Functional Analysis of the Antigen Binding Site on the T Cell Receptor α Chain

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Summary

We have identified residues on a T cell receptor (TCR) α chain that are important for interaction with antigen/major histocompatibility complex (MHC). Using site-directed mutagenesis, we modified DNA encoding the postulated antigen/MHC binding loops on the TCR α chain expressed by the T cell clone D5, which recognizes *p*-azobenzenearsonate–conjugated antigens presented by cells bearing I-A^d. These variant TCR α chains were expressed in conjunction with the wild-type D5 TCR β chain on the surface of hybridoma cells, and were tested for the ability to recognize hapten-conjugated antigens presented by I-A^d. Individual amino acid substitutions in each of the three antigen binding loops (α1, α2, α3) of the D5 TCR α chain affected antigen recognition, demonstrating that all three loops are important in recognition of antigen/MHC. A subset of the single amino acid substitutions completely eliminated antigen recognition, thus identifying the residues that are particularly important in the recognition of antigenic peptide/MHC by the D5 TCR. Because the wild-type D5 TCR recognizes arsonate and certain structural analogues of arsonate conjugated to a variety of protein antigens, we were able to test whether the TCR substitutions affected the specificity of the D5 TCR for hapten or carrier antigen. One substitution introduced into antigen binding loop α3 markedly altered the pattern of carrier recognition. Together, these results verify the Ig model for the TCR and are consistent with the proposition that residues forming the first and second antigen binding loops of the TCR contact the MHC, while those forming the third loop contact mainly antigenic peptides.

Recognition of foreign antigenic peptides physically associated with proteins encoded in the MHC is accomplished by the α/β (or γ/δ) polypeptide chains of the TCR (reviewed in reference 1). Sequence similarity of the amino acids in V and C domains of the TCR and Ig chains, especially in certain residues known to be critical in maintaining Ig structure, suggests that the secondary and tertiary structure of the TCR resembles that of Ig Fab fragments (2–4). By analogy to the antigen binding site on Ig, three regions of each TCR chain, formed by loops between β sheets, are thought to contact the antigenic peptide/MHC protein (5–7). Residues in antigen binding loops α1 and α2 on the α chain and β1 and β2 on the β chain are encoded by the variable gene segments, while residues in antigen binding loops α3 on the α chain and β3 on the β chain are encoded by the V-(D)-J junction.

Based on the proposed spatial similarities between TCR and Ig antigen binding loops, and their projection onto the known three-dimensional structure of a human MHC protein, it has been postulated that TCR residues in the first and second antigen binding loops contact mainly residues of the MHC protein, while TCR residues in the third antigen binding loop contact antigenic peptides (5–7). This model is consistent with frequently observed correlations between the usage of particular TCR variable gene segments (or residues therein) and antigen/MHC reactivities (reviewed in reference 1). However, the relative importance of each of the six postulated antigen binding loops in recognition of antigenic peptides/MHC by a single TCR remains unclear. Consequently, it is not known whether all six antigen binding loops simultaneously contact the antigenic peptide/MHC. Moreover, it is not known how residues on each antigen binding loop contribute to TCR contact with the antigenic peptide/MHC. These issues must be resolved in order to understand the molecular basis of TCR recognition.

We have developed a gene transfer system to test the relative importance of particular residues of the TCR in recognition of antigen/MHC (8). In this report we provide an extensive survey of residues of a TCR α chain using site-directed mutagenesis. We show that amino acid substitutions at more than one position in all three of the proposed antigen binding loops of a TCR α chain markedly affect antigen recognition. Moreover, an amino acid substitution in antigen binding loop α3 altered TCR specificity for peptide

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antigen. These results support the structural model for the TCR based upon the known structure of Ig, demonstrating that all three antigen binding loops on the TCR α chain are required for recognition of antigenic peptides/MHC.

Materials and Methods

Cells. The arsonate-reactive, I-A^d-restricted T cell clone D5 (Ar-5), which utilizes Vα3.1/JαTA20' and Vβ2/Dβ2-2/Jβ2-5 gene segments, has been described previously (9, 10). Its TCR-negative variant GLS11α^-, which contains a deletion of the Vα gene segment expressed in D5 cells yet continues to express a functional TCR β chain, has been characterized previously (8, 11). GLS11α^- was fused with the receptor-negative subline of BW5147, BW-1100.129.237 (12), to generate the hybridoma GLS11hα^- (8). Subclone 11h.3α^- of the GLS11hα^- cell line was used for transfection with plasmids described below. Transfectants expressing the double amino acid substitution D5α(Y26S,G28V) are D5αM1 cells described previously (8). Culture supernatant from the hamster hybridoma 145-2C11 (2C11) was used as a source of antibody to the murine CD3-ϵ chain (13). Culture supernatant from the rat hybridoma B20.6 (14) was used as a source of antibody to murine Vβ2 and was a gift from B. Malissen (INSERM-CNRS, France). TA3 (Ia^{dxk}) (15) cells were used to present antigens via class II MHC molecules and to crosslink anti-CD3 mAb via F_c receptors. All cells were cultured in medium containing supplemented DME (9).

DNA Constructions. The plasmid pFneoD5αWT has been described previously (8) and contains DNA encoding the wild-type D5 TCR α chain. The DNA used to express the wild-type D5 TCR α chain in the present study (m13pFneoD5αWT) was constructed by ligating the 0.6-kb NheI/SacII fragment containing the m13 origin of replication from the vector CDM8 into the EcoRI site of pFneoD5αWT. The orientation of this fragment was such that superinfection of *dut$^-$ung$^-$* CJ236 bacteria carrying m13p-FneoD5αWT as a plasmid with helper phage M13K07 generated uracil-labeled, single-stranded DNA encoding the D5 TCR in the sense orientation. Antisense mutagenic oligonucleotides, 20 nucleotides in length, were synthesized and contained one to three nucleotide differences positioned equidistant from the ends of the oligonucleotide. Mutagenic plasmids were generated by the method of Kunkel (16) using reagents supplied in the Muta-Gene Kit (Bio-Rad Laboratories, Richmond, CA). Mutant plasmids were selected by transformation into *dut$^+$ung$^+$* bacteria, MC1061 or XL-1 Blue, and were screened by sequencing with dideoxynucleotides using Sequenase (U.S. Biochemical Corp., Cleveland, OH). The integrity of plasmids containing TCR α DNA variants was confirmed by sequencing near the region of interest and by restriction mapping. Sequencing an average of 180 bp of the VJ regions, representing >50% of the entire variable regions, of eight of the variant D5 TCR that decreased antigen responses >10-fold and three that had no effect on antigen responses did not reveal any mutations other than those deliberately introduced. Variant D5 TCR DNA, and cells derived from these DNA transfected into 11hα^- cells, are designated by the amino acid substitutions they carry. For example, D5α(Y26S) represents the substitution of Ser for Tyr at position 26 of the D5 TCR α chain (numbering according to Chothia et al. [5] using the single-letter amino acid code as shown in Fig. 1).

Transfection of 11h.3α^- Cells. 11h.3α^- cells were transfected by electroporation as before with a Cell Porater (Bethesda Research Laboratories, Gaithersburg, MD) (8). In some experiments a Gene Pulser apparatus (Bio-Rad Laboratories) was used; in these cases, 6×10^6 11h.3α^- cells were suspended in 0.75 ml of serum-free DME with 25 μg plasmid DNA (diluted in 10 μl 10 mM Tris, pH 8, 1 mM EDTA), subjected to 250 V, 960 μF, and left at room temperature for an additional 10 min. Cells were cultured in DME containing 10% FCS for 2 d to allow recovery from electroporation. Cells expressing surface TCR were selected by growth in DME containing 5% FCS and 1 mg/ml G-418 sulfate (Gibco Laboratories, Grand Island, NY) for 2 wk, and were isolated after sequential incubation with 2C11 anti-CD3 mAb and goat anti-mouse antibody coupled to magnetic beads (Dynabeads; Dynal, Great Neck, NY) as suggested by the manufacturer. Single cell clones were obtained by limiting dilution and were tested for surface CD3 expression. Clones that were >95% CD3$^+$ were tested for responses to 2C11 anti-CD3 mAb and Ars-Ova, as described below. Representative clones were chosen for further analysis of antigen responses and were maintained in medium containing G418. Surface expression of CD3 was routinely tested in transfectants within 2 wk of antigen assays to ensure that clones remained CD3$^+$. Surface expression of CD4, CD45, and LFA-1 in transfectants exhibiting decreased antigen responses was comparable with that in D5αWT cells, as determined by flow cytometry, indicating that the decreases in antigen recognition observed were not due to the loss of these accessory molecules (data not shown).

Flow Cytometry. 2×10^5 cells were resuspended in 50 μl of 50% FCS in wash solution (2% FCS, 0.1% sodium azide, in PBS) and mixed with 50 μl of primary antibody diluted in wash solution. Final antibody concentrations were 10% 2C11 (or 50% B20.6 rat anti-murine Vβ2) culture supernatant and 0.05% normal hamster serum as control solution. Cells were incubated on ice for 30 min, washed, and incubated on ice for 30 min in 100 μl of secondary antibody containing 5% normal goat serum and PE-conjugated goat anti-mouse antiserum that crossreacts with hamster antibodies (Tago Inc., Burlingame, CA) diluted 1:100 in wash solution. Cells were washed and analyzed via a FACScan® flow cytometer using the FACScan® program (Becton Dickinson & Co., Mountain View, CA).

Immunoprecipitation and Electrophoresis of Iodinated Cell Surface Proteins. Surface proteins of cells expressing transfected TCR chains were iodinated, solubilized with 1% digitonin, and immunoprecipitated with 2C11 anti-CD3 mAb as described (10). The immunoprecipitate was subjected to sequential electrophoresis in a non-equilibrium pH gradient gel (NEPHGE) and 12.5% SDS-PAGE as described (17).

Preparation of Antigens. Intact chicken OVA, turkey OVA (tOVA), bovine gamma globulin (BGG), hen egg lysozyme (HEL), rabbit gamma globulin (RGG), and KLH were conjugated with diazotized *p*-azobenzenearsonate (Ars-), *p*-benzoate (C-), or *p*-azobenzenesulfonate (S-) as described previously (9).

IL-2 Secretion Assay. 10^5 hybridoma cells were cultured with 10^5 TA3 APC in the presence of several dilutions of antigen or stimulatory anti-CD3 mAb in triplicate wells of 96-well microtiter plates for a final volume of 200 μl. Since free antigen concentrations >300 μg/ml were toxic to CTLL-20 cells, APC were prepulsed with antigen when 2,000–3,000 μg/ml antigen was tested. After 20 h, supernatants were harvested and frozen before the assay on CTLL-20 cells. Proliferation of CTLL-20 cells in response to culture supernatants was carried out as described previously (18). Alternatively, viability of CTLL-20 cells was determined 24–48 h later by measuring reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO) by the cells on a Vmax microplate reader (Molecular Devices Corp., Menlo Park, CA) using a reference wavelength of 650 nm and a test wavelength of 570 nm, as described by Tada

et al. (19). Typical standard deviations of triplicate wells were <10% of sample means. As representative examples, standard deviations are indicated in Fig. 4 *B* when greater than one-half of the symbol height. Comparisons were made between a single concentration of supernatants generated from cells bearing variant TCR (usually diluted 1:4) and serial twofold dilutions of supernatants generated from cells bearing the wild-type D5 TCR (usually diluted 1:4 through 1:16). Supernatants in which responses to anti-CD3 mAb by cells bearing wild-type TCR matched most closely to supernatants from cells bearing variant TCR were chosen to calculate relative antigen recognition as: $(\text{antigen}_{WT}/\text{antigen}_{\text{variant}}) \times (\text{2C11}_{\text{variant}}/\text{2C11}_{WT})$; where antigen and 2C11 are the concentrations of antigen or 2C11 anti-CD3 mAb that stimulated identical IL-2 secretion in transfectants expressing wild-type or variant TCR. In every experiment, transfectants expressing variant TCR were tested along with a representative transfectant expressing wild-type D5 TCR. Whenever possible, comparisons in dose-response curves were made in the linear portion of the IL-2 response curve. When no antigen response was detected, the highest antigen concentration tested was used to calculate relative antigen recognition; in these cases, numbers potentially overestimate antigen responses.

Results

Outline Structure of the D5 TCR. We were interested in identifying the regions and, in more detail, particular residues of the D5 TCR that are important in recognition of antigenic peptide/MHC. We have used the proposed outline structure for the TCR based upon the known structure of Ig (5) to predict the amino acids of the D5 TCR that contact antigen/MHC (Fig. 1). Taking into account the conservation of residues predicted to form the interface between Vα and Vβ domains (*squares*) and residues buried between β strands (*triangles*) forming paired, tightly packed β sheets, we have identified amino acid residues predicted to form the β strands A to G (*dotted boxes*) and the antigen binding loops α1, α2, and α3 (*solid boxes*) of the D5 TCR α chain. We have tested the role of the residues predicted to form the antigen binding loops of the D5 TCR α chain by alanine-scanning mutagenesis.

We have chosen to substitute with Ala because its substitution removes side chains beyond the β carbon and should not alter the conformation of the main-chain polypeptide. Systematic Ala substitution has been useful in the study of a number of proteins, including the Ig-like molecule CD4 (20, and see Discussion). We did not substitute Pro-30 or Pro-54 with Ala because of the special structural constraints often dictated by Pro in protein structure: Pro has been shown to play an important role in determining the structure of the canonical antigen binding loop L3 in Ig (21, 22). We did not substitute Val-55 or Val-56 because in the Ig structures complexed with HEL that have been determined to date, light chain residues in the second antigen binding loop do not make extensive contacts with antigen (23). In addition to the amino acid residues that form the predicted antigen binding loops, we have substituted Tyr-24 with Ala; although Tyr-24 is predicted to be the last residue forming the B β strand, we thought that Tyr might be especially important in antigen recognition by the D5 TCR (see Discussion). Moreover, analogous to the situation observed with Ig, and as emphasized

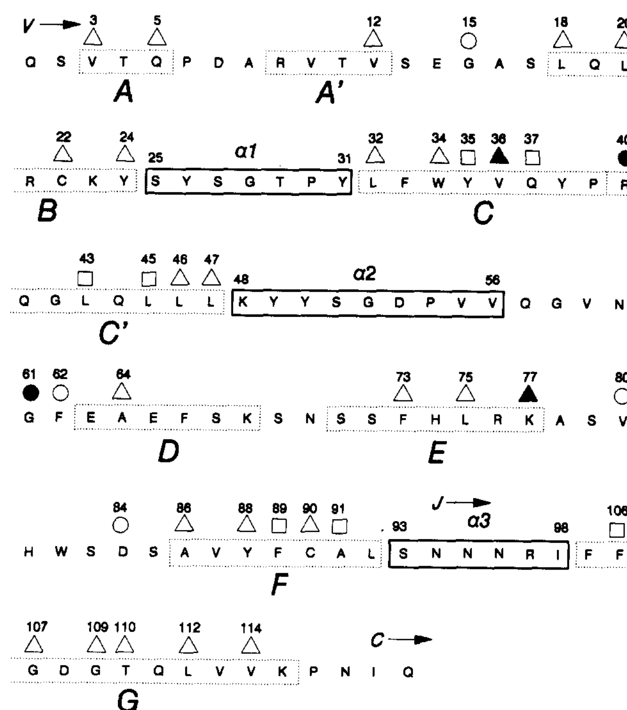

Figure 1. Outline structure of D5 TCR α chain. The structure of the D5 TCR α chain variable domain is represented according to Chothia et al. (5), highlighting the residues that determine the Vα framework structure. Arrows indicate the borders of the V, J, and C regions. Dotted boxes surround residues comprising β strands A–G (*bold letters*), while solid boxes surround residues that form the antigen binding loops α1, α2, and α3 between β strands. Symbols above the sequence indicate residues that are used in the alignment; triangles represent residues that are buried between the β sheets, squares are residues that make contact with the Vβ domain, and circles are residues in turns. Open symbols above sequences signify residues that comply with those residues that determine the Ig fold in TCR α chain; filled symbols denote those that are represented in <40% of the TCR analyzed by Chothia et al. (5). The single-letter code for amino acid residues is used. The numbering system used is that of Chothia et al. (5), except in the α3 region, where residues are numbered consecutively 93–98; the next consecutive residue, Phe-105, is the first residue of the G β strand.

by Chothia et al. (5), framework residues might also contribute to contact with antigen/MHC.

A Model TCR. We have utilized the *p*-azobenzenearsonate-specific T cell clone D5 (Ar-5) and the T cell hybridoma derived from D5 cells, D5h3, in previous studies involving T cell recognition of antigenic peptides/MHC (8–10, 24, 25). Recognition of arsonate-conjugated antigens/MHC by the antigen receptor expressed on the T cell clone D5 is similar to recognition by other TCR: D5 cells recognize haptenated peptides derived from intact hapten-conjugated proteins (E. A. Nalefski, and A. Rao, manuscript in preparation). D5 cells recognize antigens in an MHC-restricted manner; that is, D5 cells recognize antigens presented by a specific MHC protein, I-A^{d} (9). As is the case with other T hybridomas, the recognition of antigen by D5h3 cells is sensitive to specific amino acid substitutions introduced into the NH_2-terminal end of the predicted α helix on the β chain of the I-A^{d} molecule (E. A. Nalefski, unpublished results).

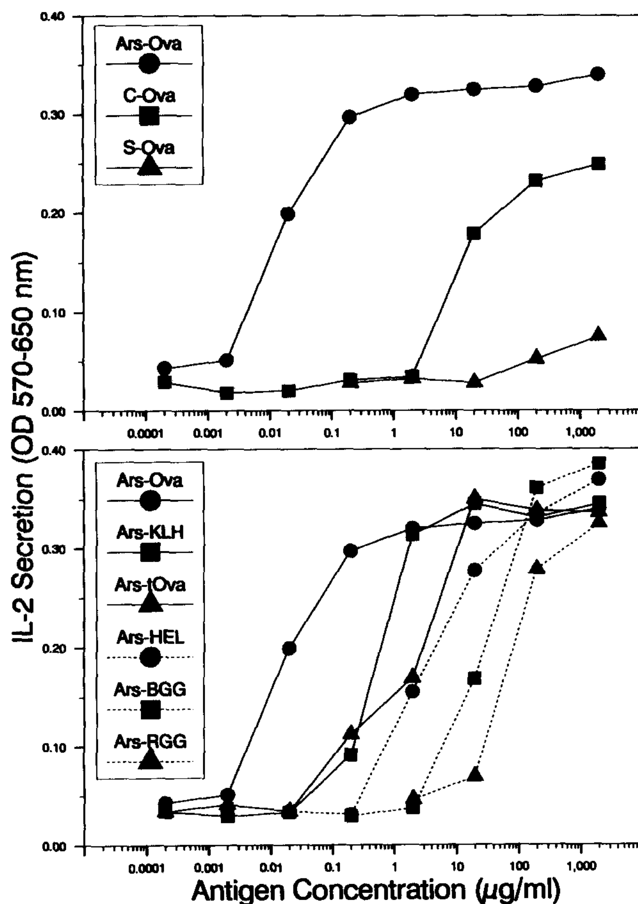

Figure 2. Antigen specificity of the D5 TCR. IL-2 secretion by transfectant expressing the wild-type D5 TCR was measured in response to OVA conjugated with arsonate or its structural analogues (*top*) or to a variety of arsonate-conjugated antigens cultured with I-A^d-bearing TA3 APC (*bottom*). Shown at top is the response to OVA conjugated with arsonate (*filled circles*), benzoate (*filled squares*), or sulfonate (*filled triangles*). Shown at the bottom is the response to arsonate conjugated to OVA (*filled circles, solid line*), KLH (*filled squares, solid line*), tOVA (*filled triangles, solid line*), HEL (*filled circles, dashed line*), BGG (*filled squares, dashed line*), and RGG (*filled triangles, dashed line*). Results represent average MTT reduction measured by OD 570–650 nm in triplicate wells of the IL-2-dependent cell line CTLL-20 in response to hybridoma culture supernatants.

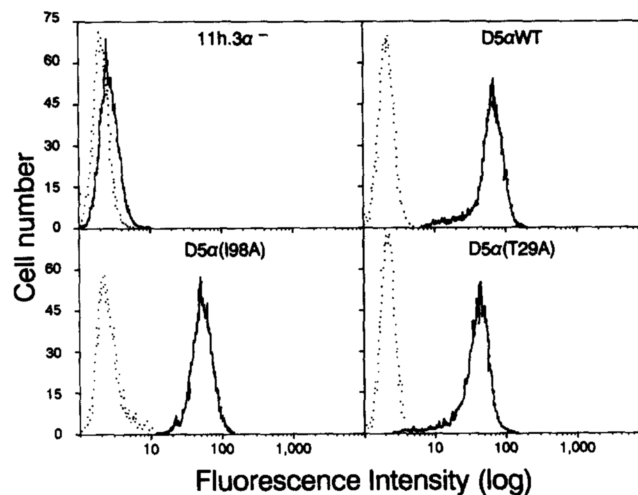

Figure 3. Surface expression of TCR/CD3 in cells expressing the wild-type or variant forms of the D5 TCR α chain. Surface expression of the TCR/CD3 complex was measured by flow cytometry of recipient and transfected cells after incubation with 2C11 anti-CD3 mAb (*solid lines*) or normal hamster serum (*dotted lines*), and fluorescent secondary antibody. D5αWT, D5α(I98A), and D5α(T29A) are representative clones derived by transfecting recipient 11h.3α$^-$ cells with m13pFneoD5αWT, D5α(I98A), and D5α(T29A), respectively. Shown is an analysis of 4,500 cells. Cells bearing other variant D5 TCR also expressed comparable levels of the surface TCR/CD3 complex. Cells exhibiting decreased antigen responses also expressed the epitope detected by the mAb B20.6 anti-murine Vβ2 at levels comparable with that expressed by cells expressing D5αWT.

Using the gene transfer system described previously (8), we reconstituted the antigen specificity of the D5 TCR by transfecting DNA encoding the D5 TCR α chain into a hybridoma derived from D5 cells that lacked expression of the D5 TCR α chain. Cells expressing the transfected wild-type D5 TCR α chain displayed the characteristic hierarchy of recognition by the D5 TCR for OVA conjugated with arsonate or its analogues (Fig. 2 *top*) or various carrier antigens conjugated with arsonate (Fig. 2, *bottom*). This hierarchy of hapten reactivity is also displayed when these haptens are conjugated to the OVA peptide spanning amino acid residues 36–50 (8, and E. A. Nalefski and A. Rao, manuscript in preparation). This hierarchy of antigen reactivity allowed us to ask whether amino acid substitutions introduced into the D5 TCR α chain selectively affected its hapten or carrier specificity.

The Effect of Amino Acid Substitutions Introduced into the D5 TCR α Chain on the Recognition of Ars-OVA/I-A^d. Surface expression of TCR/CD3 by representative cells expressing transfected wild-type or variant D5 TCR α chains is shown in Fig. 3. Although surface expression of TCR/CD3 in most transfectants was comparable, we have observed minor differences in the levels of surface TCR expression in some clones and concomitant variability in the amount of IL-2 secreted in response to 2C11 anti-CD3 mAb (data not shown). To control for such discrepancies, we normalized IL-2 secretion by each of the transfectants, as described in Materials and Methods and shown in Fig. 4 *A*. In Fig. 4 *A*, *left*, a 1:4 dilution of supernatant from transfectant D5α(N94A) contained as much IL-2 secreted in response to anti-CD3 mAb as did a 1:8 dilution of supernatant from D5αWT cells. In Fig. 4 *A*, *right*, comparison of 1:4 dilution of supernatant from D5α(N94A) cells to a 1:8 dilution of supernatant from D5αWT cells revealed that the antigen dose-response displayed by D5α(N94A) cells had been shifted to at least 5,000-fold higher concentrations of antigen.

We tested the ability of the transfectants expressing variant D5 TCR to recognize Ars-OVA presented by I-A^d-bearing TA3 cells. Several experiments were performed in which dose-response curves from each transfectant expressing variant D5 TCR α chains were compared with those from cells expressing the wild-type D5 TCR, examples of which are shown in Fig. 4 *B*. The amino acid substitution D5α(R97A) had little effect on antigen recognition (*top left*), even when relative responses to anti-CD3 mAb were taken into account. The amino acid substitution D5α(I98A) caused an ~200-fold decrease in the

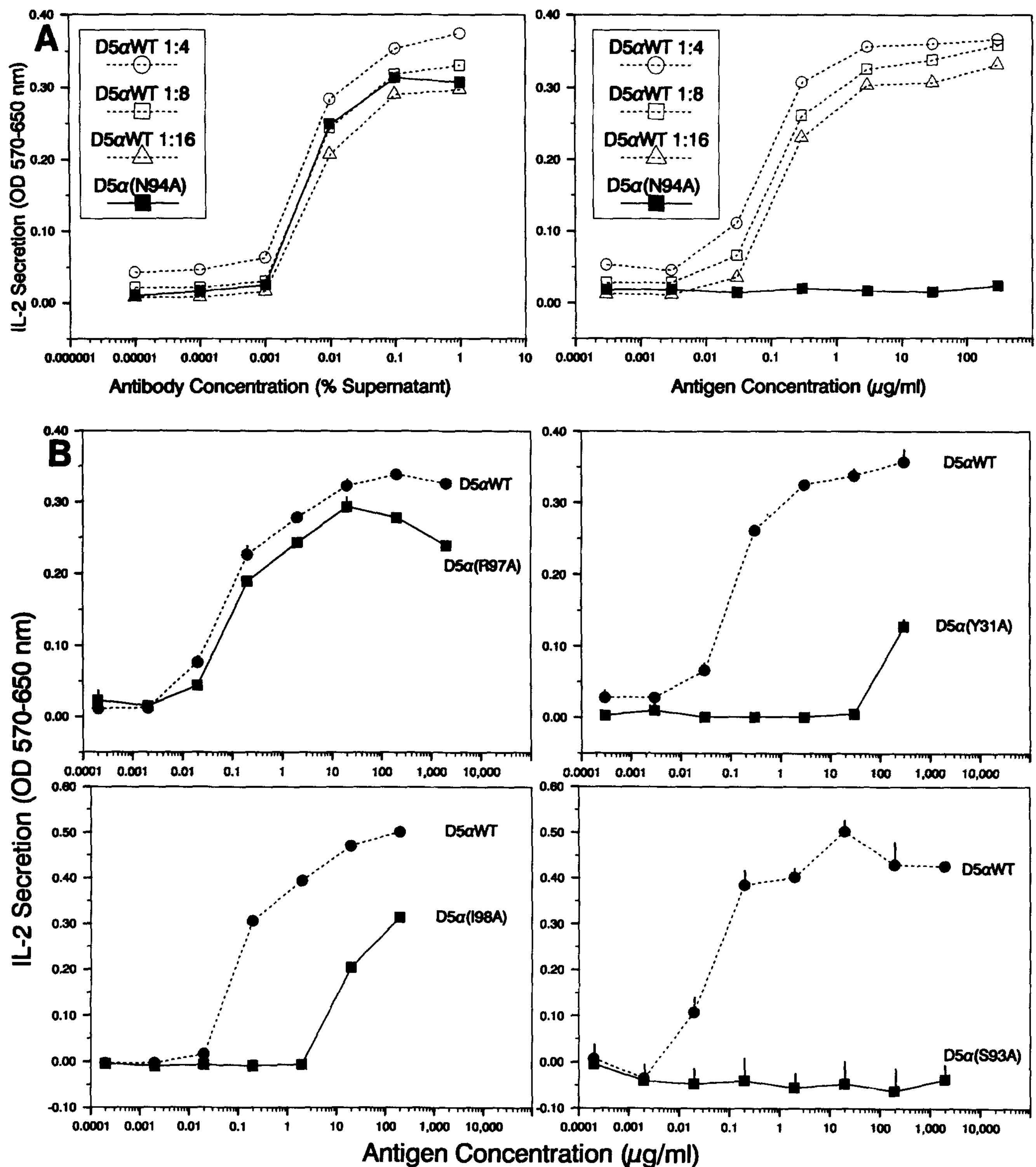

Figure 4. Responses to anti-CD3 mAb or Ars-OVA/I-A^d by representative cells expressing wild-type or variant forms of the D5 TCR α chain. (*A*) IL-2 secretion by cells expressing D5αWT or D5α(N94A) was measured in response to the indicated amounts of 2C11 anti-CD3 mAb (*left*) or Ars-OVA (*right*) cultured with TA3 cells. Shown is a 1:4 dilution of the supernatant generated from D5α(N94A) cells (*filled squares, solid lines*) compared with a 1:4 (*open circles*), 1:8 (*open squares*), and 1:16 dilution (*open triangles*) of supernatant generated from D5αWT cells (*dashed lines*). (*B*) IL-2 secretion by cells expressing D5αWT and representative transfectants expressing D5α(R97A), D5α(I98A), D5α(Y31A), or D5α(S93A) was measured in response to the indicated amounts of Ars-OVA cultured with TA3 cells. Shown is a comparison of a 1:4 dilution of supernatants from each cell line (*filled squares, solid lines*) to supernatants generated from D5αWT (*filled circles, dashed lines*) for which IL-2 secretion in response to anti-CD3 mAb was matched as closely as possible as in *A*. IL-2 secretion was determined, as in Fig. 2. Bars indicate SDs of sample means when larger than one half of the symbol size.

antigen response (*bottom left*) when relative responses to anti-CD3 mAb were taken into account. The amino acid substitution D5α(Y31A) caused an ~1,000-fold shift in the antigen dose-response curve (*top right*); when relative responses to anti-CD3 mAb are taken into account, this dose shift translates to a 6,000-fold decrease in antigen recognition. Finally, the substitution D5α(S93A) eliminated the response to antigen (*bottom right*); when relative responses to anti-CD3 mAb are taken into account, this dose shift translates to at least a 50,000-fold decrease in antigen recognition. In this manner, we calculated relative antigen recognition from multiple experiments for each of the transfectants and displayed them in Fig. 5.

Residues that appear critical for the recognition of antigen/MHC in the D5 TCR are those residues that decrease antigen recognition at least 10-fold when substituted with Ala (or Gly or Ser in the case of Tyr-26); Tyr-24, Tyr-26, Tyr-31, Lys-48, Tyr-50, Ser-93, Asn-94, and Ile-98. We are less certain about the absolute role of Tyr-49; its substitution with Ala decreased antigen recognition ~10-fold, but the decrease ranged between twofold and 50-fold in five experiments (Fig. 5). Amino acid substitutions K48A, S93A, and N94A completely eliminated antigen responses, while substitutions Y24A, Y31A, and Y50A severely affected antigen responses (displaying a slight antigen response at the highest antigen concentration tested). These cell lines expressed surface CD4, CD45, and LFA-1 at levels comparable with that expressed by D5αWT cells, as determined by flow cytometry (data not shown), demonstrating that the loss of these molecules did not account for the observed decreases in antigen recognition. Amino acid substitutions Y26S, Y26G, and I98A moderately decreased antigen responses. Amino acid substitutions S25A, Y26F, S27A, T29A, S51A, D53A, N95A, N96A, and R97A had little or no effect on antigen recognition. None of the amino acid substitutions substantially increased relative antigen recognition. In sum, these results demonstrate that residues in all three antigen binding loops are

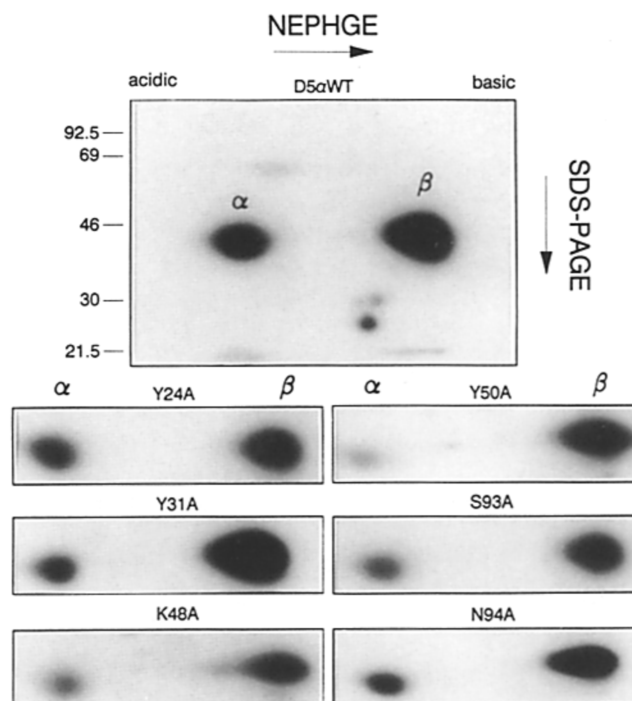

Figure 6. Surface expression of TCR α/β chains by transfectants exhibiting severely diminished responses to antigen. Cell surface proteins of transfectants D5αWT, Y24A, Y31A, K48A, Y50A, S93A, and N94A were labeled with ^{125}I using lactoperoxidase, solubilized in 1% digitonin, immunoprecipitated with 2C11 anti-CD3 mAb, and analyzed by two-dimensional NEPHGE and SDS-PAGE. The entire gel derived from D5αWT, but only the relevant portions of the autoradiograms from transfectants expressing variant TCR α/β chains (~30–50 kD), are shown. Because of differences in the numbers of cells labeled and in exposure time of autoradiograms, these results do not indicate absolute amounts of TCR α or β chains expressed on a given cell line. TCR α/β chains are indicated; molecular mass markers are shown on the left (in kD).

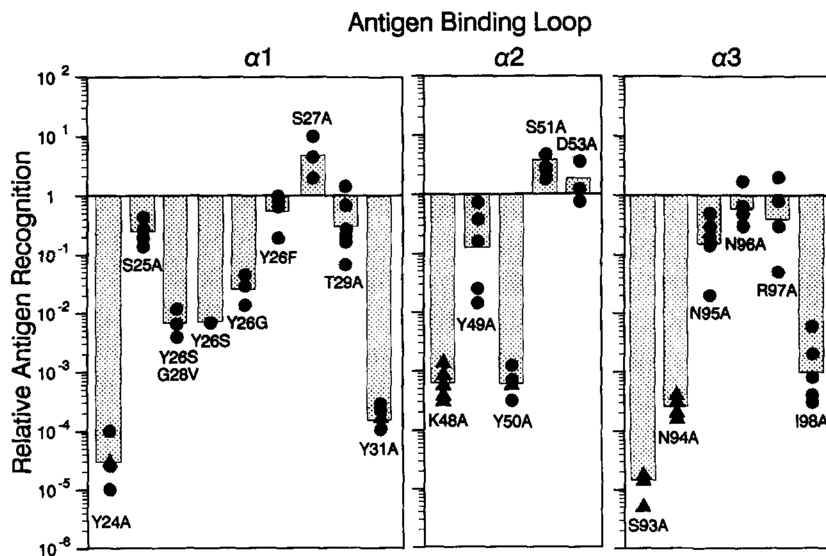

Figure 5. Relative responses to Ars-OVA/I-A^d by cells expressing the variant forms of the D5 TCR α chain. IL-2 secretion by cell expressing D5αWT or variant D5 TCR was measured in response to several doses of Ars-OVA or 2C11 anti-CD3 mAb cultured with TA3 cells, as in Fig. 4. Relative antigen recognition by transfectants was calculated as described in Materials and Methods, taking into account responses to anti-CD3 mAb, relating antigen recognition to transfectants expressing wild-type D5 TCR (WT = 1.0). Each point represents an independent experiment with the same transfectant clone or the same experiment with different clones. Bars indicate geometric mean ($\sqrt[n]{X_1X_2X_3 \ldots X_n}$) of three to six experiments, except for D5α(Y26S), which was tested in a single experiment. Experiments in which no antigen response was detected at the highest antigen concentration tested are indicated with a triangle; such experiments potentially overestimate relative antigen recognition, thus underestimating the effect of a particular amino acid substitution.

important in recognition of antigen/MHC and that significant structural alterations in side chains can be tolerated at certain positions in each loop.

Surface Expression of TCR α/β Chains in Transfectants Displaying Severely Diminished or Eliminated Antigen Responses. We examined whether transfectants displaying little or no response to antigen expressed surface TCR α/β chains. Surface proteins of wild-type transfectant and selected transfectants (Y24A, Y31A, K48A, Y50A, S93A, N94A) were labeled with ^{125}I by lactoperoxidase, lysed with 1% digitonin and immunoprecipitated with 2C11 anti-CD3 mAb. Immunoprecipitates were subjected to NEPHGE and SDS-PAGE to separate TCR α and β chains, as shown in Fig. 6. All cell lines expressed both α and β TCR chains in TCR/CD3 complexes, indicating that their decreased antigen responses were not due to improper chain association or the expression of nonclassical TCR (such as β-β or β-δ dimers). The relative decrease in the labeling of the α chain in the transfectants expressing the Y31A or Y50A variants suggests that Tyr-31 and Tyr-50 are targets for iodination in the α chain. The same panel of transfectants stained as well as the wild-type transfectant with B20.6 anti-murine Vβ2 mAb, demonstrating that the amino acid substitutions in the α chain did not affect the epitope on Vβ2 recognized by this mAb (data not shown).

The Effect of Amino Acid Substitutions Introduced into the D5 TCR α Chain on Specificity for Hapten or Carrier Antigen. We tested whether amino acid substitutions introduced into the D5 TCR α chain affected the pattern of reactivity towards various arsonate-conjugated antigens presented by cells bearing I-A^d, as displayed in Fig. 2. For most of the transfectants bearing variant D5 TCR (and showing IL-2 responses strong enough to be recorded), this pattern of relative potency was not affected: the degree of shift in the dose-response curves to the other arsonate-conjugated antigens was roughly equal to that observed for Ars-OVA (data not shown). However, cells expressing the TCR variant D5α(I98A) displayed a pattern of reactivity towards the various arsonate-conjugated antigens that was qualitatively different from that displayed by cells expressing the wild-type D5 TCR (Fig. 7; for simplicity, only the responses to the antigens Ars-OVA, Ars-tOVA, and Ars-BGG are shown). Although the dose responses of cells expressing D5α(I98A) to Ars-OVA and Ars-tOVA (Fig. 7), as well as Ars-KLH and Ars-HEL (data not shown), were equivalently shifted to 100–1,000-fold higher antigen concentrations, the response to Ars-BGG was decreased only 10-fold. Identical results were obtained in three independent experiments testing the response of cells expressing D5α(I98A) to this panel of antigens (data not shown). Thus, the substitution I98A had the net effect of creating a TCR that now recognized Ars-BGG better than it recognized Ars-tOVA.

We tested whether cells expressing variant D5 TCR exhibited a different pattern of reactivity towards OVA conjugated with arsonate or its structural analogues benzoate and sulfonate. For all of the transfectants that exhibited a response to these antigens strong enough to be detected, there was no difference in the pattern of relative potency of hapten-OVA conjugates (data not shown).

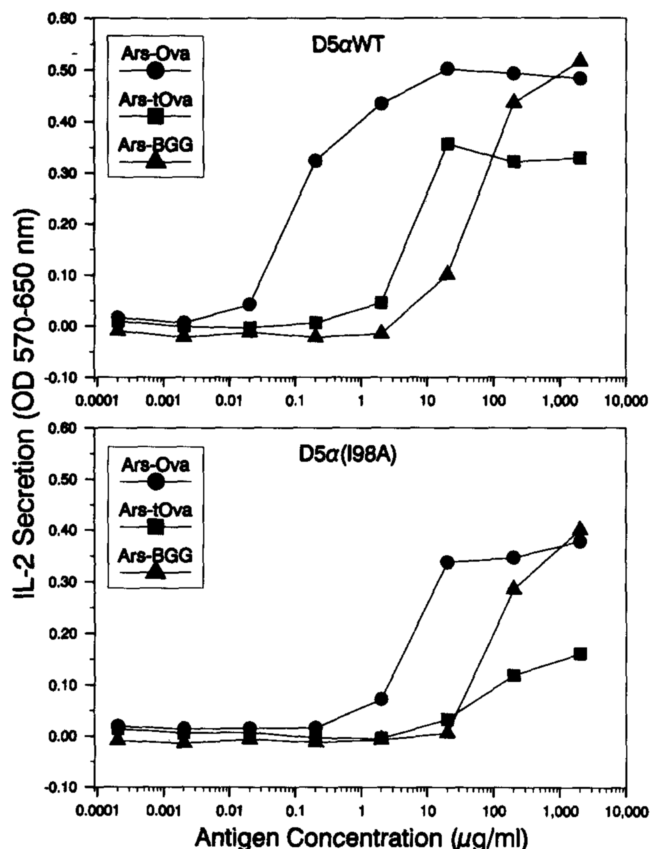

Figure 7. Responses to various arsonate-conjugated antigens/I-A^d by cells expressing the wild-type or variant I98A of the D5 TCR α chain. IL-2 secretion by cells expressing D5αWT (*top*) or D5α(I98A) (*bottom*) was measured in response to the indicated amounts of arsonate-conjugated antigens cultured with TA3 cells. The responses to Ars-OVA (*filled circles*). Ars-tOVA (*filled squares*), and Ars-BGG (*filled triangles*) were tested using dilutions of supernatants from cells for which IL-2 secretion in response to anti-CD3 mAb was matched as in Fig. 4 *A*. IL-2 secretion was determined as in Fig. 2. Identical results were obtained in two other experiments.

Discussion

Similarity between the TCR and Ig exists at several levels. Both are encoded by rearranged gene segments organized in a similar manner (reviewed in reference 2). A great deal of similarity exists at the level of primary sequence, especially in residues thought to be important in maintaining their structure (2–5). On the basis of these similarities, we have altered residues predicted to form the antigen binding loops of a model TCR that recognizes arsonate-conjugated antigens. Fig. 8 summarizes our results, which demonstrate that all three predicted antigen binding loops of the D5 TCR α chain are likely to play an important role in the recognition of antigen/MHC.

The Effect of Ala Substitutions on Overall TCR Conformation. The amino acid substitutions we have introduced into the D5 TCR α chain are unlikely to disrupt the overall conformation of the TCR. As outlined in Fig. 1, we modeled the TCR primary structure upon that of an Ig domain according to Chothia et al. (5) and then intentionally directed our amino acid substitutions to residues forming the predicted

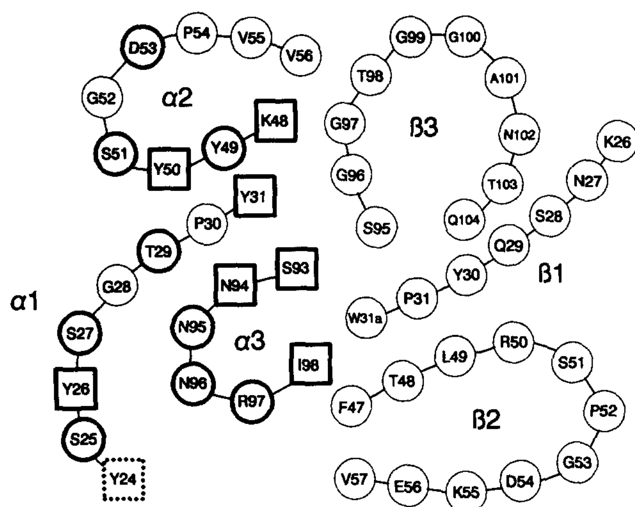

Figure 8. Summary of the effects of amino acid substitutions introduced into the predicted D5 TCR antigen binding loops. Shown is a schematic representation of the antigen/MHC binding site on the D5 TCR based on the model proposed by Chothia et al. (5) and outlined in Fig. 1, highlighting the effect of amino acid substitutions introduced into the D5 TCR α chain on Ars-OVA/I-A^d recognition. Bold symbols around residues (using a single-letter code for amino acids) denote those that were tested in the present study, the results of which are shown in Fig. 5. Squares denote residues that, when substituted with Ala (or Gly or Ser in the case of Tyr-26), cause a >10-fold decrease in response of the D5 TCR to Ars-OVA presented by cells bearing I-A^d. Continuous symbols denote the residues in antigen binding loops α1-α3 and β1-β3; a dotted symbol denotes Tyr-24, the last residue of the B β strand in the TCR α chain. The substitution of Ala for Ile-98, located in the antigen binding loop α3 predicted to contact antigenic peptides, caused a change in carrier peptide specificity in the D5 TCR. Note that this figure indicates the relative position of the antigen binding loops and the residues that form them, not necessarily the conformation of the loops.

antigen binding loop regions of the TCR α chain. As Ala is one of the most abundant amino acids and is found both buried or exposed to solvent in protein structures (26), substitution with it should be tolerated in a loop structure. In fact, surface staining with an anti-Vβ2 antibody (B20.6) demonstrated that the variant TCR exhibiting decreased antigen recognition retained the epitope recognized by this mAb (data not shown). The only substitution that might possibly influence the conformation of the α chain is Y24A. Because 93% of the TCR α chains analyzed bear His, Tyr, or Phe at position 24 (5), the substitution Y24A could disrupt the local conformation of the B β strand, rather than affecting contact with antigen.

The importance of β strand residues in determining the overall conformation of the Ig fold has been demonstrated in a similar study involving CD4. Ala was systematically substituted for each of 64 hydrophilic and hydrophobic residues in the NH_2-terminal domain of the Ig-like molecule CD4 to determine residues important in the interaction with HIV gp120 (20). 14 Ala substitutions disrupted the conformation of CD4 as judged by antibody binding: 10 of these residues are found in β sheets, while only one of the remaining four is in a position analogous to the Ig antigen binding loops between β strands (27, 28).

The Effect of Ala Substitutions on Association of TCR α and β Chains. The model proposed by Chothia et al. (5) predicts that the TCR residues that probably have the strongest influence on chain pairing are those residues in the C, C', F, and G β strands that are involved in the TCR α-β interface, and we carefully avoided making substitutions at these residues. In fact, each variant TCR generated was efficiently expressed on the surface of 11hα$^-$ cells (as judged by flow cytometry using anti-CD3ε and anti-Vβ2 mAb and immunoprecipitation of surface iodinated proteins) and could be stimulated with anti-CD3ε mAb, demonstrating that the substitutions disrupted neither association with the normal D5 TCR β chain or CD3 polypeptides, nor signaling through invariant portions of CD3.

There is a strict requirement for the presence of both α and β TCR chains for successful surface expression of the TCR/CD3 complex in 11hα$^-$ cells, which have deleted the Vα gene segment rearranged and expressed on D5 cells. This strict requirement is born out by the fact that we observe regained surface CD3 expression in the 11hα$^-$ recipient only upon transfection with DNA encoding the wild-type or variant D5 TCR α chain. Mock-transfected cultures of 11hα$^-$ cells have never been observed to show any trace of surface TCR/CD3 expression as analyzed by flow cytometry. Indeed, continuous culturing of the 11α$^-$ T cell clone and the 11hα$^-$ T cell hybridoma has never led to the development of any cells expressing detectable surface CD3. Moreover, we have shown directly by surface iodination and immunoprecipitation that surface TCR α and β chains are expressed in transfectants exhibiting low or no response to antigen, ruling out the possibility that solely β-β homodimers or nonclassical TCR heterodimers (such as β-δ) (29) appeared in our transfectants that exhibited low or no response to antigen (Fig. 6). Our observations are in complete agreement with those reported by Saito et al. (30), in which TCR β chain dimers were not detected in T cell mutants deficient in α chain expression.

Validity of the Ig Model of the TCR. Our results demonstrate that residues important in the recognition of antigen/MHC by the D5 TCR include: Tyr-24, Tyr-26, Tyr-31, Lys-48, Tyr-50, Ser-93, Asn-94, Ile-98, and possibly Tyr-49 (Figs. 5 and 8). We propose that these residues, located in or immediately adjacent to proposed antigen binding loops, either determine the conformation of the antigen binding loops on the D5 TCR or directly contact some portion of the antigenic peptide/MHC ligand. Our results are consistent with the Ig model for the TCR and imply that principles similar to those that are involved in antibody interactions with globular antigens might govern TCR recognition of its ligand. For example, the specificity of the TCR for antigen/MHC probably arises from the complementarity of TCR and antigen/MHC surfaces that span the same approximate area as those involved in antibody-antigen complexes. Because the TCR simultaneously contacts multiple residues on both α helices of the MHC molecule, it has been previously estimated that the interface between the TCR and antigen peptides/MHC molecule spans 600–780 Å^2 of surface

area (31); this estimate agrees well with the 600–900 Å^2 of surface area involved in the structures of antibody-antigen complexes determined to date (23). Our results indicate that seven to nine residues of the TCR α chain are potentially in simultaneous contact with antigen/MHC. This number agrees well with the 7–11 light chain residues that make contact with protein antigens in the four Fab structures that have been determined (23), suggesting that TCR α chain contact with antigen/MHC also involves some 300–400 Å^2 of surface area. Furthermore, it has been suggested that aromatic interactions play an important role in antigen recognition because of the high degree of solvent accessibility of aromatic residues in antibody combining sites (32). In the case of the D5 TCR, multiple Tyr residues are important in antigen/MHC reactivity (Figs. 5 and 8); Tyr may play a particularly important role in antigen/MHC recognition by the D5 TCR because of the nature of its ligand (see below).

TCR Residues That Contact Antigenic Peptides or the MHC Molecule. The current model of the TCR predicts that residues in the third antigen binding loop contact mainly antigenic peptides (5–7). Consistent with this model, the amino acid substitution D5α(I98A) selectively alters the pattern of recognition of a subset of the arsonate-conjugated antigens that were tested, suggesting that the amino acid Ile-98 in antigen binding loop α3 contacts the antigenic carrier peptide to which an arsonate hapten has been attached (Fig. 7). Our results are consistent with other instances in which particular amino acids in the third antigen binding loop of TCR chains have been implicated in contact with antigenic peptides. Amino acid residues at position 100 of antigen binding loop β3 in pigeon cytochrome *c*–specific TCR have been shown to affect the fine specificity of those TCR for antigen (33, 34). More recently, direct contacts between the TCR α chain residue 93 in α3 and β chain residue 100 in β3 and cytochrome *c* peptides bound to I-E^k have been proposed (35).

The substitution of Ala for Ile-98 results in the removal of its β-methyl and β-ethyl groups, making the side chain considerably smaller. If Ile-98 contacts an antigenic peptide, substitution of bulkier side chains for residues on the antigenic peptide might compensate for the substitution of Ala for Ile-98. We are currently testing this hypothesis with arsonate-conjugated peptide Ova(36–50).

In keeping with the current model for the TCR, Ser-93 and Asn-94, also in antigen binding loop α3, are predicted to contact some portion of the hapten-conjugated antigen recognized by the D5 TCR. Because the substitution of both these residues eliminated responses to all antigens tested, we could not establish whether they contact the hapten itself or some portion of the peptide. Nevertheless, the elimination of an antigen response by the removal of a single hydroxyl group in the substitution D5α(S93A) illustrates the remarkable influence that small structural changes can have on T cell recognition.

The current model for the TCR also predicts that residues in α1 and α2 primarily contact the α helices of the MHC molecule. In keeping with this model, Tyr-26, Tyr-31, Lys-48, Tyr-50, and possibly Tyr-24 are predicted to interact with residues on the I-A^d molecule. It is noteworthy that each of the 17 human or murine TCR Vα sequences in subgroup 4 analyzed by Chothia et al. (5), to which Vα3 family members belong, possesses either Arg or Lys at position 48, the first position of antigen binding loop α2. Since these TCR recognize various antigen/MHC combinations, it is tempting to speculate that the positively charged residue at position 48α in all of the TCR in this subgroup might interact with a conserved negatively charged residue on an α helix of the MHC molecule.

Comparison between Antiarsonate Ig and TCR. Recently the high-resolution three-dimensional structures of two antiarsonate antibodies have been determined by x-ray diffraction (36, 37). In one of the structures, an arsonate binding site was modeled at the bottom of a conical "pit" formed by antigen binding loops L3, H2, and H3 (37, 38). The presence of this hapten binding site was supported by several lines of experimental evidence: conservation of residues throughout high affinity antiarsonate antibodies, site-directed mutagenesis, and comparison of three-dimensional structures of two antiarsonate antibodies. The hapten binding site was formed by the light chain residue Arg-96, heavy chain residues Asn-35, Ser-99, and Trp-47, and a tightly bound water molecule that hydrogen bonded to the arsonate moiety; heavy chain residues Tyr-50 and Tyr-106 packed against the phenyl rings of the hapten. Substitution of Ala for each of these residues severely diminishes the binding of the antibodies to the arsonate hapten (37, 39; and S. Sompuram and J. Sharon, personal communication).

We were surprised to observe that analogous amino acid substitutions introduced into the D5 TCR α chain severely impaired the ability of the D5 TCR to recognize arsonate-conjugated antigens presented by I-A^d: substitution of Ala for Tyr-31 and Tyr-50 in the proposed antigen binding loops and Tyr-24 in an adjacent β strand; substitution of Ala for Ser-93; substitution of Ala for Asn-94; substitution of Ala for Lys-48. Even though the contribution of β chain residues has not been taken into account, this coincidence could imply that similar molecular interactions occur between arsonate and an antiarsonate antibody and the arsonate hapten conjugated to an antigenic carrier peptide and the D5 TCR. By analogy to antiarsonate antibodies, one model that can be proposed is that D5 TCR α chain residues Tyr-31 or Tyr-50 (or Tyr-24) pack against the phenyl rings of the arsonate hapten, and Lys-48, Ser-93, and Asn-94 hydrogen bond to the arsonate moiety of the hapten. A great deal of binding energy might be created through the packing of aromatic rings of TCR residues and the phenyl ring of the arsonate moiety, as has been calculated for other aromatic-aromatic interactions in protein structures (40). Because their substitutions are more easily tolerated, Tyr-26 in antigen binding loop α1 and possibly Tyr-49 in α2 probably contact some portion of the I-A^d molecule.

If this model for TCR residues that contact the arsonate hapten is correct, it would contradict the current model of the TCR that residues in the first and second antigen binding loops contact mainly residues of the MHC molecule (5–7).

The presence of these residues, all encoded by the Vα3 segment except for Asn-94 and all critical for the specificity of the D5 TCR, might explain the observed correlation between usage of the Vα3 gene segment and reactivity to arsonate (10).

It is possible that hapten-specific TCR might not recognize antigenic peptides in exactly the same manner as other "conventional" TCR, since the hapten moiety might contribute a disproportionate amount of binding energy to the TCR/MHC/haptenated peptide complex. In the case of Ig, x-ray crystallography has shown that the binding of small molecules such as haptens to antibodies is similar but not identical to the binding of larger protein antigens to antibodies: small molecules bind in clefts between antigen binding loops on the ends of antibodies while globular proteins pack against these loops, forming relatively flat surfaces without extensive protusions (reviewed in reference 23). Nevertheless, the study of hapten-specific antibodies was critical for our understanding of antibody-antigen interactions. The results provided in this report, which are consistent with the Ig model of the TCR, demonstrate how the molecular interactions involved in the ternary complex of TCR/antigenic peptide/MHC can be elucidated using a hapten-specific TCR.

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