purchased from the American Type Culture Collection (ATCC).

HIV-1 Env-expressing plasmids for generating pseudoviruses were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Pseudoviruses were produced by cotransfecting 293T cells with *env*, *rev*, and pNL4-3.Luc.R-E- or pSG3 using a ProFection kit (Promega, Madison, WI) or polyethylenimine (PEI) MAX40,000 (Polysciences, Warrington, PA). Glycan-modified $HIV\text{-}1_{JRFL}$ was generated as described above in the presence of 25 μM kifunensine or 20 μM swainsonine (Sigma, St. Louis, MO). Supernatants were harvested after 48 h and clarified by centrifugation and 0.22-μm filtration. Single-use aliquots were stored at −80°C until use.

Point mutations in JRFL Env were introduced in pCAGGS JRFL.JB gp160 using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. All mutant constructs were sequenced to confirm the correct amino acid changes. Plasmid pCAGGS JRFL.JB gp160 was kindly supplied by the NIH Vaccine Research Center.

Neutralization assay. Virus neutralization was measured as described previously (43). Briefly, serially diluted MAbs were incubated with the virus for 1 h at 37°C. TZM.bl cells were then added to virus-MAb mixtures in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and DEAE-dextran (Sigma, St. Louis, MO). After 48 h, virus infection was determined using the Bright-Glo luciferase assay system (Promega, Madison, WI). For neutralization assays with soluble CD4 (sCD4), the virus was preincubated with recombinant human sCD4 (Progenics Pharmaceuticals, Tarrytown, NY) for 30 min at 37°C, before addition of serially diluted MAbs. Virus infection was measured in TZM.bl cells using a β-galactosidase-based assay (Promega, Madison, WI).

Fab production and purification. The Fab fragment of MAb 2424 was prepared by papain digestion as described previously (35, 44). Briefly, the IgG molecule was mixed with papain (Worthington, Lakewood, NJ) at a 20:1 molar ratio in 100 mM Tris (pH 6.8) with 1 mM cysteine hydrochloride and 4 mM EDTA. The mixture was incubated for 1 h at 37°C, and the reaction was stopped with 10 mM iodoacetamide. The Fab fragment was separated from the Fc fragment and the undigested IgG by a protein A column and further purified by size exclusion chromatography. The Fab fragment was then concentrated to about 10 mg/ml for crystallization.

Crystallization, data collection, structure determination, and refinement. The $V3_{JRFL}$ peptide was synthesized by Biomatik (Wilmington, DE), dissolved in water, and mixed with Fab 2424 at a 10:1 molar ratio. Crystallization conditions were screened and optimized using the vapor diffusion hanging-drop method. Well-diffracted crystals of Fab alone were obtained with a well solution of 20% polyethylene glycol 8000, 0.1 M Tris (pH 8.5), 36% glycol, whereas those of the Fab/epitope complex were obtained in a well solution of 28% polyethylene glycol 4000, 0.17 M Li_2SO_4, 0.085 M Tris (pH 8.5), 15% glycerol. X-ray diffraction data sets were collected at the synchrotron beamline GM/CA-CAT of the Advanced

A

Relative mean of neutralizing potency of V3 mAbs against HIV-1 JRFL

V3 mAb	IC_{50} (μg/ml) Mean ± SEM
2424	7.6 ± 3.3
447-52D	18.6 ± 3.0
2219	> 100
3074	> 100
2557	> 100
3869	> 100
1418 (neg ctrl)	> 100

B

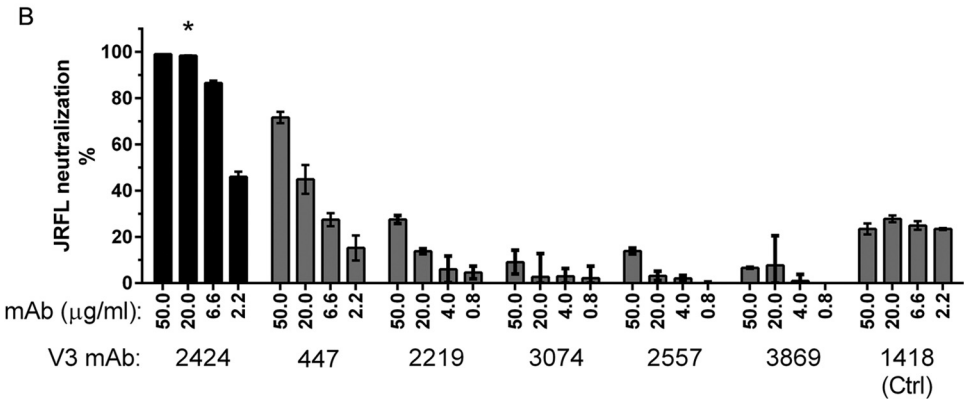

FIG 1 Neutralization potency of 2424 versus the other V3-specific MAbs against JRFL. (A) Relative neutralizing potency of 2424 and five other V3 MAbs against JRFL. These V3 MAbs were isolated from HIV-infected individuals (22, 38–40). Means and standard errors of IC_{50}s from four independent experiments are shown. (B) Titration curves of the six V3 MAbs against JRFL. Neutralization assay was performed with JRFL pseudovirus and TZM.bl target cells as previously reported (43). MAbs were titrated and incubated with virus at 37°C for 1 h. TZM.bl cells were then added to the virus-MAb mixtures in DMEM containing 10% fetal bovine serum and DEAE-dextran. Neutralization activity was assessed 48 h later using the luciferase detection system. The parvovirus-specific MAb 1418 was included as a negative control. Means and standard deviations were calculated from duplicate wells. Representative data from multiple repeat experiments are shown. Statistical analysis was performed by two-way analysis of variance (ANOVA). *, $P < 0.001$ compared to MAbs 447-52D, 2219, 3074, 2557, 3869, and 1418.

Photon Source (APS), Argonne National Laboratory. All data sets were processed using the HKL 2000 package (45) and XDS (46), and structures were determined by molecular replacement using a homologous Fab structure (PDB ID 3KDM) as the initial model. Cycles of refinement for each model were carried out in COOT and Phenix (47, 48). The NCS constraints were imposed for the three complexes in the refinement for the 3.18-Å structure of the Fab 2424/V3 complex. Final structural analyses were carried out using ICM, and figures were generated using PyMOL (Schrödinger, LLC) and ICM (49).

Protein structure accession numbers. Coordinates and structure factors of Fab 2424/V3 complex and Fab 2424 have been deposited in the Protein Data Bank under accession numbers 4XMK and 4XML, respectively.

Nucleotide sequence accession numbers. The IGHV and IGKV sequences of 2424 have been deposited in GenBank with accession numbers EU794426 and KP050787, respectively.

RESULTS

V3-specific MAb 2424 potently neutralizes HIV-1 JRFL isolate. Most V3-specific MAbs isolated from HIV-infected subjects or vaccine recipients are capable of neutralizing tier 1 HIV-1 isolates but not the more resistant tier 2 or tier 3 viruses, due to the cryptic nature of many V3 epitopes on the Env spikes of tier 2 and tier 3 viruses. However, one V3-specific MAb, 2424, shows a potent neutralizing activity against a relatively resistant HIV-1 isolate, JRFL, indicating the presence of an Ab-accessible neutralizing V3 epitope on this virus. As shown in Fig. 1A, among six V3 MAbs tested, 2424 was the most effective, with a 50% inhibitory concentration (IC_{50}) of 7.6 μg/ml, whereas the remaining five had IC_{50}s of 18.7 μg/ml or >100 μg/ml. The IC_{50}s of other V3 MAbs against JRFL were also shown previously to range from 11 to >50 μg/ml (26). More recently, when a panel of 48 V3 MAbs was tested, 2424 was also found to be the most potent against JRFL (50). The greater potency of 2424 was further shown by its ability to achieve a neutralization plateau of 100%, a level unattainable by 447-52D and the other V3 MAbs tested (Fig. 1B; also, see Fig. 3 to 5).

2424 is also unlike the other V3 MAbs in terms of its Ig gene usage. This MAb has VH and VL gene pairing not used by the other V3 MAbs (50). The 2424 VH and VL genes are somatically mutated 14.4% and 5.1% from the germ line IGHV3-53 and IGLV2-28 genes, respectively. Heavy-chain complementarity-determining region 3 (CDR H3) is composed of 17 amino acids, similar in length to normal human Igs (51). Moreover, while the majority of V3 MAbs use λ light chains, 2424 uses a κ light chain. Only 17 out of 70 human V3 MAbs with known VH and VL genes use κ light chains (23, 38, 50, 52). Together, these data suggest that 2424 is a unique MAb targeting an epitope in the V3 loop that may be more accessible or occluded differently compared to the other V3 epitopes.

2424 displays subtype B-specific binding and neutralizing activities. To better characterize MAb 2424 and its epitope, we

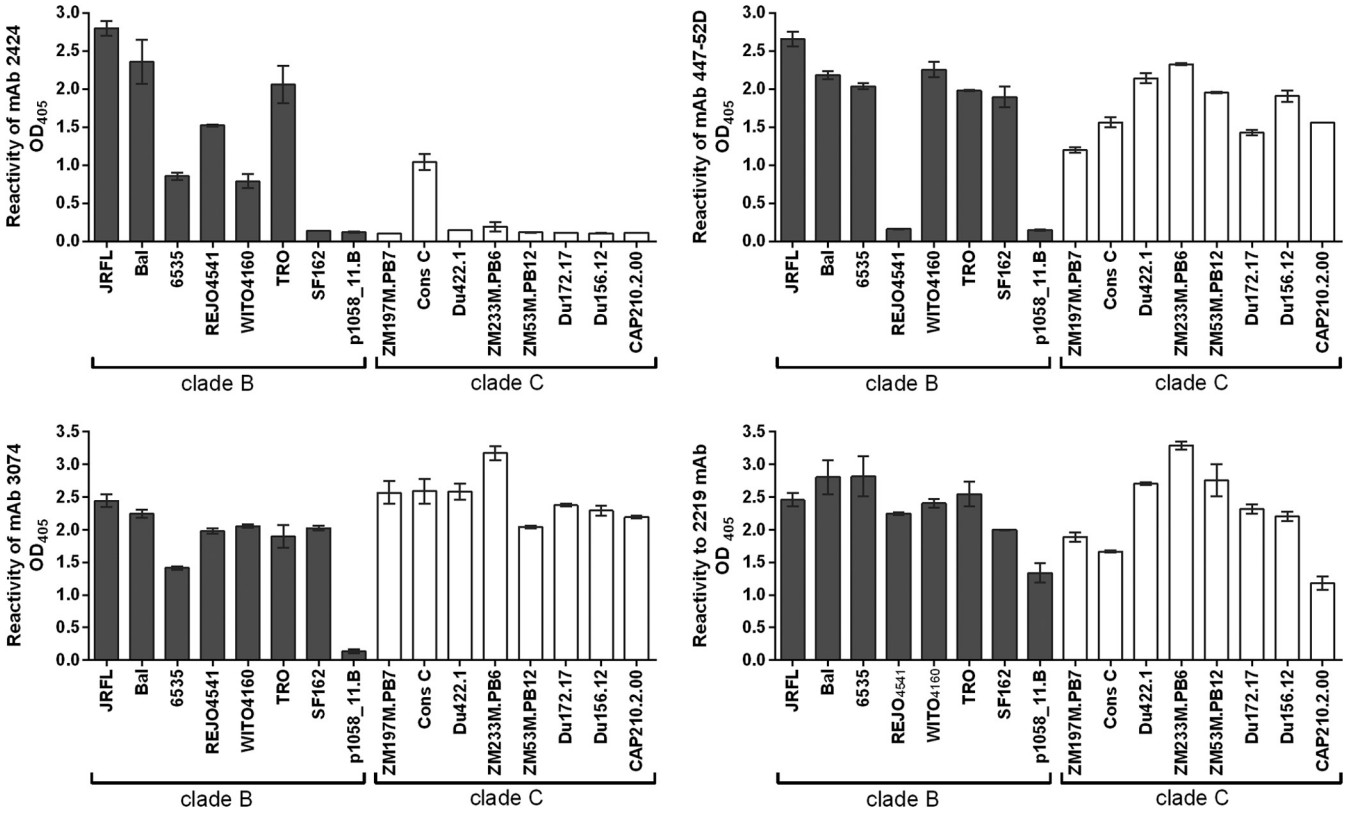

FIG 2 Binding of MAb 2424 versus the other V3 MAbs to gp120 proteins of clade B and clade C HIV-1 isolates. The binding of MAbs (1 μg/ml) to gp120 used at 1 μg/ml to coat the well surface was detected by ELISA. An irrelevant control MAb, MAb 1418, was also tested in parallel and displayed no binding to any gp120 in the panel ($OD_{405} \leq 0.2$; data not shown). Data from one representative experiment are shown; the experiments were repeated twice.

assessed the cross-reactivity of 2424 in ELISA using recombinant gp120 proteins from HIV-1 subtype B and C (Fig. 2). Three V3 MAbs with weaker JRFL neutralization activities, 447-52D, 3074, and 2219, were also tested for comparison. 2424 displayed a gp120-binding profile distinct from those of the other three MAbs. 2424 was reactive mainly with subtype B gp120s, whereas 447-52D, 3074, and 2219 recognized most of the subtype B and subtype C gp120s tested. 2424 also poorly reacted with recombinant gp140 proteins of subtype C (CN54), subtype A (UG37), and subtype A (BG505-SOSIP.664) (optical density at 405 nm [OD_{405}] = 0.28, 0.15, and 0.313, respectively; OD_{405} of irrelevant MAb 1418 = 0.15), although it was reactive with few V3 peptides of subtype A viruses, such as KE-Ken29 (OD_{405} = 1.3) and D687 (OD_{405} = 0.5).

The neutralizing breadth and potency of MAb 2424 were evaluated using TZM.bl target cells and pseudoviruses expressing 15 HIV-1 Envs from tier 1 to 3 subtype B and non-B viruses (2, 32) (Table 1). 2424 neutralized six viruses (BX08.16, BaL26, SS1196.1, REJO4541, JRCSF, and JRFL.JB), and three of these viruses (JRFL, JRCSF, and REJO4541) are relatively resistant to other V3 MAbs (26, 50). This neutralization pattern paralleled the 2424 binding to V3 peptides of the corresponding viruses. All six viruses belong to subtype B, confirming the subtype B specificity of this MAb. In contrast, the other V3 MAbs studied here (447-52D, 2219, 2557, 3074, and 3869) neutralized viruses from both B and non-B subtypes (26, 50). These data indicate that MAb 2424 is relatively potent against JRFL, JRCSF, and REJO4541, but its binding and neutralizing breadth are more restricted than those of the other V3 MAbs.

We next determined how the addition of 2424 affected virus neutralization of the other V3 MAbs. Neutralizing activities of 2424 and 447-52D were tested individually or in a 1:1 combination against eight HIV-1 pseudoviruses (Table 2). The combination of 2424 and 447-52D was able to neutralize seven of the viruses, while on its own, 447-52D neutralized six viruses. However, the IC_{50}s of the combined MAbs were generally higher than those of the individual MAbs, indicative of potential competition and steric hindrance between the two MAbs.

Neutralizing activity of 2424 is affected by soluble CD4 and removal of the N-glycan at position 301 but not by changes in N-glycan composition of the virus Env. To further characterize the 2424 epitope, we tested whether 2424 neutralizing activity was affected by virus pretreatment with soluble CD4. CD4 binding to the virus Env causes conformational changes that induce exposure of masked epitopes on V3 (53). A fixed concentration of soluble CD4, which on its own yielded ~20% neutralization, was incubated with JRFL, prior to addition of titrated amounts of 2424. Three other V3 MAbs (447-52D, 3074, and 3869) and an irrelevant parvovirus-specific MAb (1418) were tested for comparison. CD4 treatment augmented virus neutralization by 2424, similar to that observed with 447-52D, 3074, and 3869 (Fig. 3). The neutralization levels attained were significantly higher than the calculated sums of percent neutralization attained by each MAb alone and CD4. The other V3 MAbs, 2219 and 2557, were similarly affected by CD4 (54). These data indicate that CD4-induced conformational changes modulate MAb accessibility of the 2424 epitope, similar to the other neutralizing V3 epitopes.

TABLE 1 Neutralization activity of MAb 2424 against HIV-1 pseudoviruses

Virus	Tier[a]	Clade	V3 sequence[b]	MAb 2424		
				IC_{50} (μg/ml)[c]	Peptide binding[d]	Signature motif[e]
Bx08.16	1B	B	CTRPNNNTRKSI**H**IG**P**G**R**AFYTTGDIIGDIRQAHC	**<0.4**	**+++**	**+**
Bal.26	1B	B	CTRPNNNTRKSI**H**IG**P**G**R**AFYTTGEIIGDIRQAHC	**<0.4**	**+++**	**+**
SS1196.1	1B	B	CTRPNNNTRKSI**H**IG**P**G**R**AFYATGGVIGDIRQAHC	**<0.4**	**+++**	**+**
JRFL.JB	2/Chr	B	CTRPNQNTRKSI**H**IG**P**G**R**AFYTTGEIIGDIRQAHC	**5.4**	**+++**	**+**
JRCSF	2/Chr	B	CTRPSNNTRKSI**H**IG**P**G**R**AFYTTGEIIGDIRQAHC	**6.6**	**+++**	**+**
REJO4541	2	B	CTRPNNNTRKSI**H**IA**P**G**R**AFYATGEIIGDIRKAYC	**11**	**+++**	**+**
Bal.01	1B	B	CTRPNNNTRKSINIGPGRAFYTTGEIIGDIRQAHC	>50	NT	−
SF162.LS	1A	B	CTRPNNNTRKSITIGPGRAFYATGDIIGDIRQAHC	>50	±	−
6535.3	1B	B	CTRPNNNTRKSINLGPGRAFYATGDIIGDIRQAHC	>50	+	−
YU2	2	B	CTRPNNNTRKSINIGPGRALYTTGEIIGDIRQAHC	>50	−	−
MW965.26	1A	C	CTRPNNNTRKSVRIGPGQTFYATGAIIGDIRQAHC	>50	−	−
HO31.7	2	B	CTRPSNNTRKSITIGPGRAFYTTGDIIGDIRRAHC	>50	NT	−
DJ263.8	1B	A	CTRPNNNTRRSVRIGPGQTFYATGDIIGDIRQAHC	>50	−	−
HO61.14	3	B	CTRPNNNTRKSIPIGPGRAFYTTGDIIGDIRQAHC	>50	NT	−
ZM109F	1B	C	CIRPGNNTRKSIRLGPGQTFYATGDVIGDIRKAYC	>50	−	−

[a] Chr, chronic.
[b] Boldface indicates the 2424 epitope signature motif.
[c] MAb concentration needed to reach 50% inhibition in the TZM.bl neutralization assay. IC_{50}s of <50 μg/ml are in bold.
[d] ELISA reactivity against biotinylated V3 peptides. Reactivity with scrambled peptide was an OD_{405} of ≤0.5. Average OD_{405}s are classified as follows: −, 0.2 to 0.5; ±, 0.5 to 0.75; +, 0.75 to 1.5; ++, 1.5 to 2.0; +++, 2.0 to 3.5. NT, not tested.
[e] His^{308}, Pro^{313}, and Arg^{315} constitute a signature motif for the 2424 epitope.

Next we examined the contribution of N-glycan composition in influencing the accessibility of 2424 epitope vis-a-vis the other V3 epitopes. Our previous studies demonstrated that V3 epitopes on JRFL were better exposed when the virus was produced in the presence of a mannosidase inhibitor, kifunensine or swainsonine, which enriches the viral Env glycoproteins with high mannose-type N-linked glycans (27). Hence, we evaluated neutralization of JRFL produced with or without kifunensine by 2424 and five other V3 MAbs (Fig. 4A). JRFL neutralization by 2424 was unaltered whether the virus was produced with or without kifunensine. Kifunensine treatment also had minimal effect on 3074, but this MAb did not neutralize JRFL. In contrast, 447-52D, 2219, 2557, and 3869 were more effective against JRFL produced in the presence of kifunensine than untreated JRFL. Swainsonine treatment also had no effect on JRFL neutralization by 2424, although it enhanced virus sensitivity to 447-52D (Fig. 4B). MAb 2G12, which recognizes a cluster of N-glycans bearing the high-mannose type (55), and the CD4-binding site-specific MAb b12 were tested for comparison (Fig. 4A). 2G12 displayed higher neutralization against $JRFL^{Kif}$ than untreated JRFL, whereas neutralization by b12 was changed slightly, consistent with previously published data (56). The irrelevant control MAb 1418 had no neutralizing activity against treated or untreated virus.

Although various levels of neutralization were observed with the different V3 MAbs against untreated JRFL, the six V3 MAbs had similar activity of binding to the JRFL gp120 protein (Fig. 4C). These data demonstrated that the epitopes recognized by the six V3 MAbs are present on the monomeric JRFL Env but are masked to different degrees when expressed on the virus. Neutralization data of JRFL produced with or without a mannosidase inhibitor further indicate that, while the exposure of many V3 epitopes was modulated by the N-glycan composition of the virus Env, the 2424 epitope was not affected. Two N-glycans previously implicated as controlling V3 exposure include those at position 197 at the C-terminal base of V1V2 and position 301 at the N-terminal base of V3 (57–59), and both glycans were assigned to be the complex type

TABLE 2 Virus neutralization by 2424 and 447-52D individually or in combination

Virus	Tier[a]	Clade	IC_{50} (μg/ml)[b]		
			2424	447-52D	2424 + 447-52D
SF162	1A	B	>25	**<1**	**<1**
Bal.01	1B	B	>25	**<1**	**<1**
DJ263.8	1B	A	>50	**8.5**	**25.0**
SS1196.1	1B	B	**<1**	**<1**	**<1**
6535	1B	B	>50	**2.5**	**4.5**
JRFL.JB	2/Chr	B	**4**	**15**	**10.5**
REJO	2	B	**18**	>25	**20**
PVO.4	3	B	>50	>50	>50
No. positive/total (%)			3/8 (37.5)	6/8 (75)	7/8 (87.5)

[a] Chr, chronic.
[b] Total MAb concentration needed to reach 50% neutralization in the TZM.bl neutralization assay. IC_{50}s of ≤25 μg/ml are in bold.

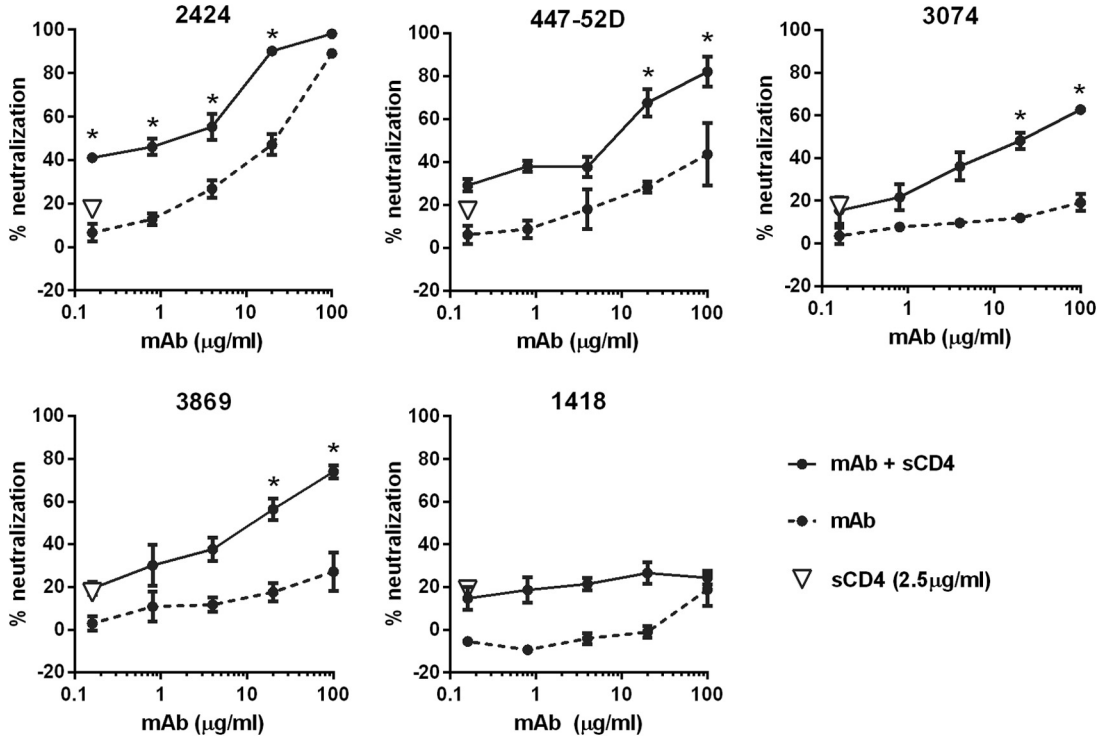

FIG 3 Effects of CD4 engagement on virus neutralization by 2424 versus the other V3 MAbs. Neutralization of JRFL pseudovirus by V3 MAbs 2424, 447-52D, 3074, and 3869 was evaluated after treatment with or without sCD4. Virus was pretreated with 2.5 μg/ml of sCD4 for 30 min at 37°C or left untreated and then incubated with serially diluted MAb for 1 h at 37°C. TZM.bl cells were added to virus-MAb mixtures in DMEM containing 10% fetal bovine serum and DEAE-dextran. After 48 h, virus infection was measured based on β-galactosidase activity. Means and standard errors from two independent experiments are shown. *, $P < 0.01$ based on the two-way ANOVA to show a synergistic difference above the calculated sum of percent neutralization attained by MAb and sCD4 on their own.

(60). However, for JRFL, there is a glycosylation site at position 301 but not at position 197. To evaluate the importance of N-glycan at these positions, mutations were introduced into JRFL Env at position 301 (N to D or N to Y) to remove the specific glycan or at position 197 (D to N) to add a glycosylation site. The mutations at position 301 rendered the virus sensitive to all V3 MAbs tested, including 2424 (Fig. 5). Nonetheless, mutations that remove the glycan at residue 301 also enhanced sensitivity of different HIV-1 isolates to antibodies against the CD4-binding site and the CD4-induced epitopes, although the MPER epitopes in the gp41 subunit were unaffected (57, 59, 61, 62), indicating that mutations at this position induce global structural alterations which affect not only V3 but also other distant sites. In contrast, the D197N mutation did not affect virus neutralization by any of the six V3 MAbs tested. The contribution of residue 197 in shielding V3 epitopes is not associated with N-glycan; rather, this residue is involved in stabilizing the interprotomer interactions between V1V2 and V3 that allow V1V2 to shield V3 in the Env trimeric spike. Only specific mutations that disrupt these interactions (e.g., D197H and D197Q) are able to release V3 from the V1V2 masking (S. Zolla-Pazner, unpublished data).

The crystal structure of MAb 2424 and its epitope reveals that 2424 targets a vulnerable region at the apex of the V3 crown. To define the epitope of MAb 2424, we determined the structure of the 2424 antigen-binding fragment (Fab) cocrystallized with a 23-mer $V3_{JRFL}$ peptide, NNTRKSIHIGPGRAFYTTGEIIG (residues 301 to 325 in the HxB2 numbering system [63]). The structure of the 2424 Fab-epitope complex was solved by molecular replacement and refined to 3.18-Å resolution with an R_{work}/R_{free} value of 22.5%/28.3% (Fig. 6A and Table 3). The crystals grew in an orthorhombic space group with three Fab-epitope complexes in the asymmetric unit. Since the noncrystallographic symmetry constraints (NCS) were imposed in the refinement, only one complex is described in detail here. The residues of the light and heavy chains are numbered following the convention of Kabat and Wu (64), preceded by "L" and "H," respectively, and the residues of the V3 peptide are preceded by a "P." Although a 23-mer peptide was used in the crystallization, only 11 residues, with the sequence IHIGPGRAFYT (residues 307 to 319), were observed in the electron densities and thus built into the final model. We also determined the structure of Fab 2424 alone and refined it to a 2.68-Å resolution (R_{work}/R_{free} = 20.0%/28.5%) (Table 3). The Fab structure is very similar to that in the Fab-epitope complex, with only one residue, Phe^{H58}, in the antigen-binding site of the Fab alone having a different side chain orientation.

The Fab/V3 epitope complex revealed that 2424 binds the very apex of the V3 crown (Fig. 6 and 7), forming direct contacts with 9 residues, ^{P308}HIGPGRAFY^{P318}. However, Ile^{P309} and Phe^{P317} have only very little backbone contact (~6.5 $Å^2$ and 4.3 $Å^2$, respectively) with the antibody, and their side chains point away from the antigen-binding surface of 2424. The antibody binds the epitope using the ladle mode, one of the two binding modes typically used by anti-V3 crown MAbs (65). In this binding mode, the arch of the V3 crown (four residues at the apex, typically GPGR for

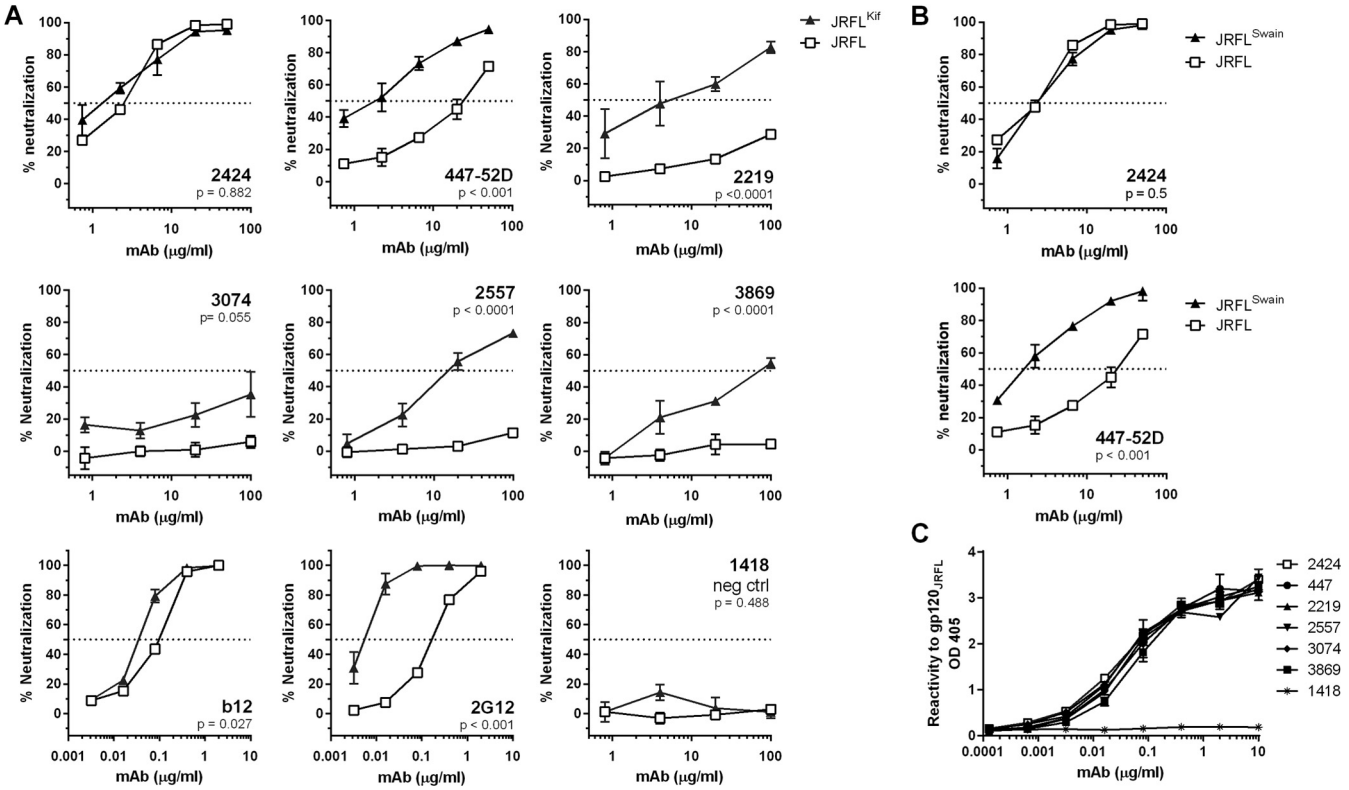

FIG 4 Neutralizing activities of MAb 2424 versus the other V3 MAbs against JRFL produced in the presence or absence of a mannosidase inhibitor. (A) HIV-1 JRFL pseudoviruses were produced in transfected 293T cells in the absence or presence of 25 μM kifunensine ($JRFL^{Kif}$) and tested for neutralization by V3 MAbs (2424, 447-52D, 2219, 2557, 3869, and 3074), MAb 2G12, CD4bs MAb b12, or an irrelevant MAb, MAb 1418. (B) HIV-1 JRFL pseudoviruses were produced in 293T cells with or without 20 μM swainsonine ($JRFL^{Swain}$) and tested for neutralization by V3 MAbs 2424 and 447-52D. Serially diluted MAbs were incubated with virus at 37°C for 1 h. TZM.bl cells were then added to the virus-MAb mixtures in complete DMEM containing DEAE-dextran. Neutralization activity was assessed after 48 h. Results with 2424 and 447-52D were averages from duplicate wells, and data from one representative experiment are shown. Results with other MAbs are shown as averages from two to three experiments. *P* values were determined by the two-way ANOVA. (C) V3 MAb reactivity with gp120 JRFL was assessed in ELISA. Recombinant gp120 protein (1 μg/ml) was coated on 96-well plates and reacted with serially diluted V3 MAbs. Data from a representative experiment with averages and standard deviations from duplicate wells are shown.

subtype B or GPGQ for subtype C) points directly toward the antigen-binding site (Fig. 6A). This is different from the other binding mode, called the cradle mode, in which the V3 crown lies sideways in the long antigen-binding groove of the antibody (35, 66). Like other anti-V3 crown MAbs, the antigen-binding site is negatively charged, so that it can accommodate the positively charged V3 crown. Interestingly, residue P315-P317 forms a small 3_{10} helical turn, which is rarely observed in V3 crown structures despite the early prediction from a sequence analysis that the C-terminal part of V3 has a propensity to be helical (67). All CDR loops participate in the antigen-antibody interactions, and they form a relatively small but deep binding site basin. There are three specific binding pockets that interact with the side chains of His^{P308}, Pro^{P313}, and Arg^{P315}, respectively (Fig. 7A). The contact areas of these three residues (240 $Å^2$) account for over 60% of the contact areas of all the epitope residues with 2424 (383 $Å^2$). In agreement with these structural data, the presence or absence of these three specific residues dictates whether 2424 binds strongly or poorly to V3 from diverse HIV-1 isolates (Table 1). Thus, the side chains of these three residues dominate the antigen-antibody interaction for 2424, forming the signature motif for 2424 recognition. This is consistent with the 2424 specificity for subtype B viruses, which often have His^{P308} and Arg^{P315}.

Comparison of the antigen-antibody interactions of 2424 with 447-52D. We compared structurally the antigen-antibody interaction of 2424 with that of 447-52D, a well-characterized anti-V3 crown MAb. The most obvious similarity between these two MAbs is their mode of antigen binding: both use the ladle mode, but 447-52D has a 22-amino-acid CDR H3 that serves as the handle of the ladle (Fig. 6B). They also have some similarities in the details of their antigen binding. For example, the side chain Arg^{P315} of the epitope in 2424 forms a salt bridge with a heavy-chain aspartic acid, and it is also surrounded by several Tyr residues from both heavy and light chains (Fig. 7A). Similarly, the side chain Arg^{P315} in 447-52D also forms a salt bridge with a heavy-chain aspartic acid, and it is also sandwiched between the side chains of two aromatic amino acids of the antibody (Fig. 7B). In addition, Pro^{P313} of V3 in both MAbs is placed in a hydrophobic environment with its side chain stacked against the side chain of either a Phe (2424) or a Trp (447-52D). However, the two MAbs also have several differences in their antigen-antibody interactions. First, the epitope of 2424 is two residues shorter in the N terminus than that of 447-52D; this focuses 2424 binding to the very apex of the V3 crown. Second, 2424 is highly side chain specific, burying the side chains of His^{P308} and Arg^{P315} in deep pockets in the antigen-binding site, while 447-52D utilizes a beta-sheet main-chain interaction with the N terminus of the V3 crown, rendering 447-52D

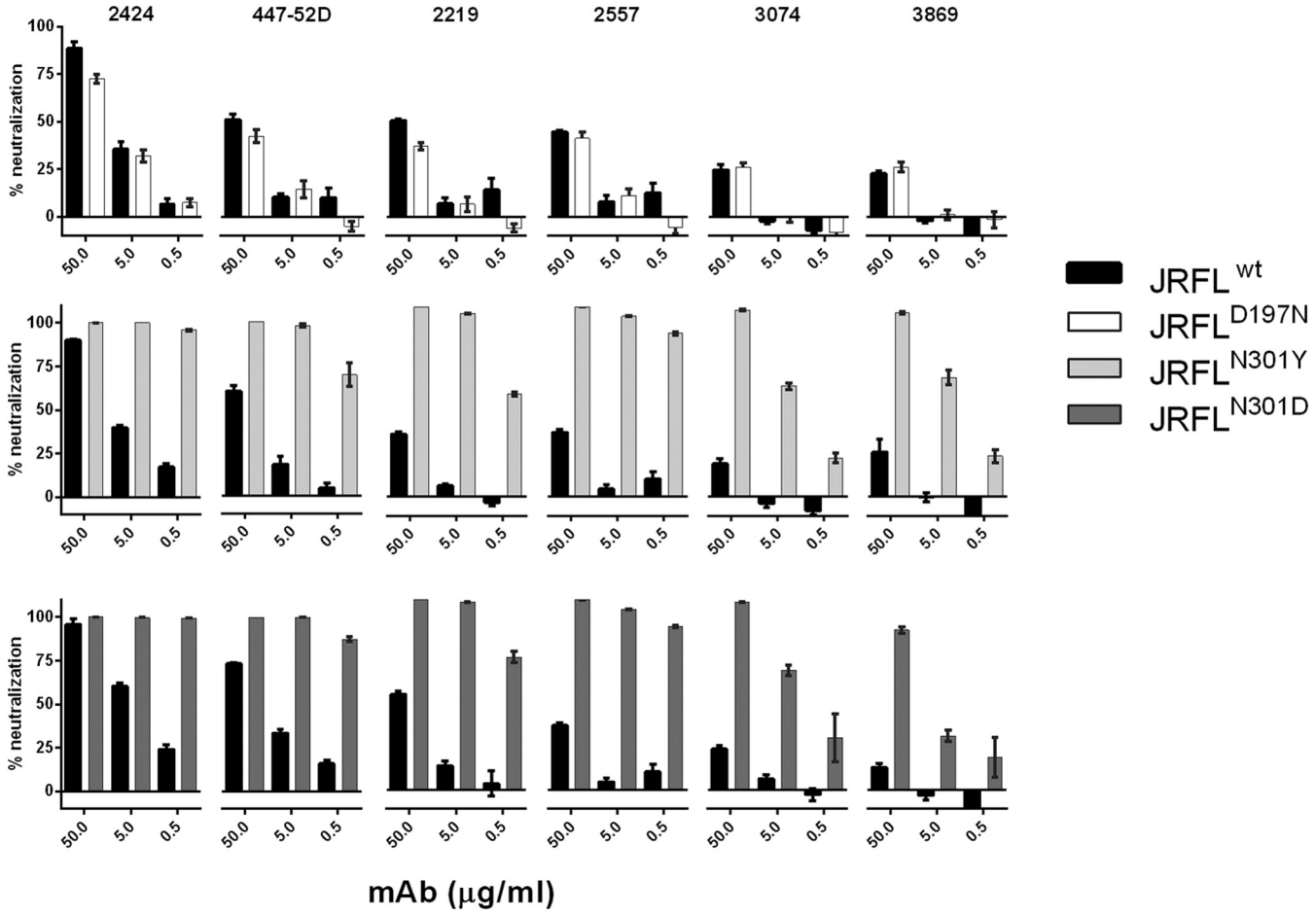

FIG 5 Neutralization activities of 2424 versus the other V3 MAbs against HIV-1 pseudoviruses expressing wild-type or mutant JRFL Envs. HIV-1 JRFL pseudoviruses bearing wild-type or mutated Envs were produced in transfected 293T cells and incubated by titrated V3 MAbs (2424, 447-52D, 2219, 2557, 3074, and 3869) for 1 h at 37°C. TZM.bl cells were then added to the virus-MAb mixtures in complete DMEM containing DEAE-dextran. Neutralization activity was assessed after 48 h. Results are means and standard errors from two experiments.

more broadly reactive. The limited contacts between 2424 and the V3 crown and the 2424 epitope being farther away from nearby glycans may explain how its binding is less affected by the glycan composition of gp120 (Fig. 4). However, its side chain specificity is likely to limit the neutralization breadth of 2424.

DISCUSSION

This study presents the crystallographic structure of MAb 2424 in complex with its V3 epitope, elucidating the distinctive capacity of this V3 MAb to neutralize HIV-1 JRFL, a virus isolate resistant to many other V3 MAbs (26, 52). The presence of such a MAb indicates that, although epitopes on the V3 crown region of tier 2 or tier 3 viruses are often occluded, some are accessible to Abs. The broadly neutralizing MAb PGT 128 that is highly potent against tier 2 and tier 3 viruses also binds to a complex epitope consisting in part of a short beta-strand segment of the V3 loop and N-linked glycans at the V3 base (17). 2424 uses a different approach to access its epitope in that it targets the very tip of the V3 crown, away from the many N-glycans shrouding the HIV Env surface. In support of this idea, we demonstrate that treating the virus with a mannosidase inhibitor, which changes the sugar composition of N-glycans on the virus Env, did not affect virus neutralization by 2424. On the other hand, most other V3 MAbs were more potent against viruses bearing Env with homogenously high mannose-type glycans, suggesting the important contribution of complex-type N-glycans in masking the epitopes of these V3 MAbs, either by direct steric hindrance or by facilitating formation of more compact Env trimers. All together, the data presented herein indicate that, unlike other V3 epitopes, the 2424 epitope is devoid of and is minimally masked by N-glycans; thus, it constitutes a distinct target which would complement the other neutralizing epitopes on the virus Env.

Another distinctive feature of MAb 2424 is its ability to mediate 100% neutralization against JRFL. Although the mechanistic explanations for this activity remain unknown, the result suggests the possibility that MAb 2424 can attain binding saturation of the virus Env due to the relative distance of its epitope from the surrounding glycans. Nonetheless, the neutralizing activity of MAb 2424 was further enhanced after CD4 binding and also affected by the removal of N-glycan from residue 301 at the base of V3, indicating that the 2424 epitope is not completely exposed on the prefusion Env spikes. The binding affinity of MAb 2424 is not noticeably different from that of the other V3 MAbs when tested with soluble gp120 monomer. On the Env trimers, the V3 distal tip

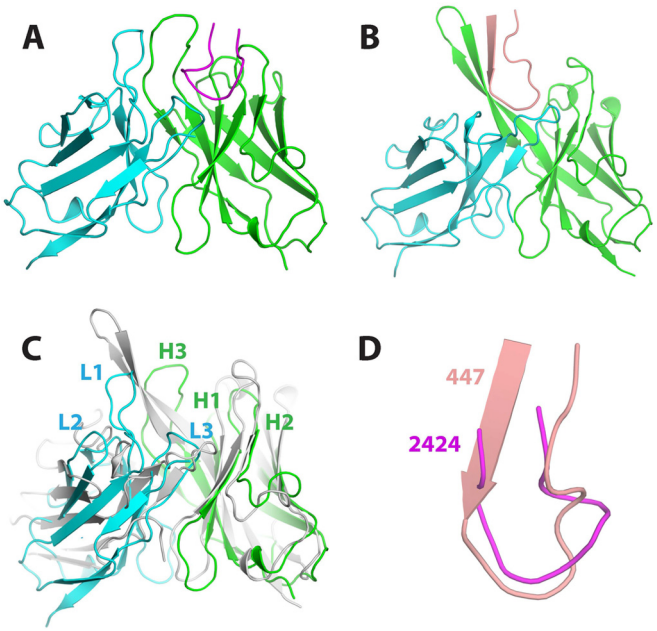

FIG 6 Structural comparison of MAbs 2424 and 447-52D. (A) Ribbon representation of the Fab 2424/$V3_{JRFL}$ complex. The epitope and the light and heavy chains are magenta, cyan, and green, respectively. For simplicity, only the Fv region is shown. (B) Fab 447-52D/$V3_{MN}$ complex. The epitope is shown in salmon. (C) Superimposition of the two Fabs. Both light and heavy chains of 447-52D are in gray. (D) Superimposition of the V3 peptides bound to 2424 and 447-52D. The N terminus of 2424 epitope is shorter than that of 447-52D.

may be constrained to preferentially adopt the conformation recognized by 2424 and/or may be better oriented for binding by 2424 compared to the other V3 crown MAbs.

Together with the current 2424-V3 structure, many crystal structures of human V3 MAbs in complex with their cognate V3 epitopes have been resolved (35, 68–70). They reveal three major modes of Ab-epitope interactions, designated ladle, cradle, and Janus. The ladle mode is exemplified by 447-52D, which grasps V3 by the long CDR H3 handle of the ladle. 2424 belongs to this same category but is distinct from 447-52D in that it interacts only with the tip of the V3 crown with the bowl portion of the ladle. The potency of 2424 to neutralize relatively resistant viruses such as JRFL and REJO and its indifference to mannosidase inhibitors imply that the very tip of V3 targeted by 2424 is minimally shielded by glycans in these viruses. In contrast, the cradle mode is represented by MAbs 2219 and 2557, whose antigen-binding sites are shaped like a cradle, and the V3 crown lies in it. MAbs in this category are effective only when the virus Env is enriched with the high-mannose-type N-glycans, indicating the critical contribution of N-glycans in shielding these MAbs' epitopes. In addition, there is a third binding mode, the Janus mode, which is characterized by MAbs 3074 and 268; they approach V3 from two opposing sides to contact, respectively, the conserved hydrophobic core and the strain-specific hydrophilic face, in the middle segment of the V3 crown (35). MAb 3074 neutralizes JRFL poorly whether the virus is produced with or without a mannosidase inhibitor, indicating that this MAb cannot reach into the hydrophobic core of V3 when presented on the JRFL Env spike and that this conserved site is concealed by factors other than N-glycans.

The distinct epitope and neutralization profile of MAb 2424 correspond to its Ig constant and variable gene usage. MAb 2424 has a kappa light chain and is encoded by IGHV3-53 and IGLV2-28 genes. Among 51 human anti-V3 MAbs evaluated by Gorny et al. (38), the majority (76.5%) use lambda light chains. Only 12 (23.5%) utilize the IGHV3 gene family, and these IGHV3-encoded V3 MAbs are paired preferentially with kappa light chains. However, although V3 Abs are skewed away from using

TABLE 3 Crystallographic data collection and refinement statistics[a]

Parameter	Fab 2424/$V3_{JRFL}$	Fab 2424
Data collection		
Space group	$P22_12_1$	$P2_1$
Cell dimensions		
a, *b*, *c* (Å)	95.71, 121.62, 139.84	69.59, 45.04, 74.78
α, β, γ	90, 90, 90	90, 96.75, 90
Resolution (Å)	3.18 (3.37–3.18)	2.68 (2.84–2.68)
CC (1/2)	99.2 (76.5)	99.3 (70.4)
I/σI	11.6 (2.0)	11.5 (2.0)
Completeness (%)	99.9 (99.9)	99.1 (98.6)
Refinement		
Resolution (Å)	48.28–3.18	47.86–2.68
Unique reflections	53,260	13,118
R_{work}/R_{free}	22.5/28.3	20.0/28.5
No. of atoms		
Protein	10,085	3,287
Solvent	0	33
B factors (Å^2)		
Protein	69.7	59.7
Solvent		47.3
RMS deviations		
Bond length (Å)	0.009	0.009
Bond angle (°)	1.183	1.225

[a] Numbers in parentheses refer to the outer resolution shell. RMS, root mean square.

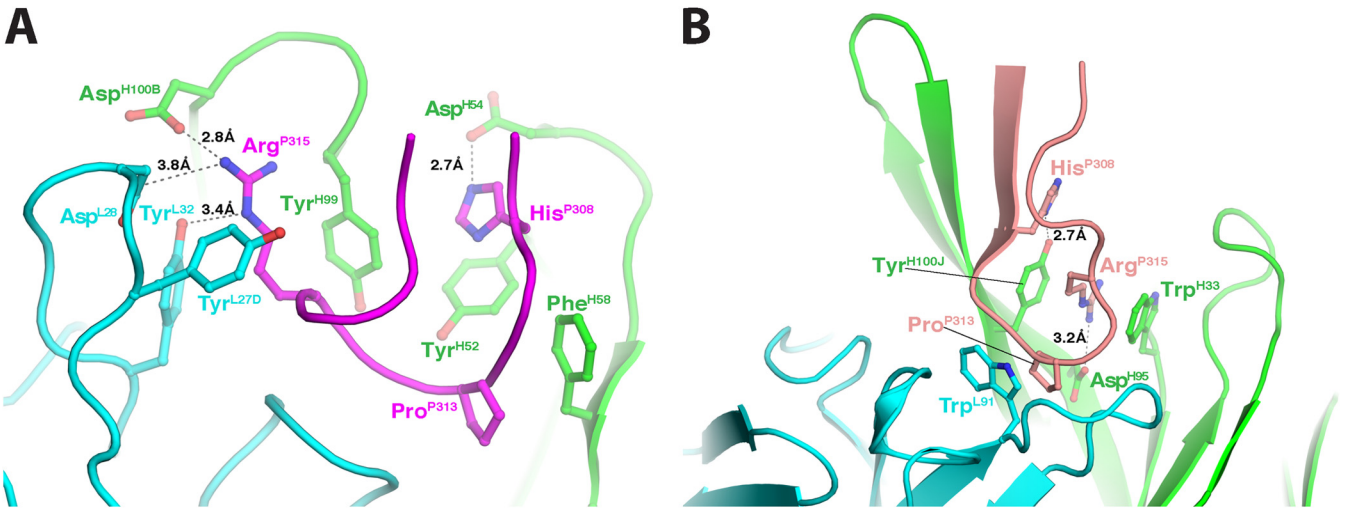

FIG 7 Details of the antigen-antibody interactions in the 2424 and 447-52D Fab/V3 peptide complexes. The color scheme is the same as for Figs. 6A and B. The key residues involved in the antigen-antibody interactions are shown as sticks. (A) Fab 2424/epitope complex. Note that three residues, His^{P308}, Pro^{P313}, and Arg^{P315}, of V3 play key roles in the antigen-antibody interaction: (i) the side chain His^{P308} is stacked with that of Tyr^{H52} and forms a potential hydrogen bond with Asp^{H54}, (ii) the side chain of Pro^{P313} is stacked with that of Phe^{H58}, and (iii) the side chain of Arg^{P315} is surrounded by three Tyrs and forms a salt bridge with Asp^{H100B}. (B) 447-52D Fab/epitope complex. Note again that three residues, His^{P308}, Pro^{P313}, and Arg^{P315}, of V3 play key roles in the antigen-antibody interaction.

IGHV3, this gene family is common among Abs from healthy uninfected individuals. As shown by Tiller et al. (51), 54.8% of 183 recombinant Abs isolated from uninfected individuals belong to the IGHV3 gene family. It remains to be determined if such V3 Abs can be generated in these individuals by vaccination. 2424-like Abs were not the dominant response elicited in mice upon immunization with gp120 JRFL alone or in complex with MAb, as indicated by the inability of serum Ab pools from the immunized animals to neutralize 2424-sensitive viruses such as JRFL or REJO (27), although the composition of dominant and nondominant Ab responses were not yet delineated and the gp120/MAb complex vaccines tested were not selected and optimized for presenting the 2424 epitope. Nonetheless, many epitopes in the V3 crown has been shown to be highly immunogenic in humans and in animal models. MAb 2424 also is not as highly mutated, with somatic hypermutation rates of about 14% and 5% in its VH and VL genes and a CDR H3 length of 17 amino acids. This level of affinity maturation is lower than those required to generate the extremely potent and broadly neutralizing MAbs targeting other Env sites, such as PG6 and PG19 (15), PGT 141-145, PGT121, PGT127, PGT128, and PGT135 (16), VRC01 (66), NIH45-46 (71), 10E8 (72), and the most recently reported 35O22 (73). Rather, it is in the range observed with influenza virus-specific Abs after repeated administration of influenza vaccines (74), although it remains above the mutation rates achieved after immunization with gp120 protein alone or with prime-boost vaccines of ALVAC and gp120 protein (58).

The crystallographic structures of 2424 and its V3 epitope reveal the important contribution of His^{P308} and Arg^{P315}, both of which are characteristic of subtype B V3, and provide an explanation for the 2424 specificity for subtype B Env. In contrast, the consensus Env sequences of subtype A and subtype C viruses, which encompass the vast majority of HIV-1 isolates circulating in the areas of epidemicity in Africa and Asia, commonly contain an Arg at position 308 and a Gln at position 315. Induction of Abs directed to the 2424 epitope site at the apex of V3 with the subtype B and non-B sequences would be desirable. No MAb has been identified thus far that targets the non-B counterpart of the 2424 epitope to determine whether this site is also accessible on non-B HIV-1 isolates. Nonetheless, targeting this site would constitute an additional approach to the other V3-targeting vaccine strategies that focus on the complex PGT128-like peptidoglycan epitopes or the V3 epitopes recognized via the cradle or Janus mode. Abs against disparate targets on V3 and other sites on the HIV Env are needed to prevent the transmission of a diverse array of HIV-1 isolates and block escape variants that would readily emerge under pressure from only one Ab class.

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