Differential Roles of T Cell Receptor α and β Chains in Ligand Binding Among H-2K\(^d\)-restricted Cytolytic T Lymphocyte Clones Specific for a Photoreactive Plasmodium berghei Circumsporozoite Peptide Derivative*

To study the interaction of T cell receptor with its ligand, a complex of a major histocompatibility complex molecule and a peptide, we derived H-2K\(^d\)-restricted cytolytic T lymphocyte clones from mice immunized with a Plasmodium berghei circumsporozoite peptide (PbCS) 252–260 (SYIPSAEKI) derivative containing photoreactive \(N^\alpha\)-[4-azidobenzoyl] lysine in place of Pro-255. This residue and Lys-259 were essential parts of the epitope recognized by these clones. Most of the clones expressed BV181A1 encoded \(\alpha\) chains along with specific complementary determining region (CDR) 3β regions but diverse \(\alpha\) chain sequences. Surprisingly, all T cell receptors were preferentially photoaffinity labeled on the \(\alpha\) chain. For a representative T cell receptor, the photoaffinity labeled site was located in the Va C-strand. Computer modeling suggested the presence of a hydrophobic pocket, which is formed by parts of the VaJa C- and G-strands and adjacent CDR3α residues and structured to be able to avidly bind the photoreactive ligand side chain. We previously found that a T cell receptor specific for a PbCS peptide derivative containing this photoreactive side chain in position 259 similarly used a hydrophobic pocket located between the junctional CDR3 loops. We propose that this nonpolar domain in these locations allow T cell receptors to avidly and specifically bind epitopes containing non-peptidic side chains.

Crystallographical studies showed that some peptide side chains intrude into allele specific pockets of the MHC molecule, whereas others are surface exposed and may interact with TCR (2, 3). Conversely, crystallographical studies on a BV882A1 encoded TCR \(\beta\) chain and an AV4S2 TCR \(\alpha\) chain fragment confirmed that TCR are folded in an Ig-like manner but also indicated significant structural differences, such as different hinge structures and interstrand hydrogen bond formation (4, 5). Little is still known on how TCR bind their ligand (MHC-peptide complexes). Based on theoretical considerations, it has been proposed that V-encoded complementary regions CDR1 and CDR2 primarily interact with the MHC molecule, such that the junctional CDR3 are located over the MHC peptide binding groove (6). Support for this concept mainly derives from experiments indicating that CDR3 loops interact with residues of MHC bound peptides (7, 8). One study using immunization of single chain TCR transgenic mice with peptide variants suggested that CDR3α interacted with an N-terminal residue of an MHC class II bound peptide and CDR3β with a C-terminal one (9). A more recent study, using a similar strategy, showed that an N-terminal peptide residue interacted with CDR1α or CDR2α, while a C-terminal one interacted with CDR3β, implicating a diagonal orientation, e.g. about 30 °C. In contrast, analysis of a K\(^\alpha\)-restricted OVA-specific TCR indicated that the most C-terminal residue of this epitope interacted with CDR3α (11).

While these studies are in accordance with the current concept of TCR-ligand interactions (6), there are observations that are difficult to explain this way. For example, MHC class II-restricted tetanus toxin t830–844 reactive T cells exhibited preferential BV2S1 usage but lacked junctional sequence conservation on both chains (12). Furthermore, an L\(^\delta\)-restricted arsotane-reactive CTL clone using the same AV3S1 sequence as an I-A\(^d\)-restricted arsotane-reactive one, but an unrelated CDR3α junction, also recognized this epitope, suggesting that in this system, CDR3 sequences may not be important (13, 14). Moreover, we found that a TCR specific for the Plasmodium berghei circumsporozoite PbCS peptide 252–260 (SYIPSAEKI) conjugated with 4-azidobenzoic acid (ABA) at Lys-259 bound the photoreactive ligand side chain with CDR3α and V\(\beta\) C′-strand/CDR2β residues (15).

In this previous study, we modified the H-2K\(^d\) (K\(^\alpha\))-restricted PbCS 252–260 by replacing Ser-252 with photoreactive iodo-4-azidosaliclylic acid (IASA) and by conjugating the TCR contact residue PbCS Lys-259 with ABA, to make IASA-YIPSAEK(ABA)I. CD8\(^+\) CTL clones were derived from mice immunized with this conjugate. These recognized IASA-YIPSAEK(ABA)I as well as YIPSAEK(ABA)I in a K\(^\alpha\)-restricted manner, but not IASA-YIPSAEKI or the parental PbCS peptide. Selective pho-
toactivation of the IASA group allowed covalent attachment of the conjugate to Kd molecules. Incubation of these complexes with cloned CTL and photoactivation of the ABA group resulted in TCR photoaffinity labeling (15). Unlike PbCs-specific CTL, these clones preferentially expressed BV151A1 encoded TCR β chains that were paired with J5Ta28 encoded α chains (15). Sequences encoded by these TCR gene elements formed a hydrophobic pocket, which was structured and located such that it could efficiently accommodate the photoactive ligand side chain. In the present study, we replaced PbCs Pro-255 with K(ABA) and produced likewise peptide derivative-specific CTL clones. This residue was chosen because it is surface exposed and critical for T cell recognition (16). Here we report that these CTL clones were remarkably different from the ones described previously in terms of antigen recognition, TCR sequences, and binding of the photoactive ligand side chain. For a representative TCR, we show a novel binding principle by which TCR can efficiently and specifically bind a chemically modified epitope.

EXPERIMENTAL PROCEDURES

Peptide and Conjugate Synthesis and Analysis—Reagents for chemical synthesis were obtained from Bachem Finechemicals (Bubendorf, Switzerland), Pierce, and Sigma. All synthetic and analytical procedures were performed essentially as described (17, 18). In brief, peptides were synthesized on an Applied Biosystem Instruments 431 peptide synthesizer (ABI, Foster City, CA) using Fmoc (N-(9-fluorenyl)methoxycarbonyl) for transient protection. The deprotected peptides and peptide derivatives were purified by HPLC on a C-18 column (1 × 25 cm, Macherey Nagel, Oensingen, CH) using a Waters 600E HPLC system equipped with an in-line 1000 S diode array UV spectrophotometer (Applied Biosystem Instruments). The column was eluted by a linear gradient of acetonitrile on 0.1% trifluoroacetic acid in water, rising within 1 h from 0 to 75%. All compounds displayed the expected UV spectra (17, 19, 20). ANBA-YIKSAEKI displayed UV absorption at 280 nm. The mass of all purified compounds was verified by mass spectrometry on an LDI 7000 mass spectrometer (Linear Scientific, Reno, CA). Radioiodination of ASA-YIK(A-PO3H2)SAEKI and Dap(ISA)-YIK(ABA)SAEKI was performed by radioiodination of ASA-YIKO3H2H2O/ABA/SAEKI and Dap(ISA)-YIKO3H2H2O/ABA/SAEKI, respectively, followed by enzymatic dephosphorylation. The HPLC-purified peptides were lyophilized and immediately used for photoaffinity labeling experiments.

Kd and TCR Photoaffinity Labeling—Soluble monomeric Kd-peptide derivative complexes were prepared by incubating purified Kd with 125I-labeled YIK-ABA-SAEKI followed by UV irradiation at ≥350 nm and FPLC gel filtration as described by Luescher et al. (15, 17). For TCR photoaffinity labeling, 107 cpm of Kd-125I-YIK(A-PO3H2)SAEKI were incubated with 5 × 106 CTL, resuspended in 1 ml of Dulbecco’s modified Eagle’s medium supplemented with 2% fetal calf serum and 20 mM HEPES, on ice for 3 h followed by UV irradiation at 312 ± 40 nm. For two-dimensional gel analysis and peptide mapping, peptide derivative-Kd cross-linking was prevented by using the monofunctional PbCs derivative Dap(ISA)-YIKABA/SAEKI as described previously (15). 1 mCi of Dap(125I)-YIKABA/SAEKI was incubated with 50 μg of Kd for 2 h at ambient temperature prior to addition of 4 × 106 clonal CTL in 2 ml of medium (see above) containing β-2 microglobulin (2.5 μg/ml). After 2–3 h of additional incubation at 0–4 °C, the incubations were UV irradiated at 312 ± 40 nm.

Immunoprecipitation and Gel Electrophoresis—The photoaffinity labeled cells were washed twice with cold PBS and lysed on ice in PBS (1 × 106 cells/ml) supplemented with 0.7% Nonidet P-40, HEPES, phenylmethylsulfonyl fluoride, leupeptin, and iodoacetamide as described (17). After centrifugation (3 min at 15,000 × g), the detergent-soluble fractions were membrane filtrated and subjected to immunoprecipitation with the anti-TCR Cβ mAb H57-597 or the anti-Kd-β2 mAb SF1−1.1.1 as described (17). For two-dimensional gel analysis and peptide mapping, TCR photoaffinity complexes immunoprecipitated with H57-597 mAb on albumin-Sepharose as described (17, 19, 20). The washed and lyophilized Sepharose was resuspended in sample buffer for IEF, and after a 1-h incubation at 37 °C, the supernatants were subjected to disc IEF followed by SDS-PAGE as described (21). All antibodies were obtained from American Type Culture Collection (ATCC, Rockville, MD).

Peptide Mapping—The photoaffinity labeled and immunoprecipitated W13 TCR was reduced and denatured in 200 μl of 100 mM Tris buffer, pH 8.0, supplemented with 8 μl urea, 100 mM 2-mercaptoethanol, and 1% Nonidet P-40, at 37 °C for 1 h. To the supernatant, 400 μl of 500 mM Tris, pH 8.0, containing 100 mM iodoacetate were added. After incubation for 1 h at 20–24 °C, the samples were dialyzed at 4 °C in 25 mM sodium phosphate buffer, pH 7.0, containing 1 mM guanidine hydrochloride, both containing 10% acetonitrile, and subjected to tryptic or protease V8-5 peptide mapping as described (15) except that a C18 column was used (0.4 × 65 mm particle size, Vydac, Hesperia, CA). In brief, 10 μl aliquots of trypsin or protease V8-5 (Boehringer Mannheim) were added in 12-h intervals, and after 48 h of incubation at 37 °C, the digests were subjected to HPLC. Radioactivity was monitored by γ counting of 1 ml fractions and chromatography by measuring the A1340 of standards including Nε-(2,4-dinitrophenyl)lysine (elution time 29.1 min), respectively, approximately YIPSASEKI (25.6 min), MAVDPIGHLY (28.7 min), ASNENMDAM (30.1 min), and oxidized insulin β chain (37.7 min) (Sigma). The maximal variation tolerated was ±1%. For secondary peptide mapping, labeled digest fragments were reconstituted in 300 μl of 100 mM phosphate buffer, pH 7.4, containing 50 μg of bovine γ-globulin and 10% acetonitrile. Endoprotease Pro-C (gift from Dr. J. Gagnon, Biologie Structurale, C.E.A. and C.N.R.S., URA 1333, Grenoble, France) was added in 12-h intervals, and after 36–48 h, the samples were subjected to HPLC. SDS-PAGE analysis of labeled digest fragments was performed as described (22).

Generation of ANBA-YIK/ABA/SAEKI-specific CTL Clones—Cloned CTLs were obtained as described previously (15, 16). In brief, (BALB/c × C57BL/6) F1 mice were immunized with ConA spleen blasts expressing cognate Kd-ANBA-YIK/ABA/SAEKI complexes. Cultures of peritoneal exudate lymphocytes were stimulated in vitro over two 7-day intervals. Viable cells were cloned 7 days after the second in vitro stimulation by fluorescence-activated cell sorting of CD8+ cells into 96-well plates. The clones were stimulated weekly with 2 × 105 γ-irradiated P815 pulsed before hand with 1 μM of ANBA-YIK/ABA/SAEKI and 3 × 103 γ-irradiated BALB/c splenocytes. All rapidly expanding cultures were tested as described below. The U, V, and W clones were derived from three different mice.

Cytolytic Assays—The cytolytic activity of the CTL clones under study was assessed in a standard 51Cr release assay as described previously (16, 23). In brief, 51Cr-labeled P815 cells or Kd-transfected L cells (L-Kd) (2 × 105 cells/well) were incubated with a constant number of CTLs (6–8 × 103 cells/well) and serial dilutions of peptide derivative or peptide variant, respectively, in 96-well microplates. The L-Kd mutants have been described previously (24). After 4 h of incubation at 37 °C, the released 51Cr was measured, and the specific lysis was calculated in percent as 100 × (experimental-spontaneous release)/ (total-spontaneous release). The relative antigenic activities were calculated by dividing the concentration of ANBA-YIK/ABA/SAEKI required for half-maximal lysis by that required for the peptide derivative variants. The ability of a peptide derivative to bind to Kd, was assessed in a recognition-based competition assay as described (23). In brief, 51Cr-labeled P815 cells were incubated with three-fold dilutions of the test peptide derivatives prior to addition of the antigenic HLA-Cw3 peptide 170–179 (RYLKNGKETL) and Cw3-specific cloned CW3/1.1 CTL. After 4 h of incubation, the 51Cr release was measured. The Kd competitor activity of each compound was calculated relative to the one of ANBA-YIK/ABA/SAEKI, which was defined as 1. For the sake of comparison, the relative antigenic activities of the different peptide variants were normalized by dividing the relative antigenic activity by the corresponding relative Kd competitor activities.

TCR Sequence Analysis—Total RNA extraction was performed using the isothiocyanate acid-phenol method. cDNA synthesis was carried out on total RNA with AMV reverse transcripts following the supplier instructions (Boehringer Mannheim, Rotkreuz, Switzerland). Screening of junctional regions of α and β chains was performed by PCR using primers as described in Ref. 25. Purified PCR products were sequenced with Sequenase Version 2.0 kit (U. S. Biochemicals, Cleaveland, OH) and a 96-well ATP following the supplier instructions of the kit. The sequence of W13 and U4, the primers W135 and W139 (GACCGAATTCGATGACGATCAATAGATGGAC) and W133 (CAACCGTGGTACCACAGGGGAAGCTGTAAGCTG) were used.

Molecular Modeling—Models of the W13 TCR and Kd-ANBA-YIK/ABA/SAEKI complex were built using the ICM software (26). For the β chain, we used as template the crystal coordinates of a BV8S2A1 encoded β chain (4). A crude model of the W13 Vβ chain was obtained.
TABLE I
Recognition of PbCS peptide derivatives by seven independent CTL clones

| CTL clone | ANBA-YIKSAEKI | YIKSAEKI | YIKSAEKI | YIKSAEAI | YIKSAEI | YIKSAEI | YIKSAEAI | YIKSAEAI | YIKSAEAI |
|-----------|----------------|----------|----------|----------|----------|----------|----------|----------|----------|
| U3        | 150<sup>a</sup> | 4        | <10<sup>-4</sup> | <10<sup>-4</sup> | 0.03 | 0.3 | 0.3 |
| U4        | 20             | 2        | <10<sup>-5</sup> | <10<sup>-5</sup> | <10<sup>-5</sup> | 0.6 |
| V8        | 0.5            | 0.3      | <10<sup>-6</sup> | 10 <sup>-6</sup> | <10<sup>-6</sup> | 0.01 |
| V13       | 3              | 0.2      | <10<sup>-5</sup> | 0.002 | <10<sup>-5</sup> | 0.1 |
| V17       | 10             | 0.4      | <10<sup>-6</sup> | <10<sup>-6</sup> | 0.05 | <10<sup>-5</sup> | 0.003 |
| V19       | 1              | 0.1      | <10<sup>-6</sup> | <10<sup>-4</sup> | <10<sup>-4</sup> | 0.08 |
| W13       | 27             | 5        | <10<sup>-5</sup> | <10<sup>-5</sup> | 0.003 |

<sup>a</sup> The normalized relative antigenic activities of the peptide derivatives were calculated by dividing the relative antigenic activities by the relative K<sup>b</sup> competitor activities (see “Experimental Procedures”).

<sup>b</sup> The relative K<sup>b</sup> competitor activities were calculated relative to the one of ANBA-YIK(ABA)/SAEKI.

<sup>c</sup> The numeric values indicate the concentration (pm) of ANBA-YIK(ABA)/SAEKI required for 50% maximal lysis (<sup>51</sup>Cr release) by the corresponding CTL clones. Dilutions (3-fold) of the peptide derivative were made in the range of 10<sup>-5</sup>–10<sup>-15</sup> m. These values were used to calculate the relative antigenic activity of the other conjugates.

using the ProMod package (27) and was subjected to a standard energy minimization with X-PLOR and followed by a regularized minimization of the geometry with ICM (the RMSB between the initial and the regularized structures was 0.49 Å for non-hydrogen atoms). Similarly, the model of the W13 TCR V<sub>b</sub> chain was obtained by homology modeling using the x-ray structure of 1J49 (5) as a template. The resulting V<sub>b</sub> model (RMSB between the template and the modeled structure was 1.0 Å for 106 Ca atoms) was associated with the V<sub>j</sub> model to form a V<sub>a</sub>-V<sub>j</sub> complex, starting with the geometry of an Ig V<sub>j</sub>V<sub>i</sub> domain. After minimization of the complex, possible conformations of the CDR3 loops were searched using a local deformation Monte-Carlo procedure. The K<sup>b</sup>-ANBA-YIK(ABA)/SAEKI complex was modeled on the basis of the x-ray structure of 1J49 (5) as described before (15). As the PbCS peptide derivative contains non-canonical amino acids, namely K(ABA) and ANBA, models for these residues were generated using the molecular editor of the HyperChem package. Atom charges were calculated using the CND02 method. The files containing the resulting coordinates and charges were imported and processed by ICM to produce ICM library entries used in subsequent modeling. The structure of the trimolecular complex, consisting of W13 V<sub>a</sub>-V<sub>j</sub> docked to the K<sup>b</sup>-peptide complex, was obtained by several cycles of the following procedures and guided by the experimental data derived from the mapping of the photoaffinity labeled site and the effects of mutations in K<sup>b</sup>: 1) local deformation Monte-Carlo search of an optimal conformation for V<sub>a</sub>-V<sub>j</sub> CDR loops within the field of the K<sup>b</sup>-peptide surface; 2) Monte-Carlo search of the optimal position of the side chains of all residues of the interface between TCR and ligand; 3) a constrained Brownian-like “walk” of the ligand around the TCR; and 4) exhaustive minimization of the whole structure. The resulting structure was checked visually as well as by calculation of the local electrostatic interactions in the K(ABA) binding pocket.

RESULTS

To produce a new family of “photoprobe”-specific CTL clones, we prepared a photoactive derivative of the PbCS peptide 252–260 by replacing Pro-255 with K(ABA). To cross-link the peptide derivative to K<sup>b</sup>, Ser-252 was replaced with IASA to make IASA-YIK(ABA)/SAEKI. As assessed in a recognition-based competition assay, this derivative bound to K<sup>b</sup> nearly 100-fold less efficiently than the parental PbCS peptide, but replacement of IASA with ANBA, N<sub>2</sub>-IASA-2,3,1-diaminopro-pionic acid (Dap(lASA)), or Nβ-(iodosalicyloyl)-2,3,1-diaminopro-pionic acid (Dap(lSA)A) restored efficient K<sup>b</sup> binding (2–3-fold less than the PbCS peptide). However, since ANBA-YIK(ABA)-SAEKI was not available in radioactive form and Dap(lASA)-YIK(ABA)/SAEKI very inefficiently photoaffinity labeled K<sup>b</sup> (12), IASA-YIK(ABA)/SAEKI was used for radioactive photoaffinity labeling experiments.

Antigen Recognition by ANBA-YIK(ABA)/SAEKI-specific CTL Clones—From 14 independent CTL clones derived from mice immunized with ANBA-YIK(ABA)/SAEKI, seven that exhibited efficient TCR photoaffinity labeling were selected for further studies. As shown for a representative experiment in Table I, the concentration of ANBA-YIK(ABA)/SAEKI required for half-maximal lysis of P815 target cells was in the range of 0.5 (V8 clone) to 150 pm (U3 clone), which is comparable with other K<sup>b</sup>-restricted CTL clones (15, 16). The recognition by all clones was inhibited by the anti-K<sup>b</sup> α1 mAb 20–8,4S, indicating that they were K<sup>b</sup>-restricted (data not shown).

All clones efficiently recognized the derivative lacking the N-terminal photoactive group (YIK(ABA)/SAEKI), but none detectably recognized the derivative lacking ABA (YIKSAEKI) except for a very inefficient recognition by the V17 clone (Table I). Interestingly, all clones recognized, though some inefficiently, the variant containing acetyl in place of ABA (YIK(ABA)/SAEKI), suggesting that the labeling site and the effects of mutations in K<sup>b</sup> demonstrated that K(ABA) and Lys-259, but not the N-terminal photoactive group, were essential parts of the epitope recognized by these clones and that generally the full spacer length of these side chains was required for efficient antigen recognition.

To obtain information on TCR-K<sup>b</sup> contacts, we assessed the ability of the CTL clones to recognize ANBA-YIK(ABA)/SAEKI...
FIG. 1. Effect of K\textsuperscript{d} mutations on ANBA-YIK(ABA)SAEKI recognition by specific CTL clones. L cells expressing K\textsuperscript{d} molecules containing single alanine substitutions in the indicated positions of the α1 and α2 helices were tested in \textsuperscript{51}Cr release assay as described under “Experimental Procedures.” White boxes indicate normalized antigenic activities of 1–0.1, hatched boxes indicate activities of 0.1–0.001, and black boxes indicate activities of <0.001. For normalization, the specific lysis measured on L cells expressing wild-type K\textsuperscript{d} (K\textsuperscript{d} wt) was used as reference. No lysis was detectable on untransfected L cells (LM1).

TCR Photoaffinity Labeling—For TCR photoaffinity labeling, covalent K\textsuperscript{d}-peptide derivative complexes were used, which were obtained by photoaffinity labeling of soluble K\textsuperscript{d} with \textsuperscript{125}IASA-YIK(ABA)SAEKI. This derivative, upon correction for its reduced K\textsuperscript{d} binding, was recognized by all clones as efficiently as ANBA-YIK(ABA)SAEKI. Following incubation of the cloned CTL with K\textsuperscript{d}-peptide derivative complexes and photoactivation of the ABA group, the cells were detergent solubilized and the lysates subjected to immunoprecipitation. The immunoprecipitates with an anti-TCR mAb migrated on SDS-PAGE under reducing conditions with an apparent molecular mass of 87–92 kDa (Fig. 2A). Since TCR are composed of disulfide-linked α and β chains, 38–45 kDa each (21), the molecular mass of these materials corresponded to trimolecular complexes consisting of K\textsuperscript{d} heavy chain (45 kDa), the peptide derivative (~1,400 Da), and one TCR chain.

To assess the specificity of TCR photoaffinity labeling, cloned W13 CTL were incubated with \textsuperscript{125}IASA-YIK(ABA)SAEKI in the absence or presence of a 300-fold molar excess of peptide PbCS 252–260 or the D\textsuperscript{d}-restricted peptide Ad5 E1a 234–243 (Fig. 2B, lanes 1–3). Following UV irradiation at 312 ± 40 nm, which activates the IASA and ABA groups, the cells were analyzed as in the previous experiment. The TCR photoaffinity labeling observed in the absence (lane 1) or presence of the D\textsuperscript{d} binding peptide (lane 3), was abolished in the presence of the K\textsuperscript{d} binding PbCS peptide (lane 2), indicating that TCR photoaffinity labeling required peptide derivative binding to K\textsuperscript{d} (of CTL) and that free conjugate was unable to detectably label the TCR. The same findings were obtained for the other clones (data not shown).

Analysis of total lysate of W13 cells labeled with K\textsuperscript{d}-\textsuperscript{125}IASA-YIK(ABA)SAEKI on SDS-PAGE under reducing conditions showed two labeled species of apparent molecular masses of 45 and 90 kDa, respectively (lane 4). These materials correspond to the K\textsuperscript{d}-peptide derivative complex and the TCR-ligand complex, respectively. The absence of other labeled species shows that the TCR photoaffinity labeling was remarkably selective. The same labeled species were observed following immunoprecipitation with anti-K\textsuperscript{d} α3 mAb SF1–1.1.1 (lane 5), which precipitates free as well as ligand cross-linked with TCR (17). Upon immunoprecipitation with anti-TCR mAb, only the trimolecular complex was observed (lane 6). This TCR photoaffinity labeling was inhibited by the anti-K\textsuperscript{d} α1 mAb 20–8-4S (lane 7), which prevents K\textsuperscript{d}-TCR interactions (17), but not by the anti-D\textsuperscript{d} mAb 34–4-20S (lane 8). Finally, when analyzed by
SDS-PAGE under nonreducing conditions, labeled material of approximately 130 kDa was observed (lane 9). This increase in molecular mass corresponds to the second TCR chain, which is part of the trimolecular complex under these conditions. These results demonstrate that the TCR photoaffinity labeling was specific and required peptide derivative presentation by K\({b}\).

Assessment of the Photoaffinity Labeled TCR Chain—To identify which TCR chain was photoaffinity labeled, W13 TCR was labeled with K\(d\)-associated Dap\(^{125}\)ISA)-YIK(ABA)SAEKI. This conjugate was recognized by all CTL clones as efficiently as ANBA-YIK(ABA)SAEKI (data not shown) but, due to lacking an N-terminal photoreactive group, was unable to cross-link to K\(d\). The immunoprecipitated TCRs were analyzed by two-dimensional gel electrophoresis in which the first dimension was IEF and the second SDS-PAGE. As shown in Fig. 3A, labeled material with an IP of about 4.9 and an apparent molecular mass of approximately 43 kDa was observed. Alternatively, W13 cells were surface radioiodinated and analyzed likewise. The same analysis showed two labeled materials, one of which migrated very similarly to the one in the previous experiment while the other had an IP of about 6.5 and an apparent molecular mass of approximately 44 kDa (Fig. 3B). Since both TCR chains have similar molecular mass but the IP of \(\alpha\) chains are acidic (IP 4–5) and those of \(\beta\) chains nearly neutral (21), these results indicate that the W13 TCR was photoaffinity labeled selectively at the \(\alpha\) chain. The same analysis was performed with the other clones. As shown in Fig. 3C, all clones were labeled selectively at the \(\alpha\) chain except for the V17 TCR, which was labeled 70% at the \(\alpha\) chain and 30% at the \(\beta\) chain.

TCR Sequencing by PCR—PCR using V region-specific primers showed that four of the seven clones (U4, V8, V19, and W13) expressed BV1S1A1 encoded \(\beta\) chains (Table II, left). Sequenc- ing indicated that their junctional, CDR3\(b\) equivalent regions, were 11–12 residues long and contained Ser in position 1, Gln in position 2, Asp or Glu in position 3, Gly in positions 5 and 6, usually Glu in position 10, and Leu in PC (Table II, left). The corresponding J\(\beta\) were different, but all, except one (U4), belonged to the J\(\beta_2\) family. Of the remaining clones, two expressed BV8S1 (U3 and V17) and one expressed BV13S1 (V13). The corresponding CDR3\(\beta\)s were more diverse but always acidic. The same analysis performed on the \(\alpha\) chains indicated that the Va, Jo, and CDR3\(\alpha\) sequences were diverse. For the V8 clone, none of the Vo-specific primers (22) used for screening (25) detected an in-frame transcript. Performing the same analysis on six additional ANBA-YIK(ABA)SAEKI-specific CTL clones showed a very similar pattern (data not shown).

The finding that specific TCR—\(\alpha\) chain (Fig. 3C).

Mapping of the TCR Photoaffinity Labeled Site on the W13 TCR—To elucidate this paradox, we mapped the photoaffinity labeled site on a representative TCR, the W13 TCR, which was BV1S1A1 and AV4S10 encoded (Table II). To this end, W13 TCR was photoaffinity labeled with K\(d\)-associated Dap\(^{125}\)ISA)-YIK(ABA)SAEKI and following reduction and alkylation, was extensively digested with protease V-8. The resulting digest fragments were separated by HPLC on a C-18 column. As shown in Fig. 4A, the major labeled fragments reproducibly eluted from the column after approximately 33 min. On SDS-PAGE, this material was homogeneous and migrated with an apparent molecular mass of about 8,300 Da (Fig. 4F, lane 1). The later eluting labeled materials migrated slower on SDS-PAGE (apparent molecular masses of about 4,300 Da, 38 min, and 13,000 Da, 44 min, respectively) and were more abundant after shorter digest periods, suggesting that they were incomplete digest products.

**FIG. 3.** Two-dimensional gel electrophoresis of labeled TCR. W13 cells were photoaffinity labeled with Dap\(^{125}\)ISA)-YIK(ABA)SAEKI (A) or were surface radioiodinated (B). The immunoprecipitated TCR were analyzed by two-dimensional gel electrophoresis in which the first dimension (horizontal) was IEF and the second (vertical) SDS-PAGE (10%), both under reducing conditions. The same analysis was performed on the other clones. The densitometric evaluation of the autoradiograms are summarized in panel C. 100% is the total TCR photoaffinity labeling.

When trypsin was used for digestion, the major labeled fragments also eluted after about 33 min (Fig. 4B). On SDS-PAGE, this material was homogeneous but migrated slightly faster (apparent molecular mass of about 3,800 Da) than the major labeled V-8 digest fragment (Fig. 4F, lane 2). A second labeled tryptic digest fragment eluted from the HPLC column after 38–39 min and migrated on SDS-PAGE with an apparent molecular mass of about 7,600 Da. This component again was more abundant after shorter digest periods, suggesting that it was an incompletely digested fragment (data not shown). Digestion of the labeled primary V-8 digest fragment with trypsin resulted in a new fragment that by HPLC and SDS-PAGE was indistinguishable from the labeled primary tryptic fragment (Fig. 4, C and F, lane 3). Treatment of the primary
suggests the presence of a deep hydrophobic pocket between the heavy and light chain of the ligand (Fig. 6A). This prediction is consistent with the finding that the photoaffinity labeled sites were confined in essence by the side chains of CDR3 segments 33–39, and the primary tryptic fragment would correspond to residues 17–39. This is in accordance with the observed molecular mass and the finding that the photoaffinity labeled sites were contained in the Va segment 33–39 (Figs. 4 and 5).

To study TCR-ligand interactions by TCR photoaffinity labeling, we derived CTL clones from mice immunized with the Aβa azido substituent with the phenyl moiety of Va Phe-35, and iii) hydrogen bond formation of the ABA carbonyl oxygen, the most polar atom of this side chain, with CDR3α Ser-94 (Fig. 6B). This prediction is consistent with the finding that the photoaffinity labeled sites were contained in the Va segment 33–39 (Figs. 4 and 5).

According to our ligand model, the K(ABA) side chain of Kα-bound ANBA-YIK(ABA)AEKI is very extended and “leans over” to the Kβ α2 helix (Fig. 6D). A very similar orientation was obtained when the ligand was modeled alone or together with the W13 TCR (data not shown). The Kβ residues Glu-62, Ser-69, and Tyr-155, which upon alanine substitution dramatically affected the antigen recognition by the W13 clone (Fig. 1), are located on the surface of the α1 and the α2 helices, respectively.

While the available crystallographical coordinates permitted modeling of the TCR framework, including the hydrophobic pocket, with good accuracy, conclusive modeling of CDR3 loops was elusive because of the high number of possible, energetically equivalent conformers. We therefore modeled these loops by using the ligand surface as “template” to define loop conformations that provide the lowest energy of the TCR-ligand complex. These docking experiments were based on the assumptions that 1) the ligand K(ABA) side chain inserts in the hydrophobic pocket of the W13 TCR, 2) the Kβ residues Glu-62, Ser-69, and Tyr-155, but not Gln-65, Gln-72, Gln-149, Glu-154, or Glu-163, interact with the TCR, and 3) Asp-95 or Glu-101 of CDR3β interact with PbCS Lys-259. The latter assumption was made based on the finding that the W13 TCR (and most other TCR of that specificity) has two acidic residues in CDR3, or Glu-163, interact with the TCR, and 3) Asp-95 or Glu-101 of CDR3β interact with PbCS Lys-259. The latter assumption was made based on the finding that the W13 TCR (and most other TCR of that specificity) has two acidic residues in CDR3, but none in CDR1 and CDR2 of both chains (Fig. 5). These experiments suggest that the W13 TCR interacts with its ligand in the orientation shown in Fig. 6A, in which the C-terminal portion of the Kα-bound peptide is located underneath CDR3β, and the N-terminal portion is underneath CDR3α. It is noteworthy that the helices of Kα, especially the α2 helix, are slanted, and the W13 TCR has a complementary surface contour, which provides a maximal surface contact in this orientation (data not shown).

**DISCUSSION**

To study TCR-ligand interactions by TCR photoaffinity labeling, we derived CTL clones from mice immunized with the PbCS peptide derivative ANBA-YIK(ABA)SAEKI. As in previous studies with other photoreactive PbCS peptide derivatives (15, 16), ANBA-YIK(ABA)SAEKI-specific CTL were readily obtained. The “photoprobe”-specific CTL described here, as well as those described previously, displayed all the hallmarks of

| CTL clone | Vβ | TCR-β | TCR-α |
|-----------|----|-------|-------|
| U4        | 1S1A1 | CAS | SQDVGSSNEERL | FFG | 1.4 | 4S3 | CAL | GDTGSSGKEL | TLG |
| V8        | 1S1A1 | CAS | SQETGGAELT | YFG | 2.3 | 4 | CAL | Out of frame | TFG |
| V19       | 1S1A1 | CAS | SQDWGGAGQNTL | YFG | 2.4 | 2S1 | CA | Out of frame | TFG |
| W13       | 1S1A1 | CAS | SQDLGGAELT | YFG | 2.3 | 10 | CAM | Out of frame | TFG |
| U3        | 8S1 | CAS | SDNQDTQ | YFG | 2.5 | 11S2 | CA | EANTKVK | VFG |
| V117      | 8S1 | CAS | SDSDGTDNQP | LFG | 1.5 | 5S1 | CA | SRKIL | IFG |
| V13       | 13S1 | CAS | SYYQANTEV | FFG | 1.1 | 10S1 | CA | PDTNAYKV | IFG |

**TABLE II**

TCR gene elements usage and TCR junctional amino acid sequences

Seven ANBA-YIK/ABA/SAEKI-specific CTL clones are listed on the vertical axis. The TCR-β (left) and α (right) segments consistent with an open reading frame, encoding for junctional, putatively CDR3-like, regions are shown. For Va and Vβ, the nomenclature described in Ref. 36 was used.

The TCR model suggests the presence of a deep hydrophobic pocket between CDR1α, CDR2α, CDR3α, and CDR3β (Fig. 6A), which is confined in essence by the side chains of CDR3β Ser-94 and Ala-100 and CDR3α Ser-94 and Leu-102 and the Va residues Asn-33 and Phe-35 (C-strand) and Ala-92 (F-strand) (Fig. 6, B and C). As suggested by docking experiments, this pocket, upon small structural changes, can avidly bind the K(ABA) side chain of the ligand (Fig. 6, A and C, and data not shown). The main forces driving this interaction include: i) hydrophobic interactions (e.g., the pocket and K(ABA) side chain evading contact with water), ii) significant Van der Waals interactions due to high complementarity of pocket and side chain, iii) 8π–8π interaction of the ABA azido substituent with the phenyl moiety of Va Phe-35, and iii) hydrogen bond formation of the ABA carbonyl oxygen, the most polar atom of this side chain, with CDR3α Ser-94 (Fig. 6B). This prediction is consistent with the finding that the photoaffinity labeled sites were contained in the Va segment 33–39 (Figs. 4 and 5).

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**DISCUSSION**

To study TCR-ligand interactions by TCR photoaffinity labeling, we derived CTL clones from mice immunized with the PbCS peptide derivative ANBA-YIK(ABA)SAEKI. As in previous studies with other photoreactive PbCS peptide derivatives (15, 16), ANBA-YIK(ABA)SAEKI-specific CTL were readily obtained. The “photoprobe”-specific CTL described here, as well as those described previously, displayed all the hallmarks of
antigen recognition by "conventional" CTL, including MHC restriction and dependence on auxiliary molecules (i.e. CD8 and LFA-1) (Fig. 2 and data not shown) (17, 29). Similarly, numerous reports described CD8+ as well as CD4+ T cells that recognize antigens modified with haptens such as trinitrophenyl, benzoxarsonate, or fluorescein but also with carbohydrates (13, 30–32). The epitopes recognized by these T cells, as far as is known, are MHC bound peptides that contain nonpeptidic entities. Thus, while antigen recognition by T cells is MHC restricted, it is not limited to conventional peptides but clearly can include a vast array of structures. In several cases, such T cell reactivities have been shown to play a role in disorders such as drug allergies or delayed type hypersensitivities (33).

The epitope recognized by ANBA-YIK(ABA)SAEKI-specific CTL clones included two peptide derivative residues, K(ABA) and PbCS Lys-259 (Table I). The latter residue was also essential for the recognition of the PbCS 252–260 peptide by most PbCS-specific CTL clones (16). Similarly, the antigen recognition by IASA-YIPS(ABA)I-specific CTL clones required K(ABA) in position 8 (15). These findings demonstrate that for the PbCS system, the residue in position 8 is a primary TCR contact residue. This is consistent with computer modeling suggesting that this ligand residue is fully solvent exposed (Fig. 6D and Ref. 15). However, the three systems differed regarding the residue in position 4. For PbCS and IASA-YIPSAEK(ABA)I-specific CTL PbCS, Pro-255 was a secondary
TCR contact residue (15, 16), but for ANBA-YIK(ABA)SAEKI-specific ones, K(ABA), in this position, was essential (Table I). Since the N-terminal photoreactive group was not required for antigen recognition by ANBA-YIK(ABA)SAEKI and IASA-YIPSAEK(ABA)I-specific clones (Table I and Ref. 15), the epitopes recognized by these families of clones essentially differed by the absence or presence of K(ABA) in positions 4 and 8, respectively.

The TCR expressed by the three families of clones were also different. Those expressed by PbCS-specific CTL clones were highly diverse both in terms of CDR3 sequences and TCR gene element usage, except that 57% of the clones expressed

**Fig. 5. Amino acid sequence of W13 TCR α chain (A) and β chain (B) variable domains.** The protease V-8 cleavage sites (E and D) are indicated by triangles, and those of trypsin (R and K) are indicated by squares and circles, respectively. The endoprotease Pro-C cleavage sites (P) are indicated by a diamond. Open boxes indicate CDR1, CDR2, and CDR3 loops, and the hatched box indicates the segment containing the photoaffinity labeled sites.

**Fig. 6. Computer models of the W13 TCR and K(ABA)-ANBA-YIK(ABA)SAEKI complex.** A, the surface contour of the W13 TCR ligand binding site is shown. The α chain is in light blue and the β chain is in dark blue. Yellow domains indicate hydrophobic regions. The carbon backbone of ANBA-YIK(ABA)SAEKI is shown in white, and the side chains of K(ABA) and Lys-259 are in red. B and D show details of the K(ABA) side chain (in red) bound in the hydrophobic pocket formed by the Va C-strand (carbon backbone in light blue and side chains of F-35 and Tyr-37 in orange), F-strand-CDR3-Ja (carbon backbone same color and side chains of Tyr-90, Ala-92, Leu-93, Ser-94, Leu-102, and Leu-104 in green). CDR3α spans from Ser-94 to Leu-102. CDR3β (carbon backbone in dark blue and side chains of Ser-99 and Ala-100 in purple) confine, in part, the opening of the pocket. D, shown is the K(ABA)-ANBA-YIK(ABA)SAEKI complex as seen from the TCR. The carbon backbone of K(ABA) is in gray with the side chains of Glu-62, Ser-69 (on a1 helix), and Tyr-155 (on a2 helix) in yellow. The carbon backbone of ANBA-YIK(ABA)SAEKI and the side chains of K(ABA) and Lys-259 are shown in red.
BV3S1A1 (25). In contrast, about 80% of IASA-YIPSAEK- (ABA)I-specific clones expressed BV1S1 encoded β chains that were paired with JoTA28 encoded α chains (15). On the other hand, the ANBA-YIK(ABA)SAEK-specific CTL also preferentially expressed BV1S1, though less frequently, but the α chains lacked any apparent sequence consensus (Table II).2 Remarkably, however, the ANBA-YIK(ABA)SAEK-specific TCR, unlike the IASA-YIPSAEK(ABA)I-specific ones, were selectively labeled at the α chain (Fig. 3). Peptide mapping showed that the W13 TCR photoaffinity labeling took place in a site-specific manner (Fig. 4), which implies that the ABA group specifically bound to this TCR.

As suggested by computer modeling, the W13 TCR ligand binding site contains a deep hydrophobic pocket, which is located and structured such that it can avidly bind the ligand K(ABA) side chain (Fig. 6). This is consistent with the localization of the photoaffinity labeled site in the Va C-strand segment 33–39, which contains Phe-35, that according to our docking experiments intimately interacts with the ABA azide substituent (Figs. 4 and 6, B and C). In accordance with that is the finding that the derivative lacking this substituent (YIK(BASAEEKI) was recognized by W13 CTL less efficiently.2 In addition, the most polar atom of this side chain, the carboxyl oxygen of the ABA group, according to our modeling forms a stabilizing hydrogen bond with CDR3α Ser-94, which is in agreement with the observation that W13 CTL recognized, though inefficiently, the variant YIK(Ac)SAEKI (Table I).

The binding of the K(ABA) side chain in a hydrophobic pocket, formed mainly by CDR3α and Va framework residues, which are more conserved than CDR residues (34, 35), may explain why these TCR were preferentially labeled at the α chain even though they lacked any apparent sequence consensus (Table II). The finding that a side chain of an MHC-bound peptide interacts with TCR Va framework residues is not in agreement with the current concept of TCR-ligand interactions, which postulates that such residues interact primarily with CDR3 loops (6). It is, however, important to note that the K(ABA) side chain is considerably longer than a conventional amino acid side chain (Fig. 6D). This long spacer is required for the ABA group to interact with the TCR, as demonstrated by the observation that shortening of this side chain by only one methylene group (1.5 Å) abolished the antigen recognition by the W13 as well as most other CTL clones (Table I). Moreover, the K(ABA) side chain is remarkably hydrophobic and hence preferentially interacts with nonpolar domains on TCR. Since these TCR were selected for avid binding of the photoreactive ligand, it can be expected that their ligand binding includes an avid binding of the K(ABA) side chain. The accommodation of the ABA group in a hydrophobic pocket, as proposed by our modeling, constitutes such a binding principle (Fig. 6, A-C).

These findings are reminiscent of a previous study, in which we showed that a BV1S1A1/JoTA28 encoded TCR, specific for IASA-YIPSAEK(ABA)I, utilized a similar principle to bind the K(ABA) side chain (15). However, in this case, the hydrophobic pocket was located and formed by the BV1S1A1 C- and C′-strands and the JoTA28 encoded portion of CDR3α and adjacent G-strand residues (15). Based on these findings, we propose that TCR can express hydrophobic domains in the indicated locations; since they are formed by TCR framework and CDR3α residues, their shape and physicochemical nature may vary significantly according to TCR sequences. While normal amino acid side chains of MHC-bound peptides are unlikely to interact with these domains, we suggest that they enable TCR to efficiently and specifically bind epitopes containing modified side chains. Such side chains usually are long and often amphipatic or hydrophobic, which allows them to efficiently interact with these sites.

It remains to be explained why ANBA-YIK(ABA)SAEK-specific TCRs were selectively photoaffinity labeled at the α chain, while the IASA-YIPSAEK(ABA)I-specific ones were labeled at the β chain or both chains (Fig. 3) (15). This labeling pattern may be explained by either of two different orientations of TCR-ligand interactions. i) The K4 bound peptide runs approximately diagonal across the TCR binding site, such that K(ABA) in position 8 interacts mainly with the β and in position 4 with the α chain (Fig. 6A). Strong evidence for this orientation has been reported by Sant’Angelo et al. (10) and Sun et al. (37). ii) Alternatively, the MHC-bound peptide could be located underneath CDR3α and CDR3β, e.g. rotated by 30–40° relative to the orientation shown in Fig. 6A, in which the orientation of the K(ABA) side chain determines with which TCR chain the ABA group preferentially interacts. This orientation corresponds to the one predicted on theoretical grounds (6) and by studies in which TCR single chain transgenic mice were immunized with peptide variants (9).

The system described here and the one described previously (15) allows assessment of TCR-ligand interactions by TCR photoaffinity labeling. This permits rapid and conclusive mutational analysis of TCR-ligand interactions but also makes possible the preparation of covalent TCR-ligand complexes for x-ray crystallographic studies. In addition, as TCR photoaffinity labeling is applicable on living cells, these systems are very suitable for structure-function studies and assessment of co-receptor participation in TCR-ligand interactions (29).

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