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The production, purification and crystallization of a soluble heterodimeric form of a highly selected T-cell receptor in its unliganded and liganded state

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T-cell antigen receptors (TcRs) are heterodimeric cell-surface receptors that play a pivotal role in the cellular immune response. The TcR interacts specifically with a peptide-laden major histocompatability complex (pMHC). A human TcR has been characterized that interacts with an immunodominant epitope, FLRGRAYGL, from the Epstein–Barr virus, a ubiquitous human pathogen, in complex with HLA-B8. Despite the vast TcR repertoire, this TcR is found in up to 10% of the total T-cell population in seropositive HLA-B8+ individuals. In this report, this highly selected TcR is characterized by expressing in *Escherichia coli*, refolding, purifying and crystallizing the receptor. In addition, the HLA-B8–FLRGRAYGL complex has been expressed in *E. coli*, refolded and shown to be functionally active. Using native gel electrophoresis, the refolded TcR is shown to be capable of binding specifically to the refolded HLA-B8–FLRGRAYGL and this TcR has been crystallized in complex with the pMHC. The crystals of the unliganded and liganded TcR diffract to 1.5 and 2.5 Å, respectively.

1. Introduction

The clonally distributed antigen receptors (TcRs) of T lymphocytes play a fundamental role in the cellular immune response (Davis & Bjorkman, 1988; Garcia *et al.*, 1999). The majority of T cells express heterodimeric disulfide-bonded $\alpha\beta$ receptors that specifically co-recognize structural determinants of microbial peptides complexed with products of the major histocompatibility complex (MHC). Recently, the structures of a number of TcR–p-MHC complexes have been determined by X-ray crystallography (for a recent review, see Rudolph & Wilson, 2002). This work has given fundamental insights into the mode of TcR–p-MHC binding; however, it is likely that not all the key structural features of this recognition process have been elucidated.

Partly to address this, we have undertaken the study of a highly selected T-cell receptor from a human cytotoxic T lymphocyte (CTL) clone with dual specificity for a cognate viral ligand and an allogeneic human leukocyte antigen (HLA) molecule (Burrows *et al.*, 1994, 1995). The CTL clone, LC13, specifically recognizes HLA-B8 in complex with the FLRGRAYGL peptide determinant from EBNA 3, a latent Epstein–Barr virus (EBV) antigen. EBV is a ubiquitous member of the herpes virus family and establishes a persistent infection in up to 90% of adults. The LC13 TcR comprises highly restricted gene segments that are found in the same combination in most CTL specific for HLA-B8–FLRGRAYGL from unrelated individuals (Argaet *et al.*, 1994; Callan *et al.*, 1998) and this specificity can comprise up to 10% of circulating CD8+ T cells (Callan *et al.*, 1998). Hence, the LC13 clonotype is referred to as a 'public' TcR (Argaet *et al.*, 1994). This conserved receptor usage is remarkable given the enormous repertoire of approximately 10^7–10^8 unique T-cell receptors that exist to cope with the huge number of foreign peptides that might be presented by polymorphic MHC molecules.

However, despite this vast database of TcRs there are only five examples of complete heterodimeric extracellular $\alpha\beta$ TcR three-dimensional structures: two murine TcR structures termed 2C and N15 (Garcia *et al.*, 1996; Wang *et al.*, 1998) and three human structures, termed A6, B7 and HA1.7 (Garboczi, Ghosh *et al.*, 1996; Ding *et al.*, 1998; Hennecke *et al.*, 2000). The major impediment to the structural study of TcRs is the production of large quantities of correctly folded $\alpha\beta$ heterodimers. A variety of approaches have been used to overcome the difficulties associated with expression of TCRs, including the expression of an isolated chain or single domains, as well as expression of TCR extracellular domains in bacterial and eukaryotic systems.

In this report, we successfully express in *E. coli* and refold the HLA-B8–EBV complex and the public TcR and show that they are both functional. We have crystallized the unliganded public TcR and these crystals

diffract to 1.5 Å resolution. Furthermore, we have crystallized the LC13 TcR in complex with the HLA-B8–FLRGRAYGL complex and these crystals diffract to 2.5 Å resolution. This work will significantly extend our knowledge of TcR form and function.

2. Materials and methods

2.1. Construction of expression vectors

RNA prepared from LC13 T cells with Trizol (Life Technologies) was reverse transcribed. A DNA fragment containing the sequence encoding either the LC13 α- or β-chain was obtained by PCR amplification of cDNA using combinations of either the 5′ primer CGCCATATGAAAACCACACAGCCAAATTCA and the 3′ primer CCCAAGCTTTTAGGAGCTCTCTGGGCTGGGGAAGAAG (α-chain) or the 5′ primer CGCCATATGGGTGTCTCCCAGTCCCCTAGG and the 3′ primer CCCAAGCTTTTAGTCTGCGCGCCCCCAGGCCTCGGCGCTG (β-chain). Each PCR-derived α or β gene was cloned as a *Nde*I–*Hin*dIII fragment into the pET-30 expression vector (Novagen) and sequenced for verification. Translation was engineered to terminate immediately before each of the α- or β-chain constant-region cysteines normally forming an interchain disulfide bond. The codon encoding the unpaired cysteine at position 186 of the Cβ region was changed to encode alanine by site-directed mutagenesis (QuikChange, Stratagene). The predicted amino-acid sequence for LC13 α-chain is (accession No. L26455) MKTTQPNSMESNEEEPVHLPCNHSTISGTDYIHWYRQLPSQGPEYVIHGLTSNVNNRMASLAIAEDRKSSTLILHRATLRDAAVYYCILPLAGGTSYGKLTFGQGTILTVHPNIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSDVYITDKTVLDMRSMDFKSNSAVAWSNKS DFACANAFNNSIIPEDTFFPSPESS. The predicted amino-acid sequence for LC13 β-chain is (accession No. L26456; mutation of C186 to A is in bold) MGVSQSPRYKVAKRGQDVALRCDPISGHVSLFWYQQALGQGPEFLTYFQNEAQLDKSGLPSDRFFAERPEGSVSTLKIQRTQQEDSAVYLCASSLGQAYEQYFGPGTRLTVTEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVSTDPQPLKEQPALNDSRYALSSRLRVSATFWQNPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRAD.

2.2. Expression and purification of LC13

Inclusion-body protein of the LC13α- and LC13β-chain was prepared essentially as per the method of Garboczi, Utz *et al.* (1996). The recombinant expression plasmids were transformed into BL21 (DE3) *E. coli*. A positive transformant was selected and grown at 310 K for 16 h in LB plus kanamycin. The inoculate was diluted 1/100 into a litre of LB medium and grown to an OD_{600} of 0.6. The culture was induced with 1 m*M* IPTG and grown for a further 4 h. Bacteria were pelleted, resuspended in 5 ml of resuspension buffer containing 50 m*M* Tris pH 8.0, 25%(*w*/*v*) sucrose, 1 m*M* EDTA, 10 m*M* DTT, 0.2 m*M* PMSF and 1 µg ml^{-1} pepstatin A and then frozen at 203 K.

The bacterial pellet was thawed and then lysed by addition of 22.5 ml of lysis buffer containing 50 m*M* Tris pH 8.0, 1%(*v*/*v*) Triton X-100, 1%(*w*/*v*) sodium deoxycholate, 100 m*M* NaCl, 10 m*M* DTT, 1 mg DNAse I, 5 m*M* $MgCl_2$. After 20 min of continuous rocking at room temperature, the samples were homogenized for 30 s using a Polytron homogenizer, after which 10 m*M* EDTA was added. Inclusion bodies were isolated by centrifugation at 277 K, 10 000 rev min^{-1} for 15 min in a Sorvall GSA rotor. The pellets were resuspended in 150 ml of wash buffer containing 50 m*M* Tris pH 8.0, 0.5%(*v*/*v*) Triton X-100, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* PMSF and 1 µg ml^{-1} pepstatin, homogenized and centrifuged. This wash step was repeated four times. A final wash in a buffer containing 50 m*M* Tris pH 8.0, 1 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* PMSF and 1 µg ml^{-1} pepstatin A was then performed.

The inclusion bodies were resuspended in 20 m*M* Tris pH 8.0, 8 *M* urea, 0.5 m*M* EDTA, 1 m*M* DTT, homogenized and centrifuged at 277 K, 15 000 rev min^{-1} for 30 min in an SS34 rotor. Inclusion-body protein present in the supernatant was quantified by comparison of Coomassie blue-stained SDS–PAGE fractionated aliquots and protein standards, before freezing at 203 K.

50 mg each of LC13α and LC13β inclusion-body protein was thawed, mixed with 4 µ*M* ml^{-1} DTT, combined, added to 1.5 *M* guanidine–HCl, 5 m*M* sodium acetate, 5 m*M* EDTA and injected into 700 ml of stirring refolding buffer containing 100 m*M* Tris pH 8.5, 0.4 *M* arginine, 0.5 m*M* oxidized glutathione, 5 m*M* reduced glutathione, 2 m*M* EDTA, 0.2 m*M* PMSF and 1 µg ml^{-1} pepstatin A at 377 K. An equal amount of LC13α and LC13β inclusion-body protein was added 16 h later. 24 h later, refolded protein was dialysed (Spectrum; molecular-weight cutoff 6–8000 kDa) once against 17 l of 5 m*M* Tris–HCl, 0.1 *M* urea pH 8 for 6–8 h and once against 17 l of 10 m*M* Tris–HCl, 0.1 *M* urea for 16 h. Dialysed protein was captured on a column containing 30 ml of DE52 resin (Whatman), (pre-equilibrated with 10 m*M* Tris–HCl pH 8) and eluted with 10 m*M* Tris–HCl, 0.4 *M* NaCl. Eluted LC13 TCR was concentrated to 2–4 ml and loaded onto a HiLoad 16/60 Superdex 200 pg gel-filtration column (Amersham Pharmacia, Uppsala, Sweden) in the presence of 10 m*M* Tris–HCl, 150 m*M* NaCl. Fractions containing the TCR were pooled and purified further on a MonoQ column (Pharmacia). Peak fractions were pooled and concentrated to 24 mg ml^{-1}.

2.3. Preparation of soluble class I HLA–peptide complexes

Soluble class I heterodimers consisting of amino-acid residues 1–276 of HLA-B8 heavy chain (hc) bound non-covalently to β_2m and complexed with the peptide FLRGRAYGL were produced as for the LC13 TcR, with the following exceptions. The hc and β_2m were expressed separately in BL21 *E. coli*. Inclusion-body aliquots containing 31 and 24 mg of hc and β_2m, respectively, were combined separately with 1.5 *M* guanidine–HCl, 5 m*M* sodium acetate pH 5.3 and 5 m*M* EDTA. 30 mg of FLRGRAYGL peptide, β_2m and hc were added in that order to 1 l of refolding buffer pH 8.0. Two additional aliquots containing 31 mg of hc were added to the refolding buffer at 12 and 24 h. 24 h after the last addition of hc, the refold mix was dialysed once against 20 l of 5 m*M* Tris pH 8.0 for 24 h. Captured refolded protein was eluted from DE.52 resin with 150 m*M* NaCl. Gel filtration was performed with a HiLoad 16/60 Superdex 75 pg gel-filtration column (Amersham Pharmacia, Uppsala, Sweden).

2.4. Preparation of tetrameric MHC–peptide complexes

Soluble HLA-B8–FLR tetramers were prepared essentially as per the method of Altman *et al.* (1996). The hc (HLA-B8.birA) encodes amino-acid residues 1–278 of HLA-B8 and includes, in addition, a carboxy-terminal sequence encoding for a BirA biotinylation site. HLA-B8.birA hc and β_2m were expressed separately in BL21 *E. coli*. 12, 4 and 6 mg of hc, β_2m and FLRGRAYGL peptide, respectively, were combined in 200 ml of refolding buffer. Following capture on a DE.52 anion-exchange column and gel filtration on a HiLoad 16/60 Superdex 75 pg column, HLA-B8.birA–FLRGRAYGL complexes were biotinylated using BirA enzyme at 5 µg ml^{-1} for 15 h at 303 K, then mixed with

extravidin-PE (Sigma–Aldrich, St Louis, MO, USA) in a 4:1 ratio to form tetramers.

2.5. Cell surface staining with tetramers

Fresh peripheral blood was diluted with an equal volume of iso-osmotic PBS and peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Hypaque (Amersham Pharmacia Biotech) density-gradient centrifugation. 5×10^5 PBMC were incubated for 30 min at room temperature with 0.5 µg of tetrameric complex labelled with PE and saturating amounts of anti-CD8 antibodies conjugated to Tri-color (Caltag Laboratories, South San Francisco, CA, USA) in PBS containing 0.1% sodium azide and 1% fetal calf serum (FCS). Cells were washed and resuspended in PBS/azide/FCS and analysed on a Becton Dickinson FACS using *CELL QUEST* software.

2.6. Native gel band-shift assay

Graded amounts of LC13 TcR were added to 1 µg of HLA-B8–FLR in a final volume of 5 µl of 10 m*M* Tris, to which 1.5 µl of 50% glycerol was added. The proteins were then subjected to electrophoresis under non-denaturing conditions (10% polyacrylamide gel; Garboczi, Utz *et al.*, 1996) at 277 K. Proteins were visualized by staining with Coomassie blue R-250.

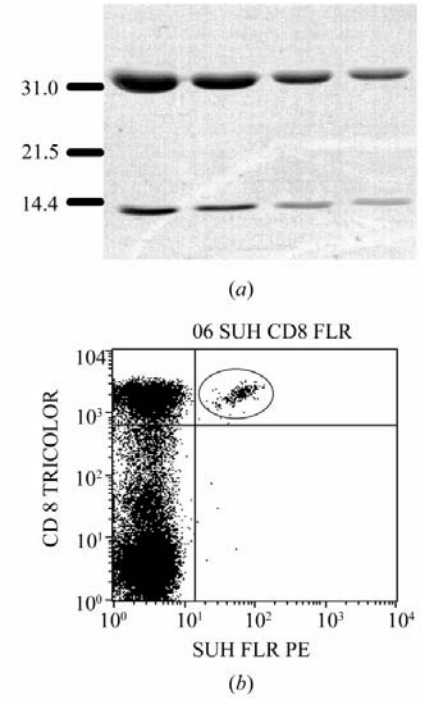

Figure 1
B8–EBV refolding and functional testing. (*a*) SDS–PAGE analysis of refolded B8–FLRGRAYGL complexes following anion-exchange and size-exclusion chromatography. (*b*) Tetrameric complexes of B8–FLRGRAYGL stain antigen-specific T cells (circled), indicating that our refolded complexes can functionally recognize their corresponding TCR expressed on the surface of antigen-specific T-cells.

2.7. Crystallization and data collection

Crystallization experiments were performed at room temperature using the hanging-drop vapour-diffusion technique. Data were collected on a Quantum 4 CCD at beamline 14 BM-C within the BioCars station at the Advanced Photon Source. The crystals were flash-frozen prior to data collection using 10–15% glycerol in the precipitant buffer as the cryoprotectant. The data were processed and scaled using the *HKL* package (Otwinowski, 1993).

3. Results and discussion

3.1. Expression, refolding of HLA-B8–EBV complex and functional verification

Two forms of HLA-B8 hc (HLA-B8 destined for crystallography and HLA-B8.birA destined for tetramer formation) and β_2m were expressed individually in BL21 *E. coli* and were then refolded in the presence of an excess of the EBV peptide FLRGRAYGL. Following capture of refolded HLA-B8–β_2m–FLRGRAYGL complexes on a DE.52 anion-exchange column, purification by gel filtration (HLA-B8 and HLA.B8.birA complexes) and a further Mono-Q anion-exchange purification step (HLA-B8 complex) were performed. Fig. 1(*a*) shows SDS–PAGE of purified HLA-B8–FLRGRAYGL complexes, the 35 and 12 kDa bands corresponding to hc and β_2m evident. SDS–PAGE analysis of purified HLA-B8.birA–FLRGRAYGL complexes showed the expected molecular-weight increase and comparable purity of the preparation (not shown). Tetramers produced using HLA-B8.birA–FLR complexes were used to stain PBMC from an EBV seropositive HLA-B8+ patient (Fig. 1*b*). A subpopulation of CD8+ T-cells stained positively with the HLA-B8.birA–FLR tetramers, consistent with the results of other workers (Burrows *et al.*, 1994), confirming that HLA-B8 had refolded correctly with β_2m and the FLRGRAYGL peptide to a conformation capable of interacting with the LC13 TcR.

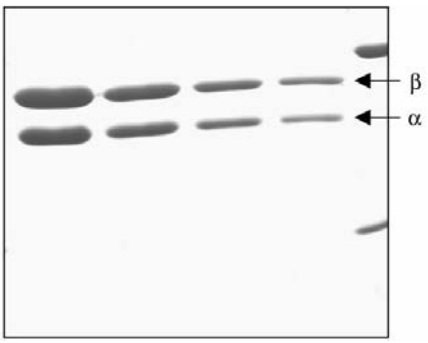

Figure 2
LC13 was refolded and purified by anion exchange and gel filtration as described in the text. The α- and β-chains are clearly visible on a 12% acrylamide SDS gel under reducing conditions (first lane). The second lane is of the dissolved crystals.

3.2. Expression, refolding and purification of the LC13 TcR

The TcR α- and β-chains of the human T-cell clone LC13 were expressed separately in *E. coli* as denatured inclusion-body protein. Stop codons were introduced at the C-terminus of the extracellular domains, immediately preceding the cysteines normally forming interchain disulfide bonds, to produce polypeptides lacking the transmembrane and cytoplasmic domains. To reduce mismatched disulfide bonding, the codon encoding the unpaired cysteine at position 186 of the Cβ region was changed to encode alanine. Yields of both polypeptides were typically 80 mg of protein per litre of LB culture. Purified inclusion bodies of α- and β-chains were solubilized in urea and guanidine–HCl, combined and refolded in an oxidized/reduced glutathione redox buffer. Typically, 2–5% of the total protein refolded correctly, resulting in a yield of approximately 4–10 mg LC13$\alpha\beta$ per litre of starting culture. Refolded protein was concentrated by binding to and elution from an anion-exchange column. The unfolded protein eluted at a different salt concentration. The protein was further purified by gel-filtration chromatography followed by a final anion-exchange step. SDS–PAGE analysis of the peak fractions demonstrated the presence of both α- and β-chains (Fig. 2).

3.3. Specific binding of LC13 TcR with HLA-B8–FLR complex

The structural integrity of the refolded receptor was first determined by a gel-shift assay following electrophoresis in a native agarose gel in the presence and absence of specific HLA-B8–FLRGRAYGL ligand. As shown in Fig. 3, the refolded LC13 receptor underwent gel retardation in the presence of prior incubation with either recombinant HLA-B8–FLRGRAYGL complexes or BiRA-tagged HLA-B8–FLRGRAYGL complexes used for tetramer formation. However, there was no gel retardation of soluble LC13 co-incubated with BiRA-tagged HLA-B8–RAK complexes (the

control peptide), another immunodominant HLA-B8-restricted EBV determinant.

3.4. Crystallization of the TcR alone and in complex

Large plate-like crystals were grown (Fig. 4*a*) using the hanging-drop vapour-diffusion technique at room temperature. The initial Hampton Screen yielded no crystals; however, a PEG/pH screen yielded a shower of needles overnight (10–20% PEG 8000 pH 7–8). The morphology of these initial crystals was improved by the addition of salt (200–400 m*M* NaCl). The diffraction-quality crystals were grown by mixing three volumes of 12 mg ml^{-1} LC13 with the reservoir buffer (12–15% PEG 8000, 300 m*M* NaCl pH 6.5). The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 45.8, b = 68.1, c = 160.5 Å. The crystals were flash-frozen prior to data collection using 15% glycerol as the cryoprotectant. The data were processed and scaled using the *HKL* package. The space group and unit-cell parameters are consistent with one molecule per asymmetric unit. The crystals diffract to 1.5 Å resolution (overall $R_{\rm merge}$ 7.8%). Dissolution of the crystals and running the sample on the gel confirmed that the intact heterodimer was crystallized (Fig. 4*b*).

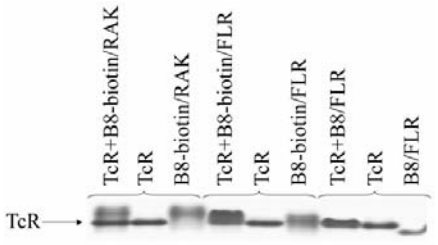

Figure 3
Characterization of the LC13–HLA-B8–EBV complex. The native gel band-shift assay was performed essentially as described in Garboczi, Utz *et al.* (1996), except a stacking gel was included. 1 µg of B8 + FLR was loaded singly or in combination with graded amounts of LC13 TCR (final volume was 6.5 µl) on a 10% polyacrylamide gel in the absence of SDS. Samples were preincubated for 20–30 min prior to loading and electrophoresed at 4–5 mA for 13–18 h at 277 K. RAK represents a peptide that binds to HLA-B8 but that the LC13 TcR does not recognize.

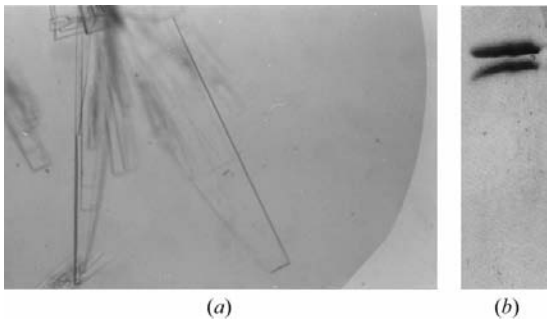

Figure 4
(*a*) Large plate-like crystals of the unliganded LC13 TcR. (*b*) Single crystals were washed three times in mother liquor, boiled in SDS–PAGE loading buffer and loaded on a 12% acrylamide SDS gel.

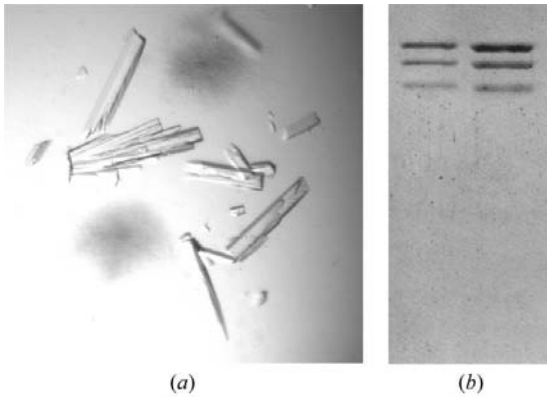

Figure 5
(*a*) Crystals of the LC13–HLA-B8–EBV complex. (*b*) Single crystals were washed three times in mother liquor, boiled in SDS–PAGE loading buffer and loaded on a 12% acrylamide SDS gel (lane 1) to verify the crystals contained the complex. Lane 2 is of the starting material.

Small rod-shaped crystals of the LC13–HLA-B8–EBV complex were formed by the hanging-drop vapour-diffusion technique at room temperature (Fig. 5*a*). Equimolar amounts of each component (overall concentration 4 mg ml^{-1}) were mixed with an equal volume of reservoir buffer (25% PEG 4000 pH 7.5, 0.2 *M* ammonium acetate). These final conditions were arrived upon following the initial Hampton Screen. The crystals were dissolved and run out on a gel to confirm that the complex was crystallized (see Fig. 5*b*). The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 56.65, b = 105.95, c = 144.89 Å. The crystals were flash-frozen prior to data collection using 10% glycerol as the cryoprotectant. The data were processed and scaled using the *HKL* package. The space group and unit-cell parameters are consistent with one molecule per asymmetric unit. The crystals diffract to 2.5 Å resolution (overall $R_{\rm merge}$ = 9.1%).

In this report, we demonstrate that we have expressed and refolded an immunodominant TcR and the HLA-B8–EBV peptide complex and have shown both receptors to be functionally active. The crystals of the unliganded TcR diffract to very high resolution, whereas the previous benchmark for an unliganded TcR (2C) structure is 2.5 Å (Garcia *et al.*, 1996). Furthermore, preliminary diffraction studies on the LC13–HLA-B8–EBV complex have been undertaken. Structure determination of the liganded and unliganded forms of the TcR are under way. This work allows us to begin to address the immunodominance of this public receptor and ascertain any conformational changes that may take place upon complexation.

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