Assembly, Specific Binding, and Crystallization of a Human TCR-$\alpha\beta$ with an Antigenic Tax Peptide from Human T Lymphotropic Virus Type 1 and the Class I MHC Molecule HLA-A2[1]

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T lymphocytes use TCR-$\alpha\beta$ to bind and to recognize complexes of antigenic peptides bound to MHC proteins located at the surface of APCs. We have assembled and crystallized this intercellular complex of TCR/peptide/MHC from soluble human TCR-$\alpha\beta$ and soluble peptide/HLA-A2 complexes. The soluble TCR-$\alpha\beta$ binds specifically to its in vivo ligand, the complex of HLA-A2, and a peptide from the Tax protein of human T lymphotropic virus type 1. The soluble TCR also binds in vitro to an altered peptide ligand, which appears to be a partial agonist in T cell assays as determined by its ability to elicit different cytolytic and lymphokine secretion responses. Heterodimerization and the antigenic specificity of the TCR do not require its interchain disulfide bond, transmembrane segments, or glycosylations. Crystals of the TCR/peptide/HLA-A2 complex diffract x-rays, providing the means to study in atomic detail the mechanism of Ag-specific cell-cell recognition between T cells and target cells. *The Journal of Immunology,* **1996, 157: 5403–5410.**

In the cellular immune response, Ag-specific T cell recognition results from the binding of a TCR to the complex of an antigenic peptide bound to a class I or class II MHC glycoprotein located on the surface of an APC (reviewed in Ref. 1). Specific binding by the TCR triggers signals within the T cell that are central to the development of the T cell repertoire, the regulation of the immune response, and the activation of CTLs. Human cells infected by viruses display on their cell surfaces peptides from viral proteins bound to MHC molecules. CTLs expressing a TCR specific for a viral peptide/class I MHC complex are activated to cause lysis of infected cells and/or release of cytokines following specific recognition of the complex.

X-ray crystal structures of several peptides complexed with class I and class II molecules describe the interaction surface of the MHC/peptide-half of the "molecular recognition complex," consisting of the TCR and the peptide/MHC molecule (2, 3). Models of the three-dimensional structure of the TCR have been proposed based on the sequence homology with Ab Fab fragments (4–7) and on the existing structures of the TCR β-chain and of the TCR Vα domain (8, 9). The structure of a TCR in complex with a peptide/MHC molecule is required to reveal the interactions between a bound TCR and its ligand.

One difficulty hampering structural studies of the TCR has been the lack of sufficient amounts of homogeneous TCR protein, as the TCR has proven to be difficult to overexpress in several systems. The production of soluble human and mouse TCR-$\alpha\beta$ have been reported: in mammalian cells (10–20), in insect cells (21, 22), and in bacteria (23–29). Soluble TCR-$\alpha\beta$ proteins produced by a few of these recombinant systems have been crystallized.

For biochemical, binding, and structural studies, we chose a human TCR-$\alpha\beta$ specific for the Tax peptide from human T lymphotropic virus type 1 (HTLV-1)[6] in complex with HLA-A2. The x-ray structure of the HLA-A2/Tax peptide complex has been determined and predicts peptide positions that may be recognized directly by a TCR (30). HTLV-1 is associated with adult T cell leukemia and a slowly progressive neurologic disease, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (31). In peripheral blood from patients with HAM/TSP, the frequency of HTLV-1-specific $CD8^+$ precursor CTL is at least 40-fold higher than in asymptomatic HTLV-1-infected individuals, and in $HLA\text{-}A2^+$ patients, the great majority of $CD8^+$ HTLV-1-specific CTL recognize a peptide (LLFGYPVYV) from residues

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[6] Abbreviations used in this paper: HTLV-1, human T lymphotropic virus type 1; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; MWCO, membrane m.w. cutoff; TNB, thionitrobenzoic acid; RT, room temperature; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MIP, macrophage inflammatory protein.

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11–19 of the HTLV-1 Tax protein (32). αβ T cell clones from HTLV-1-infected HLA-A2$^+$ patients diagnosed with HAM/TSP have been isolated, and one such Tax 11–19-specific CD8$^+$ clone, identified as A6, exhibits peptide-specific lysis of target cells transfected with HLA-A2 and pulsed with Tax 11–19 peptide (33).

Using engineered cDNAs of the TCR from clone A6, we have expressed the extracellular domains of the TCR-α and -β polypeptides separately as insoluble inclusion bodies in *Escherichia coli*. When refolded separately and then mixed, the α- and β-chains formed soluble heterodimers, whether linked by an interchain disulfide bond or not. When α- and β-chains expressed without the cysteines that form the interchain disulfide bond were refolded together, they formed heterodimers spontaneously and at higher yields. The peptide Ag specificity of the soluble TCR appears identical to that of the T cell from which it was isolated. The ternary complex, assembled from the soluble TCR-αβ and the Tax peptide bound to soluble HLA-A2, forms crystals that diffract x-rays and will enable the determination of the structure of this Ag-specific cell-cell interaction complex.

Materials and Methods

cDNA isolation and construction of expression plasmids

PCR V-region screening and DNA sequence analysis determined that the TCR of T cell clones A6 and 2G4 uses the genes Vα2.3 Jα24 and Vβ12.3 Dβ2.1 Jβ2.7 Cβ2. cDNAs for the α and β subunits were isolated by PCR with V-region-specific and C-region-specific primers (33). The DNA sequence of the α and β subunits of A6 and 2G4 are identical. The Vα2.3 and Vβ12.3 gene segments have been renamed AV2S1A2 and BV13S1, respectively (34).

For bacterial expression, PCR was used to insert an initiating methionine codon just before the codon for the predicted first amino acid residue of each mature subunit. The 5′ primers also encoded a ribosome binding site and the cleavage site for the restriction enzyme *Eco*RI. An internal *Eco*RI site at the seventh codon of the α subunit was removed by a silent single base change that was also encoded in the 5′ PCR primer. A stop translation codon (TAA) and a *Hin*dIII restriction site encoded on the 3′ PCR primers were placed to yield α and β subunits of two lengths (Fig. 1). Two codon changes were made to increase the level of bacterial expression. The third codon of the mature α subunit was changed (GAG to GAA) to increase the number of A and T nucleotides in the codons specifying the N-terminal residues of the protein. The first codon of the mature β subunit was changed (AAT to AAC) to yield a more frequently used codon in *E. coli*. The unpaired cysteine residue at position 191 in the Cβ region (8) was changed to alanine by overlap PCR and restriction fragment replacement. All coding regions used for expression had the intended nucleotide sequences as determined by DNA sequencing. The engineered α and β coding regions were ligated into plasmid pLM1 bearing the T7 polymerase promoter (35) and protein was expressed in the bacterial strain BL21(DE3)plysS (36). Previously described bacterial strains and methods were used to prepare HLA-A2 molecules bound to synthetic peptides (37).

Inclusion body protein preparation

Insoluble protein was prepared similarly as described in Reference 37, but with modifications. LB media (6 liters, 37°C) containing 0.4% (w/v) glucose, 30 mg/L chloramphenicol, and 100 mg/L ampicillin was inoculated with a single bacterial colony. Isopropyl-β-D-thiogalactopyranoside (0.5 mM) was added at an OD_{600} of 0.7 to 1.0, and cells were harvested after 2 h. The cell pellets were suspended in a buffer containing 50 mM Tris-HCl, 25% sucrose, 1 mM sodium EDTA, 0.1% sodium azide, and 10 mM DTT, pH 8.0. Lysozyme (1 mg/ml), DNase I (25 μg/ml), and $MgCl_2$ (5 mM) were added. For lysis, a buffer (2.5 ml per ml of cell suspension) was added containing 50 mM Tris-HCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 100 mM NaCl, 0.1% sodium azide, and 10 mM DTT, pH 8.0. After the viscosity decreased, sodium EDTA (10 mM) was added, and the suspension was frozen and then thawed. $MgCl_2$ (10 mM) was added for DNase I activity and then, after adding 10 mM sodium EDTA, cell debris was centrifuged. Cell pellets were suspended in and pelleted from 50 mM Tris-HCl, 0.5% Triton X-100, 100 mM NaCl, 1 mM sodium EDTA, 0.1% sodium azide, and 1 mM DTT, pH 8.0, four times. The inclusion bodies were then suspended in 50 mM Tris-HCl, 1 mM sodium EDTA, 0.1% sodium azide, and 1 mM DTT, pH 8.0; centrifuged; dissolved in 10 to 20 ml of 25 mM 2-(*N*-morpholino)ethanesulfonic acid, 8 M urea, 10 mM sodium EDTA, and 0.1 mM DTT, pH 6.0; and ultracentrifuged at 140,000 × *g*. Aliquots of the supernatant were stored at −70°C. The protein concentration of the supernatant was estimated with a Coomassie dye-binding assay (Bio-Rad, Richmond, CA).

Alpha (Vα2.3--N--Jα24--Cα):

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MQKEVEQNSG PLSVPEGAIA SLNCTYSDRG
SQSFFWYRQY SGKSPELIMS IYSNGDKEDG
RFTAQLNKAS QYVSLLIRDS QPSDSATYLC
A
--VT--TDSWGKLQFGAGTQVVVTP--
DIQNPDPAVY QLRDSKSSDK SVCLFTDFDS
QTNVSQSKDS DVYITDKTVL DMRSMDFKSN
SAVAWSNKSD FACANAFNNS IIPEDTFFPS
PESScdvklv eksfetdtnl nfqnls
```

Beta (Vβ12.3--N--Dβ2.1--N--Jβ2.7--Cβ2):

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MNAGVTQTPK FQVLKTGQSM TLQCAQDMNH
EYMSWYRQDP GMGLRLIHYS VGAGITDQGE
VPNGYNVSRS TTEDFPLRLL SAAPSQTSVY
FCAS
--RP--GLAGG--RP--EQYFGPGTRLTVT--
EDLKNVFPPE VAVFEPSEAE ISHTQKATLV
CLATGFYPDH VELSWWVNGK EVHSGVSTDP
QPLKEQPALN DSRYCLSSRL RVSATFWQNP
RNHFRCQVQF YGLSENDEWT QDRAKPVTQI
VSAEAWGRAD cgftsesyqqg
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FIGURE 1. Amino acid sequences of the expressed α and β subunits of the TCR from T cell clone A6. Amino acid residues encoded by different gene segments and by N-addition nucleotides are separated by two hyphens. Two length forms of the α and β subunits were used in this study, one composed of the residues shown here in uppercase (short form) and one composed of the residues shown here in uppercase and lowercase (long form). The cysteine at position 191 in Cβ (in boldface type) within the sequence ...SRYCLSS... is present in the long form of β, but has been changed to alanine in the short form of β. The N-terminal methionine residues were added for bacterial expression. Comparing the N termini of the mature proteins shown here with the extensive Vα and Vβ sequence comparisons in Arden et al. (34), it appears that these constructs include an extra N-terminal residue in α and two extra N-terminal residues in β from the respective leader peptide sequences.

Refolding the TCR by dialysis and forming an interchain disulfide bond

The denatured α and β subunits dissolved in 8 M urea were separately diluted into a buffer containing 100 mM Tris-HCl, 400 mM L-arginine-HCl, 5 M urea, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.2 mM PMSF, pH 8.3, to a final protein concentration of 2 μM. This was dialyzed (membrane m.w. cutoff (MWCO) = 6,000–8,000) for 16 h at 4°C against the same buffer with 1 to 2 M urea and with 5 mM β-mercaptoethanol, but without glutathione. The two solutions were then dialyzed for 16 h against 20 mM Tris-HCl, 150 mM NaCl, 1 mM sodium EDTA, and 0.5 mM DTT, pH 7.5. The dilute proteins were concentrated in several ways: by ammonium sulfate precipitation (50% saturated at 0°C), by acetone precipitation (50% v/v at 0°C), or in an ultrafiltration cell (MWCO = 10,000; Amicon, Beverly, MA).

The refolded β subunit was treated with 2 to 5 mM DTNB (Ellman's reagent; 5,5′-dithiobis(2-nitrobenzoic acid); Sigma Chemical Co., St. Louis, MO) in 100 mM sodium phosphate and 1 mM sodium EDTA, pH 6.8, to derivatize the free cysteine at the C-terminus of the protein with thionitrobenzoic acid (TNB). It is likely that the unpaired cysteine within the Cβ domain also reacted with DTNB. β was then dialyzed (MWCO = 14,000) against 100 mM sodium phosphate and 1 mM sodium EDTA, pH 6.8, and α was dialyzed against 10 mM sodium phosphate, 150 mM NaCl, and 1 mM EDTA, pH 5.8, to remove excess DTNB and DTT. α and β were mixed at a 1:1 molar ratio to form a disulfide bond between the free C-terminal cysteine on the α subunit and the TNB-derivatized C-terminal cysteine on the β subunit. The mixture of α and β was dialyzed against 10 mM Tris-Cl, pH 7.5, concentrated, and used directly in a band-shift assay.

Native gel band-shift assay

Native gels were 12 or 15% polyacrylamide of 0.75-mm thickness without a stacking gel, used standard Tris/glycine buffers without SDS, and were stained with Coomassie blue R-250. Proteins (10 μg each) were placed in 10 μl of Tris or PBS, and then mixed with 3 μl of 50% glycerol for loading

the gels. Samples were not heated or reduced. Preparative native gels were 10% acrylamide, 3-mm thick, and were loaded along the entire width (16 cm) with 6 to 10 mg of total protein. The gel region containing the shifted complex band was excised and placed back in the 3-mm gel apparatus. Acrylamide (5%) was polymerized around the gel piece, leaving a space at the top of the gel. A piece of dialysis membrane was clamped across the top of the gel. With the anode and cathode reversed, the protein moved up and into the space at the top of the gel, retained there by the dialysis membrane. The TCR/Tax/HLA-A2 complex was concentrated and buffer exchanged in a Centricon-10 concentrator (Amicon).

Refolding the TCR by dilution

α (45 mg) and β (30 mg) inclusion body proteins dissolved in 8 M urea were added together to ~15 ml of 6 M guanidine-HCl, 10 mM sodium acetate, and 10 mM EDTA, pH 4.2, at room temperature (RT). A liter of refolding buffer consisting of 100 mM Tris-HCl, 1 M L-arginine-HCl, 2 mM sodium EDTA, 6.3 mM cysteamine (2-mercaptoethylamine), 3.7 mM cystamine (Ref. 38), and 0.5 mM PMSF was adjusted to pH 8.5 at RT and cooled to 10°C. The 15-ml guanidine-HCl solution containing α and β was diluted into the refolding buffer with vigorous stirring. The refolding solution was incubated at 10°C for 6 to 12 h, and then a second 15-ml mixture of α and β was added (39). After 6 to 12 additional hours, a third identical 15 ml of α and β mixture was added and incubation at 10°C continued for 24 h. The refolding buffer was then dialyzed (MWCO = 6,000–8,000) against 10 liters of 100 mM urea at 4°C for 24 h and then against 10 liters of 10 mM Tris-HCl, 100 mM urea, pH 7.5, again for 24 h. PMSF (0.5 mM) was added and the protein was adsorbed to DE52 anion exchange resin (20 g; Whatman, Hillsboro, OR) prepared in 10 mM Tris-HCl, pH 8.0, at RT. The protein was eluted with 10 mM Tris-HCl, 400 mM NaCl, pH 8.0. After further concentration in a Centriprep-30 concentrator (Amicon), the TCR was purified on Superdex-75 16/60 and Sephacryl S-100 gel filtration columns (Pharmacia, Piscataway, NJ). The purified protein was concentrated to 36 to 100 mg/ml in a Centriplus-30 concentrator (Amicon) and buffer exchanged to 10 mM Tris-HCl, 0.2% sodium azide, pH 7.5.

Cellular assays

$CD8^+$ CTL clones A6 and 2G4 are sister clones derived from the PBL of HAM/TSP patient no. 3, as described (33). CTL activity and cytokine/chemokine production of clone 2G4 were measured using peptide-pulsed HLA-A2-transfected Hym2.C1R (40) as APCs. CTL activity is presented as percent specific lysis (Ref. 33). Cytokine/chemokine secretion was induced by culture of 10^5 2G4 cells and 10^5 HLA-A2-transfected Hmy2.C1R cells treated with mitomycin C (200 μg/ml; Sigma Chemical Co.) and either unpulsed or pulsed with varying concentrations of peptides for 2 h at 37°C. APCs were washed three times and cultured for 48 h with 2G4 cells in round-bottom 96-well microtiter plates in Iscove's Modified Dulbecco's Medium (Life Technologies, Gaithersburg, MD) supplemented with 5% heat-inactivated FCS. Supernatants were collected and assayed for IFN-γ, macrophage inflammatory protein (MIP)-1α, and MIP-1β secretion by sandwich ELISA assay kits as described by the manufacturer (R&D Systems, Minneapolis, MN). Values reported are in pg/ml with background values of 2G4 cells cultured with unpulsed APCs subtracted.

Results

Expression of TCR-α and -β polypeptides

The TCR-α and -β polypeptides of the human T cell clone A6, specific for HLA-A2 complexed with the Tax peptide of HTLV-1, were expressed separately in *E. coli*. To create polypeptides that lack the transmembrane sequences, translation stop codons were placed at the C-terminus of the extracellular domains (Fig. 1, long form) or stop codons were substituted for the C-terminal-most cysteine codons (Fig. 1, short form). The α and β polypeptides produced by the long-form constructs each include the cysteine residue most proximal to the transmembrane segment that is involved in the interchain disulfide bond. The TCR-$\alpha\beta$ heterodimer as isolated from T cells has been observed to be disulfide bonded (41–44). Both polypeptides were expressed in large quantities as inclusion bodies. Lysing the bacterial cells with lysozyme and detergents and washing the insoluble protein with detergent-containing buffer produced highly purified proteins (data not shown), typically yielding 40 mg of protein per liter of bacterial culture.

Refolding and assembly of disulfide-bonded $\alpha\beta$ heterodimers

Without further purification, α- and β-chains solubilized in denaturant could be refolded separately by removal of the denaturant through dialysis under dilute conditions in the presence of an oxidized/reduced glutathione redox buffer. A small shift in the mobility on SDS-PAGE between reduced and nonreduced samples indicated that intrachain disulfide bonds formed in both chains (data not shown). The specific formation of interchain disulfide-bonded $\alpha\beta$ heterodimers rather than homodimers was accomplished by treating the refolded β-chains with the sulfhydryl reagent DTNB, which forms a labile TNB-cysteine disulfide bond with the free cysteine of β that later will form the interchain disulfide bond. When DTNB-treated β and refolded α are mixed, the TNB-cysteine bond of the derivatized β is attacked by the free cysteine of the α-chain, displacing TNB and forming a cysteine-cysteine bond between α and β. This procedure is similar to methods used to assemble bifunctional Abs by specific heterodimerization of IgG heavy chains (45).

Specific binding of $\alpha\beta$ heterodimers to HLA-A2/Tax using a band-shift assay

The specific interaction of soluble TCR-$\alpha\beta$ with the HLA-A2/Tax peptide complex was demonstrated using native (nondenaturing) PAGE (Fig. 2*A*). A mixture of the refolded TCR and HLA-A2/Tax peptide complex exhibited a prominent shifted band that migrated at a position (Fig. 2*A*, *lane 2*, arrow marked "Complex") distinct from either TCR (*lane 8*) or HLA-A2/Tax (*lane 3*) alone. To demonstrate that this band was the ternary complex, the shifted band was isolated from a preparative native gel. The eluted complex migrated as a single band when reloaded on a native gel (Fig. 2*A*, *lane 1*). SDS-PAGE analysis of the eluted protein under nonreducing conditions revealed a TCR band of approximately 60 kDa (Fig. 3, *lane 1*, arrow marked "TCR") in addition to the two subunits of HLA-A2, the heavy chain at 31 kDa and β2 m at 11 kDa. Under reducing conditions (Fig. 3, *lane 2*) the 60-kDa band is converted to a 29-kDa band likely containing both the α- and the β-chains of the TCR, since as shown below, both the α and β subunits are required for the band-shift (Fig. 2*B*). These long form α and β polypeptides (Fig. 1) have nearly identical mobilities on SDS-PAGE.

Native-PAGE analysis confirmed that the binding specificity of the TCR-$\alpha\beta$ was preserved in the soluble, refolded molecule. No shifted band appeared when the TCR was mixed with HLA-A2 complexed with a variant of the Tax peptide (Fig. 2*A*, *lane 4*), having acetylated lysine residues substituted for glycine at position 4 and for tyrosine at position 8. These two residues of the peptide are exposed and accessible to recognition by a TCR as seen in the x-ray structure of the HLA-A2/Tax complex (30). Similarly, no complex was formed when TCR was mixed with HLA-A2 complexed with an unrelated peptide (Fig. 2*A*, *lane 9*).

The complex of HLA-A2/Tax and the disulfide-linked TCR heterodimer produced by the method described above did not crystallize, probably due to the heterogeneity of the TCR, as reflected by the several bands seen on native-PAGE. Native-PAGE can separate proteins based on very slight differences in charge and size. The difference in mobility of HLA-A2 between *lanes 3* and *5* in Figure 2*A*, for example, results from sequence differences in the bound 9-mer peptides that are not differences in charge. Although the part of the TCR preparation that bound HLA-A2/Tax and shifted its mobility was relatively homogeneous (Fig. 3, *lane 1*, arrow marked "TCR"), even the TCR/Tax/HLA-A2 complex isolated from preparative native gels did not crystallize. One source of heterogeneity is the formation of nonnative disulfide bonds. The

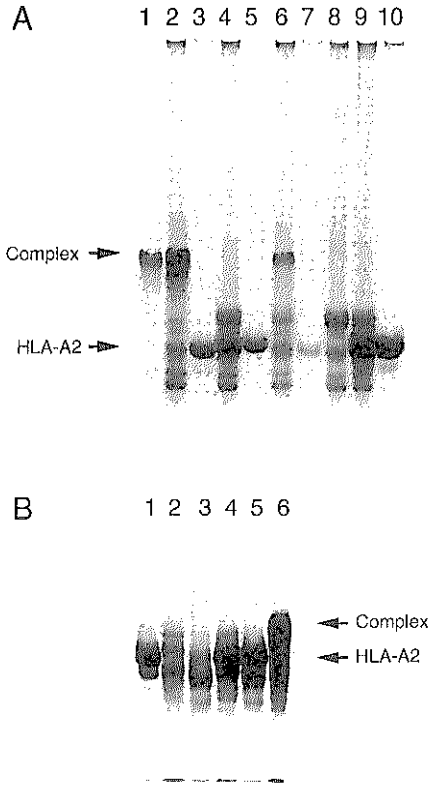

FIGURE 2. *A*, Native gel band-shift assay reveals that refolded and disulfide-bonded TCR-αβ forms a specific complex with the HLA-A2/Tax peptide molecule. *Lane 1*, TCR/Tax/HLA-A2 (5 μg) eluted from a preparative native gel. *Lane 2*, HLA-A2/Tax (12 μg) and TCR (27 μg). *Lane 3*, HLA-A2/Tax (12 μg). *Lane 4*, HLA-A2/Taxkk (12 μg) and TCR (27 μg). *Lane 5*, HLA-A2/Taxkk (12 μg). *Lane 6*, HLA-A2/Tax (3 μg) and TCR (27 μg). *Lane 7*, HLA-A2/Tax (3 μg). *Lane 8*, TCR (27 μg). *Lane 9*, HLA-A2/M1 (20 μg) and TCR (27 μg). *Lane 10*, HLA-A2/M1 (20 μg). Peptide sequences are: Tax (LLFGYPVYV), Taxkk (LLFkYPVkV with both lysines acetylated), and M1 (GILGFVFTL from the matrix protein of influenza A virus). Note the absence of complex formation (*lanes 4* and *9*) with peptides other than the Tax peptide. The TCR preparations used here represent the disulfide-bonded long forms of the α and β subunits (see Figure 3). The TCR used in *lanes 2* to *10* was concentrated by ammonium sulfate precipitation. The TCR in *lane 1* is a sample of the same preparation as in Figure 3. *B*, Both the α and β subunits are required for the band-shift with HLA-A2/Tax. *Lane 1*, HLA-A2/Tax. *Lane 2*, α subunit. *Lane 3*, β subunit. *Lane 4*, α subunit and HLA-A2/Tax. *Lane 5*, β subunit and HLA-A2/Tax. *Lane 6*, α and β and HLA-A2/Tax. A new band representing the complex appears only in *lane 6*, in the presence of both of the subunits. Iodoacetamide-treated long forms of both subunits are used here (see text). Control experiments (not shown) confirm that the band that migrates a little faster than HLA-A2 on native gels is free β_2-microglobulin.

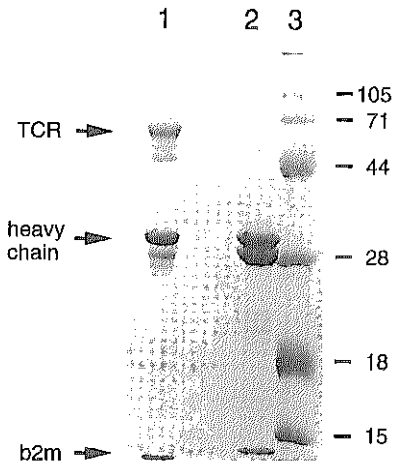

FIGURE 3. The shifted band on native gels contains TCR-αβ and HLA-A2. *Lane 1*, TCR/Tax/HLA-A2 complex (8 μg, nonreducing conditions). *Lane 2*, TCR/Tax/HLA-A2 complex (8 μg, reducing conditions). *Lane 3*, standard proteins. SDS-PAGE gel (15%) with TCR/Tax/HLA-A2 complex isolated from a preparative native gel. The TCR used here was prepared with a disulfide bond between the α and β subunits (*lane 1*). Under reducing conditions, the ~60-kDa TCR band from *lane 1* becomes a new band at 29 kDa in *lane 2*, migrating ahead of the heavy chain of HLA-A2. The long forms of the α and β subunits have very similar gel mobilities under reducing conditions. When the interchain disulfide bond is absent, but the intrachain disulfide bonds are present, the subunits have distinct mobilities (data not shown). Before forming the interchain disulfide of the TCR and loading on the preparative native gel, the α and β subunits were concentrated from the dilute refolding buffer by precipitation with 50% acetone. Samples for this gel contained 10 mM iodoacetamide. Molecular mass standards are listed on the *right* in kilodaltons.

refolded α subunit usually yielded three bands on nonreducing SDS-PAGE (data not shown), an indication of mispairing of cysteines.

To confirm that the band-shift of the TCR/Tax/HLA-A2 complex was caused by the TCR-αβ heterodimer and not by αα or ββ homodimers, and to test whether the interchain disulfide was required, α and β were refolded separately and the free cysteines of both chains were reacted with DTNB. Each refolded chain was purified as a sharp peak at its expected m.w. by gel filtration chromatography. Then, both α and β were separately treated with DTT, followed by excess iodoacetamide to block all free cysteines, thereby eliminating the potential for interchain disulfide bond formation. The TCR-αβ lacking the interchain disulfide bond still bound to HLA-A2/Tax as observed by the shift in mobility on native-PAGE (Fig. 2*B*, *lane 6*). Neither the α subunit alone (Fig. 2*B*, *lane 4*) nor the β subunit alone (Fig. 2*B*, *lane 5*) showed any binding to HLA-A2/Tax. This preparation of TCR was still very heterogeneous (Fig. 2*B*, *lanes 2* and *3*).

The α subunit showed a marked tendency to aggregate. Gel filtration chromatography of separately refolded α usually revealed a large aggregate peak. In PAGE in 8 M urea without SDS, conditions under which β migrates as a discrete band, α migrated as a smear of several bands (data not shown).

α and β associate without an interchain disulfide bond and bind specifically to HLA-A2/Tax

Based on the knowledge (Fig. 2*B*) that α and β without an interchain disulfide bond can associate and bind HLA-A2/Tax, we substituted stop codons for the codons in the expression plasmids that encode the C-terminal-most cysteines (Fig. 1, short forms). The α- and β-chains were shortened by 22 and 11 residues, respectively, and now could be clearly distinguished by SDS-PAGE (Fig. 4). In addition to removing the cysteines involved in the interchain disulfide, the free cysteine in the Cβ domain was replaced by an alanine residue to help prevent the mispairing of cysteines during folding. We also decided to refold the α- and β-chains together in an attempt to avoid the aggregation of the α subunit with itself.

Rapid dilution of a mixture of the denatured α- and β-chains resulted in both refolding and heterodimerization. Typically, refolding was conducted on about 100 mg of each subunit and approximately 20% of the diluted α and β protein refolded and dimerized. The dilute refolded protein was concentrated by binding to and elution from an anion exchange column and was further

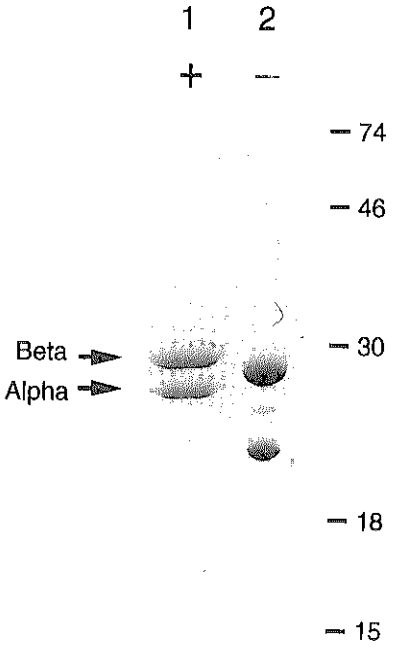

FIGURE 4. The 40-kDa peak from gel filtration chromatography contains the noncovalently associated TCR-$\alpha\beta$. *Lane 1*, TCR-$\alpha\beta$ (15 μg, reducing conditions). *Lane 2*, TCR-$\alpha\beta$ (15 μg, nonreducing conditions). The short form (see legend of Fig. 1) of the β subunit used here is larger (27.5 kDa) and runs more slowly than the short form of the α subunit (22.7 kDa). Note the faster mobility of both chains under the nonreducing conditions of *lane 2*, indicating the presence of intrachain disulfide bonds maintaining a more compact structure. Molecular mass standards are listed on the *right* in kilodaltons.

purified by gel filtration chromatography. The refolded noncovalently linked $\alpha\beta$ heterodimer elutes during gel filtration chromatography at a volume expected for a 40-kDa protein when compared with the elution of standard proteins (data not shown). The calculated molecular mass of the heterodimer is 50.2 kDa.

SDS-PAGE analysis of the peak fractions revealed the presence of both α- and β-chains in apparent equimolar amounts (Fig. 4). As expected, under nonreducing conditions no disulfide-bonded heterodimer was seen, and the difference in mobilities of reduced and unreduced α and β subunits on SDS-PAGE is consistent with the formation of intrachain disulfide bonds in both polypeptides (Fig. 4, compare *lanes 1* and *2*). No indication of the formation of noncovalent homodimers was observed. The homogeneity of these refolded α and β subunits on nonreducing SDS-PAGE was better than the previous preparations and suggested that the proper disulfide bonds were being formed. An initial screen of crystallization conditions with a mixture of this TCR protein and the HLA-A2/Tax complex yielded crystals (see below and Fig. 7).

The refolded noncovalently associated TCR is stable and very soluble and is routinely prepared at 100 mg/ml (2 mM). Gel filtration chromatography of the TCR after 2 mo of storage at 4°C revealed little or no formation of aggregates. The α subunit is more sensitive to proteolysis than β, since during refolding experiments and occasionally after long-term storage at 4°C, degradation of α occurred while β remained intact (data not shown).

Interaction of TCR with altered peptide ligands

The TCR-$\alpha\beta$ formed from the noncovalently associated and shortened α- and β-chains retained binding specificity for HLA-A2/Tax. Varying the ratio of HLA-A2/Tax to TCR yielded the band-shift results shown in Figure 5*A*. On native-PAGE, the TCR

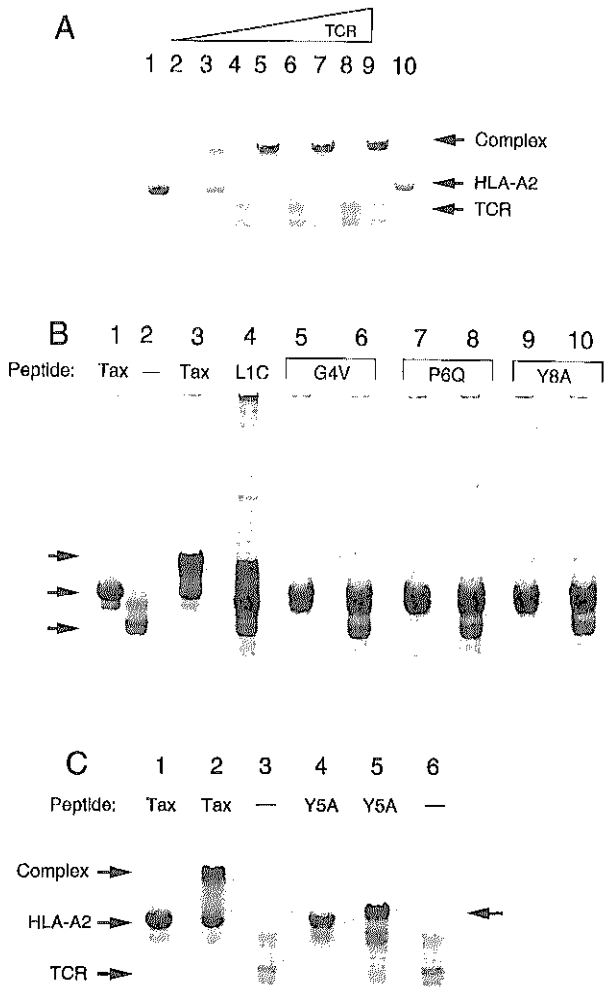

FIGURE 5. *A* to *C*, Noncovalently associated TCR-$\alpha\beta$ forms a complex with HLA-A2/Tax and a Tax peptide altered at P1 or P5, but not with other altered Tax peptides bound to HLA-A2. *A*, Band-shift assay with HLA-A2/Tax peptide. *Lanes 2* and *3*, TCR (6 μg). *Lanes 4* and *5*, TCR (12 μg). *Lanes 6* and *7*, TCR (18 μg). *Lanes 8* and *9*, TCR (24 μg). *Lanes 1*, *3*, *5*, *7*, *9*, and *10*, HLA-A2/Tax (6 μg). Note that the TCR runs on a native gel as two bands, both of which shift in the presence of HLA-A2/Tax (compare *lanes 4* and *5*). *B*, Band-shift assay using the A6 TCR and HLA-A2 complexed with altered Tax peptides. *Lane 1*, HLA-A2/Tax peptide (LLFGYPVYV). *Lane 2*, TCR. *Lane 3*, HLA-A2/Tax and TCR. *Lane 4*, HLA-A2/(L1C) and TCR. *Lane 5*, HLA-A2/(G4V). *Lane 6*, HLA-A2/(G4V) and TCR. *Lane 7*, HLA-A2/(P6Q). *Lane 8*, HLA-A2/(P6Q) and TCR. *Lane 9*, HLA-A2/(Y8A). *Lane 10*, HLA-A2/(Y8A) and TCR. Note the shifted band with the Tax peptide in *Lane 3* (topmost arrow) and the shifted band in *Lane 4* with an altered Tax peptide in which the leucine at position 1 of the peptide is replaced by cysteine (L1C). The three other altered Tax peptides did not induce gel shifts (*lanes 6*, *8*, and *10*). The HLA-A2/(L1C) preparation loaded in *lane 4* with TCR contains some soluble aggregates of HLA-A2. Ten micrograms of each protein were loaded. Middle arrow, position of HLA-A2; lower arrow, position of TCR bands. *C*, Band-shift assay with the HLA-A2/Y5A ligand. *Lane 1*, HLA-A2/Tax peptide. *Lane 2*, HLA-A2/Tax and TCR. *Lane 3*, TCR. *Lane 4*, HLA-A2/Y5A. *Lane 5*, HLA-A2/Y5A and TCR. *Lane 6*, TCR. The different mobility of the HLA-A2/Y5A/TCR complex (*lane 5*) may be caused by differences in net charge, in its size, and/or in its shape compared with the HLA-A2/Tax/TCR complex (*lane 2*). Ten micrograms of each protein were loaded.

exhibits two bands (Fig. 5*A*, *lanes 2*, *4*, *6*, *8*), both of which shift mobility in the presence of HLA-A2/Tax (Fig. 5*A*, compare *lanes 2* and *3*, *4* and *5*, etc.). The soluble TCR-$\alpha\beta$ also formed a complex

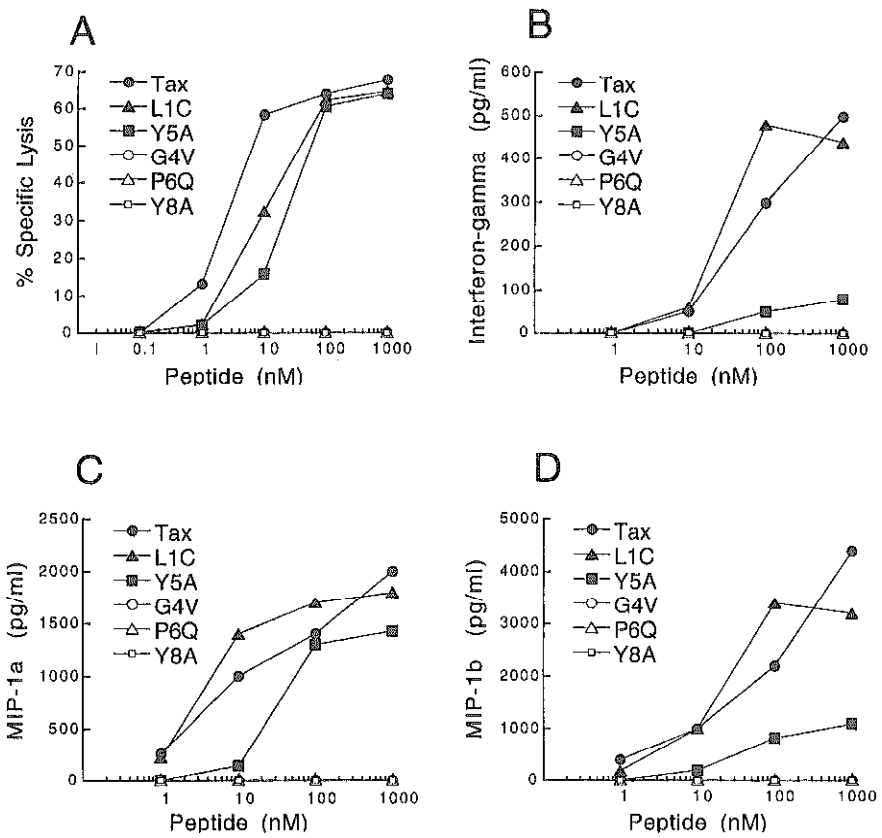

FIGURE 6. Functional T cell assays with altered Tax peptide ligands. *A*, $CD8^+$ CTL clone 2G4 was assayed for lytic activity on HLA-A2-transfected Hmy2.C1R target cells pulsed for 1 h at 37°C with peptides at the indicated concentrations. Percent specific lysis was obtained at an E:T ratio of 2:1. *B*, *C*, and *D*, Secretion of IFN-γ, MIP-1α, and MIP-1β was induced with the same APCs as in *A*, pulsed with or without peptides as described in *Materials and Methods*.

with HLA-A2 complexed with a Tax peptide modified at position 1 of the peptide from leucine to cysteine (L1C) (Fig. 5*B*, *lane 4*) and with HLA-A2 complexed with a Tax peptide altered from tyrosine to alanine at position 5 of the peptide (Y5A) (Fig. 5*C*, *lane 5*). Tax peptides modified at position 4 (G4V), 6 (P6Q), or 8 (Y8A), all of which bind to and promote the refolding of HLA-A2, did not mediate binding of soluble HLA-A2 to soluble TCR under the conditions used in the band-shift assay (Fig. 5*B*, *lanes 5–10*).

The binding of soluble TCR to the altered Tax peptides complexed with soluble HLA-A2 correlates with results from T cell assays. CTL clone 2G4, a sister clone of A6 that expresses the identical TCR as A6 (33), was assayed for the ability of its TCR to be engaged by HLA-$A2^+$ APCs pulsed with varying concentrations of the Tax peptide or the altered Tax peptides described above. TCR engagement was assessed either by lysis of target cells or by induction of secretion of the cytokine IFN-γ or the chemokines MIP-1α or MIP-1β. Peptide L1C induced lysis of target cells and IFN-γ, MIP-1α, and MIP-1β secretion to similar levels as those induced by the Tax peptide and at similar peptide concentrations (Fig. 6). Peptide Y5A induced detectably less lysis of target cells, though near the level induced by Tax, and induced significantly less secretion of IFN-γ, MIP-1α, and MIP-1β compared with Tax (Fig. 6). No target cell lysis or cytokine/chemokine secretion was induced by APCs pulsed with peptides G4V, P6Q, or Y8A even when using peptide concentrations of up to 1 μM. These results indicate that the TCR of clone 2G4 could be functionally engaged by complexes of HLA-A2 with the peptides Tax, L1C, and Y5A but not detectably by complexes with the peptides G4V, P6Q, or Y8A.

Crystals of the TCR-αβ/Tax peptide/HLA-A2 complex

Soluble TCR-αβ (36 mg/ml) and Tax peptide/HLA-A2 complex (12 mg/ml) were mixed at a 1:1 molar ratio and crystallized by vapor diffusion in hanging drops. The crystallization experiment used drops consisting of 1 μl of protein solution and 1 μl of each precipitant from a screen of precipitant conditions (46). Crystals appeared under the buffer conditions of 10% polyethylene glycol 8000, 100 mM magnesium acetate, 50 mM sodium cacodylate, pH 6.5. Microseeding new hanging drops with 10-fold dilutions of a suspension formed by crushing the original small crystals readily produced large (400 × 400 × 200 μm) crystals. SDS-PAGE of the washed crystals revealed that four polypeptides, the α- and β-chains of the TCR and the two chains of HLA-A2, are present in the crystals (Fig. 7, *lane 1*).

Crystals diffracted x-rays from a rotating anode x-ray generator to about 3.5 Å resolution at RT but appeared to disorder after overnight exposure to x-rays. Crystals could be flash cooled to −160°C in a stream of cold nitrogen gas after being soaked in the crystal growth solution supplemented with 20% glycerol as cryoprotectant. Diffraction was observed to about 3.3 Å from frozen crystals. Using the synchrotron x-ray source at CHESS (Cornell High Energy Synchrotron Source), diffraction data to beyond 2.8 Å resolution could be collected from crystals at −160°C. Analysis of the diffraction intensities of a partial dataset collected on a rotating anode source (90% complete to 3.5 Å, 46% complete from 3.5 to 3.3 Å) indicated the space group to be C2 with unit cell dimensions: a = 228.2 Å, b = 49.6 Å, c = 94.7 Å, β = 91.3°. The size and symmetry of the unit cell are consistent with the presence of one TCR/Tax peptide/HLA-A2 complex per asymmetric unit assuming 56% solvent content.

Discussion

Soluble TCR-αβ has been assembled by refolding the extracellular domains of the α- and β-chains expressed as insoluble inclusion bodies in *E. coli*. Inclusion body yields of 40 mg per liter of bacteria and a 20% refolding efficiency allow routine purification of 40 mg of pure TCR-αβ. Refolding of TCR-αβ heterodimers was

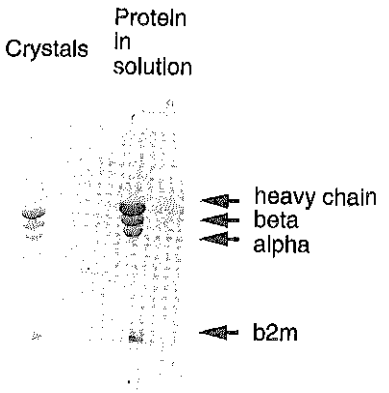

FIGURE 7. Crystals contain the four protein subunits of the TCR-$\alpha\beta$Tax/HLA-A2 complex. *Lane 1*, Crystals washed two times with the crystallization buffer. *Lane 2*, TCR/Tax peptide/HLA-A2 sample (1 μl, 16 μg) used to produce the crystals. From *top* to *bottom* of this 15% SDS-PAGE gel: heavy chain of HLA-A2 (31 kDa), TCR-β subunit (27.5 kDa), TCR-α subunit (22.7 kDa), β_2-microglobulin (11 kDa).

accomplished from α- and β-chains either with or without the capacity to form the single interchain disulfide bond found in vivo. Furthermore, the individual α- and β-chains could either be refolded separately before being mixed to form TCR or could be mixed while denatured and allowed to refold and heterodimerize together. The soluble TCR-$\alpha\beta$ produced in either case binds specifically to the complex of HLA-A2 with the Tax peptide of HTLV-1 as shown in band-shift assays (Figs. 2 and 5). Thus the specificity of the cell-cell interaction has been retained in the pure, soluble, recombinant molecular components. The band-shift native gel assay was also very useful as a functional test in guiding the development of the procedure for refolding recombinant TCR-$\alpha\beta$. Assembly of the complex of TCR/peptide/HLA-A2 does not require any of the carbohydrate of either glycoprotein, nor does it require the presence of the interchain disulfide bond of the TCR-$\alpha\beta$.

The soluble TCR was shown to bind to HLA-A2 complexed with the Tax peptide, but not with HLA-A2 complexed with unrelated or modified peptides (Figs. 2*A* and 5*B*), and the specificity of binding correlated with that seen in cellular assays (Fig. 6). It is particularly notable that the behavior of altered peptide ligands in CTL lysis and cytokine secretion assays correlated with the binding of soluble TCR to soluble MHC/peptide complexes in band-shift assays. Tax peptide, Tax L1C, and Tax Y5A, which band-shift, induce CTL lysis and lymphokine secretion, while Tax G4V, P6Q, and Y8A, which do not band-shift, are inactive in CTL lysis and do not stimulate the secretion of the lymphokines IFN-γ, MIP-1α, or MIP-1β. These data argue that the three peptides that mediate TCR binding between pure proteins also engage the TCR in cellular assays, and those that fail to bind as pure proteins (at least detectably in the band-shift assay) fail to engage the TCR detectably in cellular assays. Altered peptide Y5A appears to be a partial agonist (47, 48) by mediating CTL lysis to an extent comparable to that elicited by Tax itself, but by inducing lymphokine secretion at significantly reduced levels.

The refolding methods developed during this work may be of general utility in refolding other TCRs, since the sequences of TCR-$\alpha\beta$ are highly homologous and are expected to have essentially the same structure. In one instance, a class II-restricted TCR has been produced in bacteria and refolded using these methods. The resulting TCR-$\alpha\beta$ could be shown to bind both a specific class II/peptide complex and a superantigen using band-shift assays (J. Park, D. N. Garboczi, A. Seth, D. C. Wiley, unpublished observations). If, in the absence of the interchain disulfide bond, low affinities between some pairings of the α- and β-chains should limit heterodimer stability, high concentrations of the polypeptides now available from bacterial expression could be used to overcome this for structural studies.

A study of the crystals of the TCR-$\alpha\beta$/peptide/HLA-A2 complex reported here should reveal the atomic details of the Ag-specific cell-cell recognition that is central to the activation of T cells. This should also define the conserved and hypervariable TCR contacts to the MHC molecule itself that underlie alloreactivity and positive selection. Finally, it may allow a better definition of cross-reactivity (a potential basis of autoimmunity) and provide a physical basis for defining the parameters required for mechanisms of immunologic tolerance.

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