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Neutralizing activity of antibodies to the V3 loop region of HIV-1 gp120 relative to their epitope fine specificity

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Abstract

The V3 loop of HIV-1 gp120 is considered occluded on many primary viruses. However, virus sensitivity to neutralization by different V3 mAbs often varies, indicating that access to V3 is not restricted equally for all antibodies. Here, we have sought to gain a better understanding of these restrictions by determining the neutralizing activities of 7 V3 mAbs (19b, 39F, CO11, F2A3, F530, LA21, and LE311) against 15 subtype B primary isolates and relating these activities to the fine specificity of the mAbs. Not surprisingly, we found that most mAbs neutralized the same 2–3 viruses, with only mAb F530 able to neutralize 2 additional viruses not neutralized by the other mAbs. Epitope mapping revealed that positively-charged residues in or near the V3 stem are important for the binding of all the mAbs and that most mAbs seem to require the Pro residue that forms the GPGR β hairpin turn in the V3 tip for binding. Based on the mapping, we determined that V3 sequence variation accounted for neutralization resistance of approximately half the viruses tested. Comparison of these results to those of select V3 mAbs with overall better neutralizing activities in the light of structural information suggests that an antibody's mode of interaction with V3, driven by contact residue requirements, precludes the antibody from accessing its epitope on different viruses. Based on the data we propose an angle of interaction with V3 that is less stringent on access for antibodies with cross-neutralizing activity compared to antibodies that neutralize relatively fewer viruses.

Keywords

V3 antibodies; cross-neutralization; V3 accessibility; epitope mapping; HIV vaccine design

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INTRODUCTION

An HIV vaccine will likely need to incorporate a component that elicits a humoral response capable of neutralizing a broad array of circulating virus isolates (Gallo, 2005). The target for neutralizing antibodies (NAbs) is the viral envelope spike (Burton and Montefiori, 1997) and many strategies are being pursued to elicit antibodies with the desired level of cross-neutralizing activity (reviewed in (Haynes and Montefiori, 2006; Hu and Stamatatos, 2007; Phogat and Wyatt, 2007)). However, as a result of viral evolution, driven perhaps in most part by host NAb pressure (Beaumont et al., 2001; Frost et al., 2005; Wei et al., 2003), the envelope spike harbors a remarkable plethora of features to shield conserved regions from antibody recognition (reviewed in (Karlsson Hedestam et al., 2008; Wyatt et al., 1998)). Collectively, these features represent a substantial scientific hurdle towards formulating a NAb-inducing HIV vaccine component, as evident from the relatively low cross-neutralizing activity of NAbs elicited by the large majority of immunogens developed to date.

To aid the design of an effective B-cell immunogen, and given HIV's defenses, the antigenicity of the envelope spike on different HIV isolates from different subtypes needs to be elucidated. This process has been aided greatly by the existence of a handful of rare mAbs that can neutralize a broad range of primary viruses and protect against viral challenge in animal models, which have allowed also for the identification of potential target sites for vaccine design (Burton et al., 2004; Kramer, Siddappa, and Ruprecht, 2007; Zolla-Pazner, 2004). Unfortunately, the inability to elicit NAbs with similar broad neutralizing activity or even with modest cross-neutralizing activity suggests that there are still gaps in our understanding of features that affect efficient antibody recognition of epitopes on the envelope spike and of how to design immunogens to effectively elicit antibodies to target sites (Hu and Stamatatos, 2007).

One of the potential, albeit often debated, target sites for vaccine design is the V3 loop on the gp120 subunit of the envelope spike (Hartley et al., 2005; Haynes and Montefiori, 2006; Zolla-Pazner, 2005). The V3 loop has been studied extensively. Lower enthusiasm for seeking to target V3 is based largely on the general observation that V3 antibodies often exhibit poor cross-neutralizing activity (Hartley et al., 2005) and several factors, for example steric hindrance by glycans, V3 sequence variability, and masking of the V3 loop by V1/V2 (Gram et al., 1994; Krachmarov et al., 2005; Krachmarov et al., 2006; Pinter et al., 2004; Schonning et al., 1996), help explain why many V3 mAbs may exhibit little neutralizing activity. A recent study has suggested also that the presence (or absence) of certain V3 residues may greatly affect the neutralizing activity of V3 mAbs and the presence of subtype-specific epitopes within V3 has been proposed (Patel, Hoffman, and Swanstrom, 2008). There exists, however, a small selection of V3 mAbs that exhibit relatively better cross-neutralizing activity than other V3 mAbs (Eda et al., 2006; Gorny et al., 2006; Pantophlet et al., 2007). The neutralizing activity of these select mAbs suggests that they may be hampered less by at least some of the access-restrictive factors outlined above. The notion that such restrictions do not apply equally to all V3 antibodies is supported also by the often noted spectrum of virus sensitivities to different V3 mAbs (Gorny et al., 2004; Gorny et al., 2002).

Here, we wanted to investigate the extent to which the fine specificity of V3 mAbs affects their neutralizing activity, as part of efforts to better comprehend the often observed resistance of HIV isolates to V3-directed antibodies and broad antibody recognition in general. Efforts were made in a recent study to map the epitopes of different V3 mAbs using a phage-display peptide library expressing random dodecamer sequences (Patel, Hoffman, and Swanstrom, 2008). However, for most mAbs that were investigated, no consensus sequence could be identified that resembled V3. In this study, we applied a commonly applied scanning mutagenesis approach, utilizing a panel of V3 mutants that previously allowed mapping, with high accuracy,

of residues important for the binding of the modestly cross-neutralizing V3 mAb B4e8 (Bell et al., 2008; Pantophlet et al., 2007).

RESULTS

Neutralizing activity of V3 mAbs against subtype B primary viruses

To assess the cross-neutralization potential of the V3 mAbs, we tested them against a panel of 15 viruses in a robust and highly sensitive pseudovirus-based single-round infectivity assay with luciferase readout (Table 1). The viruses were selected from a previously published 30-member panel of subtype B primary viruses (Binley et al., 2004) because they exhibit diverse sensitivities to V3 mAbs (Binley et al., 2004;Li et al., 2005). The test conditions were the same as described in the Binley et al. study. IC_{50} values are reported because they allow for a more reliable comparison between the potency and cross-reactivity of different antibodies, as argued previously (Binley et al., 2004).

Overall, the mAbs were only able to neutralize a very limited number of viruses; most mAbs neutralized 2–3 viruses, whereas only mAb F530 neutralized 5 viruses (Table 1). The 92BR020 molecular clone was neutralized by all 7 mAbs and was shown previously as neutralization-sensitive to V3 mAb B4e8 (Pantophlet et al., 2007). Strikingly, the quasispecies pool of this virus was not neutralized by V3 mAbs 58.2 or 447-52D (Table 1) (Binley et al., 2004), even though the amino acid sequences of the quasispecies pool and the molecular clone share ~95% homology; the clone is also similarly sensitive to neutralization by mAbs b12, 2G12, 2F5, and 4E10 and a broadly neutralizing HIV^+ serum as the quasispecies pool (T. Wrin et al., unpublished results). Although we cannot readily explain the discrepancy between the quasispecies pool and the clone for mAbs 58.2 and 447-52D, the results suggest that virus 92BR020 is likely sensitive to neutralization by several V3 antibodies.

Viruses 93TH305 and 5768-p27 were neutralized by 5 and 4 of the mAbs tested here, respectively (Table 1). For virus 93TH305 the IC_{50} values ranged from 13–37 μg/ml, in the same range as observed previously for V3 mAbs 447–52D, 58.2 and B4e8 (Table 1). For virus 5768-p27, IC_{50}s ranged from 3–41 μg/ml. Virus 5768-p27 is not sensitive to neutralization by mAbs 447-52D, 58.2, or B4e8 (IC_{50}s>50 μg/ml; Table 1), most likely due to the rather uncommon Lys at position 315; mAbs 447-52D, 58.2, and B4e8 prefer an Arg at this position for efficient binding (Keller et al., 1993;Pantophlet et al., 2007;Seligman et al., 1996;White-Scharf et al., 1993;Zolla-Pazner et al., 2004).

Aside from viruses 92BR020, 93TH305, and 5768-p27, only mAb F530 also neutralized viruses 91US056 and 92HT594 (Table 1). Virus 91US056 is not sensitive to neutralization by mAbs 447-52D, 58.2, and B4e8 (Binley et al., 2004;Pantophlet et al., 2007), because of the Ala at position 315 instead of an Arg. Virus 92HT594 is neutralized also by mAbs 447-52D and B4e8 (IC_{50}s: 8 and 4 μg/ml, respectively; Table 1), but is resistant to neutralization by mAb 58.2. None of the 7 V3 mAbs tested here neutralized (IC_{50}>50 μg/ml) any of the other viruses that are neutralization-sensitive to mAbs 58.2, 447-52D, and/or B4e8 (Table 1).

MAb fine specificity as determined by V3 alanine scanning

The same 18 alanine point mutants generated previously for mapping the specificity of mAb B4e8 (Pantophlet et al., 2007) were used to map the specificity of the mAbs tested here; this approach proved highly accurate for mapping B4e8's fine specificity, as evidenced by the interactions observed between B4e8 and V3 from the subsequent structure of a B4e8:V3 peptide complex (Bell et al., 2008). As with mAb B4e8, apparent antibody binding affinities were determined and related to the apparent antibody affinity for wild-type gp120 (Pantophlet et al., 2007). We found that the binding of all mAbs could be reduced by several substitutions.

None of the patterns resembled the effects of alanine substitutions on B4e8 binding (Table 2). Unexpectedly, we also observed that the binding of several mAbs –19b, CO11, F2A3, F530, and LA21—was increased upon changing select residues to Ala; the largest number of increased binding was observed for mAb F2A3 (Table 2). The following specific observations were also made:

a. Binding of mAb 19b was strongly reduced (>10-fold) upon substitution of Arg^{304}, Ile^{307}, Pro^{313}, Arg^{315}, Phe^{317}, or Tyr^{318}, and moderately reduced (~4-fold) upon substitution of Lys^{305} (Table 2). These results are consistent overall with those from previous mapping studies of mAb 19b utilizing Ala-substituted peptides and various gp120s (Moore et al., 1995;Scott et al., 1990). We note that Arg^{304} and Lys^{305}, identified here as important for 19b binding, have not been investigated in the previous Scott et al. or Moore et al. studies. We did not find the side chain of Thr^{320} to be important for 19b binding, in agreement with results from the Moore et al. study showing that residues with physicochemical properties different from threonine can occupy position 320 in phage-displayed peptides selected with mAb 19b (Moore et al., 1995). A recent study by Patel et al. proposed Ile^{307}, His^{308}, Gly^{312}, Pro^{313}, and Ala^{316} as important for mAb 19b binding, based on a peptide sequence selected with 19b from a peptide phage display library (Patel, Hoffman, and Swanstrom, 2008). However, this sequence was only 1 of 10 different sequences selected and, thus, may not have been representative of the 19b epitope. We also found that substituting Asp^{325} with Ala increased the binding affinity of mAb 19b by 2-fold (Table 2). Asp^{325} forms a salt bridge with Arg^{419} located within the coreceptor binding site on the gp120 core (Huang et al., 2005). This interaction may restrict the structural flexibility of the C-terminal stem of the V3 loop; introducing the Ala may prevent formation of this salt bridge, thus, allowing for a better formation or presentation of the 19b epitope.

b. The apparent binding affinity of mAb 39F, unlike all the other mAbs, was diminished solely by substitutions at the juncture of the V3 stem and tip but not by substituting residues located central to the V3 tip, such as Pro^{313} or Arg^{315} (Table 2). Specifically, binding affinity was strongly diminished by replacing Lys^{305} or Ile^{307} with Ala, and moderately decreased upon substitution of Ser^{306} and Ile^{309}, suggesting that this mAb interacts principally with the N-terminal flank of the V3 loop. This notion is supported further by the observation that substitution of Arg^{298} and Arg^{304} also diminished binding, although not substantially (Table 2).

c. The binding of mAbs CO11, F2A3, LA21, and LE311 was diminished by many of the same substitutions (Table 2). The apparent affinities of all four mAbs were diminished upon substitution of His^{308} and binding of mAbs CO11, LA21 and LE311 was substantially reduced upon substitution of Pro^{313}. The extremely strong reduction in the apparent binding affinity of mAbs CO11 and LA21 for mutant P313A (500- and 250-fold, respectively; Table 2) suggests that interaction with the aliphatic side chain of the Pro and/or a precise conformation of the V3 β-hairpin turn may be critical for efficient binding of these two mAbs. The apparent binding affinities of the two other mAbs, F2A3 and LE311, for mutant K305A were strongly diminished (>10-fold) relative to wild-type binding. This observation suggests that the epitopes of these two antibodies may overlap mostly with the N-terminal flank of the V3 region. It should be remarked that the residues identified here as important for binding of mAb CO11 (His^{308}, Pro^{313}, and Arg^{315}) are consistent with the sequence proposed recently in a Patel et al. study as the (core) epitope of mAb CO11 (Patel, Hoffman, and Swanstrom, 2008); contrary to the above-noted low frequency of the proposed 19b epitope sequence, the CO11 sequence was identified in 11 of 20 phage-displayed peptide sequences selected with mAb CO11.

d. As noted above, the binding of mAb F2A3 was substantially enhanced by several substitutions: I309A, F317A, Y318A and D325A (Table 2). We posit that the influence of Asp^{325} is the same as postulated above for mAb 19b. Based on the projection of the mapping results onto the gp120+V3 structure (see below), we posit that the other 3 residues affect the precise alignment of critical residues required for the interaction with F2A3; as with Asp^{325}, their interaction with neighboring residues likely affects how well the F2A3 epitope is presented.

e. The effects of the Ala-substitutions on mAb F530 binding were very similar to the previous four mAbs (Table 2), which likely explains its ability to neutralize the same viruses as the other 4 mAbs (cf. Table 1). However, unlike the other 4 mAbs, the apparent binding affinity of mAb F530 was not reduced as substantially by the His^{308}→Ala change.

V3 mAb fine specificity related to neutralizing activity

We ranked, for each mAb, the viruses that were most potently neutralized followed by those viruses that were not neutralized, to determine which residues are permissible (Fig. 2). Because some of the viruses used are from quasispecies pools, we listed all variant V3 sequences reported for a given virus; in our panel, this applied to viruses QH0515 and 92BR021.

Only mAb 19b neutralized virus 5768-p27 more potently than virus 92BR020 (Table 1; Fig. 1); although the residues identified as important for 19b binding are present in the V3 sequence of both viruses, a notable difference between them is the residue at position 309, which is a Met in virus 5768-p27 and an Ile in virus 92BR020. It has been shown previously that consensus peptide sequences selected with 19b from a phage-display library predominantly contain a Met at position 309 (Moore et al., 1995); thus, 19b seems to require an aliphatic side chain at position 309, with greater preference for Met. Based on the mapping, the inability of mAb 19b to neutralize the 6 viruses QH0515, BG1168, 6101-p15, 91US056, 92BR021, and ME1 could be explained by substitution of important contact residues (Fig. 1), whereas inability of the mAb to neutralize the other 6 viruses (QH0692, SS1196, 92US712, 93TH305, JR-CSF, and JR-FL) could not be explained by this.

For mAb 39F, the sequence of the two viruses neutralized (92BR020 and 93TH305) also correlated well with the mapping results (Fig. 1); the presence of Ser at position 306 (virus 92BR020) seems preferred over the smaller Gly (virus 93TH305). Of the 13 viruses resistant to neutralization by mAb 39F, the resistance of 6 viruses (BG1168, 5768-p27, 6101-p15, 92BR021, 92HT594, and ME1) could be explained by alterations of residues important for binding (Fig. 1), whereas neutralization of the 7 other viruses (QH0515, QH0692, SS1196, 91US056, 92US712, JR-CSF, and JR-FL) could not be accounted for by sequence incompatibility.

For the remaining 5 mAbs (CO11, F2A3, F530, LA21, and LE311) we observed also that the V3 sequence of some viruses could not account for neutralization resistance. Specifically, we observed that the V3 sequences of viruses QH0692, SS1196, 92US712, JR-CSF, and JR-FL consistently could not account for neutralization resistance (Fig. 1). It should be noted that all of these viruses are sensitive to neutralization by V3 mAbs B4e8, 447-52D, and/or 58.2 (Binley et al., 2004;Pantophlet et al., 2007), indicating that their V3 loop is not inaccessible to antibody. We observed also that the V3 sequence of other select viruses could not account for neutralization resistance of some mAbs tested here, for example virus BG1168 for mAbs CO11 and LE311, virus 91US056 for mAb LA21, and virus 6101-p15 for mAb F530.

MAb fine specificity mapped onto V3 in the structural context of gp120

To obtain a better understanding of the spatial orientation of the residues identified as important for V3 mAb binding, we projected the results of the mapping onto the structure of a soluble CD4-, Fab X5-complexed gp120 core with the V3 loop attached (Huang et al., 2005). We chose this structure because the conformation of the V3 stem is not substantially influenced by interactions with the antibody (Huang et al., 2005).

The results of the projected maps are shown in Fig. 1. For mAb 19b, we observed a distinct binding site formed by Arg^{304} in the N-terminal V3 stem and Arg^{315}, Phe^{317}, and Tyr^{318} in the C-terminal half of the V3 tip. Pro^{313}, which was also important for binding, is somewhat distant from the distinctive formation of the other 4 residues, suggesting that the primarily role of this residue is to properly juxtapose the residues that form the 19b epitope, as suggested previously (Moore et al., 1995). The side chain of Arg^{315} is not critical for 19b binding, given that efficient binding is also observed to monomeric $gp120_{JR\text{-}CSF}$ with a Gln substitution at the same position (Pantophlet et al., unpublished observations). Thus, the presence of both the Pro and Arg residues is required to form the V3 tip hairpin turn and juxtapose the 'true' contact residues. Ile^{307} lies somewhat distant from the epitope map and, thus, it seems unlikely that this residue interacts with mAb 19b directly. However, based on the substantial decrease in 19b binding to I307A mutant, its presence seems crucial for proper presentation of the epitope. This notion is supported by previous results showing that mAb 19b prefers residues with aliphatic side chains at position 307 (Moore et al., 1995).

Residues important for the binding of mAb 39F –Lys^{305}, Ser^{306}, Ile^{307}, and Ile^{309}—also form a distinct binding site on the N-terminal flank of V3, along the length of the V3 structure (Fig. 2), supporting the notion that this antibody interacts with the N-terminal part of V3. Projection of the Ala-scanning results for mAbs CO11, F2A3, LA21, and LE311 onto the V3 structure showed that the maps for CO11 and LA21 share a higher similarity than the maps for mAbs F2A3 and LE311, which are similar to each other. The maps for CO11 and LA21 are somewhat similar to the map for B4e8, with the notable difference that B4e8 binding is not critically dependent on Pro^{313} (Fig. 2). Although the residues important for CO11 and LA21 binding form a somewhat disjointed pattern, these mAbs likely also contact neighboring residues. A similar disconnected pattern is observed with residues important for the binding of mAb B4e8 (Fig. 2), yet structural analysis later revealed that B4e8 forms less-critical interactions with neighboring residues (Bell et al., 2008).

As expected, the epitope maps for mAbs F2A3 and LE311 are similar to those of mAbs CO11 and LA21, most notably His^{308}, Pro^{313} and Arg^{315} (Fig. 2). For both mAbs F2A3 and LE311 we observed that Lys^{305}, Ile^{307}/His^{308}, and Pro^{313}, all of which are important for mAb binding, form a nearly linear arrangement on the V3 structure. In the case of mAb F2A3, the location of residues that, upon substitution to alanine, increased antibody binding are crowded around the 4-residue linear arrangement, supporting the notion that residues at the adjacent positions play a substantial role in the spatial positioning of the contact residues.

For mAb F530 we obtained a map that resembled the maps obtained with mAbs CO11, F2A3, LA21, and LE311 (Fig. 2). However, the notable ability of mAb F530 to bind V3 without requiring the presence of Arg^{315} suggests that this antibody, like mAb 39F, interacts mostly with the N-terminal flank of the V3 loop.

DISCUSSION

Our results illustrate how the epitope fine specificity of antibodies to V3 may affect their level of cross-neutralizing activity. All 7 mAbs tested here neutralized very few viruses; common among all mAbs was the requirement for one or more positively-charged residues at the

juncture of the N-terminal flank of the V3 stem with the V3 tip for efficient binding. The mAbs also depended, to varying extents, on the presence of the Pro at the V3 tip for efficient binding. We posit that the requirement for interaction with certain residues near the V3 stem and in the V3 tip angles the mAbs such that they are restricted from interacting with their cognate epitope on many viruses, exemplified here by viruses QH0692, SS1196, 92US712, JR-CSF, and JR-FL. These 5 viruses do not have alterations in residues identified by our scanning mutagenesis approach as being important for binding but nevertheless were not neutralized by the 7 mAbs that were studied here. However, we know that the V3 loops on these viruses are not completely inaccessible to antibody, given that these viruses are sensitive to neutralizing by V3 antibodies such as B4e8 and 447-52D.

The factor(s) responsible for restricting V3 access of the 7 mAbs tested here remain to be identified. Recent studies using chimeric viruses have shown that the V1/V2 loop on some primary viruses, e.g. JR-FL an YU2, may greatly limit antibody recognition of V3 (Krachmarov et al., 2005; Krachmarov et al., 2006; Pinter et al., 2004). It is therefore tempting to speculate that V1/V2 may also be limiting V3 access on the other viruses tested here but this will need to be determined experimentally; for example, it is conceivable that specific residues outside of V3 (and V1/V2) affect the presentation of V3 antibody epitopes. It should be noted that we did not investigate further the relative importance of residues identified as critical for antibody binding in the context of different V3 sequences. It is possible that the contribution of each residue to binding affinity may vary in the context of V3 sequences from different viruses. More detailed studies, for example, by investigating changes in the sensitivity of neutralization-resistant viruses used here in which important antibody binding residues in V3 are introduced may help to further elucidate the relationship between sequence variability and neutralization sensitivity.

Only structural information can provide accurate insight into how the 7 mAbs tested here interact with V3. Nevertheless, even in the absence of such information, we believe structural information from other V3 mAbs for which important binding residues have been determined supports this hypothesis. For example, mAbs 59.1 (epitope: ^{306}RIHIGPGRAFYTT^{320}), 50.1 (epitope: ^{305}KRIHIGP^{313}), 83.1 (epitope: ^{306}RIHIGPGR^{315}), and 58.2 (epitope: ^{306}RIHIGPGRAFY^{318}) bind overlapping epitopes on V3 (Seligman et al., 1996) and require many of the same residues for efficient binding (important contact residues are underlined; critical residues are double underlined (White-Scharf et al., 1993)). Nonetheless, these 4 mAbs interact from markedly different angles with V3 (Fig. 3). MAbs 59.1, 50.1, and 83.1, which only neutralize viruses that are generally sensitive to antibody neutralization (Bou-Habib et al., 1994), seem to require a particular β hairpin turn conformation at the center of the V3 tip (GPGR) for binding, as judged by the substantial reduction in binding efficiency upon substituting one or both glycine residues. This hairpin turn requirement mirrors the strong dependence of mAbs 19b, LE311, CO11, and LA21 on the presence of the proline in the GPGR turn for binding, presumably to juxtapose important contact residues. Thus, mAbs 19b, LE311, CO11, and LA21 may need to interact with V3 from angles, perhaps similar to mAbs 59.1, 50.1, and/or 83.1, which do not permit access to V3 on many different primary viruses. MAb 58.2, which neutralizes more primary viruses than mAbs 19b, LE311, CO11, or LA21 (Table 1; (Binley et al., 2004)), seems less dependent on the precision of the turn given that its binding is only substantially diminished upon substituting Arg^{315} (White-Scharf et al., 1993), and interacts with V3 from an angle that is notably different from mAbs 59.1, 50.1, and 83.1 (Fig. 3).

The association of low cross-neutralizing activity with binding to the residues in or near the V3 stem segment is supported by other observations. For example, it has been shown that isolate-specific V3 responses in subtype B- and D-infected individuals are directed to residues located in the stem segments of the V3 loop (Lawoko et al., 2000; Schreiber et al., 1997) and

weakly neutralizing serum antibody responses to V3 in individuals immunized with recombinant gp160 are preferentially directed to the N-terminal stem of V3 (Coeffier et al., 1997). Furthermore, in a recent study seeking to elicit 447-52D-like NAbs by utilizing fusion proteins containing the 447-52D contact epitope ^{305}KSIHIGPGRAFYTT^{320} (Chakraborty et al., 2006), immune sera seemed to require for binding the N-terminal segment of the engrafted motif and neutralized the generally-sensitive virus MN but not the more resistant viruses BaL or JR-FL.

Fig. 3 shows that mAbs 58.2, 447-52D, and B4e8, which all neutralize several primary viruses (Binley et al., 2004;Pantophlet et al., 2007), interact with V3 within nearly the same plane of orientation; antibodies interacting with V3 from other angles tend to exhibit lower cross-neutralizing activity (Bou-Habib et al., 1994;Gorny et al., 2004;York et al., 2001). The importance of the angle of antibody interaction for effective neutralization of HIV is exemplified by insight from recent crystal structures of the broadly neutralizing mAbs 2F5, 4E10, and b12 in complex with antigen (Cardoso et al., 2005;Zhou et al., 2007), which suggest that such antibodies need to interact in with their target sites from specific angles to gain access to their epitopes on infectious virions; the difficulty in eliciting 2F5-, 4E10-, and b12-like antibodies supports the notion that these sites are not easily accessed. Our results illustrate how similar restrictive factors might apply to V3 antibodies. MAb 58.2 exhibits the lowest cross-neutralizing activity among the 3 mAbs that neutralize different primary viruses, despite interacting with V3 from nearly an identical angle as mAb 447-52D. This difference in neutralizing activity may be due to mAb 447-52D forming main-chain contacts with residues in the V3 stem region, which has been suggested to enhance its ability to neutralize a notable fraction of viral isolates (Stanfield et al., 2004). MAb B4e8, which seems to neutralize slightly better than mAb 447-52D (Keele et al., 2008;Pantophlet et al., 2007), interacts with V3 from a slightly elevated angle relative to the 2 other mAbs.

In sum, we have presented here an extensive analysis in an attempt to better understand how epitope fine specificity affects the neutralizing activity of V3 antibodies. Previous studies have shown that V3 may be shielded by the V1/V2 region (Cao et al., 1997; Krachmarov et al., 2006; Pinter et al., 2004; Wyatt et al., 1993), but such studies did not provide a clear rationale for the spectrum of sensitivities exhibited by a given primary isolate to the neutralizing activity of different V3 mAbs. Antibodies that interact with V3 from certain angles may indeed be greatly restricted from accessing their epitope on several viruses, depending on the stringency of the steric constraints. However, certain parts of V3 may still be accessible from select angles. The V3 tip in particular seems somewhat better accessible on different viruses, as judged from the appreciable neutralizing activity exhibited by mAb B4e8 (Pantophlet et al., 2007). The apparently unique binding mode of mAb B4e8, and potentially those of other V3 mAbs (Gorny et al., 2006), may provide an avenue for further investigations into V3 tip accessibility on different viruses, for example by engineering variants with specificities for V3 sequences not recognized by the parental antibody.

MATERIALS AND METHODS

Antibodies

Seven human mAbs directed to the V3 loop on HIV-1 gp120 were studied here: 19b, 39F, CO11, F2A3, F530, LA21, and LE311. MAb 19b was isolated from an asymptomatic individual infected with a clade B virus by Ebstein-Barr virus (EBV) transformation of peripheral blood lymphocytes and was shown to be specific for the V3 loop by mapping with peptides (Scott et al., 1990). MAbs 39F and F2A3 were isolated from two subtype B-infected long-term non-progressors, and mAbs CO11, LA21 and LE311 were isolated from three different patients (AC-01, AC-05, and AC-033, respectively) on anti-retroviral therapy during acute infection and who then underwent several periods of structured treatment interruptions

(Montefiori et al., 2001; Rosenberg et al., 2000). These mAbs were selected as described elsewhere (Xiang et al., 2003) and have been defined as being directed to V3 based on their reactivity with select clade B V3 peptides (J. Robinson, unpublished data). MAb F530 was isolated from an HIV-1 infected patient by direct fusion of mononuclear splenocytes with HMMA human myeloma cells (Posner et al., 1989; Posner, Elboim, and Santos, 1987). Hybridomas were selected based on specific reactivity hybridoma supernatants with HIV-infected cells and ability of the antibodies to capture a relatively high degree of virions in a virus capture assay (L. Cavacini, unpublished results). The general specificity of mAb F530 for the V3 loop was determined based on its binding profile with variable-loop deleted gp120 mutants (L. Cavacini, unpublished results). Most mAbs described above have been utilized in previous antigen binding studies and were selected for study here based on their overall broad reactivity with envelope-based preparations from different clade B viruses and consensus sequences (Grundner et al., 2002; Haynes et al., 2006; Koch et al., 2003; Liao et al., 2006; Moore et al., 1994; Pantophlet, Wilson, and Burton, 2004; Patel, Hoffman, and Swanstrom, 2008; Selvarajah et al., 2005; Yang et al., 2002).

Viruses

Pseudovirus preps were produced by co-transfection of HEK293 cells with an *env*-expressing plasmid (pCXAS) and a subgenomic plasmid (pHIVlucΔU3) as described previously (Binley et al., 2004). The test viruses (n=15; Table 1) were selected from the 30-member panel of subtype B primary viruses used in a previous study by Binley et al. (Binley et al., 2004). Eight of the test viruses were quasispecies derived from primary virus cultures and are as follows: QH0515 (Binley et al., 2004; Cleghorn et al., 2000; Hu et al., 2000; Li et al., 2005), QH0692 (Binley et al., 2004; Cleghorn et al., 2000; Li et al., 2005), 5768-p27 (Binley et al., 2004; Li et al., 2005), 6101-p15 (Binley et al., 2004; Li et al., 2005), 91US056 (Binley et al., 2004; Trkola et al., 1995), 92BR021 (Binley et al., 2004; Gao et al., 1994; Torre et al., 2000), 93TH305 (Binley et al., 2004), and ME1 (Binley et al., 2004; Chen et al., 1997); the noted references are for *env* sequences belonging to these viruses that have been reported in the Binley et al. study and elsewhere. The remaining 7 viruses were molecular clones. Three of these molecular clones (92HT594, JR-CSF, and JR-FL) have been described previously (Binley et al., 2004), whereas the remaining four clones (BG1168, SS1196, 92BR020, 92US712) have not been reported previously; each molecular clone was selected from a panel of 6 clones derived from the respective viral quasispecies pools reported previously (Binley et al., 2004). Each clone was chosen based on the overall similarity between its sensitivity to neutralization by the broadly neutralizing mAbs b12, 2G12, 2F5, and 4E10, and a broadly neutralizing HIV^+ serum and the neutralization sensitivity profiles of the corresponding viral quasispecies to the same inhibitors (T. Wrin et al., unpublished results).

Neutralization assays

Neutralization assays were performed at Monogram Biosciences using their high-throughput neutralization assay with U87 target cells expressing CD4, CCR5, and CXCR4 and pseudotyped viruses (Richman et al., 2003; Schweighardt et al., 2007). Assay conditions were the same as described previously (Binley et al., 2004); serial dilutions of mAb, starting at 50 μg/ml, were incubated for 1 hour with virus after which the mixture was added to target cells.

Generation of V3 mutants

The V3 mutants, generated in the JR-CSF background, were the same as described recently for mapping the epitope specificity of mAb B4e8 (Pantophlet et al., 2007). All mutations were verified by DNA sequencing.

Epitope mapping by ELISA

Binding assays to determine apparent antibody binding affinities were performed using viral lysates of supernatants collected from transiently-transfected 293T cells as described (Pantophlet et al., 2007; Pantophlet et al., 2003). The mAbs were added to the ELISA plate wells in 5-fold serial dilutions and binding was detected with a peroxidase-conjugated secondary antibody and TMB substrate (Pierce). Apparent binding affinities (K_{app}) were defined as the antibody concentrations at half-maximal binding; percentage changes in affinity relative to wild-type gp120 were calculated as: [K_{app}wild-type/K_{app}mutant]×100%.

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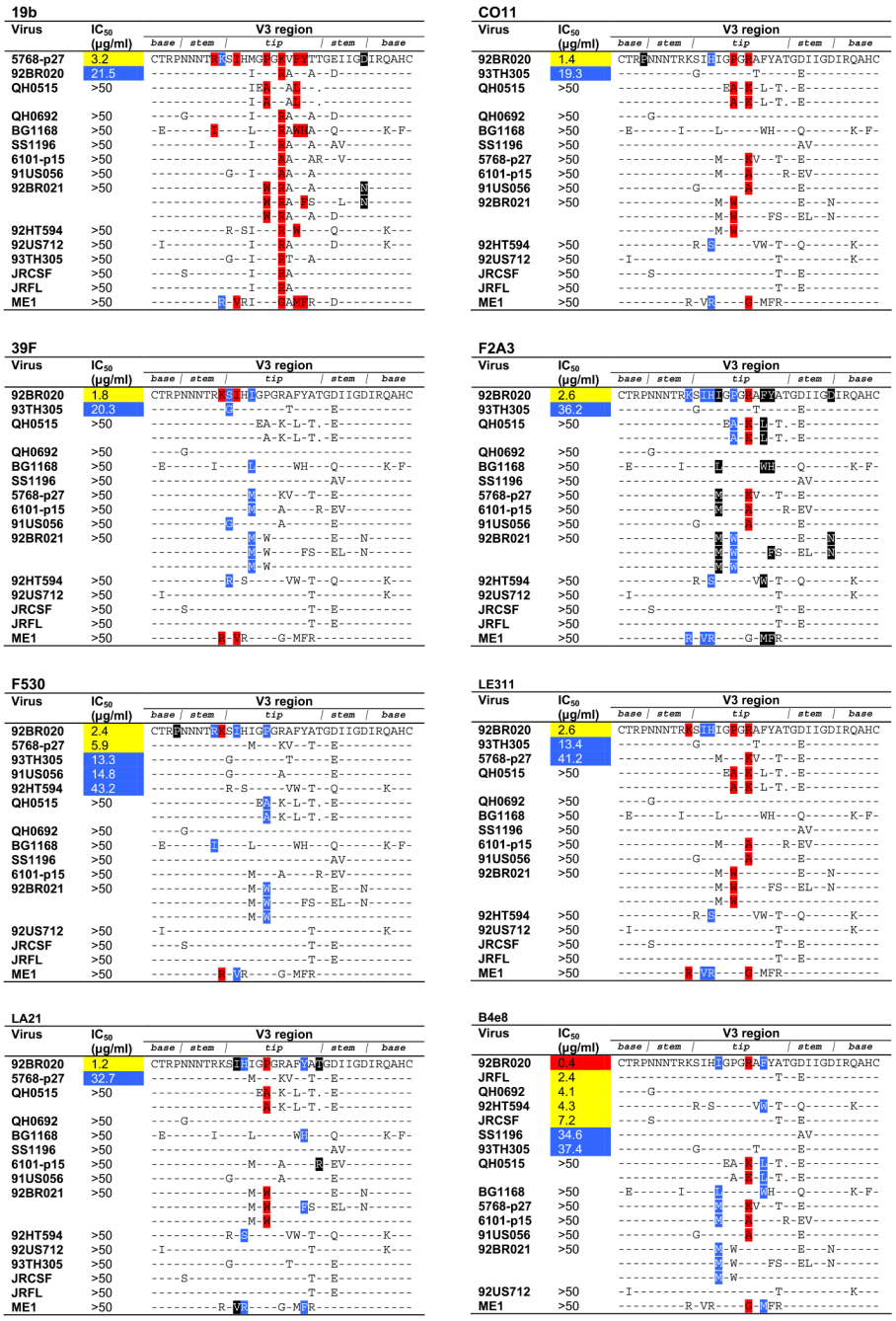

Fig. 1.

Epitope specificity of V3 mAbs related to neutralizing activity. The V3 sequences of the 15 subtype B viruses investigated in this study are shown. For viruses QH0515 and 92BR021, multiple sequences are listed to represent the different V3 sequences that have been reported from quasispecies pools of these 2 viruses; for the other viruses for which quasispecies pools were used only a single sequence (shown) has been reported. To depict which residues are most permissible for each mAb, viruses are ranked according to their neutralization sensitivity; viruses not neutralized are ranked in the order as they appear in Table 1. The locations of those residues that substantially diminish or enhance mAb binding are colored as in Table 2; dashes indicate sequence homology with the virus that was neutralized most potently and, to maintain

clarity, are not colored. Sequence gaps relative to the V3 sequence of the most potently neutralized virus are denoted by a dot. Sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/clustalw/) (Larkin et al., 2007) and formatted for publication using the sequence alignment publishing tool SeqPublish (http://www.hiv.lanl.gov/content/hiv-db/SeqPublish/seqpublish.html).

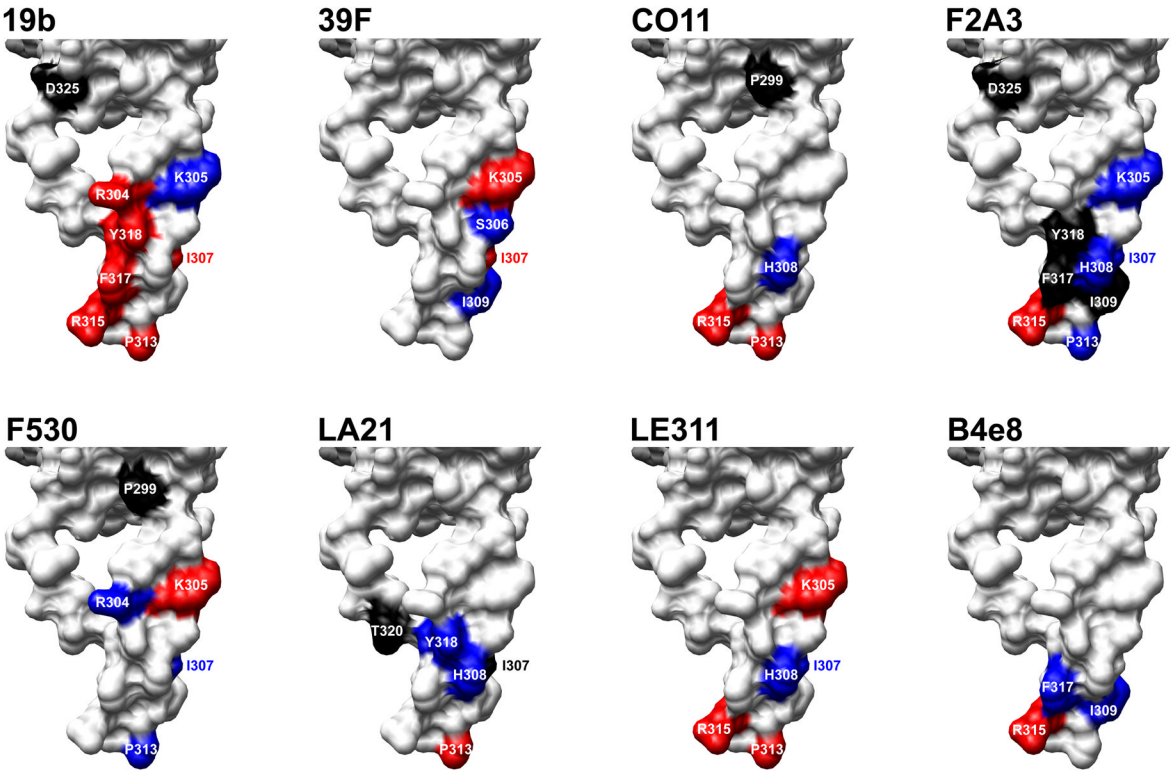

Fig. 2.

Effects of alanine substitutions on antibody binding mapped onto the structure of V3 in the context of gp120. Substitutions that significantly diminished or increased binding of the V3 mAbs tested here (Table 2) were mapped onto the structure of $gp120_{JR\text{-}FL}$-core with V3 attached (Huang et al., 2005); only the V3 structure is depicted (surface rendering). The color scheme is the same as in Table 2. Molecular graphics images were produced using the UCSF Chimera package (http://www.cgl.ucsf.edu/chimera), then compiled and labeled in Adobe Photoshop.

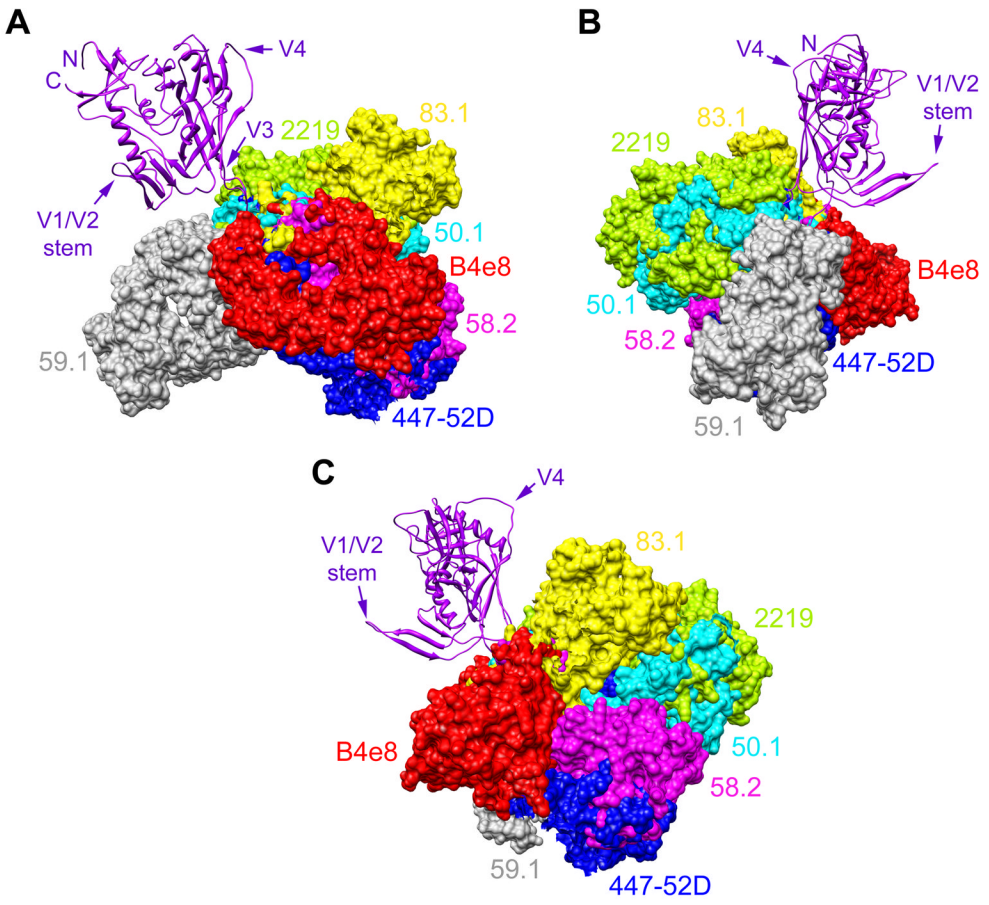

Fig. 3.

Angle of interaction between V3 antibodies and V3. Structure of $gp120_{JR-FL}$-core containing V3 (Huang et al., 2005) (purple; ribbon representation) shown from 3 perspectives with the Fab fragments of 7 different mAbs (surface rendering) superimposed to depict their different angles of interaction with V3. (A) Gp120 from the perspective of CD4; (B) Gp120 from the perspective of gp41 and the gp120:gp41 interface; (C) Gp120 from the approximate perspective of a coreceptor molecule (e.g. CCR5). To establish the angle for each mAb, the V3 peptide (not shown for clarity purposes) from each mAb:peptide complex structure was superimposed onto the V3 structure of gp120 using the MatchMaker feature in UCSF Chimera, which constructs a sequence alignment and then performs a least-squares fit to superimpose the aligned residue pairs; the sequence alignment is based on a combination of residue identity/similarity and secondary structure correspondence. The V3 mAbs shown are: B4e8 (red), 447-52D (blue), 58.2 (magenta), 59.1 (grey), 50.1 (cyan), 83.1 (yellow), and 2219 (green). Aside from V3, additional regions of gp120 (N- and C termini, V1/V2 stem, and V4) are denoted to orientate the reader.

Table 1

Neutralizing activity of V3 mAbs against a panel of subtype B viruses

Virus[a]	IC_{50}(μg/ml)[b]									
	19b	**39F**	**C011**	**F2A3**	**F530**	**LA21**	**LE311**	**B4e8**	**447D**	**58.2**
QH0515	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
QH0692	>50	>50	>50	>50	>50	>50	>50	4.1	40.3	39.3
BG1168*c*	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
SS1196*c*	>50	>50	>50	>50	>50	>50	>50	34.6	1.9*	0.5*
5768-p27	3.2	>50	>50	>50	5.9	32.7	41.2	>50	>50	>50
6101-p15	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
91US056	>50	>50	>50	>50	14.8	>50	>50	>50	>50	>50
92BR020*c*	21.5	1.8	1.4	2.6	2.4	1.2	2.6	0.4	>50*	>50*
92BR021	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
92HT594*c*	>50	>50	>50	>50	43.2	>50	>50	4.3	7.9	>50
92US712*c*	>50	>50	>50	>50	>50	>50	>50	>50	0.6*	1.4*
93TH305	>50	20.3	19.3	36.2	13.3	>50	13.4	37.4	8.5	25.4
JR-CSF*c*	>50	>50	>50	>50	>50	>50	>50	7.2	>50	32.8
JR-FL*c*	>50	>50	>50	>50	>50	>50	>50	2.4	32.6	>50
ME1	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50
aMLV	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50

[a] All viruses are quasispecies pools except where noted. Virus designations are generally denoted as year (of isolation), country (of isolation), and name/number. aMLV, amphotrophic murine leukemia virus (negative control virus). *c*, molecular clone.

[b] IC_{50}s are color-coded as follows: white, IC_{50} >50 μg/ml; blue, 50 μg/ml> IC_{50} >10 μg/ml; yellow, 10 μg/ml> IC_{50} >1 μg/ml; red, IC_{50} <1 μg/ml. The IC_{50}s for mAbs B4e8, 447-52D (447D), and 58.2 are taken from a previous studies (Binley et al., 2004; Pantophlet et al., 2007).

*, IC_{50} obtained with quasispecies pool (mAbs 447D and 58.2 only); for these viruses the IC_{50} against the molecular clone listed has not been determined.

Table 2

Epitope mapping of V3 mAbs

MAb	Apparent affinity (%)[a] relative to wild-type gp120JRCSF for indicated V3 mutants located in noted V3 segments																	
	base[b]			stem			tip										stem	
	R298A	P299A	N301A	N302A	R304A	K305A	S306A	I307A	H308A	I309A	P313A	R315A	F317A	Y318A	T319A	T320A	E322A	D325A
19b	71	146	113	66	5	23	167	0.3	128	54	4	7	0.3	0.3	72	123	95	201
39F	66	156	148	73	66	0.5	40	6	75	50	108	95	75	119	107	164	104	160
CO11	130	258	58	114	74	79	190	84	17	71	0.2	0.2	79	162	97	120	90	177
F2A3	76	168	135	114	86	11	90	50	40	219	38	0.1	211	257	161	176	148	223
F530	57	218	151	59	11	7	143	30	55	59	36	78	158	158	103	171	162	89
LA21	77	118	124	97	130	135	128	216	41	197	0.4	54	56	29	137	220	66	169
LE311	68	140	109	78	64	2	127	44	35	106	3	3	136	127	122	103	108	138
B4e8	79	96	124	85	88	83	87	74	88	23	91	3	49	76	81	127	77	117

[a] Apparent affinities were determined as the antibody concentration at half-maximal binding based on ELISA binding curves using the program Graphpad Prism (v.4.0). Apparent affinities relative to wild-type gp120JRCSF were calculated with the formula (apparent affinity for wild type/apparent affinity for mutants) ×100%. Red: <10% relative binding affinity; blue: >10% to <50% relative binding affinity; white: >50% to <200% relative affinity between; black: >200% relative affinity. The mapping results for mAb B4e8 are taken from (Pantophlet et al., 2007). Residue numbering is based on the HXB2 reference sequence (http://www.hiv.lanl.gov/). The mutants are arranged from the N- to C-terminal portion of the V3 region (left to right).

[b] V3 is divided into 3 structural segments based on the V3 structure in the context of gp120 (Huang et al., 2005): (I) the base (residues 296–300 (N-terminal portion) and 326–331 (C-terminal portion)), (II) the stem (residues 301–305 (N-terminal portion) and 321–325 (C-terminal portion)), and (III) the tip (residues 306–320).