Helper T-cell antigenic site identification in the acquired immunodeficiency syndrome virus gp120 envelope protein and induction of immunity in mice to the native protein using a 16-residue synthetic peptide

(human immunodeficiency virus/human T-lymphotropic virus type III/vaccine/epitopes/amphipathicity)

KEMP B. CEASE*, HANAH MARGALIT†, JAMES L. CORNETTE†, SCOTT D. PUTNEY‡, W. GERARD ROBEY§, CECILIA OUYANG*, HOWARD Z. STREICHER¶, PETER J. FISCHINGER§, ROBERT C. GALLO¶, CHARLES DELISI‖, AND JAY A. BERZOFSKY*,**

*Metabolism Branch, †Laboratory of Mathematical Biology, and ¶Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; ‡Repligen Corp., Cambridge, MA 02139; §Office of the Director, National Cancer Institute–Frederick Cancer Research Facility, Frederick, MD 21701; and ‖Office of Health and Environmental Research, Department of Energy, Washington, DC 20545

ABSTRACT   Much effort has been devoted to the analysis of antibodies to acquired immunodeficiency syndrome virus antigens, but no studies, to our knowledge, have defined antigenic sites of this virus that elicit T-cell immunity, even though such immunity is important in protection against many other viruses. T cells tend to recognize only a limited number of discrete sites on a protein antigen. Analysis of immunodominant helper T-cell sites has suggested that such sites tend to form amphipathic helices. An algorithm based on this model was used to identify two candidate T-cell sites, env T1 and env T2, in the envelope protein of human T-lymphotropic virus type III$_B$ that were conserved in other human immunodeficiency virus isolates. Corresponding peptides were synthesized and studied in genetically defined inbred and F_1 mice for induction of lymph node proliferation. After immunization with a 426-residue recombinant envelope protein fragment, significant responses to native gp120, as well as to each peptide, were observed in both F_1 combinations studied. Conversely, immunization with env T1 peptide induced T-cell immunity to the native gp120 envelope protein. The genetics of the response to env T1 peptide were further examined and revealed a significant response in three of four independent major histocompatibility haplotypes tested, an indication of high frequency responsiveness in the population. Identification of helper T-cell sites should facilitate development of a highly immunogenic, carrier-free vaccine that induces T-cell and B-cell immunity. The ability to elicit T-cell immunity to the native viral protein by immunization with a 16-residue peptide suggests that such sites represent potentially important components of an effective vaccine for acquired immunodeficiency syndrome.

Although much work has been done on the antibody response to acquired immunodeficiency syndrome (AIDS) virus proteins (1–9), relatively little information is available on the T-lymphocyte responses. Yet T-cell immunity is a major defense against other viral infections, and helper T-cell immunity is necessary for a memory antibody response. The response of helper T cells has been found to be highly focused on a limited number of discrete sites on protein molecules rather than broadly directed at all segments of an antigen (10–17). This general feature of the response, known as immunodominance, is observed in both mice and humans with membrane-associated and soluble antigens. Moreover,

it has been found to be true even for noneukaryotic proteins such as influenza hemagglutinin (18, 19) or staphylococcal nuclease (20) for which self-tolerance cannot account for the limited number of antigenic sites. Specific segments of a protein, furthermore, may induce active suppression that abrogates the response to all other regions of a molecule (21). For these reasons, the rational design of a vaccine against the AIDS virus requires identification of those segments of protein sequence that elicit T-cell immunity. Such knowledge would be especially valuable in situations where recombinant fragments or synthetic peptides are employed.

An ideal vaccine would be highly immunogenic, would induce both T-cell and B-cell virus-specific immunity, and would be free of irrelevant carrier proteins. While traditional approaches using whole virion or virion subunits can generally achieve this, practical considerations such as safety and availability of native antigen have led many to consider more highly engineered vaccine constructs for AIDS (1, 22–24). As a first step in identifying potentially important T-cell sites, we have initially focused on the gp120 envelope protein of human immunodeficiency virus (HIV). A T-cell response to the gp120 envelope protein has been demonstrated by Zarling *et al.* (25) in macaques immunized with vaccinia constructs containing gp120 coding sequence. However, identification and characterization of immunodominant T-cell sites within this 484-residue protein or other HIV proteins have not been reported, to our knowledge.

MATERIALS AND METHODS

Sequence Analysis. Our approach to the problem of finding T-cell sites in this large protein was to apply a method developed in our laboratory that identifies ≈75% of known immunodominant helper T-cell epitopes from 12 model proteins (26–28). This strategy is based on the observation that immunodominant T-cell sites tend to have an amino acid sequence consistent with formation of an amphipathic helix with hydrophilic residues on one face and hydrophobic residues on the opposite face (26). In an amphipathic α-helix, the hydrophobicity varies sinusoidally with a period of 3.6 residues per turn of the helix or a frequency of 100° per residue. An amphipathic 3$_{10}$-helix is a helix with 3 residues per turn and thus has a frequency of 120°. Based on this model, an algorithm, entitled AMPHI, has been developed

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Abbreviations: HIV, human immunodeficiency virus; HTLV-III, type III human T-cell lymphotropic virus; AIDS, acquired immunodeficiency syndrome; PPD, purified protein derivative.
**To whom reprint requests should be addressed.

for identification of such sequences in proteins given only primary sequence data (28). The AMPHI algorithm was used to examine HIV envelope protein amino acid sequences (29–33) for sites with periodic variation in the hydrophobicity consistent with formation of an amphipathic helix.

As five potential sites were identified in the gp120 sequence, we chose to study first those potential sites from more conserved regions (based on a comparison of the six available sequences), as these might be more broadly useful. Furthermore, because glycosylation might mask potential T-cell epitopes, we focused on segments lacking N-linked glycosylation sites.

Synthetic Peptides. Synthetic peptides corresponding to these selected sites were prepared using standard methods of solid-phase peptide synthesis on a Vega 250 peptide synthesizer using double dicyclohexylcarbodiimide-mediated couplings (34, 35) and butyloxycarbonyl (Boc)-protected amino acid derivatives. Hydroxybenzotriazole preactivation couplings were performed when coupling glutamine or asparagine. The extent of coupling was monitored using the qualitative ninhydrin test and recoupling was performed when <99.4% coupling was observed. Peptides were cleaved from the resin using the low/high hydrogen fluoride (HF) method (36). For peptide env T2, standard HF cleavage was employed as removal of the tryptophan formyl protecting group was found not to be required for antigenic activity. Peptides were purified to homogeneity by gel filtration and reverse phase HPLC (37). Composition was confirmed and concentration determined by amino acid analysis (kindly performed by Robert Boykins, Food and Drug Administration).

Purified and Recombinant Proteins. Native gp120 was purified from virus-infected cells as described (1). The recombinant proteins R10 and PB1 were produced by cloning restriction fragments *Kpn* I (nucleotide 5923) to *Bgl* II (nucleotide 7197) or *Pvu* II (nucleotide 6659) to *Bgl* II (nucleotide 7197) from the BH10 clone of type III human T-cell lymphotropic virus (HTLV-III$_B$) into the Repligen expression vector, followed by expression in *Escherichia coli* and purification as described (3). R10 was initially solubilized in 20 mM Tris·HCl, pH 8.0, with 10 mM 2-mercaptoethanol at 0.22–0.66 mg/ml. PB1 was solubilized in 8 M urea at 1.9 mg/ml. Protein R10 represents residues 49–474 of the HTLV-III$_B$ envelope protein with 25 non-HTLV-III vector-derived residues at the N terminus and 440 such residues at the C terminus. Protein PB1 represents residues 294–474 of gp120 with 30 and 24 non-HTLV-III residues at the N and C termini, respectively. The N-terminal residues of PB1 are partially shared with those of R10, whereas the C-terminal residues are unrelated.

Mice. As a genetically defined model of an outbred population, we studied the immune response to these proteins in (C57BL/6 × C3H/HeJ)F$_1$ and (A.SW × BALB/c)F$_1$ mice (*H-2*^{bxk} and *H-2*^{sxd}, respectively). This strategy provides for H-2 complementation in the context of four different strain backgrounds. In some studies, the corresponding parental strains or C57BL/10 congenics, B10.A(5R), B10.BR, B10.S(9R), and B10.D2, were studied. The C3H/HeJ and BALB/c mice express both I-A and I-E molecules of the *H-2*^{k} and *H-2*^{d} haplotypes, respectively. The C57BL/6 and A.SW mice express only I-A molecules of the *H-2*^{b} and *H-2*^{s} haplotypes, respectively, because they produce only an I-E$_\beta$ and no I-E$_\alpha$ chain. In the F$_1$ hybrids selected (*H-2*^{bxk} an *H-2*^{sxd}), I-E$_\beta$ pairs with the other parental (nonpolymorphic) I-E$_\alpha$, resulting in surface expression of an I-E molecule via trans complementation with a phenotype predominantly determined by the I-E$_\beta$ chain. These molecules are also expressed in the B10.A(5R) and the B10.S(9R) congenics (*H-2*^{i5} and *H-2*^{t4}, respectively) as a result of cis complementation.

Lymph Node T-Cell Proliferation Assays. Mice were immunized with either 10 μg (0.1 nmol) of the large recombinant protein R10 or 5.2 μg (3 nmol) of peptide env T1 in 50 μl of complete Freund's adjuvant (Difco) subcutaneously at the base of the tail. Eight days later, the draining lymph nodes were removed, and a single-cell suspension was assayed in quadruplicate cultures at 3×10^5 cells per well in complete medium as described (12, 14). Thymidine incorporation into DNA during the last 18 hr of a 5-day culture was determined as a measure of proliferation. The background without antigen was subtracted to obtain the difference in cpm (Δcpm).

RESULTS

AMPHI parameters for the two most favorable sites are shown in Fig. 1. Candidate T-cell sites were selected by including appropriate flanking residues. Candidate T-cell sites env T1 and env T2 were defined as residues 428–443 and residues 112–124, respectively. The standard epitope nomenclature employed consists of viral isolate, protein designation, site type, and assigned number or residue number [e.g., HTLV-III$_B$(BH10)env T1].

Quantities of purified gp120 available precluded use in immunization, and thus, the R10 protein containing the majority of the gp120 sequence in nonglycosylated form was the largest immunogen used. In both F$_1$ hybrids immunized with R10, a strong response was observed not only to the immunogen R10, but also to gp120 and the env T1 peptide (Fig. 2 *A* and *B*). Therefore, the response was largely directed at envelope sequence and not at the irrelevant vector-derived residues in the recombinant protein. Thus, the recombinant R10 fragment is an effective immunogen for priming for a response to the native gp120. The response to the synthetic
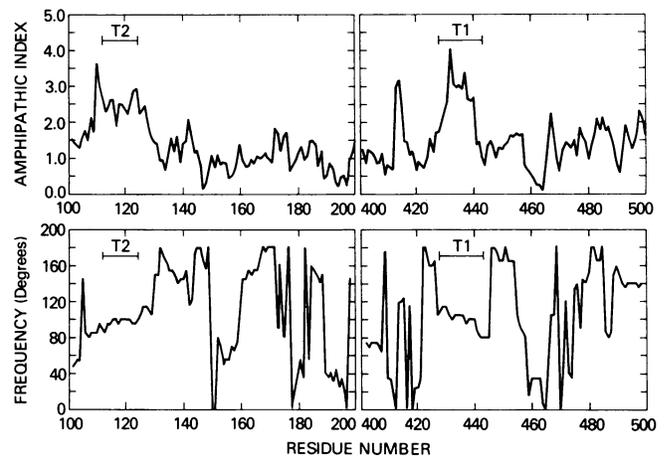
FIG. 1. Results of AMPHI analysis in the region of the env T1 and env T2 sites. Overlapping blocks of 11 residues were examined, and resultant parameters were assigned to the middle residue. The results for the residues 100–200 and 400–500, encompassing the env T2 and T1 sites, respectively, appear on the *Left* and *Right*, respectively. (*Upper*) The amphipathic index, a measure of intensity of amphipathicity, determined at a frequency of 100° or 120° per residue. The amphipathic index at 100° or 120° is defined as the fractional area under the power spectrum curve (intensity as a function of frequency) in the range of 85°–110° or 105°–135°, respectively, divided by the fractional width of the interval (28). The index for the block is the maximum of these two values. (*Lower*) The frequency (degrees turn of putative helix per residue) where the maximum amphipathic signal is observed. The amino acid sequences of the indicated sites are: env T2 (residues 112–124), His-Glu-Asp-Ile-Ile-Ser-Leu-Trp-Asn-Gln-Ser-Leu-Lys; env T1 (residues 428–443), Lys-Gln-Ile-Ile-Asn-Met-Trp-Gln-Glu--Val-Gly-Lys-Ala-Met-Tyr-Ala.

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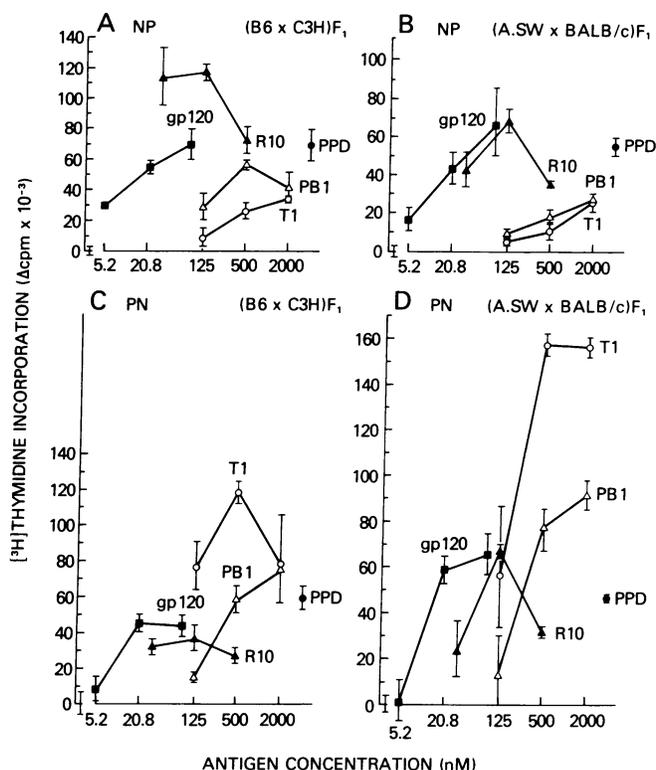
FIG. 2. Lymph node proliferation assays of HTLV-III envelope gp120 and related recombinant and synthetic peptide antigens in F_1 hybrid mice. (A and B) Mice were immunized with recombinant R10 protein. (C and D) Mice were immunized with peptide env T1. The experiments shown in A and B used the near-native-size immunogen and peptide test antigen and are thus labeled NP experiments, whereas in C and D experiments used a peptide immunogen and native test antigen and are thus labeled PN. The no-antigen backgrounds were: A, 21,771 cpm; B, 17,844 cpm; C, 30,674 cpm; D, 29,298 cpm. The confidence intervals for the background values are shown at the zero position of each vertical axis ($n = 8$). The panels are scaled according to the magnitude of the purified protein derivative (PPD)-positive control response. Δcpm equals total cpm minus background.

peptide env T1 indicates that the T-cell response to the 425-residue R10 is in fact partially focused on the 16-residue env T1 site. T cells from mice immunized with PB1, the smaller of the two recombinant fragments, also responded to env T1 peptide (data not shown). In other experiments with R10-immune lymph node cells, a response to peptide env T2, similar to that to peptide env T1, was observed (Table 1).

Given that immunization with a large fragment spanning

Table 1. Response to env T2 peptide in R10(residues 49–474)-immune F_1 hybrid mice relative to native gp120 and env T1 peptide

Antigen	[³H]Thymidine incorporation, cpm	
	(B6 × C3H)F_1	(A.SW × BALB/c)F_1
gp120	69,738 (1.01)	65,949 (1.07)
env T1	17,686 (1.12)	25,140 (1.15)
env T2	20,703 (1.18)	23,332 (1.14)
Medium	10,864 (1.09)	13,381 (1.07)

[³H]Thymidine incorporation is shown for each group expressed as the geometric mean cpm with the standard error term for quadruplicate samples shown in parentheses. ($n = 8$ for the medium controls.) Antigen concentrations were 0.075 μM for gp120 and 4.8 μM for the env T1 and T2 peptides. The responses to antigen are statistically significant relative to the medium control in each case ($P < 0.025$ by one-tailed Student's t test).

most of the gp120 sequence elicits a response partially focused on a small site defined by a synthetic peptide, a native immunogen/peptide test antigen or "NP" experiment (i.e., one in which we immunize with a near-native-size fragment and test with the peptide), we next asked whether immunization with the synthetic peptide would elicit immunity to the native protein, a peptide immunogen/native test antigen or "PN" experiment. Immunogenicity resulting in a positive PN test would appear to be a prerequisite for efficacy as a vaccine site. F_1 mice immunized with env T1 peptide showed substantial immunity, not only to the env T1 immunogen, but also to the native gp120 as well as to the recombinant proteins (Fig. 2 C and D). Thus, a 16-residue synthetic peptide can elicit T-cell immunity to the native AIDS virus protein.

To further characterize genetic restriction of the response to env T1, we studied the independent H-2 disparate parental strains from which the F_1 hybrids had been derived: C57BL/6, C3H/HeJ, A.SW, and BALB/c. Mice were immunized with env T1 peptide and studied with native and peptide antigens. C57BL/6 ($H-2^b$ haplotype) was found to be a nonresponder, whereas the other strains (haplotypes $H-2^k$, $H-2^s$, and $H-2^d$) were responders to the env T1 peptide (Fig. 3). The response to native gp120 paralleled that to the peptide. A corresponding pattern of responsiveness is also observed in congenic strains of mice (Table 2). Thus, peptide env T1 represents a 16-residue peptide that can prime T cells for a secondary response to the 484-residue glycosylated native gp120 in multiple but not all major histocompatibility haplotypes.

An unexpected finding was the striking crossreaction between env T1 and env T2 peptides. The env T1 immune cells responded to env T2 as well as to the immunizing peptide (Fig. 3). Crossreactivity of env T2 was most pronounced on the $H-2^s$ haplotype. Prompted by this finding, we compared the two sequences and observed a degree of homology that was even more evident when considered in the context of possible α-helical structure, as shown in Fig. 4. Not only do env T1 and env T2 share the hydrophobic Ile-Ile-Xaa-Yaa-Trp cluster on the hydrophobic face and the lysine on the hydrophilic face of the helix but also the spatial relationship between these is identical. Glutamine and acidic amino acids (glutamic acid and aspartic acid) neighboring the
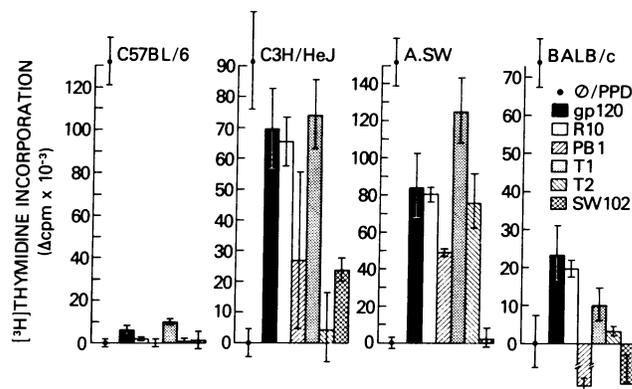
FIG. 3. Response of env T1 peptide immune lymph node cells to gp120 and related antigens in the independent parental mouse strains. C57BL/6, C3H/HeJ, A.SW, and BALB/c mice were immunized with the 16-residue env T1 peptide, and lymph node proliferation assays were performed. SW102 is a peptide representing sperm whale myoglobin residues 102–118 (37). The negative and positive controls with no antigen (∅) and PPD, respectively, are shown in the first position of each panel. The panels are scaled according to the magnitude of the PPD response. The no-antigen backgrounds were: B6, 16,334 cpm; C3H, 74,253 cpm; A.SW, 28,771 cpm; BALB/c, 34,600 cpm.

Table 2.   Response to gp120 in env T1 peptide-immune H-2 congenic mice

Antigen	[³H]Thymidine incorporation, cpm			
	B10.A(5R)	B10.BR	B10.S(9R)	B10.D2
PPD	85,511 (1.06)	100,872 (1.07)	71,006 (1.05)	44,564 (1.17)
gp120	45,857 (1.02)	69,456 (1.05)	68,219 (1.06)	64,858 (1.10)
Medium	29,715 (1.03)	40,639 (1.04)	22,863 (1.04)	19,665 (1.06)

[³H]Thymidine incorporation is shown for each congenic group of mice expressed as the geometric mean cpm with the standard error term for quadruplicate samples shown in parentheses. ($n = 8$ for the medium controls.) Antigen concentrations were 0.075 μM for gp120 and 32 μg/ml for PPD.

lysine are observed in both cases as well. The poor reactivity to peptide 102–118 of sperm whale myoglobin, which is derived from an unrelated protein and shares minimal homology with env T1, indicates that the property of being an amphipathic α-helical peptide is not sufficient for crossreactivity (37). As an additional specificity control, gp120, env T1, and env T2 were tested using lymph node cells from the high responder C3H and (A.SW × BALB/c)F$_1$ mice immune to an unrelated antigen, sperm whale myoglobin, and were found to be nonstimulatory (data not shown).

DISCUSSION

Induction of T-cell immunity may contribute in several ways to protection against HIV infection. Though AIDS progresses despite the presence of detectable antibody to viral proteins in most patients, neutralizing antibody of variable titer has been demonstrated in many such patients (39–41). Neutralizing titers that are group specific are substantially higher in healthy AIDS-related complex patients and in HIV antibody-positive hemophiliacs (39, 40). Whether this relationship is causal or simply correlative is as yet unknown. If these antibodies or others induced by prior vaccination are in fact protective, provision of optimum T-cell help at the time of immunization, as well as when faced with an infectious challenge, would appear essential. Substantial T-cell help should also be required for an effective cell-mediated response to infected cells. Natural killer cells have been shown to selectively kill HIV-infected cells *in vitro* (42). Given that a major mode of viral transmission in the infected patient is thought to be cell to cell (43, 44), a vaccine that primes helper T cells for production of lymphokines (45) that augment natural killer cell and possible lymphokine-activated killer cell activities, as well as virus-specific cytotoxic T lympho-

cyte immunity, may be essential for an effective vaccine. The AMPHI algorithm was developed to identify helper T-cell sites, and consequently, its relevance to cytotoxic T lymphocytes specificity is unknown. However, it does successfully identify the two characterized sites in influenza nucleoprotein recognized by human and murine cytotoxic T lymphocytes (46). *In vivo* expression of antigens, such as in a vaccinia or adenoviral vector, may be required for efficient elicitation of a classical cytotoxic T lymphocyte response (47, 48). The demonstrations that helper T cells can kill major histocompatibility complex class II-positive antigen-presenting cells suggest an additional mechanism whereby helper T-cell immunity might help prevent or contain viral infection (49, 50).

Though species differences are certain to influence the T-cell repertoire, the molecules and mechanisms leading to a T-cell response are conserved across species, and thus, the factors determining immunodominance would be expected to be similar as well. The one helper T-cell site (from influenza virus) that has been characterized at the synthetic peptide level in humans is in fact immunodominant in mice as well and has an amino acid sequence consistent with formation of a highly amphipathic α-helix (18, 19).

While it is encouraging that amphipathicity analysis has aided in the successful identification of two T-cell epitopes from HIV gp120, the present results are not intended to be a statistical test of the method. Further validation will require the testing of a large number of peptides from many proteins. Rather, the goal of this study was to localize T-cell sites from the HIV envelope that might be useful in vaccine development, as, to our knowledge, no T-cell epitopes have been identified in any AIDS virus protein. Studies with AIDS virus peptides have been directed at antibody specificities (1–9) or pharmacologic blocking of gp120 binding to CD4 (51) and have dealt with sites distinct from those of the present study.

The fact that helper T-cell immunity can generally be induced with short peptides as well or better than with native protein stands in sharp contrast to the situation with B-cell immunity for which tertiary structure is frequently important (52, 53) and indicates that peptide vaccines aimed at T-cell immunity may be more successful than those aimed at antibody production. In a synthetic peptide or recombinant fragment-based construct, one could selectively include important helper T-cell sites, in multiple copies if desired, and exclude suppressor T-cell sites (21). Sites associated with specific functions or possible undesirable side effects, such as the CD4 binding site(s), the site(s) mediating syncytia formation, or the neuroleukin homology site (54, 55), may be systematically included or excluded. For vaccines designed to induce antibodies as well as T-cell immunity, incorporation of pathogen-derived T-cell sites along with important B-cell sites obviates the need to chemically couple small peptides to irrelevant carriers and, consequently, disposes of coupling and carrier-derived problems and enables a natural secondary immunization on exposure to the pathogen. Thus, the T-cell sites identified in this study may be potentially important components of an effective AIDS vaccine. If, in fact, a vaccine can be developed that substantially enhances cell-
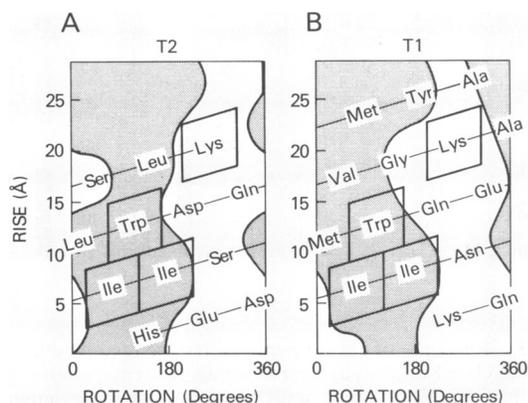
FIG. 4. α-Helical net representation of the env T2 and env T1 sites. This display can be thought of as slicing the cylinder of the helix length-wise down one face, opening, and flattening it (38). There are 3.6 residues per turn of the helix. The hydrophobic residues are shaded. Residues common to both sites are boxed. Regions outside of the peptides are shaded according to hydrophobicities of residues in the gp120 sequence.

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mediated immunity as well as the antibody response, it might be effective therapeutically in the prodromal stages of the disease as well as for prevention. Prior to initiation of human trials, it is anticipated that any potential vaccine preparation would be assessed in the chimpanzee, a species that is readily infected with HIV and in which prevention of initial infection is potentially demonstrable.

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