Localization of CD4$^+$ T cell epitope hotspots to exposed strands of HIV envelope glycoprotein suggests structural influences on antigen processing

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The spectrum of immunogenic epitopes presented by the H2-IA^b MHC class II molecule to CD4$^+$ T cells has been defined for two different (clade B and clade D) HIV envelope (gp140) glycoproteins. Hybridoma T cell lines were generated from mice immunized by a sequential prime and boost regime with DNA, recombinant vaccinia viruses, and protein. The epitopes recognized by reactive T cell hybridomas then were characterized with overlapping peptides synthesized to span the entire gp140 sequence. Evidence of clonality also was assessed with antibodies to T cell receptor Vα and Vβ chains. A total of 80 unique clonotypes were characterized from six individual mice. Immunogenic peptides were identified within only four regions of the HIV envelope. These epitope hotspots comprised relatively short sequences ($\approx$20–80 aa in length) that were generally bordered by regions of heavy glycosylation. Analysis in the context of the gp120 crystal structure showed a pattern of uniform distribution to exposed, nonhelical strands of the protein. A likely explanation is that the physical location of the peptide within the native protein leads to differential antigen processing and consequent epitope selection.

The primary role of any vaccine is to generate sustained immune memory to antigenic epitopes that are expressed on, or by, the pathogen in question. Vaccines designed to prevent the development of virus-induced pathology must promote the clonal expansion of CD4$^+$ T cells specific for those complexes of nonself peptide and self MHC class II glycoprotein that will be encountered again as a consequence of natural virus challenge. Experiments in a variety of mouse model systems have established that the virus-specific CD4$^+$ (T_h) subset functions both to enhance antibody production by cognate interaction with B cells (1, 2) and to promote the development of effector CD8$^+$ T cells (3, 4). Studies of readily eliminated viruses further indicate that the development of long-term memory in both lymphocyte compartments depends substantially on a concurrent T_h response (5), and CD8$^+$ T cell-mediated control of persistent infections requires the continued presence of virus-specific CD4$^+$ T cells (6, 7). This CD4$^+$ T_h for the CD8$^+$ subset is thought to operate via the intermediary of the activated dendritic cell (8). In some infections, particularly with the large DNA viruses, INF-γ-producing CD4$^+$ T cells are also important effectors of immunity (9–11). An ongoing CD4$^+$ T cell response also is thought to be important for the CD8$^+$ T cell-mediated control of HIV infection (12). Effective priming of the CD4$^+$ T cell response would thus seem to be a priority for any HIV vaccine.

What is known about the antigenic epitopes recognized by HIV-specific CD4$^+$ T cells? Previous studies have sought to characterize immunogenic peptides from the HIV envelope (env) protein (13–20) for a variety of mammalian species expressing a spectrum of MHC class II phenotypes. Much of the available information was generated by scoring heterogeneous CD4$^+$ T cell responses in bulk proliferation assays (13, 15, 16, 18). Some of these responses were very weak, and the assignment of antigenic epitopes from such studies to almost every region of the HIV env protein is open to question. The present experiments use a prime and double-boost vaccination protocol in H2^b mice to characterize the HIV env-specific CD4$^+$ T cell response for a single MHC class II glycoprotein (H2-IA^b). These results generated with a sensitive hybridoma T cell assay provide insights into the importance of viral glycoprotein structure for peptide selection and antigenicity.

Materials and Methods

Mice. Female C57BL/6 (B6) mice were purchased from The Jackson Laboratory and housed under specific pathogen-free conditions in a BL2 containment area. All mice were under the age of 3 months at the initiation of the immunization protocol. Experiments were reviewed by the institutional animal care and use committee and were performed under American Association of Laboratory Animal Care guidelines.

Immunogens. The prime and boost immunization regime used two different HIV gp140 envs, each of which was prepared as recombinant DNA, a recombinant vaccinia virus (VV), and protein purified from transfected Chinese hamster ovary (CHO) cells. The two HIV envs were primary isolates, 1007 (a clade B virus from an HIV-infected individual in Memphis, TN) and UG92005 (a clade D virus from the World Health Organization; ref. 21). The env genes were isolated by PCR (22–25) from HIV-infected peripheral blood mononuclear cells. Unique 1007 and UG92005 sequences of $\approx$570 aa [positions 51–618, see Ratner *et al.* (26) for sequence and numbering system] were individually substituted into the BH10 env sequence in a pSC11-based VV recombination vector. Substitutions used unique *Kpn*I and *Bsm*I enzyme restriction sites and standard molecular methods (22). The resultant chimeric envs initiated at amino acid 38 (the approximate junction of signal sequence and gp120 protein) and encompassed full gp120 and external gp41 segments. Plasmids were transfected into VV (Western reserve, wrwt)-infected TK$^-$143B cells, and recombinant VVs were selected in bromodeoxyuridine, then plaqued, and further selected by Western blot analyses of infected cell lysates, using anti-HIV antibodies as developing reagents (22).

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Abbreviations: env, envelope; VV, vaccinia virus; CHO, Chinese hamster ovary; TCR, T cell receptor.

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The DNA vaccines were made (25, 27) by independently incorporating each env sequence (amplified by PCR from the pSC11 vectors) into the pJW4303 vector, containing a cytomegalovirus enhancer/promoter, cytomegalovirus intron A, tissue plasminogen activator leader, and bovine growth hormone poly(A) sequence. The resultant plasmids expressed env (initiating at residue 41), again encompassing gp120 and external gp41 sequences. Plasmids were purified (Giga Plasmid kit, Qiagen, Valencia, CA) before injection into the mice.

The HIV-env producing cell lines were prepared by cotransfection of CHO-K1 cells (American Type Culture Collection) with the pSV2-neo (28) and the pJW4303 env plasmids. Recombinant cells were selected with G418 (GIBCO/BRL), and env expression was established by Western blot analysis of cell lysates, again using anti-HIV antibodies as developing reagents. The transfected CHO cells subsequently were cloned by limiting dilution. Purified gp140 proteins were prepared from culture supernatants by standard methods with anti-HIV antibody-coated immunoaffinity columns.

Vaccination. Separate groups of mice were immunized with the 1007 and UG92005 constructs. The prime and boost schedule (27) used DNA, followed by VV and CHO-derived protein. The first immunization was with 100 μg of the recombinant DNA vaccine given i.m. 24 h after a bupivacaine (Astra Pharmaceutical, Westborough, MA) injection at the priming site. After 3–4 weeks, these mice were boosted i.p. with 1.0×10^7 plaque-forming units of the VV recombinant. After an additional 3–4 weeks, mice received 1–10 μg of purified env from the CHO cell supernatants. The env protein was emulsified in complete Freund's adjuvant and injected into the base of the tail.

Hybridoma Production. Inguinal and *para*-aortic lymph nodes and spleens were obtained from immunized mice sampled 10 days after the final boost with the env protein. Cell suspensions were prepared from all tissues, and red blood cells were lysed from splenic samples. Lymph node cells (1.0×10^6 cells/ml) were stimulated with splenocytes (5.0×10^6 cells/ml), and recombinant CHO cell lysates were matched with the vaccine (either 1007 or UG92005; 1×10^5 lysed CHO cells per ml) for 2–4 days at 37°C in complete medium (29). Activated T cells (1.0×10^7) then were fused with an equal number of BW5147 $\alpha^-\beta^-$ (30) or BWZ.36 (31) cells. Fused cells were plated by limiting dilution for selection in microtiter cultures. The hybridoma lines subsequently were harvested from plates in which fewer than one-third of wells scored positively for growth.

IL-2 Assays. Hybridomas were screened *in vitro* with either B6 spleen cells or -IA^b-transfected L cells (AF7–1C6; ref. 32) as antigen-presenting cells. These stimulator cells were incubated with either peptides or lysates of vaccinia virus-infected cells (see below). Hybridomas were plated at 1.0×10^5 cells/well with antigen-presenting cells in 96-well plates. After 18–24 h, supernatants were removed and assayed for IL-2 production on an IL-2-dependent HT-2 cell line by using an oxidation-reduction indicator (Alamar Blue, Alamar Biosciences, Sacramento, CA) read at 570 nm with a reference wavelength of 595 nm (33). Recombinant human IL-2 (R & D Systems) served as a positive control.

Hybridoma Screening. Hybridomas were tested for IL-2 production after stimulation with antigen-presenting cells and env protein, recombinant VV (1007 or UG92005 to match the vaccine), or control VV (either wild-type VV or VV-HN expressing the hemagglutinin-neuraminidase protein of parainfluenza virus type 1). The VV-infected stimulators were prepared by incubating spleen cells with virus (multiplicity of infection = 5) for 1 h in serum-free medium and for an additional 4 h with medium containing serum. These antigen-presenting cells then were washed three times before plating. Hybridomas that responded specifically to the HIV env recombinant VV were expanded for further screening with overlapping peptides (8–15 mers) representing the full env protein used for vaccination. The peptides, which were made by Chiron or at the Hartwell Center at St. Jude Children's Research Hospital, were tested first as pools, then individually at concentrations of 1–10 μg peptide/ml.

Structural Analysis. Peptide determinants were mapped within the envelope crystal structure (34). Figures were prepared with MOLSCRIPT (35) and rendered with RASTER3D (36).

Flow Cytometry for T Cell Receptor (TCR) Typing. A panel of unconjugated, biotinylated, and phycoerythrin-labeled mAbs was used to characterize the spectrum of TCR expression on the env-specific hybridoma cell lines (37). Individual mAbs were specific for the constant region of the TCR β-chain, Vβ 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14, and Vα 2, 8 and 11. In some cases, hybridomas expressing a V$\beta 8^+$ TCR were further defined as being V$\beta 8.1^+$, V$\beta 8.2^+$, V$\beta 8.1^+$ or 8.2^+ (Vβ8.1–2), or V$\beta 8.3^+$. The secondary staining reagents included fluorochrome-labeled avidin, goat anti-rat IgM, goat anti-mouse IgG, and goat anti-rat IgG. These reagents were prepared from hybridoma supernatants (38) or were purchased from PharMingen, Caltag, Jackson ImmunoResearch, or Biosource International (Camarillo, CA).

Results

The Analytical Approach. These experiments defined the H2-IA^b-restricted response for two HIV env glycoproteins from primary isolates of a clade B (1007) and a clade D (UG92005) virus. The mice were first immunized by using a sequential prime and double-boost regime (DNA, VV-recombinant and protein), then cells from spleen and lymph nodes were fused with a stable T cell line for hybridoma production. A total of three independent fusions were done for each env, using lymphocytes from different mice in every case. More than 200 hybridomas were tested with IL-2 assays, initially against pools of overlapping peptides, then for reactivity to individual peptides within positive pools. Responses of $\geq$40 units/ml IL-2 were considered positive, with no antigen controls scoring at $\leq$20 units/ml. The majority of the hybridomas secreted $\geq$1,280 units/ml IL-2 after stimulation with 10 μg/ml peptide. Specificity for HIV env peptides was demonstrated for 85% of these hybridoma cell lines. Evidence of clonality in this panel of hybridomas was established, in part, by the fact that 75% were found to stain with one or another of a panel of mAbs to different TCR Vβ and Vα chains.

Discrete Hotspots Define T_h Epitopes for HIV env Glycoproteins. The complete list of H2-IA^b-restricted T_h epitopes identified in the course of these experiments is shown in Table 1. The results are tabulated to show the sequence of each epitope, the particular env used for immunization, the TCR usage of hybridomas within each set, and the number of individual clonotypes with a given specificity. A total of 80 distinct clonotypes were identified, distinguished from one another based on differences in the peptide specificity, differences in TCR usage, or derivation from different mice. The T_h epitopes mapped to heavily glycosylated regions (39) in V2 (amino acids 162–196, see ref. 26 for numbering system), C2 (amino acids 205–237), V3 (amino acids 300–345), V4-C4 (amino acids 412–454), and gp41 (amino acids 613–632). Further characterization of T_h epitope position within the env protein (Fig. 1) revealed a striking, nonrandom congregation of peptide sequences. All of the epitopes were found to cluster in four relatively short, discrete polypeptide regions (*A–D*), whereas large expanses of env sequence were found to contain no T_h determinants.

Table 1. T_h-epitope hotspots in the HIV env protein

Priming antigen*	Sequence 1007/UG92005†	
	KNCSFNITTSIRDKMQQEYALFYKLDVVPIENANA KNCSFNITTELIDKKQQVEALFYKLDVVQIDNSTN	**-V2**
UG	■■■■■■■■■■■■■■■■ **Vβ‡5**	(1)§
UG	■■■■■■■■■■■■■■■■ **Vβ-ND**	(1)
	NTSTITQACPKVSFEPIPIHYCAPAGFAILKCNDKKFN NTSVITQACPKITFEPIPIHYCAPAGFAILKCNDKKFN	**-C2**
1007	■■■■■■■■■■■■■■■■ **Vβ3** ■■■■■■■■■■■■■■■■	(1)
1007	■■■■■■■■■■■■■■■■ **Vβ3,8.1-2**	(2)
1007	■■■■■■■■■■■■■■■■ **Vβ6**	(1)
1007	■■■■■■■■■■■■■■■■ **Vβ4,6,7,8.1-2,8.3,11,12,ND**	(12)
1007	■■■■■■■■■■■■■■■■ **Vβ-ND** ■■■■■■■■■■■■■■■■	(1)
1007	■■■■■■■■■■■■■■■■ **Vβ-ND** ■■■■■■■■■■■■■■■■	(1)
UG	■■■■■■■■■■■■■■■■ **Vβ12,ND** ■■■■■■■■■■■■■■■■	(2)
UG	■■■■■■■■■■■■■■■■ **Vβ5,8.2,12,ND**	(5)
UG	■■■■■■■■■■■■■■■■ **Vβ-ND** ■■■■■■■■■■■■■■■■	(1)
	VINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISREKW TINCTRPYNNTRKGIHIGPGRAYYTT.NIVGNIRQAHCNVSKAKW	**-V3**
1007	■■■■■■■■■■■■■■■■ **Vβ6,ND**	(3)
UG	■■■■■■■■■■■■■■■■ **Vβ-ND** ■■■■■■■■■■■■■■■■	(1)
UG	■■■■■■■■■■■■■■■■ **Vβ-ND,ND‖**	(2)
UG	■■■■■■■■■■■■■■■■ **Vβ8.2,ND** ■■■■■■■■■■■■■■■■	(2)
UG	■■■■■■■■■■■■■■■■ **Vβ5,7,8.1,8.1‖,8.2,8.2‖,11,ND,ND¶**	(11)
UG	■■■■■■■■■■■■■■■■ **Vβ6,8.1,ND**	(3)
	SNNTVGNPIILPCRIKQIINMWQEVGKAMYAPPIRGQIKCSSN NNET....ITLQCRIKQIINMWQGVGKAMYAPPIAGLIQCSSN	**-V4C4**
1007	■■■■■■■■■■■■■■■■ **Vβ3,4,7,8.1,8.2,8.2¶,10,12,ND**	(13)
UG	■■■■■■■■■■■■■■■■ **Vβ6,8.1,8.2,13,14,ND,ND¶,ND‖,ND****	(10)
	TTVPWNASWSNKSLEQIWNN TNVPWNASWSNKSLEQIWNN	**-gp41**
1007	■■■■■■■■■■■■■■■■ **Vβ14** ■■■■■■■■■■■■■■■■	(1)
UG	■■■■■■■■■■■■■■■■ **Vβ4** ■■■■■■■■■■■■■■■■	(1)
UG	■■■■■■■■■■■■■■■■ **Vβ8.1,8.1‖,14,ND**	(5)

*Mice were immunized with either the 1007 or UG92005 (UG) envs using a prime and double boost regime.

†Alignment of epitopes in sequences from the V2, V3, V4, C4 and GP41 regions of the 1007 (*Upper*) and UG (*Lower*) env sequences. The shaded residues identify potential sites for N-linked glycosylation. The black boxes show the precise location of the epitopes.

‡The spectrum of Vβ usage is shown for each hybridoma set, with ND designating TCRs that could not be identified with the available panel of mAbs.

§The bracketed figures give the number of distinct clonotypes distinguished based on differences in peptide specificity, differences in TCR usage, or derivation from different mice. Positive IL-2 responses indicating peptide binding activity were approximately 1,280 units/ml, with backgrounds of 0–20 units/ml.

¶‖**Identify the Vα chains that could be characterized with the available mAbs to Vα2 (¶), Vα8 (‖), and Vα11 (**).

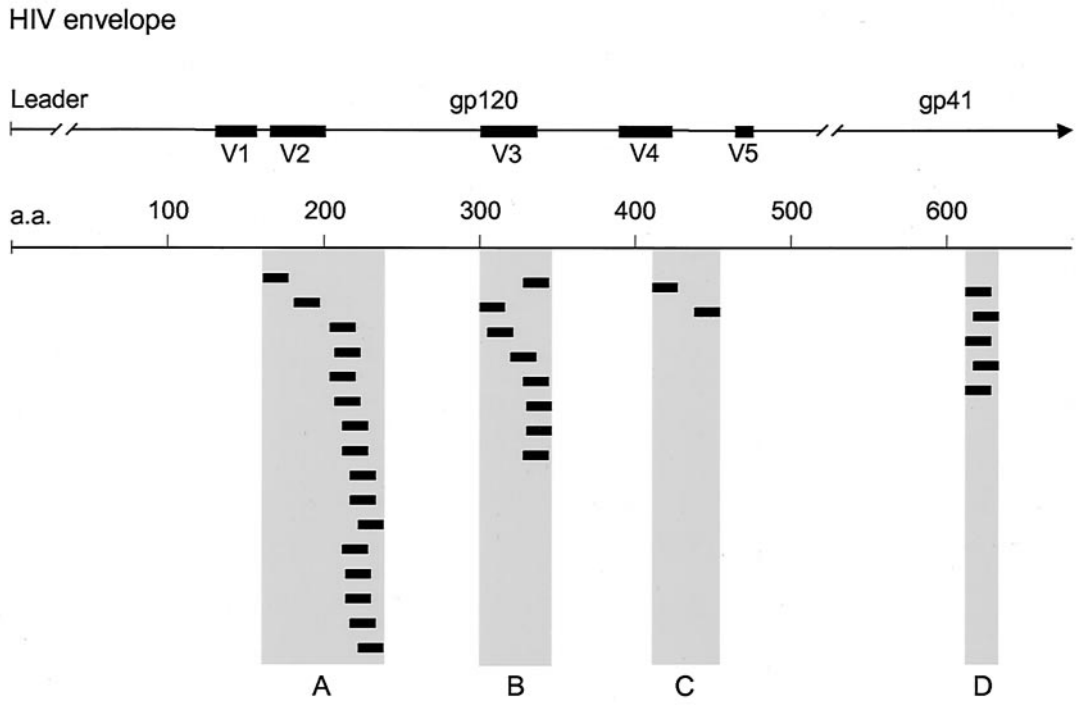

Fig. 1. The T_h epitopes are located in distinct hotspots. The antigenic peptides defined in Table 1 are restricted to four distinct regions of the env sequence. The hotspots are identified as regions *A–D* through the gp120 (*A–C*) and gp41 (*D*) sequence. Note that immunogen sequences initiated at the junction of env signal sequence (leader) and gp120 coding sequence.

Influence of and Location in Exposed, Nonhelical Loops or Strands of the HIV env. The positions of three of the four hotspots (Fig. 1 *A–C*) were then mapped onto the crystal structure (34) of the gp120 env protein. The fourth hotspot (Fig. 1*D*) is in the external domain of the transmembrane gp41. The complex of the gp120 core (excluding V1, V2, and V3 loops) and CD4 described by Wyatt and coworkers (34, 37) is illustrated in Fig. 2. The hotspots in Fig. 1 *A–C* are highlighted in Fig. 2 *A*, *B*, and *C*, respectively. Composite front and side views of the position of these three hotspots are shown in Fig. 2 *D* and *E*, respectively. All three hotspots appeared in exposed loops or strands on one face of the gp120 molecule. Some of the epitopes continued into the V2 and V3 loops that are not shown in Fig. 2, but these variable regions also are exposed on the surface of the glycoprotein. Generally, helical sequences did not contribute to the T_h epitope panel.

Discussion

These results provide definitive evidence that the selection of antigenic epitopes for $CD4^+$ T cells is influenced by the three-dimensional structure of the native protein. Although the accepted wisdom is that position determines immunogenicity, position generally refers to the ordering of amino acids within a particular sequence rather than to the physical location of that peptide in the protein. The peptide sequence obviously determines both the capacity to form an appropriate interaction with the groove in the MHC molecule on the one hand and the TCR on the responding lymphocyte on the other. Clearly, no antigenic epitope can be formed unless both requirements are satisfied. Such considerations have led to the development of predictive algorithms (40, 41), which tend to be somewhat more useful for MHC class I than MHC class II-restricted T cell responses. The present findings identify an additional constraint, which could explain why some predicted epitopes emerge in the context of an immune response, whereas others do not.

How might the location of the peptide in the original protein modify antigenicity? A distinguishing feature of all of the peptides highlighted in Fig. 2 is that they can essentially be "peeled away" from the protein without unduly disturbing the structure of the underlying core. In the folding pathway of the protein, these regions are probably among the last to become structured, and they would consequently be easily unfolded and preferentially accessible to fragmentation by the proteases in the antigen-processing pathway. This, in turn, would lead to a more rapid trafficking of the polypeptides within the endosomal/lysosomal compartments and preferential displacement of invariant chain from MHC class II glycoprotein (42–45).

Enhanced antigen processing also might result from antibody binding to exposed regions of the env glycoprotein. There is some evidence from other experimental systems that the same peptides are recognized by both Ig molecules and the TCRs of $CD4^+$ T cells (46, 47). Two recent studies of human hepatitis C virus have mapped both Ig binding and T_h epitopes to the hypervariable region of the env protein (48, 49). In support of this theory, antibodies and $CD4^+$ T cells have been shown to recognize epitopes on the HIV env V2, V3, and V4 loops. However, the exposed loops of V1 and V5 regions of HIV env are also known to elicit antibody responses (50–52), yet neither contributed to the T_h-epitope panel defined here. The question of a role, or otherwise, for antibody binding as a determinant of $CD4^+$ T cell responsiveness could be addressed experimentally by repeating the present analysis in $Ig-/-$ μMT mice.

What is the significance of epitope hotspot location in regions of heavy glycosylation? The correlation could simply be fortuitous, reflecting that the HIV env is very heavily glycosylated and that the carbohydrates tend to be on the exposed surface of the native protein. An alternative idea is that carbohydrates may bind lectins that promote trafficking through endosomal/lysosmal compartments. Such lectin effects may determine the subsequent molecular associations of degraded polypeptides with class II molecules in antigen-loading compartments (53, 54). An additional possibility is that carbohydrates may dictate favorable patterns of proteinase activity (55). In any case, the antigenic peptides need not incorpo-

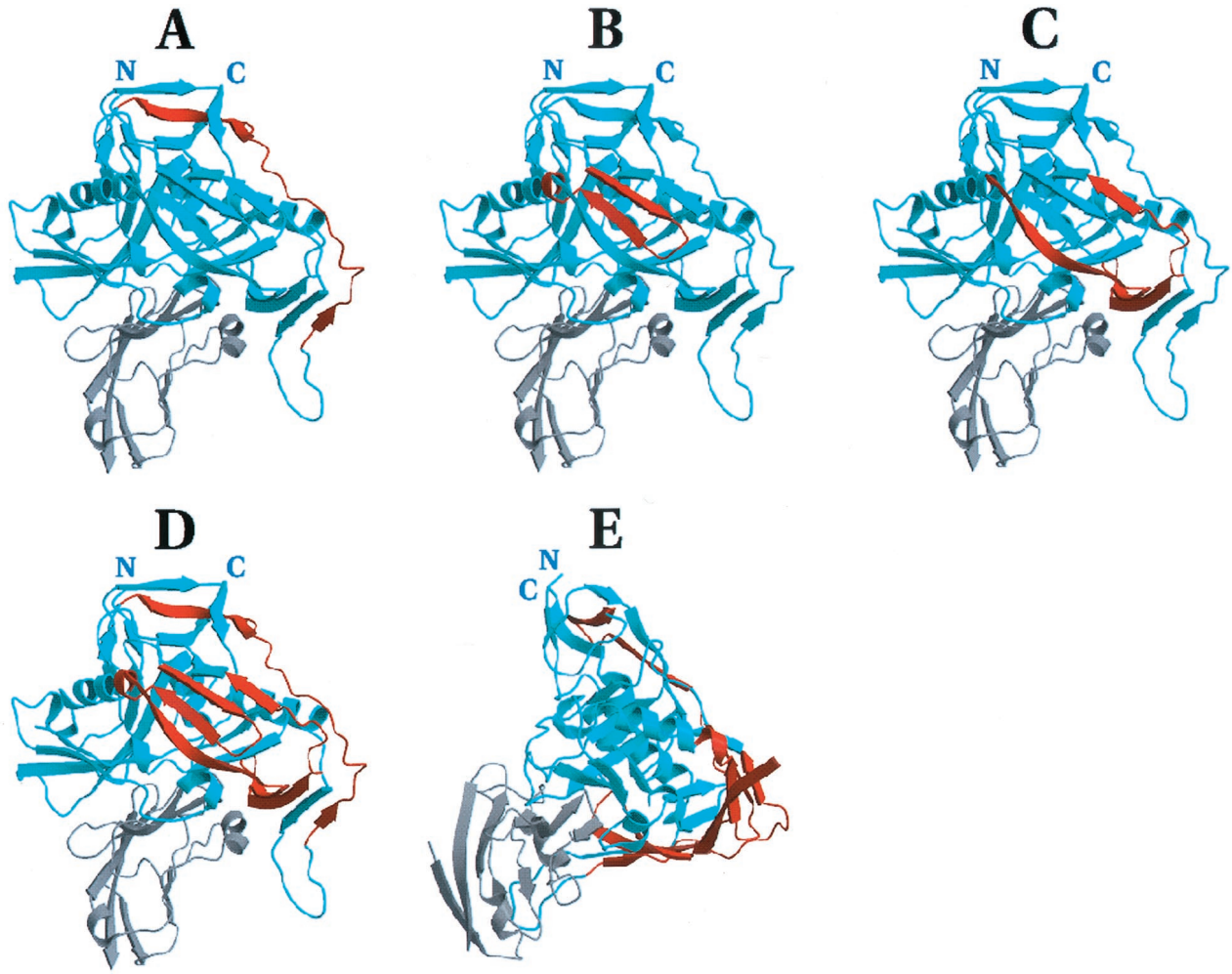

Fig. 2. Anatomical location of the hotspots to exposed, nonhelical loops and strands of the env protein. The T_h hotspots are highlighted (red) on the crystal structure (34, 37) of gp120 (blue) complexed with CD4 (gray). The structure can be oriented by the position of the N- and C-terminal residues of gp120 and the CD4 binding site. The regions *A–C* in this figure correspond to *A–C* in Fig. 1 with the omission of V2 and V3 sequences, absent from the crystal. The composite of all three hotspots is shown from front (*D*) and side (*E*) views.

rate a glycosylation site, but may simply be bordered by sites of glycosylation. These T cell responses were assayed by using synthetic peptides, so glycosylation is not critical either for MHC class II binding or TCR contact.

It should be recognized that the HIV env is unusual in that carbohydrate residues account for more than 50% of the total molecular weight of this oligomeric, globular, disulfide-bonded, membrane glycoprotein (39). Perhaps the complex three-dimensional configuration of this HIV protein accentuates an otherwise subtle influence of protein structure on subsequent antigen processing.

Although this positional effect is not explained, the present findings with the HIV env indicate that the potential influence of peptide location within a protein merits consideration during any process of vaccine design. The spectrum of antigenicity could well be modified when, for example, artificial strings are made that link a number of different peptides (56). Approaches that involve either the production or injection of whole proteins are likely to preserve antigenic clusters in the conformation that will be encountered on any subsequent challenge with live virus (24, 27, 57, 58). Immunizations with recombinant DNA, recombinant viruses, and purified env proteins should achieve this end.

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