The structural basis of antigen recognition by antibody molecules has been intensely studied using x-ray crystallography. Crystal structures of a large array of antibodies and antibody-antigen complexes have demonstrated that the hypervariable regions of Ig genes encode solvent-exposed loops that cluster to form the antigen-binding site of the Ig V domains. These loops are known as CDRs. Analysis of Ig crystal structures and CDR sequences has shown that the spatial orientation and conformation of the loops is influenced by two factors.

The first concerns the CDR itself. Each of the CDR loops appears to adopt a limited range of main chain conformations despite their diverse amino acid sequences (1). These conformations are shaped by a small number of relatively well-conserved amino acid residues within the loops. The second factor is the V domain scaffolding upon which the CDRs rest. It consists of two β sheets packed face to face and anchored by a structurally conserved core of “framework” residues. The conformations of CDR loops are partially determined by the manner in which they pack with the side chains of certain framework residues (1, 2). An understanding of these factors has resulted in a limited ability to predict CDR loop conformations of IgS of unknown crystal structure from the sequences of their heavy and light chain genes (1). This is a first step towards the engineering of antibodies specific for antigens of interest.

The α/β T cell receptor (TCR) recognizes peptide fragments bound in the groove of major histocompatibility complex (MHC) molecules. We modified the TCR α-chain from a mouse T cell hybridoma and tested its ability to reconstitute TCR expression and function in an α-chain-deficient variant of the hybridoma. The modified α-chain differed from wild type only in its leader peptide and mature NH$_2$-terminal amino acid. Reconstituted cell surface TCR complexes reacted normally with anti-TCR and anti-CD3 antibodies. Although cross-linking of this TCR with an antibody to the TCR idotype elicited vigorous T cell hybridoma activation, stimulation with its natural MHC + peptide ligand did not. We demonstrated that this phenotype could be reproduced simply by substituting the glutamic acid (E) at the mature NH$_2$ terminus of the wild type TCR α-chain with aspartic acid (D). The substitution also dramatically reduced the affinity of soluble α/β-TCR heterodimers for soluble MHC + peptide molecules in a cell-free system, suggesting that it did not exert its effect simply by disrupting TCR interactions with accessory molecules on the hybridoma. These results demonstrate for the first time that amino acids which are not in the canonical TCR complementarity determining regions can be critical in determining how the TCR engages MHC + peptide.
Materials and Methods

Cell Lines. The mouse T cell hybridoma used in these studies was 2B10.D2-22.3 (22.3) specific for chicken OVA peptides 323–339 or 327–339 presented by IA<sup>d</sup> (IA<sup>d</sup>/OVA). It was generated by fusing the AKR thymoma fusion partner BW-1100, 129.237 (BW α<sup>-</sup>/β<sup>-</sup>∶18) to lymph node cells from a B10.D2 mouse immunized with OVA 327–339 (Kushnir, E., unpublished data). The TCR α and β chains of 22.3 are identical in amino acid sequence to those of DO-11.10, a previously described T cell hybridoma with the same specificity (19, 20). Therefore, these chains are designated DO α<sup>a</sup> and DO β<sup>a</sup> in this paper. Unlike DO-11.10, however, 22.3 did not express any other functional TCR α<sup>b</sup> or β<sup>b</sup> genes. 22.3 was recloned to isolate two subclones, 22.3.111 and 22.3.145, which bear lower levels of surface TCR. In addition, a spontaneous TCR α chain loss variant of 22.3 was isolated, 22.3x<sup>−</sup>. This variant expresses DO β mRNAs, but does not contain DNA encoding DO α<sup>a</sup> and therefore was useful as a recipient for chimeric transfection studies.

Two-color Flow Cytometry. T cell hybridomas were stained for cell surface molecules and analyzed by flow cytometry as previously described (21) using the following mAbs: KJ1-26 (22) is specific for the idotype of the TCR on DO-11.10 and 22.3, and H57-597 (23), 145-2C11 (24), and GK1.5 (25) are specific for mouse TCR C<sup>b</sup>, CD3<sup>e</sup>, and CD4, respectively. In brief, 2–5 × 10<sup>5</sup> hybridoma cells were incubated with biotinylated KJ1-26 or H57-597 at 4°C for 20 min. Cells were washed three times and incubated at 4°C for 20 min with FITC-conjugated 145-2C11 or GK1.5 and streptavidin-R–conjugated phycoerythrin (Sigma Chemical Co., St. Louis, MO). Finally, cells were washed three times and two-color fluorescence data was acquired on either an Epics C or a Profile flow cytometer (Coulter Corp., Hialeah, FL).

Cell Hybridoma Stimulation Assays. T cell hybridoma activation in response to TCR ligation was assayed by measuring IL-2 secretion, as described previously (26). Stimulation assays were performed in 96-well flat-bottomed microtiter plates. In peptide stimulations 10<sup>5</sup> IA<sup>a</sup>-expressing A20.1-11.B5 B cell lymphoma cells (27) were added per well with different concentrations of OVA 327–339. For anti-TCR mAb stimulation, Immulon-3 (Dynatech Labs, Chantilly, VA) microtiter plates were coated overnight at 4°C with serial dilutions of protein A–purified KJ1-26 or GK1.5 streptavidin (Sigma Chemical Co., St. Louis, MO). Cells were washed three times and incubated at 4°C for 20 min with FITC-conjugated 145-2C11 or GK1.5 and streptavidin-R–conjugated phycoerythrin (Sigma Chemical Co., St. Louis, MO). Finally, cells were washed three times and two-color fluorescence data was acquired on either an Epics C or a Profile flow cytometer (Coulter Corp., Hialeah, FL).

Characterization of the V<sub>α</sub>13.1 leader from 22.3. Using an anchored PCR strategy modified from Loh et al. (30), DNA encoding the V<sub>α</sub>13.1 (AV13S2) leader was cloned to determine the unknown sequence of its 5′ end. Total RNA was prepared from the 22.3 hybridoma using the Ultraspec reagent (Biotex Labs, Houston, TX). Oligo(dT)-primed first strand cDNA was synthesized using the SuperScript reverse transcriptase cDNA synthesis kit (GIBCO BRL, Gaithersburg, MD) and was tailed at the 5′ end with dG residues using terminal deoxynucleotidyl transferase (International Biotechnologies Inc., New Haven, CT). TCR α chain gene sequences were PCR amplified from tailed cDNA using the anchored sense primer 5′-ATCGAGTCGACG-CCCCCC-3′ and the antisense primer 5′-CCCGAGACGCGCGTCCTTGACG-3′, synthesized at the National Jewish Molecular Resource Center (Denver, CO). The latter primer anneals to an insertion in the 5′ untranslated region of TCR α chain transcripts that is present in C57 mouse strains, but not AKR or BALB/c strains (31, 32, and Seibel, J., unpublished data). This avoided amplification of the nonfunctional TCR α chain transcripts contributed to the 22.3 hybridoma by the AKR thymoma fusion partner BW α<sup>-</sup>/β<sup>-</sup> (33, 34). This anchored PCR product was reamplified using the same anchored sense primer and the antisense primer 5′-TCTCGAATTTGCGAGAGAGGCAGG-3′ (35), yielding a product that was digested with SalI and EcoRI and cloned into the pT2Z18R vector (Pharmacia, Piscataway, NJ) for sequencing. The complete cDNA sequence of the V<sub>α</sub>13.1 leader and its 5′ untranslated region are presented in Fig. 1.

Production of Soluble TCRs and IA<sup>d</sup> Protein. A vector plasmid was constructed for expression of the DO<sup>a</sup> chain using a XhoI site at the 5′-end of the DO<sup>a</sup> segment and an introduced AccIII site at the 5′-end of C<sub>α</sub> (36), such that the final complete α chain was flanked by EcoRI sites. To express these variant DO<sup>a</sup> constructs in the 22.3x<sup>−</sup> hybridoma, the EcoRI fragments were isolated and cloned into the EcoRI site of the murine retrovirus-based vector, LXSN (38). Using methods modified from Miller et al. (38), DO<sup>a</sup> RNAs were packaged into retroviruses in the GP<sub>=</sub>env AM12 (39) and PA-317 (40) cell lines, and were introduced into the 22.3x<sup>−</sup> hybridoma by infection. Infected with stably integrated DO<sup>a</sup> genes were selected and maintained with culture medium containing 1–2 mg/ml G418 (Geneticin; GIBCO BRL).

Figure 1. Complete nucleotide sequence of the V<sub>α</sub>13.1 leader and its 5′ untranslated region. The sequence of the V<sub>α</sub>13.1 leader was assembled from six independently cloned PCR products of different lengths, which were obtained by anchored reverse transcriptase PCR from the B10.D2-derived T cell hybridoma. 22.3. Deduced amino acid residues are shown. Underlined triplets in the 5′ untranslated region indicate stop codons, which are found in all three reading frames. The V<sub>α</sub>13.1 leader is shown for comparison. A sequence alignment of the mature variable domains is based on standard mouse TCR α chain sequence alignments (54).
Recombinant baculoviruses prepared from these vectors coexpressing the TCR-α and -β genes were used to infect Hi5 insect cells (Invitrogen, San Diego, CA). Soluble heterodimers were purified from cell supernatants by immunoaffinity chromatography on an antiidiotype column, followed by a HiLoad 26.60 Superdex200 size exclusion column (Pharmacia). The proteins were further purified to a single detectable species using Mono Q Superdex200 size exclusion column (Pharmacia). The proteins were purified from cell supernatants by immunoaffinity chromatography on an antiidiotype column, followed by a HiLoad 26.60 Superdex200 size exclusion column (Pharmacia). The proteins were further purified to a single detectable species using Mono Q Superdex200 size exclusion column (Pharmacia). The proteins were further purified to a single detectable species using Mono Q Superdex200 size exclusion column (Pharmacia). The proteins were further purified to a single detectable species using Mono Q Superdex200 size exclusion column (Pharmacia). The proteins were further purified to a single detectable species using Mono Q Superdex200 size exclusion column (Pharmacia).

Results

Expression of a TCR α Chain in an α Chain-deficient Mouse T Cell Hybridoma. To study the effects of mutations in the TCR α chain on MHC + peptide recognition by T cells, we used a mouse T cell hybridoma, 22.3, which could be stimulated by either IA<sup>α</sup> class II MHC molecules bound to chicken OVA 327-339 (IA<sup>α</sup>/OV A) or by the anti-TCR idiotype mAb, KJ1-26 (22). In an initial experiment, receptor expression in an α loss variant of this hybridoma, 22.3<sup>α−</sup>, was restored by infection with a retrovirus carrying a version of the full-length Vα13.1 bearing DO α chain gene. This construction, DO αL11terD, differed from the natural DO α chain in two respects. First, it contained a Vα11.2 leader (L11) in lieu of the then unknown native Vα11.1 leader (L113). Second, after cleavage of its leader peptide, the mature NH<sub>2</sub> terminus of the DO αL11terD chain was aspartic acid (terD), instead of the glutamic acid (terE) of wild-type DO α. This conservative substitution was introduced to maintain a convenient cloning site. It was not expected to affect TCR specificity, since we had previously shown that it had no detectable effect on the binding of a number of anti-TCR mAbs, including KJ1-26, an mAb specific for the receptor idiotype (Kappler, J., unpublished data).

The infectants were screened for cell surface TCR expression by staining with mAbs to the DO TCR idiotype and CD3ε chain. Their levels of idiotype-reactive TCR were slightly lower than that of the parental hybridoma, 22.3, but were equal to or greater than those on two low TCR -expressing subclones of 22.3, 22.3.111, and 22.3.145 (Fig. 3). Cell surface levels of CD3ε suggested proper TCR association with CD3 components (data not shown). These staining results suggested that the DO αL11terD protein folded properly, associated normally with the TCR β chain and CD3 components, and reconstituted cell surface TCR complexes of normal structure.

To determine whether the structural integrity of the TCR complexes on the infectants was also reflected in functional competence, the infectants were cultured in plastic wells coated with antiidiotype mAb. In response to this TCR ligation and cross-linking stimulus, the infectants secreted IL-2 in a manner similar to that of the wild-type

![Figure 2](image-url)  
**Figure 2.** DO α chain variant constructs. Circled letters indicate the predicted NH<sub>2</sub>-terminal amino acid of the mature α chain.

![Figure 3](image-url)  
**Figure 3.** Infectants bearing the variant TCR α chain DO αL11terD respond to immobilized antiidiotype mAb as well as the parental hybridoma, 22.3, does. 22.3.111 and 22.3.145 are low TCR-expressing subclones of 22.3. Data are representative of multiple experiments. TCR level indicates the mean linear fluorescence of antiidiotype mAb staining of the hybridoma.
hybridoma, 22.3 (Fig. 3). This suggested that the TCR complexes containing the DO\textsubscript{\alpha}\textsuperscript{L11terD} chain were sufficient for normal ligation-induced signaling within the T cell hybridoma.

Replacement of the 22.3 \(\alpha\) Chain with the DO\textsubscript{\alpha}\textsuperscript{L11terD} Chain Resulted in a Hybridoma with Dramatically Reduced Ability to Respond to IAd/OVA. Since DO\textsubscript{\alpha}\textsuperscript{L11terD}-containing TCR heterodimers mediated a vigorous IL-2 response to anti-TCR antibody stimulation, it was surprising that they did not reconstitute good IAd/OVA reactivity. As shown in Fig. 4, DO\textsubscript{\alpha}\textsuperscript{L11terD} infectants responded much more poorly to IAd/OVA than did the parental hybridoma, 22.3. Since the TCR \(\alpha\) chain in these infectants differed from that of 22.3 only in its leader peptide and the substitution of D for E at the mature NH\textsubscript{2} terminus, one or both of these changes must have caused the impaired responsiveness. The amino acid sequence of the leader might, for example, have altered the site at which the leader is trimmed from the mature protein. Such an effect has been described in several proteins (43–47), including an antidigoxin antibody in which single substitutions in the heavy chain leader peptide changed the length of the mature chain by up to 10 residues (48). Some of these length changes were sufficient to change the affinity of the antibody. An alternative hypothesis was that the NH\textsubscript{2} terminus of the TCR chain influenced directly or indirectly TCR interaction with MHC peptide.

Responsiveness to IAd/OVA was restored in infectants expressing TCR \(\alpha\) Chains with E at the predicted NH\textsubscript{2} terminus, regardless of V\textsubscript{\alpha}11.2 or V\textsubscript{\alpha}13.1 leader peptide usage. To identify individually the contributions of leader peptide and NH\textsubscript{2} terminus to DO\textsubscript{\alpha} chain function, we generated four types of 22.3 \(\alpha\) infectants with each possible combination of L11, L13, terD, and terE (Fig. 2). The previously uncharacterized L13 leader was cloned from 22.3 (Fig. 1, Materials and Methods). Infectant clones that expressed comparable levels of cell surface TCR were selected by staining with antiidiotype mAb. Four clones of each type were

**Figure 4.** Infectants bearing the variant TCR \(\alpha\) chain DO\textsubscript{\alpha}\textsuperscript{L11terD} respond poorly to IAd/OVA stimulation. cOVA 327–339 peptide was presented to the hybridomas by the IAd-expressing B cell lymphoma, A20. Data points indicate the means of duplicate wells in one representative experiment.

**Figure 5.** Infectants bearing any one of four DO TCR \(\alpha\) chain variants respond equally well to immobilized antiidiotype mAb. Data points represent the means of two independent experiments with duplicate wells. Error bars indicate standard errors of the mean. Four infectants of each variant type were tested. TCR levels of all infectants varied by less than twofold.

Amount of antiidiotype antibody offered to plate (ng/ml)
tested for their ability to respond to antiidiotype antibody and to IA\(^+\)/OVA. All four types of infectants possessed comparable signal transduction capacity, since they produced similar levels of IL-2 when stimulated with immobilized antiidiotype antibody (Fig. 5). We do not know why one DO\(\alpha\)L13terD infectant responded more poorly than the other three.

In contrast, the infectants were readily divided into two groups based on their ability to respond to IA\(^+\)/OVA (Fig. 6). Regardless of the leader peptide they used, infectants expressing D at the NH\(_2\) terminus of their TCR \(\alpha\) chains (DO\(\alpha\)L11terD and DO\(\alpha\)L13terD) responded very poorly. By contrast, infectants expressing NH\(_2\)-terminal E (DO\(\alpha\)L11terE and DO\(\alpha\)L13terE) responded strongly.

The Mature NH\(_2\) Termini of Variant DO\(\alpha\) Chains Produced as Soluble Proteins in Baculovirus Were as Predicted. These experiments demonstrated that the leader peptide sequence did not affect the function of the DO\(\alpha\) chain variants, and highlighted the importance of the predicted NH\(_2\) terminus of the mature protein for IA\(^+\)/OVA responses. However, it was still formally possible that the E and D containing \(\alpha\) chains might have had their signal peptides cleaved at different sites. This would have made the two types of chains different in length. Some structural aspect of the length disparity could have directly affected the interaction of the TCR with MHC + peptide, but not with anti-TCR antibodies.

To test this hypothesis, genes encoding soluble DO\(\alpha\) chains with predicted NH\(_2\) termini of either E or D were coexpressed in insect cells with a gene encoding a soluble DO\(\beta\) chain. Purified DO\(\alpha/\beta\) heterodimers were sequenced by Edman degradation. Both DO\(\alpha\) variants were found to bear the predicted NH\(_2\)-terminal amino acid, E for DO\(\alpha\)L13terE and D for DO\(\alpha\)terD. Since insect signal peptidases typically cleave at the same site as their mammalian counterparts (49), we concluded that TCR \(\alpha\) chains in our DO\(\alpha\)terE and DO\(\alpha\)terD infectant hybridomas were identical in length, differing only in the identity of the amino acid at their NH\(_2\) termini. This difference alone must have been responsible for their contrasting responses to IA\(^+\)/OVA.

Substitution of the NH\(_2\)-terminal Amino Acid of the DO\(\alpha\) Chain Affects the Affinity of the DO TCR for MHC + peptide. The simplest explanation for our results was that the substitution of D for E at the NH\(_2\) terminus of DO\(\alpha\) \(\alpha\) directly altered the ability of the TCR to bind to IA\(^+\)/OVA. However, it was also possible that the substitution acted indirectly. For example, it could have disrupted TCR interactions with a T cell accessory molecule such as CD4. Such an interaction may not have been required for activation of the hybridoma during stimulation with antiidiotype antibody, where large numbers of TCRs were ligated. However, it may have been critical for responses to IA\(^+\)/OVA stimulation, where only a few TCRs on each hybridoma were engaged. To distinguish between these mechanisms, we used surface plasmon resonance to measure directly the binding of soluble IA\(^4\)-OVA (40) to DO\(\alpha/\beta\) heterodimers.
Anti-C β mAb was used to immobilize DO αβ heterodimers containing either DO αterE or DO αterD chains in separate flow cells of a biosensor chip. A flow cell in which the free DOβ chain was immobilized was used as a negative control. Various concentrations of IAγ-OVA were passed through the flow cells and the binding kinetics followed (Fig. 7). Obvious binding of IAγ-OVA to a TCR containing DO αterE was seen at all class II concentrations. The interaction had a relatively slow on rate, k\text{a} = 1.60 \times 10^3 \text{ M}^{-1} \text{s}^{-1}, and a fast off rate, k\text{d} = 0.05 \text{ s}^{-1}, resulting in a dissociation constant (K\text{d}) of 31 \text{ μM}. These kinetic and thermodynamic constants are similar to those observed for other TCRs binding to MHC class II + antigen complexes (50). In contrast, IAγ-OVA bound very poorly to a TCR containing DO αterD. Very weak specific binding detected only at the highest concentration of IAγ-OVA. This suggested a dissociation constant \text{>300 μM}. The results showed that the NH2 terminal E to D substitution in DOα disrupted TCR binding to class II + peptide in the absence of accessory molecules. We conclude that N terminal E must play a direct role in the interaction of this TCR with IAα/OVA.

**Discussion**

We have demonstrated that an E to D change in the NH2-terminal amino acid of a mouse TCR α chain significantly altered the affinity of the αβ-TCR heterodimer for its MHC + peptide ligand. This conservative change simply shortened the amino acid side chain by a single methylene group without changing its negative charge. This effect did not result in a gross alteration in TCR structure, since detection of the TCR complexes by anti-TCR mAbs was unaffected by the substitution. Moreover, overall TCR-mediated signal transduction was not impaired by the substitution, since hybridomas bearing it responded normally to TCR cross-linking by immobilized antidiotypic mAb.

The E to D substitution could have affected MHC + peptide responsiveness either directly, by disrupting a contact between this amino acid and the ligand, or indirectly, by altering the shape or position of amino acids on one of the receptor CDR loops. The recent crystal structures of TCR Vαs both free and as part of TCR + MHC complexes suggest that either of these possibilities is feasible (Fig. 8, references 12–14). These structures include a free Vα domain, mouse Vα4 (AV4S1), and two TCRs bound to their MHC class I + peptide ligands. These latter TCRs contained mouse Vα3 (AV3S1) and human Vα2 (AV2S1).

In all three structures, the Vα NH2-terminal amino acid is solvent exposed and in contact with the receptor CDR loops. The recent crystal structures of TCR Vαs both free and as part of TCR + MHC complexes suggest that either of these possibilities is feasible (Fig. 8, references 12–14). These structures include a free Vα domain, mouse Vα4 (AV4S1), and two TCRs bound to their MHC class I + peptide ligands. These latter TCRs contained mouse Vα3 (AV3S1) and human Vα2 (AV2S1).

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Support for this notion comes from the study of Ig structures. One study comparing Ig crystal structures concluded that the conformation and position of CDR2 of the Ig heavy chain is largely shaped by the nature of the side chain on framework residue 71 (2). In another study, a spontaneous variant of an antidigoxin antibody with 580-fold less affinity for digoxin than wild type was found to have a substitution from S to R at position 94 in its heavy chain, a residue predicted to lie in the framework at the base of CDR3 (48, 51–53). Computer modeling suggested that the substitution increased hydrogen bonding between CDR3 of the heavy chain and CDR2 of the light chain so that the digoxin binding surface of the Ig was altered. Interestingly, most of the substitution's effect was reversed if two residues from the NH2 terminus of the heavy chain were removed. Modeling suggested that the deletion increased the solvent accessibility of R94, destabilizing the aberrant hydrogen bonding and returning the CDR loop structures to wild type.
In the two TCR + MHC class I crystal structures, the side chain of the Vα NH2-terminal amino acid does not appear to make direct contacts with the MHC ligand and, in fact, the side chain of the NH2-terminal lysine of human Vα2 appears disordered in the structure (14). However, the free NH2-terminal amino group of the Vα2 chain itself is connected by a salt bridge to a conserved glutamic acid in the α helical region of the MHC class I α1 domain (14). Such a salt bridge is not found in the TCR + MHC class I structure containing mouse Vα3 (13), and probably cannot be a general feature of TCR + MHC class II complexes since class II lacks the conserved glutamic acid residue in the α helix of its α1 domain. However, the overall positions of the Vα NH2-termini in the crystals suggest that depending on the exact orientation and pitch of the TCR on its MHC ligand, direct interaction between the Vα NH2-terminal amino acid side chain and the MHC ligand may not be an infrequent feature of the complex.

Although further work will be necessary to confirm the generality of our results with this particular α/β-TCR, our findings suggest that gene constructs used to express soluble TCRs for use in binding assays and x-ray crystallography must be designed carefully. Substitutions at non-CDR residues must be introduced with the knowledge that they have the potential to disrupt MHC + peptide recognition. A detailed understanding of the role of TCR framework residues in MHC + peptide recognition will be difficult to attain until numerous TCR and TCR + MHC + peptide crystal structures have been solved.

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