Stable, soluble T-cell receptor molecules for crystallization and therapeutics

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Antibody and T-cell receptors (TCRs) are the primary recognition molecules of the adaptive immune system. Antibodies have been extensively characterized and are being developed for a large number of therapeutic applications. This has been possible because of the ability to manufacture stable, soluble, monoclonal antibodies which retain the antigen specificity of B cells. Unlike antibodies, TCRs are not expressed in a soluble form, but are anchored to the T-cell surface by an insoluble transmembrane domain. Characterization and development of TCRs has been hampered by the lack of suitable methods for producing them as soluble and stable proteins. Here we report the engineering of soluble human TCRs suitable for crystallization studies and potentially for in vivo therapeutic use.

Keywords: molecular engineering/monoclonal TCRs/peptide–HLA interactions/protein crystallization/soluble T-cell receptors

Introduction

T-cell receptors (TCRs) are specialized antigen recognition molecules expressed on the surface of T cells and capable of specifically recognizing peptide–major histocompatibility complex (MHC) antigens (Davis et al., 1998; Garcia et al., 1999). TCRs are highly variable, enabling T cells to recognize a huge number of peptide–MHC antigens, although individual T cells generally express only one type of TCR. The TCR consists of an αβ heterodimer, each chain of which comprises a variable domain linked to a constant domain.

Many strategies have been proposed for making soluble versions of the αβ TCR. Most of these work for a very limited number of TCRs, e.g. single-chain TCR designs (Chung et al., 1994; Schodin et al., 1996; Khandekar et al., 1997). A more generally applicable method for producing soluble TCRs has recently been developed, which involves stabilizing the αβ heterodimer by fusion of the TCR extracellular domains to the jun/fos coiled-coil domains (Willcox et al., 1999a,b). Although this method is highly successful at producing soluble TCRs which retain specific ligand binding activity, these soluble TCRs have proved difficult to crystallize. Furthermore, no method has yet been reported that allows TCRs to be used for therapeutic antigen targeting.

The native TCR heterodimer is stabilized by a membrane-proximal disulphide bridge, but strategies to produce soluble TCRs incorporating this bond have not been successful (Garboczi et al., 1996). Therefore, we sought to design a generic method for producing soluble human TCRs, stabilized by a non-native disulphide bond between the extracellular α and β constant domains. Molecular modelling was used to determine the optimal site for the disulphide bond, and three recombinant soluble TCRs were produced in Escherichia coli and refolded with high efficiency. The disulphide-linked TCRs (dsTCRs) were highly stable and displayed authentic binding activity demonstrated by Biacore™ surface plasmon resonance (SPR) experiments.

The design of the dsTCRs makes them highly amenable to crystallization and we report the preliminary crystallization of one dsTCR along with crystallographic analysis confirming the inter-chain disulphide bond position. These dsTCRs are likely to be amenable for in vivo therapeutic applications, and candidates are currently being developed for clinical applications. The design of the dsTCR is subject to a patent application (Jakobsen and Glick, 2001).

Materials and methods

Molecular modelling

The crystal structure of the B7 human αβ TCR, specific for human leukocyte antigen (HLA)-A*0201 in complex with the human T-trophic lymphocyte virus type-1 (HTLV-1) tax 11–19 peptide, solved at 2.5 Å resolution and deposited in the Protein Data Bank (Berman et al., 2000) under the name 1BD2 (Ding et al., 1998), was used for the disulphide bond predictions. Residues forming the interface between the constant domains were examined, and side chains whose β-carbons were closer than 7 Å were mutated in silico to cysteine. The χ1 dihedral angle (of the cysteine side chain) was then rotated in order to confirm that the distance between the sulphur atoms was optimal for a disulphide bond and did not perturb the tertiary structure of the protein.

Cloning of TCR chains

TCR chains were cloned into the bacterial expression vector pGEMT7 by polymerase chain reaction (PCR) cloning using a 5′ primer containing an NdeI site which incorporates the ATG start codon, and a 3′ primer containing a HindIII site and a TAA stop codon which replaces the native inter-chain cysteine codon. A free cysteine in the constant domain of the β-chain was mutated to alanine in order to facilitate in vitro refolding.

Cysteine codons were introduced by PCR mutagenesis. Complementary primers were designed which annealed 5′ and 3′ of the desired mutation, but contained an altered codon encoding a cysteine. These were used in a pfu-driven PCR using a template encoding the relevant TCR chain produced as above. After 16 rounds of PCR with an extension time of 8 min, the reaction was digested with DpnI to digest methylated DNA.
After 1 h digestion at 37°C, 10 µl of the reaction was transformed into E.coli XL1-blue cells which were plated out onto LB/100 µg/ml ampicillin plates. Plates were incubated overnight at 37°C, then single colonies were grown to stationary phase in 10 ml of LB containing 100 µg/ml ampicillin. A Qiagen miniprep kit was used to purify DNA from these clones. The sequence of the insert was verified by automated sequencing.

The β-chain constant region has a natural BglII site 5’ of the engineered cysteine codon which allows cloning of other β-chains into the mutated construct. In order to be able to clone different α-chains into the mutated α-chain construct, we introduced a silent BamHI site by PCR mutagenesis at position TRAC P$_3$D$_5$P$_7$ cctgaccct→ccggacct. This site may then be used to PCR clone new α-chains into the construct containing the mutant cysteine codon.

**TCR expression and refolding (Willcox et al., 1999b)**

TCR chains were expressed separately as inclusion bodies in the E.coli strain BL21-DE3(pLysS) by induction in mid-log phase with 0.5 mM IPTG. Inclusion bodies were isolated by sonication, followed by successive wash and centrifugation phases with 0.5 mM IPTG. Inclusion bodies were dissolved in 6 M guanidine, 10 mM dithiothreitol (DTT), 0.5 M ethylenediaminetetra-acetate (EDTA), buffered with 50 mM Tris pH 8.1 and stored at −80°C. Soluble TCR was refolded by rapid dilution of a mixture of the dissolved α- and β-chain inclusion bodies into 5 M urea, 0.4 M L-arginine, 100 mM Tris pH 8.1, 3.7 mM cysteamine, 6.6 mM β-mercaptoethanol (4°C) to a final concentration of 60 mg/ml.

**Purification of the soluble TCR**

The refold mixture was dialysed for 24 h against 10 vol of demineralized water, then against 10 vol of 10 mM Tris pH 8.1 at 4°C. The refolded protein was then filtered and loaded onto a POROS 50HQ column (Applied Biosystems). The column was washed with 10 mM Tris pH 8.1 prior to elution with a 0–500 mM NaCl gradient in the same buffer. Fractions were analysed by Coomassie-stained sodium dodecyl sulphate (SDS)–10% NuPAGE (Novagen, WI), and TCR-containing fractions were pooled and further purified by gel filtration on a Superdex 75PG 26/60 column (Amersham Biosciences, Uppsala, Sweden) pre-equilibrated in phosphate-buffered saline. Fracions comprising the main peak were pooled and analysed further. The final purified 1G4 dsTCR was analysed by Coomassie-stained SDS–10% NuPAGE under reducing and non-reducing conditions, and an aliquot of protein was buffer exchanged into HBSE (HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA) and concentrated prior to activity determination by BIACore™ SPR.

**Preparation of soluble biotinylated HLA complexes**

Peptide–HLA-A*0201 complexes were prepared by in vitro refolding from bacterially expressed inclusion bodies and synthetic peptide (Garboczi et al., 1992), followed by enzymatic biotinylation with BirA enzyme (O’Callaghan et al., 1999).

**Determination of binding activity by BIACore™ SPR**

CM5 BIACore™ chips (BIACore AB, St Albans, UK) were coated with streptavidin using amine coupling, and pHLA complexes were flowed over individual flow cells at a concentration of ~50 µg/ml until the response measured ~1000 response units (RU). Soluble TCR was concentrated to >10 mg/ml and the concentration was determined by measurement of OD$_{280}$ using an extinction coefficient calculated from the sequence. Serial dilutions of the TCR were flowed over the different pMHC complexes and the response values at equilibrium were determined for each concentration. Dissociation constants (K$_D$) were determined by plotting the response over background against the protein concentrations followed by a least-squares fit to the Langmuir binding equation, assuming a 1:1 interaction (Willcox et al., 1999a).

**Evaluation of stability**

Aliquots of sterile 1G4 dsTCR in phosphate-buffered saline were incubated at −65 and 25°C and analysed using Coomassie-stained SDS–polyacylamide gel electrophoresis (PAGE), isoelectric focussing, and ion-exchange and size-exclusion high-pressure liquid chromatography.

**Crystallization and structural analysis of dsTCRs**

1G4 dsTCR was concentrated to 10 mg/ml and crystallized by the hanging drop method. Two microlitre aliquots of the concentrated protein were mixed with an equal volume of a precipitating solution, and equilibrated against 500 µl of the precipitant. The precipitant solution was made up of 0.2 M ammonium acetate, 0.1 M tri-sodium citrate dihydrate pH 5.6, 30% (v/v) polyethylene glycol (PEG) 4000 in water.

**Results**

**Molecular modelling**

Table I shows the β-carbon side chain distances for selected pairs of residues in the α/β constant domain interface. TRAC threonine 48 and TRBC serine 57 (residue numbering according to the International Immunogenetics Database (IMGT) (Lefranc and Lefranc, 2001)) were predicted to be optimal because of their close proximity, the direction of their side chains, which are oriented towards each other, and the relative chemical similarity of their side chains to cysteine. Indeed, mutating these residues to cysteine in silico and orienting the sulphur atoms towards each other by rotating the χ1 dihedral angle yields a disulphide distance as close as 1.5 Å without perturbing the tertiary structure of the protein.

**Production of dsTCRs**

A6 TCRs (Utz et al., 1996) (specific for HLA-A*0201 in complex with the HTLV-1 tax 11-19 peptide) with engineered inter-chain disulphide linkages in various positions, described in Table I, were produced using PCR mutagenesis, followed by

| Table I. Analysis of β-carbon distances for selected pairs of residues in the interface between the α- and β-chain TCR constant domains |
|-----------------|-----------------|---------------|
| TRAC residue   | TRBC residue    | β-Carbon distance (Å) |
| Threonine 48   | Serine 57       | 4.73           |
| Threonine 45   | Serine 77       | 5.33           |
| Serine 61      | Serine 57       | 6.24           |
| Leucine 50     | Serine 57       | 3.38           |
| Tyrosine 10    | Serine 17       | 3.59           |
| Serine 15      | Valine 13       | 4.97           |
| Serine 15      | Glutamate 15    | 5.93           |
| Threonine 45   | Aspartate 59    | 5.60           |
| Leucine 12     | Serine 17       | 5.68           |
| Serine 61      | Arginine 79     | 5.43           |
| Leucine 12     | Phenylalanine 14| 3.87           |
| Valine 22      | Phenylalanine 14| 4.95           |
| Tyrosine 43    | Leucine 63      | 6.31           |
expression as *E. coli* inclusion bodies, and *in vitro* refolding. Of these, the TCR with an engineered disulphide linkage between cysteines introduced at positions TRAC 48 and TRBC 57 produced the best yields of soluble TCR with clean BIAcore binding data (data not shown).

TCR chains from human T-cell clones JM22 (Lehner et al., 1995) (specific for HLA-A*0201-influenza matrix peptide) and 1G4 (Chen et al., 2000) (specific for HLA-A*0201-NY-ESO peptide) were also cloned into the α- and β-chain constructs containing engineered cysteines at positions TRAC 48 and TRBC 57, respectively. The A6, JM22 and 1G4 dsTCRs refolded with >40% efficiency to produce protein preparations which were >95% pure after ion-exchange and gel-filtration chromatography, as judged by Coomassie-stained SDS–PAGE analysis.

Figure 1 shows a typical purification for the 1G4 dsTCR on (i) anion-exchange and (ii) gel-filtration columns. The purified protein was analysed by Coomassie-stained SDS–PAGE under reducing and non-reducing conditions (Figure 2). The dsTCR α- and β-chains run separately under reducing conditions, but the introduced disulphide bond holds the chains together under non-reducing conditions and they run as a single band of higher molecular weight.

**BIAcore SPR binding analysis**

BIAcore SPR equilibrium binding analysis for 1G4 dsTCR binding to the HLA-A*0201-NY-ESO peptide complex is shown in Figure 3. The estimate for the dissociation constant (K_D) of 15.5 μM compares with a value of 14 μM obtained for

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**Fig. 1.** (a) Anion-exchange chromatography of *in vitro* refolded 1G4 dsTCR on an 8 ml POROS 50HQ column eluted with a gradient of NaCl (conductivity shown as dotted line). The protein elutes as a single major peak at ~600 mM NaCl. Fractions corresponding to the main peak were collected and pooled. (b) Gel-filtration chromatography of peak fractions from (a) on a Superdex 75PG 26/60 column (Amersham Biosciences) isocratically eluted with phosphate-buffered saline. The protein elutes as a single major peak at an elution volume of ~160 ml. Fractions corresponding to the main peak were collected and pooled.

**Fig. 2.** Coomassie-stained SDS–PAGE of pooled fractions from Figure 1b. Lane 1, standard proteins of known molecular weight; lane 2, contains 10 μg of non-reduced 1G4 dsTCR; lane 3, contains 10 μg of reduced (with 50 mM DTT) 1G4 dsTCR. The reduced 1G4 TCR runs as separate α- and β-chains of ~23 and ~28 kDa, respectively. The non-reduced 1G4 dsTCR runs as a single disulphide-linked band of ~45 kDa, the anomalously low apparent molecular weight being due to its more compact disulphide-linked structure under non-reducing conditions.

**Fig. 3.** BIAcore SPR analysis of the binding of purified 1G4 dsTCR to the HLA-A*0201-NY-ESO peptide complex. Response values were calculated by subtracting specific from non-specific (HLA-A*0201 tax 11-19 peptide) response readings and were plotted against protein concentration calculated from absorbance at 280 nm using an extinction coefficient derived from the amino acid sequence. The K_D was calculated using a least-squares fit to the data assuming a 1:1 interaction which was confirmed by a Scatchard plot.
the same TCR refolded as a jun/fos fusion (N.Lissin, unpublished results).

K_D estimates for the dsTCRs are compared with the similar jun/fos fusions in Table II. In all cases, the binding affinity is comparable with the jun/fos fusions indicating that the binding domains of the dsTCRs are not significantly affected by the constant domain cysteine linkage.

**Stability analysis of the 1G4 dsTCR**

The 1G4 dsTCR was highly stable, showing no significant chemical or physical degradation at −65°C over a period of 7 months and at 25°C over a period of 4 weeks, using the analytical methods described (data not shown). The protein was also stable over the course of four freeze–thaw cycles.

**Crystallization and structural analysis of the 1G4 dsTCR**

Single crystal X-ray diffraction data of 1G4 dsTCR were collected at Station 9.6 of the SRS Daresbury Laboratory, UK. The effective resolution of the data was 2.5 Å. The structure was solved by molecular replacement using the 1BD2 structure of the B7 TCR (Ding et al., 1998) as the starting model. The structure of the constant domains was refined at 2.5 Å resolution. The bond distance between the sulphur atoms of the engineered disulphide bond is 2.03 Å, and the distance between the sulphur atoms of the native structure is 2.5 Å. This implies that there is very little local disturbance of the natural structure around the introduced disulphide bond.

**Discussion**

The investigation of TCRs has been hampered by difficulties in making soluble versions of these cell-surface molecules. The intrinsic instability of the αβ heterodimer has made it difficult to produce in a soluble truncated form, except in a very limited number of cases. In order to stabilize the αβ heterodimeric pairing, a number of approaches have been reported. Formation of single-chain TCRs, analogous to scFv antibody fragments, has had limited success despite numerous attempts (Chung et al., 1994; Schodin et al., 1996; Khandekar et al., 1997; Plaksin et al., 1997). For example, the Vα-linker-Vβ single-chain TCR construct yields fully functional A6 TCR but not 1G4 TCR, when produced by in vitro refolding from E.coli inclusion bodies (P.Molloy, unpublished results).

A more successful approach has been to produce soluble TCR in which the extracellular domains are stabilized in the heterodimer by a jun/fos coiled-coil domain (Willcox et al., 1996). Although this construct allows production of a wide variety of antigen-specific soluble TCRs (Avidex, unpublished results), the flexible fusion to the coiled-coil domains makes the construct generally difficult to crystallize for structural analysis. The presence of a non-native fusion domain would also make this construct unsuitable for in vivo therapeutic applications.

The dsTCR described here provides a solution to both of these problems. It is a completely globular structure which favours formation of well ordered protein crystals. The difference between the dsTCR and the natural TCR is minimal, consisting of two mutations to cysteine that are buried in the inaccessible interface between the α- and β-chain constant domains. The dsTCR should therefore also present a minimal risk of antigenicity and immunogenicity in vivo.

This method of engineering soluble TCRs has been tested in a number of different TCRs specific for different peptide–HLAs. Here we present data on three different dsTCRs specific for class I HLA–peptide antigens. All have shown specific binding activity to their cognate peptide–HLA complex in a
The antigen binding domains of the jun/fos fusion TCRs and the dsTCRs are unlikely to have been affected by the engineering involved in producing the soluble proteins. Indeed, the affinity measurements for dsTCRs compare closely with those obtained with jun/fos fusion TCRs (Table II).

The globular structure of the dsTCRs makes them generally amenable for crystallization studies. The 1G4 dsTCR was readily crystallized which enabled us to confirm the presence of the engineered disulphide bond. Further dsTCRs and dsTCR–pHLA complexes have been crystallized, and we are currently in the process of gathering data for these crystals. dsTCRs should provide a reliable tool for generating a general understanding of the molecular details of antigen recognition by T cells. Furthermore, dsTCRs may provide the basis for generating targeted therapeutic agents retaining the specificity of human T cells for peptide–HLA antigens. This could make a large number of new antigen targets, particularly those derived from intracellular proteins, accessible to ‘monoclonal’ protein therapy.

Acknowledgements

We would like to thank Andrew Johnson for conducting the evaluation of stability, Corneli van der Walt for technical assistance running SDS–PAGE, and Nikolai Lissin and Peter Molloy for allowing inclusion of unpublished data.

References


Received March 11, 2003; revised July 1, 2003; accepted July 30, 2003

Note added in proof

We have recently engineered higher affinity TCRs, based on the disulphide-linked TCRs reported herein, with K_Ds as low as 2.5 nM, which are being developed as therapeutic targeting agents.