Identification of Class II Major Histocompatibility Complex and T Cell Receptor Binding Sites in the Superantigen Toxic Shock Syndrome Toxin 1

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Summary

Superantigens, in association with class II major histocompatibility complex (MHC) molecules, activate T cells bearing particular β chain variable domains of the T cell receptor (TCR). Unlike conventional peptide antigens, superantigens bind as intact proteins to TCR and MHC molecules outside their peptide binding sites. To characterize these interactions at the molecular level, random point mutations were generated in the gene encoding toxic shock syndrome toxin 1, a bacterial superantigen associated with toxic shock syndrome. Functionally impaired mutants were identified based on their lack of murine and human T cell stimulatory activities, and experiments analyzing binding to human histocompatibility leukocyte antigen-DR molecules differentiated residues involved in MHC from TCR binding. The results showed that the great majority of mutations are clustered in two distinct regions of the toxic shock syndrome toxin 1 molecule. The class II MHC binding site is located in the hydrophobic region of the NH2-terminal domain, and the TCR binding site is primarily in the major central groove of the COOH-terminal domain. These studies provide insight into the interactions necessary for superantigen-mediated disease in humans.

Staphylococcus aureus and group A streptococci secrete a number of exotoxins expressing superantigen activity, including toxic shock syndrome toxin (TSST)1-1, staphylococcal enterotoxins A to E, and streptococcal pyrogenic exotoxins A and C (1, 2). TSST-1 has been shown to be the major cause of toxic shock syndrome (2-4). The development of this disease appears to be dependent on superantigen-induced T cell stimulation and consequent systemic release of cytokines such as TNF and IL-2 (2, 5). TSST-1 activates human T cells bearing β chain variable domains (Vβ)-2, which represent ~10% of the total T cell repertoire (5-7), and stimulation is dependent upon antigen-presenting cells expressing HLA-DR molecules (8, 9). Although x-ray crystallographic studies have suggested that TSST-1 consists of two separate domains (10, 11), the TCR and MHC binding sites have not yet been defined. The present study used a comprehensive mutagenesis of TSST-1 to functionally define these binding sites.

Materials and Methods

Cloning of the Tst-1 Gene from S. aureus. Total genomic DNA was extracted from several strains of TSST1-producing S. aureus (kindly provided by Dr. John James and Dr. Jim Todd, Children’s Hospital, Denver, CO) using a protocol (12) modified for small bacterial cultures. For cloning, the wild-type tst-1 gene (13) was amplified by PCR using flanking primers A and B shown in Table 1. Forward primer A anneals to the first seven codons of the mature wild-type tst-1 gene of S. aureus. Preceding these codons in primer A is an initiation codon (ATG), a ribosome binding site (GGAGG) and a spacer region (As) for efficient expression in Escherichia coli (14), along with a KpnI restriction site for cloning. Reverse primer B anneals to the final 10 codons of tst-1 (including the ochre stop codon TAA) and also contains an XbaI restriction site for cloning. Reverse primer B anneals to the final 10 codons of tst-1 (including the ochre stop codon TAA) and also contains an XbaI restriction site for cloning. After agarose gel electrophoresis and purification by GeneClean (Bio 101, Inc., Vista, CA), the PCR product was digested with KpnI and XbaI and cloned into E. coli as described (15). Several wild-type clones were sequenced, confirming the published sequence (13) as corrected by Lee et al. (16).

Generation of Mutant TSST1 Proteins. PCR primers carrying random mutations (Table 1) were used to generate mutant TSST1 molecules. The primers were synthesized such that, at each position, the three incorrect nucleotides were incorporated at a frequency of 0.25-1.0% (17). Mutations were introduced into tst-1

Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; TSST, toxic shock syndrome toxin; Vβ, β chain variable domain.
The primers A and B are forward and reverse flanking oligonucleotide primers, respectively. The 13 indicated primers, which were randomized with incorrect nucleotides as indicated in the text, were used for mutagenesis of the entire \textit{tst-1} gene. Underlined nucleotides represent regions of overlap between consecutive primers to ensure mutagenesis at the junction and hence not incorporated into the initial PCR product.

via the "megaprimer" PCR method (18, 19). The first PCR reaction was performed using 1 of the 13 mutagenic primers indicated in Table 1 paired with reverse primer B and with the cloned wild-type \textit{tst-1} gene as template. After agarose gel electrophoresis and purification, this PCR product (megaprimer) was used directly in a second PCR reaction with primer A and \textit{tst-1} template to reconstruct the full-length gene. Finally, using gel-purified full-length product, a third PCR reaction was performed with flanking primers A and B to generate sufficient material for cloning. Full-length \textit{tst-1} genes with mutations were then cloned into either pTZ18R or pUC18 plasmids and transformed into \textit{E. coli} for production and screening of mutant proteins (15).

\textbf{Quantitation of TSST-1.} Individual colonies of \textit{E. coli} transformants harboring mutated \textit{tst-1} genes were grown in 96-well plates containing 0.2 ml of 2x YT liquid medium (15) and carbenicillin. The production of recombinant TSST-1 was induced by the addition of 1 mM isopropyl-\beta-D-thiogalactoside, and bacterial lysates were prepared as described (17). To measure the amount of toxin in each lysate, a sandwich ELISA was developed using mAbs to TSST-1, produced by standard methods from BALB/c mice immunized multiple times with TSST-1. The properties of these antibodies will be described in detail elsewhere (Shimonkevitz, R. S., E. Boen, S. Malmstrom, E. Brown, J. M. Hurley, B. Kotzin, and M. Matsumura, manuscript submitted for publication). Two of these mAbs specific for different sites on TSST-1 were adsorbed overnight to microtiter plates, each at a concentration of 1.0 \mu g/ml in PBS. After washing to remove unbound antibody, lysates or known concentrations of TSST-1 were incubated for 2.0 hr. After a second wash, a horseradish peroxidase-conjugated rabbit anti-TSST-1 (Toxin Technology, Sarasota, FL) was used as a secondary reagent to detect bound toxin. The color reaction was developed using o-phenylene-diamine and detected using a microplate ELISA reader.

\textbf{Purification of Mutant TSST-1 Proteins.} Typically, 1 liter of \textit{E. coli} was grown overnight and the production of TSST-1 was induced for 24 hr as described above. Cells were harvested by centrifugation, suspended in 20 ml of 10-mM Tris-HCl/1-mM EDTA buffer, pH 7.4, and disrupted by ultrasonication. The bacterial extract was centrifuged and dialyzed overnight against 10 mM potassium phosphate buffer, pH 6.1. The TSST-1 protein was then purified by HPLC using a cation exchange column (SP-5PW; Bio-Rad Laboratories, Hercules, CA) with a linear NaCl gradient from 0 to 500 mM in 10 mM potassium phosphate buffer, pH 6.1. The wild-type TSST-1 was eluted at \sim 120 mM NaCl. This method yielded \sim 10 mg of purified TSST-1 protein/liter of bacterial culture. The wild-type and mutant: TSST-1 proteins prepared by this method were judged to be >95% pure by analysis on gels after SDS-PAGE.

\textbf{Assays for T Cell Stimulatory Activity.} Bacterial lysates were screened for superantigen activity by stimulation of a murine T cell hybridoma as described (17). Since murine T cells responding to TSST-1 are contained within the subset expressing VB15, a hybridoma cell line expressing VB15 (KOX 15-4.95) was used as responder cells. Various dilutions of lysate in 0.2 ml culture medium (RPMI 1640 or IMDM containing 10% fetal bovine serum, 2 mM t-glutamine, 2 mM pyruvate, 10 mM Heps, 50 U/ml penicillin, and 50 mg/ml streptomycin) were added to microtiter culture wells containing \sim 1 \times 10^5 T hybridoma cells and 5 \times 10^5 HLA-DR-expressing antigen-presenting cells (either LG2, a human B lymphoma cell line homozygous for DR1, or Raji-DR, described below). T cell stimulation was assayed after the overnight release of IL-2 using the IL-2-dependent HT-2 cell line bioassay.

Human PBL were prepared by Ficoll-Hypaque centrifugation, and \sim 1 \times 10^5 cells were added to microtiter wells containing various dilutions of the wild-type or mutant TSST-1 proteins in 0.2 ml culture medium (described above). Cultures were incubated at 37°C for 3 d, pulsed overnight with 0.5 \muCi tritiated thymidine, and harvested. Cellular proliferation was measured by the amount of incorporated radioactivity as quantitated with a beta scintillation counter.

In selected experiments, stimulation of a human VB2+ cell line was also studied. Human PBL were stained for VB2 and CD4 as described (20) and sorted on a cytofluorograph. Double-positive cells were expanded by culture at 2 \times 10^7/ml in the presence of 10^5 unseparated autologous PBL (previously irradiated to 4,000 rad) and 1 \mu g/ml PHA-P (Murex Diagnostics, Ltd., Dartford, UK). After 5 d of culture, PHA was washed out, and IL-2 was added for an additional 10 d before using the cells in a stimulation assay.

For response to wild-type and mutant TSST-1 proteins, VB2+ T cells were cultured at 5 \times 10^5 cells per well in 0.2 ml of the culture medium described above with 1 \times 10^5 LG2 cells.

\textbf{Class II MHC Binding Assay.} Mutant proteins were tested for binding to class II MHC using the Raji-DR cell line (American Type Culture Collection, Rockville, MD), which expresses a high and homogeneous level of cell surface HLA-DR, detected by immunofluorescence. HPLC-purified wild-type and mutant TSST-1 protein were biotinylated, and various concentrations were incubated in the presence of 5 \times 10^5 Raji cells on ice for 2 hr in HBSS containing 0.1% BSA and 0.1% sodium azide. After washing, bound TSST-1 was detected by incubation with PE-avidin, and fluorescence intensity was analyzed on a cytofluorograph. At least 2 \times 10^5 viable cells were evaluated for binding and compared to cells with the wild-type TSST-1.
incubated without TSST-1, and then stained and analyzed in a similar manner.

Results and Discussion

To identify the class II MHC and TCR binding sites on TSST-1, we generated random mutations throughout the entire 194-residue mature protein. The initial screening for mutant proteins was based on the ability of a murine Vβ15-expressing T cell hybridoma to secrete IL-2 in response to the wild-type recombinant TSST-1 expressed in E. coli. Lysates of individual bacterial clones that failed to stimulate IL-2 production were then screened by ELISA for the presence of toxin. Clones that failed to produce toxin or produced toxin at <25% of the wild-type levels were eliminated from further analysis.

The frequency of mutations resulting in nonstimulatory toxins varied greatly in different regions of the molecule (Table 2). Two functionally important regions were revealed. The first region, defined by libraries 1–3, corresponds to residues 1–47 of the mature TSST-1 protein. Of 846 transformants screened within this region, 35 clones (4.1%) failed to stimulate the murine T cell hybridoma. The second region comprises residues 91–152 (libraries 7–10). Here, 155 of 1,646 clones (9.4%) were negative for routine T cell hybridoma activation. We found no mutants affecting T cell stimulation between residues 46 and 92 and only one mutant between residues 151 and 194.

Clones of interest were analyzed by DNA sequencing to identify mutations and then tested for their capability to stimulate human PBL (Table 3). Since TSST-1 stimulates human Vβ2+ T cells, many of the mutants were also tested against a human Vβ2+ T cell line with results identical to those obtained with human PBL (data not shown). Although many mutants that were negative in the murine T cell hybridoma stimulation assay showed partial to full activity with human responding PBL, several of the mutant toxins were completely devoid of human T cell stimulatory activity. These included mutants with amino acid substitutions of Tyr13 → Leu and Ser15 → Trp (designated Y13L/S15W), G16V, G31S/S32P, all mutants at residues H135 and L137V, and several at residue Q139.

The differences in murine and human T cell stimulation by certain TSST-1 mutants may reflect the extensive amino acid polymorphism between the human Vβ2 and murine Vβ15 TCR (5) and/or differences in the two assay systems used to measure T cell proliferation. A similar observation was made for a number of staphylococcal enterotoxin B (SEB) mutants that failed to stimulate any murine T cell hybridomas (17) but strongly stimulated human T cells (Kappler, J. W., and B. L. Kotzin, unpublished work). The structural similarities between murine Vβ15 and human Vβ2 that allow subsets expressing these Vβ to be selectively targeted by TSST-1 are not currently known.

To further characterize the mutations that abolished human PBL stimulatory activity and to dissect the effects of the mutations on TCR and MHC binding, several mutant proteins of interest (Y13L/S15W, G16V, I45V, G31S/S32P, T128S/V88A, H135Q, H135R, L137V, Q139K, and Q139P) were purified by ion-exchange HPLC. A quantitative proliferation assay of the purified mutant toxins with responding human PBL revealed that all of these mutants were 104–106-fold less active than the wild-type TSST-1 (Fig. 1 A), except for I45V, which showed 100-fold decreased activity (Fig. 1 B). Interestingly, even very conservative side-chain changes such as G16V, H135Q, and L137V in TSST-1 dramatically decreased its T cell-stimulatory activity.

Table 2. Frequency of Mutant TSST-1 Proteins Unable to Stimulate Murine Vβ15+ T Cells

| Library number | TSST-1 residues subject to mutation | Murine Vβ15 stimulation | Percentage |
|---------------|-----------------------------------|-------------------------|------------|
|               |                                    | Functionally defective/total studied |            |
| 1             | 1–17                               | 10/282                  | 3.5        |
| 2             | 16–32                              | 17/282                  | 6.0        |
| 3             | 31–47                              | 8/282                   | 2.8        |
| 4             | 46–62                              | 0/94                    | 0.0        |
| 5             | 61–77                              | 0/94                    | 0.0        |
| 6             | 76–92                              | 0/60                    | 0.0        |
| 7             | 91–107                             | 6/282                   | 2.1        |
| 8             | 106–122                            | 7/282                   | 2.5        |
| 9             | 121–137                            | 35/429                  | 8.2        |
| 10            | 136–152                            | 107/653                 | 16.4       |
| 11            | 151–167                            | 0/94                    | 0.0        |
| 12            | 166–182                            | 1/107                   | 0.9        |
| 13            | 181–194                            | 0/35                    | 0.0        |
### Table 3. Functional Effects of TSST-1 Mutations

| Murine Vβ15 nonstimulatory mutants | Stimulation of human PBL* | Binding to HLA-DR† |
|-----------------------------------|--------------------------|------------------|
| L10Q/D11V                         | + + + +                   | + + + +          |
| Y13L/S15W                         | –                        | + + + +          |
| S14I                              | +                        | + + + +          |
| S15G                              | + + + +                   | + + + +          |
| G16V                              | –                        | –                |
| T21R                              | + + + +                   | + + + +          |
| G31S/S32P                         | –                        | –                |
| I45V                              | + + + +                   | + + + +          |
| P97L/L102V                        | + + + +                   | + + + +          |
| L102S                             | + + + +                   | + + + +          |
| L113F/K114T                       | + + + +                   | + + + +          |
| W116R, S                          | + + + +                   | + + + +          |
| S127F                             | +                        | + + +            |
| T128S/V88A‡                       | +                        | + + +            |
| F131I, L, V, Y                    | + + + +                   | + + + + (F131Y)  |
| E132A, G, I, P, R, V, W           | + + (E132W), + + + +     | + + + +          |
| E132A/Q136L                       | + + + + +                 | + + + +          |
| R134L                             | +                        | + + + +          |
| H135A, D, I, Q, R                 | –                        | + + + + (H135Q, R)|
| Q136R                             | + + + +                   | + + + +          |
| L137V                             | –                        | + + + +          |
| T138I                             | +                        | + + + +          |
| Q139E, H, K, L, P, R              | + +, – (Q139K, P)        | + + + + (Q139K, P)|
| Q139E/L143S                       | –                        | + + + +          |
| Q139E/S147R                       | +                        | + + + +          |
| Q139L/Y144F                       | –                        | + + + +          |
| Q139L/Y144N                       | +                        | + + + +          |
| I140K, R, T, V                    | + + + +                   | + + + + (I140R, T)|
| H141D, L, N, P, R, Y              | + + + +                   | + + + + (H141N)  |
| H141L/S147G                       | + + + +                   | + + + +          |
| R145H, P, S                       | + + + +                   | + + + + (R145H)  |
| T150P                             | + + + +                   | + + + +          |

All mutant proteins listed here, except for mutations at residues 132, failed to stimulate the murine Vβ15 T cell hybridoma yet produced wild-type levels of TSST-1 proteins as determined by ELISA. Mutant proteins with more than two amino acid substitutions were eliminated from the table.

*Toxin concentrations ranging from 2.5 pg to 250 ng/ml were tested. Data from the 2.5-ng/ml dose (see Fig. 1) are presented as: + + + +, 75-100%; + + +, 50-75%; + +, 25-50%; +, 10-25%; and -, <10% of the response to wild-type toxin.

†Toxin concentrations from 0.1 ng to 100 ng/ml were tested, with data obtained using the 0.1 ng/ml dose presented as: + + + +, 75-100%; + + +, 50-75%; + +, 25-50%; +, 10-25%; and -, <10% of the binding obtained using wild type TSST-1. Blank spaces indicate that those mutants were not tested for binding to HLA-DR.

‡Although only residues 121-137 were targeted for mutagenesis, a second mutation (V88A) was found by DNA sequencing, presumably due to a PCR error.

HLA-DR binding studies using biotinylated TSST-1 directly demonstrated that the G31S/S32P mutations markedly diminished class II MHC binding, whereas the I45V mutation had only a slight effect (Fig. 2). The residual MHC binding of the G31S/S32P mutant may account for its residual T cell-stimulatory activity at high concentration on human PBL (Fig. 1 B). To dissect the effect of mutations at residues 31 and 32 on MHC binding, single amino acid mutants G31S and S32P were generated. The former mutant stimulated human PBL similarly to the wild-type TSST-1,
Figure 1. Proliferative response of human PBL to HPLC-purified mutant and wild-type TSST-1 proteins. Stimulation assays were performed as described in Materials and Methods. (A) Comparison of mutants that affect TCR binding (except for G31S/S32P) -- O--, Y13L/S15W; -- A-, G16V; -- ~-, G31S/S32P; -- l-, T128S/V88A; -- H135Q; -- ~-, H135R; -- ~-, L137V; -- ~-, Q139K; -- ~, Q139P; -- &-, wild-type fTSST-1. (B) Comparison of mutants that affect MHC binding. -- O-, G31S; -- O-, S32P; -- ~-, G31S/S32P; -- ~-, 145V; -- ~-, wild-type fTSST-1.

whereas the latter was 104-fold less active than the wild-type protein (Fig. 1 B). Likewise, binding experiments showed that S32P was impaired in HLA-DR binding by four orders of magnitude (Fig. 2), whereas G31S showed little difference from wild type (data not shown). Thus, residue 32 accounts for most, but not all, of the impaired interaction with class II MHC observed with the double mutant.

Fig. 3 illustrates the locations on the three-dimensional structure of TSST-1 (11) of mutations that had the greatest effect on human T cell-stimulatory activity (see mutants indicated as negative for human PBL-stimulatory activity in Table 3). These mutations are unambiguously clustered within two sites: a TCR binding site comprising both the proximal NH2- and COOH-terminal domains (residues 13–16 and 135–139, respectively) and a class II MHC binding site within the NH2-terminal domain (residues 31–45). As shown in Table 3, additional mutants that knocked out murine but not human T cell stimulation were identified. If these mutations are also considered, the TCR binding site consists of the central long α helix (residues 127 to 150) and the NH2-terminal short α helix along with its subsequent loop (residues 16–10). In addition, a portion of the loop between the β7 and β8 sheets (residues 97–116) influences TCR binding. Thus, the TCR binding site identified by murine T cell responses precisely overlaps with the region comprising the major groove in the TSST-1 molecule (10, 11). Our results indicate that amino acid residues at positions 13 and/or 15, 16, 135, 137, and 139 appear to be the most important for TCR binding, because mutations in these positions almost completely abolished the stimulatory activity of TSST-1 for both murine and human T cells. Most notably, the residues at positions 16, 135, and 139 are located on the protein surface (10, 11), and therefore these residues might directly interact with the TCR. On the other hand, residue 137 is buried within the protein and, therefore, the effect of this mutation on TCR binding would be indirect, presumably because of a distortion of local structure.

The MHC binding site localizes to the region in the vicinity of the NH2-terminal portion of the second β strand (Fig. 3). The five β strands in this region form a structure.
known as a β-barrel motif (10, 11). Mutations G31S/S32P, which greatly impair HLA-DR binding, are located in a solvent-exposed hydrophobic surface formed by the concave face of β-barrel strands 1, 2, and 3 in the NH2-terminal domain. The β-barrel motif in the NH2-terminal domain of TSST-1 is similar to that in the corresponding domain of SEB. In SEB, nonpolar residues F44, L45, and F47 form a ridge that protrudes from the loop between β-strands 1 and 2 in the NH2-terminal domain, and this ridge interacts with a hydrophobic depression in the HLA-DR1 molecule (21, 22). Mutations in SEB at F44 disrupt binding to HLA-DR (17). Similarly, our study suggests that this hydrophobic region of TSST-1 may form the MHC binding site. The I45V mutation (on β-strand 3) also affects MHC binding, although its effect is not as significant as G31S/S32P (Fig. 1 B and Fig. 2). In contrast to the mutations of G31S and S32P, which locate on the protein surface, the isoleucine at position 45 is within the protein interior (10, 11). It is likely, therefore, that the I45V mutation indirectly affects MHC binding by perturbing the conformation of the hydrophobic surface.

Binding of both TSST-1 and SEB to HLA-DR are affected by the same mutations in the DR α-chain, most notably at residue K39 (23, 24). Although we found only a single MHC binding site in TSST-1, mutational analysis of SEB predicted two class II MHC binding sites on opposite sides of the molecule (17). This may suggest that, whereas TSST-1 and SEB interact with the same region of the HLA-DR1 α-chain, SEB can use a second distinct binding site.

Our findings are consistent with and greatly extend previous studies of TSST-1 mutations that affect T cell mitogenicity and toxicity (16, 25–27). For example, TSST-ovine is a naturally occurring variant that is different from TSST-1 at seven amino acids, including E132K and I140T. These changes have been predicted to be responsible for its reduced toxicity in rabbits (25). Furthermore, the mutant H135A was identified in a previous study as lacking murine T cell–stimulatory activity (27). Our studies show that these mutations are centrally located in the TCR binding site.

TSST-1 has been implicated in ~90% of menstruation-related and nearly half of nonmenstrual cases of toxic shock syndrome (2, 4). In addition, some studies have suggested that TSST-1 may be an etiologic agent in some cases of Kawasaki syndrome (20, 28), an inflammatory syndrome of children, although this remains controversial (29). Different superantigens have also been suggested as triggers for other autoimmune diseases (30–32). The present studies provide a basis for designing immunomodulatory molecules that interfere with superantigen activity and vaccines that prevent the harmful effects of these potent immunostimulatory molecules.

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