Conformational Integrity and Ligand Binding Properties of a Single Chain T-cell Receptor Expressed in Escherichia coli*

Sanjay S. Khandekar, Brian M. Bettencourt, Daniel F. Wyss, Jerome W. Naylor, Pamela P. Brauer, Kevin Huestis, Donard S. Dwyer, Albert T. Profy, Marcia S. Osborne, Julian Banerji, and Barry Jones

From Procept, Inc., Cambridge, Massachusetts 02139

We recently showed that a soluble, heterodimeric murine D10 T-cell receptor (TCR) (Vα2Ca, Vβ8.2Cβ) expressed in insect cells binds both Vβ8.2-specific bacterial superantigen staphylococcal enterotoxin C2 (SEC2) and a soluble, heterodimeric major histocompatibility complex class II I-Aβ-conalbumin peptide complex with a low micromolar affinity. To define further the structural requirements for the TCR/ligand interactions, we have produced in Escherichia coli a soluble, functional D10 single chain (sc) TCR molecule in which the Vα and Vβ domains are connected by a flexible peptide linker. Purified and refolded D10 scTCR bound to SEC2 and murine major histocompatibility complex class II I-Aβ-conalbumin peptide complex with thermodynamic and kinetic binding constants similar to those measured for the baculovirus-derived heterodimeric D10 TCR suggesting that neither the TCR constant domains nor potential N- or O-linked carbohydrate moieties are necessary for ligand recognition and for expression and proper folding of the D10 scTCR. Purified D10 scTCR remained soluble at concentrations up to 1 mg/ml. Circular dichroism and NMR spectroscopy indicated that D10 scTCR is stabilized predominantly by β-sheet secondary structure, consistent with its native-like conformation. Because of its limited size, high solubility, and structural integrity, purified D10 scTCR appears to be suitable for structural studies by multidimensional NMR spectroscopy.

Immune activation of T lymphocytes is normally initiated by the specific interaction between an antigenic peptide presented by the major histocompatibility complex (MHC)1 (1) and the α and β (or γ and δ) clonotypic chains of the T-cell receptor (TCR) complex (2, 3). T-cells can also be stimulated by superantigens, although the TCR/superantigen interaction differs from that of TCR/MHC peptide in that specificity for a particular superantigen appears to be determined only by the sequence of the germ line-encoded Vβ segment of the TCR (4, 5).

Because of its central role in immune recognition, detailed structural information on the TCR is of great interest. Recently, three-dimensional structures of a heterodimeric, glycosylated murine αβ TCR (2C), the corresponding 2C TCR/class I MHC-peptide complex (6), and a nonglycosylated human αβ TCR (A6) bound to a class I MHC-peptide complex (7) were solved using x-ray crystallography. The overall orientation of the CDR regions of both of these TCRs is similar. Moreover, the folded variable domain structures of both TCRs resemble the antigen binding region of antibodies, although differences exist in the Cα domain and in the interdomain pairing of Cα and Cβ (6). Crystal structures of an isolated TCR β-chain (8) and a Vα homodimer (9) also showed immunoglobulin-like folding patterns. Three-dimensional crystal structures of staphylococcal enterotoxin C2 and C3 bound to the extracellular portion of the β-chain (Vβ8.2Jβ2.1Cβ1) of the mouse 14.3.d TCR were also recently reported (10). Unlike the TCR/MHC interactions (6, 7), only the CDR2 and to lesser extents CDR1 and hypervariable region 4 (HV4) of the Vβ-chains are involved in binding to SEC.

Three-dimensional high resolution structures can also be derived from NMR-based approaches (11, 12). The NMR structures have the potential advantage of being obtained in solution and can provide insights into conformational flexibility and structural dynamics (13, 14). Although the NMR approach is mostly limited to proteins with molecular masses less than ~30 kDa that can be uniformly enriched with 13C and 15N isotopes and remain soluble at high submillimolar concentrations (12), deuteration in concert with 13C, 15N multidimensional NMR techniques can push this limit to higher molecular masses (15). Because of their limited size (~30 kDa), single chain (sc) TCR molecules in which the Vα- and Vβ-chains are connected by a flexible polypeptide linker (reviewed in Ref. 16) might be amenable to NMR structure determination, and various scTCR constructions have been produced previously using Escherichia coli expression systems (reviewed in Ref. 16). Despite this, the conformational integrity and ligand binding characteristics of these proteins have not been well established (Ref. 17, and for review see Ref. 16).

We recently reported ligand binding properties of a soluble, heterodimeric murine αβ TCR derived from the D10 T-cell clone (Vα2, Vβ8.2) of the AKR (H-2k) mouse and expressed in baculovirus-infected insect cells (18). Using a surface plasmon resonance (SPR) biosensor (19, 20), we showed that this protein binds both Vβ8.2-specific bacterial superantigen staphylococcal enterotoxin C2 (SEC2) and a soluble, heterodimeric MHC class II I-Aβ-conalbumin peptide (residues 134–146) complex with a low micromolar affinity (18).

To define further the structural requirements for D10 TCR binding, and to obtain material suitable for NMR structural studies, we produced a soluble D10 scTCR molecule using an E.
coli expression system. Purified and refolded protein bound to SEC2 and IA\(^4\)-conalbumin peptide complex with thermodynamic and kinetic binding constants similar to those measured previously for the insect cell-derived, heterodimeric D10 TCR (18), suggesting that neither the TCR constant domains nor potential N- or O-linked carbohydrate moieties are necessary for ligand binding. In addition, based on circular dichroism and one-dimensional NMR spectroscopy, purified D10 scTCR appears to be suitable for structural studies by multidimensional NMR spectroscopy. NMR studies on the D10 scTCR can be helpful in assessing the flexibility of its various CDR regions and may provide further insight into whether the scTCR undergoes conformational changes as a result of its interactions with superantigens and class II MHC-peptide complex.

**MATERIALS AND METHODS**

**Antibodies**—Hybridomas expressing D10 cloneotype-specific mAb 3D3 (21) (provided by Dr. Al Bothwell, Yale Medical School, New Haven, CT) and ACID-p1/BASE-p1 leucine zipper (LZ)-specific mAb 1Veleroh211 (2H11 (18, 22)) were grown in hollow fiber reactors, and the proteins were purified by immobilized metal affinity chromatography using immobilized protein A (Repligen, Cambridge, MA) as described (23). TCR V\(\alpha\)2-specific mAb B20.1 (24) and V\(\beta\)8.1/2-specific mAb MR5-2 (25) were obtained from Pharmingen (San Diego, CA).

**Proteins**—Heterodimeric D10 TCR expressed in baculovirus-infected insect cells was purified using 3D3 immunofinity chromatography as described (18). Similarly, baculovirus-derived soluble, heterodimeric murine MHC class II \(\alpha\)-chain and \(\beta\)-chain genes, resulting in a gene encoding D10 scTCR in the context of D10 scTCR, a small amount of purified protein was concentrated by size exclusion chromatography using a Superdex 200PG 60 column (Pharmacia Biotech Inc.). To obtain D10 scTCR, purified fusion protein was first digested with thrombin (Calbiochem) at 40.1 (w/w) for 16 h at 37 °C in 50 mM Tris-HCl (pH 8.0) containing 2 mM CaCl\(_2\) and 0.02% sodium azide. The digested sample was then applied to the Ni-NTA metal affinity column using a 240-min linear gradient with 2 column volumes of buffer A, a refolding gradient was initiated with 2 column volumes of buffer A, and applied to a Ni-NTA metal affinity column (Qiagen, Chatsworth, CA) at a flow rate of 3.7 ml/min. After washing the column with 2 column volumes of buffer A, a refolding gradient was initiated and maintained at a flow rate of 3.7 ml/min using fast protein liquid chromatography (Pharmacia Biotech Inc.). A 240-min linear gradient was formed from 0% buffer A to 100% buffer B containing 50 mM Tris-HCl (pH 8.0), 0.2 M GdnHCl, 20% glycerol, and 0.5 M NaCl. After washing the column with 1 column volume of buffer B, the bound material was eluted with buffer B containing 250 mM imidazole. The eluted MBP-D10 scTCR fusion protein was further purified by immunoaffinity chromatography on an immobilized 3D3 column, which specifically recognizes a D10 TCR conformational epitope (18, 21, 29).

**Protein concentrations** were determined by spectrophotometry at 280 nm using extinction coefficients of 1.7 and 1.2 \(\text{m}^{-1} \cdot \text{cm}^{-1}\) for MBP-10 scTCR and D10 scTCR, respectively. Quantitative amino acid analysis was performed using an Applied Biosystems (Perkin-Elmer) model 423 amino acid analysis system. To test the solubility characteristic of D10 scTCR, a small amount of purified protein was concentrated using Centriprep-10 concentrator (Amicon, Beverly, MA). Protein concentrations were determined by spectrophotometry at 280 nm using extinction coefficients of 1.7 and 1.2 \(\text{m}^{-1} \cdot \text{cm}^{-1}\) for MBP-D10 scTCR and D10 scTCR, respectively. Quantitative amino acid analysis was performed using an Applied Biosystems (Perkin-Elmer) model 423 amino acid analysis system. To test the solubility characteristic of D10 scTCR, a small amount of purified protein was concentrated using Centriprep-10 concentrator (Amicon, Beverly, MA). 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structural and ligand binding properties of an scTCR

A flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. Molecular weight of D10 scTCR was also determined by electrospray-mass spectrometry using a VG Biotech Bio-Q instrument with a quadrupole mass analyzer (M-Scan Inc., West Chester, PA).

Circular Dichroism (CD) Spectroscopy—CD analysis was performed on purified D10 scTCR (0.15 mg/ml in 2 mM HEPES (pH 7.2)). Far-UV CD spectra were recorded on a CD instrument model 62 DS (Aviv Associates, Lakewood, NJ) using a 2-mm path length cell. Data were collected using a time constant of 1 s at every 0.2 nm and with a 1 nm constant spectral bandwidth at 25 °C. The CD data were analyzed for secondary structure prediction by an algorithm Proser: V3.1 provided by the manufacturer (Aviv Associates, Lakewood, NJ).

NMRSpectroscopy—One-dimensional NMR experiments were carried on purified D10 scTCR (~80 μM in 95% (v/v) H2O/D2O and 20 mM deuterated acetate (pH 4.5)) at 25 and 37 °C on a Varian UNITYplus750 MHz spectrometer with a 1H resonance frequency of 750.079 MHz. The spectral width was 23,995 Hz, and the carrier was placed on the H2O resonance which was suppressed by low power presaturation during the recycle delay (1.3 s). 8,192 complex points were obtained, and 1,024 transients were collected for signal averaging. Data processing was carried out using Felix software ( Biosym Technologies, San Diego). The residual water signal was removed by the time domain convolution technique. Data were multiplied with a residual water signal was removed by the time domain convolution technique. Data were multiplied with a 2π/2 shifted squared sine bell apodization function and zero-filled to 16,384 points prior to Fourier transformation. Chemical shifts were referenced relative to the water resonance, calibrated in turn at 4.755 and 4.631 ppm at 25 and 37 °C, respectively, on an external 2,2-dimethyl-2-silapentamethylsulfonic acid standard.

Surface Plasmon Resonance (SPR) Binding Experiments—Binding studies were performed using a commercial biosensor instrument (BLAcoreX™, Pharmacia BioSensor, Uppsala, Sweden). All proteins used in these experiments were purified by size exclusion chromatography. Immobilizations were carried out in 10 mM sodium acetate at pH 5.5 for D10 scTCR or at pH 4.0 for superantigens using the Amine Coupling Kit (Pharmacia BioSensor, Uppsala, Sweden) as described (18, 19). Heterodimeric, soluble D10 TCR (18) and BDC2.5 TCR (33) derived from baculovirus-infected insect cells were used as control proteins for specificity experiments. HEPES-buffered saline (HBS; 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20) was used as the running buffer. CD spectra were recorded on a CD instrument model 62 DS (Aviv Associates, Lakewood, NJ) using a 2-mm path length cell. Data were analyzed using the BI4Evaluation software package (BLAcoreX Evaluation 2.1, Pharmacia Biosensor) which also allows the parameters to be extracted from a single run (18, 34–36). To determine the apparent association (k on) and dissociation (k off) rate constants, the association and dissociation phases of the sensograms were fitted on single site models, A + B ↔ AB, and A + B → A + B, respectively. The apparent dissociation constant (K D) was determined from the ratio of k off/k on.

RESULTS

Expression and Purification of D10 scTCR—The construct used to produce recombinant D10 scTCR is shown schematically in Fig. 1A. The TCR Vβ and Vα domains were connected by a 27-residue flexible peptide linker (Fig. 1B), and the E. coli maltose-binding protein (MBP) was fused to the N terminus of the Vβ domain via a linker containing a thrombin cleavage site. In addition, six histidine residues were added to the C terminus to allow binding to a Ni-NTA affinity chromatography column. The expressed protein was purified under nonreducing conditions to preserve the disulfide bonds formed in vivo. A similar approach was utilized to refold an E. coli-expressed single-chain antibody (37).

Monomeric MBP-D10 scTCR fusion protein was purified from E. coli lysates of supernatants using a combination of amylose, Ni-NTA, and mAb 3D3 affinity chromatography steps. Purified fusion protein was then subjected to Superdex 200PG size exclusion chromatography to remove the small amounts of aggregates. Purity of the 70-kDa fusion protein was monitored after each step by SDS-PAGE performed under reducing (Fig., 2A, lanes 1–4) and nonreducing (Fig. 2A, lanes 5–8) conditions. The N-terminal sequence determined for the purified product (KIEEGKLV), was as expected for the correctly processed maltose-binding protein (38).

Thrombin digestion of size exclusion chromatography, purified monomeric fusion protein resulted in efficient cleavage (Fig. 2B). Cleavage of the aggregated fusion protein isolated following size exclusion chromatography, by contrast, was inefficient and resulted in significant precipitation (not shown). Thrombin-cleaved D10 scTCR was purified by Ni-NTA affinity chromatography carried out under nonnaturating conditions (Fig. 2B). The overall yield of purified D10 scTCR was 0.7–1.0 mg/liter of E. coli cell culture.

Characterization of D10 scTCR—The N-terminal amino acid sequence of purified D10 scTCR was determined to be GSAYQSQP, corresponding exactly to the amino acid sequence predicted following thrombin cleavage of the fusion protein (Fig. 1B). On SDS-PAGE analysis, purified D10 scTCR migrated faster under non-reducing than under reducing conditions (Fig. 3A), suggesting the existence of intramolecular disulfide bonds that lead to a more compact structure. Furthermore, TCR family-specific mAbs B20.1 (Vβ2) and MR5.2 (Vβ8.1/2) recognized only the non-reduced protein on Western blot analysis (not shown). Similar results were obtained with baculovirus-expressed, disulfide-linked, heterodimeric D10 TCR. Together, these results indicate that purified D10 scTCR is in a correctly disulfide-bonded conformation. The isoelectric point (pI) of purified D10 scTCR was 5.80 ± 0.03, as determined from the ratio of the integrated signal for each sample at every 0.2 nM in HBS, pH 7.4, at 25 and 37 °C, respectively. The overall yield of purified D10 scTCR was 0.7–1.0 mg/liter of E. coli cell culture.
relative percentage of secondary structural elements: 11%

digitized CD data provided the following predictions for the Ni. Chrom.

using Ni-NTA column (MBP-D10 scTCR (reducing conditions).

Methods.” The samples were analyzed by 12% SDS-PAGE under non-exclusion chromatography.

Monomeric MBP-D10 scTCR isolated following Superdex 200 PG size exclusion chromatography. B, purification of D10 scTCR. Monomeric MBP-D10 scTCR (MBP-D10) was cleaved with thrombin and purified using Ni-NTA column (Ni. Chrom.) as described under “Materials and Methods.” The samples were analyzed by 12% SDS-PAGE under non-reducing conditions. Lane 1, purified monomeric MBP-D10 scTCR; lane 2, thrombin digestion of MBP-D10 scTCR; lane 3, Ni-NTA flow-through (FT) of the thrombin-digested monomeric fusion protein; lane 4, D10 scTCR eluted (El) from Ni-NTA column using 250 mM imidazole. Presence and absence of thrombin is indicated by + or −.

The conformation of purified D10 scTCR was examined by CD and NMR spectroscopy. The far UV-visible CD spectrum indicated that purified D10 scTCR contained predominantly β-sheet secondary structure (Fig. 4A). Deconvolution of the digitized CD data provided the following predictions for the relative percentage of secondary structural elements: 11% α-helix, 72% β-sheet, 14% random coil, and 3% turn. These results are in agreement with the recently solved crystal structures of the heterodimeric TCRs (6, 7) and the immunoglobulin domain model for the Vα/Vβ TCR structure as proposed by others (17, 39–42).

To probe further the secondary structure of the purified protein, a series of one-dimensional NMR experiments were performed. A representative one-dimensional NMR spectrum is shown in Fig. 4B. The following observations strongly suggest that purified D10 scTCR predominantly adopts a β-sheet secondary structure: 1) the amide signals (∼11.0 to −6.0 ppm) show a dispersion, which is typical for folded proteins (11); 2) there are many low field shifted Hα resonances, which confirm that the scTCR is predominantly folded (A in Fig. 4B); 3) a few isolated signals upfield from the methyl resonances of the thrombin-cleaved D10 scTCR containing the C-terminal hexahistidine extension.

Our previous SPR binding studies demonstrated that Vβ8.2-specific bacterial superantigen SEC2 binds to insect cell-derived solu-

FIG. 3. Characterization of purified D10 scTCR. A, SDS-PAGE analysis of purified protein. D10 scTCR (6 μg) was subjected to 12% SDS-PAGE under reducing (R, lane 1) and nonreducing (NR, lane 2) conditions. B, isoelectric focusing analysis. Purified D10 scTCR (5 μg) was analyzed by isoelectric focusing on Servalyte precoat gels (pH 3–10) at 4 °C under nonreducing conditions (lane 2). The protein bands were visualized using Serva Blue stain. The known pI values (lane 1) for the marker proteins (Pharmacia) are as follows: amylglucosidase (3.5), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.2), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin (6.85, 7.35), lentil lectin (8.15, 8.45, 8.65), and tryptosinogen (9.3). C, size exclusion chromatography of isolated D10 scTCR. A 100-μg aliquot of purified D10 scTCR (solid line) was passed over a TSK G2000 size exclusion chromatography column. Elution profile of molecular weight standards is shown (dashed line, arrows indicate molecular weights). The molecular mass of D10 scTCR was determined to be approximately 28 kDa.
change in baseline during injection of TSST-1 is due to a change in the bulk refractive index resulting from differences in the salt concentrations of the protein solutions and the running buffer (17, 18). In addition, the sensogram shown in Fig. 5A indicates that, like baculovirus-derived D10 TCR, *E. coli*-expressed D10 scTCR binds to SEC2 with higher affinity than SEB due to a slower dissociation rate.

The binding of solution phase D10 scTCR to immobilized superantigens was also examined. D10 scTCR (5 to 1 μM) bound to an SEC2-coated (Fig. 5B) surface in a concentration-dependent manner but did not bind to TSST-1-coated (Fig. 5C) or control (Fig. 5D) surfaces. Moreover, similar to our previous findings (18), D10 clone-specific mAb 3D3 completely blocked the sD10 TCR/SEC2 interaction (Fig. 5E). Antibody 3D3 (21) recognizes a conformational epitope formed by the juxtaposition of the Vα and Vβ domains (18). These results therefore imply that the epitope for mAb 3D3 may overlap the binding site for SEC2. Alternatively, it may be that the excluded volume of 3D3 sterically blocks the binding of SEC2.

Kinetic constants for the D10 scTCR-SEC2 interaction were also determined using SPR measurements (34, 35). For D10 scTCR binding to immobilized SEC2, *Kₐ* and *Kₘₑₜ* were determined to be 2.45 ± 0.72 × 10⁴ M⁻¹ s⁻¹ and 1.36 ± 0.15 × 10⁻² s⁻¹, respectively. From these values, a *Kₐ* of about 0.6 μM was calculated. These values are similar to those determined for the baculovirus-derived, heterodimeric D10 TCR/SEC2 interaction (Table I).

**Binding of D10 scTCR to Soluble MHC Class II-Peptide Complex**—Our previous studies showed that an insect cell-derived, soluble, heterodimeric murine class II MHC I-A<sup>d</sup> derivative containing fused antigenic conalbumin peptide and complementary leucine zipper sequences (I-A<sup>d</sup>-CA-LZ) specifically stimulates proliferation of the D10 T-cell clone and binds to the soluble, heterodimeric D10 TCR with a *Kₐ* of about 2.1 μM (18). As expected, this interaction was not seen with the soluble, heterodimeric BDC2.5 TCR (18), a control TCR protein specific for a murine pancreatic β-cell antigen presented by murine MHC class II I-A<sup>d</sup> (33, 47). Moreover, neither unloaded I-A<sup>d</sup>-LZ nor human HLA-DR1-βHA peptide complex showed detectable binding to the heterodimeric D10 TCR, indicating that the bimolecular interaction between D10 TCR and I-A<sup>d</sup>-CA-LZ is specific (18).

To understand better the structural requirements for immune recognition, SPR experiments were performed to examine the binding interactions between D10 scTCR and various MHC class II-peptide complexes. Purified I-A<sup>d</sup>-CA-LZ at 5 μM bound specifically to D10 scTCR immobilized on the sensor surface (Fig. 6A). The sensogram spike at the end of the injection is due to differences in salt concentration between the protein solution and running buffer. Neither I-A<sup>d</sup>-LZ nor human HLA-DR1-βHA peptide complex showed detectable binding. These specificities are identical to those observed for heterodimeric D10 TCR (18).

Kinetic and equilibrium binding constants for I-A<sup>d</sup>-CA-LZ binding to immobilized D10 scTCR were also measured using the SPR biosensor. For these studies, I-A<sup>d</sup>-CA-LZ was injected over a D10 scTCR-coated surface at various concentrations (5 to 1 μM) (Fig. 6B), and association and dissociation rate constants were determined to be 1.71 ± 0.47 × 10⁴ M⁻¹ s⁻¹ and 2.58 ± 0.38 × 10⁻² s⁻¹, respectively. The ratio of these kinetic constants gave an equilibrium dissociation constant of 1.5 μM. These values are nearly identical to those determined for the heterodimeric D10 TCR/I-A<sup>d</sup>-CA-LZ interaction (Table I) (18).

**DISCUSSION**

In the present study, milligram amounts of refolded, native-like D10 scTCR were produced for structural and functional
Fig. 5. Interactions between D10 scTCR and bacterial superantigens analyzed by SPR. A, sensorgram showing binding of superantigens to immobilized D10 scTCR. Superantigens TSST-1, SEB, and SEC2, each at 0.5 mg/ml in water, were passed over coupled D10 scTCR (5300 RU). B–E, sensorgrams showing binding of D10 scTCR to immobilized superantigens. D10 scTCR (5.0 to 1.0 μM) was passed over immobilized SEC2 (B, 1313 RU), TSST-1 (C, 1253 RU), and control (D) surfaces. Between injections, surfaces were regenerated with 5 μl of 10 mM HCl (not shown). E, sensorgram showing mAb 3D3 blocks the D10 scTCR/SEC2 interaction. D10 scTCR (2.5 μM) was passed over immobilized SEC2 (983 RU) either in the absence or presence of mAb 3D3 (5.0 μM). Between injections, surfaces were regenerated with HCl.
Purified D10 scTCR possessed native-like properties based on several analytical techniques. CD and one-dimensional NMR spectra indicated that purified protein is stabilized predominantly by β-sheet secondary structure. Recently, similar results were reported for an *E. coli*-derived Vα domain of a murine B4.2.3 TCR (42). Importantly, purified D10 scTCR remains soluble at concentrations to 30 mg/ml, further indicating a native-like conformation. This has not been reported for other *E. coli*-derived scTCR molecules (17, 40, 41, 50, 51). Previously, it was suggested that the solubility of *E. coli*-derived scTCR molecules is enhanced by the presence of >20 charged amino acid residues in the variable domains (particularly Vα) and by a small (1–2) number of potential N-linked glycosylation sites (52). The solubility of purified D10 scTCR is consistent with the first criterion (there are 23 charged amino acids in the α-chain) but not with the second (there are 3 potential N-linked glycosylation sites in D10 scTCR).

The ability of *E. coli*-derived D10 scTCR to interact with Vβ8.2 TCR-specific bacterial superantigens in the absence of MHC class II molecules is consistent with our previous finding (Ref. 18, also see Table I) and also with other published reports (17, 53–54). The kinetic and binding constants for the D10 scTCR/SEC2 interaction are similar to those of the baculovirus-derived, heterodimeric (VαCoNpβCβ) D10 TCR/SEC2 interaction (Table I), indicating that only VαVβ domains are required for SEC2 binding. Previously, the affinity of SEC2 for the myeloma cell-derived, soluble VβCβ-chain of the 14.3.d TCR (Vα4.1, Vβ8.2) was determined to be 5.4 μM using the SPR biosensor, and 2.32 μM by a sedimentation equilibrium binding technique (55). Together, these results further imply that residues in the common V8.2 segment alone are sufficient for binding to superantigens, at least in the absence of MHC (17, 55–57). The recently solved three-dimensional crystal structures of the β-chain of the 14.3.d TCR bound to both SEC2 and SEC3 further support this conclusion (10).

Kinetic and dissociation constants for the interactions between different TCRs and MHC-antigen complexes have been determined by several laboratories (58, 59, also reviewed in Ref. 16). Our recent studies (18) showed that the affinity of soluble, heterodimeric D10 TCR for soluble I-AK-CA peptide complex is about 2.1 μM. The *Kₐ* for *E. coli*-derived D10 scTCR/I-AK-CA peptide complex was found to be nearly identical (1.5 μM) to this value (Table I), further implying that the variable domains of the heterodimeric D10 TCR are sufficient for their interaction with the murine MHC class II I-AK-CA peptide complex.

Malchiodi and co-workers (55) showed that an unglycosylated mutant form of the murine 14.3.d TCR β-chain in which four out of five potential Asn-linked glycosylation sites were eliminated through site-directed mutagenesis bound to various

| Complex          | Apparent *kₐ* | Apparent *kₜ* | Apparent *Kₐ* |
|------------------|---------------|---------------|--------------|
| D10 scTCR/SEC2   | 2.45 ± 0.72 x 10⁶ | 1.36 ± 0.15 x 10⁻² | 0.6          |
| D10 TCR/SEC2     | 1.69 ± 0.12 x 10⁶ | 1.86 ± 0.47 x 10⁻² | 1.1          |
| D10 scTCR/I-AK-CA-LZ | 1.71 ± 0.47 x 10⁴ | 2.58 ± 0.38 x 10⁻² | 1.5          |
| D10 TCR/I-AK-CA-LZ | 1.07 ± 0.19 x 10⁴ | 2.20 ± 0.65 x 10⁻² | 2.1          |

* Taken from Ref. 18.

**FIG. 6.** Interactions between D10 scTCR and soluble class II MHC molecules analyzed by SPR. A, sensorgram showing that 1-AK-CA-LZ specifically interacts with immobilized D10 scTCR. Purified 1-AK-CA-LZ (5 μM), I-AK-LZ (5 μM), and soluble HLA-DR1/HA peptide complex (15 μM) proteins were passed over immobilized D10 scTCR (2004 RU). B, sensorgram showing concentration-dependent binding of 1-AK-CA-LZ to immobilized sD10 TCR. Purified 1-AK-CA-LZ (5.0–1.0 μM) was passed over immobilized sD10 TCR (2768 RU). Between injections, surfaces were regenerated with 5 μl of 10 mM HCl.
forms of SEC with affinities similar to those of fully glycosylated form. Although these results suggest that N-linked glycans do not contribute to superantigen binding, the possible involvement of the fifth remaining site as well as the potential yet unidentified O-linked carbohydrate sites (60) in binding to SEC could not be ruled out. Since the E. coli-derived D10 scTCR was not glycosylated, our studies provide a direct evidence that the carbohydrates do not play a significant role in the binding of D10 TCR to SEC2 and also to murine MHC class II I-A\textsuperscript{\textalpha}-CA peptide complex. In addition, these results suggest that carbohydrates are also not required for the expression and conformational stability of the D10 TCR.

Sequence alignments (39) and more recently the x-ray crystallographic data (6–9) suggest that TCRs and Igs are built on the same principles. Interestingly, the interdomain contact residues that are present in the corresponding V\textsubscript{\textgamma}-V\textsubscript{\textdelta} antibody interface are also conserved between Va and V\textbeta domains of TCR (6). Moreover, similar to Ig molecules, within each Va and V\textbeta domains of 2C TCR about 24 residues form the core of the \beta-sheet sandwich (6). The CD and one-dimensional NMR spectra of purified D10 scTCR described here also indicate that scTCR is stabilized predominantly by \beta-sheet secondary structure, consistent with its native-like conformation.

Because of its size, high solubility, and structural integrity, purified D10 scTCR should be suitable for structural studies by multidimensional NMR spectroscopy. Such studies may help in determining the packing of the Va and V\textbeta domains in scTCR and in assessing the flexibility of its various CDR regions in solution. In addition, the structural and dynamic studies with superantigens and class II MHC/peptide should be useful in further understanding the molecular interactions between TCR and its ligands.

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