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Specific Binding of a Soluble Recombinant β-Chain T-Cell Antigen Receptor to Toxic Shock Syndrome Toxin-1, a Superantigen

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Toxic shock syndrome toxin-1 (TSST-1) is a bacterial superantigen that causes toxic shock in humans, probably by polyclonal activation of T-cells resulting from the cross linking of class II major histocompatability complex (MHC) molecules of antigen-presenting cells to the antigen receptors of T-cells (TCRs). The X-ray crystal structures of the complexes between superantigens and the human class II MHC molecule HLA-DR1 have revealed the molecular basis of the interaction between two proteins. However, structural studies of the TCR:superantigen complex have been hampered primarily by the lack of sufficient amounts of homogeneous TCR protein. Here we report the preparation of large amounts of a soluble β-chain TCR and its interaction with TSST-1. The β-chain TCR was expressed in *E. coli* as inclusion bodies. The inclusion body protein was refolded with an efficiency of 10-15% by a dialysis method in the presence of a reduced/oxidized glutathione redox buffer. The refolded β-chain TCR binds specifically to TSST-1 with a binding interaction strong enough to be detected by native gel shift assay and to make it possible to purify the complex by gel-filtration chromatography.

Toxic shock syndrome toxin-1 (TSST-1) is a bacterial superantigen that causes toxic shock in humans, probably by polyclonal activation of T-cells (Marrack *et al.*, 1990) that results in overproduction of lymphokines (Miethke *et al.*, 1992). Selective expansion of T-cells expressing the Vβ2 family of T-cell antigen receptor (TCR) β-chains was observed in patients with toxic shock syndrome (Choi *et al.*, 1990). The activation of large numbers of mature T-cells by TSST-1 and other superantigens results from the cross linking of class II major histocompatability complex (MHC) molecules of antigen presenting cells to the antigen receptors of T-cells (Fleischer and Schrezenmeier, 1988; Marrack and Kappler, 1990). Earlier studies indicate that superantigens function as intact molecules (Fleischer and Schrezenmeier, 1988), and bind primarily outside of the peptide binding groove of class II MHC molecules and to the fourth hypervariable loop (HV4) of TCRs (Fleischer and Mittrücker, 1991; Pullen *et al.*, 1991; Seth *et al.*, 1994).

The X-ray crystal structures of the human class II MHC molecule HLA-DR1 complexed with the superantigen staphylococcal enterotoxin B (SEB; Jardetzky *et al.*, 1994) and TSST-1 (Kim *et al.*, 1994) revealed the molecular basis of the interaction between superantigen and class II MHC molecules. SEB binds exclusively to the α-chain of DR1 off one edge of the peptide binding groove. Interestingly, one loop of SEB contacts polymorphic residues of DR1 recognized by the TCR during conventional antigen recognition, suggesting an uncoventional model for the interaction between the TCR and MHC during superantigen activation. The TSST-1 binding site on DR1 overlaps that of SEB, but the two binding modes are very different, although the tertiary structures of SEB and TSST-1 are similar (Acharya *et al.*, 1994; Prasad *et al.*, 1993; Swaminathan *et al.*, 1992). While SEB binds primarily off one edge of the DR1 peptide binding site, TSST-1 extends over almost one-half of the binding site contacting both the flanking MHC α-helices and the bound peptide. This suggests

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The abbreviations used are: MHC, major histocompatability complex; TSST-1, toxic shock syndrome toxin-1; TCRs, receptors of T-cells.

that TCR would bind to TSST-1:DR1 complex very differently than to DR1:peptide complex or SEB:DR1 complex.

However, structural studies of the TCR and TCR: superantigen complex have been hampered primarily by the lack of sufficient amounts of homogeneous TCR protein. As a result, our understanding of the molecular basis of the interaction between superantigen and TCR is very limited, compared to that of superantigen: MHC complex. Here we report the preparation of large amounts of a soluble β-chain TCR and its interaction with TSST-1. The β-chain TCR was expressed in *E. coli* as inclusion bodies. The inclusion body protein was refolded *in vitro*. The refolded β-chain TCR binds specifically to TSST-1 and the binding interaction is strong enough to be detected by native gel shift assay and to allow purification of the complex by gel-filtration chromatography.

Materials and Methods

Gene sources and bacterial strains

The human TCR β-chain (Vβ2.10, Jβ2.1 and Cβ2) was obtained by PCR cloning from peripheral blood lymphocytes that had been repetitively stimulated with TSST-1 (Utz *et al.*, unpublished results). The TCR β-chain was expressed in *E. coli* with the expression vector pLM1 containing a T7 promoter (Sodeoka *et al.*, 1993). The *E. coli* strain DH5α was used for DNA manipulation and the BL21 (DE3) plysS was used for protein expression (Studier *et al.*, 1990). The recombinant DNA of the TCR β-chain (pB2.10) contains the protein coding region of the extracellular domain (amino acids 1-254, Fig. 1a).

Materials

TSST-1 and SEB were purchased from Toxin Technology (Tampa, FL).

Truncation of the β-chain TCR

The C-terminal region of the β-chain TCR (amino acids 233-254) was truncated by using PCR with pB2.10 as the template and the oligonucleotide primers 5'-GATACTGCCTGAGCAGCCGCCTGAGG-3' and 5'-GTTTCAAAGCTTTACTGGGTGACGGGTTTGGC-3', containing the underlined *Bsu*36I and *Hin*dIII restriction sites, respectively. The stop codon TAA at codon 233 was included in the *Hin*dIII recognition site. The amplification mixture (100 μl) contained dGTP, dATP, dTTP, dCTP (each 200 μM), oligonucleotide primers (each 1 μM), template DNA (20 ng) and *Taq* polymerase (Boeringer Manheim) in 50 mM Tris-HCl (pH 9.0)/50 mM NaCl/10 mM $MgCl_2$. The reaction mixture was subjected to 25 cycles of 94 ℃ for 1 min, 55 ℃ for 1 min, 72 ℃ for 1 min, and a final 2 min at 72 ℃. The amplified DNAs were gel-purified, digested with *Bsu*36I/*Hin*dIII, and ligated into pB2.10 that had been digested with *Bsu*36I/*Hin*dIII and gel-purified. The plasmid was transformed into *E. coli* DH5α, and clones containing the correct insert were identified by agarose gel electrophoresis. One clone was subjected to DNA sequencing to verify its sequence and the plasmid (pB2.10-S) was transformed into *E. coli* BL21 (DE3) plysS for expression of the truncated form of β2.10 TCR (amino acid 1-232).

Another recombinant plasmid of the β-chain TCR (pB2.10-M) that expresses a truncated form of β-chain TCR (amino acid 1-243) was constructed with the same method. The oligonucleotide primers used were 5'-GATACTGCCTGAGCAGCCGCCTGAGG-3' and 5'-GTTTCAAAGCTTTAGTCTGCTCTACCCC-AGGCCTC-3', containing the under-lined *Bsu*36I and *Hin*dIII restriction sites, respectively.

Preparation of inclusion bodies

Six liters of bacteria transformed with pB2.10-S and pB2.10-M, respectively, were incubated at 37 ℃ and induced with 0.5 mM isopropyl-β-D-thiogalactoside at OD_{650} of 0.6-0.8. Four hours after the induction, bacteria were harvested by centrifugation, and inclusion bodies (insoluble protein aggregates) were isolated as described by Nagai and Thogersen (1987), with the modification of a freeze and thaw step after detergent treatment of the bacteria (Garboczi *et al.*, 1996). After washing four times with the Triton solution and one time with the Tris solution, the inclusion body pellet was dissolved in 20 ml of 8 M Urea in 50 mM MES (pH 6.5) with 1 mM EDTA and 1 mM DTT. Insoluble material was pelleted by centrifugation at 15,000 rpm for 30 min at room temperature, and the protein solution was stored at −70 ℃ in aliquots.

Refolding of the β-chain TCR

Inclusion body protein of the TCR β-chain was refolded by a dialysis method in the presence of a reduced/oxidized glutathione redox buffer (Garboczi *et al.*, 1996). About 20 mg of inclusion body solution was diluted with 30 ml of 8 M urea in 20 mM Tris (pH 8.0), and further diluted with 400 ml of 0.1 M Tris-HCl (pH 8.0)/0.4 M L-Arginine/4.5 M Urea/2 mM EDTA/5 μg/ml leupeptin/5 mM reduced glutathione/0.5 mM oxidized glutathione. The solution was dialyzed against 4 liters of 0.1 M Tris-HCl (pH 8.0)/0.4 M L-Arginine/2 M Urea/2 mM EDTA for 2 days at 4 ℃, and was further dialyzed against 4 liters of 20 mM Tris-HCl (pH 8.0)/0.15 M NaCl/1 mM EDTA/0.5 mM DTT for 1 day at 4 ℃, and finally against 4 liters of 20 mM Tris-HCl (pH 8.0)/1 mM EDTA/0.5 mM DTT for 1 day at 4 ℃.

Purification of refolded protein

The refolding mixture was applied to a DEAE Sepharose column equilibrated in 20 mM Tris-HCl (pH 8.0) and eluted with 20 mM Tris-HCl (pH 8.0)/0.4 M NaCl. The fractions were analyzed by SDS-PAGE under non-reducing conditions, and the frac-

tions containing the correctly folded protein were pooled and concentrated with a Centriprep-30 and a Centricon-30 (Amicon) to 0.5 ml. The concentrated protein was subjected to a FPLC gel filtration column equilibrated in 20 mM Tris buffer (pH 7.5), and the peak with the appropriate mobility was collected. The peak fractions were concentrated with a Centricon-30 to 250 μl.

Results

cDNA cloning of a TCR β-chain

A TCR β-chain cDNA was cloned from peripheral blood lymphocytes by PCR with V-region-specific and C-region-specific primers (Utz *et al.*, 1996). The peripheral blood lymphocytes from a normal blood donor were stimulated repeatedly with TSST-1 and the activated lymphocytes were shown to be TSST-1 specific in proliferation assays prior to PCR analysis. The amino acid sequence of the cloned TCR β-chain shown in Figure 1a indicates that the TCR β-chain uses the genes Vβ2.10, Jβ2.1, and Cβ2 (Rowen *et al.*, 1996). This is consistent with the observation that patients with toxic shock syndrome exhibit selective expansion of T cells expressing the Vβ2 family of TCR β-chains (Choi *et al.*, 1990).

TCR and the Fab domain of immunoglobulin

α- and β-chains of TCR belong to the immunoglobulin (Ig) super-family. Particularly, the am-

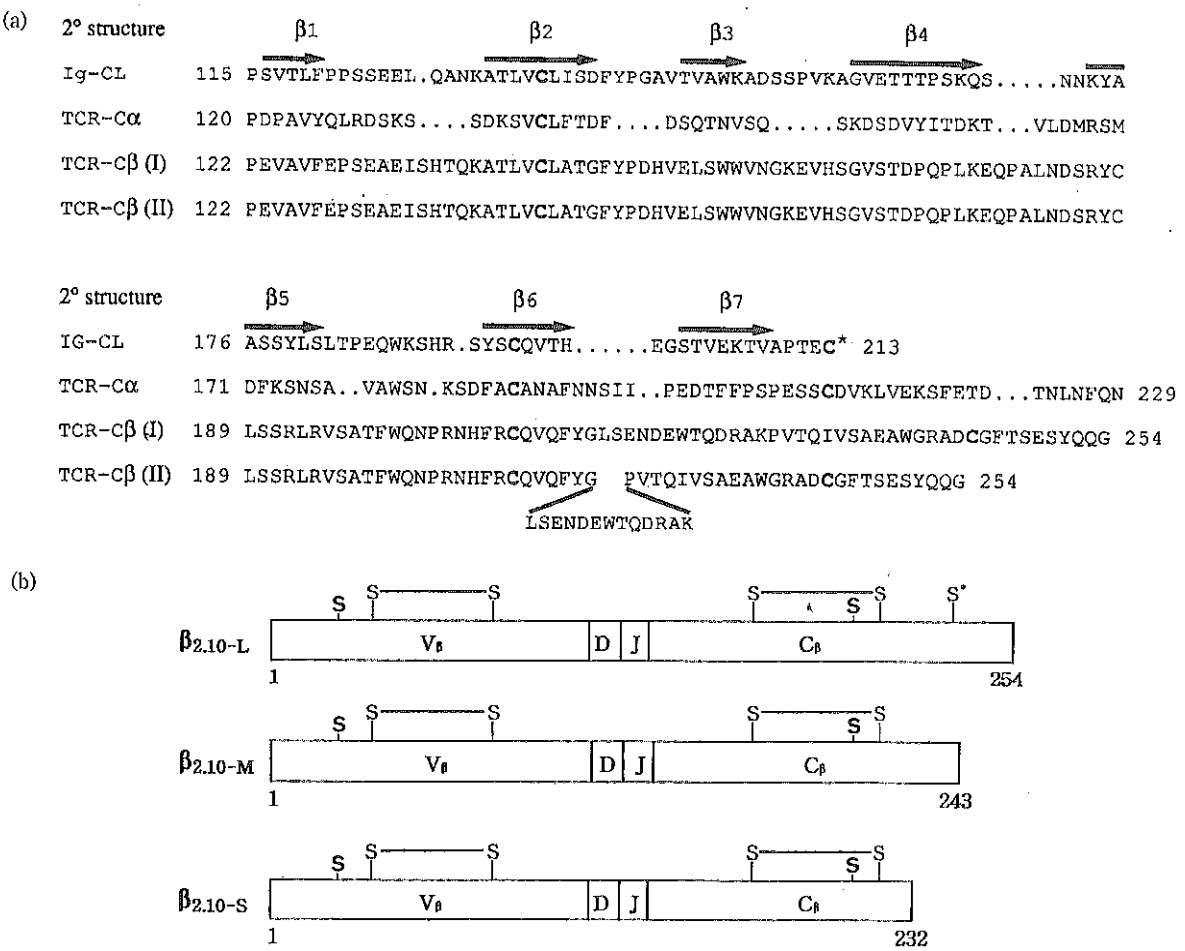

Figure 1. (a) Amino acid sequence alignment of the constant domains of the Ig light chain, TCR α-chain, and TCR β-chain. Amino acid sequences were aligned with the BESTFIT program of the GCG package, and edited manually based on the secondary structural information of the Ig light chain. Cysteine residues which form an intra-molecular disulfide bond are bold and the cysteine residue which forms an inter-chain disulfide bridge to the α-chain is marked with an asterisk. TCR β-chain is aligned two ways, I and II, depending on the location of the inserted sequence element found in TCR β-chains. The amino acid sequence of the variable domain of the cloned TCR β-chain (Vβ2.10) is MGAVVSQHPS RVICKSGTSV KIECRSLDFQ ATTMFWYRQF PKQSLMLMAT SNEGSKATYE QGVEKDKFLI NHASLTLSTL TVTSAHPEDS SFYICSAAFN EQFFGPGTRL TVLEDLKNVF P 121. (b) Map for the three β2.10 TCR constructs. Translational stop codons were substituted for the amino acid residue 255 in β2.10-L, for the C-terminal-most cysteine codons (Cys244) in β2.10-M, and for the amino acid residue 233 in β2.10-S. Cysteine residues are labeled -S, disulfide bonds are -S-S-, and the cysteine residue that forms an inter-chain disulfide bridge to the α-chain is marked with an asterisk.

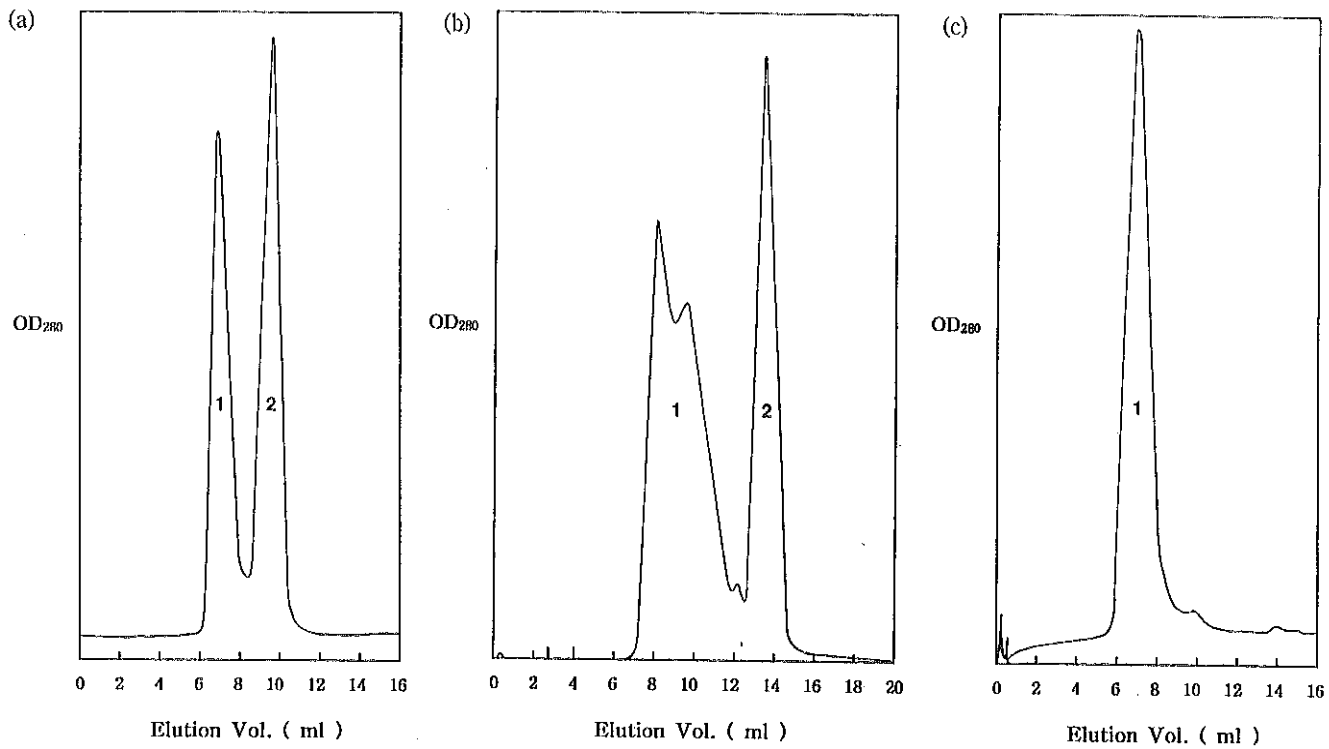

Figure 2. (a) FPLC gel-filtration profile of the β2.10-L TCR. A Superdex 75 column (Pharmacia) equilibrated in 20 mM Tris buffer (pH 7.5) was used for final purification. SDS PAGE analysis indicates that peak 1 consists of aggregated β-chain TCR, and peak 2 consists of correctly folded β-chain. (b) FPLC gel-filtration profile of the β2.10-M TCR. Superdex 200 column (Pharmacia) equilibrated in 20 mM Tris buffer (pH 7.5) was used for final purification. SDS PAGE analysis indicates that peak 1 consists of aggregated β-chain TCR, and peak 2 consists of correctly folded β-chain. (c) FPLC gel-filtration profile of the β2.10-S TCR. Superdex 75 column (Pharmacia) equilibrated in 20 mM Tris buffer (pH 7.5) was used for final purification. SDS PAGE analysis indicates that peak 1 consists of aggregated β-chain TCR.

ino acid sequence of TCR is homologous to the Fab domain of antibody. The structural model of TCR has been proposed based on the tertiary structure of the Fab domain (Chothia *et al.*, 1988; Davis and Bjorkman, 1988; Claverie *et al.*, 1989; Novtny *et al.*, 1986) and the structure of some TCR domains (Bentley *et al.*, 1995; Fields *et al.*, 1995). In order to determine the exact position of the extracellular domain of TCR, we aligned the amino acid sequences of TCR α- and β-chains with that of Ig light chain whose three-dimensional structure is known. We found that TCR β-chain has a unique inserted sequence element between the second cysteine residue of the constant domain and the last cysteine residue, which forms a disulfide bridge with the α-chain (Fig. 1a). We made three constructs of β2.10 TCR (Fig. 1b) to address where the inserted sequence element is located on the three dimensional structure and whether the inserted sequence element is important for maintaining the three dimensional structure of β-chain TCR (see below).

Expression of the β-chain TCR

The human TCR β-chain (Vβ2.10) was expressed in *E. coli*. To create polypeptides of the extracellular domain of the TCR β-chain, translation stop codons were substituted for the C-terminal-most cysteine codons (Cys 244) in our second construct (Fig. 1b, β2.10-M, mid-size form) and stop codons were placed at amino acid residue 233 in our third construct (Figure 1b, β2.10-S, short form). This may correspond to the C-terminal-most cysteine residue of immunoglobulin light chain if the inserted sequence element found in TCR β-chain is not structurally important (Figure 1a). The β-chains produced by the second and third constructs each lack the cysteine residue that is located close to the transmembrane domain and is involved in the inter-molecular disulfide bond with the α-chain (Allison *et al.*, 1982). Both polypeptides were expressed in large quantities as inclusion bodies in *E. coli*. Lysing the bacterial cells by freeze and thaw method with lysozyme and detergents and washing the inclusion bodies several times with Triton solution yielded highly purified proteins of up to 30-50 mg per liter of bacterial culture. The purified inclusion body protein was over 80% pure as determined on SDS gels.

Refolding of the β-chain TCR

The β-chain TCRs solubilized in urea solution were refolded by removal of the denaturant by dialysis under dilute condition in the presence of a

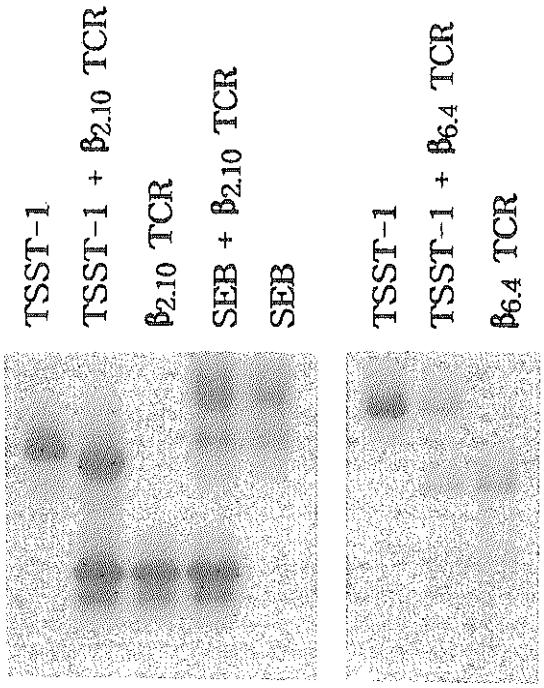

Figure 3. Native gel-shift assay reveals that the refolded β-chain TCR (β2.10-M) forms a specific complex with TSST-1.

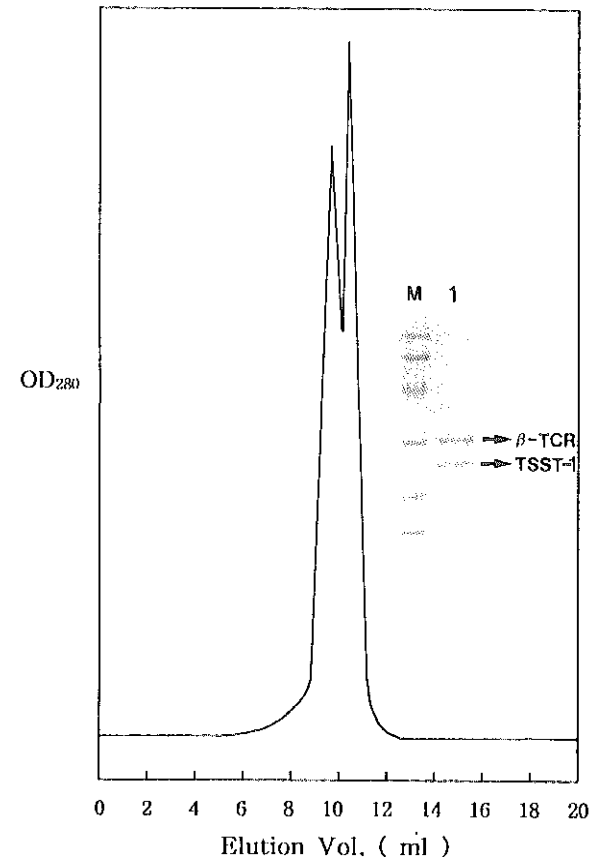

Figure 4. Purification of TSST-1:β2.10 TCR complex by FPLC gel-filtration chromatography. Superdex 75 column equilibrated in 20 mM Tris buffer (pH 7.5) was used and the peak fractions were analyzed by SDS PAGE. The first peak from gel-filtration chromatography contains both the β-chain TCR and TSST-1 (lane 1), and the second peak contains the β-chain TCR or TSST-1 depending on which component is added in excess. Lane M, size marker proteins (97, 66, 45, 31, 21.5 and 14.5 kDa from the top).

reduced/oxidized glutathione redox buffer. The extent of refolding was analyzed by FPLC gel-filtration chromatography and SDS PAGE. Figure 2 shows the typical FPLC gel-filtration profiles of three refolded β-chain TCR proteins (β2.10-L, M and S). In figure 2a, peak 1 at 7 ml, the void volume of the column consists of aggregated β-chain, and peak 2 at 9 ml consists of correctly folded β-chain (β2.10-L). FPLC gel-filtration profile of β2.10-M also exibits two major peaks, 1 for aggregates and 2 for correctly folded β-chain (Figure 2b). β2.10-L and β2.10-M were refolded with an final efficiency of 10-15% but β2.10-S was not refolded at all (figure 2c). In Figure 2c, peak 1 at the void volume of the column consists of aggregated β2.10-S TCR. Refolding of β2.10-S by the dilution method (Garboczi *et al.*, 1992) gave the same result. The refolding method described here may be of general utility in refolding other TCR β-chains. In fact, two other TCR β-chains (β6.4 and β 12.1) were efficiently refolded using this method (Fig. 3; Garboczi *et al.*, 1996).

Binding of TSST-1 to the β-chain TCR

The interaction of a soluble β-chain TCR (β2.10-M) with TSST-1 was observed using non-denaturing PAGE (Fig. 3). The complex of the β-chain TCR and TSST-1 appeared as a prominent shifted band that migrated at a position, distinct from either β-chain TCR or TSST-1 alone. A similar band shift was observed with the longer version of β2.10 TCR (β2.10-L) and TSST-1. The presence of β-chain TCR at the shifted band was confirmed by immunoblot analysis using an anti-TCR antibody (data not shown). However, the binding interactions of the β2.10 TCR with SEB and that of the β6.4 TCR with TSST-1 (Fig. 3) were not detected by the gel shift assay, indicating that the interaction between the β2.10 TCR and TSST-1 is highly specific.

Purification of TSST-1:β-chain TCR complex

The complex formation of soluble β-chain TCR with TSST-1 was also observed using FPLC gel-filtration chromatography. The FPLC gel-filtration chromatography profile of a mixture of the β-chain TCR and TSST-1 usually exhibited two well separated peaks (Fig. 4). SDS-PAGE analysis of the peaks revealed that the first peak contained both the TCR β-chain and TSST-1 proteins (Fig. 4), and the second peak contained the β-chain TCR or TSST-1 depending on which component was added in excess.

Interestingly, the Coomassie blue staining intensity of β-chain TCR and TSST-1 bands looked similar, suggesting that the molar ratio of the β-chain TCR and TSST-1 may be 1:1.

Discussion

Amino acid sequence comparison of TCR and immunoglobulin (Ig) has indicated that TCR is homologous to the Fab domain of Ig. Based on the sequence homology with the Fab domain, a structural model of TCR has been proposed (Chothia *et al.*, 1988; Davis and Bjorkman, 1988). We also compared the amino acid sequence of TCR with that of Ig light chain to determine exactly which portion of TCR may correspond to the Fab domain of Ig (Fig. 1a). Interestingly, however, TCR β-chain contains a unique inserted sequence element between the second cysteine residue of the constant domain and the last cysteine residue which forms an inter-chain disulfide bridge with the α-chain. Whether this inserted sequence element is located within the constant domain or in the stem loop region between the constant domain and the transmembrane segment was not clear due to low sequence homology around this region. Therefore we made two additional constructs (β2.10-M and β2.10-S) to see whether the inserted sequence element is important for proper folding of the β-chain TCR. Both β2.10-M and β2.10-S polypeptides were expressed in large quantities as inclusion bodies in *E. coli*, but unlike β2.10-L and β2.10-M, β2.10-S was not refolded at all, suggesting that β2.10-S is lacking an essential component for proper folding. This suggests that the unique inserted sequence element found in TCR β-chains could be located within the constant domain and the sequence element must be important for maintaining the tertiary structure of β-chain TCR. X-ray crystal structure of a murine β-chain TCR determined recently (Bentley *et al.*, 1995) clearly shows that the inserted sequence element forms a long loop between the sixth and seventh β-strands of the constant domain, and the loop is located at the interface between the variable domain and constant domain.

This study showed that TCR β-chain could be refolded *in vitro* without α-chain. Garboczi *et al.* (1996) has also observed that TCR α-chain and β-chain could be refolded separately and assembled properly by mixing refolded α- and β-chains together. These results suggest a possibility that the TCR α-chain and β-chain are capable of forming a homodimer and heterodimer depending on the circumstances although the propensity could be different. To address the question about the oligomeric state of the β-chain TCR, we determined the molecular weight by gel-filtration chromatography and performed chemical cross-linking experiments with DSP. The apparent molecular mass of β-chain TCR determined by FPLC gel-filtration chromatography was about 60 kDa suggesting that it could exist as a dimer. However, intermolecular cross-linking of β-chain TCR was not observed by chemical crosslinking reactions with DSP. In addition, X-ray crystal structure of a murine β-chain TCR shows that it exists as a monomer in crystals (Bentley *et al.*, 1995). Further experiments are necessary to clarify the oligomeric state of β-chain TCR.

The refolded β-chain TCR seems to be folded correctly judging from the following observations. First, the refolded β-chain TCR was very soluble and stable for at least one month, and it behaved like a normal soluble protein on gel-filtration chromatography and nondenaturing/denaturing PAGE. Second, the β-chain TCR crystallized under the buffer conditions of 15% PEG4K, 5 mM DTT, and 100 mM HEPES (pH 7.5). Third, the β-chain TCR binds specifically to TSST-1, a natural ligand (Fig. 3). Fourth, ELISA results indicate that the β-chain TCR binds an anti-TCR monoclonal antibody that recognizes the constant domain of TCR β-chain (data not shown).

The refolded β-chain TCR (β2.10) was shown to bind to TSST-1, but not to SEB (Fig. 3). This result suggests that the soluble form of the β-chain TCR is a proper ligand for TSST-1 and the binding interaction is specific. The specificity of the binding interaction of β2.10 TCR and TSST-1 is further supported by the fact that the β6.4 TCR prepared similarly does not bind to TSST-1.

The binding affinity of superantigen and a soluble form of αβ TCR or β-chain TCR has been measured by surface plasmon resonance detection technique and sedimentation equilibrium technique (Malchiod *et al.*, 1995; Seth *et al.*, 1994). The equilibrium dissociation constant (Kd) of αβ TCR:SEB complex is 0.82 μM and those of a murine β-chain TCR and SEC1, 2, and 3 are 2.51 μM, 2.32 μM, and 0.86 μM, respectively. The binding constant of TSST-1:β-chain TCR complex formation has not been determined, but our data indicate that the binding affinity of TSST-1 and the β-chain TCR is strong enough to be detected by native gel shift assay and to allow purification of the complex by gel-filtration chromatography. Furthermore, TSST-1:β-chain TCR complex crystallized under the buffer conditions of 15% PEG4K, 5% isopropanol, and 100 mM HEPES (pH 7.5). SDS PAGE analysis of the washed crystals revealed that two proteins, TSST-1 and β-chain TCR, are present in the crystals (data not shown). This result also suggests that the binding affinity of TSST-1 and β-chain TCR is strong enough to be analyzed by X-ray crystallography.

TCR β-chain is known to play an important role in the early development of T-cells in the thymus (Groettrup *et al.*, 1992; Kishi *et al.*, 1995; Mombaerts *et al.*, 1992; Shinkai *et al.*, 1993). The nature of the β-chain TCR expressed on premature T-cells awaits further characterization. However, the observation

that the TCR β-chain can be refolded by itself supports the notion that it could exist stably on the surface of certain T-cells and that it could have a functional role in the development of T-cells. The availability of cDNA and soluble protein of TCR β-chain will also be useful in investigating the structure and functional role of the β-chain TCR in the development of T-cells.

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