T Cell Receptor and Coreceptor CD8<sub>αα</sub> Bind Peptide-MHC Independently and with Distinct Kinetics

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Summary

The T cell surface glycoprotein CD8 enhances T cell antigen recognition by binding to MHC class I molecules. We show that human CD8<sub>αα</sub> binds to the MHC class I molecule HLA-A2 with an extremely low affinity (K<sub>d</sub> ~ 0.2 mM at 37°C) and with kinetics that are between 2 and 3 orders of magnitude faster than reported for T cell receptor (TCR)/peptide-MHC interactions. Furthermore, CD8<sub>αα</sub> had no detectable effect on a T cell receptor (TCR) binding to the same peptide-MHC class I complex. These binding properties provide an explanation as to why the CD8/MHC class I interaction is unable to initiate cell-cell adhesion and how it can enhance TCR recognition without interfering with its specificity.

Introduction

CD8 and CD4 are T cell surface glycoproteins that enhance T cell antigen recognition by binding to major histocompatibility complex (MHC) class I and II molecules, respectively, on antigen-presenting or target cells (Janeway, 1992; Zamoyska, 1994, 1998). Both CD8 and CD4 are thought to bind to the same peptide-MHC molecules as the T cell receptor (TCR) and are thus commonly referred to as coreceptors (Janeway, 1992). While TCR/peptide-MHC interactions have been extensively studied (Davis et al., 1998a), the interactions between coreceptors and MHC molecules are less well characterized. CD8 exists on the cell surface as a homo (αα) or hetero (αβ) dimer (Zamoyska, 1994). Recently, crystal structures have been obtained of complexes between soluble human and mouse CD8<sub>αα</sub> and the MHC class I molecules HLA-A2 and H-2K<sup>α</sup>, respectively (Gao et al., 1997; Kern et al., 1998). In the present study, we use surface plasmon resonance (SPR) to study the affinity and kinetics of the interaction between human CD8<sub>αα</sub> and HLA-A2 and its effects on TCR binding to the same peptide-MHC class I complex.

Results and Discussion

CD8<sub>αα</sub> Binds HLA-A2 with an Exceptionally Low Affinity

SPR has proved to be a reliable method for measuring the affinity and kinetics of very weak protein-protein interactions, provided that certain pitfalls are avoided (van der Merwe and Barclay, 1994; Davis et al., 1998b). Optimal SPR analysis requires immobilization of one of the interacting molecules onto a sensor surface in a consistent orientation without affecting its binding properties. This was achieved by immobilizing HLA-A2 molecules that had been selectively biotinylated on β2-microglobulin onto streptavidin-coated surfaces. Soluble CD8<sub>αα</sub>, which binds to HLA-A2 with a 1:1 stoichiometry (Gao et al., 1997), eluted at the expected position (M, ~28000, calculated M, 26826) when run at high concentrations (~300 μM) on a gel-filtration column (Figure 1A), indicating that it does not form higher-order aggregates and is therefore suitable for affinity and kinetic analysis. This CD8<sub>αα</sub> produced a larger response when injected over a surface presenting immobilized HLA-A2 than when injected over a control surface, indicating binding (Figure 1B). A CD8<sub>αα</sub> variant with mutations in the CDR-2-like loop (Q54E/N55D), which contacts the α3 domain of HLA-A2 (Giblin et al., 1994; Gao et al., 1997), did not bind HLA-A2 (Figure 1B), confirming the specificity of this interaction.

The affinity of the CD8<sub>αα</sub>/HLA-A2 interaction was determined by equilibrium binding analysis, as previously described (van der Merwe et al., 1993, 1994, 1997). CD8<sub>αα</sub> was injected at a range of concentrations through flow cells with either HLA-A2 or a control protein immobilized (Figure 2A). The binding at each concentration was obtained by subtraction of the control response from the HLA-A2 response (Figure 2B). Both direct nonlinear curve fitting and Scatchard analysis gave a K<sub>d</sub> of ~150 μM at 25°C (Figure 2B; Table 1). In order to address the possibility that biotinylation of β2-microglobulin, which makes some contacts with CD8<sub>αα</sub> (Gao et al., 1997; Kern et al., 1998), changed the binding properties of the CD8<sub>αα</sub>/MHC class I interaction, the affinity was also measured using HLA-A2 that had been enzymatically biotinylated at its carboxyl terminus (O’Callaghan et al., 1999). CD8<sub>αα</sub> bound with a similar affinity to either form of HLA-A2 (Table 1), indicating that immobilization via biotinylation of β2-microglobulin had little effect on the CD8<sub>αα</sub>/MHC class I interaction. At 37°C, the HLA-A2 coupled via β2-microglobulin rapidly lost binding activity, apparently as a result of dissociation of the heavy chain from the sensor surface, leading to poor quality data (Table 1). In contrast, HLA-A2 immobilized via biotinylated heavy chain was stable at 37°C, enabling affinity measurements to be performed (Figure 2C). CD8<sub>αα</sub>

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Figure 1. Specific Binding of CD8αα to HLA-A2
(A) Gel-filtration of CD8αα. CD8αα (50 μL at ~300 μM) was separated on a Superdex S-200 column. The elution positions of albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12 kDa) are shown.

(B) CD8αα binds specifically to immobilized HLA-A2. Similar concentrations (~300 μM) of wild-type CD8αα or the CD8αα mutant Q54E/N55D were injected (solid bars) for 90 s through flow cells with HLA-A2 (solid trace) or the control protein OX68 (dotted trace) immobilized. HLA-A2 presented an influenza matrix peptide (HLA-A2-flu, see Table 1) and was coupled via biotinylated β2-microglobulin. This experiment was performed at 25°C using a flow rate of 5 μl/min with ~1500 RU of HLA-A2 or the control protein OX68 immobilized.

The high background responses seen in the control flow-cell reflect the high refractive index of the injected CD8αα samples, a consequence of very high protein concentrations.

bound HLA-A2-peptide complexes with a Kd of ~200 μM at 37°C (Figure 2C; Table 1). Similar affinities were measured for CD8αα binding to several different peptide/HLA-A2 complexes (Table 1).

CD8αα Dissociates Rapidly from HLA-A2
Since preliminary analysis indicated that the kinetics of the CD8αα/MHC-peptide interactions are extremely rapid, data were collected at 0.1 s intervals, the fastest rate possible on the BIAcore2000 (Figure 3A). When CD8αα was injected over HLA-A2 at 25°C, equilibrium was reached within 0.4 s (Figure 3A), making direct determination of the association rate constant (kass) impossible. Dissociation was also extremely fast, with the response returning to baseline within 0.4 s of the end of the injection (Figure 3A). Because the dissociation time was of the same order as the time taken to wash protein from the cell (van der Merwe et al., 1994), the flow-rate was increased to 1.67 μl/s, the maximal rate possible on the BIAcore2000. At this flow-rate, CD8αα dissociated with an apparent koff of 18 s⁻¹. The rate at which the background response falls in the control flow cell (~24 s⁻¹) provides an estimate of the washing time.

This agrees well with the theoretical value (~28 s⁻¹) calculated for this flow rate (flow rate × flow cell volume)/(koff/μM). The true koff for the CD8αα/MHC class I interaction may well be greater than 18 s⁻¹ since we could not exclude rebinding following dissociation, a phenomenon commonly seen in BIAcore experiments (van der Merwe et al., 1993, 1994, 1997). In addition, the koff at 37°C is likely to be higher than at 25°C because the affinity is lower (Table 1). If the koff is taken to be ~18 s⁻¹ and the Kd ~126 μM (at 25°C), the kass can be calculated to be ~140000 M⁻¹ × s⁻¹ (kass = koff/Kd). This

Figure 2. The Affinity of CD8αα Binding to HLA-A2
(A) CD8αα was injected at the indicated concentrations for 30 s through flow cells with ~8000 RU of either HLA-A2-flu (solid trace) or the control protein OX68 (dotted trace) immobilized. HLA-A2-flu was coupled via biotinylated β2-microglobulin. The amount of CD8αα that bound HLA-A2-flu at each concentration was calculated as the difference between the responses at equilibrium in the HLA-A2-flu and control flow cells and is plotted in (B). (C) A similar experiment was performed at 37°C using HLA-A2-HY (~2400 RU) coupled via biotinylated heavy chain and OX68 (~4000 RU) as a control protein. In (B) and (C) the solid lines in the main plots represent nonlinear fits of the Langmuir binding isotherm to the data. These yielded Kd values of 156 and 205 μM, respectively. The insets show Scatchard transformations of the same data; the Kd values shown were obtained from the slopes by linear regression (Kd = 1/Kslope). These experiments were performed at a flow-rate of 10 μl/min.
**Table 1. Summary of Affinity Constants**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Immobilization</th>
<th>Kd (µM)</th>
<th>25°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flu</td>
<td>β2-microglobulin</td>
<td>159 ± 18 (n = 5)</td>
<td>&gt;207 (n = 2)</td>
<td></td>
</tr>
<tr>
<td>Pol</td>
<td>β2-microglobulin</td>
<td>126 ± 34 (n = 7)</td>
<td>&gt;236 (n = 1)</td>
<td></td>
</tr>
<tr>
<td>H-Y</td>
<td>heavy chain</td>
<td>173 ± 21 (n = 1)</td>
<td>211 ± 11 (n = 1)</td>
<td></td>
</tr>
<tr>
<td>HA-1</td>
<td>heavy chain</td>
<td>107 ± 2 (n = 2)</td>
<td>132 ± 10 (n = 2)</td>
<td></td>
</tr>
</tbody>
</table>

a Flu, Influenza matrix protein 58-66 (GILGFVFTL); Pol, HIV-1 polymerase 476-484 (ILKEPVHGIV); H-Y, male-specific, minor histocompatibility antigen from the protein SMCY (FIDSYICQV); HA-1, minor histocompatibility antigen from one allele of the KIAA0223 gene (VLHDDLLLEA).

b Mean ± SD or, for n = 2, mean ± range of n independent determinations.

c Saturation was not achieved in these experiments. The values shown are the concentrations of CD8αααα that gave 50% of the binding obtained with the highest concentration of CD8αααα. The actual Kd values are likely to be higher than this.

koff is no slower than measured for other protein-protein interactions (Mason and Wallace, 1986; van der Merwe and Barclay, 1994; Davis et al., 1998b), indicating that the low affinity of the CD8αααα/MHC class I interaction is not a consequence of an unusually slow koff.

**Comparison with the Mouse CD8αααα/MHC Class I Interaction**

The affinity and kinetic values obtained in this study are very different to the values previously reported for the mouse CD8αααα/MHC class I interaction. Garcia et al. (1996) reported an affinity ~5-fold higher for the latter interaction (Kd 30-39 µM at 25°C). The most striking difference, however, is in the binding kinetics. Garcia et al. (1996) measured koff (~10^5 M^-1 s^-1) and koff (~0.05 s^-1) values between 2 and 3 orders of magnitude slower than the values obtained in the present study. In contrast, we have recently found that soluble mouse MHC class I binds a soluble, recombinant form of mouse CD8αααα with affinity and kinetic constants (Kd > 200 µM, koff > 10 s^-1) very similar to those reported here for the human CD8αααα/MHC class I interaction (G. F. G. et al., unpublished data). This indicates that the discrepancies between our findings and those of Garcia et al. (1996) are not the result of species differences. Neither are the discrepancies the result of allelic differences, since Garcia et al. (1996) obtained very similar results with H-2K, H-2D, and H-2L alleles, and we have measured similar affinities for CD8αααα binding to several HLA-A, -B, and -C alleles (G. F. G. et al., unpublished data). A third possibility is that the differences result from the absence of the mucin-like stalk region from the form of CD8 used in this study. This seems unlikely, however, because CD8 binds MHC class I in an orientation that makes it difficult to envisage a direct interaction between the stalk region and MHC class I (Gao et al., 1997; Kern et al., 1998). A fourth possibility is that the discrepancy results from the presence of multivalent aggregates, a pitfall commonly encountered when using high protein concentrations in SPR experiments (van der Merwe et al., 1993, 1994; Davis et al., 1998b). The binding of aggregated material has certain characteristic features (van der Merwe et al., 1993, 1994; Davis et al., 1998b) that are consistent with the data presented by Garcia et al. (1996). First, the apparent koff is very slow because binding is multivalent. Second, because multivalent aggregates are usually minor contaminants (van der Merwe et al., 1993, 1994), their concentration is much lower (~5%) than the total concentration of the protein. Because the total concentration (C) is used when calculating the association rate constant (koff) from the association rate (koff = association rate/C), the calculated apparent koff is much lower than the true koff. Finally, because of the mixture of monovalent and multivalent material, the association and dissociation phases will exhibit both very fast and slow phases (see Figure 1A, Garcia et al. [1996]), with large differences in rate constants for each phase. While mass transport limitations and rebinding can lead to biphasic binding kinetics (Schuck, 1997), it is unlikely to result in phases with such markedly different rate constants. Although Garcia analyzed their CD8 by gel-filtration, aggregates may have been present at below detectable levels. We have observed that very low levels of such aggregates (~2%)...
can dominate the binding if the monomeric interaction has a low affinity and high concentrations of the protein are injected (van der Merwe et al., 1993, 1994; Davis et al., 1998b).

CD8αα and a TCR Bind Independently to HLA-A2-flu

We next examined whether the binding of CD8αα to a peptide-MHC class I complex influenced the binding of a T cell receptor (TCR) to the same peptide-MHC (Figure 4). In order to simplify interpretation of the experiment, this analysis was performed with peptide-MHC immobilized and the TCR and CD8αα in solution. The affinity of a soluble TCR specific for HLA-A2 presenting the influenza matrix peptide (HLA-A2-flu, Table 1) was unaffected by the presence of soluble CD8αα; the binding of the TCR and CD8αα to HLA-A2-flu appeared to be simply additive (Figure 4A). Similarly, CD8αα binding had no detectable effect on kinetics of the TCR/HLA-A2-flu interaction (see Figure 4B, dissociation phase). This lack of effect of CD8αα on the TCR/peptide-MHC class I interaction is consistent with structural data showing that while the α3 domains of mouse and human MHC class I do move upon CD8αα binding, no discernable conformational changes are evident on the TCR binding platform (Gao et al., 1997; Kern et al., 1998). Our finding that CD8αα and TCR bind to peptide-MHC class I independently in solution does not rule out the possibility that CD8 will enhance TCR binding to peptide-MHC class I on cells. Clearly, indirect interactions between CD8 and the TCR/CD3, mediated by shared associations with signaling molecules (Thome et al., 1996), would also enable CD8 to stabilize the TCR/peptide-MHC interaction (see below).

Our results differ from Garcia et al. (1996), who reported that purified soluble CD8αα and CD8αβ enhance the binding of soluble peptide-MHC class I to TCR immobilized onto a sensor surface. However, interpretation of their experiment is complicated by the fact that SPR detects changes in mass and so CD8 will enhance the SPR response simply by increasing the mass of the peptide-MHC complex that binds TCR. Furthermore, if multivalent aggregates of CD8 are indeed present (see above), they may form multivalent peptide-MHC/CD8 complexes with enhanced avidity for (and slow dissociation rates from) immobilized TCR. Another possible explanation for the discrepancy between our results and those of Garcia et al. (1996) is that the mucin-like stalk regions of CD8, which are absent from the CD8 used in the present study, interact directly with the TCR. However, unless the CD8 stalk displays remarkable flexibility, the orientation of CD8 binding to MHC class I would place it some distance from the TCR (Gao et al., 1997; Kern et al., 1998). This lack of effect of CD8αα on the TCR/peptide-MHC class I interaction reported by Garcia et al. (1996) is not a general feature of CD8 function.

Implications for CD8 Function

Our results indicate that CD8 binds MHC class I with a lower affinity than measured between conventional cell-cell recognition molecules (Table 2) (van der Merwe and Barclay, 1994; Davis et al., 1998b). The latter tend to have affinities with Kd values ≤ 100 μM, suggesting that an affinity of Kd ~ 100 μM might be close to the lower limit of what is needed for adhesion mediated by molecules that interact with a 1:1 stoichiometry. In support of this, a recent study of the rat CD2/CD48 interaction (Dustin et al., 1997) suggests that its solution affinity (3DkD 60–90 μM) results in a membrane-attached or two-dimensional affinity (2DkD ~ 45 molecules/μm²) that is just sufficient to drive adhesion at physiological surface densities of CD48 (~100 molecules/μm²). Perhaps more striking than the low affinity of the CD8αα/MHC class I interaction are the extremely fast kinetics. The koff (≈ 18 s⁻¹) is considerably faster than the koff values reported for TCR/peptide-MHC interactions, which range from 0.01 to 0.1 s⁻¹ at 25°C for agonist peptides (Davis et al., 1998a). These differences have
Table 2. Affinity and Kinetic Constants for Molecular Interactions Involved in T Cell Antigen Recognition

<table>
<thead>
<tr>
<th>Interaction</th>
<th>k&lt;sub&gt;on&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt; × s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;off&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (µM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8&lt;sub&gt;223&lt;/sub&gt;/HLA-A2</td>
<td>≥100 000</td>
<td>≥18</td>
<td>~200</td>
<td>this study</td>
</tr>
<tr>
<td>TCR/peptide-MHC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>600-22 000</td>
<td>0.01-0.1</td>
<td>1-90</td>
<td>(Davis et al., 1998a)</td>
</tr>
<tr>
<td>CD2/CD58</td>
<td>≥400 000</td>
<td>≥4</td>
<td>~16</td>
<td>(van der Merwe et al., 1994)</td>
</tr>
<tr>
<td>CD28/CD80</td>
<td>≥660 000</td>
<td>≥1.6</td>
<td>4</td>
<td>(van der Merwe et al., 1997)</td>
</tr>
<tr>
<td>L-selectin/GlyCAM-1</td>
<td>≥100 000</td>
<td>≥10</td>
<td>~105</td>
<td>(Nicholson et al., 1998)</td>
</tr>
<tr>
<td>LFA-1/ICAM-1</td>
<td>—</td>
<td>—</td>
<td>~100</td>
<td>(Lollo et al., 1993)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All interactions shown are between human molecules except for TCR/peptide-MHC interactions, which have only been studied with murine molecules. All measurements were performed at 37°C except for TCR/peptide-MHC interactions, which were measured at 25°C.

<sup>b</sup> Kd values shown were obtained by equilibrium binding but the range of Kd values shown for TCR interactions includes some that were calculated from the k<sub>on</sub> and k<sub>off</sub>.

<sup>c</sup> The range of values reported for several non-alloreactive interactions are shown.

important implications for the mechanism of CD8 function (see below), since recent data (Alam et al., 1996; Lyons et al., 1996) support models (McKeithan, 1995; Davis and van der Merwe, 1996; Rabinowitz et al., 1996) of TCR triggering in which the K<sub>d</sub> of the TCR/peptide-MHC interaction is a primary factor determining the outcome of TCR ligation.

The very low solution affinity of the CD8/MHC class I interaction provides an explanation for its inability to independently initiate cell-cell adhesion, except when these molecules are expressed at unphysiologically high surface densities (Norment et al., 1988). Interestingly, CD8 is able to mediate adhesion to surface-immobilized MHC class I molecules, but only following tyrosine-kinase-dependent signaling through the TCR (O'Rourke et al., 1990; O'Rourke and Mescher, 1992). It is now known that this signaling leads to lck-mediated recruitment of CD8 into tyrosine-phosphorylated TCR/CD3 complexes (Beyes et al., 1992; Thome et al., 1996). We suggest that the affinity of CD8 for MHC class I is so low that significant engagement only occurs following recruitment of CD8 into the TCR/CD3 complex. Importantly, this recruitment will follow initial, coreceptor-independent activation of the TCR/CD3 complex, as previously suggested (Thome et al., 1996). Once recruited, CD8 could enhance peptide-MHC ligation by adding to the much stronger TCR/peptide-MHC interaction. We suggest that peptide specificity is maintained because the very low affinity and fast kinetics of the TCR/MHC class I interaction mean that TCR/CD3/CD8 complexes bind only very transiently to nonspecific peptide-MHC class I complexes. Only when TCR binds to specific peptide-MHC class I is the interaction between the TCR/CD3/CD8 and peptide/MHC class I complexes sufficiently stable for T cell receptor triggering.

This mode of coreceptor function, in which the coreceptor first associates with a previously activated TCR/CD3 complex before engaging peptide-MHC, was first proposed for CD4 (Xu and Littman, 1993) in order to explain the results of experiments with CD4-lck chimeric proteins. Xu and Littman’s (1993) findings suggested that lck-mediated recruitment of CD4 into the TCR/CD3 complex is necessary in order for CD4 to enhance T cell antigen recognition (Xu and Littman, 1993). However, lck-mediated recruitment is not essential when CD4 is expressed at very high levels (Killeen and Littman, 1993), consistent with the notion that it is the low affinity of the CD4/MHC class II interaction that makes recruitment necessary. More recent data (Hamp et al., 1997), which show that CD4 enhances T cell hybridoma recognition of agonist but not antagonist peptides, are consistent with this mode of coreceptor function if one postulates that TCR triggering with an antagonist peptide does not lead to recruitment of coreceptor to the TCR/CD3 complex.

In summary, the binding properties reported here for the CD8/MHC class I interaction support the suggestion (Xu and Littman, 1993; Thome et al., 1996) that a functionally significant CD8/peptide-MHC class I interaction can only occur when CD8 is recruited into a TCR/CD3/CD8 complex so that physically associated CD8 and TCR/CD3 bind simultaneously to the same peptide-MHC class I complex. We suggest that the distinct binding properties of the TCR and CD8 for peptide-MHC class I are necessary to ensure that the specificity of this interaction is dominated by the TCR.

Experimental Procedures

Soluble CD8<sub>223</sub> Preparation

Purified soluble human CD8<sub>223</sub> (residues 1-120) was expressed in Escherichia coli, refolded, and purified as described (Gao et al., 1998). The extinction coefficient was determined by amino acid analysis to be 32480 M<sup>-1</sup> × cm<sup>-1</sup>. DNA encoding the CD8<sub>223</sub> mutant Q54E/N55D was produced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and checked by dideoxy DNA sequencing of the entire coding portion. The mutant protein was expressed and refolded as for wild-type CD8<sub>223</sub> and purified by gel filtration as an approximately 30 kDa homodimer. At least 90% of the purified wild-type and mutant CD8<sub>223</sub> could be immunoprecipitated by the MAbs OKT8 and 51.1 (data not shown), both of which block MHC class I binding in adhesion assays (Norment et al., 1988), suggesting that the bulk of these proteins were correctly folded and active. Thus, the CD8<sub>223</sub> concentration was calculated from the extinction coefficient, assuming 100% activity.

Soluble HLA-A2 Preparation

HLA-A2 heavy chain was expressed in E. coli and refolded as previously described together with synthetic peptides (Genosys) and biotinylated β2-microglobulin (Garboczi et al., 1992; Vessey et al., 1997). Prior to refolding with HLA-A2, β2-microglobulin was chemically biotinylated as described (Vessey et al., 1997) using N-hydroxysuccinimido-biotin (Sigma). HLA-A2 that had been enzymatically biotinylated on the heavy chain C terminus was produced using the QuikChange Site-Directed Mutagenesis Kit and checked by dideoxy DNA sequencing of the entire coding portion. The mutant protein was expressed and refolded as for wild-type CD8<sub>223</sub> and purified by gel filtration as a homodimer. The CD8<sub>223</sub> concentration was calculated from the extinction coefficient, assuming 100% activity.

Soluble TCR Preparation

The TCR studied derives from the JM22 T cell clone (Moss et al., 1991; Lehner et al., 1995). It is specific for an HLA-A2-restricted
peptide (GLGFWFTL) from the influenza matrix protein (58-66) and utilizes gene segments TCRAV0102(2)911C1 and TCRBV17S1J2 757C2. Expression, refolding, and purification of a soluble form of this TCR is to be described in detail elsewhere (B. E. W. et al., unpublished data). In brief, TCR α and β chains carrying c-jun (α) and c-Fos (β) heterodimerisation motifs at their carboxyl termini were expressed separately in E. coli, solubilized from inclusion bodies, and refolded together. The αβ heterodimers were purified on a POROS 10 HQ column using a BioCAD/SPRINT Perfusion Chromatography system (Perceptive Biosystems Inc., Framingham, MA), before analysis on the BIAcore.

At least 84% of the TCR could be precipitated by sepharose beads coated with HLA-A2-flu (data not shown), indicating that the bulk of the TCR was correctly folded. The TCR concentration was calculated from the extinction coefficient (105500 M$^{-1}$ cm$^{-1}$), determined by amino acid analysis, assuming 100% activity.

Surface Plasmon Resonance

SPR studies were performed using a BIAcore 2000 (BIAcore AB, St Albans, UK) in HBS (BIAcore AB). HBS controls 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Surfactant P20. Streptavidin (Sigma) was covalently coupled to Research Grade CM5 sensor chips (BIAcore) via primary amines using the Amine Coupling kit (BIAcore). For coupling, the streptavidin was dissolved in 10 mM sodium acetate (pH 5.5) and injected at 0.5 mg/ml. Immobilization levels ranged from 6000 to 11000 Response Units. Biotinylated HLA-A2 and control proteins were then immobilized at the indicated levels by injection at 0.05 to 0.15 mg/ml for 0.5 to 10 min over streptavidin-coupled surfaces. The biotinylated control proteins used included mouse (OX68, IgG2a, anti-rat CD4) and rat (CAMPATH-1, IgG2b, anti-CD52) monoclonal antibodies and biotinylated soluble CD5. Kd values were obtained either by Scatchard plots or by nonlinear curve fitting of the Langmuir binding isotherm (bound – C$ \times $max/[Kd + C]), where C is CDBox concentration and max is maximum CDBox binding) to the data using the Levenberg-Marquardt algorithm as implemented in the Windows 95 application Origin (version 5; MicroCal Software, Northampton, MA, USA).

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