The crystal structure of staphylococcal enterotoxin type D reveals Zn^{2+} -mediated homodimerization

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Bacterial superantigens, including the staphylococcal enterotoxins, are the most potent activators of T cells known and have been suggested as a causative factor in Gram-positive shock in humans. Staphylococcal enterotoxin D (SED) is dependent upon Zn^{2+} for high affinity interactions with MHC class II molecules and thus SED was co-crystallized with Zn^{2+} . The crystal structure of SED has been determined in two different space groups, at 2.3 and 3.0 Å resolution respectively. The three-dimensional structure of SED is similar to structures of other bacterial superantigens, although this study has revealed that SED has the unique capability of forming dimers in the presence of Zn^{2+} . The high affinity Zn^{2+} site used in dimer formation is located on the surface of the β -sheet in the C-terminal domain. Two bound metal ions are coordinated by residues from both molecules in the dimer interface and thus contribute directly to formation of the dimer. A second Zn^{2+} site is located on the surface close to the domain interface of the molecule. The unique feature of SED in forming a Zn^{2+} -dependent homodimer seems to facilitate novel and biologically relevant multimeric interactions with MHC class II molecules, as shown by the induction of cytokine mRNA in human monocytes when exposed to SED and SED mutants.

Keywords: MHC class II molecules/staphylococcal enterotoxin D/superantigen/X-ray crystallography

Introduction

Superantigens differ from conventional antigens in that they bind as non-processed proteins with high affinity to major histocompatibility (MHC) class II molecules on antigen-presenting cells (APC) and activate a high frequency of T lymphocytes expressing certain T cell receptor (TCR) variable β -chains (V β). The activated T cells proliferate vigorously, regardless of antigenic specificity, and subsequently T cell- and monocyte-derived cytokines, such as interleukin-1 and -2 (IL), interferon- γ and tumour necrosis factor- α and - β (TNF) are produced in large amounts. The released cytokines most likely contribute to the development of staphylococcal and streptococcal toxin-

induced disease processes (reviewed in Marrack and Kappler, 1990).

The staphylococcal superantigens, excluding the functionally and structurally related toxic shock syndrome toxin 1 (TSST-1), can be divided into two subgroups based on sequence similarity. The first subgroup consists of staphylococcal enterotoxins A, D, E and H (SEA, SED, SEE and SEH) and the second of staphylococcal enterotoxins B and C1–C3 (SEB, SEC1, SEC2 and SEC3). The sequence identity of SED with the other staphylococcal superantigens ranges from 16% for TSST-1 to 53–55% for SEA and SEE respectively. SEA, SED and SEE are all dependent on Zn^{2+} for high affinity binding to MHC class II molecules, in contrast to SEB and SEC1–3 (Fraser *et al.*, 1992).

In recent years, elucidation of the crystal structures of free forms of SEA (Schad *et al.*, 1995), SEB (Swaminathan *et al.*, 1992), SEC2 (Papageorgiou *et al.*, 1995) and TSST-1 (Prasad *et al.*, 1993; Acharya *et al.*, 1994), as well as the SEB–MHC class II (Jardetzky *et al.*, 1994) and TSST-1–MHC class II complexes (Kim *et al.*, 1994), have revealed some of the structural constraints for superantigen-mediated protein–protein interactions. The structure of SEB bound to a MHC class II molecule showed that the superantigen binds to the α -chain of the MHC class II molecule outside the peptide antigen-binding groove. A comparison of the crystal structures of the non-complexed MHC class II molecule and the free form of SEB with the SEB–MHC class II complex showed that no large conformational change in either component occurred upon complex formation. The three-dimensional structure of TSST-1 complexed with the MHC class II molecule shows that the TSST-1 binding site on MHC class II molecules overlaps that of SEB but covers a larger area on the MHC molecule.

The recently determined crystal structures of the free forms of SEA and SEC2 revealed a metal binding site in each protein. The metal binding site in SEA is located on the surface of the β -sheet of the C-terminal domain, whereas the SEC2 Zn^{2+} site is partly buried in the interface between the N- and the C-terminal domains. Site-directed mutagenesis of SEA was recently used to confirm that coordination of Zn^{2+} is required for high affinity binding of this superantigen to MHC class II molecules. SEA most likely binds bivalently to both the α - and the β -chains, utilizing the SEB-like site in the first case and the Zn^{2+} -binding site in the latter (Abrahmsén *et al.*, 1995; Hudson *et al.*, 1995), in contrast to SEB, which binds monovalently to only the α -chain.

In this study, the crystal structure of SED solved in two different space groups is presented and shown to be a Zn^{2+} -dependent homodimer. Further, the biological relevance of the observed crystal structure homodimer was supported by studies showing that SED induced

cytokine mRNA expression in a monocytic cell line using unique multimeric modes of cross-linking MHC class II molecules.

Results

Structure determination

Two out of the three high affinity Zn^{2+} coordinating residues seen in SEA are conserved in SED and the third, a histidine, is replaced by an aspartate. Consequently, it was expected that SED would also contain a Zn^{2+} -binding site. In order to study the structure of such a protein-metal ion complex, SED was pre-incubated with 100 μM Zn^{2+} prior to crystallization. Protein pre-incubated with Zn^{2+} crystallized readily, whereas metal-depleted material did not.

The three-dimensional structure of SED was first determined using data from crystals in the space group P4_3 grown at pH 6.75. The initial structure was solved using modified structures of SEB and SEA as search molecules in a molecular replacement procedure. The asymmetric unit contained two SED molecules and their structures were partly refined to 3.0 Å resolution. This partly refined P4_3 SED structure was used to solve the second crystal form belonging to space group C2 , grown at pH 7.5. Here data had been collected to 2.3 Å resolution. As in the former case, the asymmetric unit contained two SED molecules. The structural features of SED discussed below are derived from this latter crystal form, although a comparison between the two SED structures revealed that they were virtually identical.

At the present stage of refinement the SED model consists of residues 6–53, 58–91, 98–166 and 173–228 for both molecules in the asymmetric unit. Furthermore, three Zn^{2+} ions and 143 solvent molecules have been included. The bound Zn^{2+} ions in the asymmetric unit were easily identified as pronounced densities in F_oF_c electron density maps. The crystallographic R factor is 21.4% (R free 29.2%) in the resolution interval 10–2.3 Å.

The SED monomer

The expected two domain architecture of the molecule is seen, although the designation of SED as a two domain protein might be misleading, since this and all other staphylococcal superantigens are compact molecules. A β -barrel is seen in the N-terminal domain (residues 15–102) and a β -grasp motif constitutes the major part of the C-terminal domain (residues 117–228). The two domains are connected by a 15 residue loop. The N-terminal five residues are not visible in the electron density map, while residues 6–14 pack against the C-terminal domain (Figure 1). Secondary structure elements are listed in Table I.

The refined structure reveals an expected close similarity in general architecture to other structures determined for staphylococcal superantigens, with an overall root mean square (RMS) deviation of 0.89 Å to SEA, 1.69 Å to SEB and 1.98 Å to TSST-1, comparing 201, 175 and 151 structurally equivalent $\text{C}\alpha$ positions respectively. The major differences between SED and SEA/SEB resides in flexible loop regions. The RMS deviation between the two SED molecules in the asymmetric unit is 0.41 Å.

The first five residues in the N-terminus, loop 54–57, the disulfide loop 92–97 and the loop 167–177 are

Fig. 1. A schematic picture of the SED monomer. Secondary structure elements are shown as arrows (β -strands, $\beta 1$ – $\beta 12$) and spirals (α -helices, $\alpha 1$ – $\alpha 4$). Two bound Zn^{2+} ions are shown as spheres. The N- and C-termini are labelled. This figure was drawn using Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

Table I. Secondary structure elements

Residue number	Type	Residue number	Type
1–5	not visible	117–126	strand 6
6–14	loop 1	127–128	loop 9
15–25	helix 1	129–133	strand 7
26–30	loop 2	134–137	loop 10
31–38	strand 1a	138–140	strand 8
39	glycine	141–146	loop 11
40–42	strand 1b	147–160	helix 3
43–45	loop 3	161–166	loop 12a
46–50	strand 2	167–172	not visible
51–53	loop 4a	173–175	loop 12b
54–57	not visible	176–183	strand 9
58–61	loop 4b	184–186	loop 13
62–66	strand 3	187–192	strand 10
67–68	loop 5	193–200	loop 14
69–75	helix 2	201–205	helix 4
76–79	loop 6	206–210	loop 15
80–83	strand 4	211–215	strand 11
84–91	loop 7	216–217	loop 16
92–97	not visible	218–227	strand 12
98–102	strand 5	228	C-terminus
103–116	loop 8		

Secondary structure assignments from the program DSSP (Kabsch and Sander, 1983).

apparently unordered or have multiple conformations and could not be model built. It might be worthwhile to point out that the disulfide loop in SEB was also found to be unordered in the MHC class II complex (Jardetzky *et al.*, 1994).

The SED homodimer

In contrast to previously determined structures for superantigens, SED crystallizes as a Zn^{2+} -dependent dimer (Figure 2). A high affinity Zn^{2+} site corresponds to the metal binding site in SEA and is located on the surface

Fig. 2. The SED homodimer. Two SED molecules are bridged by two Zn^{2+} ions, drawn as spheres, in the homodimer interface. This figure was drawn using Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

of the C-terminal β -sheet. The C-terminal β -sheets of two SED molecules pack against each other and bury a large solvent-inaccessible interface area, of $\sim 1200 \text{ \AA}^2$, in which two Zn^{2+} ions are located. Thus the two C-terminal domain β -sheets pack against each other in an antiparallel fashion.

In order to verify whether the SED dimer also appears in solution, gel filtration chromatography was performed in the presence (100 μM) and absence of Zn^{2+} . The major part of the SED protein eluted as an apparent dimer in the presence of Zn^{2+} . In contrast, an EDTA (100 μM)-treated sample of SED eluted only as monomer (data not shown). In a second approach, covalent cross-linking of SEA and SED was performed in the presence and absence of Zn^{2+} . From these experiments it was clear that SED formed dimers (and possibly trimers) in solution at a concentration equal to or lower than 5 μM Zn^{2+} . In contrast, SEA did not show any tendency to form dimers in either experimental approach. To confirm that the ability

Fig. 3. Biochemical analysis of homodimerization. SDS-PAGE of cross-linked SEA and SED. Following di-succinimidyl suberate treatment, SEA and SED were electrophoresed in a 12% SDS-polyacrylamide gel and Coomassie stained. Lane 1, SEA_{wt} with 100 μM EDTA; lane 2, SEA_{wt} with 50 μM Zn^{2+} ; lane 3, SED_{wt} with 100 μM EDTA; lane 4, SED_{wt} with 10 μM Zn^{2+} ; lane 5, SED_{F42A} with 10 μM Zn^{2+} ; lane 6, SED_{D182A} with 10 μM Zn^{2+} ; lane 7, SED_{H218A} with 10 μM Zn^{2+} ; lane 8, SED_{D222A} with 10 μM Zn^{2+} .

Fig. 4. The homodimer interface of SED. The ligands that coordinate Zn^{2+} tetrahedrally are shown. Ligands from one molecule are shown in yellow and from the other in green. This figure was drawn using Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

of SED to form homodimers was dependent upon Zn^{2+} ion coordination, we introduced alanine substitutions of residues predicted to contribute to homodimerization. Mutants with alanine substitutions in the high affinity Zn^{2+} ligands, Asp182, His218 and Asp222, all lacked the ability to form dimers in cross-linking experiments. In contrast, alanine substitution of Phe42, in the SEB-like MHC class II binding site, did not affect homodimerization (Figure 3).

The Zn^{2+} binding sites

Two Zn^{2+} ions are symmetrically coordinated in the homodimer interface. Each is tetrahedrally coordinated by His18 from one SED molecule and Asp182, His220 and Asp222 from the other, and vice versa (Figure 4). Thus, tetrahedral binding of two Zn^{2+} ions in the homodimer interface is seen. These latter three ligands are in the same position as the corresponding high affinity ligands in SEA, i.e. His187, His225 and Asp227. His218 corresponds to Asn223 in SEA. In contrast, the metal ion in SEA co-crystallized with Cd^{2+} (SEA- Cd^{2+}) was octahedrally

Fig. 5. (A) Proliferation of human PBM and (B) superantigen-dependent cellular cytotoxicity of SED_{wt} (◆), SED_{F42A} (■), SED_{D182A} (▲), SED_{H218A} (●) and SED_{D222A} (□). Human PBM were stimulated for 96 h and proliferation was measured as incorporation of [³H]thymidine after a 24 h pulse. Cytotoxicity was analysed in a 4 h ⁵¹Cr release assay using a SED-reactive human T cell line and Raji as the target cell lines.

Fig. 6. Cross-linking of MHC class II molecules by SED_{wt} or SED_{H218A} is required for induction of IL-1β and TNF-α mRNA expression. The inhibition by SEA mutants of (A) SED_{H218A}- and (B) SED_{wt}-induced cytokine expression in the human THP-1 monocyte cell line is shown. THP-1 cells (5×10^6 cells/ml) were stimulated for 1 h with SED_{H218A} or SED_{wt} (0.5 μg/ml). Pre-treatment of the cells for 30 min with medium alone, SEA_{F47A} or SEA_{F47A} + SEA_{D227A} (5 μg/ml) was performed prior to the addition of SED proteins. The SEA_{F47A} and SEA_{D227A} mutants are expected to bind monovalently to the HLA-DR β- or α-chain respectively.

coordinated involving the N-terminal Ser1 amino nitrogen and γ oxygen and a water molecule in addition to the three high affinity ligands discussed above. Based on a preliminary crystal structure of SEA-Zn²⁺ in the same space group as the SEA-Cd²⁺ structure, Schad *et al.* suggested that Zn²⁺ could also be octahedrally coordinated (Schad *et al.*, 1995).

A comparison between the published SEA-Cd²⁺ structure and the SED structure presented here has to take into account that the heterologously expressed superantigens involved differ in the N-terminal part. SED lacks the first five N-terminal residues compared with SEA and, accordingly, the N-terminus cannot participate in Zn²⁺ coordination. Further, tetrahedral Zn²⁺ coordination, as seen for SED, is the norm in metal binding proteins (reviewed in Vallée and Auld, 1990). Other important residues around the Zn²⁺ binding motif are Asn123, Trp125, Lys188 and Tyr224, whose side chains create a network of polar and non-polar interactions.

A second metal binding site, located in a similar position as reported for SEC2 (Papageorgiou *et al.*, 1995), is seen

in both crystal forms, but here the metal ion is shared between the two SED molecules in the asymmetric unit. The Zn²⁺ ion is coordinated by two ligands from one molecule, His8 and Glu12, and His109 and Lys113 from the second molecule in the asymmetric unit. The use of lysine as a Zn²⁺ ligand is unusual, but has previously been observed in aminopeptidases where the primary amine is a Zn²⁺ ligand in a two metal co-catalytic site (Thompson and Carpenter, 1976).

SED is a potent superantigen

SED_{wt} stimulates proliferation in human peripheral blood mononuclear cells (PBM) with an EC₅₀ value of 0.6 pM, compared with 0.8 pM for SEA_{wt}. The SED mutant Phe42→Ala (SED_{F42A}) showed a decreased ability to induce proliferation in PBM (EC₅₀ 49 pM), which corresponded well with the results obtained for the analogous mutant in SEA Phe47→Ala (SEA_{F47A}; EC₅₀ 59 pM). Disruption of the SED high affinity zinc binding site by the mutants Asp182→Ala (SED_{D182A}) and Asp222→Ala (SED_{D222A}) decreases the ability to induce proliferation in PBM dramatically. The EC₅₀ values for SED_{D182A} and SED_{D222A} were 430 and 5200 pM respectively, which is substantially higher compared with the corresponding SEA mutants His187→Ala (SEA_{H187A}; EC₅₀ 8.2 pM) and Asp227→Ala (SEA_{D227A}; EC₅₀ 790 pM). In this assay substitution of the SED residue His218→Ala (SED_{H218A}) had no effect on proliferation. In a T cell cytotoxicity assay against MHC class II-positive Raji cells, a similar pattern is observed, where the corresponding EC₅₀ values for SED_{wt}, SED_{F42A}, SED_{D182A}, SED_{H218A} and SED_{D222A} are 0.1, 6.3, 46, 0.7 and 81 pM respectively (Figure 5).

We have previously shown that cross-linking of MHC class II on the surface of human monocyte THP-1 cells by SEA induces mRNA expression of the inflammatory cytokines TNF-α and IL-1β (Mehindate *et al.*, 1995). This effect most likely involves cross-linking of MHC class II α- and β-chains, since cytokine induction can be inhibited by substituting either Phe47 or any high-affinity zinc ligand in SEA with alanine. In addition, pre-treating the monocytes with the mutants SEA_{F47A} (which is expected to bind only the HLA-DR β-chain) or SEA_{D227A} (which is expected to bind only the HLA-DR α-chain) inhibited the effect of SEA_{wt}. In this study we show that SED_{wt} and SED_{H218A} efficiently induced TNF-α and IL-1β mRNA

Fig. 7. A comparison between the α -chain binding motif in SEB with the corresponding region in SED. SEB residues are in green and SED residues in yellow. The structural alignment was performed using the least squares fit option in the program O (Jones *et al.*, 1991). The figure was drawn using Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

expression in THP-1 cells, suggesting that this superantigen cross-links MHC class II molecules. Interestingly, the addition of SEA_{F47A} blocked cytokine induction by SED_{H218A} but failed to influence SED_{wt} (Figure 6). The results are compatible with the view that the SED mutant His218→Ala induces cytokine mRNA expression using α - β cross-linking, whereas SED_{wt} activates monocytes by either α - α or α - β cross-linking. Indeed, combined addition of SEA_{F47A} and SEA_{D227A} mutants completely blocked the ability of SED_{wt} to induce cytokine mRNA production (Figure 6).

The SEB-like MHC class II binding site

In the structure of the SEB-MHC class II complex, favourable hydrogen bonds and hydrophobic interactions are observed between the SEB residues Gln43, Phe44, Tyr46, Glu63, Tyr85 and Tyr99 and the α -chain of the receptor. The corresponding binding epitope in SEA has been verified by site-directed mutagenesis, where a substitution Phe47→Ala markedly reduced the ability to induce MHC class II-dependent T cell proliferation and impaired cross-linking of MHC class II molecules on human monocytes (Abrahamsén *et al.*, 1995; Mehindate *et al.*, 1995).

Similar effects are seen with the substitution Phe42→Ala in SED (data not shown). The structural homology between SEB and SED in this region is remarkable, including the orientation of the corresponding side chains (Figure 7). Thus the same binding mode for SED and SEB to the MHC class II α -chain seems likely.

Discussion

The SED monomer is a compact 'two domain' protein consisting of a β -barrel in the N-terminal domain and a variant of a β -grasp motif (a β -sheet packing towards a central helix) in the C-terminal domain. The crystal structure of SEA, co-crystallized with Cd²⁺, revealed that it was highly homologous in topology to SEB and SEC2 and to a lesser extent to TSST-1. As expected, SED is more similar to SEA in its three-dimensional structure than to SEB or TSST-1. However, SED has a unique feature amongst superantigens; it forms Zn²⁺-dependent homodimers. In the interface of the homodimers two Zn²⁺ ions are identically coordinated by Asp182, His220 and Asp222 from one molecule and by His218 from the other, thereby the two metal ions are symmetrically coordinated. The SED homodimers observed in both crystal forms also occur in solution in the presence of Zn²⁺. The crystal structure interpretation is supported by the observation that alanine substitutions of either Asp182, His218 or Asp222 prevent dimer formation.

A second Zn²⁺-binding site in SED is observed in both crystal forms, similar to that reported for SEC2. The metal ion is coordinated by two ligands from each molecule in the asymmetric unit. Consequently, the two SED molecules in the asymmetric unit bind three Zn²⁺ ions, of which two are found in the homodimer interface. One might therefore argue that the second Zn²⁺ site is a metal binding site induced by the crystallization conditions, rather than a specific Zn²⁺ binding site. However, a stoichiometry of close to two Zn²⁺ ions bound per SED monomer in solution was found using titration calorimetry. In similar experiments, SEA bound Zn²⁺ in equimolar amounts (D.Hallén, personal communication). The functional role of these two Zn²⁺ binding sites, as well as the metal ion itself, remains to be shown. However, a fair assumption would be that a major role for the SEA-like Zn²⁺ site in the dimer interface is to participate in SED dimer formation or SED-MHC class II cross-linking. The role of the second site, if any, is more uncertain, but it could have a 'structural' function in the maintenance of a preferable local structural conformation.

The three homodimer interface Zn²⁺ ligands in SED (Asp182, His220 and Asp222) residing in the same molecule correspond to the three high affinity Zn²⁺ ligands in SEA (His187, His225 and Asp227). Despite this similarity, SEA does not form homodimers in the presence of Zn²⁺. One difference between the SEA and SED structures is that the SEA N-terminus participates in metal ion coordination. A Cd²⁺ ion was used in the published structure, but unpublished data suggest that the same coordination was observed with Zn²⁺ (Schad *et al.*, 1995).

SEA has been shown to contain two binding sites for the MHC molecule. One low affinity site resembled the hydrophobic binding motif seen in the SEB-MHC class

Fig. 8. A Schematic picture of a model of two MHC class II molecules cross-linked by the SED Zn^{2+} -dependent homodimer. Two SEB–MHC class II complexes were independently structurally aligned with the SED homodimer using the least squares fit option in the program O (Jones *et al.*, 1991). The SEB molecules of the complex were removed assuming a close similarity in binding of SED to the α -chain of the MHC class II molecule. The figure was drawn using Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

II complex and a second site was located around the Zn^{2+} ion. It has been postulated that the metal ion could act as a bridge between the β -chain of MHC class II molecules and the SEA molecule. In the formation of this SEA–MHC class II complex three Zn^{2+} ligands are postulated to be derived from the superantigen and the fourth from the receptor (Abrahmsén *et al.*, 1995). The postulated coordinating residue in the MHC class II β -chain is an exposed histidine residue, His81 (Herman *et al.*, 1991). Judged from the SEA– Cd^{2+} structure, the SEA N-terminus would have to disengage from the metal ion in order to allow the suggested ligand function of the MHC class II residue. However, SEA pre-incubated with Zn^{2+} can co-crystallize with the metal ion without involvement of the N-terminal serine. In this crystal form the Zn^{2+} coordination is tetrahedral and the N-terminus is apparently unordered (Sundström *et al.*, 1996). The reported N-terminus of SEA (Betley and Mekalanos, 1988) corresponds with the expected product from signal peptide processing (von Heijne, 1986). In contrast, signal peptide processing of SED should contain five additional residues compared with the reported N-terminus (Bayles and Iandolo, 1989). To resolve this question, we performed N-terminal sequencing of SEA and SED produced in

Table II. Results of N-terminal sequencing of SEA and SED produced by their native host *S. aureus*

Processing region	Percentage of sample with indicated terminus		
	1	2	3
TLTTSPLVNG—SEKSEEINEK	<5	70	30
LVISPLNVKA—NENIDSVKEK	30	40	30

SEA (top) and SED around the signal peptide processing site. Ten amino acid residues on either side of the predicted processing sites are shown.

Staphylococcus aureus. The results showed that both SEA and SED were mixtures of three forms, one having the expected N-terminus resulting from signal peptide processing and two additional forms with three or five amino acid residues deleted (Table II). Thus, it was confirmed that the SED used in this study is biologically relevant, but raises the question whether longer forms of SED as well as the shorter forms of SEA could have the ability to form homodimers similar to SED in the presence of Zn^{2+} ions. In this case the fourth ligand in SEA, residing on a postulated neighbouring molecule, would be

Asn223, in contrast to His218 in SED. Although histidine is a superior ligand, asparagine could also participate in metal ion coordination.

A comparison of the three-dimensional structures of SEB and TSST-1 complexed with MHC class II molecules showed that the TSST-1 binding site overlaps that of SEB but that the two binding modes differ. As seen in the SEB-MHC class II structure (Jardetzky *et al.*, 1994) and shown by SEA mutagenesis studies (Abrahmsén *et al.*, 1995), a motif in domain I centred around a conserved phenylalanine (Phe44 in SEB, Phe47 in SEA and Phe42 in SED) represent a common binding site for the MHC II class II α -chain. This region has a remarkable structural similarity in the SEA-Cd²⁺, SEB and SED structures. SED Phe42→Ala and SEA Phe47→Ala mutants (Abrahmsén *et al.*, 1995; Mehindate *et al.*, 1995) displayed reduced activity in MHC class II-dependent T cell activation assays, suggesting a similar role for this motif in these superantigens.

Thus, the staphylococcal superantigens have developed multiple binding modes for MHC class II molecules (HLA-DR), as exemplified by the SEB-HLA-DR α -chain dimeric complex, the TSST-1 interaction with the HLA-DR α -chain and antigenic peptide on a single HLA-DR molecule and the Zn²⁺-dependent SEA-HLA-DR β -chain binding. The dual binding sites of SEA imply the possibility of trimeric HLA-DR α -SEA-HLA-DR β complex formation. This hypothesis was strongly supported by the results showing that binding of SEA to MHC class II-positive monocytes induces production of the pro-inflammatory cytokines IL-1 β and TNF- α , in contrast to mutants lacking either the SEB-like MHC class II binding site (Phe47→Ala) or the Zn²⁺-dependent site (Asp227→Ala) (Mehindate *et al.*, 1995). As expected, binding of SED to MHC class II molecules seems to be dependent on Zn²⁺ ions (P. Antonsson, unpublished observation). Thus it is reasonable to suggest that SED has the ability to cluster MHC class II molecules, similar to that shown by SEA. Indeed, in this study we could confirm that SED efficiently induces activation of monocytes. The data further suggest that both α - α and α - β chain cross-linking of MHC class II molecules was achieved by SED. Pre-treatment of monocytes with SEA_{F47A} failed to interfere with the ability of SED_{wt} to induce IL-1 β and TNF- α mRNA expression. In contrast, the effects of SED_{H218A} and SEA_{wt} (Mehindate *et al.*, 1995) on cytokine expression were completely blocked by addition of SEA_{F47A}. Thus, it is reasonable to argue that Zn²⁺-dependent SED homodimerization as observed in the crystal structure facilitates novel multimeric superantigen-MHC class II molecule interactions. The MHC class II α -chain is monomorphic, whereas the β -chain can be highly polymorphic. In certain β -chain alleles, such as HLA-DRw53, His81 is substituted by tyrosine and thus the binding of SEA is abrogated (Karp and Long, 1992). The ability of SED to utilize either α - α or α - β chain interactions may preserve the capacity to induce MHC class II cross-linking, irrespective of polymorphism within the MHC class II β -chain. The properties of SED_{H218A} differ only marginally from SED_{wt} in the proliferation and cytotoxicity assays. However, the SED_{H218A} molecule still has the capability to cross-link MHC class II molecules

efficiently through α - β chain interactions which seems to be sufficient for activation of these T cell responses.

Taking the above observations into account it seems plausible that SED could have multimeric modes of interacting with target receptors. As seen in the cytokine induction assay, the SED dimer could be present in plasma but dissociate upon binding to MHC class II molecules and bind in the SEA-like manner discussed above. In addition, the SED dimer can offer a further mode of clustering MHC class II molecules: a tetrameric HLA-DR α -SED-SED-HLA-DR α complex (Figure 8). The differentiated binding modes, either as a dimer or a monomer, could possibly activate different subsets of T cells by interactions with separate V β -chains, rendering the SED mechanism of action highly complex.

Material and methods

Chemicals and equipment

If otherwise not stated, all chemicals were purchased from Sigma (USA) or Fluka (Switzerland). All protein purification equipment and materials were from Pharmacia Biotechnology (Sweden). SEA and SED from *S.aureus* used in N-terminal sequencing were purchased from Toxin Technology Inc. (USA).

Cloning, in vitro mutagenesis and expression of SED

SED was cloned using the polymerase chain reaction (PCR) on a crude DNA extract from a SED-positive *S.aureus* strain (ATCC No. 23235). The sequence of the cloned SED was identical to earlier reported SED sequences (Bayles and Iandolo, 1989) except for a single point mutation, C→G, substituting Pro84 in the mature SED protein by alanine. Direct sequence analysis of the PCR product revealed the same point mutation, indicating that this is a naturally occurring variant of SED. Unique restriction enzyme sites were introduced by site-directed mutagenesis to be used as recombination sites for fragments containing PCR-generated point mutations. PCR reactions were performed with AmpliTaq (Perkin-Elmer, USA) according to the recommendations of the manufacturer. PCR-produced fragments were cloned in PCR-script (Stratagene, USA) and sequenced. The SED variants were subcloned in the expression vector pKP1055 (Abrahmsén *et al.*, 1995). *Escherichia coli* K12 strain UL635 harbouring the different pKP1055-SED constructs was grown overnight at 24°C. Periplasmic extracts from the cultures were subjected to further purification.

Protein purification

Following fermentation the culture medium was clarified by centrifugation, the pH adjusted to 7.0 and Tween 80 added to 0.02%. Flow-through from a DEAE column was applied to a SP Sepharose HP column. Following washing the column was eluted with a gradient of 20 mM ammonium acetate, pH 7.5, 0.02% Tween 80 to 500 mM ammonium acetate, pH 7.5, 0.02% Tween 80. Wild-type SED was recovered from the flow-through from a DEAE column and applied to a SP Sepharose HP column. Following washing the column was eluted with a gradient of 20 mM ammonium acetate, pH 7.5, 0.02% Tween 80 to 500 mM ammonium acetate, pH 7.5, 0.02% Tween 80. Culture medium containing SED mutants was affinity purified on an anti-SED rabbit IgG column, coupled to an NHS-activated HiTrap column. Bound material was eluted as a single peak with 0.1 M sodium citrate, pH 2.5, 0.02% Tween 80 and subsequently neutralized with 1 M Tris, pH 9.0, 0.02% Tween 80. The material was concentrated using Centrprep-10 (Amicon, USA) and transferred to 10 mM ammonium acetate, pH 6.8, 0.02% Tween 80 using PD-10 columns. Wild-type SED as well as SED mutants were shown to be >95% pure when run on pre-cast 10–15% gradient SDS-polyacrylamide gels.

N-terminal sequencing

N-terminal amino acid sequence determinations were according to Coull *et al.* (1991), using an automated solid-phase Prosequencer 6600 instrument equipped with a Maxima data handling system (Millipore Corporation, USA). The proteins were covalently bound to Sequelon DITC membranes from the same company.

Biochemical analysis of homodimerization

Aliquots of 1–5 µg SED_{wt} or SED mutants in 10 mM ammonium acetate were added to a final volume of 20 µl 20 mM HEPES buffer, pH 7.4, with 150 mM NaCl and the desired amount of Zn²⁺ ions or EDTA. To this buffered protein solution 1 mM cross-linking agent (di-succinimidyl glutarate or di-succinimidyl suberate; Pierce, USA) dissolved in dimethylsulfoxide was added and the reaction carried out at 37°C for 5–10 min. To terminate the reaction, 1 µl 2 M Tris was added. The cross-linked protein was separated using SDS-PAGE gel electrophoresis and stained using standard techniques. Gel filtration chromatography of SED and SEA (2 mg/ml) pre-incubated with either ZnSO₄ (100 µM) or EDTA (100 µM) was performed at pH 7.5 in 20 mM Tris with 100 mM NaCl using a HR 5/20 G-50 Sephadex superfine gel filtration column. The elution times of monomeric SEA/SED and dimeric SED were compared with that of a molecular weight standard, containing commercially available protein in the molecular weight range of 13–66 kDa, used to calibrate the columns in the presence of Zn²⁺ or EDTA.

Cells

The human B cell lymphoma cell line Raji was cultured in complete R medium RPMI-1640 supplemented with 10% fetal calf serum (Gibco, USA) 1 mM glutamine, 5×10^{-5} M β -mercaptoethanol, 0.1 M NaHCO₃, 1×10^{-2} M HEPES buffer, 0.1 mg/ml gentamycin (Biological Industries, Israel), 1×10^{-3} M sodium pyruvate. Peripheral blood mononuclear cells were prepared from heparinized blood from normal donors. The cells were isolated by density centrifugation over Ficoll-Paque as previously described (Dohlsten *et al.*, 1991). The human SED-reactive cell line was generated as previously described (Dohlsten *et al.*, 1994). The THP-1 monocytic cell line was obtained from the American Type Tissue Collection (Rockville, MD) and cultured as described earlier (Mehindate *et al.*, 1995).

Cytotoxicity assay

Cytotoxicity was measured in a standard ⁵¹Cr release assay after 4 or 6 h as previously described (Dohlsten *et al.*, 1991). Human Raji cells were used as target cells. The effector cells, SED-reactive human T cell lines, were added at an effector:target ratio of 30:1. ⁵¹Cr-labelled target cells were used in the cytotoxicity assays at 2500 cells/200 µl complete medium in V-bottomed microtitre wells. SED_{wt} or SED mutants were added at various concentrations as indicated and ⁵¹Cr release was measured in a γ counter. The percentage specific cytotoxicity was calculated as $100 \times [(c.p.m. \text{ experimental release} - c.p.m. \text{ background release}) \div (c.p.m. \text{ total release} - c.p.m. \text{ background release})]$.

Lymphocyte proliferation assays

To measure proliferation 10^5 human PBM were incubated at 37°C in 200 µl complete medium in U-shaped 96-well microtitre plates with varying amounts of SED_{wt} or SED mutants for 72 h. Proliferation was estimated by incorporation of [³H]thymidine.

Northern blot analysis

THP-1 cells were resuspended in RPMI containing 5% foetal calf serum (5×10^6 cells/ml), pre-treated for 30 min with medium alone, SEA_{F47A} or SEA_{F47A} + SEA_{D227A} (5 µg/ml), then stimulated for 1 h with SED_{H218A} or SED_{wt} (0.5 µg/ml). The reaction was stopped and RNA purified as previously described (Sambrook *et al.*, 1989). RNA (10 mg/well) was loaded onto 1% agarose gels and after electrophoresis transferred onto Hybond-N filter paper and hybridized with random primer-labelled cDNA probes for IL-1 β and TNF- α (Mehindate *et al.*, 1995). Equal loading of RNA were confirmed by hybridization with glyceraldehyde 3-phosphate dehydrogenase cDNA. The mRNA hybridizing with the cDNA probes was visualized by autoradiography.

Crystallization of SED

SED was crystallized using vapour diffusion. Two forms of crystals were optimized. The first, which crystallized in space group P₄₃, was grown by mixing 3 µl protein solution (10 mg/ml) containing 100 µM ZnSO₄ with 3 µl 3–8% (w/v) polyethylene glycol (PEG) 20000 in 0.1 M MES buffer, pH 6.75, in a sealed tissue culture 24-well plate (Falcon, USA). The crystallization droplets were equilibrated at 18°C with 1 ml of the mother liquor for 2–3 weeks to obtain optimal diffraction quality crystals. Crystals were $0.3 \times 0.3 \times 0.3$ mm in size and diffracted to 2.9 Å with a conventional X-ray source. The second form in space group C2 were grown by the same method, mixing 3 µl protein solution (10 mg/ml) containing 100 µM ZnSO₄ with 3 µl 500 mM NH₄SO₄, 9–15% PEG 6000, 25 mM MgCl₂ in 0.1 M HEPES buffer, pH 7.5. The crystallization droplets were equilibrated at 18°C with 1 ml of the mother liquor for

Table III. Crystallographic data for the P₄₃ and C2 crystal forms of SED

	P ₄ ₃	C2
No. of crystals	1	2
Resolution	3.0 Å	2.3 Å
Completeness	98.1% (20–3.0 Å)	98.6% (20–2.3 Å)
Multiplicity	3.0	2.8
<i>R</i> _{merge}	9.8% (20–3.0 Å) 33.2% (3.1–3.0 Å)	6.7% (20–2.3 Å) 26.6% (2.4–2.3 Å)
Cell	77.57 77.57 126.41 Å 90° 90° 90°	172.98 40.40 93.69 Å 90° 120.8° 90°
No. of solvent molecules	0	143
Unique reflections	14736	24746
RMS bond deviations (Å)	ND	0.011
RMS angle deviations (°)	ND	1.64
Model <i>R</i> factor/ <i>R</i> free	24.9/35.6%	21.4/29.7%

1–2 weeks to obtain optimal diffraction quality crystals. Crystals were $1.0 \times 0.5 \times 0.25$ mm in size and diffracted to at least 2.3 Å with a conventional X-ray source.

Data collection and processing

All data were collected using a MAR image plate system. The data were processed with MOSFLM (CCP4, 1994) using the reflex algorithm (Kabsch, 1988) for indexing and point group determination and then further reduced and scaled using the CCP4 program package (CCP4, 1994). For crystallographic data see Table III.

Structure determination

At the time this work was initiated the only superantigen coordinates available were for the SEB-HLA-DR1 complex. A modified search molecule for molecular replacement was created where SEB was converted to a polyalanine model except for those residues that were identical in SED and SEB (~80 residues). The AMORE (Navaza, 1994) molecular replacement solution obtained with this modified SEB model was verified using a similarly modified SEA-Cd²⁺ model when these coordinates were made available to us. The highest scoring solution in the resolution interval 8–4 Å was found in space group P₄₃ with two SED molecules in the asymmetric unit. A rigid body refinement in X-plor (Brünger, 1992a) preceded a careful cyclic process of model building in O (Jones *et al.*, 1991), making corrections for main chain differences as well as introduction of clearly visible side chains and POWELL minimizations in X-plor using data between 15 and 3.0 Å. This cyclic procedure was repeated until ~80% of the side chains were visible at 3.0 Å resolution. The *R* factor for this model was 24.9% (*R* free 33.6%).

Using this 3.0 Å model as a search model for the C2 crystal form revealed the highest scoring solution in the resolution interval 10–3.5 Å with two SED molecules in the asymmetric unit. All data between 15 and 2.5 Å were used in a process similar to the initial minimization and model building process for the P₄₃ crystal form. In addition, NCS restraints as well as simulated annealing (Brünger *et al.*, 1989) refinement steps in X-plor were included, to create a refined model with an *R* value of 21.4% (*R* free 29.7%) after a final *B* value refinement. Solvent molecules were manually introduced into F_o-F_c densities above 3.0 σ . After three cycles, 180 solvent molecules had been introduced. A final POWELL minimization followed by a dynamics run from 2500 to 300 K in 50 ps steps including data between 10 and 2.3 Å was performed. *B* value refinement was added as the final step and solvent molecules with high temperature factors (>45 Å²) as well as those with absent 2F_o-F_c electron densities at 1 σ cut-off were removed, leaving 143 solvent molecules in the final model. The free *R* value (Brünger, 1992b) was used to validate the progress of the entire refinement. Solvent-accessible areas were calculated using the program DSSP (Kabsch and Sander, 1983), the quality of the model assessed using PROCHECK (Laskowski *et al.*, 1993) and structural alignments performed using the least square fit procedure in the program O. The coordinates of the SED structure will be deposited in the Brookhaven Protein Data Bank.

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