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Production of soluble $\alpha\beta$ T-cell receptor heterodimers suitable for biophysical analysis of ligand binding

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Abstract

A method to produce $\alpha\beta$ T-cell receptors (TCRs) in a soluble form suitable for biophysical analysis was devised involving in vitro refolding of a TCR fusion protein. Polypeptides corresponding to the variable and constant domains of each chain of a human and a murine receptor, fused to a coiled coil heterodimerization motif from either c-Jun (alpha) or v-Fos (beta), were overexpressed separately in *Escherichia coli*. Following recovery from inclusion bodies, the two chains of each receptor were denatured, and then refolded together in the presence of denaturants. For the human receptor, which is specific for the immunodominant influenza A HLA–A2-restricted matrix epitope (M58-66), a heterodimeric protein was purified in milligram yields and found to be homogeneous, monomeric, antibody-reactive, and stable at concentrations lower than 1 μM. Using similar procedures, analogous results were obtained with a murine receptor specific for an influenza nucleoprotein epitope (366–374) restricted by H2-D^b. Production of these receptors has facilitated a detailed analysis of viral peptide–Major Histocompatibility Complex (peptide–MHC) engagement by the TCR using both surface plasmon resonance (SPR) and, in the case of the human TCR, isothermal titration calorimetry (ITC) (Willcox et al., 1999). The recombinant methods described should enable a wide range of TCR–peptide–MHC interactions to be studied and may also have implications for the production of other heterodimeric receptor molecules.

Keywords: coiled coil; isothermal titration calorimetry; protein refolding; surface plasmon resonance; T-cell receptor

A critical event in the adaptive immune response is the binding of antigen-specific T-cell receptors (TCRs) on the surface of a T-cell with peptide-MHC complexes on an antigen presenting cell (APC) (Davis et al., 1998a). Such interactions are known to have low affinities compared to antibody-antigen binding (Williams & Barclay, 1986; Fremont et al., 1996), but in combination with other accessory interactions are able to mediate highly sensitive recognition of foreign antigens. The kinetic properties of TCR–peptide–MHC interaction are postulated to be critical parameters in many models of TCR triggering (McKeithan, 1995; Rabinowitz et al., 1996; Davis et al., 1998b), and experimentally measured TCR dissociation rates have indeed been correlated with T-cell responsiveness (Matsui et al., 1994). The cell surface TCRs that mediate these interactions are heterodimeric proteins of the immunoglobulin superfamily (IgSF). They are associated with invariant proteins of the CD3 complex, which is involved in signal transduction to the inside of the cell. The extracellular portion of each TCR chain consists of a membrane-proximal constant domain and a membrane-distal variable domain bearing highly variable loops analogous to the complementarity determining regions (CDRs) of antibodies (Bentley & Mariuzza, 1996). These form the binding site for the peptide–MHC complex. The ligands of $\alpha\beta$ TCRs are the MHC class Ia and class II gene products (Madden, 1995). These are highly polymorphic proteins that also belong to the IgSF and possess a peptide binding groove, enabling them to present a diverse array of short peptide fragments at the APC cell surface.

Biochemical and structural analysis of TCR–peptide–MHC interaction requires relatively large amounts of soluble forms of TCR. Despite the existence of numerous methods for the production of particular murine and human $\alpha\beta$ TCRs in soluble form (Davis et al., 1998a), these have not proved to be applicable to all receptors. Here, we report a new method for the production of soluble TCR involving *E. coli* expression and protein refolding. The previous use of such techniques has established that the im-

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munoglobulin domains of the TCR are capable of mediating specific noncovalent α-β chain association, allowing human TCRs to be produced in ligand-specific form suitable for crystallization (Garboczi et al., 1996). However, such interaction is often weak, as is the case with the human TCR studied here and other human TCRs (Garboczi et al., 1996). Consequently, TCRs assembled in this way are often unstable. To circumvent such problems, we have incorporated the heterodimerization domain of the c-Jun and v-Fos transcription factors onto the C-terminal of the TCR α and β chains, respectively. Such leucine zipper sequences have been employed previously to produce soluble TCR, but in a eucaryotic system to decrease nonspecific covalent chain pairing (Chang et al., 1994) rather than to increase the stability of the heterodimeric species. Our approach allows production of relatively high quantities of correctly folded receptor that is stable even at low concentrations and can be used for ligand binding analyses. These methods should be applicable to a range of TCRs and will assist molecular analysis of TCR–MHC interaction.

Results and discussion

Recombinant receptor was designed (Fig. 1) so that the two chains of the heterodimer pair with each other solely by noncovalent interactions. A short linker was used to connect the leucine zipper heterodimerization motif from c-Jun and v-Fos to the C-terminal of the TCR α and β sequences, respectively. When refolded together in isolation, the Fos-Jun peptides have been shown to heterodimerize specifically without the need for a covalent interchain linkage (O'Shea et al., 1989) and are known to assemble as a coiled coil domain (Glover & Harrison, 1995). In the native TCR, the α and β chains form an interchain disulfide bond immediately C-terminal to the last residue of the constant domain we have included in our construct. Furthermore, the Fos-Jun peptides have been shown to be able to form an interchain disulfide bond at the level of the proline in the linker we have used (O'Shea et al., 1989). Consequently, despite the absence of an interchain disulfide bond in our recombinant receptor, the alignment of the two chains was expected to be optimal. Two TCRs were produced as TCR zippers. One (termed JM22z) is a human receptor (JM22) (Lehner et al., 1995) specific for the immunodominant influenza A matrix epitope (M58–66) presented by HLA–A2 (HLA–A2–Flu), and one (termed F5z) a murine receptor (F5) (Palmer et al., 1989) specific for an influenza nucleoprotein epitope (366–374) presented by H2-D^b (H2-D^b-NP).

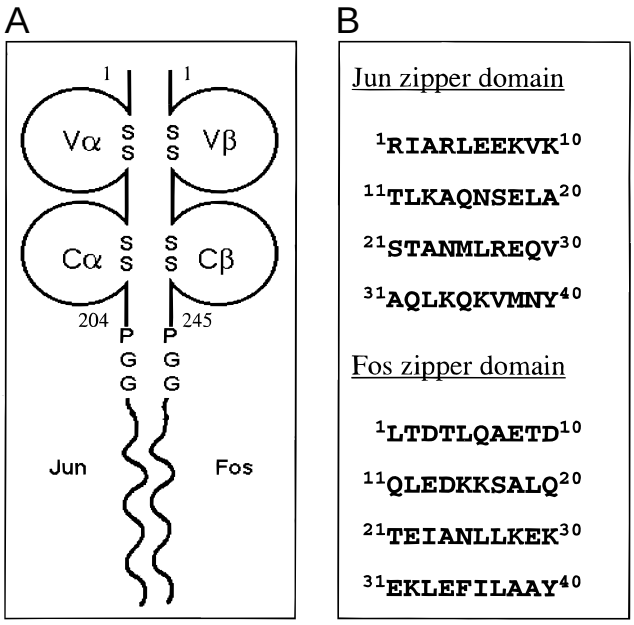
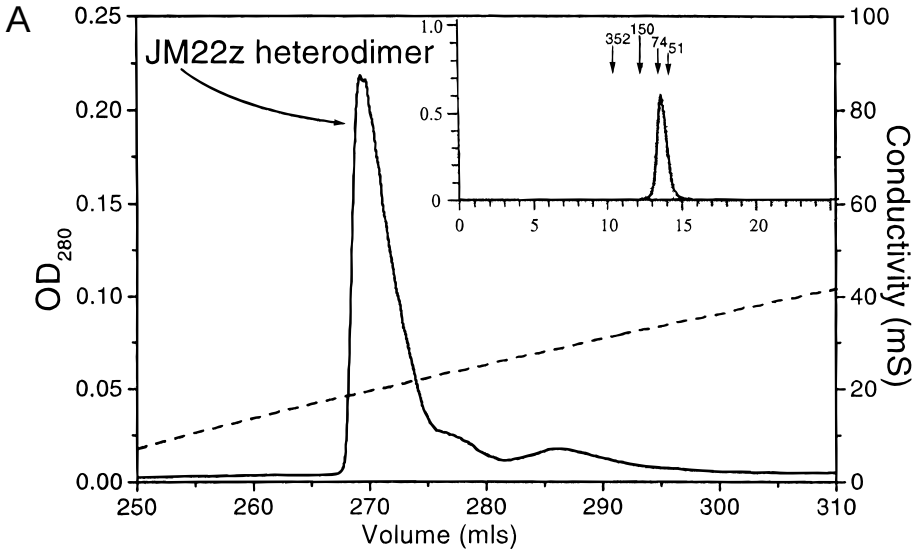


Fig. 1. A: Design of the TCR zipper construct. The design incorporates the two IgSF domains of each TCR chain [shown as semicircles and marked C (constant) and V (variable)]. Intrachain disulfide bonds are shown as SS. For each chain, the constant domain is encoded up to but not including the native interchain cysteine residue. The C-terminal of each chain is fused via a short linker to a leucine zipper heterodimerization motif from either c-Jun (α) or v-Fos (β). PGG refers to the amino acid sequence of the linker (Pro-Gly-Gly). The N-terminal sequence of the TCR chains was predicted using the program SIGCLEAVE from the GCG package. The length of the TCR sequences is shown, numbered for the JM22z receptor. **B:** Leucine zipper domains. The sequences from the c-Jun and v-Fos proteins that have been incorporated into the α and β chains of the recombinant receptor, respectively. These derive from amino acids 285–324 of Jun and 162–201 of Fos (O'Shea et al., 1989).

Each TCR zipper chain was expressed individually in *E. coli* and accumulated in inclusion bodies. Following purification, inclusion bodies were solubilized at high concentrations in either urea or guanidine. For each receptor, the α and β chains were renatured together by dilution refolding of the denatured protein to form heterodimeric TCR. Initial attempts to refold TCR zipper in conditions used previously (100 mM Tris pH 8.5, 1 M L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.2 mM PMSF) to refold human TCR (Garboczi et al., 1996) resulted in severe protein precipitation. This was dependent on the presence of the zipper sequences, provoking the suggestion that the entirely alpha-helical zipper domains folded first and permitted heterodimerization before the more complex immunoglobulin domains had folded correctly. The refolding buffer was, therefore, altered to include denaturant (Orsini & Goldberg, 1978), in the hope that this would prevent hydrophobic interactions between partially folded immunoglobulin domains, but allow individual chains to fold completely before stable heterodimerization. A 5 M urea buffer was chosen because this level of denaturant has been shown to be compatible with TCR chain refolding (Garboczi et al., 1996). This step was sufficient to prevent precipitation occurring and allowed correctly folded TCR zipper heterodimers to assemble with acceptable yields using an optimized protocol.

Following dialysis, the refolded TCR zipper was purified using anion exchange and gel filtration steps (Fig. 2A). The JM22z heterodimer which had been purified by anion exchange eluted as an ~70 kDa protein from a Superdex 200 gel filtration column (Pharmacia, Uppsala, Sweden), indicating the protein was monomeric, even at relatively high concentrations (Fig. 2A, inset). A similar elution volume was observed with the F5z receptor. SDS-PAGE analysis of the JM22z receptor (Fig. 2B) showed that it comprised approximately equimolar amounts of α and β chains. Under reducing conditions, the two chains ran either side of the 31 kDa marker. Under nonreducing conditions, each chain migrated independently, but with an increased mobility, consistent with the presence of intradomain disulfide bonds (Garboczi et al., 1996). Purified F5z receptor had similar properties.

Surface plasmon resonance (SPR) was used to test the reactivity of the recombinant JM22 receptor to an antibody specific for BV17 chains (because JM22 uses the variable region segment BV17S1). The E17.5F3.15.13 antibody (Immunotech, Marseilles, France) was



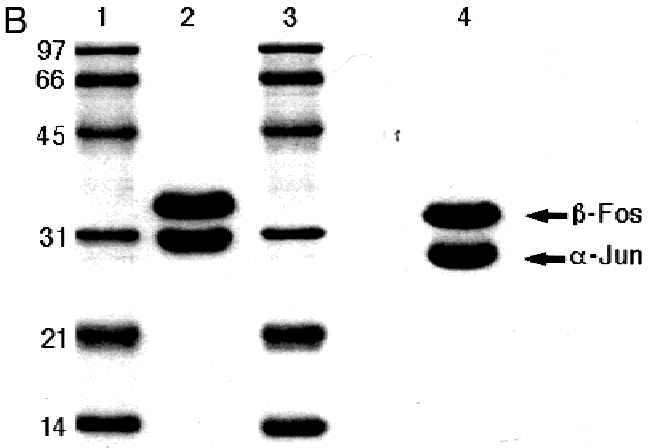


Fig. 2. Purification and characterization of JM22z. **A:** Anion exchange purification. A 200 mL sample of refolded JM22z was loaded onto a POROS 10 HQ column and eluted as described in the text. The main peak of protein was then analyzed by gel filtration and SDS-PAGE. Gel filtration (inset). Anion-exchange purified JM22z receptor eluted from a Superdex 200 HR 10/30 column (Pharmacia, Uppsala, Sweden) at a volume of 13.6 mL, indicating a molecular weight of ~74 kDa. No aggregate peak is observed. The elution volumes of standard proteins ranging from 10.7 mL (352 kDa) to 14.3 mL (51.4 kDa) are shown. The protein concentration of the peak fraction was 26 μM. This behavior is consistent with the molecular weight of ~60 kDa and indicates that in solution the receptor is monomeric and does not self-associate under these conditions. **B:** Reducing/nonreducing gel analysis. Identical samples of anion-exchange purified JM22z were loaded onto a 15% acrylamide SDS gel, either under nonreducing (lane 4) or reducing (lane 2) conditions. Marker proteins are shown in lanes 1 and 3. Molecular weights are shown in kilodaltons. Under both sets of conditions, the noncovalently associated heterodimer is dissociated into separate chains in approximately equimolar amounts. The α chain has a higher mobility than the β chain due to a smaller constant domain. In lane 4, each chain has a higher mobility compared to lane 2 and migrates independently, suggesting a single species of intrachain disulfide bonding is present. No higher molecular weight cross-linked species are observed. This behavior is consistent with correct disulfide bond formation.

found to bind JM22z immobilized to a flow cell surface by direct amine coupling (data not shown) and to a modified form of the TCR (JM22zbt), immobilized via a biotinylated tag (Fig. 3). JM22zbt incorporates a short sequence at the C-terminal of the β chain, which can be recognized and specifically biotinylated by the BirA enzyme. Following biotinylation, JM22zbt was immobilized onto streptavidin-coated flow cells. SPR was also used to show that soluble HLA–A2–Flu bound specifically to both amine coupled JM22z (Willcox et al., 1999) and JM22zbt immobilized to streptavidin (Fig. 3). Significantly higher levels of the antibody and the HLA–A2–Flu complex were bound by JM22zbt immobilized to streptavidin than by JM22z immobilized by amine coupling. This is probably because the use of a C-terminal biotinylated tag allows JM22zbt to be immobilized in a uniform orientation suitable for ligand binding, whereas amine-coupled JM22z may be randomly orientated, and consequently, largely inactive. The observations that JM22z and JM22zbt both retain ligand specificity and are able to bind to the antibody that recognizes functional $BV17^{+ve}$ T cells are consistent with the TCR domains of both forms of recombinant JM22 adopting a native conformation.

We have recently used SPR to carry out a detailed affinity and kinetic analysis of the binding of JM22z and F5z TCRs to their peptide/MHC ligands (Willcox et al., 1999). Both JM22z and F5z yielded greater responses when injected over flow cells coated with their cognate peptide–MHC vs. irrelevant peptide complexes or control proteins, indicating specific binding. Such responses were observed at 40 μg/mL of TCR, implying TCR heterodimers were stable at low concentrations. The interaction exhibited similar affinity and kinetics in either orientation, suggesting that the activity of the JM22z TCR was similar to that of the HLA–A2–Flu complex. The biological activity of the JM22z TCR preparation was also measured directly by binding TCR from solution onto agarose beads coated with the HLA–A2–Flu complex. At least 84% of the TCR was precipitated, indicating that, for this TCR, affinity and kinetic measurements calculated on the basis of 100% activity were likely to be essentially correct.

The in vitro refolding methods described have enabled a murine and a human TCR to be produced as soluble recombinant fusion proteins. Both receptors appear to be correctly folded, and bind specifically to their cognate peptide–MHC complex. This has fa-

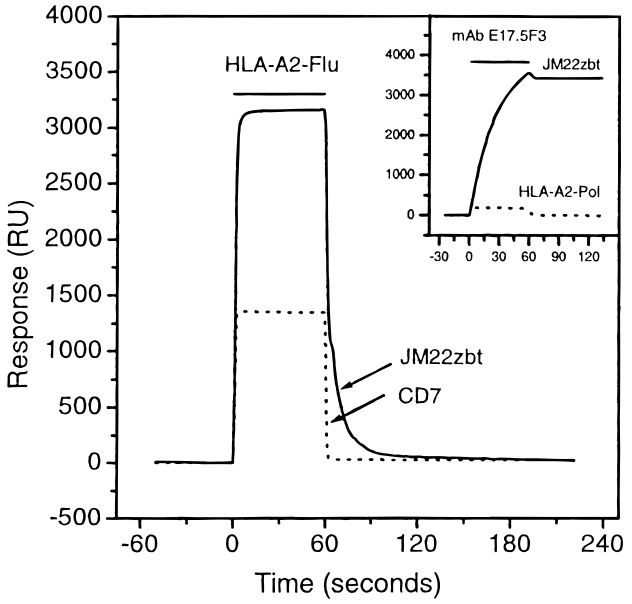


Fig. 3. SPR characterization of TCR-zipper. **Main panel:** Specific binding of the HLA–A2–Flu complex to immobilized JM22zbt. The HLA–A2–Flu complex (514 μM) was injected for 1 min over flow cells coated with JM22zbt (4980 RU) or CD7 control protein (9720 RU). The difference in the responses in each flow cell indicate that 1,800 RU of HLA–A2–Flu was specifically bound to the JM22zbt-coated surface. **Inset:** Binding of anti-BV17 antibody to immobilized JM22zbt. The E17.5F3.15.13 antibody was injected for 1 min at a concentration of ~100 μg/mL over two flow cells, one of which contained the JM22zbt receptor (4086 RU), and one of which contained the HLA–A2–Pol complex (2794 RU) as a control. The response in the two flow cells is shown above. The immobilized JM22zbt specifically bound ~3,400 RU of antibody during a 5 μL injection.

cilitated an affinity and kinetic analysis of TCR/viral–peptide–MHC interaction using SPR, a technique that allows direct affinity and kinetic measurements of weak protein–protein interactions. Yields of the human receptor JM22z were sufficient for a thermodynamic analysis of TCR–peptide–MHC binding using Isothermal Titration Calorimetry (ITC) (Willcox et al., 1999). The effects of T-cell coreceptor/MHC and TCR–peptide–MHC interactions on each other have also been studied (Wyer et al., 1999). The recombinant techniques used should in principle be applicable to other $\alpha\beta$ TCRs, including those restricted by MHC class II molecules. Hopefully, they will allow further questions to be addressed, such as the range of TCR affinities within different T-cell responses, the properties of dominantly selected receptors, and the kinetic requirements for receptor triggering. The methods described here also provide a starting point for the production of soluble $\gamma\delta$ T-cell receptors.

Materials and methods

Cloning of TCR zipper expression constructs

The regions of the c-*jun* and v-*fos* genes encoding the leucine zippers were generated by PCR reactions from cDNA generously provided by Gerard Evan and Richard Treisman, respectively. The c-*jun* and v-*fos* fragments were ligated into pBluescript II KS- (Stratagene, La Jolla, California) using unique XhoI and XmaI restriction sites to obtain constructs BJ107 and BJ108, respectively. To generate DNA fragments encoding JM22α-Jun and JM22β-Fos, the TCR chain sequences were amplified from plasmids containing the JM22α and JM22β sequences previously generated using PCR from human cDNA obtained from the JM22 CTL clone. For the F5 TCR, the α and β chain sequences were amplified from plasmids mpK19α6 and mpK19β6, respectively (Palmer et al., 1989). For both receptors, the 5′ primers used were designed to incorporate a high AT content immediately 3′ of the initiation codon and using *E. coli* codon preferences to maximize expression (Gao et al., 1998; O'Callaghan et al., 1998). TCR-α and -β fragments were inserted into BJ107 and BJ108, respectively, using the unique XbaI and SmaI restriction sites. At this stage, an internal NdeI site in the F5 α chain was removed by silent mutagenesis. Also, to prevent incorrect disulfide bonding occurring during refolding, the codon coding for the unpaired cysteine in the TCR β constant domain (residue 188 of the recombinant JM22β-Fos and F5β-Fos proteins) was mutated to serine. Both of these steps were carried out by PCR mutagenesis, using the Quickchange™ Mutagenesis Kit (Stratagene). The sequence of the inserted fragments was verified by dideoxy DNA sequencing. From these plasmids the TCR zipper fragments were subcloned as NdeI-BamHI fragments into pGMT7, an expression vector that uses the T7 promoter (Rosenberg et al., 1987), to obtain plasmids expressing JM22α-Jun (GFG020) and JM22β-Fos (GFG021), and F5α-Jun (GFG089) and F5β-Fos (GFG092). The plasmid JMB002, which encodes the JM22β-Fos chain with a biotinylation tag (JM22β-Fosbt), was generated by PCR amplifying the entire JM22β-Fos-encoding sequence using pGFG021 as a template, and cloning NdeI-BamHI fragments into BJ193, a pGMT7-derived plasmid that incorporates the amino acids GSGGGLNDIFEAQKIEWH at the C-terminal.

TCR zipper expression and inclusion body purification

TCR zipper chains were overexpressed in *E. coli* and purified as follows. GFG020, GFG021, JMB002, GFG089, and GFG092, the pGMT7 expression plasmids encoding the JM22α-Jun, JM22β-Fos, JM22β-Fosbt, F5α-Jun, and F5β-Fos proteins, respectively, were transformed separately into *E. coli* strain BL21pLysS (DE3), and single colonies were grown at 37 °C in TYP (ampicillin 100 μg/mL) medium to OD_{600} of 0.4 before inducing protein expression with 0.5 mM IPTG. Cells were harvested 3 h postinduction by centrifugation for 30 min at 4000 rpm in a Beckman J-6B. Cell pellets were resuspended in a buffer containing 50 mM Tris-HCl, 25% (w/v) sucrose, 1 mM EDTA, 0.1% (w/v) sodium azide, 10 mM DTT, pH 8.0. After an overnight freeze–thaw step, resuspended cells were sonicated in 1 min bursts for a total of around 10 min in a Milsonix XL2020 sonicator using a standard 12 mm diameter probe. Inclusion body pellets were recovered by centrifugation for 30 min at 13,000 rpm in a Beckman J2-21 centrifuge. Three detergent washes were then carried out to remove cell debris and membrane components. Each time the inclusion body pellet was homogenized in a Triton buffer (50 mM Tris-HCl, 0.5% Triton X-100, 200 mM NaCl, 10 mM EDTA, 0.1% (w/v) sodium azide, 2 mM DTT, pH 8.0) before being pelleted by centrifugation for 15 min at 13,000 rpm in a Beckman J2-21. Detergent and salt were then removed by a similar wash in the following buffer: 50 mM Tris-HCl, 1 mM EDTA, 0.1% (w/v) sodium azide, 2 mM DTT, pH 8.0. Finally, the inclusion bodies were solubilized in denaturant for 3–4 h at 4 °C. JM22α-Jun, JM22β-Fos, JM22β-Fosbt, and F5α-Jun pellets were dissolved separately in a urea solution

(50 mM MES, 8 M urea, 10 mM EDTA, 2 mM DTT, pH 6.5), whereas F5β-Fos pellets were dissolved in a guanidine solution containing 50 mM MES, 6 M guanidine, 10 mM EDTA, 2 mM DTT, pH 6.5. Insoluble material was then pelleted by centrifugation for 30 min at 13,000 rpm in a Beckman J2-21, and the supernatant was divided into 1 mL aliquots and frozen at −70 °C. Solubilized, purified inclusion bodies were quantitated using a Bradford dye-binding assay (Biorad, Richmond, California). For each chain a yield of around 100 mg of purified inclusion body was obtained from 1 L of culture. From SDS-PAGE analysis, the purity of each inclusion body was estimated to be around 90% (data not shown).

Refolding and purification of the TCR zipper

Approximately 30 mg (i.e., 1 μmol) of each solubilized inclusion body chain (JM22α-Jun and either JM22β-Fos or JM22β-Fosbt; F5α-Jun and F5β-Fos) was thawed from frozen stocks and a further pulse of DTT (4 μmol/mL) was added to ensure complete reduction of cysteine residues. Samples were then mixed and the mixture diluted into 15 mL of a guanidine solution (6 M guanidine-hydrochloride, 10 mM sodium acetate, 10 mM EDTA, pH 5.5), to ensure complete chain denaturation. The guanidine solution containing fully reduced and denatured TCR zipper chains was then injected into 1 L of the following refolding buffer: 100 mM Tris pH 8.5, 400 mM L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 5 M urea, 0.2 mM PMSF. The solution was left for 24 h. The refolded protein was then dialyzed twice to remove denaturant. The JM22z receptor was dialyzed firstly against 10 L of 100 mM urea, secondly against 10 L of 100 mM urea, 10 mM Tris pH 8.0. The F5z receptor was dialyzed first against 10 L of 2 M urea, and second against 10 L of 100 mM urea, 10 mM Tris pH 8.0. Both refolding and dialysis steps were carried out at 6–8 °C. Dialyzed JM22z or JM22zbt receptor was separated from degradation products and impurities by loading the dialyzed protein onto a POROS® 10 HQ analytical anion exchange column in seven 200 mL aliquots, and each time eluting bound protein with a gradient of 0–400 mM NaCl over 50 column volumes using a BioCAD®/SPRINT™ Perfusion Chromatography® system (Perseptive Biosystems, Inc., Framingham, Massachusetts). Noncovalently associated heterodimer eluted in a single peak at approximately 100 mM NaCl. Peak fractions (typically containing heterodimer at a concentration of 100–300 μg/mL) were stored at 4 °C before being pooled and concentrated. The yield of JM22z heterodimer was ~15%. Purified JM22zbt was biotinylated using the enzyme BirA as described (O'Callaghan et al., 1999). Dialyzed F5z protein was concentrated down in a stir-cell (Amicon, Beverly, Massachusetts) before being purified on a Superdex 200 gel filtration column (Pharmacia, Uppsala, Sweden). Fractions corresponding to this peak were purified further on a POROS® 10 HQ anion exchange column using a BioCAD®/SPRINT™ Perfusion Chromatography® system (Perseptive Biosystems, Inc.). Heterodimer was eluted as a single peak, and peak fractions pooled and concentrated. The yield of F5z heterodimer was estimated to be not more than 10%.

Preparation of soluble peptide–MHC complexes

Soluble HLA–A2–Flu complexes were prepared by refolding HLA–A2 heavy chain with β_2-microglobulin (both expressed in *E. coli*) and the synthetic peptide corresponding to Influenza matrix protein 58–66 (GILGFVFTL) (Genosys, Woodlands, Texas), essentially as described (Garboczi et al., 1992). The refolded complexes were then purified by both gel filtration and anion exchange before being used in SPR experiments.

Surface plasmon resonance

Binding studies were carried out at 25 °C using the BIAcore™ 2000 (BIAcore AB, St. Albans, United Kingdom) as described (Willcox et al., 1999). Streptavidin was immobilized onto CM5 binding chips using a standard amine coupling at pH 5.0. For testing binding of the E17.5F3.15.13 antibody to recombinant JM22 receptor proteins, JM22z was immobilized by amine coupling, whereas biotinylated JM22zbt was immobilized to separate streptavidin-coated flow cells by injection at 5 μL/min. Biotinylated CD7 protein and OX68 antibody were immobilized to streptavidin-coated flow cells and used as controls in JM22zbt and JM22z antibody binding experiments, respectively.

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