Crystallographic and Mutational Data Show That the Streptococcal Pyrogenic Exotoxin J Can Use a Common Binding Surface for T-cell Receptor Binding and Dimerization*§

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The protein toxins known as superantigens (SAgs), which are expressed primarily by the pathogenic bacteria Staphylococcus aureus and Streptococcus pyogenes, are highly potent immunotoxins with the ability to cause serious human disease. These SAgS share a conserved fold but quite varied activities. In addition to their common role of cross-linking T-cell receptors (TCRs) and major histocompatibility complex class II (MHC-II) molecules, some SAgS can cross-link MHC-II, using diverse mechanisms. The crystal structure of the streptococcal superantigen streptococcal pyrogenic exotoxin J (SPE-J) has been solved at 1.75 Å resolution (R = 0.209, Rfree = 0.240), both with and without bound Zn²⁺. The structure displays the canonical two-domain SAg fold and a zinc-binding site that is shared by a subset of other SAgS. Most importantly, in concentrated solution and in the crystal, SPE-J forms dimers. These dimers, which are present in two different crystal environments, form via the same face that is used for TCR binding in other SAgS. Site-directed mutagenesis shows that this face is also used for TCR binding SPE-J. We infer that SPE-J cross-links TCR and MHC-II as a monomer but that dimers may form on the antigen-presenting cell surface, cross-linking MHC-II and eliciting intracellular signaling.

The common human pathogens Staphylococcus aureus and Streptococcus pyogenes secrete a number of potent protein toxins known as superantigens. These toxins derive their name from their primary functional attribute, which is to bind simultaneously to T-cell receptors (TCRs)¹ and MHC class II (MHC-II) molecules, outside the MHC peptide-binding groove and as intact molecules rather than processed peptides. This can cause massive overstimulation of the cellular immune response, with the overproduction of cytokines such as tumor necrosis factor α and interleukin-2, as a result of uncontrolled T-cell activation (1–3). This activity is central to their involvement in many human diseases, such as toxic shock, scarlet fever, food poisoning, and possibly others such as rheumatoid arthritis (2, 4, 5).

The SAg family comprises staphylococcal enterotoxins (SEs) such as SEA, SEB, SEC1–3, and SED, streptococcal pyrogenic exotoxins (SPEs) such as SPE-A and SPE-C, and toxic shock syndrome toxin-1 (TSST-1). The sequencing of the complete genomes of several strains of S. aureus (6) and S. pyogenes (7) has led to the discovery of many more sag genes, including spej, and the realization that in these two organisms this is a widespread protein family that must play a major role in their pathogenicity. The SAgS share widely different levels of sequence identity. Some are so similar (for example SEA and SEE, with ~ 90% sequence identity) as to make allelic variants between different strains difficult to distinguish, but many share much lower sequence identity, around 20%. Structurally, however, the SAgS share a highly conserved fold (8, 9), comprising an N-terminal β-barrel domain with the well known OB-fold (10, 11), and a C-terminal β-grasp domain comprising a β-sheet that wraps around a long central helix.

A striking feature of the SAg family, however, is that this conserved fold supports a wide variety of different binding modes. Most SAgS (for example SEB and TSST-1) have a single MHC-II-binding site, located on their N-terminal domains (12–14), often referred to as the generic MHC-II-binding site, whereas others (such as SMEZ and SPE-H) have instead a site on their C-terminal domains, mediated by a bound Zn²⁺ ion (9). Still others, such as SEA, have both sites (15–17), giving them the ability to cross-link MHC-II on antigen-presenting cells (APCs) and thus elicit intracellular signaling in the APCs. A variation on this theme is given by several other SAgS, including Sed and SPE-C, which can cross-link MHC-II by formation of homodimers. Thus, SED forms zinc-dependent homodimers through its C-terminal domain and can cross-link MHC-II through the N-terminal domain sites at each end of the homodimer (18). On the other hand, SPE-C dimerizes via its N-terminal domain and can cross-link MHC-II by the two C-terminal domain Zn²⁺ sites of the dimer (19).

In contrast to the varied MHC-II-binding modes, the evidence so far suggests that most, if not all, SAgS bind to TCR via a common site, at the interface between the N- and C-terminal domains (20, 21). The ability to select particular TCR Vβ subtypes appears to derive from sequence and structural diversity.
Structure and Function of the Superantigen SPE-J

Relative molecular mass of SPE-J calculated from light scattering data

| Concentration | Mass from $R_h$ | Cpl/$R_h$ |
|---------------|----------------|-----------|
| mg/ml         | kDa            | %         |
| 1.0           | 23.3           | 27        |
| 2.0           | 33.4           | 25        |
| 4.0           | 44.2           | 19        |
| 5.5           | 54.6           | 14        |
| 6.0           | 52.5           | 17        |
| 10.0          | 61.1           | 14        |

$R_h$ = hydrodynamic radius.

Experimental Procedures

Protein Expression and Purification—SPE-J was cloned and expressed in *Escherichia coli* as described by Proft et al. (24). The protein was overexpressed as a glutathione S-transferase fusion protein and was initially purified using glutathione/agarose. After cleavage of glutathione S-transferase from the toxin with protease 3c, the protein was further purified by cation exchange chromatography (MonoS HR 5/5 column, Amersham Biosciences) followed by gel filtration (Superdex 75 HR 10/300 column, Amersham Biosciences). Small fractions were taken across the protein peak, and dynamic light scattering (see below) was used to determine which fractions were to be used for crystallization trials. Only those with a Cpl/$R_h$ ratio of less than 14% were used.

Light Scattering Analysis—Dynamic light scattering was performed by using a Protein Solutions (Charlottesville, VA) DynaPro molecular sizing instrument to determine not only the monodispersity of protein samples, prior to crystallization, but also to determine the relative molecular mass of the protein at various concentrations. Samples ranged in concentration from 0.8 to 12.0 mg/ml, and 30 measurements were made at each concentration. Results are summarized in Table I.

Crystallization—Crystals were grown at 18 °C in the hanging drop method by mixing 1 μl of protein solution (10 mg/ml protein in 50 mM HEPES/KOH, 100 mM NaCl, pH 7.0) with 1 μl of reservoir solution (210 mM lithium acetate, 17% PEG 3350, pH 5.5). Small, needle-shaped crystals of maximum dimension of 0.03 mm grew over a period of 2–3 weeks. These crystals were monoclinic, space group C2, with unit cell dimensions $a = 186.6$, $b = 46.4$, $c = 72.2$ Å, $\beta = 90.6^\circ$. This gave $V_m$ values of 3.0 Å$^3$/Da (59% solvent) assuming two molecules per asymmetric unit, or 2.0 Å$^3$/Da (39% solvent) assuming three molecules per asymmetric unit; the structure determination showed the latter to be correct.

Crystals of zinc-bound SPE-J (Zn-SPE-J) were obtained by soaking crystals in 100 mM zinc acetate, 20% PEG 3350, 250 mM lithium acetate, pH 5.8, for 1 h. This short, sharp soak gave much better diffraction than from crystals soaked in lower zinc concentration (1 mM) for a longer period (4–24 h). Crystals were mounted in a cryoloop and flash-frozen by plunging into liquid $N_2$ after a rapid pass through a cryoprotection solution. The latter comprised 0.23 M lithium acetate, 18% PEG 3350, and 20% ethylene glycol, pH 5.8, for SPE-J and 100 mM zinc acetate, 0.23 M lithium acetate, 20% PEG 3350, and 20% ethylene glycol, pH 5.8, for Zn-SPE-J.

Data Collection—X-ray diffraction data to 1.7 Å resolution were collected for SPE-J at 110 K at the Stanford Synchrotron Radiation Laboratory. Zn-SPE-J data to 2.0 Å resolution were collected at 110 K using CuK$_\alpha$ radiation from a Rigaku RU-H3R x-ray generator equipped with Osmonic mirrors, an Oxford cryostream, and a Marx/345 imaging plate system. Raw data were processed using MOSFLM (25) and scaled and merged with SCALA (26). Data collection statistics are summarized in Table II.

Structure Determination and Refinement—The structure of SPE-J was solved by molecular replacement using AMoRe (27) with the closely related SAg structure SPE-C (19) (Protein Data Bank code 1AN8), as search model; SPE-J and SPE-C share 49% sequence identity. Two molecules were found and used for phasing to 2.0 Å resolution, after which an initial model was built with ARP/WARP (28). This gave an almost complete model for both molecules (391 of 422 residues) and also revealed the position of a third molecule, which was added to the model. Further refinement was with CNS (29), with cycles of refinement being interspersed with manual model building into the electron density using the graphics program TURBO FRODO (30). Solvent molecules, all treated as water, were added using the WATERPACK facility in CNS and were retained if they had spherical density and appropriate hydrogen bond geometry. The quality of the model was checked periodically with PROCHECK (31), and hydrogen bonds were identified following the distance and angle criteria of Baker and Hubbard (32). The Zn-SPE-J structure was solved using the final SPE-J structure as a starting model and was refined in the same way.

Site-directed Mutagenesis—Single-site mutants of SPE-J were generated by overlap PCR. Oligonucleotide primers pGEX.fw/SpeJmut.rev and pGEX.rev/SpeJmut.fw were used for 12 cycles of PCR with pGEX-3cSpeJ (24) as template. See supplemental Material for primer sequences. The PCR products were then purified from agarose gels and used as templates for 18 cycles of PCR with pGEX.fw/pGEX.rev primer pairs. The PCR products were cloned into pGEX-3c vectors, and the recombinant SPE-J mutant proteins were produced as described previously for wild type SPE-J (34). The DNA sequences of the cloned SPE-J mutants were confirmed using a Licor automated DNA sequencing (model 4200).

Toxin Proliferation Assay—The mitogenic activity of the SPE-J mutants was determined in a peripheral blood lymphocyte (PBL) stimulation assay as described previously (22). In brief, PBLs were purified from blood of healthy donors and incubated with varying dilutions of SPE-J mutants (100 ng/ml to 1 fg/ml). After 3 days of incubation at 37 °C, 0.1 μCi of $[^3H]$thymidine was added. After another 24 h, the PBLs were harvested and counted on a Cobra scintillation counter. The decrease in T-cell mitogen activity was calculated as the amount of mutant toxin needed to achieve half-maximum stimulation ($P_{50}$ value) of wild type SPE-J.

RESULTS

Crystal Structure of SPE-J—The three-dimensional structure of SPE-J was determined by molecular replacement and refined at 1.75 Å resolution to an $R$ factor of 0.209 and free $R$ factor of 0.240. The model has excellent geometry with 87.2% of non-glycine residues falling in the most favored regions of the Ramachandran plot, as defined in PROCHECK (31), with no outliers. The three molecules in the asymmetric unit of the crystal are organized in such a way that A and B form a putative dimer (see below) and C also forms a dimer, with another molecule C, related by 2-fold crystallographic symmetry. The final model for molecule A comprises the complete polypeptide for mature SPE-J, residues 1–209, but with two additional residues (Gly-2 and Ser-1) also modeled at the N terminus, left after cleavage of the glutathione S-transferase fusion domain. Sequence numbering here follows that of the mature protein. Molecule B lacks residues 97–101, and molecule C lacks residues −2, −1 and 1; these have no interpretable electron density and are assumed to be disordered. Further details are given in Table II.

Molecular Structure—SPE-J has the characteristic two-domain SAg fold (8, 9), shown in Fig. 1. Following an N-terminal helix α2 (residues 2–17), which ends in the inter-domain cleft, the
**Table II**

Data collection, refinement, and model details

| SpecJ | Zn-SpecJ |
|-------|---------|
| Data collection | | |
| Resolution (Å) | 40–1.6 | 30–1.9 |
| Multiplicity | 4.8 (4.6) | 5.4 (5.3) |
| Unique reflections | 72,162 | 44,941 |
| Completeness (%) | 99.4 (99.4) | 99.9 (99.9) |
| R <sub>free</sub> (%) | 5.5 (45.3) | 6.4 (40.0) |
| R <sub>i/o</sub> | 7.9 (1.9) | 8.4 (1.9) |
| Refinement | | |
| Resolution limits (Å) | 1.75 | 2.0 |
| No. reflections | 51,993 | 36,793 |
| R (R<sub>free</sub>) (%) | 20.9 (23.9) | 21.8 (24.9) |
| Protein atoms | 5162 | 5162 |
| Ions | | |
| Water molecules | 314 | 159 |
| Geometry | | |
| r.m.s.d. * bond lengths (Å) | 0.005 | 0.006 |
| r.m.s.d. bond angles (degree) | 1.22 | 1.19 |
| % most favored in Ramachandran plot | 87.2 | 85.9 |

* r.m.s.d., root mean square deviation.

**Fig. 1. Structure of the SpecJ monomer.** Ribbon diagram in which the major secondary structural elements are labeled in accord with the nomenclature first used for SEB (42). In this orientation the N-terminal domain is on the right and the C-terminal domain on the left. Secondary structural elements are as follows: α2, 2–19; β1, 21–32; β2, 35–40; β3, 48–54; β4, 66–72; β5α, 81–87; β5β, 88–92; β6, 102–108; β7, 111–118; β8, 125–127; α4, 128–145; β9, 155–162; β10, 166–172; α5, 180–186; β11, 194–196; and β12, 201–208. Figure was drawn with Pymol.

N-terminal OB-fold domain has a β-barrel structure comprising five highly curved β-strands, β1 to β5, which create a concave outer surface that is used in many SAgs for MHC-II binding. The C-terminal β-grasp domain is based on a mixed, five-stranded, β-sheet that wraps around a central α-helix, α4 (residues 128–145). When SPE-J is compared with other SAgs by using the program SSM (www.ebi.ac.uk/msd-ssm/), its closest homolog is SPE-C, with which it shares 49% sequence identity and 199 residues that can be matched with a root mean square (r.m.s.) difference in Ca positions of 1.24 Å. This gives a Z score of 14.8, with the only significant differences in the polypeptide chain conformation being in the β5–β8 loop, where a single residue (Asn-96) is inserted in SPE-J and small changes in strand β2 and the β4–β5 and β10–α5 loops. The next closest hits are SMEZ-2 (Z score 11.0, 32% sequence identity, 196 Ca atoms matching with an r.m.s. difference of 1.41 Å) and SPE-H (Z score 9.0, 25% sequence identity, 182 Ca atoms matching with an r.m.s. difference of 1.92 Å). These are all streptococcal SAgs.

**Molecular Packing, Evidence for Dimerization**—Examination of the crystal packing shows that molecules A and B share a significant interface (Fig. 2), which is considerably more extensive than any of their other packing interactions in the crystal, and has many of the properties expected of a protein dimer. This interface buries a total of 1360 Å² of accessible surface area (680 Å² per monomer, or 6.5% of the monomer surface), calculated using the Protein-Protein Interaction Server (www.biochem.ucl.ac.uk/bsm/PP/server); this uses the algorithm of Lee and Richards (33) with a probe radius of 1.4 Å. The interface is formed by the C-terminal half of helix α2 and its connection to strand β1, the β2–β3 loop, the β4–β5 loop, all from the N-terminal domain, and the end of helix α4 and start of helix α5, both from the C-terminal domain. The residues that make the greatest contribution to the interface are Tyr-14, Glu-17, and Ile-19 from α2, Phe-77, Arg-79, and Tyr-83 from β4–β5, Gln-142 from α4, and Arg-181 from the start of α5, which hydrogen bonds across the interface to the carbonyl oxygen of Gly-17.

The third molecule in the asymmetric unit, molecule C, forms a very similar interaction with another molecule C, related by crystallographic symmetry. The dimerization of these two molecules buries a somewhat larger surface area of 2130 Å² (1065 Å² per monomer, 10.4% of the monomer surface). The structural elements that comprise it are the same as for the A-B dimer, however, involving residues in and around the interdomain cleft (Fig. 2). The principal contributors to the interface are Tyr-14, Glu-17, and Ile-19 from α2, Tyr-43, Lys-44, and Lys-45 from β2–β3, Phe-77, Tyr-80, and Tyr-83 from β4–β5, Gln-142 from α4, and Arg-181 from α5. In both the A-B and C-C dimers there are 6–10 direct protein-protein hydrogen bonds across the interface, and a number of water molecules make bridging interactions.

**Zinc Binding**—SPE-J has been shown to bind to MHC-II in a zinc-dependent manner. The native SPE-J structure contained no bound zinc, however, and of the three residues proposed to form the Zn²⁺-binding site (24), the side chains of His-201 and Asp-203 were close together but that of the third putative ligand, His-167, was turned away. In the Zn-SPE-J structure, however, after soaking the crystals very briefly in 100 mM Zn²⁺, the side chain of His-167 had moved, and these three residues are bound to a fully occupied Zn²⁺ ion, with bond lengths of 2.1–2.2 Å. A water molecule is bound as a fourth ligand, completing a tetrahedral coordination site. The zinc site is located on the concave surface of the C-terminal domain and is equally accessible for MHC-II binding in both the monomeric and dimeric forms of SPE-J dimer. In the latter the Zn atoms are ~60 Å apart, at the two ends of the dimer.

**Functional Analysis of the TCR-binding Site in SPE-J**—SPE-J is most closely related to SPE-C by amino acid sequence (49% identity) and, like SPE-C, primarily stimulates T-cells carrying the Vβ2 TCR (24). We therefore selected for mutagenesis those residues in SPE-J that were equivalent to the SPE-C residues shown to contact Vβ2 TCR (24). We therefore selected for mutagenesis those residues in SPE-J that were equivalent to the SPE-C residues shown to contact Vβ2 TCR (24). We therefore selected for mutagenesis those residues in SPE-J that were equivalent to the SPE-C residues shown to contact Vβ2 TCR (24). We therefore selected for mutagenesis those residues in SPE-J that were equivalent to the SPE-C residues shown to contact Vβ2 TCR (24). We therefore selected for mutagenesis those residues in SPE-J that were equivalent to the SPE-C residues shown to contact Vβ2 TCR (24). We therefore selected for mutagenesis those residues in SPE-J that were equivalent to the SPE-C residues shown to contact Vβ2 TCR (24). We therefore selected for mutagenesis those residues in SPE-J that were equivalent to the SPE-C residues shown to contact Vβ2 TCR (24).
**DISCUSSION**

Solution studies, using dynamic light scattering, show clearly that SPE-J forms dimers at higher protein concentrations (>3 mg/ml) and in fact has a somewhat greater propensity for dimerization than SPE-C. In contrast, another SAg, SMEZ-2, showed only monomers under the same conditions. SPE-J was also shown to stimulate the rapid aggregation of LG-2 cells, presumably by cross-linking of MHC-II molecules (24). Further functional studies indicate that SPE-J binds to MHC-II only through the C-terminal zinc site that it shares with other zinc-dependent SAgS (24), and the conclusion must be that, as in the case of SPE-C (23), it is its ability to form dimers that enables SPE-J to cross-link MHC-II.

The mode of dimerization found in our SPE-J crystals was a surprise, however. It does not involve the N-terminal OB-fold binding face, as is the case for dimers of SPE-C (19), nor does it involve the face of the C-terminal β-sheet as for SED (18). Instead, the dimer interface in SPE-J, as seen in the crystal structure, involves residues in and around the interdomain region, residues 10–19 from helix α2 and the α1-β1 loop, 42–45 from the β2-β3 loop, 77–83 from the β4-β5 loop, Gln-138 and Gln-142 from helix α4 in the C-terminal domain, and Arg-181 from helix α5, also in the C-terminal domain. Although it cannot necessarily be inferred that a mode of association seen in crystals also occurs in solution, especially if the surface area buried by this association is not great (34), in the present case SPE-J has been shown to form dimers in solution, at similar concentrations as were used to grow the SPE-J crystals. Most significantly, the same dimer is found in two completely different crystal environments, namely the A-B dimer between two independent molecules in the asymmetric unit and the C-C dimer between SPE-J molecules related by crystallographic symmetry. This argues strongly that the same dimer would be seen in solution. The buried surface area (1360 Å² for A-B and 2130 Å² for the more closely packed C-C dimer) is at the low end of the range for functional dimers (34, 35) but is consistent with the solution data that shows dimerization only at higher concentrations.

The SPE-J mutagenesis results indicate that the TCR binding surface is very similar to that for SPE-C, consistent with their high structural similarity. The strongest decrease in T-cell mitogenicity was observed in the Y14A and R181Q mutants.
molecules as a dimer, and so stimulate intracellular signaling and cytokine expression by antigen-presenting cells, but independently of its TCR activation ability. Which of these activities is expressed may depend on local concentration effects.

Seen in this light, the ability of SPE-J to express different activities under different conditions, stimulating T-cells as a monomer and cross-linking MHC-II as a dimer, is yet another expression of the diverse behavior of this family. It is reasonable to suppose that other activities will be uncovered, even for apparently well characterized family members. In this connection, we note that in at least one crystal form of TSST-1 (41), a crystal dimer is found that uses essentially the same binding surface as in the SPE-J dimer. The relative orientations of the two TSST-1 molecules are different from those of the two SPE-J molecules, but the interaction could imply that TSST-1, too, could express other activities through this association, if replicated in solution. Again by analogy with SPE-J, the interaction in the TSST-1 crystal dimer could provide a model for its TCR binding surface, which has not yet been defined crystallographically.

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