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Crystal Structure of a SEA Variant in Complex with MHC Class II Reveals the Ability of SEA to Crosslink MHC Molecules

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Summary

Although the biological properties of staphylococcal enterotoxin A (SEA) have been well characterized, structural insights into the interaction between SEA and major histocompatibilty complex (MHC) class II have only been obtained by modeling. Here, the crystal structure of the D227A variant of SEA in complex with human MHC class II has been determined by X-ray crystallography. SEA_{D227A} exclusively binds with its N-terminal domain to the α chain of HLA-DR1. The ability of one SEA molecule to crosslink two MHC molecules was modeled. It shows that this SEA molecule cannot interact with the T cell receptor (TCR) while a second SEA molecule interacts with MHC. Because of its relatively low toxicity, the D227A variant of SEA is used in tumor therapy.

Introduction

Superantigens are microbial toxins that activate the immune system by binding as unprocessed molecules to major histocompatibility (MHC) complex class II [1] and T cell receptor (TCR) molecules [2]. They activate a large fraction of human T cells (~20 % of all T cells) and stimulate them to secrete inflammatory cytokines, such as interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). These properties are attributed to their unique ability to crosslink MHC class II and TCR by recognizing a specific subset of TCR Vβs. Superantigens are known to be involved in a number of diseases characterized by fever and shock and are important virulence factors for two human commensal organisms, *Staphylococcus aureus* and *Streptococcus pyogenes*, as well as for some viruses [3]. The bacterial superantigens, streptococcal pyrogenic exotoxins (SPEs), streptococcal mitogenic exotoxins (SMEZ), staphylococcal enterotoxins (SEs), streptococcal superantigen (SSA), and toxic shock syndrome toxin (TSST-1) can be divided into three genetic subfamilies (class 1, SPE-C, SPE-J, SPE-G, and SMEZ; class 2, SEB, SEC1, SEC2, SEC3, SSA, SPE-A, and SEG; class 3, SEA, SEE, SED, SEH, SEI, SEJ, SEK, and SEL) as well as two individual branches (SPE-H and TSST-1), according to biochemical structural characterization [4]. All these superantigens are globular proteins of 22–29 kDa and contain two domains with a remarkable structural conservation (an N-terminal β barrel, a C-terminal β grasp motif, and an α helices-spanning center), considering their low sequence identity [4].

Crystal structures of superantigens in complex with MHC class II have shown that they either interact with the α chain, as in SEB and TSST-1 [5, 6], or with the β chain, in a zinc-dependent manner, as in SEH and SPE-C [7, 8] of MHC class II. Both SEH and SPE-C coordinate a zinc ion in a similar manner, with one aspartate and one or two histidine residues. The zinc ion bridges His81 on MHC class II, which is the third ligand (the fourth in the case of SPE-C). SEA coordinates zinc in a similar manner to SEH and SPE-C, using Asp227, His225, and His187, and is believed to interact with His81 on MHC class II in a similar manner, as well [7–9]. Alanine substitution studies have shown that SEA is able to interact with either the α chain or the β chain of MHC class II [10, 11]. SEA binds with its N-terminal domain, with low affinity (~10 μM), to the α chain of MHC class II and with its C-terminal domain, in a zinc-dependent manner, with medium affinity (~100 nM), to the β chain [10].

It is well established that superantigens, in general, and SEA, in particular, may be used clinically. Most extensive studies have been performed in the field of cancer therapy [12]. Because of its low systemic toxicity, the SEA_{D227A} variant is successfully used in clinical trials in the treatment of lung cancer patients [13]. Although SEA is one of the most extensively studied superantigens, no three-dimensional structure information of the complex between SEA and MHC class II is currently available.

We have crystallized the D227A variant of SEA in complex with HLA-DR1 and determined the three-dimensional structure to 3.2 Å resolution using X-ray crystallography. The substitution of the zinc-coordinating Asp227 to an alanine has been shown to reduce the binding affinity to human MHC class II more than 1000-fold [10]. The crystal structure shows that SEA_{D227A} interacts with the α chain of HLA-DR1 with its N-terminal domain and that the interaction with the β chain of HLA-DR1 is completely abrogated. We also suggest a model of the quaternary complex where one SEA molecule crosslinks two MHC class II molecules while interacting with TCR.

Results

Overall Structure

The molecular complex investigated consists of the D227A variant of SEA (~27 kDa), the extracellular domains of HLA-DR1 (~44 kDa) containing the α chain

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Key words: MHC class II; protein-protein interaction; SEA; staphylococcal enterotoxin; superantigen; X-ray crystallography

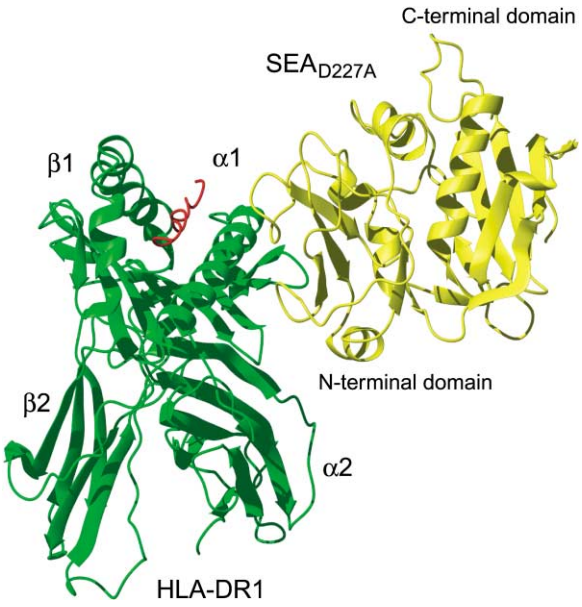

Figure 1. Ribbon Representation of the DR1-SEA$_{D227A}$ Complex with HLA-DR1, in Green, Ha-Peptide, in Red, and SEA$_{D227A}$, in Yellow

Table 1. Crystal Data Collection and Structure Refinement Statistics

Data Collection		
Resolution range (Å)	30–3.2	
R_{sym} (%)[a]	10.1	24.6[b]
Completeness (%)	91.6	87.9[b]
Unique reflections, total	38,082	4,777[b]
Redundancy, total	3.1	2.8[b]
I/σ	6.4	3.1[b]
Refinement Statistics		
R factor (%)	24.5	
R_{free} (%)[c]	33.9	
B factor (Å^2)	46.2	
Water molecules	23	
Model Statistics		
Bond length rmsd (Å)	0.009	
Bond angles rmsd (°)	1.59	

[a] $R_{sym} = \Sigma_h\Sigma_i |I(h,i) - \langle I(h)\rangle|/\Sigma I_h\Sigma_i(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of h and $\langle I(h)\rangle$ is the corresponding average value of all i measurements.
[b] Last shell, 3.2–3.37.
[c] R_{free} was determined with 8% of all reflections.

(~21 kDa) and the β chain (~22 kDa), and the hemagglutinin peptide (Ha-peptide), PKYVKQNTLKLAT.

Alanine substitution studies have shown that SEA has two binding sites for human MHC class II, one N-terminal site and one C-terminal zinc-dependent site [10, 11]. We have chosen to crystallize a variant of SEA in which the C-terminal zinc-dependent site is believed to be abrogated. In this way, we have been able to capture the complex in which SEA uses its low affinity site when binding to HLA-DR1. The major interactions between SEA$_{D227A}$ and HLA-DR1 are between the N-terminal β barrel of SEA$_{D227A}$ and the α chain of HLA-DR1. The superantigen binds to one side of HLA-DR1, outside the peptide binding groove, and does not contact the peptide (Figures 1 and 2; Table 1).

The overall structure of SEA$_{D227A}$ in complex with HLA-DR1 has minor conformational changes compared with the uncomplexed structure of SEA [9]. The major difference is in the C-terminal domain in the zinc binding area, where no electron density for a zinc ion is seen in the 3.2 Å $2F_o - F_c$ map. This is expected, since Asp227 in SEA is known to be one of the three zinc ligands, according to mutagenesis studies [10]. Hence, when Asp227 is substituted to an alanine, the SEA molecules lose their affinity for zinc as well as their ability to bind HLA-DR1 in a zinc-dependent fashion. The N terminus of SEA has been suggested to provide one of the coordinates to the zinc ion [9]. This residue, Ser1, is seen in the DR1-SEA$_{D227A}$ complex structure and is positioned in such a way that it could coordinate a zinc ion. However, residues 3–6 in the N-terminal region of SEA$_{D227A}$ are not seen, possibly due to disordering of the protein.

A second zinc site, which is situated between the two domains of the protein, has been observed in SEA by X-ray crystallography [14]. Thermal denaturation also shows that zinc ions can stabilize the structure of SEA$_{D227A}$ [15]. However, no such zinc site is detected in the DR1-SEA$_{D227A}$ structure, although the crystallization was performed in the presence of 1 mM $ZnCl_2$. This second zinc binding site may therefore only be relevant at higher concentrations of zinc. A similar zinc site was also observed in the crystal structure of SEC_2, and it has been suggested to be important for SEC_1 when interacting with MHC class II [16, 17]. Two of these corresponding and putative zinc coordinating ligands in SEA, His114 and Glu39, have previously been substituted to investigate the consequence for MHC class II binding [10]. Neither the MHC class II binding nor the ability to mediate T cell cytotoxicity was affected, and,

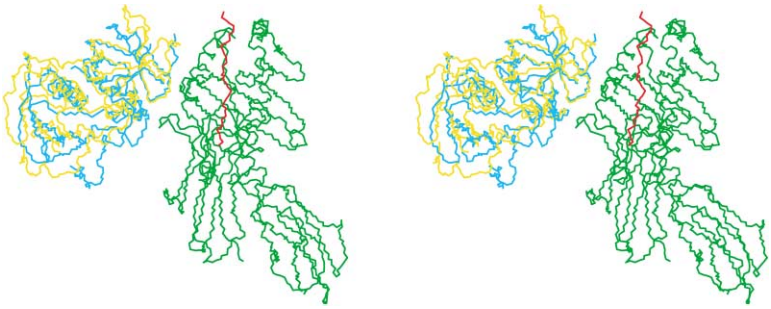

Figure 2. Stereoview of the Cα Chain Representation of the DR1-SEA$_{D227A}$ and DR1-SEB Complexes

HLA-DR1 from both complexes has been superimposed. Areas in SEB that are not defined in the crystal structure are not displayed. HLA-DR1, green; antigenic peptide, red; SEA$_{D227A}$, yellow; SEB, cyan.

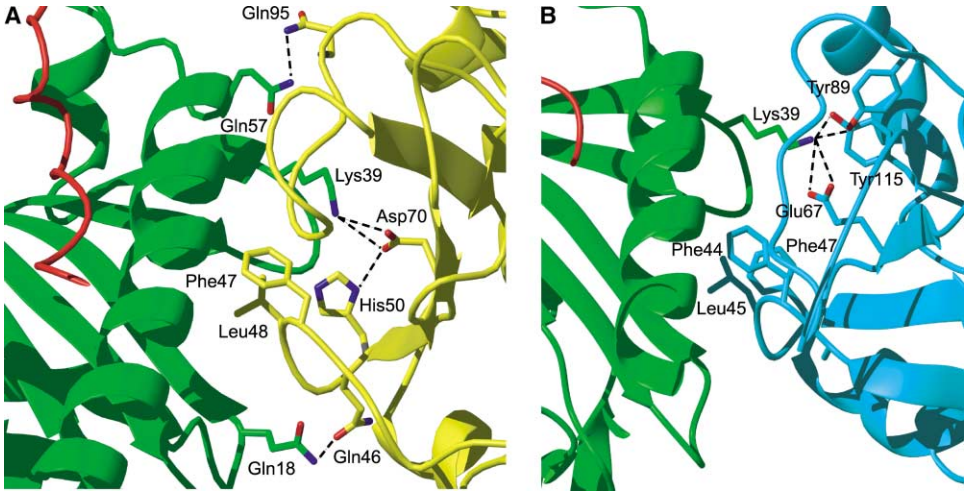

Figure 3. Ribbon Representations of the Interfaces between (A) HLA-DR1, in Green, and SEA$_{D227A}$, in Yellow, and (B) HLA-DR1, in Green, and SEB, in Cyan, as Well as Electrostatic Interaction and Hydrogen Bond Pattern of Selected Residues in the Interface

therefore, we conclude that the proposed second zinc site is not important for the interaction with MHC class II, in accordance with the DR1-SEA$_{D227A}$ structure.

The overall HLA-DR1 structure in this complex is very similar to the published uncomplexed structure of HLA-DR1 [18]. This agrees well with other solved complexes between superantigens and MHC class II, where no major changes in the structures of the MHC class II molecules have been reported [5, 7].

The Interface between SEA$_{D227A}$ and HLA-DR1

The total accessible area in the complex is 29,896 Å^2, calculated with GRASP [19], with an interface burying 554 Å^2 and 567 Å^2 of the HLA-DR1 and SEA$_{D227A}$ solvent-accessible surfaces, respectively [20]. The interface between SEA$_{D227A}$ and HLA-DR1 consists of a ridge of nonpolar residues (Phe47, Leu48, and His50) situated on the loop between the β1 and β2 strands of SEA$_{D227A}$, which protrudes their side chains into the cavity between the α1 helix and the β sheet that creates the peptide binding groove of HLA-DR1. The amino acids creating the ridge, in particular, Phe47, interact with several nonpolar amino acids (Leu60, Ile63, and Ala64) located on the α1 helix of HLA-DR1 as well as with Lys39 and Met36, situated on the loop between the β3 and β4 strands on the α chain of HLA-DR1. In addition, Asp70 of SEA$_{D227A}$ makes a salt bridge with Lys39. A hydrogen bond is seen between His50 and Asp70, both on SEA$_{D227A}$, which possibly stabilizes the interface by directing Asp70 to Lys39 on HLA-DR1, hence facilitating the salt bridge formation. Two hydrogen bonds are likely to be formed between Gln46 and Gln95 on SEA$_{D227A}$ and Gln18 and Gln57 on the α chain of HLA-DR1, respectively. The interaction between Gln46 and Gln18 is close to the hydrophobic interacting area between SEA$_{D227A}$ and HLA-DR1 (described above), while the interaction between Gln95 (SEA$_{D227A}$) and Gln57 (HLA-DR1) is situated between the disulphide loop of SEA$_{D227A}$ and the α1 domain of the α helix of HLA-DR1 (Figure 3A). The importance of Phe47 on SEA and Lys39 and Gln18 on HLA-DR1 for complex formation as well as for T cell activation (IL-2 production) has earlier been shown by substitution studies [21].

The crystal structure of DR1-SEA$_{D227A}$ clearly demonstrates that one SEA molecule cannot interact with both its N- and C-terminal sites with the same MHC class II molecule at the same time. This is because the C-terminal domain of SEA$_{D227A}$ is oriented away from the HLA-DR1 molecule and is therefore too far away to establish an interaction (Figure 1).

Discussion

In this study, the crystal structure of SEA$_{D227A}$ in complex with HLA-DR1 was determined at 3.2 Å resolution. SEA$_{D227A}$ binds to the α chain of HLA-DR1 and does not contact the presented Ha-peptide. The D227A substitution of SEA prevents the superantigen from interacting with the β chain of HLA-DR1 through the zinc-dependent site on the C-terminal domain.

Comparison with SEB

The overall structure of the DR1-SEA$_{D227A}$ complex mimics the binding between SEB and HLA-DR1, in general [5, 22]. However, the contacting region between SEA$_{D227A}$ and HLA-DR1 is only around 70% of the contacting region of DR1-SEB (burying 788 Å^2 and 764 Å^2 of the HLA-DR1 and SEB solvent-accessible surfaces, respectively). The major difference between the two superantigen-HLA-DR1 complexes is that the SEA$_{D227A}$ is partially rotated away from the HLA-DR1 molecule compared with SEB (Figure 2). The rotation is mainly due to movement of the disulphide loop caused by a replacement of Tyr94 in SEB, which stabilizes the DR1-SEB complex, to the corresponding Ala97 in SEA$_{D227A}$. In addition, Ser96 in SEB, also situated on the disulphide loop, contacts HLA-DR1, as well. In the interface of DR1-SEB, there are seven hydrogen bonds between the molecules, while, in the DR1-SEA$_{D227A}$ complex, there are only two hydrogen bonds. For instance, in the DR1-SEB structure, two hydrogen bonds are formed from Tyr89 and Tyr115 on SEB to Lys39 of HLA-DR1 (Figure 3B). These are in close

proximity to the salt bridge between Glu67 of SEB and the same lysine. In the DR1-SEA_{D227A} structure, a similar salt bridge is formed between Asp70 of SEA_{D227A} and Lys39 of HLA-DR1, but the two corresponding residues (Tyr92 and Tyr108), although structurally aligned with the two tyrosines in SEB, are too far from HLA-DR1 to be able to form hydrogen bonds. This is due to a small conformational change of the side chain of Lys39 (2 Å movement of the nitrogen; Figure 3) as well as a larger overall distance between the SEA_{D227A} and the HLA-DR1 molecules than in the DR1-SEB complex. The comparably small contact region for the DR1-SEA_{D227A} complex is correlated to the ability for residues on SEA_{D227A} to make van der Waals contacts with HLA-DR1. Hence, this is in full agreement with SEB binding 40-fold stronger to MHC class II [10, 23].

Interaction with Other MHC Molecules

All published complex structures between superantigens and MHC molecules have so far been with HLA-DR molecules [5–8]. However, it has been shown that the SSA superantigen binds HLA-DQ molecules on the cell surface [24]. A sequence comparison between HLA-DR, HLA-DQ, and HLA-DP shows that Lys39 in the α chain of HLA-DR1, which is important for the low-affinity site between SEA and HLA-DR1, is conserved between the different HLA-DR, -DP, and -DQ molecules. In addition, His81 on the β chain of HLA-DR1, which is the most important amino acid for the zinc-dependent medium-affinity site between SEA and HLA-DR1, is also conserved between all three HLA molecules [25]. Hence, SEA is very likely to be presented by both HLA-DQ and HLA-DP in a similar way to HLA-DR, which is supported by cell-based studies [26].

Crosslinking of MHC

The ability of SEA to simultaneously bind two separate MHC class II molecules, utilizing both the low- and medium-affinity sites, results in a high-affinity interaction with MHC class II. The DR1-SEA_{D227A} structure demonstrates that SEA is able to crosslink two MHC class II molecules because the C-terminal domain is directed away from the bound HLA-DR1 and is completely free to interact with another MHC class II molecule with no steric hindrance. The ability of SEA to use both sites for MHC class II binding has earlier been shown to be important for optimal T cell activation and cytokine induction [27–29]. The interaction between the C-terminal domain of SEA and HLA-DR1 is suggested to be very similar to the interaction between SEH and HLA-DR1. This is supported by the fact that both superantigens have a zinc-dependent interaction with the β chain of HLA-DR1 as well as an interaction with the antigenic peptide [7, 11, 30, 31]. A model of SEA crosslinking two MHC class II molecules was created by performing structural alignment between the complexes DR1-SEA_{D227A} and DR1-SEH (1HXY) [7], superimposing the HLA-DR1 molecules to model the medium-affinity site between SEA and HLA-DR1 (Figure 4). Sequence alignment with Clustal W [32] shows that, in addition to SEA, there are three other bacterial superantigens, SED, SEE, and SEJ, that may crosslink MHC class II molecules by using its N-terminal and C-terminal domains. This is in agreement with previous results demonstrating the importance of simultaneous ligation of both the α chain and the β chain of two different MHC class II molecules for optimal T cell response by SED and SEE in cell-based studies [33].

T Cell Activation

Substitution studies have been performed with SEA to explain the consequence of the two MHC class II binding sites for optimal T cell activation. Eliminating the low-affinity class II binding site had a stronger influence on the Vβ profile than did the removal of the medium-affinity zinc-dependent site [28]. However, the potency of SEA to mediate T cell cytotoxicity is more affected by eliminating the zinc site [10]. Through the use of the structure solved here, it was possible to model the quaternary complex (MHC_2-SEA_1-TCR_1) to find out how the crosslinking of two MHC class II molecules by one SEA molecule affects the interaction interface between APC and the T cell. The model was made by combining the crosslinking model of SEA, described above, and a model of SEA interacting with the β chain of TCR, based on the structure of the SEB-TCR complex (1SBB) [34], since it has been shown that SEA and SEB have overlapping TCR binding regions [35, 36]. Finally, the TCR-HLA-DR1 complex (1FYT) [37] was used to model the α chain of TCR, which is not present in the SEB-TCR structure (Figure 4A). The overall geometry of the complex, with the C termini of MHC and TCR in opposite directions, results in a very plausible contacting region between APC and the T cell (Figure 4A). The overall distance between the C termini is in agreement with the MHC-TCR complex (~130 Å) [37]. This clearly shows that it is possible for TCR and MHC to interact in the quaternary complex formation, as has previously been suggested [38] (Figure 4A). The model presented here rules out the possibility for one MHC class II molecule to simultaneously interact with TCR and with two SEA molecules. The Vα chain of TCR and the second SEA molecule that interacts with the β chain of MHC class II will totally overlap (Figure 4B). However, trimeric complexes (SEA_2-MHC_1) have been seen in solution [39], and these complexes might appear on the cell's surface, but the first SEA molecule will not be able to interact with the TCR because of the overlap between the second SEA and TCR (Figure 4). Consequently, it is not possible for SEA and MHC class II to oligomerize and create chains of complexes on the cell surface for an optimal T cell activation.

The model for crosslinking two MHC molecules by SEA suggests a mechanism where the medium-affinity zinc-dependent site is the first and most important interaction between SEA and MHC class II. When that binding has been established, SEA binds a second MHC class II molecule on the cell surface using its low-affinity site to induce an optimal T cell response (SEA_1-MHC_2). As a consequence of crosslinking, there must be more MHC class II molecules present than SEA on the cell surface, which agrees well with data where less than 0.3 % of the cell surface MHC class II needs to be occupied by SEA to generate an optimal T cell response

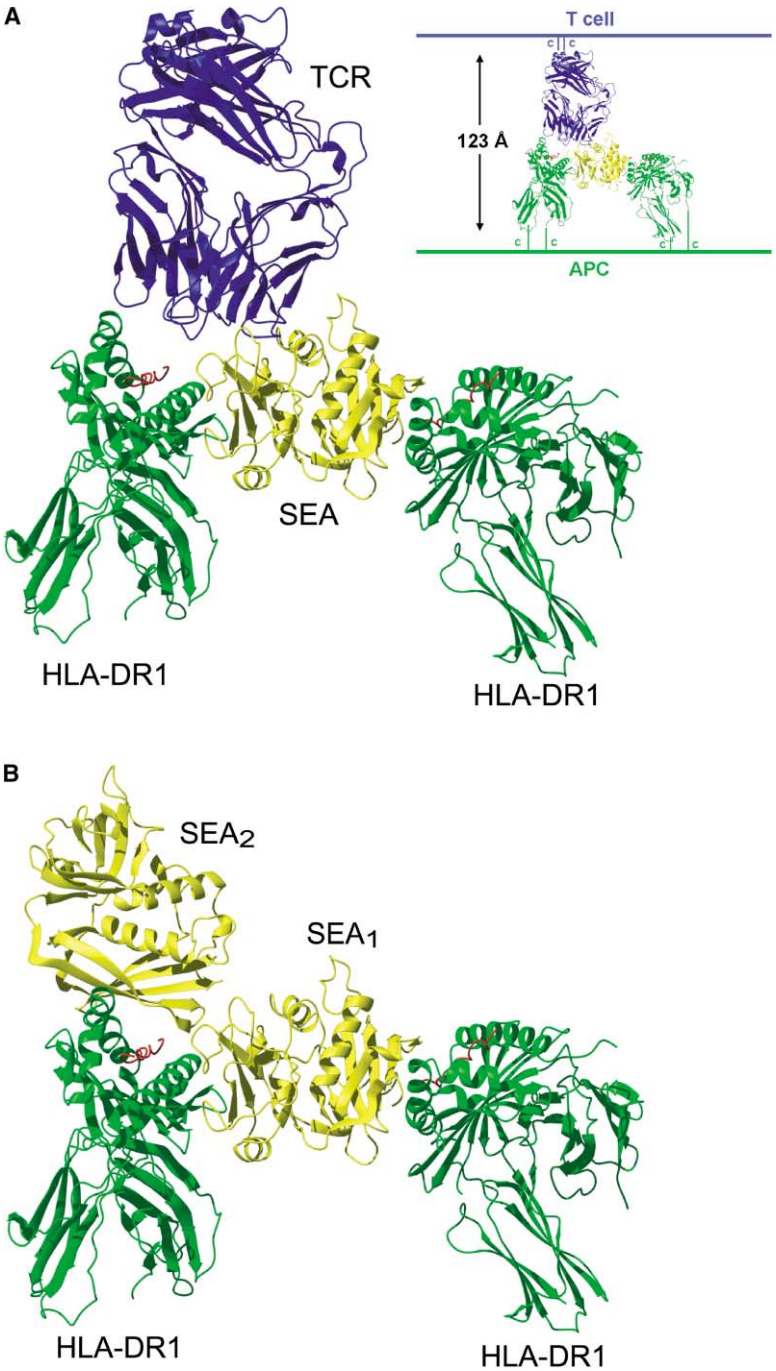

Figure 4. Structural Overview of Modeled Complexes

(A) Model of the quaternary complex, which illustrates SEA (yellow) crosslinking two HLA-DR1 molecules (green) as well as interacting with the T cell receptor (blue). The insertion illustrates the ability of the quaternary complex to create a regular distance between the APC and the T cell.

(B) Model of an HLA-DR1 (green) molecule presenting two SEA (yellow) molecules, where the first SEA molecule will be unable to interact with TCR because of the Vα chain of TCR and the second SEA molecule will totally overlap, according to Figure 4A.

[40]. However, SEA may be presented to TCR, although less optimally, using only its medium-affinity site. In this case there will be no direct interaction between TCR and MHC, according to the quaternary complex (MHC_2-SEA_1-TCR_1) model. It has been demonstrated that T cells activated when SEA is presented only by its medium-affinity site are also activated by SEA in the absence of MHC class II molecules [41]. This concludes that, when SEA is suboptimally presented by MHC, no interaction between MHC and TCR will occur. The suboptimal presentation of SEA will lead to stimulation of a more selective TCR Vβ repertoire than when both the medium- and low-affinity sites interact with MHC class II molecules [28]. Hence, the zinc-dependent site is important for SEA to find the MHC class II molecules on the cell surface of APCs, while the low-affinity site is more important for activating a broad Vβ repertoire of T cells.

Tumor-Targeted Superantigens

Bacterial toxins have been known since the beginning of the twentieth century to have antitumor effects [42]. An approach of superantigen-based tumor therapy has been developed where a superantigen is fused to fragments of tumor-reactive monoclonal antibodies [43]. In

this way, the T cells can be directed to MHC class II-negative tumor cells and kill the target cells [12]. Initial clinical studies used wild-type SEA [44, 45], but, because of toxicity, there has been a need to find novel variants. SEA_{D227A} is successfully used in fusion with the Fab moiety of the tumor-reactive antibody 5T4 in phase II clinical trials [13]. The advantage of the mutant, compared with wild-type SEA, is a reduced accumulation of superantigen in MHC class II-expressing organs, leading to reduced systemic shock [46]. The crystal structure described here explains the ability of SEA_{D227A} to bind MHC class II in a more moderate fashion than does wild-type SEA, which is desirable for activating a sufficient amount of T cells to evoke an antitumor response, but this prevents the superantigen from homing in on other organs than the tumor.

Biological Implications

Superantigens are a group of highly potent immunostimulatory proteins of bacterial or viral origin that bind MHC class II and activate a large number of T cells. Activation results in a vigorous T cell proliferation and production of cytokines. Determining the structural basis on presentation of superantigens by MHC class II, as well as how the ability of T cell activation is influenced by the presentation, is essential to understanding the biological properties of superantigens. While SEA is one of the best biologically characterized superantigens, the structural knowledge of its MHC interaction is based on other superantigens, like SEB. The structure presented here reveals how SEA interacts with MHC class II through its N-terminal domain. In contrast to other related superantigens, such as SEB and SEH, SEA is known to bind and crosslink MHC class II bivalently, using both its N-terminal and C-terminal domains. In this study, the C-terminal interaction with MHC class II has been abrogated by an alanine substitution to capture the low-affinity interaction with MHC. The crosslinking of MHC molecules is essential for SEA to be able to activate a large population of T cells and, hence, become one of the most potent superantigens. Crosslinking also stimulates antigen-presenting cells to secrete inflammatory cytokines. In addition, the low-affinity site is here shown to be important for the proper presentation of SEA to TCR, since SEA's ability to stimulate a broad population of T cells is reduced if this site is eliminated. Superantigens have, for a long time, been known to have antitumor effects. The variant studied here has shown very promising results in clinical trials because of its ability to bind MHC in a more moderate fashion. This leads to a reduced accumulation of superantigen in MHC-expressing organs, leading to reduced systemic shock compared with wild-type SEA.

Experimental Procedures

Cloning, Protein Overexpression, and Purification

Recombinant SEA was expressed in the *Escherichia coli* K12 strain UL635 with a plasmid, which contained the gene for SEA_{D227A}, a synthetic signal peptide, a kanamycin resistance gene, and a constitutive promoter [10].

Bacteria from frozen (−70°C) stock solution in 20 % glycerol were incubated at 25°C for 22–24 hr in shaker flasks containing (per liter) 2.5 g of $(NH_4)_2SO_4$, 3 g of KH_2PO_4, 2 g of K_2HPO_4, 0.5 g of trisodium citrate, 1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of kanamycin, 12 g of glucose, and 1 ml of trace element solution [47], however, without $Na_2MoO_4 \cdot 2H_2O$. The cells were grown to an Abs_{620} of 2–3, and 10 ml of the cultivation medium was used to inoculate a 1 liter fermenter (Belach Bioteknik, Sweden) with a starting volume of 800 ml growth medium. This medium contained (per liter) 2.5 g of $(NH_4)_2SO_4$, 9 g of KH_2PO_4, 6 g of K_2HPO_4, 0.5 g of trisodium citrate, 1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of kanamycin, 20 g of glucose, and 1 ml of trace element solution, as above. The pH was kept constant at 7.0 by titration of 25% NH_3, the aeration was 1 l/min, and the temperature was 25°C. The dissolved O_2 was kept at 30% by regulating the agitation from 400 rpm to 2000 rpm during the batch phase and by regulating the feed of glucose (60% w/v) during the fed batch phase. After cultivation the cells were removed by centrifugation at 6 000 × g for 45 min at 4°C, and the clarified medium containing SEA_{D227A} was stored at −20°C prior to purification. The pH of the medium was adjusted to 6.0 and applied to a HiLoad 26/10 SP Sepharose column (Amersham Pharmacia Biotech). The sample was eluted with a linear gradient of 0–400 mM of NaCl with a running buffer containing 10 mM KH_2PO_4 and 0.025% Tween-20 at pH 6.0. The yield was 80 mg/l of growth medium.

The extracellular parts of the HLA-DR1 α chain (DR*0101) and β chain (DRB1*0101) were both expressed in *E. coli* as inclusion bodies and refolded in the presence of Ha-peptide by a standard dilution protocol as described in Petersson et al. [7].

SEA was transferred to TBS (25 mM Tris and 150 mM NaCl [pH 7.4]) with a PD-10 column, while the folded HLA-DR1 complex was exchanged to 30 times-diluted TBS. The protein samples were concentrated, SEA with Centriprep-10 (Amicon, Beverly, MA) and HLA-DR1, 30 times, by slow evaporation.

Crystallization and Data Collection

Concentrated HLA-DR1 peptide complex (3.2 mg/ml, according to UV absorbance measurements at 280 nm) was mixed with highly concentrated SEA_{D227A} (10 mg/ml, according to UV absorbance measurements at 280 nm) to a final concentration of 4.5 mg/ml protein (molar ratio 1:1) in the presence of 1 mM $ZnCl_2$. Crystals were grown with vapor diffusion by mixing 1.5 μl protein solution with 1.5 μl well solution containing 0.2 M $(NH_4)_2SO_4$, 0.1 M MES, and 24% (w/v) polyethylene glycol monomethyl ether 5000 (pH 6.5). The crystals grew in 3 weeks, belonged to space group $P2_12_12_1$, with cell dimensions $a = 52.0$ Å, $b = 75.8$ Å, $c = 198.1$ Å, $\alpha = \beta = \gamma = 90°$, and contained one complex per asymmetric unit. The crystals were flash-frozen in 0.2 M $(NH_4)_2SO_4$, 0.1 M MES, 24% (w/v) polyethylen glycol monomethyl ether 5000 (pH 6.5), and 20 % (v/v) glycerol.

A data set was collected at beamline I711 at the MAX laboratory in Lund, Sweden, with a MarCCD detector. The data set was evaluated in MOSFLM [48] and scaled with the CCP4 suite of programs [49]. Statistics on data collection and quality are presented in Table 1.

Structure Determination and Refinement

The structure of the DR1-SEA_{D227} complex was solved by molecular replacement technique with the program AMORE [50], with SEA, 1ESF [9], and the HLA-DR1 peptide complex, 1DLH [18], as search models. Model building and checking of electron density maps were done with Xtalview [51], and all refinement was performed with CNS [52]. The final model includes A3–A182 in the α chain and B3–B190 in the β chain of HLA-DR1, C306–C318 in the Ha-peptide, and residues D1–D233 in SEA_{D227A}. The final R factor (R_{free}) is 0.245 (0.339), and good geometry in the model indicates a well-determined structure. More than 97% of all residues are in the most favored or allowed regions in the Ramachandran plot, and only 0.7% are in disallowed regions, according to the PROCHECK program [53]. Details of the structure are given in Table 1.

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References

1. Fraser, J.D. (1989). High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. Nature *339*, 221–223.
2. White, J., Herman, A., Pullen, A.M., Kubo, R., Kappler, J.W., and Marrack, P. (1989). The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. Cell *56*, 27–35.
3. Fraser, J., Arcus, V., Kong, P., Baker, E., and Proft, T. (2000). Superantigens—powerful modifiers of the immune system. Mol. Med. Today *6*, 125–132.
4. Sundberg, E.J., Li, Y., and Mariuzza, R.A. (2002). So many ways of getting in the way: diversity in the molecular architecture of superantigen-dependent T-cell signaling complexes. Curr. Opin. Immunol. *14*, 36–44.
5. Jardetzky, T.S., Brown, J.H., Gorga, J.C., Stern, L.J., Urban, R.G., Chi, Y.I., Stauffacher, C., Strominger, J.L., and Wiley, D.C. (1994). Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. Nature *368*, 711–718.
6. Kim, J., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1994). Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1. Science *266*, 1870–1874.
7. Petersson, K., Hakansson, M., Nilsson, H., Forsberg, G., Svensson, L.A., Liljas, A., and Walse, B. (2001). Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence. EMBO J. *20*, 3306–3312.
8. Li, Y., Li, H., Dimasi, N., McCormick, J.K., Martin, R., Schuck, P., Schlievert, P.M., and Mariuzza, R.A. (2001). Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II. Immunity *14*, 93–104.
9. Schad, E.M., Zaitseva, I., Zaitsev, V.N., Dohlsten, M., Kalland, T., Schlievert, P.M., Ohlendorf, D.H., and Svensson, L.A. (1995). Crystal structure of the superantigen staphylococcal enterotoxin type A. EMBO J. *14*, 3292–3301.
10. Abrahmsén, L., Dohlsten, M., Segren, S., Bjork, P., Jonsson, E., and Kalland, T. (1995). Characterization of two distinct MHC class II binding sites in the superantigen staphylococcal enterotoxin A. EMBO J. *14*, 2978–2986.
11. Hudson, K.R., Tiedemann, R.E., Urban, R.G., Lowe, S.C., Strominger, J.L., and Fraser, J.D. (1995). Staphylococcal enterotoxin A has two cooperative binding sites on major histocompatibility complex class II. J. Exp. Med. *182*, 711–720.
12. Dohlsten, M., Abrahmsen, L., Bjork, P., Lando, P.A., Hedlund, G., Forsberg, G., Brodin, T., Gascoigne, N.R., Forberg, C., Lind, P., et al. (1994). Monoclonal antibody-superantigen fusion proteins: tumor-specific agents for T-cell-based tumor therapy. Proc. Natl. Acad. Sci. USA *91*, 8945–8949.
13. Forsberg, G., Ohlsson, L., Brodin, T., Bjork, P., Lando, P.A., Shaw, D., Stern, P.L., and Dohlsten, M. (2001). Therapy of human non-small-cell lung carcinoma using antibody targeting of a modified superantigen. Br. J. Cancer *85*, 129–136.
14. Schad, E.M., Papageorgiou, A.C., Svensson, L.A., and Acharya, K.R. (1997). A structural and functional comparison of staphylococcal enterotoxins A and C2 reveals remarkable similarity and dissimilarity. J. Mol. Biol. *269*, 270–280.
15. Cavallin, A., Arozenius, H., Kristensson, K., Antonsson, P., Otzen, D.E., Bjork, P., and Forsberg, G. (2000). The spectral and thermodynamic properties of staphylococcal enterotoxin A, E, and variants suggest that structural modifications are important to control their function. J. Biol. Chem. *275*, 1665–1672.
16. Hoffmann, M.L., Jablonski, L.M., Crum, K.K., Hackett, S.P., Chi, Y.I., Stauffacher, C.V., Stevens, D.L., and Bohach, G.A. (1994). Predictions of T-cell receptor- and major histocompatibility complex-binding sites on staphylococcal enterotoxin C1. Infect. Immun. *62*, 3396–3407.
17. Papageorgiou, A.C., Acharya, K.R., Shapiro, R., Passalacqua, E.F., Brehm, R.D., and Tranter, H.S. (1995). Crystal structure of the superantigen enterotoxin C2 from Staphylococcus aureus reveals a zinc-binding site. Structure *3*, 769–779.
18. Stern, L.J., Brown, J.H., Jardetzky, T.S., Gorga, J.C., Urban, R.G., Strominger, J.L., and Wiley, D.C. (1994). Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature *368*, 215–221.
19. Nicholls, A., Sharp, K.A., and Honig, B. (1991). Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. Proteins *11*, 281–296.
20. Jones, S.T.J.M. (1998). Protein-Protein Interaction Server (http://www.biochem.ucl.ac.uk/bsm/PP/server/index.html).
21. Thibodeau, J., Dohlsten, M., Cloutier, I., Lavoie, P.M., Bjork, P., Michel, F., Leveille, C., Mourad, W., Kalland, T., and Sekaly, R.P. (1997). Molecular characterization and role in T cell activation of staphylococcal enterotoxin A binding to the HLA-DR alpha-chain. J. Immunol. *158*, 3698–3704.
22. Bolin, D.R., Swain, A.L., Sarabu, R., Berthel, S.J., Gillespie, P., Huby, N.J., Makofske, R., Orzechowski, L., Perrotta, A., Toth, K., et al. (2000). Peptide and peptide mimetic inhibitors of antigen presentation by HLA-DR class II MHC molecules. Design, structure-activity relationships, and X-ray crystal structures. J. Med. Chem. *43*, 2135–2148.
23. Mollick, J.A., Chintagumpala, M., Cook, R.G., and Rich, R.R. (1991). Staphylococcal exotoxin activation of T cells. Role of exotoxin-MHC class II binding affinity and class II isotype. J. Immunol. *146*, 463–468.
24. Sundberg, E., and Jardetzky, T.S. (1999). Structural basis for HLA-DQ binding by the streptococcal superantigen SSA. Nat. Struct. Biol. *6*, 123–129.
25. Marsh, S.G.E. (2002). HLA Class I and II Sequence Alignment (http://www.anthonynolan.org.uk/HIG/data.html).
26. Wallgren, A., Festin, R., Gidlof, C., Dohlsten, M., Kalland, T., and Totterman, T.H. (1993). Efficient killing of chronic B-lymphocytic leukemia cells by superantigen-directed T cells. Blood *82*, 1230–1238.
27. Tiedemann, R.E., and Fraser, J.D. (1996). Cross-linking of MHC class II molecules by staphylococcal enterotoxin A is essential for antigen-presenting cell and T cell activation. J. Immunol. *157*, 3958–3966.
28. Newton, D.W., Dohlsten, M., Olsson, C., Segren, S., Lundin, K.E., Lando, P.A., Kalland, T., and Kotb, M. (1996). Mutations in the MHC class II binding domains of staphylococcal enterotoxin A differentially affect T cell receptor Vbeta specificity. J. Immunol. *157*, 3988–3994.
29. Mehindate, K., Thibodeau, J., Dohlsten, M., Kalland, T., Sekaly, R.P., and Mourad, W. (1995). Cross-linking of major histocompatibility complex class II molecules by staphylococcal enterotoxin A superantigen is a requirement for inflammatory cytokine gene expression. J. Exp. Med. *182*, 1573–1577.
30. Kozono, H., Parker, D., White, J., Marrack, P., and Kappler, J. (1995). Multiple binding sites for bacterial superantigens on soluble class II MHC molecules. Immunity *3*, 187–196.
31. Nilsson, H., Bjork, P., Dohlsten, M., and Antonsson, P. (1999). Staphylococcal enterotoxin H displays unique MHC class II-binding properties. J. Immunol. *163*, 6686–6693.
32. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673–4680.
33. Al-Daccak, R., Mehindate, K., Damdoumi, F., Etongue-Mayer, P., Nilsson, H., Antonsson, P., Sundstrom, M., Dohlsten, M., Sekaly, R.P., and Mourad, W. (1998). Staphylococcal enterotoxin D is a promiscuous superantigen offering multiple modes of interactions with the MHC class II receptors. J. Immunol. *160*, 225–232.
34. Li, H., Llera, A., Tsuchiya, D., Leder, L., Ysern, X., Schlievert, P.M., Karjalainen, K., and Mariuzza, R.A. (1998). Three-dimensional structure of the complex between a T cell receptor beta

chain and the superantigen staphylococcal enterotoxin B. Immunity *9*, 807–816.
35. Antonsson, P., Wingren, A.G., Hansson, J., Kalland, T., Varga, M., and Dohlsten, M. (1997). Functional characterization of the interaction between the superantigen staphylococcal enterotoxin A and the TCR. J. Immunol. *158*, 4245–4251.
36. Irwin, M.J., Hudson, K.R., Fraser, J.D., and Gascoigne, N.R. (1992). Enterotoxin residues determining T-cell receptor V beta binding specificity. Nature *359*, 841–843.
37. Hennecke, J., Carfi, A., and Wiley, D.C. (2000). Structure of a covalently stabilized complex of a human alphabeta T-cell receptor, influenza HA peptide and MHC class II molecule, HLA-DR1. EMBO J. *19*, 5611–5624.
38. Redpath, S., Alam, S.M., Lin, C.M., O'Rourke, A.M., and Gascoigne, N.R. (1999). Cutting edge: trimolecular interaction of TCR with MHC class II and bacterial superantigen shows a similar affinity to MHC:peptide ligands. J. Immunol. *163*, 6–10.
39. Tiedemann, R.E., Urban, R.J., Strominger, J.L., and Fraser, J.D. (1995). Isolation of HLA-DR1.(staphylococcal enterotoxin A)2 trimers in solution. Proc. Natl. Acad. Sci. USA *92*, 12156–12159.
40. Lavoie, P.M., McGrath, H., Shoukry, N.H., Cazenave, P.A., Sekaly, R.P., and Thibodeau, J. (2001). Quantitative relationship between MHC class II-superantigen complexes and the balance of T cell activation versus death. J. Immunol. *166*, 7229–7237.
41. Lando, P.A., Olsson, C., Kalland, T., Newton, D., Kotb, M., and Dohlsten, M. (1996). Regulation of superantigen-induced T cell activation in the absence and the presence of MHC class II. J. Immunol. *157*, 2857–2863.
42. Coley, W.B. (1910). The treatment of inoperable sarcoma by bacterial toxins. Proc. R. Soc. Med. *3*, 1–48.
43. Dohlsten, M., Hedlund, G., Akerblom, E., Lando, P.A., and Kalland, T. (1991). Monoclonal antibody-targeted superantigens: a different class of anti-tumor agents. Proc. Natl. Acad. Sci. USA *88*, 9287–9291.
44. Alpaugh, R.K., Schultz, J., McAleer, C., Giantonio, B.J., Persson, R., Burnite, M., Nielsen, S.E., Vitek, L., Persson, B., and Weiner, L.M. (1998). Superantigen-targeted therapy: phase I escalating repeat dose trial of the fusion protein PNU-214565 in patients with advanced gastrointestinal malignancies. Clin. Cancer Res. *4*, 1903–1914.
45. Dohlsten, M., Lando, P.A., Bjork, P., Abrahmsen, L., Ohlsson, L., Lind, P., and Kalland, T. (1995). Immunotherapy of human colon cancer by antibody-targeted superantigens. Cancer Immunol. Immunother. *41*, 162–168.
46. Hansson, J., Ohlsson, L., Persson, R., Andersson, G., Ilback, N.G., Litton, M.J., Kalland, T., and Dohlsten, M. (1997). Genetically engineered superantigens as tolerable antitumor agents. Proc. Natl. Acad. Sci. USA *94*, 2489–2494.
47. Forsberg, G., Forsgren, M., Jaki, M., Norin, M., Sterky, C., Enhorning, A., Larsson, K., Ericsson, M., and Bjork, P. (1997). Identification of framework residues in a secreted recombinant antibody fragment that control production level and localization in Escherichia coli. J. Biol. Chem. *272*, 12430–12436.
48. Leslie, A.G.W. (1992). Joint CCP4 and ESF-EACMB Newsletter on Protein Crystallography No. 26 (Warrington, UK: Daresbury Laboratory).
49. CCP4 (Collaborative Computational Project 4) (1994). The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. *50*, 760–763.
50. Navaza, J. (1994). AMoRe: an automated package for molecular replacement. Acta Crystallogr. A *50*, 157–163.
51. McRee, D.E. (1999). XtalView/Xfit—a versatile program for manipulating atomic coordinates and electron density. J. Struct. Biol. *125*, 156–165.
52. Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. (1998). Crystallography and NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr. D Biol. Crystallogr. *54*, 905–921.
53. Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M. (1993). PROCHECK: a program to check stereochemical quality of protein structures. J. Appl. Crystallogr. *26*, 283–291.

Accession Numbers

The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession code 1LO5.