Epitope Mapping of the Human Immunodeficiency Virus Type 1 gp120 with Monoclonal Antibodies

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A soluble form of recombinant gp120 of human immunodeficiency virus type 1 was used as an immunogen for production of murine monoclonal antibodies. These monoclonal antibodies were characterized for their ability to block the interaction between gp120 and the acquired immunodeficiency syndrome virus receptor, CD4. Three of the monoclonal antibodies were found to inhibit this interaction, whereas the other antibodies were found to be ineffective at blocking binding. The gp120 epitopes which are recognized by these monoclonal antibodies were mapped by using a combination of Western blot (immunoblot) analysis of gp120 proteolytic fragments, immunoaffinity purification of fragments of gp120, and antibody screening of a random gp120 gene fragment expression library produced in the lambda gt11 expression system. Two monoclonal antibodies which blocked gp120-CD4 interaction were found to map to adjacent sites in the carboxy-terminal region of the glycoprotein, suggesting that this area is important in the interaction between gp120 and CD4. One nonblocking antibody was found to map to a position that was C terminal to this CD4 blocking region. Interestingly, the other nonblocking monoclonal antibodies were found to map either to a highly conserved region in the central part of the gp120 polypeptide or to a highly conserved region near the N terminus of the glycoprotein. N-terminal deletion mutants of the soluble envelope glycoprotein which lack these highly conserved domains but maintain the C-terminal CD4 interaction sites were unable to bind tightly to the CD4 receptor. These results suggest that although the N-terminal and central conserved domains of intact gp120 do not appear to be directly required for CD4 binding, they may contain information that allows other parts of the molecule to form the appropriate structure for CD4 interaction.

The human immunodeficiency viruses (HIV) are a family of retroviruses which are the probable causative agents of the acquired immunodeficiency syndrome (4, 26). These retroviruses fall into the lentivirus class, and they appear to induce immunodeficiency by infecting a subclass of immune system cells which are responsible for T-cell help. These T helper cells, in addition to other cells which are infectible by these viruses, all bear a common surface protein, the CD4 antigen (23). Several lines of evidence suggest that CD4 functions as the receptor for HIV. These include (i) the demonstration that monoclonal antibodies to CD4 are able to block virus infection (5, 12); (ii) the formation of a high-affinity complex between the external envelope glycoprotein of HIV, gp120, and the CD4 molecule (16, 18); and (iii) the conversion of noninfectible cells to HIV-infectible cells by gene transfection and surface expression of CD4 (17).

The viral protein that is responsible for binding of the virion to the receptor is the large external envelope glycoprotein, gp120 (16, 18). The most convincing demonstration of this fact is the formation of a high-affinity complex between this viral glycoprotein and the CD4 receptor. A fascinating aspect of the structure of gp120 is the high degree of variability found between different isolates of HIV (1, 7, 25, 28). One hypothesis for the function of these variable regions suggests that they are the result of immunoselection of neutralization-resistant viruses during the course of infection (9). In agreement with this possibility, cross-neutralization studies have shown that antibodies to a given isolate of HIV gp120 are incapable of neutralizing many divergent

isolates (11, 14, 27). This variability is confined to clearly delineated regions that are interspersed with domains which are highly conserved between different isolates. The high degree of interisolate conservation suggests a functional role for these interspersed conserved domains. For example, one such domain was found by in vitro mutagenesis to be critical for CD4 binding (16), whereas a mutation in a second conserved domain was found to disrupt virus infection but not to grossly inhibit binding of the glycoprotein to its receptor (29). In addition, mutations in other conserved domains were found to affect envelope processing and gp120-gp41 interactions (13). These results suggest that HIV glycoprotein gp120 contains conserved structural domains which have several different functions.

In this study, we used gp120-specific monoclonal antibodies as probes for the investigation of one functional aspect of gp120, binding of the glycoprotein to CD4. These monoclonal antibodies were used in a gp120-CD4-binding assay to determine those that are capable or incapable of inhibiting this interaction. The epitopes recognized by both types of monoclonal antibodies were mapped by using a combination of techniques, and a region critical for receptor binding was found. Interestingly, we also mapped monoclonal antibody-binding sites to two other highly conserved domains that do not appear to be directly involved in CD4 interaction. Production of secreted, soluble deletion mutants that lack these conserved regions of gp120 results in glycoproteins which are unable to bind tightly to CD4, suggesting that these domains contribute structurally important information to the formation of an appropriately structured carboxy-terminal CD4 interaction site.

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MATERIALS AND METHODS

Monoclonal antibodies. A soluble form of recombinant gp120 (rgp120) was produced and purified to homogeneity essentially as previously described (15). Mice or rats were injected seven times with 30 μg of purified rgp120 in Freund adjuvant over a period of 7 months. Spleens from immunized mice or rats were disrupted, and the cells were fused with NP3×63.Ag8.653 myeloma cells and selected in hypoxanthine-aminopterin-thymidine medium. Individual wells were analyzed for reactivity with recombinant gp120 in a solid-phase enzyme-linked immunosorbent assay. Cells in positive wells were cloned by limiting dilution, and the monoclonal antibody produced by each cell was purified by staphylococcal protein A column chromatography. Monoclonal antibody isotypes were determined by using anti-immunoglobulin antibodies. Epitope groups were determined by competition between unlabeled antibodies (15 μg/ml) and either ^{125}I- or horseradish peroxidase-labeled antibodies for binding to immobilized recombinant gp120.

gp120-CD4 receptor-binding assay. The source of CD4 receptor was a Chinese hamster ovary (CHO) cell line which was transfected with an expression plasmid containing the human CD4 cDNA sequence (16). This line was previously shown to produce large amounts of a membrane-bound form of human CD4. Binding experiments with N-terminal deletion mutants of gp120 (see below) were performed with a highly purified soluble form of CD4 (6, 24). Purified rgp120 was radioiodinated by the lactoperoxidase method to a specific activity of 0.53 nCi/ng. We incubated 100.0 ng (~100,000 dpm) of ^{125}I-labeled gp120 with the CD4-CHO cell line for 20 h at 4°C in the presence of increasing quantities of purified gp120 monoclonal antibodies, after which the specifically bound radioactivity was quantitated by counting in a gamma counter. Approximately 7.5 to 10% of the total input radioactivity bound specifically to the CD4-expressing CHO cells in the absence of any added monoclonal antibodies. Background binding of radiolabeled rgp120 to CHO cells not expressing CD4 was found to be <5% of specific binding.

Purification of rgp120 fragments by immunoaffinity chromatography. Fragmentation of rgp120 was accomplished with either trypsin or mild acetic acid. For trypsin digestion, rgp120 was incubated with 1% (wt/wt) gp120-tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin at 37°C for 2 h. Another portion of trypsin was added, and incubation was continued for an additional 2 h. The reaction was stopped by addition of excess soybean trypsin inhibitor. Acetic acid hydrolysis was performed as previously described (16). Monoclonal antibodies were immobilized by coupling to periodate-oxidized glycerol-coated control pore glass (16). Each monoclonal antibody resin was shown to bind intact rgp120 and elute the bound protein under acid conditions (16). Tryptic or acetic acid digests of rgp120 were applied to monoclonal antibody columns, washed, and eluted. Bound peptides were analyzed by quantitative amino acid analysis.

Mapping of monoclonal antibody-binding sites to gp120 proteolytic fragments by Western blotting (immunoblotting). Initial monoclonal antibody mapping was done with naturally occurring proteolytic fragments of rgp120. These fragments were purified by sodium dodecyl sulfate-gel electrophoresis, and their N-terminal sequences were determined by gas phase microsequencing to orient their positions in the intact protein. Western blot transfers of sodium dodecyl sulfate-polyacrylamide gels containing partially degraded gp120 were probed with the various monoclonal antibodies, after which the bound antibodies were detected with perox-

idase-labeled rabbit anti-mouse immunoglobulin G (IgG) as previously described (3).

Mapping of monoclonal antibody-binding sites with a lambda gt11 expression library. A library containing randomly expressed portions of the gp160 glycoprotein precursor fused to β-galactosidase was constructed essentially as previously described (20, 30). Briefly, the ~2.6-kilobase human T-lymphotropic virus type 3b (HTLV-3b) gp160 gene region was treated with increasing quantities of DNase I, blunted with the Klenow fragment of DNA polymerase and all four deoxynucleoside triphosphates, and ligated to EcoRI oligonucleotide linkers. The material was run on a 5% acrylamide gel, and the 100- to 1,100-base-pair region of the gel was isolated. DNA in this region was eluted and ligated to 1 μg of lambda gt11 EcoRI-cut bacteriophage arms. The ligated DNA was packaged in vitro and amplified in Escherichia coli 1088 cells. The library consisted of 1.6 × 10^7 independent phage.

The library was screened by plating approximately 4 × 10^3 phage onto E. coli Y1090 cells. After plaques developed, a nitrocellulose filter impregnated with isopropyl-β-D-thiogalactopyranoside was placed onto the plate and incubated overnight. The filters were blocked for 1 h in 0.7% gelatin in NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.6]) buffer. A 1:600 dilution of the monoclonal antibody was incubated with the filters for 3 h at room temperature in 0.3% gelatin–0.2% Tween 20–NET. After 1 h of incubation with monoclonal antibody, a 1:1,000 dilution of 10 mg of peroxidase-labeled goat anti-mouse IgG per ml was added. The filters were washed in 0.1% Tween 20–NET for 1 h. The reactive phage was detected colorimetrically with horseradish peroxidase-conjugated antibodies as previously described (3).

Positively reacting phage was plaque purified, and the DNA was isolated as previously described (20). Double-stranded lambda DNA sequencing was performed at 50°C with Klenow DNA polymerase and dideoxynucleotides in the presence of the β-galactosidase gene-specific oligonucleotide primers 5'-TTGACACCAGACCAACTGGTAATG-3' (reverse or carboxy terminal) and 5'-ATGGGGATTGGTG GCGACGACTCCTGGAGCCCG-3' (forward or amino terminal) (20, 30). The primers were boiled at 100°C for 5 min with the bacteriophage DNA, after which the reaction was allowed to cool to 50°C before addition of the enzyme. The observed DNA sequence resulted in the coordinates of the inserted gp120 fragment.

Construction and expression of deletion mutants of gp120. Deletion mutants were constructed by ligating restriction enzyme-digested fragments of the gp120 gene in the proper reading frame to a cassette consisting of the signal sequence and first nine N-terminal amino acids of the herpes simplex virus glycoprotein D gene (15; D. Dowbenko, unpublished data). The gp120 gene from the HTLV-3b strain of HIV was cleaved at the HincII site (amino acid 164), the PvuII site (amino acid 257), the DraI site (amino acid 313), and the ScaI site (amino acid 370) and incorporated, together with the signal sequence cassette, into a previously described expression vector (8). The mutants were then expressed by transfection into a mammalian kidney cell line (8), and the secreted glycoproteins, labeled with [^{35}S]methionine, were tested in the CD4-binding assay as previously described (16, 24).

RESULTS

gp120 monoclonal antibodies. Eleven gp120 monoclonal antibody-producing lines were derived from either rat-mouse

TABLE 1. Epitope grouping by monoclonal antibody competition

Labeled monoclonal antibody	Competition by unlabeled monoclonal antibody[a]:										
	1F9	9F6	7F11	7G11	5C2	3E10	5G9	6D8	1D10	8G4	8D8
1F9[b]	+	+	−	−	−	−	−	−	−	−	
9F6[b]	+	+	−	−	−	−	−	−	−	−	−
7F11[b]	−	−	+	+	+	−	−	−	−	−	−
7G11[b]	−	−	+	+	+	−	−	−	−	−	−
5C2[b]	−	−	+	+	+	−	−	−	−	−	−
3E10[b]	−	−	−	−	−	+	+	−	−	−	−
5G9[b]	−	−	−	−	−	+	+	−	−	−	−
6D8[c]	−	−	−	−	−	−	−	+	−	−	−
1D10[c]	−	−	−	−	−	−	−	−	+	+	+

[a] Unlabeled competitor monoclonal antibody was added at a concentration of 15 μg/ml. Recombinant gp120 was coated onto individual wells of 96-well microtiter plates at a concentration of 1.5 μg/ml and used as the antigen. Pluses show where competition was found; minuses show the absence of competition.

[b] Monoclonal antibodies were labeled with ^{125}I to a specific activity of ~6,000 cpm/ng and used at 15 ng/ml.

[c] Monoclonal antibodies were conjugated with horseradish peroxidase (3) and used at 20 ng/ml.

or mouse-mouse spleen cell-myeloma fusions. Table 1 illustrates competition results with these 11 monoclonal antibodies. The eleven monoclonal antibodies appeared to fall into five noncompeting epitope groups. Table 2 summarizes these data. These antibodies were all of the IgG1 isotype.

Monoclonal antibody blocking of gp120-CD4 interaction. The ability of monoclonal antibodies representing each of the epitope groups to block the interaction between gp120 and the viral receptor, CD4, was tested next. The assay system used ^{125}I-labeled rgp120 and recombinant CD4 expressed on the surface of transfected CHO cells. This system has been previously shown to mimic the interaction between the HIV virion and CD4 on the surface of lymphocytes (16), and it provided a convenient virus-free system for investigating the blocking ability of the gp120 monoclonal antibodies.

Most of the murine gp120 monoclonal antibodies were unable to significantly block the gp120-CD4 interaction (Fig. 1; Table 2). These included antibodies from four of the five different epitope groups (Tables 1 and 2). These results suggested that only a relatively confined region of gp120 is directly responsible for CD4 binding, since the large degree

TABLE 2. gp120 monoclonal antibody characteristics

Monoclonal antibody	Animal species[a]	Epitope group[b]	Inhibition of CD4 binding[c]	Monoclonal antibody:gp120 ratio for 50% blocking
1F9	M-M	a	−	
9F6	M-M	a	−	
7F11	M-M	b	+	~30:1
7G11	M-M	b	+	~30:1
5C2	M-M	b	+	~30:1
3E10	M-M	c	−	
5G9	M-M	c	−	
6D8	R-M	d	+/−	>1,000:1
1D10	R-M	e	+/−	>1,000:1
8G4	M-M	e	−	
8D8	M-M	e	−	

[a] Animal species from which spleen cells were derived for fusion. M, Mouse; R, rat. All spleen cells were fused to a murine hybridoma line (M). The isotypes, determined with rabbit anti-mouse isotype-specific IgG were all IgG1.

[b] Epitopes were grouped as shown in Table 1.

[c] Increasing quantities of purified monoclonal antibodies were added to the ^{125}I-labeled gp120-recombinant CD4 interaction assay, after which the radioactivity specifically bound to the receptor was determined by counting in a gamma counter.

of potential steric hindrance caused by the bound nonblocking monoclonal antibodies did not seem to affect CD4 binding. However, with monoclonal antibodies 1D10 and 6D8, a low level of blocking was found at extremely high antibody levels. It is probable that this was an artifact of nonspecific binding, since detectable nonspecific cross-reactivity to a number of other gp120 epitopes has been found with these monoclonal antibodies at very high antibody-antigen ratios (data not shown). Three of the gp120 monoclonal antibodies were found to block the interaction between gp120 and CD4. These antibodies, termed 5C2, 7F11, and 7G11, fell into the same epitope group (group b). Interestingly, blocking by the 5C2 and 7F11 monoclonal antibodies occurred over a wide range of antibody concentrations, with only relatively high antibody-antigen ratios (~30:1) giving significant blocking of receptor binding. This result suggests that these monoclonal antibodies compete directly with the high-affinity binding of rgp120 with the CD4 receptor and agrees with the finding that the blocking monoclonal antibodies are ineffective at neutralizing virus infectivity (unpublished data). In addition, this result complements earlier experiments with a soluble form of the CD4 receptor which showed that a large excess (~1,000 fold) of the viral receptor was necessary to inhibit the binding and subsequent infectivity of the virus (6, 24). In summary, these results suggest that a relatively limited region of gp120 is involved in direct CD4 interaction and that there are several epitopes on the molecule which, even with the potential steric hindrance contributed by a bound monoclonal antibody, did not seem to be involved with CD4 binding.

Mapping of monoclonal antibody-binding sites on rgp120 proteolytic fragments. gp120 monoclonal antibody-binding sites were initially mapped by Western blot analysis with naturally occurring proteolytic fragments of the glycoprotein. Two proteolytic fragments of gp120, with molecular masses of 75,000 and 55,000 daltons, were routinely obtained during purification (Fig. 2). N-terminal sequencing of the partially proteolyzed rgp120 preparation demonstrated that the 75,000-dalton fragment was derived from the N terminus of gp120 and corresponded to amino acids 1 to 295, whereas the 55,000-dalton fragment was derived from the C terminus and corresponded to amino acids 296 to 471 (the numbering of the glycoprotein corresponds to that for the HTLV-3b–lymphadenopathy-associated virus strain [21] and begins at the mature N terminus of the protein [2]).

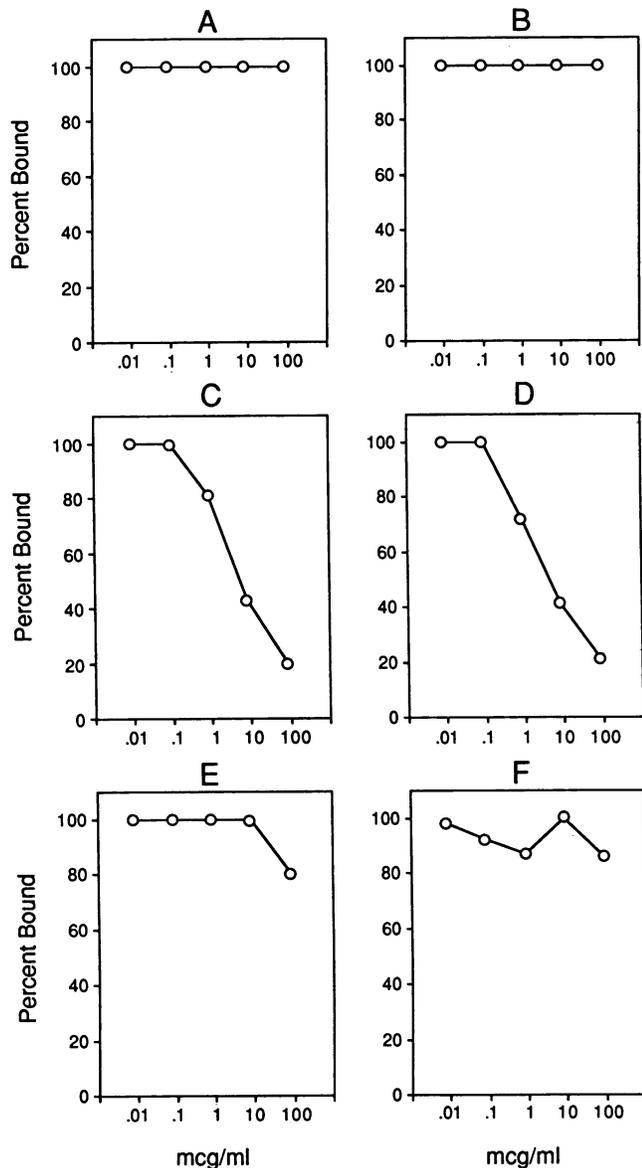Western blot analysis of the proteolytic fragments of rgp120 probed with the various monoclonal antibodies al-

FIG. 1. Blocking of the ^{125}I-labeled gp120-CD4 interaction by gp120 monoclonal antibodies. Increasing amounts of gp120 monoclonal antibodies were incubated with 100,000 cpm (~100 ng) of gp120 and CHO cells expressing CD4 on their surfaces for 20 h at 4°C. The unbound gp120 was washed with phosphate-buffered saline, and the specifically bound radioactive glycoprotein was determined by counting in a gamma counter. The abscissa shows the amounts of gp120 monoclonal antibodies added to the binding reactions. Panels: A, 1F9; B, 5G9 and 3E10; C, 5C2; D, 7F11; E, 6D8; F, 1D10. mcg, Micrograms.

lowed for the grouping of these antibodies to various regions of the glycoprotein. An example of these Western blots is shown in Fig. 2. At least one of the monoclonal antibodies bound to each of the proteolytic fragments (Table 3). For example, the 5C2, 7F11, and 7G11 blocking monoclonal antibodies bound to the C-terminal 55,000-dalton fragment. In agreement with earlier work, these results suggested that a critical part of the CD4-binding domain of gp120 is localized to the C-terminal region of gp120. Immunoaffinity chromatography has previously shown that antibody 5C2
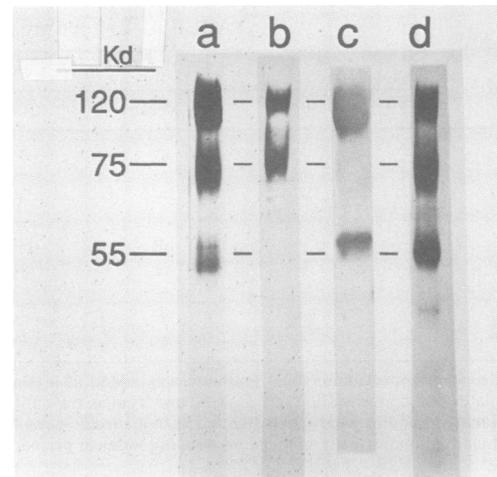

FIG. 2. Western blot analysis of binding of various gp120 monoclonal antibodies to proteolytic fragments of gp120. Lane a shows a silver-stained 8 to 15% gradient polyacrylamide gel of gp120 and proteolytic fragments of this glycoprotein produced during isolation. The 75,000- and 55,000-dalton fragments were identified by their apparent molecular masses and the N-terminal sequences observed when the partially degraded gp120 preparation was sequenced. Lanes: a, silver-stained gel of proteolytically degraded gp120 preparation; b, 9F6 reactivity; c, 5C2 reactivity; d, rabbit polyclonal anti-gp120 reactivity. Kd, Kilodaltons.

actually binds to a tryptic fragment corresponding to residues 406 to 417 (16). In a similar experiment, no tryptic fragments of gp120 were recovered from affinity columns of immobilized 7F11 or 7G11. Thus, even though the competition data shown above suggested that monoclonal antibodies 5C2 and 7F11-7G11 bound to the same epitope, the proteolytic mapping studies shown here, in addition to the previous tryptic mapping of the 5C2-binding site, suggest that these antibodies bind to different epitopes which are in close enough proximity to result in steric hindrance in the competition assay (Table 1).

TABLE 3. Localization of gp120 monoclonal antibody epitopes

Monoclonal antibody	Amino acid position(s) of proteolytic fragment(s)[a]	Amino acid position of lambda gt11 fusion protein[b]
1F9	1–295	187–276
9F6	1–295	210–274
7F11	296–471	393–408
7G11	296–471	ND
5C2	296–471; 406–417[c]	403–457
3E10	296–471	387–449
5G9	296–471	409–499
6D8	1–295; 18–39; 99–141[c]	21–85
1D10	1–295	34–55
8G4	1–295	ND
8D8	1–295	ND

[a] Naturally occurring proteolytic fragments were analyzed by N-terminal sequencing of sodium dodecyl sulfate-polyacrylamide gel-purified gp120 polypeptides. Western blot analysis was done to determine the proteolytic fragment containing a given epitope recognized by each monoclonal antibody. Numbering of amino acids began at the mature N terminus of the HTLV-3b isolate of HIV.

[b] Amino acid positions were determined by DNA sequencing of lambda gt11 bacteriophage carrying β-galactosidase-gp160 gene fragment fusion proteins which reacted with each monoclonal antibody in immunoscreens. ND, Not done.

[c] Proteolytic fragments were produced by either tryptic digestion or acetic acid cleavage and purified by immunoaffinity chromatography.
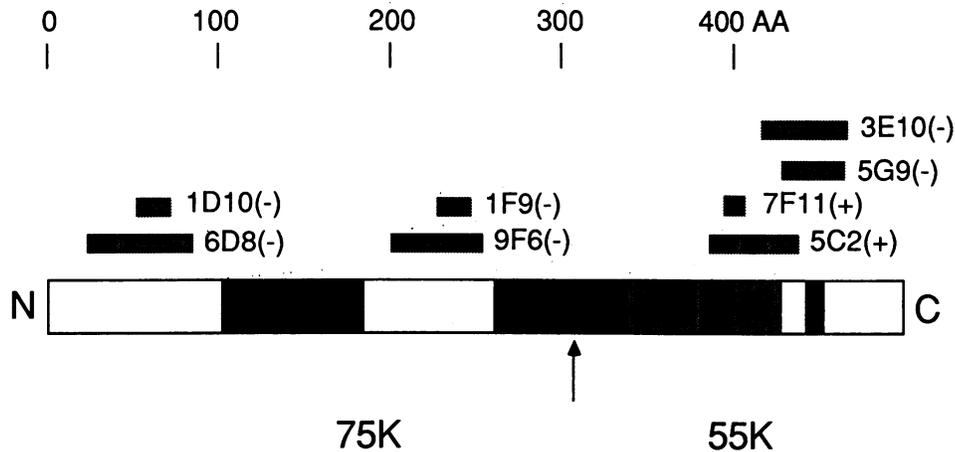
FIG. 3. Alignment of gp120 monoclonal antibody-binding sites. Antibody-binding sites were determined by Western blot mapping, immunoaffinity purification of gp120 fragments, lambda gt11 mapping, or a combination of these techniques. Although several inserts were sequenced for each positively reacting bacteriophage, only the shortest reactive fragments are shown. The bottom of the figure illustrates the nonconserved (dark), highly conserved (light), and moderately conserved (shaded) regions of gp120 (1, 7, 25, 28). The pluses in parentheses show monoclonal antibodies which block the gp120-CD4 interaction at modest antibody-to-antigen ratios, while the minuses show antibodies which do not block the interaction. The filled area within the lambda gt11-delineated site for antibody 5C2 shows a tryptic fragment which was previously found to bind to this monoclonal antibody (16), while the filled area within the 6D8 region shows the acid-cleaved peptide that bound to the 6D8 immunoaffinity column. Also shown is the proteolytic cleavage site that gives rise to the 75,000- and 55,000-dalton fragments of gp120 (75K and 55K, respectively). AA, Amino acids.

Three of the four nonblocking monoclonal antibodies were found to map to the N-terminal 75,000-dalton fragment corresponding to amino acids 1 to 295. These included the 1F9-9F6, 6D8, and 1D10-8G4-8D8 groups. These results suggested that epitopes critical for receptor binding do not reside in the N terminus of gp120. In addition, one epitope, corresponding to the non-CD4-blocking 3E10-5G9 epitope group, was found to map to the C-terminal 55,000-dalton fragment corresponding to amino acids 296 to 471. This result suggested that there was also a region in the C-terminal domain of gp120 which is dispensable for the CD4 interaction. These results are summarized in Table 3.

In addition to the overlapping tryptic and acetic acid cleavage fragments of rgp120 that purify on immobilized 5C2 and have been previously described (15), two acetic acid cleavage peptides that were linked by a disulfide bond were recovered from immobilized 6D8 (Table 3). No tryptic fragments were purified on immobilized 6D8. As described below, the lambda gt11 mapping studies with this monoclonal antibody suggest that the fragment from amino acids 18 to 39 contains the 6D8 antibody-binding site, whereas the peptide corresponding to amino acids 99 to 141 was copurified on the basis of its disulfide linkage with the peptide from amino acids 18 to 39.

Localization of gp120 monoclonal antibody-binding sites by lambda gt11 expression mapping. While the Western blot mapping data localized the binding sites for the various gp120 monoclonal antibodies to large proteolytic fragments, a second mapping technique was used to map the epitopes recognized by these antibodies to a finer degree. The lambda gt11 mapping system allows for production of a large number of random fragments which correspond to small regions throughout the gp120 coding sequence (20, 30). By screening a library of such fragments, the binding site for a given monoclonal antibody could be mapped to a relatively fine level by sequencing DNA fragments encoding fusion proteins which are recognized by the antibodies. A random library of 1.7 × 10^{7} phage was created, one-sixth of which should have the correct reading frame and orientation with

the carrier protein, β-galactosidase, and the inserted gp120 gene fragments.

All of the monoclonal antibodies tested in the lambda gt11 screening system resulted in detection of positively reacting bacteriophage. The fact that all of the anti-gp120 monoclonal antibodies were able to recognize bacterially produced, presumably denatured proteins suggests that there was an initial bias in monoclonal antibody production or screening, perhaps because of the use of recombinant, yet presumably native, gp120 for the inoculations. However, it is not clear that any conformational epitopes were demonstrated on the gp120 molecule. Most of the monoclonal antibodies recognized approximately 0.5% of the phage on a plate, while a rabbit polyclonal antibody to a recombinant gp120 antigen (15) recognized approximately 9% of the bacteriophage (data not shown). This result suggested that a relatively high percentage of bacteriophage correctly expressed gp120 epitopes which could be recognized by either monoclonal or polyclonal antibodies. While a large number of bacteriophage that reacted with each monoclonal antibody was analyzed, only the shortest reactive insert for each positively reacting virus is discussed.

A summary of the lambda gt11 mapping experiments is shown in Table 3 and Fig. 3. The lambda gt11 mapping agreed completely with the data obtained by using the gp120 proteolytic fragments on Western blots and immunoaffinity chromatography. For example, the monoclonal antibodies which resulted in blocking of the gp120-CD4 interaction were both found to map to a relatively conserved region near the C terminus of the glycoprotein. As expected, monoclonal antibodies 5C2 and 7F11 appeared to map to tightly linked regions of the molecule, in agreement with the competition analysis shown in Table 1. As described above and previously (15), the 5C2 epitope is contained in the tryptic peptide from residues 406 to 415 that is encoded within the gp120 fragment found in the phage selected by this monoclonal antibody. The 7F11 epitope was contained within a fragment spanning amino acids 393 to 408. Since the 7F11 monoclonal antibody column was unable to bind a tryptic fragment of
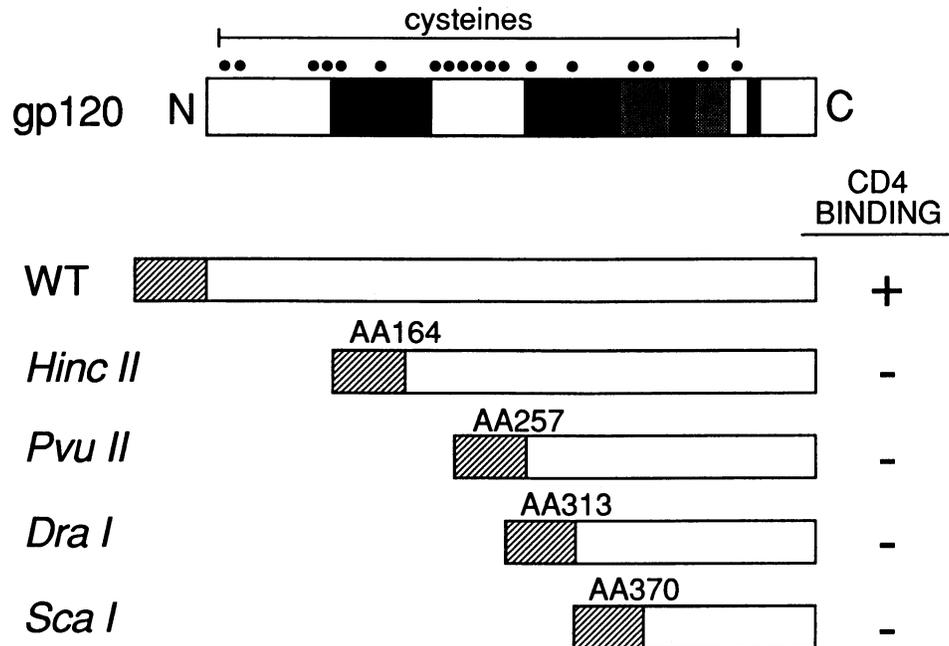
FIG. 4. N-terminal deletion mutants of gp120. The top of the figure shows the variable and conserved regions of gp120 as described in the legend to Fig. 3. The 18 conserved cysteine sites located in the glycoprotein are shown at the top. Beneath is illustrated the structure of the *Hinc*II (amino acid [AA] 164), *Pvu*II (amino acid 257), *Dra*I (amino acid 313), and *Sca*I (amino acid 370) deletion mutants. Also illustrated is the herpes simplex virus signal sequence cassette that was used to express these mutants (shaded). The processed (signal sequence-cleaved) wild-type (WT) and deletion mutant envelope glycoproteins contain nine amino acids from the N terminus of herpes simplex virus glycoprotein D (15; Dowbenko, unpublished data).

rgp120, these results suggest that the 7F11 epitope was N terminal to the 5C2 epitope. Thus, these two monoclonal antibodies defined two adjacent epitopes in the C terminus of gp120 that appeared to be contained within a region that was critical for CD4 binding.

While the exact delineation of gp120 regions which appeared to be critical for CD4 binding was important, the high-resolution localization of epitopes which could bind monoclonal antibodies without affecting gp120-CD4 interaction was also of interest. In this respect, the lambda gt11 mapping system provided some important results. For example, the two epitopes recognized by the nonblocking monoclonal antibodies 6D8 and 1D10 were found to co-map near the N terminus of gp120. This region of the glycoprotein is conserved between virus isolates, yet the lack of blocking of the gp120-CD4 interaction by monoclonal antibodies binding to this region suggests that this conserved region does not directly contribute to this interaction (Fig. 3). In addition, a second highly conserved domain contained within amino acids 180 to 250 of the glycoprotein was found to bind to two other nonblocking monoclonal antibodies corresponding to epitope group a, 9F6 and 1F9. These antibodies appeared to bind to the same highly conserved region in the central part of gp120, and their lack of blocking suggests that this region is involved with functions other than CD4 binding. Thus, these results show that two highly conserved domains of HIV gp120 appear to bind monoclonal antibodies that do not inhibit the interaction between the glycoprotein and its receptor, CD4. The highly conserved nature of these regions suggests that they are involved in other functions that are necessary for the infectivity of the virus.

The final epitope group mapped in this manner was epitope group c, which contained monoclonal antibodies

3E10 and 5G9. These antibodies were found to map to positions in the C terminus of gp120, in agreement with the proteolytic mapping data described above (Table 3). Since these antibodies neither competed for binding with epitope group b nor blocked gp120-CD4 interaction, the mapping results suggest that this epitope is C terminal to the epitope group delineated by antibodies 5C2 and 7F11-7G11. The lack of blocking of the glycoprotein-receptor interaction by these antibodies suggests that epitopes C terminal to the site delineated by epitope group b may not be critical for this binding interaction.

N-terminal deletion mutants of the envelope glycoprotein. The monoclonal antibody studies shown above, in conjunction with mutagenesis studies by Kowalski et al. (13), suggested that the N-terminal and central conserved domains of gp120 are dispensable for CD4 binding. In addition, mutagenesis studies by Willey et al. (29) revealed that mutations in the central conserved domain of gp120, while resulting in noninfectious virus, had no effect on CD4 binding, also suggesting that this region is dispensable for CD4 interaction. Finally, recent work by Ho et al. (10) has shown that antibodies directed to peptides within the central conserved domain neutralize HIV infectivity but do not block virus-receptor binding. To determine whether N-terminal regions of gp120 are dispensable for binding, N-terminal deletion mutants of gp120 were constructed and expressed as secreted proteins in transfected mammalian cells (Fig. 4). The secreted glycoproteins were immunoprecipitated with a high-titer rabbit anti-gp120 serum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All of the deletion mutants were secreted and appeared to react with the antiserum (Fig. 5). The decreased immunoreactivity of the smallest deletion mutant may have been due to a combination of poor expression levels and the
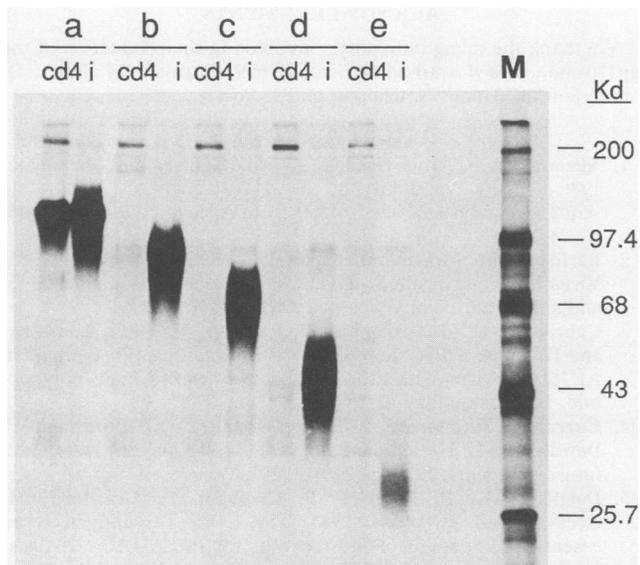
FIG. 5. Lack of CD4 binding of N-terminal deletion mutants. Mammalian cells were transfected with each mutant as well as with wild-type (Fig. 4, legend; 16) gp120 and labeled with [^{35}S]methionine, and the secreted material was used in the CD4-binding assay as previously described (16). One-tenth of the secreted material was immunoprecipitated with rabbit polyclonal serum directed to gp120 (15). The rest of the material was reacted with a soluble form of CD4 (24) and immunoprecipitated with monoclonal antibody OKT4. Each pair of lanes shows total immunoprecipitable (i) and CD4-bound (cd4) material. Lanes: a, wild-type gp120; b, HincII deletion; c, PvuII deletion; d, DraI deletion; e, ScaI deletion; M, molecular weight markers. Kd, Kilodaltons.

maintenance of only a few of the original native gp120 epitopes.

Interestingly, the deletion mutants were unable to bind to CD4 with high affinity. Approximately 5 to 10% of the immunoprecipitable wild-type gp120 was able to coprecipitate with CD4 (Fig. 5), in agreement with previous studies (16). However, none of the N-terminal deletion mutants were able to bind significantly to a soluble form of the CD4 receptor molecule. Lack of glycosylation of the secreted deletion mutants could not explain this lack of receptor-binding activity, since all of these proteins were of higher-than-expected molecular weight, appeared to be heterogeneous in size, and could be glycosylated in an in vitro system (data not shown). These results suggest that sequence information contained within the N-terminal, central, or both domains of gp120 is necessary to confer a high-affinity CD4-binding phenotype on this glycoprotein.

DISCUSSION

The external envelope glycoprotein, gp120, appears to bind HIV to its cellular receptor, CD4. The delineation of a region of gp120 which appears to be critical for binding in this report, as well as in previous reports (13, 16), suggests that a defined region of the glycoprotein serves a critical function in binding the virus to its receptor. This region shows a relatively high degree of conservation between diverse HIV isolates, as well as two other CD4-trophic retroviruses, HIV-2 and simian T-lymphotropic virus type 3 (16). However, although the number of domains necessary to accomplish high-affinity binding of gp120 to CD4 remains unknown, sequence comparisons of the different HIV iso-

lates analyzed to date suggest that there are other regions of the glycoprotein which show a high degree of homology and are, therefore, presumably of functional significance. The data reported here suggest that at least two of these conserved regions have functions other than direct participation in CD4 binding.

The mapping of the two gp120-CD4-blocking monoclonal antibodies to adjacent epitopes near the C terminus of the glycoprotein agrees with previously reported results (13, 16). Thus, the localization of the 5C2 epitope by immunoaffinity chromatography of gp120 peptides and subsequent amino acid sequencing demonstrated that the epitope recognized by this blocking monoclonal antibody was localized to a region within a relatively conserved domain of the envelope from residues 406 to 417, in agreement with the localization of this epitope by lambda gt11 mapping reported here (residues 403 to 457). The finding that a second blocking monoclonal antibody, 7F11, also binds near this site is further confirmation that this region is critical for binding of the virus to its receptor. The mapping of a nonblocking epitope, corresponding to epitope group c, C terminal to the blocking domain suggests that this critical region does not require C-terminally located sequences for high-affinity binding. C-terminal deletion and random mutagenesis of the gp120 gene have demonstrated that the CD4 interaction region of this glycoprotein may be conformationally complex, with critical regions from three different areas in the C-terminal region of the protein being important (13). One of the critical mutations that these investigators found was located in a region that mapped near the epitope which bound the nonblocking monoclonal antibodies 3E10-5G9. The nature of this discrepancy is not clear, but it may result from a combination of several variables, including, for example, a conformational change induced by the blocking mutation of Kowalski et al. (13) or only marginally effective blocking by the monoclonal antibodies used by us. In addition, it appears clear from our N-terminal deletion mapping results that deletions (13) may result in the absence of CD4 binding, even in cases in which monoclonal antibodies specific for the deleted regions do not block the interaction.

The demonstration of highly conserved domains of gp120 that do not appear to contribute directly to CD4 binding suggests that these regions have other functions important to the virus. In agreement with the results reported here, previous work (29) has demonstrated that mutations made in the centrally located, highly conserved domain of gp120 (amino acids 180 to 250) result in mutant viruses that are completely noninfectious but whose envelope glycoproteins appear to bind to the CD4 receptor in both the binding assay described here and a syncitium-forming assay. This result supports the finding shown here and suggests that an important functional domain exists in this central, highly conserved region. Recent work reported by Ho et al. (10) has demonstrated that a peptide from this region was able to induce low levels of neutralizing antibodies that were unable to block virus-receptor interaction. The potential roles for this central conserved region are many and include interaction with the transmembrane protein, gp41, uncoating of the virus, maintenance of an appropriate overall gp120 conformation, etc. (13, 16). A similar argument could be applied to the N-terminal conserved domain, which contained epitopes for two nonblocking monoclonal antibodies. Interestingly, previous work demonstrated that a mutant gp120 that lacked the N-terminal 30 amino acids bound with high affinity to the CD4 receptor (15, 16). This suggests that a minor deletion at

the very N terminus of the protein, in addition to steric inhibition brought on by the binding of monoclonal antibodies to this region, does not seem to grossly affect the ability of gp120 to bind to CD4. The potential of this N-terminal region to act as a structural component of the envelope glycoprotein is suggested by previous work which demonstrated that the noninfectious glycosylation mutant produced in the central highly conserved domain (amino acids 180 to 250) could revert to an infectious virus by a serine-to-asparagine change near the C-terminal end of the N-terminal highly conserved domain (amino acids 30 to 130) (29). Thus, it is possible that the N-terminal conserved domain interacts structurally with the central domain, with the result that both of these regions are indispensable for appropriate gp120 structure and function. In support of this possibility, preliminary mapping of disulfide linkages within rgp120 suggests that there are disulfide interactions between cysteines in the N-terminal and central conserved regions (T. Gregory and M. Spellman, manuscript in preparation). The preliminary data suggest that a complex structural interaction exists between the N-terminal conserved domain and the centrally located conserved domain of gp120.

The finding that deletion of the first 164 amino acids of gp120, including the first highly conserved domain as well as the first 6 cysteine residues of the glycoprotein, results in an apparent lack of CD4 binding suggests that there is information, either in the form of direct CD4-binding sites or structure-inducing determinants, that is contained within this region. Previous work using in vitro mutagenesis (13), in addition to work reported here, suggests that direct CD4 interaction sites do not occur in this part of the glycoprotein but are found in the C-terminal region of the molecule. Thus, it appears that the structural aspects of the C-terminally located CD4-binding regions of gp120 may in part be dependent upon sequences from the N terminus of the protein. Alternatively, removal of several cysteines in the N-terminal deletion mutants may result in aggregated proteins that are unable to bind to CD4, since their removal might result in a number of free cysteine residues remaining in the deletion mutants. In addition, the binding assay that was used here may have been too insensitive to detect deletion mutants with greatly (i.e., ~1,000 fold) decreased yet significant binding affinities for CD4, since it is likely that the levels of secreted gp120 expressed in these transient transfections were quite low (16). In conclusion, the large number of disulfide bonds in gp120, in addition to the apparent complexity of the disulfide-induced structure (19; T. Gregory and M. Spellman, unpublished data), suggests that appropriate folding of the C-terminal CD4 interaction domain is dependent upon other sequences in the molecule.

In summary, the results presented here suggest that although the highly conserved domains in the central and N-terminal regions of gp120 do not appear to be directly involved in CD4 binding, they may be required for induction of an appropriately folded envelope glycoprotein. Unfortunately, these results also suggest that attempting to produce high-titer virus-blocking antibodies to CD4-binding domains by deletion of other, nonessential regions of the HIV glycoprotein will be difficult, since the shorter gp120 molecules do not appear to retain the correct configuration that might be expected to be necessary for induction of an appropriate antiviral immune response. Finally, these results suggest that development of small-molecule therapeutics based on gp120 for the prevention of HIV binding and infection would be a difficult enterprise (22).

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