Epitope Map of Human Immunodeficiency Virus Type 1 gp41 Derived from 47 Monoclonal Antibodies Produced by Immunization with Oligomeric Envelope Protein

PATRICIA L. EARL,^{1*} CHRISTOPHER C. BRODER,^{1†} ROBERT W. DOMS,² AND BERNARD MOSS¹

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892-0445,¹
and Department of Pathology and Laboratory Medicine, University of Pennsylvania,
Philadelphia, Pennsylvania 19104²*

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The biologically relevant form of the human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein is oligomeric, with the major points of contact between oligomeric partners located in the ectodomain of gp41. To identify and map conserved epitopes and regions in gp41 where structure is influenced by quaternary interactions, we used a panel of 38 conformation-dependent and 9 conformation-independent anti-gp41 monoclonal antibodies (MAbs) produced by immunization of mice with oligomeric Env protein. By cross-competition experiments using these MAbs and several others previously described, six distinct antigenic determinants were identified and mapped. Three of these determinants are conformational in nature and dependent in part on Env oligomeric structure. MAbs to two of these determinants were broadly cross-reactive with Env proteins derived from primary virus strains. The prevalence of antibodies in HIV-1-positive human sera to the antigenic determinants was determined by the ability of such sera to block binding of MAbs to Env protein. Strong blocking activity that correlated with cross-reactivity was found.

The mature envelope (Env) glycoprotein of human immunodeficiency type 1 (HIV-1) is a complex oligomer consisting of several molecules of both gp120 and gp41 subunits (13, 37, 43, 47). During transport through the endoplasmic reticulum (ER)-Golgi network, disulfide bonds are formed and extensive glycosylation occurs, both of which contribute to the structural complexity of the protein (18, 19). Proper folding in the ER is required for efficient transport of Env to the cell surface (52), and at least three different molecular chaperones have been implicated in this process: BiP, calnexin, and calreticulin (18, 36). Oligomerization probably occurs in the ER (18, 35) and is presumably critical for intracellular transport (11). As a consequence, only oligomeric Env is expressed on the cell surface.

The oligomeric nature of Env has important functional and structural implications. Oligomeric Env can, for example, bind at least two CD4 molecules simultaneously, which may facilitate viral entry (14, 27, 30, 31). There are also significant antigenic differences between oligomeric and monomeric Env, in both the gp120 and gp41 subunits. Antibody binding to monomeric gp120 does not necessarily predict good binding to multimeric Env expressed on the viral or cell surface (28). Consequently, the ability of antibodies to neutralize HIV is determined by epitope exposure on oligomeric, rather than monomeric, Env (40, 41). Indeed, a number of epitopes present on recombinant, soluble gp120 have been shown to be completely or partially obscured on oligomeric gp120 (32).

The structure of gp41 may be even more strongly influenced by quaternary interactions, since the gp41 ectodomain is largely responsible for Env oligomerization (13, 37). Consis-

tent with this observation is the finding that a large number of monoclonal antibodies (MAbs) to gp41 bind preferentially or exclusively to oligomeric Env (4). The latter antibodies were generated by immunization with oligomeric and monomeric forms of soluble gp140, a recombinant Env protein that is truncated just prior to the transmembrane domain and from which the proteolytic cleavage site has been removed (12). Most of the MAbs produced by immunization with gp140 react with Env expressed on the surface of HIV-infected cells, indicating that the structure of gp140 mimics that of gp160. Thus, these antibodies provide an opportunity to characterize the antigenic structure of gp41 in the context of native, oligomeric protein.

In this study, we used 47 anti-gp41 MAbs and a variety of HIV-1 Env proteins to derive an antigenic map of the gp41 subunit. Nine of these MAbs bound to linear determinants and were mapped to specific peptide regions of gp41. These MAbs were then used as landmarks in a blocking assay to map the regions of gp41 that were essential for binding 38 conformation-dependent MAbs. In this manner, we defined at least three distinct conformational antigenic determinants in gp41. We found that these were (i) highly conserved in Env proteins from many clade B isolates, (ii) distinct with respect to reactivity with Env proteins from divergent clades, and (iii) immunogenic in natural HIV-1 infection.

MATERIALS AND METHODS

Cells and viruses. HIV-1 Env proteins were produced by infection of BS-C-1 cells (ATCC CCL26) with recombinant vaccinia viruses at a multiplicity of infection (MOI) of 10 to 20 PFU/cell. The following recombinant vaccinia viruses expressing gp160 from different HIV-1 isolates (names of isolate are given in parentheses) were used: vSC60 (BH8) (7), vCB28 (JRFL), vCB29 (JRCSF), vCB32 (SF162), vCB34 (SF2), vCB36 (RF), vCB39 (ADA), vCB40 (BH10), vCB41 (LAV), vCB43 (Ba-L), (2), vCB51 and vT29 (BK132), vCB52 (CM235), and vCB53 and vT27 (CM243). (vT27 and vT29 were a gift from Gail Mazzara, Therion Biologics.) Recombinant viruses vPE12B (12) and vPE18 (15) express soluble secreted forms of Env protein (IIIB) truncated after amino acids 683 (gp140) and 640 (Env_{sec}), respectively. In addition, the gp140 produced by vPE12B contains a deletion of the proteolytic cleavage site between gp120 and

* Corresponding author. Mailing address: Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Room 237, Building 4, 9000 Rockville Pike, Bethesda, MD 20892-0445. Phone: (301) 496-3654. Fax: (301) 480-1147. E-mail: pearl@atlas.niaid.nih.gov.

† Present address: Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799.

gp41. Amino acid numbering is according to the LAI sequence. vTF7-3 expresses the bacteriophage T7 RNA polymerase (20). Plasmids containing Env genes under control of the T7 promoter were used in conjunction with virus vTF7-3. Plasmids pPE32 and pPE63 contain the HIV-1 IIIIB *env* gene truncated after amino acids 640 and 579, respectively (17). Plasmids similarly truncated but containing individual mutations of Asn to Gln in each of the three potential glycosylation sites in gp41 (at amino acids 616, 624, and 637) were also used (17).

Expression of the Env proteins from 11 primary isolates of HIV-1 was obtained either by the hybrid vaccinia virus/T7 system or by expression of the *env* gene in a recombinant vaccinia virus. The following plasmids containing the *env* genes from primary HIV-1 isolates under control of the T7 promoter were obtained from B. Hahn (University of Alabama, Birmingham) (22) and the AIDS Research and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (HIV-1 *env* genetic subtype and geographical location are given in parentheses): BA301712-4 (B, Thailand), BA301715-6 (B, Thailand), MA301960-3 (C, Malawi), MA959-18 (C, Malawi), BR 25-9A (C, Brazil), Th301975-15 (E, Thailand), RW20-5 (A, Rwanda), and UG37-8 (A, Uganda). Additional *env* genes from three primary isolates of HIV-1 were the gift of F. McCutcheon (Henry M. Jackson Foundation): BK132 (B, Thailand), CM235 (E, Thailand), CM243 (E, Thailand). These three genes were cloned into the vector pSC59 under control of the vaccinia virus synthetic early/late promoter and recombinant viruses were made by standard methods (3, 16).

Antibodies. Members of a panel of 138 murine MAbs raised against soluble monomeric or oligomeric gp140 Env were used in this study. MAbs were named M (monomer), D (dimer), or T (tetramer), according to the immunogen used, and numbered sequentially (12). This paper focuses on 47 MAbs with specificity to the gp41 subunit of Env. MAbs were classified as linear or conformation-dependent on the basis of reactivity or lack of reactivity, respectively, with reduced, denatured Env protein by Western blot analysis. Rabbit polyclonal sera 2144 and R160 were raised against reduced, denatured Env protein (IIIB) produced by recombinant vaccinia viruses and baculoviruses, respectively. R160 (52) was the gift of R. Willey, NIAID. MAbs 2F5 and 25C2 (6) were from Viral Testing System Corp. MAbs 246-D and 126-6 (53) were the gift of S. Zolla-Pazner (V. A. Medical Center, New York, N.Y.). Fab L2 (1) was provided by P. Perrin and D. Burton (Scripps Research Institute, La Jolla, Calif.).

Flow cytometry. BS-C-1 cells were infected with either WR or vSC60 at an MOI of 10 for 2 h. The monolayers were trypsinized, washed, resuspended at 0.5×10^6 cells/ml in minimal essential medium containing 10% fetal calf serum (FCS), and incubated at 31°C overnight. Cells were then washed, resuspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum, and incubated with MAbs (50 µg of purified MAb/ml or unconcentrated hybridoma supernatant) for 1 h at 4°C. Cells were washed twice, incubated with goat anti-mouse-fluorescein isothiocyanate for 30 min at 4°C, and then washed two times. After fixation in 0.1 ml of PBS containing 2% paraformaldehyde, the cells were analyzed with a fluorescence-activated cell sorting (FACS) apparatus (FACScan; Becton Dickinson).

Peptide enzyme-linked immunosorbent assay (ELISA). The following peptides were used to map anti-gp41 linear MAbs. Peptides 25 (PTKAKRRVV QREKRAVGIGALFLGF), 26 (LLSDIVQOQNLLRAIEAQH), 27 (LAVE RYLKDDQQLGIWGCSCGK), 28 (GCSGKLICTAVPWNAS), 29 (DREINN YETLIHSLIEESQONQOE), 30 (IHSLLIEESQONQOEKNEOELLELDK), 31 (EK NEQELLELDKWASLWNFNITNW), and 32 (WASLWNFNITNWLWY IKLFIMIVG) were the gift of S. Koenig (MedImmune). Peptides 3800 (STMG AASMTLTVOARQLLSGI), 3801 (EGQGHLLQLTVWGKIQLOARILAVR), 3802 (PWNASWSNKSLENIWNN), and 3803 (EQIWNMTWMEWDREIN) were provided by J. Coligan (NIAID). The wells of Immulon II 96-well assay plates were coated with 50 µl of 0.05 M sodium carbonate (pH 9.5) containing 2.5 µg of peptide per ml overnight at 4°C. Plates were washed with PBS containing 0.1% Tween 20 and blocked with the same buffer plus 0.5% proteolyzed gelatin (Boehringer Mannheim Biochemicals) for 1 h at room temperature. Hybridoma supernatants were added to wells for 1 h, and bound antibody was detected with peroxidase-conjugated anti-mouse immunoglobulin G (IgG) and 2,2'-amino-di-[3-ethylbenzthiazoline sulfonate(6)] substrate (Boehringer Mannheim Biochemicals).

Metabolic labeling and immunoprecipitation. For labeling of Env proteins expressed by recombinant vaccinia viruses, BS-C-1 cells were infected at an MOI of 10 to 20 PFU per cell. For labeling of Env proteins with the hybrid vaccinia virus T7 system, BS-C-1 cells were infected with vTF7-3 at an MOI of 10. After 1 to 2 h, the virus inoculum was removed and cells were transfected with plasmid by using DOTAP (Boehringer Mannheim) according to the manufacturer's specifications. Typically, 10^6 cells were transfected with 5 µg of DNA. At 4 h post-transfection, monolayers were washed, overlaid with methionine-free minimal essential medium containing 5% dialyzed FCS and 200 µCi of [35 S]methionine (Amersham)/ml, and incubated overnight. Full-length gp160 was obtained by lysis of cells in 100 mM Tris-HCl (pH 8.0)–100 mM NaCl–0.5% Triton X-100. Soluble, secreted forms of Env protein were obtained by harvesting the medium of infected cells. Typically, 4 µg of MAb, 100 µl of hybridoma supernatant, or 1 µl of polyclonal antiserum was used per immunoprecipitation. Eight micrograms of rabbit anti-mouse IgG was included with all murine MAbs. Incubations for at least 1 h at room temperature were followed by addition of protein A-Sepharose for at least 30 min. Complexes were washed twice with Triton buffer (100 mM

Tris-HCl [pH 8.0], 100 mM NaCl, 0.5% Triton X-100) or once with Triton buffer and once with DOC buffer (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% gel) and visualized by autoradiography. Bands were quantitated with a Molecular Dynamics PhosphorImager.

IgG purification and biotinylation. Hybridomas were grown to high density in Iscove's minimal essential medium containing 10% FCS. Cells were washed, transferred to serum-free HyQ medium (HyClone) containing 50 U of interleukin-6/ml (Boehringer Mannheim Biochemicals), and maintained for several days with daily additions of medium. IgG was purified by protein G affinity chromatography (Pharmacia) and concentrated in Centricon microconcentrators. The protein concentration was determined from the A_{280} .

IgG preparations were biotinylated with NHS-SS Biotin (Pierce) according to the manufacturer's specifications. Briefly, antibody (1 to 2 mg/ml) was equilibrated in 50 mM sodium bicarbonate (pH 8.5). Freshly prepared biotin was added at 20-fold molar excess. After 45 min at room temperature, the reaction was quenched by addition of 4 to 10 volumes of 10 mM sodium phosphate–150 mM NaCl (pH 7.2). IgG was then concentrated in Centricon microconcentrators, and protein concentration was determined from the A_{280} .

MAb blocking. Aliquots of [35 S]methionine-labeled noncleaved gp140 (produced by vPE12B) were incubated with purified MAb or hybridoma supernatant at 4°C overnight. Typically, 2.0 and 0.2 µg of antibody were used per reaction, although for some experiments 10.0 and 2.0 µg were used. A second, biotinylated MAb (0.1 to 0.4 µg) was added for 1 h. Avidin agarose (Pierce) was then added, and tubes were rocked for 30 min, after which the complexes were washed twice with Triton buffer (see above). Proteins were separated by SDS-PAGE (10% gel) and visualized by autoradiography. Bands were quantitated with a Molecular Dynamics PhosphorImager.

HIV-1-positive human serum blocking. The ability of HIV-1-positive human sera to block binding of MAbs to Env protein was determined by a modification of the procedure described by Richardson et al. (39). gp140, purified by lentil lectin chromatography, was captured to Immulon II 96-well plates via interaction with concanavalin A (ConA) as follows. Wells of 96-well plates were coated with 100 µl of ConA (100 µg/ml in PBS containing 10 mM HEPES [pH 8.5] and 0.1 mM CaCl_2) for at least 2 h at room temperature. Plates were washed three times with wash buffer (9 g of NaCl and 1 ml of Tween 20/l), and gp140 was added at 40 or 80 ng/well in PBS. The plates were incubated at 4°C overnight, washed, and blocked at room temperature with block buffer (PBS containing 10% FCS) for 1 h. All subsequent steps were performed at room temperature, using block buffer as the diluent. The plates were washed three times between each step. After blocking, threefold serial dilutions of pooled HIV-positive or HIV-negative human sera were added in triplicate and incubated for 2 h. The pool of HIV-positive sera consisted of equal volumes of sera from 10 individuals with high anti-Env antibody titers (a gift from T. VanCott, Henry M. Jackson Foundation). These sera were heat inactivated at 56°C for 30 min and brought to 0.5% Triton X-100. The HIV-negative serum was from Sigma. After incubation with human sera, MAb (0.1 to 0.7 µg/well) was added and incubated for 1 h. The amount of bound MAb was detected with peroxidase-conjugated anti-mouse IgG and 2,2'-amino-di-[3-ethylbenzthiazoline sulfonate(6)]. Control wells containing no human sera were included for determination of total binding activity.

RESULTS

Reactivities of MAbs with HIV-1 Env protein on the surface of cells. Previously, we described a large panel of MAbs that were obtained by immunizing mice with monomeric or oligomeric gp140, a soluble form of the Env molecule that contains all of gp120 and the entire gp41 ectodomain (12). In addition, the gp140 used in this study contained a small deletion at the gp120/gp41 cleavage site, resulting in a protein that was not cleaved into gp120 and gp41 subunits. Therefore, it was important to determine whether the MAbs reacted with Env protein expressed on the surface of cells. Thirty-three of the 38 conformation-dependent MAbs were analyzed by FACS analysis using cells infected with a recombinant vaccinia virus (vSC60) that expresses full-length, cleaved gp160. Representative data are shown in Fig. 1. All of the anti-gp41 MAbs reacted with Env on the surface of cells, albeit to a lesser extent than observed for the CD4 binding site MAb, D20, and the V3 loop MAb, D47. In addition, incubation of the cells with soluble CD4 to induce shedding of gp120 prior to addition of MAbs increased the binding of the anti-gp41 MAbs (1a). Using HIV-1-infected cells, Pincus found a two- to sixfold increase in binding of some of the anti-gp41 MAbs following soluble CD4-induced shedding of gp120 (36a). Sattentau et al. have also

FIG. 1. Binding of MABs to Env expressed on the surface of cells. Env protein (cleaved gp160) was expressed on the surface of cells via recombinant vaccinia virus vSC60. Cells were incubated with MAB followed by anti-mouse-fluorescein isothiocyanate and subjected to FACS analysis. The no-Env control was performed with cells infected with vaccinia virus WR. The antigenic determinants of the MABs are indicated by roman numerals and are defined in Fig. 2. FACS analyses of D20, a CD4 binding site (CD4bs) MAB, and D47, a V3 loop MAB, are shown for comparison.

demonstrated increased accessibility of gp41 epitopes following gp120 shedding (42). Thus, the anti-gp41 MABs used in this study are directed to epitopes that are present on Env protein expressed on the surface cells but are blocked to various extents by the presence of gp120.

Mapping of linear MABs in the ectodomain of gp41. While most (38 of 47) of the gp41-specific MABs bound to conformational determinants, nine antibodies showed strong reactivity with reduced, denatured gp160 and gp41 by Western blot analysis, indicating that they bind to linear determinants. Five of these MABs were derived from mice immunized with dimeric Env, and four were derived from mice immunized with tetrameric Env. Two methods were used to map the regions in the ectodomain of gp41 to which these MABs bound. First, we used a set of Env proteins containing C-terminal truncations at amino acids 579, 640, and 683 (according to the LAI sequence) (15). These proteins, expressed by using a set of recombinant vaccinia viruses, have gp41 ectodomains containing 68, 129, and 172 residues. Infected cell lysates were subjected to SDS-PAGE and Western blot analyses with the nine MABs. Each

MAB bound to one or more of the truncated proteins, making it possible to localize their determinants to a stretch of amino acids as follows: D3 to amino acids 512 to 579; D49, D61, T30, T32, and T34 to amino acids 580 to 640; and D43, D50, and T3 to amino acids 641 to 683 (data not shown).

The MABs were then mapped within each region by peptide ELISA using a set of overlapping peptides spanning most of the ectodomain of gp41. The results are summarized in Fig. 2. D49, D61, T32, and T34 mapped to amino acids 597 to 613, a region that contains the two Cys residues present in the gp41 ectodomain and which has been defined as immunodominant by analysis of human sera (23, 24, 44, 53). MAB D50 mapped to amino acids 642 to 665, a region in gp41 that contains the previously defined cluster II determinant (53). Two MABs, D43 and T3, did not react with any peptide although overlapping peptides spanning amino acids 641 to 683 were used. Most likely, these MABs are somewhat conformation dependent and react in Western blots by virtue of protein refolding during binding. A human MAB to gp41 that also exhibits this property has been described (8). In addition, MAB T30 did not react

FIG. 2. Ectodomain of gp41. Positions of amino acid residues (LAI sequence) are shown above the drawing. Map positions of linear MABs defined in this paper, human MAB 2F5, clusters I and II, and the two helical regions (DP107 and DP178) are given. The disulfide loop is indicated by C-C, and potential glycosylation sites are depicted as black balloons. The locations of the six antigenic determinants (I to VI) are shown at the bottom.

FIG. 3. Mapping of MAb T30 by using glycosylation site mutants. The map position of MAb T30 was determined by using secreted Env proteins in which the Asn in each of three N-linked glycosylation sites (amino acids 616, 624, and 637) was mutated to Gln. Proteins were metabolically labeled and subjected to immunoprecipitation by rabbit polyclonal serum (R160), T30, and D61.

with any peptide, though the reactivity of T30 was found to be critically dependent on an N-linked glycosylation site in gp41 (see below).

Mapping of MAb T30 by using glycosylation site mutants.

The epitope recognized by MAb T30 which localized to amino acids 580 to 640 was further mapped by immunoprecipitation of a set of Env molecules from which each of three potential N-linked glycosylation sites in the ectodomain of gp41 were removed. In these Env proteins, the Asn residues at positions 616, 624, and 637 were individually mutated to Gln. We have previously shown that none of these mutations affect Env protein assembly (17). Proteins were metabolically labeled and subjected to immunoprecipitation with R160 (polyclonal rabbit anti-gp160 serum), T30, and D61. As shown in Fig. 3, R160 and D61 reacted equally well with all four proteins. In contrast, T30 bound to three of the proteins but did not recognize the Asn 616 mutant. Thus, the Asn at position 616, and perhaps the N-linked carbohydrate at this position as well, is critical for T30 binding.

Mapping of conformation-dependent MAbs by blocking.

The majority (83%) of the anti-gp41 MAbs obtained by immunization with gp140 bound to conformation-dependent epitopes, with nearly half reacting preferentially with oligomeric Env (4, 12). To map the regions of gp41 that are important for binding of these MAbs, five linear MAbs (D3, D61, T30, D50, and T3) mapping to distinct regions in the ectodomain of gp41 were used as landmarks (Fig. 2). A blocking assay was used as follows. Members of the panel of conformation-dependent MAbs were incubated with radiolabeled gp140 overnight. Subsequently, a biotinylated linear MAb was added for 1 h, the complexes were immunoprecipitated with avidin-agarose, and the amount of Env protein precipitated was determined by SDS-PAGE and PhosphorImager quantitation. Thus, we were able to determine if preincubation of Env with a given MAb was able to block subsequent binding of any one of five biotinylated MAbs to defined linear epitopes. Several examples of this assay are illustrated in Fig. 4, and the complete results are shown in Table 1. With this assay, 21 conformation-dependent MAbs could be classified into three major groups by their ability to block binding of three different linear MAbs. Group I contained eight MAbs (M10, D41, D54, T4, T6, T9, T10, and T35) that efficiently blocked binding of D61 but did not block binding of any other linear MAb. These antibodies also blocked binding of the human MAb 246-D, previously shown to map to this immunodominant determinant containing the disulfide loop (39, 53). Notably, all of the MAbs that exhibit complete oligomer dependence (i.e., do not react

with monomeric Env [T4, T6, T9, T10, and T35]) are in this group. In addition, M10 and D41 are oligomer dependent, although to a lesser degree. Thus, the antigenicity of the region containing the disulfide loop is dependent on tertiary and quaternary interactions. Group II contained 11 conformation-dependent MAbs that blocked the binding of linear MAb D50. The peptide region to which D50 maps has also been shown to be immunogenic and has been referred to as the cluster II determinant (53). Group III contains two conformation-dependent MAbs which blocked binding of linear MAbs T3 and D43. Although these two linear MAbs were mapped to amino acids 641 to 683 by analysis with C-terminally truncated Env proteins, neither MAb exhibited reactivity with the overlapping peptides spanning this sequence. Thus, all of the MAbs mapping to this region are conformationally dependent to some degree.

To further confirm this grouping of MAbs, at least one of the conformation-dependent MAbs from each group was biotinylated and assayed with members of the panel of conformation-dependent MAbs in the same manner. The results are given in Table 1. For the most part, each of the three epitope groups defined by blocking of binding of MAbs D61, D50, and T3 is distinct and nonoverlapping; i.e., only rarely did a MAb from one group block binding of a MAb in another group (e.g., D50 and T3 exhibit limited blocking of each other). In addition, none of the conformation-dependent MAbs blocked binding of D3 or T30 (data not shown), indicating either that the antigenicity of these two domains is not dependent on tertiary interactions or more simply that they are not very antigenic. Finally, 17 MAbs did not block binding of any of the linear MAbs in our panel. Thus, regions in the ectodomain of gp41 not defined by the five linear MAbs are important for binding of these MAbs.

At present, we are aware of only one antibody to the gp41 ectodomain that exhibits potent neutralizing activity. The anti-gp41 human MAb 2F5 has been mapped to amino acids 662 to 667 near the C terminus of the gp41 ectodomain (5, 34) (Fig. 2). This sequence overlaps the peptide to which D50 binds and is contained within the region necessary for T3 binding. However, no blocking of these MAbs was observed with 2F5. Thus, while 2F5 exhibits potent neutralizing activity, none of the 47 MAbs that we raised to gp41 bind to this region. That no other MAbs with properties similar to that of 2F5 have been described and that antibodies to the 2F5 epitope are poorly represented in human sera (11a) suggests that this epitope is either not well exposed or only weakly immunogenic.

Cross-reactivities of MAbs with divergent clade B Env proteins. The gp140 Env protein used for immunizations and

FIG. 4. Mapping of conformation-dependent MAbs by blocking. The anti-gp41 linear MAbs were used as landmarks to map the regions of gp41 necessary for binding by conformation-dependent MAbs. Each of the conformation-dependent MAbs was incubated with radiolabeled gp140 overnight. Subsequently, a biotinylated MAb was added, and complexes were immunoprecipitated with avidin agarose and analyzed by SDS-PAGE. Several examples are shown.

TABLE 1. Epitope mapping of conformation-dependent MAbs^a

Nonbio- tynilated MAb	% Blocking of indicated biotinylated MAb										
	D61	D41	T4	T6	T9	T10	T35	D50	D40	T3	D43
D61	98	97	99	98	99	97	94	0	0	0	0
M10	84	95	98		95	91		0	0	0	
D41	86	91	94		91	79		0	0	14	
D54	97	98	99	91	96	94		0	0	0	
T4	76	91	97	96	93	96	88	0	0	0	0
T6	66	89	98	92	93	93	85	0	25	0	0
T9	71	92	97	94	97	96		0	0	0	0
T10	72	83	99	98	93	96	85	0	0	0	0
T35	57	68	89	89	85	98	99	0	0	0	0
D50	0	0	0	13	0	0	0	90	96	54	63
D16	0		0	0	0	0	0	50	97	0	
D17	0	12	0					87	97		
D31	0		0					70	95	0	
D36	0		0					66	95	0	
D37	0		0					70	94	0	
D40	0	13	0	0	0	0	0	56	91	12	42
D44	0		0					62	98	16	
D55	0		0					68	97	0	
D59	0		0					57	93	0	
T37			0					61	96	0	
T45			0					64	96	16	
T3	19		0	0	0	0	0	43	53	96	90
D43	0		0	0	0	0	0	0	8	91	93
D38	0	29	0					0	11	86	67
D45	0	0	0					0	15	92	85
D3	0		0	0	0	0	0	15	0	0	0
T30	0		0	0	0	0	0	0	0	0	0
246-D	97		98	98	98	98	98				0
126-6								56	100	19	
2F5								0		0	17

^a The ability of the panel of anti-gp41 MAbs to block binding of representative biotinylated MAbs to Env was tested as described for Fig. 3. Members of each group are listed together. Human MAbs 246-D and 126-6 are representatives of clusters I and II, respectively.

hybridoma screening was derived from the BH8 clone of HIV-1 (LAI). To identify common structural features of Env that influence antigenicity, we examined the cross-reactivities of the anti-gp41 MAbs with a panel of Env proteins from different HIV-1 isolates. The gp160 proteins from nine clade B isolates were expressed by recombinant vaccinia viruses. These included the T-cell-tropic isolates BH10, LAV, and SF2; the macrophage-tropic isolates Ba-L, JRFL, ADA, and SF162; and the dual-tropic isolates RF and JRCSF. Metabolically labeled lysates of cells infected with each recombinant virus were subjected to immunoprecipitation with each of the anti-gp41 MAbs as well as with the V3 MAb, D47. Typical results are shown in Fig. 5, and full tabulation is in Table 2. Most of the MAbs reacted well with all or all but one of the Env proteins. Specifically, 100% of the anti-gp41 MAbs reacted with BH10 and LAV, 95% reacted with SF162 and Ba-L, 93% reacted with SF2, 89% reacted with JRCSF, 77% reacted with JRFL, and 59% reacted with ADA. As expected, MAb D47 reacted only with Env proteins containing the LAI V3 loop sequence. The three major antibody groups, defined by MAbs D61, D50, and T3 (Table 2), were equally cross-reactive, with two exceptions: none of the MAbs in the D50 group reacted with the ADA Env protein, and none of the MAbs in the T3 group

reacted with the JRFL Env protein. Sequence comparisons between the different Env proteins revealed that ADA contains two unique amino acid changes within the D50 determinant; E(659)D and E(662)A, changing the sequence from ELLE to DLLA. This finding suggests that these amino acids play an important role in D50 reactivity.

Cross-reactivities of MAbs with Env proteins from HIV-1 primary isolates. To extend the analysis of cross-reactivity to more highly divergent Env proteins, a set of Env proteins from 11 primary isolates was also examined by immunoprecipitation. The Env genes were expressed either by recombinant vaccinia viruses or with the hybrid vaccinia virus T7 system. These included Env proteins from the following clades and locations: clade A from Rwanda and Uganda, clade B from Baltimore and Thailand, clade C from Malawi and Brazil, and clade E from Thailand. Typical results of immunoprecipitations are shown in Fig. 6, and a tabulation of the data for all of the MAbs is given in Table 3. Reactivity was judged by the amount of divergent Env protein immunoprecipitated relative to the amount of BH8 Env immunoprecipitated (+, $\geq 40\%$; (+), 10 to 39%; and -, <10%). Many of the anti-gp41 MAbs exhibited significant cross-reactivity with these highly divergent Env proteins. In addition, MAbs in each of the three epitope groups, defined by MAb blocking, exhibited distinct patterns of cross-reactivity. Several of the members of the D61 group of MAbs cross-reacted with all 11 Env proteins, indicating that this epitope is highly conserved across clades. In contrast, members of the D50 group of MAbs exhibited relatively poor cross-reactivity. It is interesting that the ELLE sequence (residues 659 to 662) prevalent in clade B Env proteins is most frequently DLLA in clade A and C virus strains and DLLE in clade E virus strains. The two clade B Env proteins with which D50 does not react contain DLLA (ADA) and DLLE (BK132), suggesting that the E at position 659 is critical for binding. Finally, MAbs in the T3 group exhibited intermediate cross-reactivity compared to the D61 and D50 groups. Thus, this epitope is more moderately conserved.

Ability of HIV-positive human sera to block MAb binding to gp140. To determine whether antibodies in HIV-1-positive human sera could block binding of the anti-gp41 MAbs produced by immunization with oligomeric Env, we used a mod-

FIG. 5. Cross-reactivities of MAbs with Envs from clade B isolates. Metabolically labeled Env proteins were produced by infection of cells with recombinant vaccinia viruses expressing Env proteins from different clade B isolates of HIV-1. These include Envs from three T-cell-tropic (T), four macrophage-tropic (M), and two dual-tropic (dual) isolates. Lysates of infected cells were subjected to immunoprecipitation with the panel of anti-gp41 MAbs. Typical examples are shown. Cells infected with a recombinant virus that does not express Env protein were used as a negative control. D47 is an anti-V3 MAb.

TABLE 2. Cross-reactivities of MAbs with clade B env proteins^a

MAb	Reactivity with:									
	BH8	BH10	LAV	RF	SF2	ADA	BAL	JRFL	SF162	JRCSF
D61	+	+	+	+	+	+	+	+	+	+
M10	+	+	+	+	+	+	+	+	+	+
D41	+	+	+	+	+	+	+	+	+	+
D54	+	+	+	+	+	+	+	+	+	+
T4	+	+	+	+	+	+	+	+	+	+
T6	+	+	+	+	+	+	+	+	+	+
T9	+	+	+	+	+	+	+	+	+	+
T10	+	+	+	+	+	+	+	+	+	+
T35	+	+	+	+	+	+	+	+	+	+
D50	+	+	+	+	+	-	+	+	+	+
D16	+	+	(+)	+	+	-	+	+	+	+
D17	+	+	+	+	+	-	+	+	+	+
D31	+	+	+	+	+	-	+	+	+	+
D36	+	+	+	+	+	-	+	+	+	+
D37	+	+	+	+	+	-	+	+	+	+
D40	+	+	+	+	+	-	+	+	+	+
D44	+	+	+	+	+	-	+	+	+	+
D55	+	+	+	+	+	-	+	+	+	+
D59	+	+	+	+	+	-	+	+	+	(+)
T37	+	+	(+)	+	+	-	+	+	+	+
T45	+	+	(+)	+	+	-	+	+	+	+
T3	+	+	+	+	+	+	+	-	+	+
D43	+	+	+	+	+	+	+	-	+	+
D38	+	+	+	+	+	+	+	-	+	+
D45	+	+	+	+	+	+	+	-	+	+
D3	+	+	+	+	+	+	+	+	+	+
T30	+	+	+	+	+	+	+	+	+	+
M7	+	+	+	+	+	-	+	+	+	+
M9	+	+	+	+	+	+	+	+	+	-
D1	+	+	+	+	+	+	+	+	+	+
D4	+	+	+	-	-	+	-	-	+	-
D10	+	+	+	+	+	+	+	+	+	+
D11	+	+	+	+	+	+	+	+	+	+
D12	+	+	+	+	+	+	+	-	+	-
D26	+	+	+	+	+	-	+	+	+	+
D29	+	+	+	+	+	-	+	+	+	+
D30	+	+	+	+	+	-	+	+	+	+
T14	+	+	+	-	-	-	-	-	-	-
T26	+	+	+	(+)	-	-	(+)	-	-	-
T33	+	+	+	+	+	+	+	+	+	+
T39	+	+	+	+	+	+	+	-	+	+
T40	+	+	+	+	(+)	+	+	+	+	+
T41	+	+	+	(+)	(+)	+	+	-	+	(+)
T51	+	+	+	+	+	+	+	+	+	+
D47	+	+	+	-	-	-	-	-	-	-

^a Metabolically labeled Env proteins were immunoprecipitated with MAbs and analyzed by SDS-PAGE as shown in Fig. 4. +, strong reactivity; (+), decreased reactivity; -, lack of reactivity.

ification of the ELISA protocol described by Richardson et al. (39). gp140 was captured to microtiter plates with ConA, incubated with serial dilutions of pooled HIV-positive human sera, and subsequently incubated with individual MAbs. The HIV-positive serum pool was from 10 individuals with high anti-Env antibody titers. These individuals were from the Washington, D.C., area and presumably are infected with clade B viruses. The results are shown in Fig. 7. The anti-V3 MAb, D47, was not blocked by human sera, as expected since the IIIB V3 loop sequence is not prevalent in HIV-1 strains in the United States (10). In accord with the relatively high preva-

lence of CD4-blocking antibodies in human sera (29, 39), significant inhibition of binding of the CD4-blocking MAb M12 was observed. Several examples of blocking of anti-gp41 MAbs, including one from each of the antigenic determinants defined in this report, are also shown in Fig. 7. The serum concentration required to block each MAb by 50% is given in Table 4. Members of the D61 group were uniformly blocked at low serum concentrations. In contrast, members of the D50 and T3 groups showed more variability and required higher amounts of sera to block binding. MAb T30, which maps to the Asn at amino acid residue 616, was blocked by a fairly low serum concentration, but D3, which maps in the N-terminal part of the ectodomain of gp41, was blocked to only a very limited extent. In addition, several MAbs that did not map to any of the antigenic determinants defined by the linear MAbs were also examined. MAbs D10 and D12 exhibited the highest degree of blocking by human sera. Comparison of the results with the blocking of the two CD4 binding site MAbs, D20 and M12, suggests that antibodies to the epitopes recognized by these MAbs are prevalent in human sera, more so than the CD4-blocking antibodies.

DISCUSSION

Consideration of the quaternary structure of the HIV-1 Env protein is important for understanding the immunogenicity of the native, oligomeric protein and for the humoral response to HIV-1 infection. Quaternary interactions have proven to be important in the antigenic structures of several other well-characterized membrane proteins, most notably influenza virus hemagglutinin (11, 51). Several lines of evidence suggest that this is also the case for the HIV-1 Env protein. Steimer et al.

FIG. 6. Cross-reactivities of MAbs with Env proteins from primary HIV-1 isolates. Env proteins from 11 primary isolates were examined for reactivity with the panel of anti-gp41 MAbs. These include Envs from four clades as follows: clade A (RW20-5 and UG37-8); clade B (BA301712-4, BA301715-6, and BH8); clade C (MA301960-3, MA959-18, and BR-25-9A); and clade E (TH301975-15). These were expressed either by recombinant vaccinia viruses or with the hybrid vaccinia virus/T7 system. The Env proteins were metabolically labeled and subjected to immunoprecipitation with the panel of anti-gp41 MAbs. Typical examples as well as immunoprecipitation with rabbit polyclonal sera are shown.

FIG. 7. The abilities of HIV-1-positive human sera to block binding of MABs to Env protein. Oligomeric gp140 was captured in wells of 96-well plates by binding to ConA. Threefold serial dilutions of pooled HIV-positive human sera (from 10 infected individuals) were added followed by incubation with a MAB to detect serum blocking activity. Reactivity of each MAB in the absence of human sera was defined as 100%. MABs from the different antigenic determinants are shown: D54 (D61 group), D36 (D50 group), and D45 (T3 group). Anti-gp41 MAB D10, anti-gp120 CD4-blocking MAB M12, and anti-V3 MAB D47 are also shown. Binding of HIV-1-positive sera is plotted as well. O.D., optical density.

(45) showed that not all of the neutralizing activity in HIV-1-positive human sera could be accounted for by reactivity with monomeric gp120, suggesting that either epitopes in gp41 or those associated with oligomeric Env are also important. We have found that antibodies in human sera to native monomeric gp120 account for only about a third of the response to a soluble, oligomeric form of Env that contains gp120 and the gp41 ectodomain (39). In addition, Sattentau and Moore have found that antibody reactivity with monomeric gp120 does not predict neutralizing activity but that reactivity with oligomeric Env does (41). Moore and Ho have also shown that antibodies to conformational determinants are prevalent in HIV-1-positive human sera (29). Finally, we have demonstrated that immunization of mice with oligomeric Env elicits a high proportion of conformation-dependent antibodies, many of which are also oligomer dependent (4, 12). The oligomer-dependent antibodies have been mapped exclusively to gp41, confirming the importance of using oligomeric Env in analyses of anti-gp41 antibodies that arise as a consequence of HIV-1 infection.

Although much is known about the structure and immunogenicity of the gp120 subunit, the antigenic structure of the gp41 subunit remains poorly understood. This is due in part to the ease with which gp120 can be isolated: gp120 is spontaneously shed from the surface of cells expressing Env, and when expressed by itself, gp120 folds correctly and is secreted with high efficiency. In contrast, gp41 is membrane anchored and so more difficult to purify. To approach the problem, we have constructed a recombinant, oligomeric gp140 that contains the entire gp120 and the ectodomain of gp41. Because the protein is truncated just prior to the transmembrane domain, it is secreted from cells and thus can be purified to homogeneity with relative ease. An additional rationale for using this protein stems from observations that the ectodomain of gp41 is the region primarily responsible for oligomer stability (13, 17, 38, 47). Thus, in contrast to gp120, which is secreted largely in monomeric form, gp140 is oligomeric. Using gp140 as an immunogen, we generated a panel of 138 MABs, 38% of which

were specific for epitopes in gp41, including 9 directed against linear determinants and 38 directed against conformation-dependent epitopes. We demonstrated by FACS analysis that these MABs react with Env protein expressed on the surface of cells. We have used this panel of MABs to construct an antigenic map of gp41. The nine linear MABs, mapped by reactivity with C-terminally truncated Env proteins, peptide ELISA, and use of site-specific mutants, defined five distinct regions within the gp41 ectodomain. These MABs were then used to map regions of gp41 required for binding of the 38 conforma-

TABLE 3. Cross-reactivities of MABs with env proteins from primary HIV-1 isolates^a

MAB	Cross-reactivity											
	A			B			C			E		
	1	2		1	2	3	1	2	3	1	2	3
D61	+	+	+	(+)	+	+	+	+	+	+	+	+
M10	+	+	+	+	+	+	+	+	+	+	+	+
D41	+	+	+	(+)	+	+	(+)	+	(+)	(+)	(+)	(+)
D54	+	+	+	+	+	+	+	+	+	(+)	(+)	+
T4	(+)	+		(+)	(+)	(+)	(+)	(+)	+	+	(+)	(+)
T6	(+)	(+)		(+)	(+)	(+)	(+)	(+)	+	+	+	+
T9	(+)	+		(+)	+	+	(+)	(+)	+	+	+	(+)
T10	(+)	(+)		(+)	(+)	(+)	(+)	(+)	+	+	+	(+)
T35	(+)	(+)		+	+	+	(+)	+	(+)	+	+	(+)
D50	-	(+)	-	+	+	+	-	-	-	-	-	-
D16	-	-	-	+	(+)	+	-	-	-	-	-	-
D31	(+)	+	-	+	+	(+)	(+)	(+)	+	+	+	+
D36	-	(+)	-	+	+	+	-	-	-	-	-	-
D37	-	-	-	+	+	+	-	-	-	-	-	-
D40	-	-	-	+	+	+	-	-	-	-	-	-
D44	-	-	-	+	+	+	-	-	-	-	-	-
D55	-	-	-	+	+	+	-	-	-	-	-	-
D59	-	-	-	+	+	+	(+)	(+)	+	+	+	+
T37	-	+	-	+	+	+	-	-	-	-	-	-
T3	-	-	+	-	(+)	+	+	+	(+)	+	+	+
D43	-	-	+	+	(+)	+	+	+	+	+	+	+
D38	-	-	+	+	(+)	+	+	+	+	+	+	+
D45	-	-	+	+	(+)	+	+	+	+	+	+	+
D3	+	(+)	+	+	+	+	+	+	(+)	+	+	(+)
T30	+	+	+	+	+	+	+	+	+	+	+	+
M7	(+)	+	-	+	+	+	(+)	(+)	(+)	+	+	(+)
M9	+	+	+	(+)	+	+	-	-	(+)	+	+	+
D1	+	+	+	(+)	+	(+)	(+)	+	+	+	+	+
D4	-	-	+	-	-	-	-	-	-	-	-	+
D10	+	+	+	+	+	+	+	+	+	+	+	(+)
D11	+	+	+	+	+	+	+	+	+	+	+	(+)
D12	+	+	+	+	+	+	(+)	+	+	+	+	(+)
D26	-	-	-	+	+	+	-	-	-	-	-	-
D30	-	-	-	+	+	+	-	-	-	-	-	-
T14	-	-	-	-	-	-	-	-	-	-	-	-
T26	-	-	+	-	-	-	-	-	-	-	-	-
T33	+	+	+	+	(+)	+	+	+	+	(+)	+	+
T39	(+)	(+)	(+)	(+)	+	+	-	-	-	(+)	(+)	+
T40	+	+	(+)	+	+	+	+	+	+	(+)	(+)	+
T41	(+)	+	+	(+)	+	+	(+)	-	(+)	+	+	-

^a Immunoprecipitated Env proteins were quantitated with a Molecular Dynamics PhosphorImager. +, >40% of the amount immunoprecipitated by rabbit polyclonal sera; (+), 10 to 39%; -, <10%. Env proteins were derived from the primary isolates indicated in parentheses: clade A, 1 (RW20-5) and 2 (UG37-8); clade B, 1 (BK132), 2 (BA301712-4), and 3 (BA301715-6); clade C, 1 (MA301960-3), 2 (MA959-18), and 3 (BR-25-9A); and clade E, 1 (CM235), 2 (CM243), and 3 (TH301975-15).

TABLE 4. Abilities of HIV-1-positive human sera to block binding to Env protein^a

MAB	Env map location	Serum dilution required for 50% blocking
D61	597-613	1:1,000
D41	597-613	1:2,300
D54	597-613	1:1,300
T4	597-613	1:1,000
D50	642-665	1:125
D36	642-665	1:300
D40	642-665	1:300
T37	642-665	<1:100
T3	641-683	1:150
D43	641-683	1:200
D45	641-683	1:100
D3	512-579	<1:100
T30	611	1:400
D10	ND	1:3,600
D11	ND	1:4,000
D12	ND	1:2,500
T33	ND	1:300
D47	V3	No blocking
M12	CD4bl	1:450
D20	CD4bl	1:400

^a The serum dilution required to block binding of MABs to gp140 by 50% was determined from data like those shown in Fig. 6. Included are MABs to five antigenic determinants in gp41 as well as anti-gp120 MABs to the V3 loop and the CD4 binding site.

tion-dependent MABs. It appears that the ectodomain of gp41 contains at least seven distinct, nonoverlapping determinants that differ in their antigenic conservation, immunogenicity, and dependence on Env tertiary and quaternary structure. A topographical depiction of the ectodomain of gp41 is given in Fig. 8. The six antigenic determinants which have been mapped to specific peptide regions are circled.

The best-characterized antigenic determinant in gp41 is the immunodominant epitope, termed cluster I (53). Indeed, we found that a significant fraction of our MABs to gp41 mapped to this disulfide loop containing region (residues 597 to 613). In keeping with this terminology, we refer to this region as determinant I. Determinant I was recognized by four of our linear MABs, as exemplified by MAb D61. In addition, eight conformation-dependent MABs blocked the binding of D61 to oligomeric Env but not that of any other linear MAB. All of the oligomer-specific MABs (that react exclusively with oligomeric Env) are in this group, suggesting that the antigenic structure of determinant I is strongly influenced by quaternary interactions. We found that MABs to determinant I exhibited extensive cross-reactivity with primary Env proteins from divergent clades, indicating that the antigenic structure of determinant I is highly conserved and probably important in the oligomeric structure. In fact, it has been suggested that this region of gp41 forms a loop in oligomeric Env that connects two potential alpha-helical domains (48) (Fig. 8). That linear antibodies to determinant I are readily elicited by HIV infection has been shown by a number of investigators (23, 24, 44, 53). Our experiments indicate that the conformation-dependent and oligomer-dependent aspects of this epitope are also immunogenic: HIV-positive human sera strongly blocks binding of

these MABs to Env. In fact, human serum blocks binding of the D61 group of MABs more extensively than it blocks binding of several CD4 binding site MABs, suggesting that antibodies to determinant I are more prevalent than are anti-gp120 CD4-blocking antibodies. However, antibodies to this region exhibit either no or only weak neutralizing activity.

A second determinant, defined by MAB D50, mapped to amino acids 642 to 665. This region, near the C terminus of the gp41 ectodomain, overlaps one of two predicted helical domains as well as the previously defined cluster II determinant (53). Thus, we refer to it as determinant II. In accordance with the observation of Wild et al. (48) that the two alpha-helical regions interact, we have juxtaposed them in our two-dimensional representation of the ectodomain of gp41 (Fig. 8). A total of 11 conformation-dependent MABs mapped to determinant II, as defined by their abilities to block binding of D50 to oligomeric Env. Although most of the MABs in this group did not block D50 binding completely, they did fully block binding of D40, a conformation-dependent MAB that maps to this region. While the incomplete blocking of D50 could be due to differences in antibody affinities, it could also reflect the antigenic complexity of the C-terminal region of the gp41 ectodomain. While all of the antibodies to determinant II reacted with Env proteins derived from nine clade B isolates, none of them reacted with the Env protein derived from ADA (Table 2) or BK132 (Table 3). Among the clade B Env proteins tested, ADA and BK132 are unique in that they contain DLLA

FIG. 8. Epitope structure of the ectodomain of gp41. This two-dimensional depiction is modified from that of Gallaher et al. (21). The two alpha-helical regions shown to interact by Wild et al. (48) are juxtaposed. Antigenic determinants defined in this report are circled and shaded. The 2F5 epitope (determinant III) is circled with a boldface line. The linear MABs used to define each antigenic determinant are given in parentheses.

and DLLE, respectively, at residues 659 to 662 rather than the consensus clade B ELLE sequence. In addition, the determinant II MAbs reacted poorly with clade A, C, and E Env proteins, which contain either DLLA or DLLE. This finding suggests that the E at position 659 makes an important contribution to the structure of determinant II. Antibodies to determinant II were only moderately prevalent in HIV-positive human sera relative to determinant I (Table 4). The observation that determinant II is not well conserved (Table 3) could account for the low prevalence of MAbs that block binding of this epitope in HIV-positive human sera.

In addition to determinant II, the C-terminal region of the gp41 ectodomain contains at least two other distinct, antigenic regions. First is the epitope recognized by MAb 2F5 (residues 662 to 667) (5), which we term determinant III. Determinant III is of particular interest since 2F5 is the only anti-gp41 MAb described to date that exhibits significant neutralizing activity (9, 33). Surprisingly, none of the 47 anti-gp41 MAbs generated by immunization with oligomeric gp140 mapped to this region, even though 2F5 readily binds to gp140, suggesting that the epitope recognized by 2F5 is not very immunogenic. This possibility is further supported by the observation that antibodies to the 2F5 epitope are not well represented in human sera (11a). The final determinant that we identified in the C-terminal region of gp41, which we call determinant IV (amino acids 641 to 683), is best characterized by MAbs T3 and D43. While both T3 and D43 mapped to the 641–683 region in Western blots, they could not be mapped in further detail due to their failure to react with overlapping peptides spanning this region. Thus, determinant IV may be renatured following electrophoresis. Other conformation-dependent antibodies to this area have also been described (26, 46). In addition, determinant IV is clearly distinct from antigenic determinants II and III since most of the antibodies to these regions failed to compete with T3 and D43 for binding. Therefore, we think it likely that determinant IV is more C terminal than determinants II and III.

Two additional antigenic determinants were defined by 2 linear MAbs whose binding to Env was not blocked significantly by any of the 38 conformation-dependent MAbs. Thus, neither of these regions is likely to be very immunogenic. The first, defined by MAb D3 and referred to as determinant V, mapped to the amino-terminal 68 amino acids of gp41 but as yet not to any peptide within this region. Interactions of the amino-terminal region of gp41 with gp120 have been predicted (25). Consistent with this finding is the observation that antibodies to the amino-terminal region of gp41 react more strongly with Env following CD4-induced gp120 shedding (42). Thus, it is not surprising that we obtained only a single MAb that mapped to this region and that D3 binding to Env was not blocked efficiently by HIV-1-positive human sera. Human MAb 25C2 (5) maps to amino acids 526 to 543 in the N-terminal portion of antigenic determinant V. In our assay, MAb 25C2 did not block binding of D3 to Env (data not shown), suggesting that the D3 epitope is in the carboxyl end of determinant V. However, MAb 25C2 did block binding of MAb D40 (determinant II) and MAb T3 (determinant IV) by about 50% (data not shown). This partial blocking suggests a spatial proximity between the N terminus of gp41 and antigenic determinants II and IV. Finally, we detected only a single MAb that mapped to residues 611 to 640. This region of gp41, which lies between the DP107 and DP178 regions (49, 50), is highly glycosylated, perhaps accounting for its poor immunogenicity. The MAb that mapped to this region, T30, is unique in that it maps to amino acid 616, which is critical for glycosylation at that site. Like D3, T30 was poorly blocked by HIV-

positive human sera. We refer to this region as determinant VI. A group of human Fab fragments, termed cluster III by Binley et al. (1), also maps to this region of gp41. A member of cluster III, Fab L2, did not block binding of MAbs from antigenic determinant I, II, or IV (data not shown), further supporting the idea that antigenic determinant VI is not in juxtaposition with the other regions of gp41 (Fig. 8). In addition, Fab L2 did not block binding of MAb T30, indicating that antigenic determinant VI can be subdivided into two groups.

Finally, our panel contains 17 MAbs that did not map to any of the determinants defined by the linear MAbs. The epitopes recognized by these MAbs are likely to be structurally complex and may partially overlap with the six linear determinants. While many MAbs in this group cross-react with Envs from four clades, some react only within clade B. For example, MAbs M7, D10, D11, and D12 have very similar patterns of cross-reactivity, and all but one exhibit oligomer dependence. If patterns of cross-reactivity are characteristic of antigenic determinants, as observed for determinants I to VI, then these MAbs may represent another unique group. In addition, at least three of these MAbs are more potently blocked by HIV-positive human sera than any other of the MAbs tested, including anti-gp120 CD4 binding site MAbs. Thus, in addition to determinants I to VI, gp41 contains at least one and perhaps several additional determinants that are complex in nature.

In summary, we have constructed an epitope map of gp41 by using a panel of linear and conformationally dependent MAbs generated by immunization with gp140. At least seven antigenic determinants exist in the ectodomain of gp41, each varying in antigenic conservation, dependence on Env conformation, and immunogenicity, as judged by both MAb reactivity and the ability of HIV-positive human sera to bind to these determinants. In agreement with earlier studies (53), we found that antibodies to determinants I and II are readily detected in HIV-infected individuals. The other antigenic determinants appear to be less immunogenic and less highly conserved. Determinant V is likely to be masked by gp120, while determinant VI is highly glycosylated. Determinant III, defined by the broadly cross-reactive neutralizing antibody 2F5, was not recognized by any of the 47 gp41-specific MAbs that we raised to oligomeric gp41, suggesting that this region also fails to elicit a strong immune response. In general, the MAbs that we developed to oligomeric gp41 were more broadly cross-reactive with Env proteins from highly divergent isolates than were MAbs to gp120 (45a), indicating that epitopes in gp41 are highly conserved. However, the relevance of anti-gp41 antibodies in a natural HIV-1 infection is still unclear, as most anti-gp41 MAbs lack significant *in vitro* neutralizing activity, and determinants in gp41 may not be readily accessible in virus particles due to close packing of the Env protein spikes and the presence of gp120. The real significance of gp41 in the humoral immune response may therefore be due to its role in Env protein oligomerization, which may have important influences on the antigenic structure of gp120.

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