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Discontinuous, Conserved Neutralization Epitopes Overlapping the CD4-Binding Region of Human Immunodeficiency Virus Type 1 gp120 Envelope Glycoprotein

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Monoclonal antibodies have been isolated from human immunodeficiency virus type 1 (HIV-1)-infected patients that recognize discontinuous epitopes on the gp120 envelope glycoprotein, that block gp120 interaction with the CD4 receptor, and that neutralize a variety of HIV-1 isolates. Using a panel of HIV-1 gp120 mutants, we identified amino acids important for precipitation of the gp120 glycoprotein by three different monoclonal antibodies with these properties. These amino acids are located within seven discontinuous, conserved regions of the gp120 glycoprotein, four of which overlap those regions previously shown to be important for CD4 recognition. The pattern of sensitivity to amino acid change in these seven regions differed for each antibody and also differed from that of the CD4 glycoprotein. These results indicate that the CD4 receptor and this group of broadly neutralizing antibodies recognize distinct but overlapping gp120 determinants.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (3, 10). HIV-1 establishes a persistent infection in human hosts, eventually resulting in defective cellular immunity secondary to CD4 lymphocyte depletion (9).

The HIV-1 exterior envelope glycoprotein, gp120, and the transmembrane envelope glycoprotein, gp41, are derived by cleavage of the gp160 envelope glycoprotein precursor (2, 35). HIV-1 is tropic for CD4-positive cells by virtue of a high-affinity interaction between the gp120 exterior envelope glycoprotein and the CD4 glycoprotein, which acts as the virus receptor (7, 18). Following gp120-CD4 binding, the fusion of viral and host cell membranes, which involves both gp120 and gp41 envelope glycoproteins, allows virus entry (41).

The chronic nature of HIV-1 infection implies that host antiviral immunity does not indefinitely suppress virus replication. Neutralizing antibodies appear to be an important component of a protective immune response (5, 8). The fact that neutralizing antibodies generated during the course of HIV-1 infection do not provide permanent antiviral effect may in part be due to the generation of neutralization escape virus variants and to the general decline in the host immune system associated with pathogenesis. Neutralizing antibodies for HIV-1 are directed against linear or discontinuous epitopes on the gp120 exterior envelope glycoprotein. Neutralizing antibodies that arise early in infected humans and that are readily generated in animals by immunization are directed primarily against linear neutralizing determinants in the third variable (V3) loop of the gp120 glycoprotein (16, 21, 32, 34, 37). These antibodies generally exhibit the ability to neutralize only a limited number of HIV-1 strains (22, 26, 29), although some subsets of anti-V3 antibodies recognize less variable elements of the region and therefore exhibit broader neutralizing activity (1, 15, 19, 27). Envelope glycoprotein variation within the linear V3 epitope and outside of the epitope can allow escape of viruses from neutralization by these antibodies (24, 25). These antibodies do not block CD4 binding but apparently interfere with post-receptor binding events involved in virus entry and syncytium formation, presumably a component of the membrane fusion process (20, 39).

Later in the course of HIV-1 infection of humans, antibodies capable of neutralizing a wider range of HIV-1 isolates appear (4, 33, 47, 48). These broadly neutralizing antibodies have been difficult to elicit in animals and are not merely the result of additive anti-V3 loop reactivities against diverse HIV-1 isolates that accumulate during active infection (31). A subset of the broadly reactive antibodies, found in most HIV-1-infected individuals, interferes with the binding of gp120 and CD4 (17, 23, 38). At least some of these antibodies recognize discontinuous gp120 epitopes present only on the native glycoprotein (11, 12, 40). Recently, human monoclonal antibodies that are derived from HIV-1-infected individuals and that recognize the gp120 glycoproteins from a diverse range of HIV-1 isolates, that block gp120-CD4 binding, and that neutralize virus infection have been identified (14, 30, 36, 46). The epitope of one such antibody, F105, has been shown to consist of amino acids derived from at least four discontinuous regions of the gp120 glycoprotein and, in part, overlaps the discontinuous gp120 binding site for the CD4 receptor (28, 45). Neutralization-resistant HIV-1 variants could be generated by amino acid changes in any of the four regions, which are located in the second, third, and fourth conserved gp120 segments (45). These changes involved amino acids 256 to 257, 368 to 370, 421, and 470 to 477. The first two of these regions correspond precisely to regions implicated in CD4 binding (28). Receptor binding is, in addition, sensitive to changes in tryptophan 427 and aspartic acid 457, which appear to be less important for recognition by the F105 antibody (28, 45). Since F105

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recognition is sensitive to amino acid changes that do not affect the receptor binding or membrane fusion functions of the HIV-1 envelope glycoproteins (44), sequence variation within the F105 epitope can result in viruses resistant to neutralization by the F105 antibody (45).

The discontinuous epitopes recognized by three independently isolated human monoclonal antibodies, 1.5e, 2.1h, and 1125H, were chosen for characterization. The 1.5e and 2.1h antibodies were derived from the same HIV-1-infected individual, the 1.5e antibody-producing clone having been isolated earlier than the 2.1h antibody-producing clone (14, 14a). The 1125H antibody was derived from a separate HIV-1-infected individual (46). All three antibodies recognize native but not denatured gp120, block the binding of gp120 and CD4, and neutralize a relatively broad range of HIV-1 isolates (14, 14a, 46). The 1.5e, 2.1h, 1125H, and F105 antibodies neutralize viruses containing the envelope glycoproteins of the HXBc2 strain of HIV-1 with approximately equivalent efficiency (43). Since these antibodies recognize diverse HIV-1 isolates (14, 46), amino acids conserved among HIV-1 strains must constitute the critical components of the discontinuous epitopes. To identify gp120 amino acids important for recognition by these antibodies, the reactivity of each antibody with a set of HIV-1 gp120 mutants altered in conserved residues was examined. In addition, antibody reactivity with some gp120 mutants containing alterations or deletions in the variable regions of the glycoprotein was tested. The mutant glycoproteins used in this study have been characterized with respect to rate of gp160 precursor processing, subunit association, CD4-binding ability, and recognition by a number of different monoclonal antibodies, allowing an assessment of the conformational correctness of the mutant proteins (13a, 28). [^{35}S]cysteine-radiolabeled COS-1 cells transfected with plasmids expressing the wild-type or mutant envelope glycoproteins of the HXBc2 strain of HIV-1 were lysed in RIPA buffer and precipitated either with the individual human monoclonal antibodies or with a mixture of sera derived from HIV-1-infected humans (45). Since the mixed patient sera recognize multiple gp120 epitopes, most of which are not affected by the amino acid changes in the mutant glycoproteins, the latter precipitation allows an assessment of the amount of mutant envelope glycoproteins present in the cell lysate. The recognition index represents the ability of a given mutant envelope glycoprotein to be recognized by the monoclonal antibody relative to the ability of the wild-type envelope glycoprotein (Table 1, footnote *a*). The recognition index was calculated for all mutants for which precipitation by the monoclonal antibody was judged to be decreased relative to that of the wild-type glycoproteins.

The results of the immunoprecipitation studies are shown in Table 1 and Fig. 1. Both the gp160 and gp120 glycoproteins were efficiently precipitated by the 2.1h monoclonal antibody, as had been seen previously for the F105 antibody (45). Under these conditions, the 1.5e and 1125H monoclonal antibodies preferentially precipitated the wild-type gp160 rather than the gp120 glycoprotein. Mutant glycoproteins (Δ119-205 and Δ297-329) containing large deletions encompassing the V1, V2, and V3 variable regions of gp120 were efficiently precipitated by all three monoclonal antibodies (Table 1, footnote *a*). This finding indicates that amino acids critical to the formation of the epitopes for these antibodies are not found within the large gp120 variable regions. Other amino acid changes (392 N/E plus 397 N/E, 406 N/G, and 463 N/D) affecting N-linked glycosylation sites within the smaller variable regions, V4 and V5, did not significantly affect recognition by any of the three antibodies (Table 1, footnote *a*). Thus, it appears that the critical components of the antibody epitopes lie primarily within the conserved gp120 regions, consistent with the ability of all three antibodies to recognize or neutralize divergent HIV-1 isolates (14, 46).

Most of the amino acid alterations affecting the conserved gp120 regions (C1 to C5) did not abrogate recognition by the monoclonal antibodies (Table 1). However, amino acid changes in seven discontinuous gp120 regions resulted in significant decreases in recognition of the envelope glycoproteins by the three monoclonal antibodies. The seven regions include residues 88 to 102 and 113 to 117 in C1, residues 256 to 262 in C2, residues 368 to 370 in C3, residues 421 to 427 and aspartic acid 457 in C4, and residues 470 to 485 in C5. The different amino acid changes in these seven regions affected recognition by the 1.5e, 2.1h, and 1125H antibodies differently. Furthermore, several of the mutants not efficiently precipitated by one or more of the 1.5e, 2.1h, and 1125H antibodies were still able to bind CD4 or to be precipitated by the F105 antibody (Table 1). These results suggest that the effects of the amino acid changes in the seven conserved gp120 regions on recognition by the 1.5e, 2.1h, or 1125H antibody are not merely the consequence of a global disruption of gp120 conformation. Rather, the amino changes either directly disrupt the epitope or cause local conformational changes that interfere with antibody recognition.

Several of the mutants inefficiently recognized by human monoclonal antibodies directed near the CD4-binding region retain the ability to mediate virus entry. Neutralization escape mutants for the F105 antibody have been demonstrated to result from changes in either the 256–262, 368–370, 421, or 470–480 region (45). To demonstrate that neutralization escape from the antibodies studied here could also arise as a consequence of changes in the gp120 glycoprotein, we used an assay in which an *env*-defective HIV-1 provirus encoding the bacterial chloramphenicol acetyltransferase (CAT) gene was complemented for a single round of replication by the wild-type or mutant envelope glycoproteins (13) (Fig. 2). The monoclonal antibody was added to the recombinant virions for 1 h at 37°C prior to incubation of the virus-antibody mixture with target Jurkat lymphocytes. CAT transfer by recombinant viruses with the wild-type HIV-1 envelope glycoproteins was inhibited by soluble CD4, by the 110.4 and 9284 anti-V3 loop antibodies, or by the F105, 1.5e, 2.1h, and 1125H antibodies. By contrast, recombinant viruses with the 113 D/A mutant glycoprotein were relatively resistant to neutralization by the 2.1h antibody, even though these viruses were neutralized comparably to the wild-type viruses by the 110.4 and F105 antibodies or by soluble CD4. The 113 D/R alteration resulted in viruses resistant to the 1.5e, 2.1h, and 1125H antibodies but sensitive to the 110.4, 9284, and F105 antibodies. Viruses with the 257 T/G envelope glycoprotein were resistant to neutralization by the F105 and 1.5e antibodies but were neutralized by the 110.4 and 2.1h antibodies. The 313 P/S change in the V3 loop allowed escape from the 110.4 antibody but did not affect neutralization by the F105, 1.5e, or 2.1h antibody. Viruses containing the 370 E/Q mutant glycoprotein were resistant to the F105, 1.5e, and 1125H antibodies, were partially resistant to neutralization by the 2.1h antibody, and were sensitive to the 110.4 antibody. The 370 E/Q mutant was resistant to soluble CD4, as was previously reported (44). A control mutant, 420 I/R, was sensitive to neutralization by all of the antibodies tested. The 475 M/S change allowed escape from

TABLE 1. Recognition of mutant HIV-1 envelope glycoproteins by human monoclonal antibodies

Mutant (gp120 region)	Recognition index[a]				Relative CD4-binding ability[b]
	1.5e	2.1h	1125H	F105	
Wild type (C1)	1.00	1.00	1.00	1.00	1.00
40 Y/D (C1)	0.46	≥1.00	0.55	≥1.00	1.2
88 N/P (C1)	0.21	0.59	0.18	≥1.00	0.89
102 E/L (C1)	0.36	0.78	0.33	0.45	0.82
106 E/A (C1)	≥1.00	≥1.00	0.84	≥1.00	1.5
113 D/A (C1)	≥1.00	0.07	0.90	≥1.00	1.16
113 D/R (C1)	0.22	0.04	0.36	0.92	0.85
117 K/W (C1)	0.25	≥1.00	0.15	0.60	1.06
120/121 VK/LE (C1)	≥1.00	0.74	≥1.00	≥1.00	0.51
256 S/Y (C2)	<0.02	0.45	<0.004	<0.03	0.30
257 T/R (C2)	0.07	0.11	0.008	<0.007	0.16
257 T/A (C2)	0.12	0.55	0.04	<0.008	1.12
257 T/G (C2)	0.04	≥1.00	0.08	<0.03	1.04
262 N/T (C2)	0.08	0.15	0.01	0.60	0.21
266 A/E (C2)	0.52	0.64	0.54	≥1.00	0.97
368 D/R (C3)	0.06	0.14	<0.03	<0.01	<0.004
368 D/P (C3)	≥1.00	≥1.00	0.04	<0.02	0.09
368 D/T (C3)	0.51	0.48	0.03	<0.02	0.33
368 D/N (C3)	0.15	0.14	0.007	0.08	0.19
368 D/K (C3)	0.14	0.18	0.004	<0.02	<0.005
368 D/E (C3)	0.80	0.50	0.74	<0.03	0.09
370 E/Q (C3)	0.28	0.33	0.03	<0.04	0.018
370 E/R (C3)	0.02	0.12	0.02	<0.01	<0.003
370 E/D (C3)	0.37	0.33	0.35	<0.02	0.45
381 E/P (C3)	0.38	≥1.00	≥1.00	≥1.00	1.09
384 Y/E (C3)	0.53	≥1.00	0.60	0.16	0.29
386 N/Q (C3)	0.60	0.44	0.64	1.00	1.00
395 W/S (C3)	0.60	0.72	0.68	0.44	1.11
421 K/L (C4)	0.14	0.23	0.16	<0.02	0.55
427 W/V (C4)	0.35	≥1.00	0.84	≥1.00	<0.012
427 W/S (C4)	0.03	≥1.00	0.03	≥1.00	<0.006
457 D/A (C4)	0.68	0.87	0.59	0.93	0.09
457 D/R (C4)	0.24	0.26	0.26	0.42	0.15
457 D/G (C4)	0.66	0.93	0.39	0.89	0.23
457 D/E (C4)	0.48	0.72	0.46	≥1.00	0.38
470 P/G (C5)	0.41	0.33	0.25	0.19	0.82
475 M/S (C5)	0.36	≥1.00	0.42	<0.02	1.03
477 D/V (C5)	0.12	0.18	0.10	0.15	0.39
482/483/484 ELY/GRA (C5)	0.26	0.51	0.06	0.02	0.44
485 K/V (C5)	0.30	0.52	0.33	≥1.00	0.79

[a] Calculated as [(Mutant gp120 + gp160)/(wild-type gp120 + gp160) monoclonal antibody] × [(wild-type gp120 + gp160)/(mutant gp120 + gp160) patient serum]. The recognition indices are reported for all mutant glycoproteins exhibiting decreases in recognition by any of the monoclonal antibodies relative to that of the wild-type glycoproteins. Values are averages obtained in at least two independent experiments, the results of which did not differ from the mean by more than 20% of the values shown. The recognition indices of the 69 W/L, 76 P/Y, 80 N/R, 103 Q/F, 125 L/G, Δ119-205, 207 K/W, 252 R/W, 267 E/L, 269 E/L, 281 A/V, 298 R/G, 308/309/310 RIQ/RPELIPVQ, 314 G/W, Δ297-329, 380 G/F, 380/381 GE/YV, 382 F/L, 392 N/E + 397 N/E, 395 W/S, 406 N/G, 420 I/R, 430 V/S, 432 K/A, 433 A/L, 435 Y/H, 438 P/R, 456 R/K, 457 D/N, 463 N/D, 491 I/F, 493 P/K, and 495 G/K glycoproteins were at least as great as those of the wild-type glycoproteins.

[b] Derived from reference 28.

neutralization by the F105 and 1.5e antibodies but did not affect neutralization by the 110.4 or 2.1h antibody. Thus, changes in aspartic acid 113, threonine 257, glutamic acid 370, or methionine 475 can result in resistance to neutralization by some monoclonal antibodies capable of blocking receptor binding.

The effects of amino acid alterations in the HIV-1 gp120 glycoprotein on recognition of several human monoclonal antibodies that precipitate only native and not denatured gp120, that block gp120-CD4 interaction, and that neutralize divergent HIV-1 isolates were examined. These data indicate that amino acid alterations in seven discontinuous regions of the HIV-1 gp120 glycoprotein affect recognition by this group of antibodies. The discontinuity of these regions is consistent with the requirement for native gp120 structure for recognition by this group of neutralizing antibodies. The findings that all seven regions are relatively well

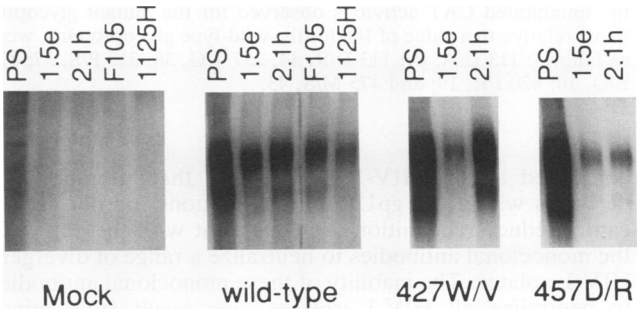

FIG. 1. Precipitation of wild-type, 427 W/V, and 457 D/R HIV-1 envelope glycoproteins by pooled patient serum (PS) and by the 1.5e, 2.1h, and 1125H human monoclonal antibodies. Precipitation of lysates derived from mock-transfected cells is also shown.

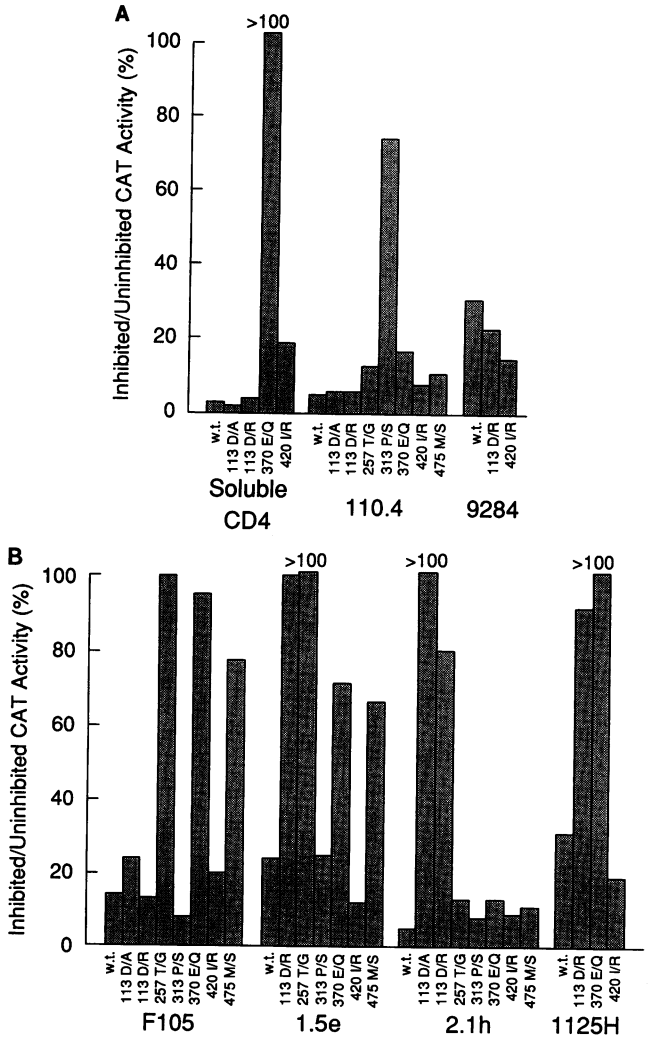

FIG. 2. Neutralization of selected mutant glycoproteins by human monoclonal antibodies. The ratio of inhibited to uninhibited CAT activity observed in target Jurkat lymphocytes incubated with recombinant virus containing either the wild-type (w.t.) or 113 D/A, 113 D/R, 257 T/G, 313 P/S, 370 E/Q, 420 I/R, or 475 M/S mutant envelope glycoprotein is shown. The inhibited CAT activities were observed following incubation of the recombinant virus for 1 h at 37°C with soluble CD4 (20 μg/ml) or the 110.4 (30 μg/ml) or 9284 (40 μg/ml) monoclonal antibody (A) or with the F105 (30 μg/ml), 1.5e (30 μg/ml), 2.1h (30 μg/ml), or 1125H (40 μg/ml) monoclonal antibody (B). In this assay, a value of 0 represents complete neutralization, while a value of 100 represents no neutralization. In this experiment, the uninhibited CAT activities observed for the mutant glycoproteins, relative to a value of 100 for the wild-type glycoproteins, were as follows: 113 D/A, 89; 113 D/R, 44; 257 T/G, 38; 313 P/S, 57; 370 E/Q, 10; 420 I/R, 19; and 475 M/S, 43.

conserved among HIV-1 isolates and that alterations or deletions within the gp120 variable regions did not significantly reduce recognition are consistent with the ability of the monoclonal antibodies to neutralize a range of divergent HIV-1 isolates. The inability of these monoclonal antibodies to neutralize all HIV-1 isolates may result from minor sequence variation occasionally observed in these seven regions, especially in the 470–485 segment, or may result from variation in gp120 regions outside those defined here secondarily influencing antibody recognition.

The seven gp120 regions defined herein to be important for recognition by anti-CD4 binding antibodies consist of both shared elements and antibody-specific elements. All of the monoclonal antibodies studied here appear to recognize a common core structure on gp120 composed of the 256–262, 368–370, and 421 regions. Changes in the first two of these regions have been shown to affect CD4 binding, suggesting that the overlap of this core antibody recognition structure with elements of the discontinuous CD4 binding site might account for the ability of the antibodies to interfere with gp120-CD4 interaction. Shared elements in the gp120 epitopes also probably account for the ability of these antibodies to compete with each other for gp120 binding.

Particular amino acid substitutions within the seven defined gp120 regions did not uniformly abrogate recognition by CD4 and all of the conformation-dependent monoclonal antibodies. This result, combined with the retention by many of these mutants of replicative or syncytium-forming function (44, 45), strongly argues against global conformational disruption of the gp120 glycoprotein accounting for decreased recognition by the antibody. Instead, it is likely that the amino acid changes affect antibody recognition by local conformational changes that either disrupt the epitope or alter the accessibility of the epitope to antibody. The inclusion of gp120 amino acids 88, 102, 113, and 117 within epitopes overlapping the CD4-binding site helps to reconcile several apparently disparate observations regarding gp120-CD4 binding. Deletions encompassing HIV-1 gp120 residues 82 to 95 or 108 to 116 have been reported to decrease CD4-binding ability (6a, 42), even though single amino acid changes in this region exerted little effect on CD4 binding (28). A polyclonal serum directed against a gp120 peptide corresponding to residues 42 to 129 blocked the binding of gp120 and CD4 (42). Our results suggest that gp120 amino acids 88, 102, 113, and 117 are proximal on the native glycoprotein to the CD4-binding region, even though they probably are not directly involved in contact with CD4.

The differential effects of the amino acid changes on gp120 recognition by the individual monoclonal antibodies and by CD4 suggest that the relevant binding regions on the gp120 glycoprotein are overlapping but distinct for each of these ligands. Even within the shared core element, particular amino acid substitutions differentially affected recognition by the individual monoclonal antibodies. Furthermore, in addition to the core structure, up to four other gp120 regions appear to be important for recognition by individual monoclonal antibodies. Figure 3 diagrams the regions important for precipitation by monoclonal antibodies or for CD4 binding. Examination of Fig. 3 reveals that the epitopes of the 1125H and 1.5e antibodies are closely related, while those of the F105 and 2.1h antibodies also exhibit significant similarity. It is noteworthy that changes in tryptophan 427 and aspartic acid 457, which have been shown to be important for CD4 binding (6, 28), alter recognition by some of the monoclonal antibodies, such as 1125H and 1.5e, and not others, such as F105 and 2.1h. Whether recognition of these gp120 residues relates to the efficiency with which the antibody interferes with the gp120-CD4 interaction is under investigation.

The ability of the mutant envelope glycoproteins to be precipitated by the monoclonal antibodies generally correlated with the neutralization of recombinant viruses carrying the mutant glycoproteins. This result suggests that the antibody recognition of the gp120 glycoprotein in detergent-containing cell lysates approximates that in the virus-associated, multimeric complex. Differences in the degree of

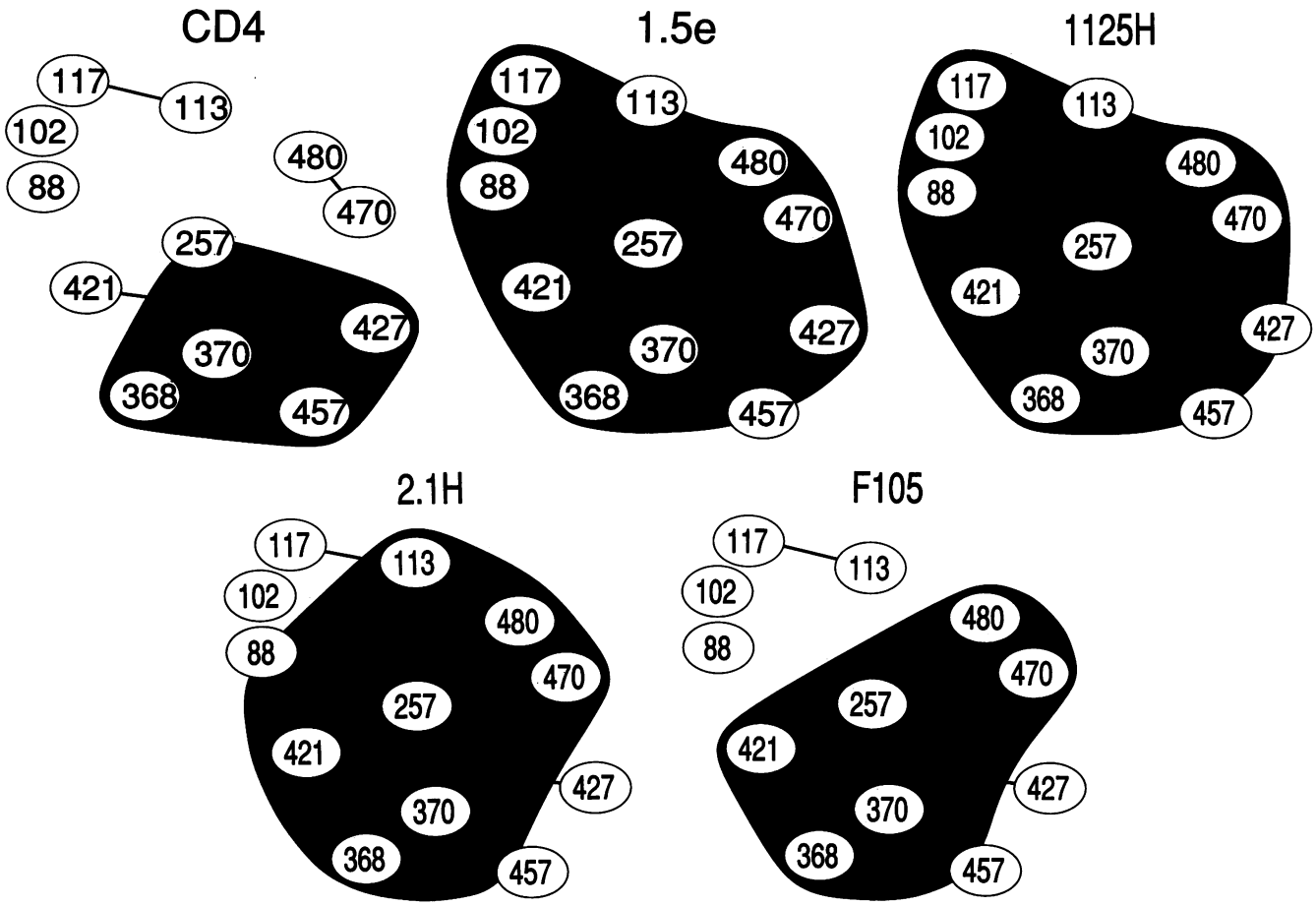

FIG. 3. Diagrams of gp120 amino acids in which alterations significantly decrease monoclonal antibody recognition or CD4 binding. The gp120 amino acids important for recognition by monoclonal antibodies or CD4 are shown, with solid lines connecting amino acids within 10 residues of each other on the linear sequence. In this numbering system, 1 represents the initiator methionine. The grey zone includes the amino acids important for recognition by the given ligand. Amino acids completely encompassed are those in which alterations result in dramatic decreases in binding. The amino acids only partially encompassed represent amino acids in which alterations result in only moderate decreases in ligand interaction or in which only some changes result in decreased ligand binding. The position of an amino acid in the diagram does not imply a necessary spatial relationship of residues on gp120.

neutralization escape and loss of antibody recognition seen for some of the mutants probably reflect subtle differences in affinity of the antibody for gp120 glycoproteins in these two contexts.

In a separate study (24a), we have identified another related pair of gp120 epitopes overlapping those identified herein. These epitopes are important for gp120 recognition by a human antibody, 448D, and by two rat monoclonal antibodies that block gp120-CD4 binding (24a). The consistency with which specific HIV-1 gp120 elements appear to be important for recognition by independently isolated monoclonal antibodies blocking gp120-CD4 interaction suggests that gp120 epitopes with these properties are limited in number. Further identification and characterization of conserved HIV-1 neutralization epitopes may reveal means to increase the efficiency of elicitation of antibodies directed against these determinants.

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