Computational Design and Crystal Structure of an Enhanced Affinity Mutant Human CD8 αα Coreceptor

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ABSTRACT Human CD8 is a T cell coreceptor, which binds to pHLA I and plays a pivotal role in the activation of cytotoxic T lymphocytes. Soluble recombinant CD8 αα has been shown to antagonize T cell activation, both in vitro and in vivo. However, because of a very low affinity for pHLA I, high concentrations of soluble CD8 αα are required for efficient inhibition. Based upon our knowledge of the wild-type CD8/pHLA I structure, we have designed and produced a mutated form of soluble CD8 αα that binds to pHLA I with approximately fourfold higher affinity. We have characterized the binding of the high affinity CD8 mutant using surface plasmon resonance and determined its structure at 2.1 Å resolution using X-ray crystallography. The analysis of this structure suggests that the higher affinity is achieved by providing a larger side chain that allows for an optimal contact to be made between the HLA α3 loop and the mutated CDR-like loops of CD8. Proteins 2007;67:65–74. © 2007 Wiley-Liss, Inc.

Key words: CD8; coreceptor; soluble protein; crystal structure; KD; protein engineering; immunotherapy; immune-suppressor

INTRODUCTION

T cells are involved in practically all immune responses and play a pivotal role in most autoimmune diseases and graft rejection. For many commonly occurring autoimmune diseases, associations with particular HLA (human leukocyte antigen) class II alleles have been established, implicating Th cells as key autoimmune protagonists. More recently, evidence has also emerged for the involvement of HLA class I (HLA I)-directed, CTL responses. Autoreactive CTLs have been detected in many autoimmune diseases and conditions, including type 1 diabetes,1 rheumatoid arthritis,2 multiple sclerosis,3 intestinal autoimmunity,7 paraneoplastic cerebellar degeneration,4 and autoimmune thyroiditis.5 In addition, the importance of CTL responses in chronic transplant organ rejection is well-established.6–9

CTL responses are specific for peptides presented by HLA I on the surface of most nucleated cells.10 T cell activation classically requires the formation of the immunological synapse,11 which is dominated by the interaction of T cell receptors (TCRs) with peptide-HLA (pHLA) complexes. Interaction of the CD8 coreceptor with pHLA I, at a site distant to the peptide binding groove, enhances synapse formation. This leads to the activation of signaling pathways involving CD8-associated p56lck tyrosine kinase and ZAP-70.12 These signaling pathways have been shown to be critical in the activation of most CTL responses13 and their involvement enhances antigen sensitivity.14 Activation of CTLs can lead to responses ranging from lymphokine secretion to target cell lysis.

CD8 exists in two structurally distinct, disulfide-linked, forms: an αα homodimer and an αβ heterodimer.15 Binding analysis of soluble recombinant forms of these two proteins indicates little difference between their affinities.16 The CD8 α-chain, but not the β-chain, interacts with p56lck, which mediates a signaling cascade via the CD3 complex.

Abbreviations: IBs, inclusion bodies; KD, equilibrium binding constant; MD, molecular dynamics; pHLA I, peptide complexed to human leukocyte antigen class I; RMSD, root mean square deviation; RU, response unit; SPR, surface plasmon resonance; TCRs, T cell receptors; wt CD8, soluble wild-type CD8 αα.

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complex leading to T cell activation.\textsuperscript{37} However, the αβ form has been shown to be more efficient in the activation of T cells,\textsuperscript{18} perhaps through improved signal transduction via lipid rafts and is the predominant CD8 form on T cells expressing an αβ TCR.\textsuperscript{19,20} The CD8 αα homodimer is predominantly expressed naturally on some NK cells, γδ T cells and intestinal epithelial T cells (iIELs). The functional significance for CD8 αα homodimer expression rather than the CD8 αβ heterodimeric form in NK cells and γδ T cells has been the subject of much scrutiny, but there is evidence for a regulatory role for CD8 αα in iIELs.\textsuperscript{20}

The crystal structure of soluble wild-type CD8 αα (wt CD8) in complex with pHLA I shows that the interaction is dominated by contacts between residues 223–229 of the HLA α3 domain and residues 51–55 on the CDR-like loops of wild-type CD8 (wt CD8).\textsuperscript{21} Additional contacts are formed between the wt CD8 and HLA I α2 and β2m domains.\textsuperscript{21} This interaction is mainly based on charge complementarity and exhibits relatively low affinity (K\textsubscript{D} = 100–223 μM) and rapid kinetics.\textsuperscript{22} Despite this low affinity, soluble forms of human\textsuperscript{23} wt CD8 can disrupt activation of some T cell clones with higher efficacy than anti-CD8 antibodies.\textsuperscript{23} This observation prompted us to further investigate the potential of soluble wt CD8 as a CTL-specific immune inhibitor.

The main limitation of soluble wt CD8 as an inhibitor of T cell activation is its low affinity for pHLA I, a feature that ensures that pHLA I binding is dominated by the antigen-specific TCR interaction. To engineer a form of CD8 αα with higher affinity for pHLA I, we designed a series of mutants modeled on the wt CD8/pHLA I complex structure, in which we isolated 23 wt CD8 candidate residues that were involved in binding to pHLA. Since each of these residues may be mutated to 19 other amino acids, testing all these mutations experimentally was not practical. To narrow the number of potential mutants, we focused on residues that are part of the CD8 CDR-like loop, since mutating them was less likely to perturb the tertiary structure of wt CD8. This left us with 11 residues that were candidates for mutation. The binding of wt CD8 to pHLA I is asymmetric; most of the candidate residues for a mutation are in subunit CD8 α1, which contains most of the pHLA I contact residues, with only two of the candidate residues (Ser-53 and Asn-55) being part of the CD8 α2 subunit. These two residues are part of the CDR-like loop formed between Ser-53 and Arg-67. Since CD8 is a homodimer, rational design of mutations must take into account that a mutation in one subunit would be also present in the second subunit, which might have an unpredictable impact upon the binding. Ser-53 and Asn-55 in the CD8 α2 domain CDR-like loop clearly interacts with pHLA I, while Ser-53 and Asn-55 of CD8 α1 domain CDR-like loop do not form any interaction, hence avoiding the undesired effect that might occur between the CD8 α1 domain CDR-like loop and pHLA I.

Additionally, to improve refolding and to reduce the likelihood of nonnative disulphide bond formation, we replaced an unpaired cysteine with an alanine residue (C33A). Here, we measure the binding affinities of these mutants and identify one with approximately fourfold higher affinity for pHLA I. This enhanced affinity CD8 mutant was crystallized and its structure was solved at 2.1 Å, indicating a potential mechanism for its higher affinity binding.

**MATERIALS AND METHODS**

**Structure Based Rational Design of Higher Affinity CD8 Variants**

Initial coordinates were taken from the wt CD8/pHLA I crystal structure solved at 2.65 Å (PDB code: 1AKJ\textsuperscript{21}), where 23 wt CD8 residues formed a contact (at least one atom at a distance lower than 4 Å) with pHLA I. We studied the inter- and intramolecular interactions, the flexibility of the CDR-like loop formed between residues 53–68 and the interactions between the solvent and the unbound CD8. A set of molecular dynamics (MD) simulations and ab inito calculations described later indicated that the S53N mutation was likely to increase the avidity between the CD8 and the pHLA I. MD simulations and free energy perturbations were performed using CHARMM (version 27\textsuperscript{24} and the standard all-atom parameter set.\textsuperscript{25} Hydrogens were added using the HBUILD module in CHARMM. Water molecules were added to the complex by superimposing a 16 Å shell of TIP3P water molecules. The water molecules and the protein hydrogens were minimized and then equilibrated by a MD simulation at 27°C for 5 ps, while keeping the remaining protein atoms fixed. The equilibration was performed using stochastic boundary conditions with a time step of 1 fs, a friction coefficient of 62 ps\textsuperscript{-1} for the water oxygens and the SHAKE algorithm.\textsuperscript{26} The system was soaked again to fill any missing cavities with water. The solvent atom positions were optimized using 500 steps of steepest descents followed by 1000 steps of conjugate gradient. At the next step, the entire system was relaxed with 500 steps of steepest descents; this was switched to conjugate gradient until the convergence criteria of root mean square deviation (RMSD) gradient of the potential energy lower than 0.25 kcal/mol Å had been achieved. A 14 Å nonbonded cut-off\textsuperscript{24} and a dielectric constant of ε = 1 were employed. The system was simulated using stochastic boundary MD.\textsuperscript{27} The reference point for partitioning the system was designated as the CD8 α2 Ser-53 Oγ atom in the wild-type structure and CD8 α2 S53N N82 atom in the S53N/C33A CD8 mutant. The system was divided into a 12 Å reaction region, a 4 Å buffer region, and a reservoir. The friction coefficients for water oxygen and heavy atoms in the protein were 62 and 200 ps\textsuperscript{-1}, respectively.\textsuperscript{28} The relaxed system was equilibrated at 27°C for 150 ps with a time step of 1 fs followed by 1 ns data collection with coordinates and energies saved to disk every 1 ps.

We calculated the relative Helmholtz free energies, ΔG\textsubscript{4} and ΔG\textsubscript{Δ} by the perturbation method.\textsuperscript{26} Since free energy is a state function, it is path-independent, and
the free energy difference ($\Delta G_4 - \Delta G_3$) is equal to the difference $\Delta G_2 - \Delta G_1$. The two perturbations were performed using 10 windows and double wide sampling. We perturbed only the partial charges on the SS3N side chain's atoms. Trajectories were produced by MD simulations with the same model and under the conditions described earlier with 150 ps of equilibration and 100 ps of data collection at every window.

Standard ab initio calculations were performed with Jaguar 4.1 using Becke 3 LYP with the 6-31G basis set. The model of the wt CD8/pHLA I complex included Asp-227 (side chain) and Val-248 (backbone) of the HLA heavy chain and Tyr-51 (side chain), Ser-53, Gln-54 (backbone), Asn-55 and Lys-56 (backbone) of the CD8 $\alpha_2$ domain CDR-like loop. We mutated Ser-53 to Asn and repeated the calculation. In both models we employed a geometry optimization with atoms Asp-227 (C$\beta$), Val-248 (N), Tyr-51 (C$\beta$), Ser-53 (N), and Lys-56 (C) held fixed at their crystal structure coordinates. Supporting information, detailing the molecular modeling procedure, is available online.

**Generation of Mutant CD8 Expression Plasmids**

The CD8 variants were generated by QuickChange PCR mutagenesis (Stratagene) of pBJ112, which contains wt CD8 $\alpha$ chain, residues 1–120, under the control of the T7 promoter. Mutant CD8 sequences were confirmed by automated DNA sequencing (Lark Technologies).

**Protein Expression, Refolding, and Purification**

One microliter of plasmid containing amino acids 1–120 of the CD8 sequence (with the relevant mutations), amino acids 1–248 of each HLA $\alpha$ chain, or amino acids 1–100 of $\beta$2m were transformed into 20 $\mu$L of Rosetta DE3 competent cells. A single colony was used to inoculate 1 L of warm TYP media (16 g tryptone, 16 g yeast extract, 5 g NaCl, and 2.5 g $K_2$HPO$_4$) containing 100 $\mu$g/mL ampicillin. The cells were incubated for 12 h at 37°C in a shaker at 250 rpm until 1 OD was reached. The transformed cells were then induced using 0.5 mM IPTG, harvested by centrifugation, and inclusion bodies were purified using 20 mM lysis buffer (10 mM Tris pH 8, 10 mM MgCl$_2$, 150 mM NaCl, and 10% glycerol), sonication, and treatment with wash buffer (0.5% w/v Triton 100, 50 mM Tris pH 8, 100 mM NaCl, and 10 mM EDTA). The inclusion bodies (IBs) were then dissolved in guanidine buffer (6 M guanidine, 50 mM Tris pH 8.1, 2 mM EDTA, and 100 mM NaCl). Prior to refolding, protein concentration was estimated using a Coomassie Plus reagent kit (Pierce) using BSA as a standard.

Refolding of CD8 was carried out as previously described with some modifications. For a 1 L refold, 60 mg of CD8 $\alpha$-chain IBs were incubated in 6 mL of 6 M guanidine buffer at 37°C for 15 min with 10 mM DTT. The denatured IBs were then added to cold refold buffer (50 mM Tris pH 8.1, 2 mM EDTA, 400 mM $\beta$-arginine, 6 mM mercaptoethanol, and 4 mM cysteamine). Each refold was incubated at 4°C for 1–2 h before dialysis. Dialysis was performed at 4°C over two days with 20 volumes of dialysis buffer (changing the dialysis buffer once) until the conductivity of the refolds was under 2 mS/cm. The CD8 refolds were filtered and diluted by half with 10 mM MES pH 6 and adjusted to pH 6 using saturated MES solution.

The CD8 protein was initially purified by cation exchange using 10 mM MES pH 6 as binding buffer and 10 mM MES pH 6, 1M NaCl as elution buffer, with a Poros50HSTM column. The protein was then gel-filtered using a Superdex200HRSTM column, into either Biacore buffer (10 mM HEPES pH 7, 3 mM EDTA, 150 mM NaCl, and 0.005% vol/vol surfactant P20) or crystallization buffer (10 mM MES pH 6, 10 mM NaCl). The pHLA I refolds were initially purified by anion exchange using 10 mM Tris pH 8.1 as binding buffer and 10 mM Tris pH 8.1, 1M NaCl as elution buffer, with a Poros50HS column. The protein was then gel-filtered into Biacore buffer (10 mM HEPES pH 7, 3 mM EDTA, 150 mM NaCl, and 0.005% vol/vol surfactant P20) using a Superdex200HR column. The purity of each protein was measured using Coomassie-stained SDS-PAGE.

**pHLA Biotinylation**

Biotinylated pHLA was prepared as previously described. Briefly, pHLA, with a biotinylation sequence tag, was treated with 400 $\mu$M biotin, 5 $\mu$M ATP, 200 $\mu$M PMSF, 1 $\mu$M leupeptin, 1 $\mu$M Pepstatin, and 15 $\mu$L of BirA enzyme. The mixture was incubated overnight at room temperature and gel-filtered using a Superdex200HR column to remove free biotin.

**Surface Plasmon Resonance (SPR)**

The binding analysis was performed using a BIAcore 3000TM equipped with a CM5 sensor chip as previously reported. Briefly, chip coupling solutions containing 100 $\mu$L of 100 mM NHS and 100 $\mu$L of 400 mM EDC were used to activate the CM5 sensor chip prior to streptavidin binding (110 $\mu$L of 200 $\mu$g/mL in 10 mM acetic acid pH 4.5). Hundred microliter of 1M ethanolamine hydrochloride was used to deactivate any reactive groups. Biotinylated pHLA at $\sim$1 $\mu$M was then coupled to the streptavidin coated chip. For HLA coupling, $\sim$600–3000 RU of protein was attached to the CM5 sensor chip. CD8 samples were concentrated to around 1 mM. For equilibrium analysis, 10 serial dilutions were carefully prepared in triplicate for each sample, which were injected over the relevant sensor chips at 25°C. All experiments were carried out in triplicate using the same chip on the same day. Results were analyzed using BIAevaluation 3.1TM, Microsoft ExcelTM and Origin.
6.1\textsuperscript{TM}. The equilibrium binding constant ($K_D$) values were calculated using a nonlinear curve fit ($y = (P_1x)/(P_2 + x)$).

Crystallization

S33N/C33A CD8 was crystallized as previously reported.\textsuperscript{31}

Diffraction Data Collection and Model Refinement

Data were collected with the rotation method at Station 14.2 of the synchrotron radiation source (SRS) (Daresbury, UK), using an ADSC Quantum 4 CCD detector system. The wavelength ($\lambda$) was set to 0.978 Å. The total number of frames recorded was 100, each covering 1° of rotation. Reflection intensities were estimated with the MOSFLM package,\textsuperscript{32} and the data scaled, reduced, and analyzed with the CCP4 package.\textsuperscript{33} Crystal data and relevant statistics are given in Tables I and II.

The refined model of the wt CD8 (PDB code 1CD8,\textsuperscript{34}) was used as a search probe in AMoRe.\textsuperscript{35} The final fitted coordinates from AMoRe were checked for close contacts using the graphics program "O".\textsuperscript{36} The model sequence coordinates from AMoRe were checked for close contacts using the graphics program "O".\textsuperscript{36} The model sequence was adjusted with O to incorporate the mutations at positions 33 and 53, prior to refinement with REFMAC.\textsuperscript{37} Manual adjustment of the model by visual inspection was performed at various stages. Remaining positive density was filled, as appropriate, with solvent molecules. The thermal parameters were allowed to refine, but were restrained so as to be similar to those of neighboring atoms. During the later stages of refinement, graphical manipulation of the model was performed with COOT. The final graphical representations of the model and electron density were prepared with PYMOL. Data collection and reduction statistics are shown in Tables I and II, respectively. The final coordinates and structure factors have been deposited at the PDB with access code 2HP4.

RESULTS AND DISCUSSION

MD Simulation Results and Binding Analysis of CD8 Variants

From the MD simulations data, a number of CD8 variants were expressed and SPR was used to determine the $K_D$ to HLA-A2 (Table III). Six of the expressed mutants showed severely reduced binding affinity such that $K_D$'s could not be determined by SPR. Q2K and L97Y bound to pHLA I with affinities approximately two-fold and fourfold less than wild-type, respectively. In contrast, S33N bound to pHLA I with an approximately fourfold higher affinity ($K_D = 30.8 \pm 1.5 \mu M$). Therefore, this mutation was the most desirable for further investigation. The MD results indicated that the CD8 α2 S53N

<table>
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<th>CD8 variants</th>
<th>CD8/HLA-A0201</th>
<th>Standard deviation</th>
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<tr>
<td>wt CD8</td>
<td>137.1</td>
<td>4.73</td>
</tr>
<tr>
<td>C33A</td>
<td>141.3</td>
<td>11.0</td>
</tr>
<tr>
<td>S53N/C33A</td>
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<td>1.5</td>
</tr>
<tr>
<td>Q2K</td>
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<tr>
<td>L97Y</td>
<td>630</td>
<td>12.7</td>
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<td>Nb</td>
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<tr>
<td>L97Q</td>
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Nb, the response was too low to determine the $K_D$.

Values in parenthesis are for the highest resolution range.

Values in parenthesis are target values.
mutation abolished the H-bonding with pHLA I Asp-227 without forming any alternative intermolecular H-bond. Instead, an intramolecular H-bond was formed with Lys-56. Free energy perturbations for the CD8a2 S53N mutant suggested that the higher affinity was a result of electrostatic interactions, raising the possibility that solvent water plays a role in the binding free energy difference.

**Improved Refolding of C33A CD8 Mutant**

The mutation of the unpaired cysteine Cys-33 to Ala-33 significantly increased the yield of in vitro refolded CD8 (data not shown) and removed the possibility of nonnative disulfide bond formation. To confirm that the overall structure and function of the protein was unaltered by this mutation, we performed SPR binding analysis, which indicated that there was no change in the binding affinity compared to wt CD8 (see Fig. 1). Initially, doublet bands were observed (at around 10 and 11 kDa) for the wtCD8 and a single band for the S53N/C33A CD8 by nonreducing SDS-PAGE analysis. However, we found that vinyl pyridine pretreatment of the wtCD8 SDS samples generated a single band, which suggests that the observed doublet bands is most likely caused by a gel artifact. Nevertheless, we observed that C33A mutation was less prone to aggregation during con-
centration, we therefore incorporated the C33A mutation on the higher affinity S53N CD8 background for further analysis.

Binding Analysis of S53N/C33A CD8

Wild-type, C33A, and S53N/C33A soluble CD8 proteins were analyzed for binding to HLA-A*0201 and HLA-A*2402 (Figs. 1 and 2). $K_D$ values were determined from binding data generated by injecting 10 serial dilutions of each CD8 variant using the same flow cells for each experiment. For wt CD8, $K_D$'s of 137.1 ± 4.73 and 166.4 ± 20.7 μM were observed for binding to HLA-A*0201 and HLA-A*2402, respectively (151.8 μM average $K_D$). For C33A CD8, similar values of 144.5 ± 11.26 and 219.3 ± 32.4 μM for HLA-A*0201 and HLA-A*2402 were calculated, respectively (181.9 μM average $K_D$). For S53N/C33A CD8, values of 30.8 ± 1.5 and 50.6 ± 4.3 μM for HLA-A*0201 and HLA-A*2402, respectively, were recorded (40.7 μM average $K_D$) (Figs. 1 and 2). This equates to an improved affinity for S53N/C33A CD8 of ~4 times compared to that of wt CD8 or C33A CD8.

X-ray Structural Analysis of Higher Affinity S53N/C33A CD8

The structure of S53N/C33A CD8 was solved at 2.1 Å and refined to an $R_{cryst}$ and $R_{free}$ of 18.2% and 23.2%, respectively (Tables I and II). The complex crystallized in space group P6_122 with unit cell parameters $a = 101.08$ Å, $b = 101.08$ Å and $c = 56.54$ Å. The overall model consists of one molecule in the asymmetric unit (AU). However, the binding stoichiometry of wt CD8 to pHLA is two CD8 molecules, forming the homodimer, to one pHLA molecule. The geometrically generated dimer would be exactly symmetric by twofold rotation. To avoid
artifacts arising from imposing exact twofold symmetry, the S53N/C33A CD8 was refined as a homodimer in the lower symmetry space group P64, thus allowing each monomer to be refined independently of the other (see Fig. 3). This was particularly important because the binding epitopes comprises the CDR-like loop containing the S53N mutation. This loop is more flexible than the rest of the structure, and can potentially adopt different conformations, perhaps seeded by the asymmetric binding motif to pHLA. A superposition of the two monomers showed small discernible differences between them, with a RMSD of around 0.13 Å when main chain atoms were compared, growing to 0.5 Å when side-chain atoms were compared. A maximum deviation of up to 7.0 Å was observed in the case of the exposed side-chain of Lys-56.

The refined model of S53N/C33A CD8 showed the same overall structural organization as the wt CD8 (see Fig. 3). The standard immunoglobulin fold was maintained, and the first mutation point at CD8 residue 33 (C33A) was clearly visible in the electron density map [Fig. 4(a,b)]. The region of disorder around residue 40–42, observed in the wt CD8 structure (PDB:1CD8), was also observed to be disordered in this model, but it was not considered to be relevant to this study as it is distant from the pHLA I binding region. More importantly, significant differences were observed in the CDR-like loops (residues 53–56) of the S53N/C33A CD8 molecule compared to the wt CD8 structure. The second region of disorder apparent in the original wt CD8 structure, residues 53–56 that form the CD8 CDR-like loops, was better ordered in the S53N/C33A CD8 structure, with significant electron density guiding the model building [Fig. 4(c,d)]. It is therefore possible to accurately describe...
the positions of the CDR-like loops. The mutated residue, S53N, fell just outside the "generously allowed" region of the Ramchandran plot, indicating a small energy cost to this mutation. It appeared to be in a tightly defined pocket, lined by well ordered bulky side chains, imposing a less than ideal conformation on the Asn side-chain. An ordered sulfate ion, originating from the crystallization solution, was observed near to the loop where this mutated residue lies.

Modeling of the Higher Affinity Interaction

The wt CD8 Ser-53 in the α1 domain does not form any interaction with pHLA I. Therefore, the prospective rational design work focused on the interaction between Ser-53 mutants in the CD8 α2 domain, which clearly interacts with pHLA I in the wt complex crystal structure.21 The S53N/C33A CD8 crystal structure revealed an unexpected potential interaction between Asn-53 of CD8 α1 and pHLA I α3 residue Asp-223 that probably contributes to the increased avidity between the two biomolecules. Therefore, the hypothesis that the rational design relied upon was not complete. It could be argued that the design of a mutation in a protein and understanding its energetic impact is a challenging task, since prediction of ΔG with an error of 1 kcal/mol is often considered as reasonable. The S53N/C33A CD8 CDR-like loops adopt a virtually identical position to the wt CD8 CDR-like loops observed in the wt CD8/pHLA I complex structure (PDB:1AKJ). The CD8 α1 and α2 domains form a symmetrical conformation in which residues 53–56, part of the CDR-like loops, are adequately exposed to allow the interactions that occur with the HLA I α3 domain (residues 223–339) upon binding.

The most salient difference between the S53N/C33A CD8 structure and that of the published wt CD8/pHLA I complex is the potential close contact of residue Asn 53 of higher affinity CD8 with Asp-227 of pHLA I [Fig. 5(a,b)]. Because of the disorder in the CDR-like loops of the free wt CD8 structure, we could not make a superposition of the S53N/C33A CD8 with wt CD8. In the wt CD8/pHLA I structure, important contacts (<4 Å) are made between the CD8 α2 domain CDR-like loop at residues Ser-53, Gln-54, and Asn-55 and pHLA I residues Asp-227, Glu-198, and Val-248/Glu-198, respectively. Similarly, strong interactions occur between the CD8 α1 domain CDR-like loop at residue Gln-54 and pHLA I residues Thr-214/Gln-262.21 These bonds form the important CD8 CDR-like loop contacts involved in the asymmetric binding mode of wt CD8 to the pHLA I α3 domain. The mutation of Ser-53 to Asn-53 introduces a longer side chain with the potential to form a more favorable contact with Asp-223 or Asp-227 of pHLA I α3 residue Asp-223 (cyan; right). This is due to the closer 5 Å approach made possible by the longer Asn side chain at residue 53.
the pHLA α3 binding loop. However, it is clear that CD8 α2 residue Asn-53 does not appear to be significantly closer to pHLA I residue Asp-227 (4 Å) compared with the Ser-53 in the wt CD8/pHLA complex. In contrast, the CD8 α1 Asn-53 residue is only 5 Å from pHLA I residue Asp-223, and thus 3.2 Å closer than Ser-53 in the wt CD8 (Fig. 5c). It is possible that this shortened distance allows the Asn-53 residue to create a new electrostatic interaction with HLA α3 residue Asp-223 (see Fig. 5). Furthermore, because of the inherent flexibility of the CD8 CDR-like loops, it is possible that upon binding to pHLA, the side-chain of Asn-53 in the S53N/C33A CD8 α1 domain could form a hydrogen-bond (2.2–3.5 Å) with pHLA residue Asp-223. Clearly, this interaction is not possible in the wt CD8/pHLA I complex due to the shortened side chains of Ser-53. This improved contact could allow the S53N/C33A CD8 to bind pHLA I with the enhanced affinity.

Furthermore, we explored the possibility of introducing an amino acid with a larger side chain than asparagine, such as glutamine, at position 53 in order to allow an amino acid with a larger side chain than aspartate to create a new electrostatic interaction with HLA α3 loops, it is possible that upon binding to pHLA, the side-chain of Asn-53 in the S53N/C33A CD8 α1 domain could form a hydrogen-bond (2.2–3.5 Å) with pHLA residue Asp-223. Clearly, this interaction is not possible in the wt CD8/pHLA I complex due to the shortened side chains of Ser-53. This improved contact could allow the S53N/C33A CD8 to bind pHLA I with the enhanced affinity.

CONCLUSIONS

Previous evidence has demonstrated human soluble wt CD8, murine soluble wt CD8, and murine wt CD8 peptide derivatives in the disruption of CTL activation. This approach is limited largely due to the low affinity of wt CD8. By developing soluble high affinity variants based on wt CD8 protein, we postulate that inhibition of CTL activation could be enhanced providing a more effective therapeutic for use in the treatment of a number of illnesses. The use of anti-IgE antibodies to treat allergic asthma has been reported, anti CD3: antibodies have been widely used as immunosuppressors, and there is evidence to suggest that soluble pHLA dimers could be used to treat autoimmune diseases such as type 1 diabetes and soluble pHLA as a general T cell immunosuppressor. The modulation of natural immune responses with soluble CD8, either wild-type or with modifications, offers an exciting and attractive prospect for therapeutic strategies.

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