The Presumptive CDR3 Regions of Both T Cell Receptor α and β Chains Determine T Cell Specificity for Myoglobin Peptides

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Summary

The T cell receptor α/β (TCR-α/β) is encoded by variable (V), diversity (D), joining (J), and constant (C) segments assembled by recombination during thymocyte maturation to produce a heterodimer that imparts antigenic specificity to the T cell. Unlike immunoglobulins (Igs), which bind free antigen, the ligands of TCR-α/β are cell surface complexes of intracellularly degraded antigens (i.e., peptides) bound to and presented by polymorphic products of the major histocompatibility complex (MHC). Therefore, antigen recognition by T cells is defined as MHC restricted. A model has been formulated based upon the similarity between TCR-α/β V region and Ig Fab amino acid sequences, and the crystal structure of the MHC class I and Ig molecules. This model predicts that the complementarity determining regions (CDR) 1 and 2, composed of TCR Vα and Vβ segments, primarily contact residues of the MHC α helices, whereas V/Jα and V/D/Jβ junctional regions (the CDR3 equivalent) contact the peptide in the MHC binding groove. Because polymorphism in MHC proteins is limited relative to the enormous diversity of antigenic peptides, the TCR may have evolved to position the highly diverse junctional residues (CDR3), where they have maximal contact with antigen bound in the MHC peptide groove. Here, we demonstrate a definitive association between CDR3 sequences in both TCR α and β chains, and differences in recognition of antigen fine specificity using a panel of I-E^d-restricted, myoglobin-reactive T cell clones. Acquisition of these data relied in part upon a modification of the polymerase chain reaction that uses a degenerate, consensus primer to amplify TCR α chains without foreknowledge of the Vα segments they utilize.

The heterodimeric TCR-α/β is encoded by V, D, J, and C segments assembled by recombination during thymocyte maturation to produce a heterodimer that imparts specificity in antigen recognition to the T cell (1). Unlike Igs, which bind free antigen, the ligands of TCR-α/β are cell surface complexes of intracellularly degraded antigens (i.e., peptides) bound to and presented by polymorphic products of the MHC (2, 3). Antigen recognition by T cells is therefore defined as MHC restricted. Based upon the similarity between TCR-α/β V region and Ig Fab amino acid sequences, and the crystal structure of the MHC class I and Ig molecules, a model of the TCR has been proposed (4, 5). This model predicts that the Ig complementarity determining region (CDR) equivalent of the TCR 1 and 2, composed of the germ line–encoded TCR Vα and Vβ segments, primarily contact residues of the MHC α helices, whereas the highly divergent V/Jα and V/D/Jβ junctional regions (the Ig CDR3 equivalent) contact the peptide in the MHC binding groove. Junctional sequence diversity is contributed by multiple factors: combinatorial association of V, D, and J segments, translation of Dβ segments in all three reading frames, and enzyme-catalyzed, template-independent addition and deletion of nucleotides between V, D, and J segments (4). Because polymorphism in MHC proteins is limited relative to the enormous diversity of antigenic peptides, the TCR may have evolved to position the highly diverse junctional residues (CDR3), where they have maximal contact with antigen bound in the MHC peptide pocket.

Using synthetic peptides, it has been possible to map the minimal length and sequence of T cell epitopes in particular MHC/antigen complexes (6–9). However, the conformational flexibility of peptides and their effects upon the MHC binding groove are as yet unknown. Furthermore, the structures of the α1 and β1 peptide–binding domains of MHC class II molecules can only be modeled from the crystallographic analysis of HLA-A2 (10), and will undoubtedly differ from their class I counterparts. The recent structural solution of a second class I molecule (Aw68) revealed small but potentially im-

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portant differences in the peptide binding pockets between the two closely related molecules (11), suggesting that generalizations about the details of MHC/peptide complexes recognized by the TCR await additional high resolution analyses. Despite these limitations, investigation of the relationship between TCR structure and ligand recognition can be informative in model systems of cloned T cells specific for defined peptides in the context of a single MHC-restricting element (12–15). By comparison of TCR sequences expressed by T cells with discrete differences in epitope fine specificity recognition, regions of the receptor chains that are important for MHC vs. peptide recognition can be identified.

Here, we demonstrate an association between junctional sequences in both TCR α and β chains, and differences in antigen fine specificity recognition using a panel of I-E^d-restricted, myoglobin-reactive T cell clones. These data substantiate the proposed TCR model described above, especially the importance of both TCR α and β CDR3 regions in peptide contact. Acquisition of these data relied in part upon a modification of the polymerase chain reaction (PCR)[1] that uses a degenerate, consensus primer to amplify TCR α chains without foreknowledge of the Vα segments they utilize.

Materials and Methods

T Cell Clones. T cell clones were derived according to established procedures (6, 16). Briefly, DBA/2 mice were immunized at the base of the tail with 100–200 μg sperm whale myoglobin (SpWMb)/50% CFA, and the draining lymph nodes (LN) were removed 8 d later. The LN cells were cultured with irradiated, syngeneic spleen cells (APC) and SpWMb for 12 d. Cells were restimulated three times with APC and the 56–131 cyanogen bromide fragment of SpWMb at 12–13-d intervals, and the bulk cultures were cloned and subcloned by limiting dilution. Clone designations refer to mouse and clone (e.g., 8.2 is clone 2 derived from mouse 8). Recognition of antigenic fine specificity by the clones was assayed by restimulation of 10^4 rested T cells with 10^6 irradiated APC and several concentrations of nested synthetic peptides. Cell proliferation was measured by [^{3}H]thymidine incorporation and is expressed as the mean of triplicate cultures in cpm $\times$ 10^3.

RNA Isolation. Total RNA was prepared from Ficoll-separated T cells 3 d after restimulation with SpWMb by homogenization of 5–10 $\times$ 10^6 cells in guanidine thiocyanate and centrifugation through cesium chloride (17). To elicit maximum TCR expression before RNA extraction and eliminate possible contamination by feeder cell RNA, resting T cells (>10 d after last antigen stimulation) were Ficoll purified, resuspended at 0.5–1.0 $\times$ 10^6/ml in media containing 10 ng/ml PMA, 250 ng/ml ionomician, and 10–25 U/ml human rIL-2 (Cetus Corp., Emeryville, CA). After 24 h, cultures were diluted 1:2 to 1:4 into media containing rIL-2 alone. A time course experiment assaying TCR mRNA levels by Northern blot hybridization to Cα and Cβ probes showed that 3–5 d of this regimen produced optimal RNA levels for further analyses.

TCR-β PCR and Sequence Analysis. A first strand cDNA synthesis was performed on 10 mg of total RNA using oligo (dT) priming of avian myeloblastosis virus reverse transcriptase (18), and 50% of the reaction was used as a template for PCR amplification. The 5′ TCR-β 8.2 probe was 5′-ATGTCTAACACTGCCTT-3′. The 3′ Cβ probe was 5′-TCA GGA ATT TTT TTT CTT GAC-3′. The primer annealing PCR segment was 1 min at 55°C. The polymerase extension segment was 1 min at 72°C. A final 10-min extension was performed at 72°C to ensure fully duplexed DNA for optimal ligation efficiency. The entire coding region of the Vβ8.2-containing transcripts was amplified using two primers, Lβ8.2 and Cβ2 (19, 20) by 25 cycles in a thermal cycler (Perkin-Elmer Corp., Norwalk, CT) (21–23). Full-length products were purified as previously described (15) and cloned into M13 vectors. Plaques hybridizing with a Vβ8.2 probe labelled with α32p-dCTP (24) were identified, and a minimum of six subclones for each TCR β chain were sequenced (25). Reactions were resolved on 40-cm wedge gels and autoradiographed overnight at room temperature. To eliminate possible sequence errors introduced either in the reverse transcription or Taq polymerization reactions, molecules were subcloned from independent PCR reactions and their sequences compared. Sequences were analyzed on a VACS computer using GenBank and EMBL data files.

TCR-α Consensus PCR. To determine the Vα segments expressed by the DBA/2 clones, Northern transfers of RNA from each clone were prepared by standard methods (26), and hybridized under conditions of high stringency to a collection of Vα region probes representing 11 of 13 known Vα families (27–32). Inclusion of a sequence in a V region family was defined by a degree of nucleotide homology ≥85% (33). Results from these studies (data not shown) defined clone 8.2 as expressing Vα4. Oligonucleotide primers for Vα4 and Cα were designed based upon published sequences (27, 32), and used in PCR amplification of the TCR-α mRNA from clone 8.2 as described for the TCR β chains. The sequence of the 5′ Vα4 oligo was 5′-CGC GAA TTC ACC ATG AAC CTT TGT CCT GAA CTG-3′. The 3′ Cα primer had the sequence 5′-TCA ACT GGA CCA CAG CCT CAG-3′. Despite crosshybridization to a Vα1 probe (TT11; reference 27), PCR primers designed from the leader sequence of this clone failed to amplify the TCR-α from clones 11.3, 12.2, 14.12, and 14.16. Consequently, the consensus primer was used to obtain the Vα sequence data. In addition to the M13 primer, sequencing primers for the α chains were Cα5R 5′-CAG AAC CCA GAA CCT GCT GTG-3′ and Cα4 5′-GCA CAT TGA TTT GGG AGT CA-3′. PCR amplification of TCR α chains from clones expressing undefined Vα was accomplished with a 216-fold degenerate "consensus" oligo for the 5′ primer and a 3′ Cα primer. The consensus Vα 5′ PCR primer, purchased from Operon Technologies (San Pablo, CA) had the sequence 5′-TAAGCG GCC GCT GGT ACZ LMC AGC ATC CXG GMG AAG GCC-3′, where Z = 40% A, 40% G, 15% C, 5% T; L = A, G, T; M = 45% A, 50% G, 5% C; X = C, T. It was used at a 1-μM final concentration in a 100-μl PCR reaction with 1 μM Cα3′ primer. The temperature of annealing segments was two cycles at 37°C, one cycle at 42°C, and 27 cycles at 55°C. Selection of these cycle programs was made empirically. Details of consensus primer specificity and sensitivity will be described elsewhere (Danska, J. S., and C. G. Fathman, manuscript in preparation). The amplified product was purified and cloned as described for the β chains. One modification was digestion of the gel purified products with Not I to exploit a cloning site in the Vα consensus primer. The material was then ligated into an M13 derivative (mp21) digested with Hinc II and Not I. Multiple subclones of the TCR α chains were sequenced as described for the β chains.

Results

Fine Specificity Analysis of SpWMb-specific T Cell Clones. Mice of the H-2^d haplotype generate class II–restricted Th

[1] *Abbreviations used in this paper:* HMb, horse myoglobin; LN, lymph nodes; PCR, polymerase chain reaction; SpWMb, sperm whale myoglobin.

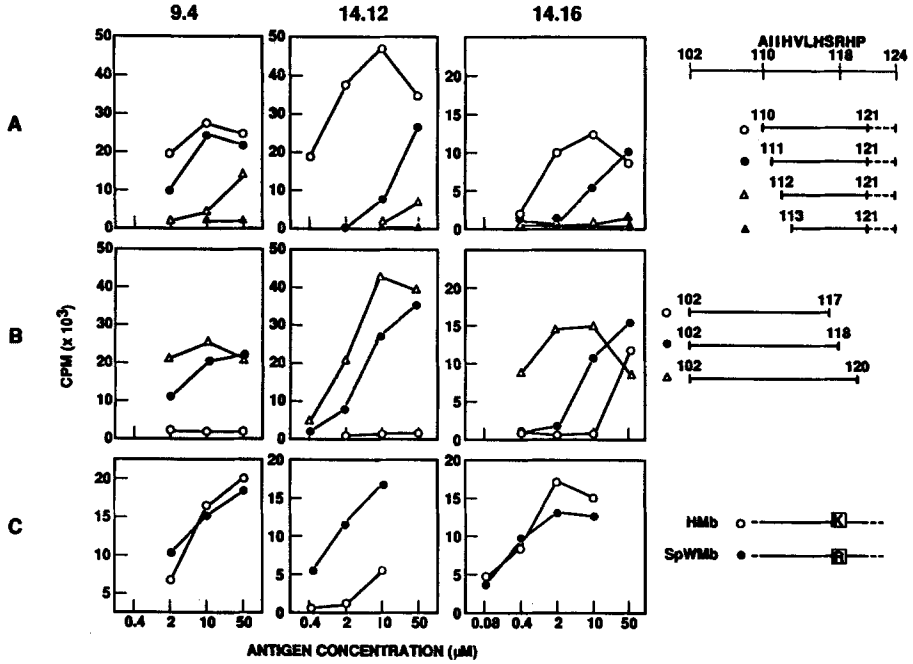

Figure 1. Antigen fine specificity of I-E^d-restricted DBA/2 clones. Cell proliferation assays were performed on T cell clones stimulated with irradiated, syngeneic APC, and truncated peptides spanning amino acids 102–124 of SpWMb. (*A*) Results for NH_2-terminal truncations; (*B*) for COOH-terminal truncations; and (*C*) for reactivity to HMb that bears a substitution (*boxed*) within the epitope recognized by these clones. Column 9.4 represents the specificity pattern of clone 9.4, characteristic of the four prototype clones, including 8.2, 11.3, and 12.2.

cells that recognize multiple epitopes of SpWMb in association with both I-A^d and I-E^d molecules (7–9). We have isolated a series of Th cell clones (6, 34) that recognize at least three distinct epitopes within SpWMb 110–121 and have determined the sequence of their TCR. Differences in recognition of antigen fine specificity among the T cell clones was examined using a nested set of synthetic SpWMb peptides serially shortened on either the NH_2 terminus or COOH terminus, as well as horse myoglobin (HMb), which has an arginine for lysine substitution at position 118. The clones were divisible into three fine specificity groups (Fig. 1). The minimal epitope of four of the six clones (8.2, 9.4, 11.3, and 12.2), represented in Fig. 1 by 9.4, consisted of amino acids 112–118, and all four "prototype clones" were equally reactive to SpW or HMb. A second fine specificity was observed in clone 14.12; this clone was 112–118 specific but showed diminished reactivity to HMb (Fig. 1 *B*). A third clone, 14.16, recognized a minimal epitope that consisted of amino acids 111–117 and recognized both species of Mb equally well (Fig. 1 *C*). Thus, among the six 110–121-reactive clones studied, there were three distinct fine specificity patterns.

To determine MHC restriction, these clones were cocultured with SpWMb and irradiated APC derived from mice of different haplotypes. All six clones responded to SpWMb and APC that expressed both I-A^d and I-E^d (DBA/2 or B10.D2), but all failed to recognize SpWMb on APC bearing only I-A^d (D2.GD), defining them as I-E^d restricted. The clones were also assayed for reactivity to APC from mice of 10 additional MHC haplotypes either without exogenous antigen (termed alloreactivity), or in the presence of SpWMb. None of the clones responded to SpWMb presented on APC from a non-H-2^d animal; clone 14.12 alone had apparent alloreactivity (I-A^k, I-E^b), further distinguishing it from the "prototypical" SpWMb 112–118–reactive clones (8.2, 9.4, 11.3, 12.2).

TCR β Chain Analysis. We undertook a molecular analysis of the TCRs used by these six SpWMb-reactive clones to ask first whether the indistinguishable antigen specificity of the four prototype clones reflected TCR primary sequence similarity, and second, whether the observed differences in epitope specificity and/or allorecognition were associated with variation in particular portions of their TCR sequences. Prior experiments using a TCR Vβ8-specific mAb had revealed that all six clones expressed either Vβ 8.1 or Vβ8.2 (34). Southern and Northern blot analyses with both Vβ8.1 and Vβ8.2 molecular probes (19, 20) confirmed that all six clones had rearranged and transcribed Vβ8.2 (data not shown). Oligonucleotide primers specific for the Vβ8.2 leader and the Cβ2 sequences were used to PCR amplify TCR-β cDNA synthesized from mRNA expressed in these clones. The nucleotide sequences are shown in Fig. 2. Among all six clones, the TCR-β diversity was limited to a single Vβ, a single Dβ, and two Jβ segments. The 112–118-specific clones including 14.12, expressed Vβ8.2/Dβ2.1/Jβ2.6, whereas clone 14.16, which recognized 111–117 as a minimal epitope, expressed a TCR-β chain containing Vβ8.2/Dβ2.1/Jβ2.5 (19). The TCR β chains of the former group differed by only two charge conservative amino acid substitutions, both encoded by template-independent, N-region nucleotides at the V/D and D/J junctions. The TCR β chains of the SpWMb 112–118–reactive clones and the SpWMb 111–117–reactive clones differ in both length and sequence of their junctional regions. Alignment of the 3′ ends of their common Vβ8.2 segment, and the residues conserved between Jβ2.6 and Jβ2.5, demonstrates that the presumptive TCR CDR3 loops of clones bearing distinct recognition specificities differ in length by four amino

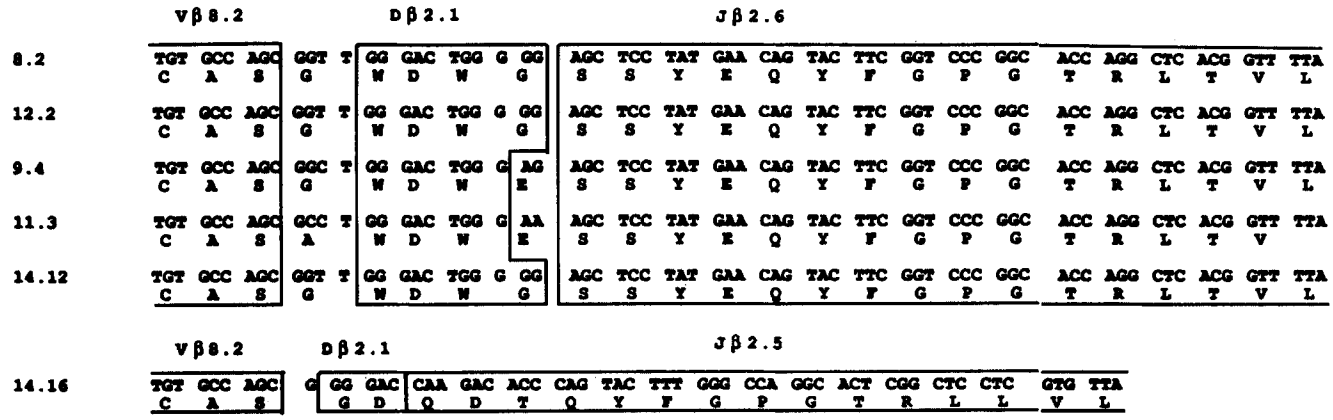

Figure 2. TCR β chain expression by the I-E^d/110–121–specific clones was quite homogeneous. The nucleotide sequences of the TCR β chains expressed by the SpWMb-reactive clones were determined. The alignment is based upon common use of the Vβ8.2 segment.

acids. These include two tryptophane residues that contribute bulky side chains to this region of the molecule. Notably, 8.2 and 14.12, which differ in recognition of SpWMb and HMb, and alloreactivity, use identical TCR β chains, indicating that these distinctions were determined by TCR α chain residues.

Consensus TCR-α-primed PCR. In contrast to the numerous mAbs directed against TCR Vβ family–specific determinants (35–39), mAb reagents reactive with individual Vα families are not currently available, and molecular probes exist for only 14 of the estimated 20 or more Vα families (27–32, 40). Northern analysis with 10 Vα family probes did not identify Vα usage in all six SpWMb T cell clones. To overcome the requirement for sequence information about the target gene to define fully homologous PCR primers flanking the region of interest for amplification, we designed a degenerate 5′ primer specific for a sequence that is relatively conserved among Vα segments (see Materials and Methods). In concert with a perfectly homologous primer specific for the 3′ terminus of Cα, the 216-fold degenerate consensus

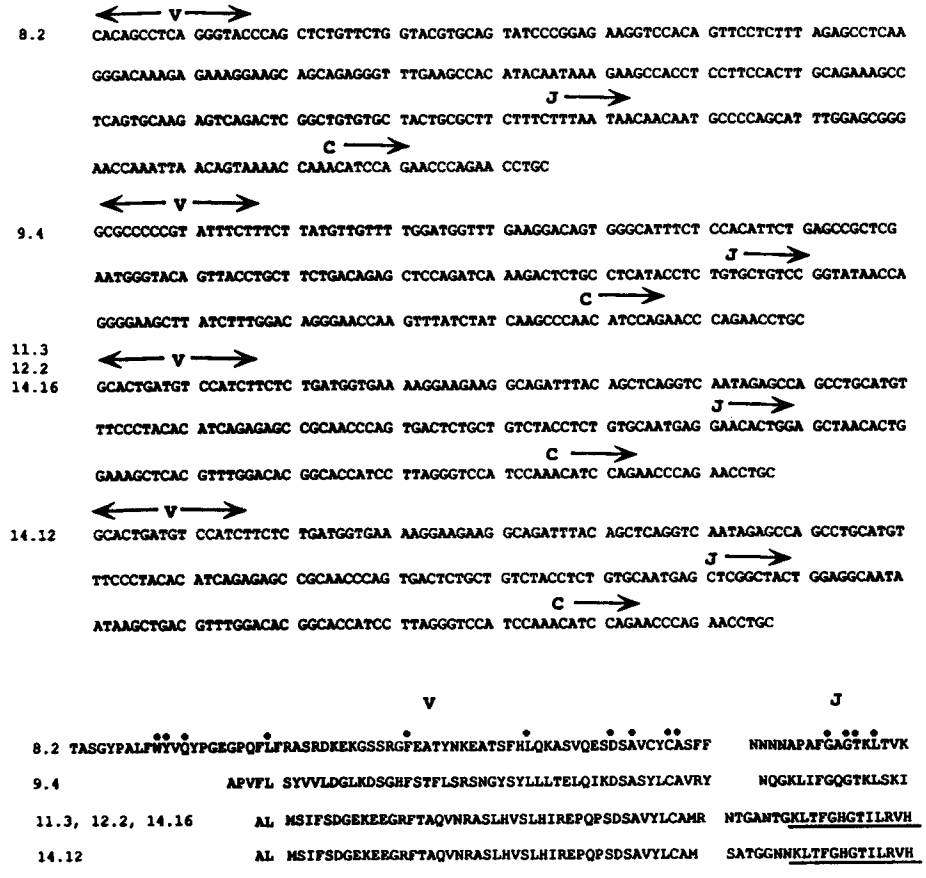

Figure 3. The TCR-α sequences of the 110–121-reactive I-E^d-restricted T cell clones were heterogeneous. Sequence analysis of the TCR α chains expressed in the T cell clones was performed. Clone 8.2 is a member of the Vα4 family, clones 11.3, 12.2, 14.12, and 14.16 are members of the Vα1 family (27, 42). Clone 9.4 defines a new family provisionally called Vα15. Given the truncation of 35 amino acids on the NH_2 terminus of the Vα regions amplified in this way, all conclusions are based upon the identity or dissimilarity of the COOH-terminal 2/3 of the Vα and entire Jα segment. Included is the CDR2 segment that differs in nucleotide sequence between different members of the same Vα family. Spaces were introduced to align amino acid residues that are highly conserved among V regions (+). A portion of the Jα of clones 11.3, 12.2, 14.16, and 14.12 is underlined to emphasize their partial homology.

primer was used to amplify cDNA from T cell clones expressing unknown TCR Vα regions (Fig. 3). To amplify many (possibly all) Vα families and include the longest possible segment of TCR α chain sequence with the consensus oligo primer, we selected a segment beginning at amino acid 33, incorporating the conserved WYXXQ motif. The resultant PCR-amplified products are truncated at their 5′ ends by ~100 bp. For applications in which full-length cDNA is desired, a primer extension reaction, using mRNA from the T cells of interest as a template, can readily incorporate the missing sequence (26). Since the consensus primer was potentially mismatched to a given Vα cDNA at multiple positions, the thermal cycling conditions were modified to permit lower stringency priming in early cycles without excessive replication of poorly matched templates later in the reaction (see Materials and Methods).

TCR α Chains of the SpWMb-specific T Cell Clones. PCR amplification of TCR-α sequences expressed by five of the SpWMb clones was performed using the consensus Vα primer (Fig. 3). Because of the consensus primer location, sequence was determined for 55–60 codons of Vα, including the putative CDR2, Jα, and any bases inserted or deleted between the V/J segments (CDR3) (Fig. 3). Because different members of the known Vα families differ in nucleotide and amino acid sequence 3′ or COOH terminal of the consensus primer location (28, 29, 32, 40), the identity of two Vα segments amplified by this method almost certainly reflects identity of their full-length sequences. In contrast to the nearly identical TCR β chains expressed by the six SpWMb-reactive clones, three Vα and four Jα sequences were found among them. Clone 8.2 expressed a member of the Vα4 family, clones 11.3, 12.2, 14.12, and 14.16 expressed a Vα included in the Vα1 family, and clone 9.4 used a novel Vα provisionally called Vα14. One of the four Jα segments expressed in the six T cell clones has been previously sequenced (41). Only one clone, 14.12, expressed a Jα that lacked an asp as the first amino acid. Since there are an estimated 50 germline Jα genes (42), many of unknown sequence, it is unclear whether this residue was encoded in the germ line or arose from N-region addition. As noted in the TCR β chain junctional regions, the lengths as well as the sequences of these Jα segments, which may influence the actual conformation of the putative TCR-α CDR3 region, differed by up to four amino acids. Clones 11.3, 12.2, 14.16, and 14.12 express indistinguishable Vα segments; however, the V/Jα junctional region of 14.12 differs in sequence over a seven-amino acid stretch, and is one residue shorter than the equivalent segment of the other three clones. In addition, clone 14.12 differs in myoglobin fine specificity and alloreactivity from clone 12.2 with which it shares a common TCR β chain sequence. Therefore, both of these differences in T cell reactivity between clones 12.2 and 14.12 map to the eight amino acids in the TCR-α junctional region.

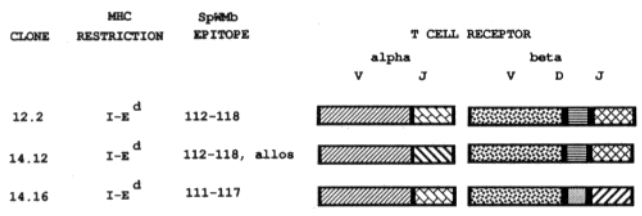

Figure 4. Differences in T cell fine specificity for SpWHb are associated with both α and β chain junctional sequences: the presumed CDR3 region of the TCR. SpWMb-reactive clones were tested for epitope specificity, MHC restriction, and alloreactivity, as described in text. The TCR from the clones were PCR amplified and sequenced as described. A schematic representation of the receptors is shown.

Discussion

Depicted in Fig. 4 are data for three clones, 12.2, 14.12, and 14.16, that illustrate the major finding of this study. Clones 12.2 and 14.16 express identical TCR-α and Vβ sequences, demonstrating that their specificity for I-E^d plus either SpWMb 112–118 or 111–117, respectively, arose from disparities in their TCR D/Jβ region: the TCR Vβ CDR3 equivalent. Clones 12.2 and 14.12 utilized identical TCR-β and Vα sequences, but distinct TCR Jα segments that conferred alloantigen and antigen specificity recognition differences between these two clones (Fig. 4). As indicated in Fig. 3, the COOH-terminal portion of the two Jα elements that distinguish these two clones are identical in sequence, further refining the critical residues to those adjacent to the V/J junction (the presumed CDR3α). Thus, differences in either the presumptive CDR3α or the CDR3β sequence generated functional alterations in T cell recognition of antigenic peptides despite identical MHC restriction of these clones.

Several additional points arise from the TCR sequence data reported here. Clones of indistinguishable MHC/antigen specificity (8.2, 9.4, 11.3, 12.2) expressed a virtually invariant TCR β chain and three Vα/Jα combinations, suggesting that similar receptor-binding sites can be generated by multiple TCR α chains paired with a given TCR β chain. However, evaluation of the reactivity of these four clones to peptides containing substitutions in the 110–121 residues (studies now in progress) is necessary to conclude that epitope recognition, conferred by all three TCR-α sequences, is identical. Previous analysis of T cell clones specific for the NH_2 terminus of myelin basic protein has also demonstrated that multiple combinations of TCR V/Jα paired with a Vβ8.2-containing TCR β chain can afford nearly identical antigen-binding sites (15, 43).

Prior studies of cytochrome *C*–reactive T cells suggested that a single, highly conserved TCR β chain amino acid at the V/D junction was important in peptide specificity (12, 44). Site-specific mutation of this residue resulted in decreased alloreactivity and cytochrome *C* dose response, without concomitant effects on MHC restriction (45). Since most of the COOH-terminal portion of the Jβ segments expressed by clones 12.2 and 14.16 are homologous, the fine specificity difference maps to the Dβ1 Jβ junction and the first few residues of the Jβ germline elements. Within this confined region, the TCR β chains of the clones vary in length by four amino acids. The Fab model of the TCR predicts that

these residues comprise the CDR3β loop; thus, its conformation should differ substantially between clones 11.3 and 14.16. Mutagenesis experiments will be required to determine how the length and amino acid composition of the putative CDR3β affects peptide recognition.

Generation of a portion of these data has been dependent upon a modification of the PCR technique that permitted amplification of TCR α chains without prior assignment of the Vα segments they contain, through use of a degenerate, consensus 5′ oligonucleotide primer. Application of this consensus primer PCR modification can be extended to questions of TCR α chain repertoire of rare cells within clonally diverse populations in vivo, as well as T cell clones. Analysis of amplified α chains from unfractionated thymocytes indicated that all Vα families for which we currently have probes (Vα 1-8, 10, 11, 13) were readily detectable in the PCR products, confirming the broad specificity of the degenerate primers. Recent experiments have indicated that this consensus PCR technique is sufficiently sensitive to allow TCR-α sequence assessment from aliquots of 10 thymocytes (Guidos, C.J., J.S. Danska, C.G. Fathman, and I.L. Weissman, manuscript submitted for publication).

to MHC/antigen complexes that align α and β CDR1 and CDR2 with MHC determinants, and the most variable CDR3 α and β chain segments with antigenic determinants (4, 5). This model provides an extremely useful framework for examining potential correlations between TCR structure and specificity but must not be mistaken for high resolution structural data. TCR CDR sequences differ from those of Ig, and the details of receptor/ligand contact will undoubtedly differ between the two classes. In addition, it is unclear whether the TCR can bind in different registers or in multiple orientations along MHC α helices, such that more than one set of amino acid contacts between TCR and ligand are possible. Furthermore, the conformational flexibility of the peptide with the groove is unknown. Resolution of these issues awaits crystallographic analyses.

Our data demonstrate the influence of both α and β chain junctional sequences (CDR3 equivalents) on T cell recognition of epitope specificity exclusive of MHC restriction, and serve to substantiate the model's presumed spacial orientation of the CDR3 regions when the TCR is engaged with its ligand.

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