Staphylococcal Enterotoxin A has Two Cooperative Binding Sites on Major Histocompatibility Complex Class II

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Summary

The superantigen staphylococcal enterotoxin A (SEA) binds to major histocompatibility complex (MHC) class II molecules at two sites on either side of the peptide groove. Two separate but cooperative interactions to the human class II molecule HLA-DR1 were detected. The first high affinity interaction to the DR1 β chain is mediated by a zinc atom coordinated by H187, H225, and D227 in SEA and H81 in the polymorphic DR1 β chain. The second low affinity site is to the DR1 α chain analogous to SEB binding and is mediated by residue F47 in SEA. Binding of one SEA to the DR1 β chain enhances the binding of a second SEA molecule to the DR1 α chain. The zinc site is on the opposite side of the SEA molecule from residue F47 so that one SEA molecule can readily bind two class II molecules. Both binding sites on SEA are required for maximal activity. Thus, unlike SEB, SEA requires two separate binding sites for optimal activity, which may allow it to stabilize SEA interaction with T cell receptors, as well as to activate the antigen-presenting cell by cross-linking MHC class II.

Superantigens such as staphylococcal enterotoxins A–E (SEA-E)[1] bind to most MHC class II antigens (1–3) and stimulate virtually all T cells bearing particular Vβ domains of the α/β TCR without conventional MHC restriction (4–7). In a generally accepted model, superantigens simultaneously bind to MHC class II and the TCR by attaching to the side of both molecules (8–10, 11). While superantigens such as SEA have an absolute dependence on presentation by MHC class II, polymorphisms in MHC and TCR as a rule do not generally affect superantigen recognition (5, 12–13), so it is uncertain what role the MHC class II molecule plays in superantigen activation.

The staphylococcal enterotoxins are small basic proteins responsible for food poisoning and toxic shock syndrome in humans. Most but not all of their toxic sequelae are attributed to their ability to induce massive cytokine secretion (16), but it is still uncertain what benefit superantigens confer to the bacteria other than to induce general immunosuppression that allows the bacteria to grow in the absence of a host immune response.

The crystal structure of staphylococcal enterotoxin B (SEB) has been resolved (17) along with a cocrystal of SEB bound to the human class II molecule HLA-DR1 (18). SEB binds to a conserved hydrophobic pocket in the α1 domain of the α chain of HLA-DR1 via key SEB residues F44 and L45, which together form an exposed hydrophobic ridge. In this position, SEB residues identified as TCR contact residues (19–21), are positioned alongside the DR1α chain in a position to ligate the TCR Vβ loop (11).

Considerable evidence from competition studies suggests that SEA and SEB do not bind in the same way to MHC class II molecules. For instance, SEA readily inhibits SEB binding to MHC class II, but excess SEB does not prevent SEA binding (2, 21, 22). Furthermore, SEA (and SEE and SED) require a single zinc atom for high affinity binding to MHC class II, and a single metal binding site with a dissociation constant of 2 μM has been located in SEA (23). In addition, SEA requires residue H81 in the β chain α helix on the opposite side from the SEB binding site (24–26). The observations that SEA and SEE both require zinc for MHC class II binding and that histidine is the most common ligand for zinc (27, 28) raised the possibility that H81 could be actively involved in a zinc coordination with SEA. Zinc actively participates in other ligand–receptor and protein–protein interactions (29–33).

In this study, we have examined the nature of SEA binding to HLA-DR1 and have identified two separate nonoverlapping MHC class II binding sites on SEA. One binding site is low affinity and appears to be identical to SEB, involving F47 in the NH_2-terminal domain. The second high affinity site involves a tetravalent zinc bridge between SEA and the H81 in the β chain of MHC class II on the opposite side of the molecule. Thus, in addition to activating T cells by

[1] *Abbreviation used in this paper:* SEA–E, staphylococcal enterotoxins A–E.

TCR Vβ ligation, SEA may also activate any cell that expresses MHC class II, including the APC.

Materials and Methods

Plasmids and Proteins. Recombinant SEA, SEB, SED, and SEE were produced in *Escherichia coli* using the expression vector pGeX. The construction of plasmids pGeX-SEA and pGeX-SEE have been described previously (18). The plasmids pGeX-SEB and pGeX-SED were constructed for this study. The SEB and SED genes were generous gifts from Dr. S. A. Khan (Department of Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, PA) and Dr. J. J. Iandolo (Microbiology Group, Division of Biology, Kansas State University, Manhattan, KS), respectively.

Plasmids encoding site directed mutants of SEA were constructed by PCR overlap and cloned into the pGeX plasmid (34, 35). All gene constructions were verified by DNA sequencing. The plasmid pGeX-SEB produces rSEB that has the wild-type NH_2-terminal glutamic acid replaced by a glycine residue, whereas the rSED produced has an additional glycine residue on the NH_2 terminus.

All recombinant proteins were purified by affinity chromatography on glutathione-agarose as described previously (18). Toxins were released from the fusion protein by cleavage with *N*-1-tosylamide-2-phenylethylcholoromethyl ketone–treated trypsin (Sigma Immunochemicals, St. Louis, MO), apart from SEB that was released by bovine thrombin (Dade International, Deerfield, IL). All recombinant proteins gave a single band on SDS-PAGE of the expected molecular weight. Soluble HLA-DR1 was released from the B-lymphoblastoid cell line LG-2 by papain cleavage and purified as described previously (36).

Peptides. Peptides were obtained from Chiron Mimotopes (Victoria, Australia) and were >95% purity by HPLC. The peptide corresponding to the region 71-85 of the β chain of HLA-DR1 had the sequence RRAAVDTYCRHNYGV. The two variants of this peptide were identical except that H81 (underlined) was substituted by glutamic acid or tyrosine. An irrelevant peptide containing histidine HIVFHTSTEPSVNYDLFG was synthesized by Immunex Corp. (Seattle, WA). The two jumbled versions of β71-85 were J1-RRAAVDTYRYHNVGC and J2-TVAYVGRYCAHNRDR. All peptides were treated at 1 mM with 2 mM iodoacetamide for 1 h before use to reduce zinc binding to the free cysteine residue.

Human PBL Stimulations. Human peripheral blood lymphocytes from healthy volunteers were purified on Ficoll (Histopaque-1077; Sigma) as recommended by the manufacturer and washed twice with RPMI 10% FCS. Recombinant toxin proteins were titrated over eight orders of magnitude by fivefold serial dilution and tested in triplicate by standard [^{3}H]thymidine incorporation assays with 2.5×10^5 PBL per well.

Determination of Relative Binding Affinities of SEA to LG-2 Cells. The binding affinity of mutant SEA proteins to MHC class II was determined as described previously (37). Comparative affinity was determined by a competitive binding assay of a fixed concentration of ^{125}I-labeled SEA binding to LG-2 cells (HLA-DR1 homozygous) at 37°C. Standard errors in these experiments were $< \pm$ 15%. The amount of each variant required to inhibit binding of ^{125}I-SEA by 50% was normalized against the amount of SEA required to cause the same level of inhibition.

Competition of SEB Binding to LG-2 Cells. 1 mg of recombinant SEB was incubated at room temperature for 2 h in 1 ml of 0.1 M borate, pH 8.5, containing 1 mg FITC then separated on an NAP10 column (Pharmacia Fine Chemicals, Piscataway, NJ). For competition assays, 5×10^4 LG-2 cells were incubated for 2 h at 37°C in 20 μl PBS/10% FCS containing 1.5 or 3.0 μM FITC SEB and 0.15–15 μM of unlabeled toxins. Cells were washed twice with ice-cold PBS/10% FCS and then analyzed immediately on a FACScan® (Becton & Dickinson Co., Mountain View, CA).

Vβ Enrichment Analysis by Anchored Multiprimer Amplification. TCR Vβ gene enrichment in human PBL cultures was determined as previously described (18). In brief, freshly isolated PBL were cultured at 10^6 cells per ml for 3 d in the presence of 10 ng/ml recombinant toxin. Human IL-2 was added on day 3 and cells were harvested on day 4. RNA was extracted (38) and reverse transcribed using a TCR β chain–specific primer. A total Vβ probe was generated by PCR using two redundant primers containing inosine. Relative expression of individual TCR Vβ cDNA species was determined by reverse dot blot hybridization of the total Vβ probe to filters containing 15 different human Vβ DNA segments.

^{65}Zinc Binding Assays. Toxins were incubated at 0.42 mg/ml (15 μM) in a final volume of 12 μl with 18 μM EDTA, followed by addition of $^{65}ZnCl_2$ (3.2 mCi/mg; New England Nuclear, Boston, MA) to 36 μM for 2 h at 37°C. The buffer used was 150 mM NaCl, 10 mM $MgCl_2$ 20 mM Tris, pH 7.3, that had been treated with chelex resin (Sigma) to remove free zinc. A Hybond C membrane (Amersham Corp., Arlington Heights, IL) was treated with 1 mM EDTA and rinsed in zinc-free buffer. Treated toxins were blotted in triplicate to the membrane for 20 min using a 96-well filtration manifold (Life Technologies, Gaithersburg, MD). Each well was rinsed under vacuum with 5× 200 μl zinc free buffer. The membrane was removed and washed twice for 2 min in the zinc-free buffer. The membrane was autoradiographed, then each dot was cut out and counted for bound ^{65}Zn. Control membranes were stained for protein with Coomassie blue R and washed in 45% methanol, 7% acetic acid.

For some experiments, purified HLA-DR1 was added to 15 μM with the toxins, and all following steps were the same as above.

The degree of zinc entrapment was determined by replacing the two final 2-min washes in zinc-free buffer with two 2-min washes in 10 μM EDTA.

For zinc-trapping experiments involving peptides, toxins were incubated at 0.42 mg/ml (15 μM) with 20-fold molar excess of the peptide in a final volume of 18 μl with 18 μM EDTA, followed by addition of $^{65}ZnCl_2$ (3.2 mCi/mg; New England Nuclear) to 36 μM for 2 h at 37°C. The zinc-free buffer consisted of 150 mM NaCl, 10 mM $MgCl_2$, 40 mM Hepes, pH 7.3. Samples were blotted in triplicate to the Hybond C membrane as described above, and the blots were washed under vacuum with 3×200 μl of the zinc-free buffer. For SEA-H225A, blots were removed washed twice with zinc free buffer before autoradiography. For SEA-H187A, blots were washed once for 2 min with 10 μM EDTA and then once with zinc-free buffer. Counts were quantified by punching out the dots and counting them for ^{65}Zn. Data does not reflect equilibrium conditions so stoichiometries of zinc binding cannot be determined.

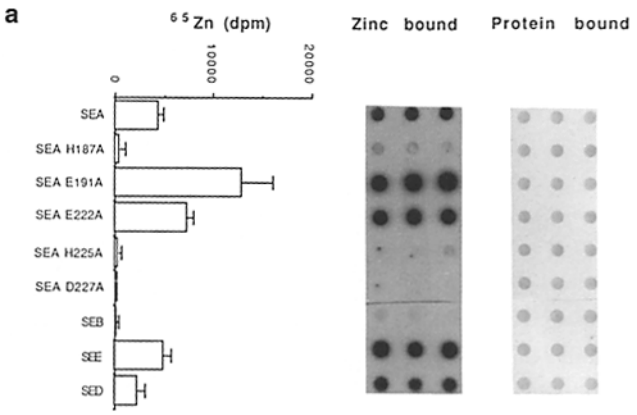
Results

Mapping of the High Affinity Zinc Site in SEA. During PCR amplification of the SEA gene, a clone was identified with a random point mutation that changed residue H187 to R187 (henceforth written H187R). This mutant SEA gene and a second deliberately constructed SEA-H187A mutant were expressed in *E. coli*, and the purified recombinant protein was assayed on PBLs and compared to wild-type SEA.

A significant loss of activity was observed, which was subsequently found to be caused by reduced MHC class II binding. Because SEA binding to MHC class II has an essential requirement for a zinc atom (23), we speculated that H187 might be a part of a zinc binding site. Histidine is the most common of zinc binding amino acids (28). The mutants SEA-H187R and a similar SEA-H187A mutant were tested for direct ^{65}Zn binding using a simple dot blot assay to comparatively measure relative ^{65}Zn binding, and the results for the latter mutant are shown in Fig. 1. SEA-H187A bound significantly lower levels of ^{65}Zn than wild-type SEA. After this finding, all other histidine, asparatic acid, and glutamic acids in the final 46 COOH-terminal region of SEA were individually mutated to alanine. There are four such positions at E191, E222, H225, and D227. The COOH-terminal domain was thought to contain all the zinc ligands because it had previously been shown that a 107-233 COOH-terminal fragment of SEA still bound to MHC class II (39). Furthermore, a common zinc binding motif L1-X_{2-3}-L2-X_{20-40}-L3 found in most zinc metalloproteins (other than zinc finger proteins) (27, 28, 40) could be applied to this region if the L1 position was assumed to be H187. Each purified mutant was examined in the direct ^{65}Zn binding assay, and the results of this analysis are shown in Fig. 1 *a*. In comparision to SEA, the mutants SEA-H187A, SEA-H225A, and SEA-D227A all had ^{65}Zn binding that was 10–>35-fold less, while SEA-E191A and SEA-E222A actually displayed a 2–3-fold increase in ^{65}Zn binding. Thus zinc was dependent on H187, H225, and D227, but not E191 and E222, even though these latter residues were in close proximity to H187. The zinc motif for SEA appears to be a mirror image of the common zinc motif. In this case, it is L1-X_{38}-X-L3.

Loss of zinc binding correlated directly with decreased HLA-DR1 binding affinity and reduced proliferation activity on human PBL (Table 1). Notably, H225A and D227A mutations abolished the ability of SEA to compete for wild-type ^{125}I-SEA binding to LG-2 cells, whereas the H187A mutation had a more moderate effect on both MHC class II binding and proliferation (Table 1). This was attributed to the fact that residue H187 was not as important to zinc binding as H225 or D227 are. Fig. 1*a* shows that the SEA-H187A mutant bound more ^{65}Zn than SEA-H225A under the same conditions. Although the total ^{65}Zn counts for SEA-H187A were only slightly more than SEA-H225A after washing of the filters, small differences in ^{65}Zn bound at nonequilibrium conditions reflect a significant difference in the dissociation rates of ^{65}Zn. This is because the monophasic dissociation of ^{65}Zn from its single site follows an asymptotic curve. Further studies have shown that SEA-H187A binding to MHC class II can be increased to wild-type levels if the solvent zinc concentration is raised above 10 μM. This concentration is only 10-fold higher than that which is required to sustain 50% of maximal binding for wild-type SEA (0.7 μM) (23). In contrast, SEA-H225A and SEA-D227A binding to MHC class II is not significantly improved by zinc levels >100 μM (data not shown).

Other staphylococcal enterotoxins were also tested for ^{65}Zn binding in the dot blot assay. Both SEE and SED bound ^{65}Zn, but SEB did not (Fig. 1 *a*). This indicated that



b

Toxin	Position		
	L1	L2	L3
SEA	H187	H225	D227
SEE	H184	H222	D224
SED	D182	H220	D222
SEB	I190	K229	E231
TSST	T157	-	-

Figure 1. Mapping the zinc coordinating residues of SEA. (*a*) Toxins were incubated with ^{65}Zn, dot blotted to nitrocellulose, washed, and autoradiographed. The individual dots were counted for ^{65}Zn. Parallel blots were stained for protein. Because of the washing steps, this assay shows comparative binding of ^{65}Zn only. The dpm values represent only a proportion of the ^{65}Zn that would normally be bound to each toxin under equilibrium conditions. (*b*) Residues in the aligned sequences of the wild-type toxins present at the equivalent position of the zinc-binding residues of SEA. The single letter amino acid code is used.

Table 1. *HLA-DR1 Affinity and Potencies of SEA and SEA Mutants*

Mutant	Relative binding affinity	P_{50} (pg/ml)
SEA	1	2
SEA-H187A	40	66
SEA-E191A	1	1.0
SEA-E222A	1	0.8
SEA-H225A	1,000	1,000
SEA-D227A	10,000	>10,000
SEA-F47S	6	130
SEA-F47S.H187A	10,000	10,000

The relative binding affinities of SEA mutants were determined by comparing inhibition of ^{125}I SEA binding to LG2 cells (DR1 homozygous) with inhibition by wild-type SEA. Stimulation potency was determined on human peripheral blood lymphocytes. The figures represent the concentration giving 50% of the maximum [^{3}H]thymidine incorporation.

SEA, SEE, and SED form a subfamily of toxins distinct from SEB based on their ability to bind zinc. This segregation into the two families is consistent with the conservation of amino acid sequence. While SEA, SEE, and SED share >70% amino acid homology, SEB is only 30% homologous to the former toxins. Fig. 1 *b* shows the amino acids at the equivalent SEA positions of 187, 225, and 227 in the aligned sequences of these toxins (9). The conserved pattern of these residues in SEA, SEE, and SED but not SEB supports the conclusion that the identified residues are involved in zinc coordination. Toxic shock syndrome toxin, which also does not require zinc for MHC class II binding, likewise does not have these residues conserved (Fig. 1 *b*; reference 9). Crystallographic analysis of zinc binding proteins shows that zinc is typically coordinated by four ligands. In all cases where the zinc ion is solvent exposed and involved in either catalytic or cocatalytic processes, the fourth ligand is a water molecule (27, 28, 30). A molecular model of SEA built from the SEB crystal structure (Homology and Discover computer programs; Biosym Technologies, San Diego, CA), reveals that the zinc atom is located on the surface of SEA and furthermore is not adjacent to any other potential zinc ligands (i.e., His, Asp, Glu, or Cys side chains) other than H187, H225, and D227. Thus the fourth zinc ligand in free SEA is most likely a water molecule.

Mutation in the High Affinity Binding Site Does Not Alter TCR Vβ Specificity. The physical separation of the zinc site from the TCR binding site was examined functionally using SEA-H187A to stimulate human PBLs at a limiting concentration. The Vβ enrichment profile of SEA-H187A was essentially identical to that of wild-type SEA, except that T cells bearing Vβ1.1 and Vβ9 were not enriched (Table 2). These particular Vβs have previously been found to be more sensitive to SEA concentration than the others and are the first to disappear as the SEA concentration becomes limiting (18) suggesting that the lack of enrichment of hVβ1.1 and hVβ9.1 in response to SEA-H187A resulted from weaker binding to MHC class II, not from an altered TCR binding site. In addition, no new Vβs were enriched by SEA-H187A, indicating that mutations that alter zinc binding do not affect the TCR binding site.

Zinc Is Sandwiched between SEA and DR1. To determine whether the zinc atom was actively involved in the SEA–MHC class II interaction, SEA, SEA-H187A, and SEA-H225A were incubated with purified HLA-DR1 in the presence of ^{65}Zn. SEA-H187A and SEA-H225A showed little if any ^{65}Zn binding alone, but the combination of these mutants with an equimolar amount of DR1 reconstituted ^{65}Zn binding (Fig. 2 *a*).

For wild-type SEA, however, the presence of purified DR1 did not significantly increase the level of ^{65}Zn binding over that seen for SEA alone. Presumably, the wild-type SEA was already saturated with zinc under these conditions.

Evidence that zinc is sandwiched in a hydrophobic interface between SEA and MHC class II is provided in Fig. 2 *b*. These results show that ^{65}Zn was not removed from the SEA/DR1 complex by EDTA washing under conditions that effectively stripped ^{65}Zn from free SEA. This resistance to EDTA was also seen with both SEA-H187A/DR1 and SEA-H225A/DR1 complexes, reflecting the strong stabilizing effect of MHC class II on zinc coordination. These results support a model where zinc bridges between SEA and MHC class II and is buried in a fourfold coordination complex within the interface of the two molecules.

Table 2. *Mutation in MHC Class II Binding Site Does Not Affect TCR Vβ Specificity*

	Vβ/Cβ (%)		
TCR (Vβ)	Unstimulated PBL	SEA	SEA-H187A
1.1	1.8 ± 0.05	**4.3 ± 0.01**	1.4 ± 0.2
2.1	5.8 ± 0.1	<1	1.1 ± 0.01
3.2	5.4 ± 0.2	1.3 ± 0.05	3.6 ± 0.2
4.1	8.8 ± 0.1	<1	1.5 ± 0.01
5.1	4.8 ± 0.1	4.2 ± 0.1	3.4 ± 0.1
5.3	5.3 ± 0.3	**15 ± 1.3**	**8.5 ± 0.1**
6.3	1.4 ± 0.2	**9.6 ± 0.01**	**9.6 ± 0.1**
6.4	1.8 ± 0.01	**9.4 ± 0.05**	**9.8 ± 0.1**
6.9	5.0 ± 1.0	**24 ± 1.0**	**22 ± 0.5**
7.4	5.8 ± 0.4	**17 ± 1.7**	**14 ± 0.5**
8.1	9.4 ± 0.3	3.2 ± 0.5	4.6 ± 1.1
9.1	1.0 ± 0.1	**4.0 ± 0.3**	1.6 ± 1.1
12.5	9.6 ± 0.5	2.7 ± 0.2	6.6 ± 0.1
15.1	1.6 ± 0.1	<1	1.4 ± 0.07
23.1	<1	**2.6 ± 0.5**	**3.4 ± 0.2**
Total (%)	68	98.6	91.6

Human PBL were stimulated with 10 ng/ml of SEA or SEA-H187A for 4 d and the TCR Vβ stimulation profile was determined (18). Data reflects the ratio of individual Vβs with respect to total Cβ. Each assay was performed in duplicate. Those Vβs in boldface were enriched with respect to unstimulated levels.

Zinc Is Coordinated by H81 of the HLA-DR β Chain in the SEA-DR Complex. Mutational studies of MHC class II have shown that the conserved H81 in the β chain of MHC class II is the only residue which significantly reduces SEA but not SEB binding (10, 26, 41). To test whether H81 in DRβ represents the fourth zinc ligand, a peptide corresponding to region 71-85 of HLA-DR1 β chain was synthesized and tested as a replacement for purified DR1 in the zinc trapping experiment. At 20-fold molar excess, DRβ71-85 partially restored high affinity ^{65}Zn binding to both SEA H187A (Fig. 3 *a*) and SEA-H225A mutants (Fig. 3 *b*). As a control, five other peptides were synthesized and tested in the same assay. Two of these control peptides were identical to the DR71-85 peptide, except that position 81 was substituted by either tyrosine or glutamic acid. Neither of these peptides restored zinc binding nor did an irrelevant histidine-containing peptide.

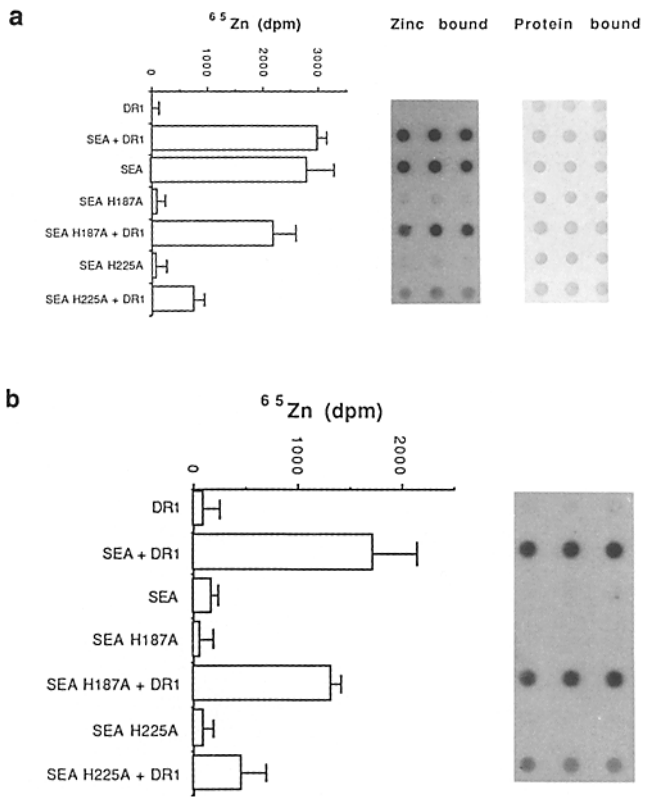


Figure 2. Zinc directly bridges binding of SEA to HLA-DR1. (*a*) Toxins were incubated with an equimolar quantity of purified HLA-DR1 and ^{65}Zn, dot blotted to nitrocellulose, autoradiographed, and the individual dots were counted for ^{65}Zn. Parallel blots were stained for protein. (*b*) Toxins were incubated with ^{65}Zn and an equimolar quantity of purified HLA-DR1, dot blotted to nitrocellulose, and washed with 10 μM EDTA before autoradiography and counting.

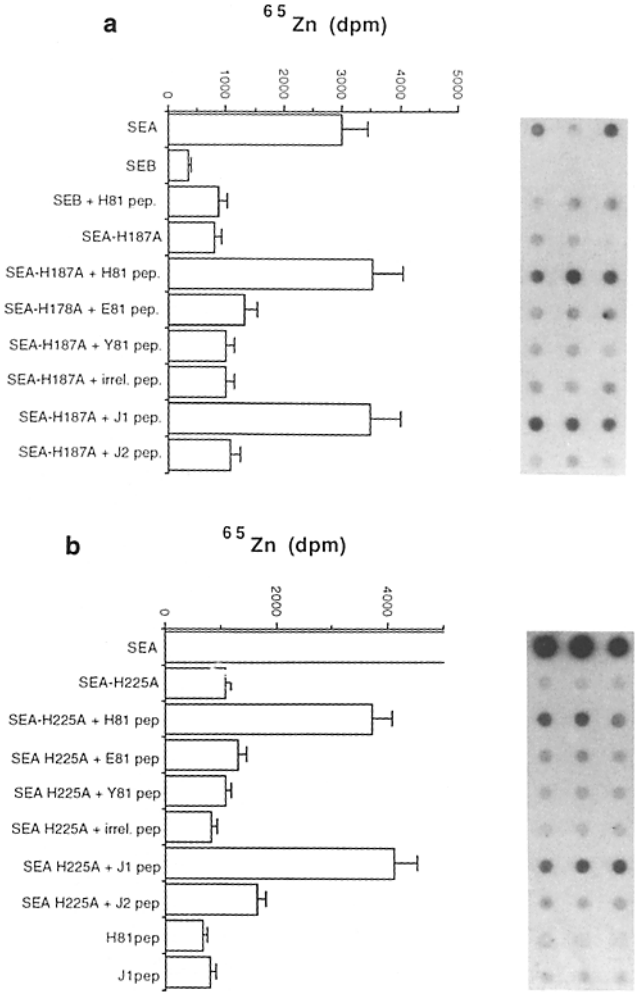


Figure 3. Identification of the HLA-DR1 residue involved in the tetravalent coordination of zinc with SEA. (*a*) SEA-H187A was incubated with 20-fold molar excess of peptides corresponding to either DR1β71-85, variants at position 81, an irrelevant histidine containing peptide, or two jumbled versions of DR71-85—J1 and J2. Incubations were performed in the presence of ^{65}Zn, dot blotted to nitrocellulose, washed briefly with 10 μM EDTA, autoradiographed, and the individual dots were counted for ^{65}Zn. (*b*) SEA-H225A was incubated with 20-fold molar excess of peptides in the presence of ^{65}Zn and treated in a similar fashion, except that the final blot was not incubated with EDTA. The final two rows were the DR71-85 and J1 peptides in the absence of any toxin.

Although glutamic acid is potentially able to coordinate zinc, replacement of histidine for glutamic acid in the DR 71-85 peptide did not reconstitute ^{65}Zn binding to either SEA-H187A or SEA-H225A. Charge or steric characteristics of glutamic acid may be responsible for this difference.

In addition to the contribution of H81 to zinc binding, the involvement of other adjacent residues in the DR71-85 peptide not directly associated with zinc binding were analyzed. Two scrambled versions of DR1β71-85 were tested. The first of these, J1, reconstituted zinc binding to both mutants as well as the native 71-85 peptide. The second scrambled peptide, J2, was ineffective. The J1 peptide differed from the original only in residue on the opposing face of the α helix away from H81 (42). That is, positions C79, R80, Y83, and V85 were scrambled. The J2 peptide on the other hand was more extensively jumbled and had changes at positions R71, R72, A74, D76, T77, R80, Y83, G84, and V85. These mutations altered residues extending out in the same direction as H81 in the DR1 β chain and would therefore be more likely to make contact with SEA. Thus, in addition to H81, residues in close proximity and pointing out in the same direction as H81 influenced specific coordination of zinc, at least in this assay. SEB plus the DR1β71-85 peptide did not bind significant levels of ^{65}Zn over and above the amount of ^{65}Zn that was bound by the peptides alone (Fig. 3 *a*). These data imply that DRβH81 represents the fourth zinc ligand in SEA-DR1 complex and would explain why mutations in DRβH81 only affect SEA and SEE binding but not SEB binding.

Location of a Second MHC Class II Binding Site on SEA. Further mutational analysis located a second contact point with MHC class II in a region of SEA quite distant from the zinc atom. While SEA and SEB appear to bind in quite different ways to MHC class II, SEA still strongly inhibits SEB binding (2). Residue F44 in the NH_2-terminal domain of SEB is cen-

tral to the SEB/DR1 interaction (11, 20). Thus the equivalent SEA residue (F47) was mutated to determine whether SEA also used this region to bind to MHC class II. A single mutant, SEA-F47S, and a double mutant, SEA-F47S.H187A, were generated and expressed in *E. coli* and tested for MHC class II binding and relative T cell potency using the standard human PBL stimulation assay. The single mutant SEA-F47S displayed a 6-fold reduction in MHC class II binding and a 250-fold reduction in T cell proliferative response (Table 1). In contrast, SEA-H187A exhibited a 40-fold drop in binding to MHC class II but retained similar T cell proliferative activity as SEA-F47S. The double mutant SEA-F47S.H187A had undetectable binding and a 10,000-fold loss in activity. Thus a mutation in the NH_2-terminal region of SEA (F47S) affected T cell proliferation more than it affected MHC class II binding compared to a mutation on the other side of SEA at the zinc site (H187A). These data suggested that binding of SEA via F47S might be less important on total binding affinity and more important for direct TCR ligation, while the zinc site might be important for initial binding to MHC class II in an orientation not directly associated with TCR ligation. To reconcile these results, a model of two SEA molecules bound to one MHC class II molecule was invoked and tested.

SEA and SEB Share the Low Affinity but Not the High Affinity Binding Site. Mutants in the COOH-terminal and NH_2-terminal binding site were tested for their ability to inhibit SEB binding to DR1 expressing LG-2 cells (Fig. 4 *a*). SEB has been shown to bind exclusively to the DR1 α chain (11). Fluoresceinated SEB was incubated at 3.0 μM and unlabeled toxins were added at 15 μM (fivefold excess). Specific SEB binding to LG-2 cells was inhibited by unlabeled SEA, SEB, and SEA-H225A. Surprisingly, SEA-H225A was the best competitor of all, reducing specific SEB binding by nearly 90%, even though this mutant had lost any high affinity zinc/DRβ chain binding. Thus, the remaining low affinity binding site in the NH_2-terminal domain of SEA-H225A was still sufficient to compete for SEB binding. This provided convincing evidence that SEA and SEB bound to the DRα chain in an identical fashion via the NH_2-terminal globular domain. The double mutant SEA-F47S.H187A, as expected, did not inhibit SEB binding at all because both sites had been mutated.

Instead of having no effect, SEA-F47S significantly increased SEB binding over that seen in the absence of any competitor. This suggested a cooperative effect between an SEA-F47S molecule bound exclusively to the β chain binding site and an SEB molecule occupying the α chain binding site. Significantly, cooperative binding occurred between two different superantigens, i.e., SEA and SEB. This cooperative effect was analyzed in more detail (Fig. 4 *b*). FITC-SEB was bound to LG-2 cells at a lower concentration than in the previous experiment in the presence of 0.1–10-fold excess of either SEA-F47S or SEA-H225A. As before, SEA-H225A effectively inhibited SEB binding at all concentrations tested while SEA-F47S progressively increased SEB binding. This increase was maximal at the molar equivalence point of SEA-F47S and SEB.

The reverse experiment was performed to show that cooperativity existed in both directions. Radiolabeled SEA-F47S was incubated with LG-2 cells in the presence of each toxin mutant and the results are shown in Fig. 5. ^{125}I-SEA-F47S binding to the DR1 β chain was inhibited by both SEA and SEA-F47S, but binding was increased by both SEA-H225A and SEB. Maximal cooperativity occurred at a molar ratio of 1:1,000, which reflected the large difference in affinity between the β chain binding site and the α chain binding site. Thus, cooperative binding was again observed beween two SEA molecules, as well as SEA-F47S and SEB.

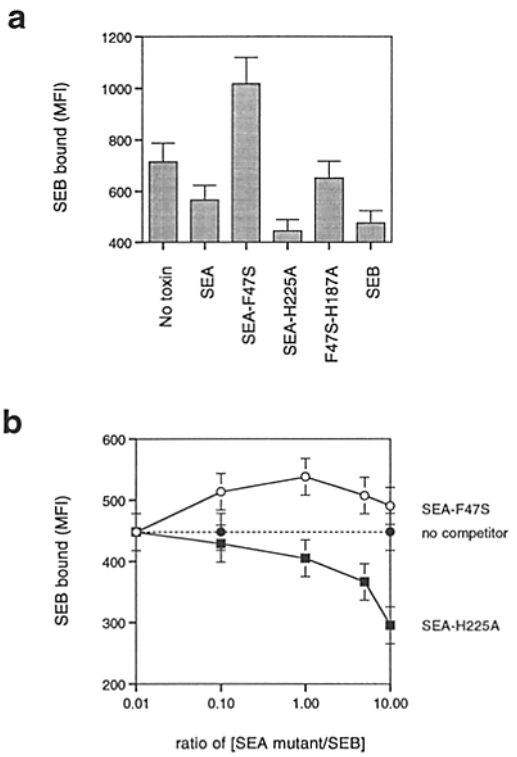


Figure 4. Competition for SEB binding by SEA mutants. (*a*) Fluoresceinated SEB was incubated at 3.0 μM with LG-2 cells in the presences of 15 μM (fivefold excess) SEA, SEA-F47S, SEA-H225A, SEA-F47S.H187A, or unlabeled SEB. Cells were incubated at 37°C for 2 h before washing and FACS® analysis. *MFI*, mean fluorescence intensity. Each value is the average of duplicate samples. (*b*) Cells were incubated with 1.5 μM FITC SEB in the presence of 0.15–15 μM SEA-H225A (■), 0.15–15 μM SEA-F47S (○), or no competitor (●).

Discussion

Two Cooperative Binding Sites on SEA for MHC Class II. Unlike SEB, SEA has a second binding site to the polymorphic β chain of MHC class II mediated by a tetravalent zinc coordination complex between H187, H225, and D227 in SEA and H81 in the DR1 β chain α helix. The zinc site is on the opposite side of the SEA molecule to the F47.L48 motif conserved between SEA, SEB, and other superantigens, which allows them to bind to the invariant MHC class II α chain (11). Zinc-mediated binding is unique to SEA, SEE, and SED (Fig. 1), and given the degree of similarity between these three toxins, it is likely that that SEE and SED also bind to MHC class II in two separate orientations.

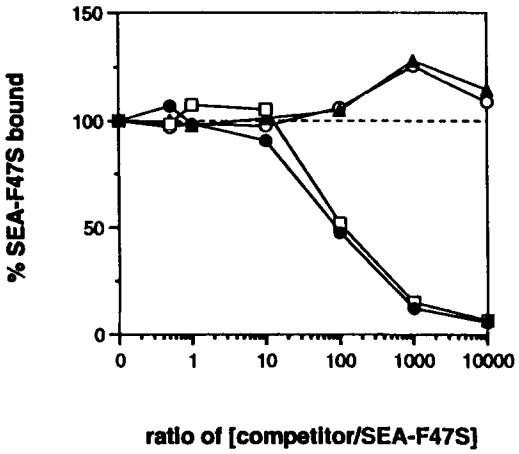


Figure 5. Competition for SEA-F47S binding to HLA-DR1. Radiolabeled SEA-F47S (50 μCi/μg) was incubated with purified DR1 in the presence of increasing amounts of either SEB (○), SEA-H225A (▲), SEA (●), or SEA-F47S (□) at 37°C. Each point is the average of duplicate samples. The percentage error was <5%.

Binding of two toxin molecules to the same DR1 molecule displayed distinct cooperativity. This was demonstrated by the ability of SEA-F47S to significantly enhance SEB binding while SEA-H225A strongly inhibited, even though zinc-mediated binding had been destroyed (Fig. 4). This was clear evidence that SEA-H225A had retained its DR α chain site while SEA-F47S had retained its DR β chain binding site. Because the affinity of SEA to the β chain is much greater than to the α chain, this would ensure that SEA first binds to the DR β chain and then assists the binding of a second molecule to the DR α chain site. The cooperative effect most likely occurs via direct contact between the two SEA molecules bound to either side of the DR α helices although this has yet to be formally proven. Dependence on initial DR β chain binding would explain why mutations that alter zinc-mediated binding (24, 26) have such a dramatic effect on SEA activity despite the fact that the DR α chain site remains intact.

These data resolve previous confusion about SEA and SEB binding to MHC class II. For example, while SEA prevents SEB binding, SEB does not inhibit SEA binding, even at 10,000-fold molar excess (21, 22). Because SEA and SEB compete only for the low affinity DR α chain site, zinc-mediated DR β chain binding is unaffected by SEB.

The Nature of the Zinc Site in SEA. The relative contribution of individual SEA residues to zinc binding varied in the order D227 > H225 > H187. The importance of D227 suggests that this residue may act as a bidentate ligand, as seen in other zinc binding proteins (43). The bridging of SEA to MHC class II via zinc most likely involves a rearrangement of the zinc coordination complex between free and bound states of SEA. We hypothesize that a water molecule bound to zinc in free SEA is replaced by H81 in the β chain of HLA-DR1. However, the potential of D227 to act as a bidentate ligand could reflect a more complex rearrangement. Notably, high affinity binding of SEA to MHC class II is strongly temperature dependent (2, 22), suggesting that the change in coordination state of the zinc atom is endothermic and may involve a structural change in one or more residues in or close to the binding site. For example, H81, which is normally hydrogen bonded to the peptide backbone in class II (42), may need to shift to interact with the zinc atom.

While the zinc atom is essential to binding, other regions of SEA have been identified, notable the very NH_2-terminal residues that also modulate high affinity binding (references 19, 44, and Hudson, K., unpublished data). In a model of SEA bound to DR1 β chain (see Figs. 6 and 7), the very NH_2 terminus of SEA would be in close proximity to DR and may also participate in the interaction. Although MHC class II β chains are highly polymorphic, these differences do not significantly alter SEA binding and/or presentation, even between species apart from the DRw53 allele, which has a H81Y change (24, 26). The recruitment of a metal atom may represent a means by which toxin binding can be sustained to a highly polymorphic protein domain with minimal dependence on surrounding residues.

Possible SEA/MHC Class II Structures and Ligation of TCR. In the absence of an SEA crystal structure, a model is provided in Fig. 6, based on the SEB crystal structure (11), that shows the location of the two MHC class II binding regions and the region that interacts with TCR (18, 19, 37). The analogous residues in SEB to the zinc site are I190 (H187) on the exposed loop between the two β strands β9 and β10, whereas K229 (H225) and E231 (D227) are on the closely associated β12 strand. The similarity between SEA and SEB in this region is sufficiently high to allow the reconstitution of a zinc site in SEB (Tiedemann, R., manuscript in preparation).

The three-dimensional structure of an SEB/DR1 cocrystal reveals that SEB contacts the DR α chain through residues F44 and L45 (analogous to F47 and L48 in SEA) (11). Residues I190, K229, and E231 in SEB, which represent the zinc site, point out and away from the DR1 structure and clearly do not interact with any portion of the same DR1 molecule. This region of the SEA molecule could interact with another

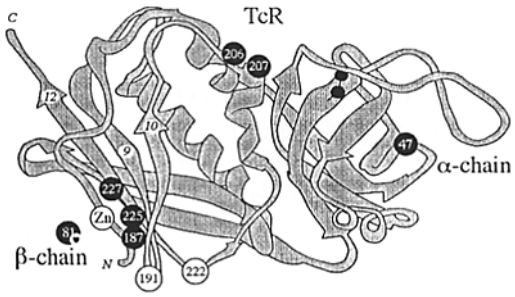


Figure 6. Relative position of the two MHC class II binding sites on SEA. The zinc atom chelated by H187, H225, and D227 in SEA. The position of these residues is based on the location of analogous residues (I190, K229, and E231) in the crystal structure of SEB (17). H187 is located on the inner descending strand of the β9-β10 loop, while H225 and D227 are located on the ascending β-12 strand. Residues E191 and E222 are also indicated, even though mutation of these adjacent residues had no negative effect on zinc binding or activity. The position of F47 in the NH_2-terminal domain of SEA is shown along with S206, N207, which are TCR contact residues.

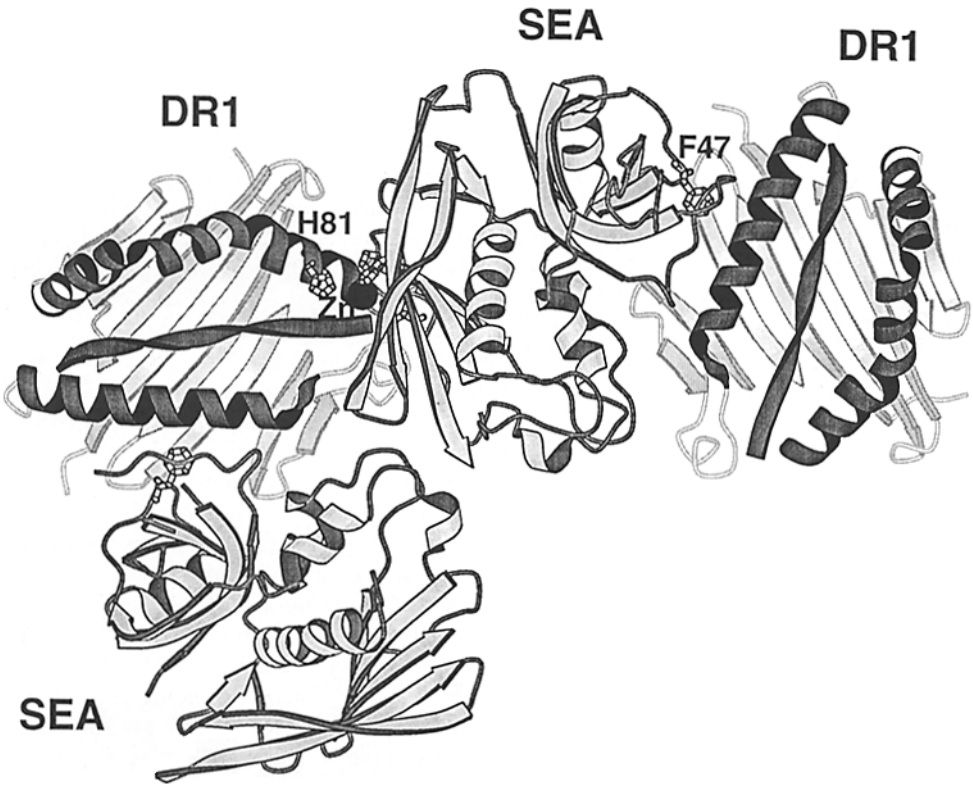


Figure 7. A hypothetical model of SEA binding to MHC class II. This figure is drawn (Insight II; Biosym) from the coordinates of the SEB/DR1 crystal structure (11). SEA is light gray and the DRα helices and peptide are dark gray. Residues in the two binding sites on opposite sides of the SEA molecule are drawn in ball-and-stick form. The low affinity interaction via F47 (labeled) to the DRα chain is shown bound in the same manner as SEB, while the zinc site is shown contacting H81 (labeled) on the DRβ chain. Two possible complexes are shown: one where SEA binds two DR1 molecules and a second where one DR1 molecule binds two SEAs. Extensive MHC class II cross-linking would result.

DR β chain via H81 and a hypothetical structure is shown in Fig. 7.

These data predict that one MHC class II molecule can bind two SEA molecules simultaneously or alternatively, one SEA molecule can cross-link two MHC class II molecules. Under normal in vivo conditions, MHC class II would be in considerable excess to SEA so the SEA.DR1$_2$ structure would be most favored. Because of the significant cooperativity evident between the two sites, however, an SEA$_2$DR1 trimer may still be favored, even at low SEA concentrations. Recent experiments with soluble DR1 and SEA have confirmed the existence of SEA$_2$DR1 complexes in solution (Tiedemann, R., manuscript submitted for publication).

Why Does SEA Have Two Binding Sites on MHC Class II? SEA requires two binding sites to activate T cells, whereas SEB needs only one. One obvious purpose of the second β chain binding site on SEA might be to recruit and stabilize the primary α chain binding at concentrations far below the dissociation constant of the α chain binding alone. This would allow SEA to be active at much lower concentrations. Two binding sites may also allow SEA to cross-link MHC class II molecules, delivering activation signals to both the APC and the T cell (40). This may upregulate surface expression of costimulatory molecules such as B7 (46) and induce cytokine secretion by the APC, which helps T cell activation. Recent evidence suggests that for optimal activity, both binding sites must be on the same SEA molecule (Tiedemann, R., unpublished data), suggesting that MHC class II cross-linking is one aspect of SEA function essential for maximal T cell activation.

It is unusual that SEB-like binding and activation have not been fully conserved by SEA despite retention of the DRα chain binding. This mechanistic difference may lie in the nature of the TCR binding site. SEA activates a broader range of TCR Vβs than SEB. In humans, SEB predominantly targets Vβ3-bearing cells while SEA targets h Vβ1.1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, 9.1, and 23.1 (18). The cost of this larger Vβ repertoire may be a reduced affinity for individual TCRs so that binding to a single site on the class II α chain is no longer sufficient to activate most T cells. Costimulatory signals may be essential to enhance the lower affinity SEA/TCR interaction, and this may have been achieved by additional cross-linking MHC class II. The fact that an SEA-H225A/MHC class II complex is insufficient for T cell activation suggests that the affinity of SEA towards TCR is too low to deliver an activation signal on its own without additional costimulation.

Despite the fact that polymorphisms in either TCR or MHC class II do not significantly alter SEA activity, MHC class II is still important for activation by SEA. This has always

been used as a strong argument favoring essential TCR. MHC class II interactions in superantigen activation and may be so for SEB. However, the more important role of MHC class II in SEA activation may be to mediate the recruitment of costimulatory molecules to the surface of the APC. This may in turn reduce the need for strict compatibility between the TCR and MHC class II peptides, again allowing SEA to activate many more T cells at much lower concentrations.

The data presented here indicate that the superantigen SEA has developed a novel mechanism to bind to the highly polymorphic MHC class II β chain. The net effect of this is to enhance binding of a second SEA molecule to the crucial class II α chain site and to cross-link MHC class II on the surface of the APC, thus activating costimulatory pathways and coalescing MHC class II on the APC surface, further enhancing its ability to activate T cells. This increased potency must provide an important advantage for *Staphylococcus aureus* strains, which produce these superantigens.

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