Identification from a Phage Display Library of Peptides That Bind to Toxic Shock Syndrome Toxin-1 and That Inhibit Its Binding to Major Histocompatibility Complex (MHC) Class II Molecules†

Atsushi Sato,*,‡ Nobuo Ida, Mayumi Fukuyama, Keishi Miwa, Jun Kazami, and Haruji Nakamura

Medical Devices and Diagnostic Research Laboratories, Toray Industries Inc., 3-3-3, Sonoyama, Otsu, Shiga, 520, Japan

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ABSTRACT: Phage display technique is a powerful tool with which to identify novel binding sequences for antibody and receptor targets. Few studies, however, have used this technology to select affinity peptides for ligand molecules. Here, we screened a peptide phage library for binding to toxic shock syndrome toxin 1 (TSST-1) to examine whether peptide ligands for TSST-1 which mimic the structure of major histocompatibility complex (MHC) class II receptors could be identified. After three cycles of biopanning, four potent sequences reactive with TSST-1 were isolated (designated phages 2, 3, 8, and 11). Selected phage were found to react specifically with TSST-1 but not with other staphylococcal exotoxins. A synthetic peptide (pep3) corresponding to the most frequently identified sequence (phage3) was shown to inhibit binding of all four isolated phage to TSST-1, suggesting that they bind to a common site on TSST-1. Furthermore, pep3 was shown to compete with MHC class II molecules for binding to TSST-1 in a concentration-dependent manner. Comparison of their sequences with MHC class II molecules revealed that phage8 shared sequence homology with two regions of the β chain of MHC class II molecules: amino acids 57−62, containing a residue (Tyr-60) involved in TSST-1 binding as suggested by X-ray crystallographic data of TSST-1−MHC class II complex; and amino acids 188−193, a region not previously known as a contact domain. These results suggest that the selected sequences recognized the MHC class II binding site on TSST-1. Thus, affinity selection for peptides binding to ligand molecules (e.g., TSST-1) rather than their cognate receptors (e.g., MHC class II) from a random phage display library represents a useful approach to understanding receptor−ligand interactions.

Toxic shock syndrome toxin 1 (TSST-1)[1] is a member of the family of staphylococcal enterotoxin (SE) superantigens produced by *Staphylococcus aureus* and is strongly implicated in toxic shock syndrome (Bergdoll et al., 1981; Schlievert et al., 1981). TSST-1 is an exotoxin with a molecular mass of 22 kDa and is composed largely of β structure. Unlike other SEs, it is a simple protein with no disulfide bonds (Blomster-Hautamaa et al., 1986). Despite low sequence homology, X-ray crystallographic analysis revealed that TSST-1 and staphylococcal enterotoxin have very similar three-dimensional structures, indicating similar biological functions (Acharya et al., 1994; Prasad et al., 1993).

In contrast to conventional protein antigens which require intracellular proteolytic processing, superantigens serving as ligands bind to major histocompatibility complex (MHC) class II receptors outside of the antigen-binding site in a nonprocessed form (Uchiyama et al., 1989; Dellabona et al., 1990) and stimulate T lymphocytes expressing particular Vβ T-cell receptor chains (Kappler et al., 1989). Thus, interaction of superantigens with MHC class II molecules plays an important role in the induction of T-cell activation.

Both crystallographic data (Acharya et al., 1994; Prasad et al., 1993) and peptide competition studies (Soos et al., 1993) suggest that the N-terminal β-barrel domain of TSST-1 is involved in MHC class II binding. The X-ray crystal structure of a complex between TSST-1 and a class II MHC molecule, HLA-DR1, was determined recently (Kim et al., 1994). Understanding the structural interaction between superantigens and MHC class II molecules may be useful in designing new therapeutic compounds for toxic shock syndrome.

Random peptide libraries constructed by fusion of random sequences to the amino-terminal region of pIII of filamentous bacteriophage are powerful tools with which to identify specific binding sequences for biologically interesting targets (Scott & Smith, 1990). Using this technique, peptide ligands for urokinase receptor (Goodson et al., 1994), immunoglobulin receptor of B-cell lymphoma (Renschler et al., 1994), concanavalin A (Oldenburg et al., 1992; Scott et al., 1992), integrins (Koivunen et al., 1993; Healy et al., 1995), and several antibodies (Scott & Smith, 1990; Miceli et al., 1994; Cwirla et al., 1990) have been identified without prior knowledge of conformational interactions. Furthermore, successful mimicry of the epitopes of a conformation-dependent monoclonal antibody (Zhong et al., 1994) and isolation of sequences binding to a target protein in a conformation-specific manner (Dedman et al., 1993) sug-

† The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the following accession numbers: D64063 for phage11, D64064 for phage2, D64065 for phage3, and D64066 for phage8.

* Address correspondence to this author. Phone: +81-6-872-8204. FAX: +81-6-872-8219. E-mail: Satou@beri.co.jp.

‡ Current address: Department of Molecular Biology, Biomolecular Engineering Research Institute, 6-2-3, Furuedai, Suita, Osaka, 565, Japan.

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[1] TSST-1, toxic shock syndrome toxin 1; MHC, major histocompatibility complex; ELISA, enzyme-linked immunosorbent assay; HLA, human histocompatibility leukocyte antigen; SE, staphylococcal enterotoxin; BSA, bovine serum albumin; IL, interleukin; MCP, monocyte chemoattractant protein; HRP, horseradish peroxidase.

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gested the possibility of isolating specific ligands for the MHC class II-binding site of superantigens.

In this study, a random phage display library was used to select ligands for TSST-1 that inhibited binding of TSST-1 to MHC class II molecules. This approach should help to improve our understanding of structural interactions between superantigens and MHC class II molecules and may help to find new drug candidates for the treatment of human toxic shock syndrome.

MATERIALS AND METHODS

Construction of a 15-Amino-Acid Random Phage Display Library. Enzymes were purchased from Takara Biochemicals (Tokyo, Japan) and New England Biolabs (Beverly, MA). The fuse vector and an *Escherichia coli* host strain (K91 kan) were kindly provided by Dr. G. P. Smith (University of Missouri, Columbia) (Scott & Smith, 1990). Degenerate DNA inserts were prepared essentially as described by Scott et al. (Scott & Smith, 1990) with the exception that the insert encodes 15 random amino acid instead of 6. The fuse 5 vector (5 μg) was cut with *Sfi*I and then ligated overnight with *Bgl*I-digested degenerate fragments with T4 DNA ligase. The ligated fuse 5 was purified with precipitation by ethanol and on a spin column (Chroma Spin-400, Clontech Laboratories, Palo Alto, CA) and then electroporated into *E. coli* MC1061 cells. The library contains 2×10^8 random peptide phage clones.

Preparation of TSST-1. TSST-1 was prepared from cultures of *S. aureus* (FRI1169) inoculated in sterile NZ-amine/yeast extract medium composed of 4% NZ-amine A, 0.15% yeast extract, and 10 mg/L nicotinic acid and 0.5 mg/L thiamin hydrochloride (adjusted to pH 6.8 with 5 N NaOH) at 30 °C for 48 h. The cells were removed by centrifugation, and TSST-1 produced in culture supernatant was purified using ion-exchange chromatography (SP-Sephadex C-25, Pharmacia Biotech, Uppsala, Sweden) and chromatofocusing (PBE94, Pharmacia Biotech, Uppsala, Sweden) essentially as described by Igarashi et al. (1984). TSST-1 (5 μg) gave a single band on an SDS−polyacrylamide gel when stained with Coomassie brilliant blue (>95% pure).

Screening of TSST-1 Binding Phage in a Random Peptide Library. A Petri dish (Falcon no. 1008, Becton Dickinson and Co., Oxnard, CA) was coated overnight at 4 °C with 10 μg of purified TSST-1 in 2 mL of phosphate-buffered saline (PBS). The dish was washed twice with PBS and then blocked with PBS containing 0.5% bovine serum albumin (BSA) for 1 h at room temperature. After removal of the blocking solution, approximately 10^{12} virions of a random phage display library in TBS-T buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Tween-20) were incubated in TSST-1-coated Petri dishes for 1 h at room temperature. The unbound phage were removed by washing 10 times with TBS-T, and bound phage were eluted with glycine hydrochloride buffer (0.1 M glycine, pH 2.2, containing 1 μg/mL BSA). Phage elutions were neutralized and then amplified in *E. coli* K91 kan. The binding and elution reactions were repeated twice except that the background yield was reduced by incubating eluted phage in a Petri dish coated with BSA only (1 μg/mL) prior to incubation in a TSST-1-coated dish. Sixteen selected phage from the third round were inoculated separately and incubated overnight at 37 °C in 3 mL of LB medium containing 20 μg/mL tetracycline. The cells were then removed by centrifugation and 1 μL of supernatant (containing 10^9-10^{10} phage particles) was used directly for analysis by a phage enzyme-linked immunosorbent assay (ELISA).

Phage ELISA. Microtiter plates (Maxisorp, Nunc, Denmark) were coated with 100 μL of PBS containing 1.0 μg/mL TSST-1, other staphylococcal enterotoxins (SEA, SEB, or SEC) or cytokines [interleukins 6 and 8 or interferon-inducible protein 10 (IP-10) or monocyte chemoattractant protein 1 (MCP-1)] at 4 °C overnight. Plates were blocked with PBS containing 0.5% BSA for 1 h at room temperature. Plates were then washed with wash buffer (2-fold diluted PBS containing 0.025% Tween-20) and 10^9-10^{10} phage particles/100 μL of assay buffer (PBS containing 0.25% BSA and 0.05% Tween-20) were incubated in each well for 1 h at 25 °C. After the wells were washed three times with wash buffer, 100 μL of horseradish peroxidase- (HRP-) labeled sheep anti-M13 IgG (Pharmacia Biotech, Uppsala, Sweden) in assay buffer at a dilution of 1:5000 was incubated for 30 min at 25 °C. After the plate was washed three times, 100 μL of substrate solution [3,3′,5,5′-tetramethyl benzidine (TMB) and peroxidase] were pipetted into the wells and incubated for 5 min at room temperature. The reaction was stopped by adding 100 μL of 1 N H_2SO_4. Absorbances at 450 nm were measured in a microplate reader. In a competitive ELISA, peptide competitors (TSST-1 or a synthetic peptide) at the indicated concentration were added to compete with immobilized TSST-1 or phage during the phage binding step.

Immunoblot Analysis. Purified TSST-1 (1 μg) and BSA (1 μg) as a negative control were spotted on the nitrocellulose membrane (Hybond C super, Amersham, England, U.K.) and dried at room temperature. Filters were blocked with blocking solution (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 3% gelatin) for 1 h at room temperature. Filters were incubated with phage3 or fuse 2 (wild type) at a concentration of 10^{11} phage particles/mL in TBS (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) containing 1% gelatin for 2 h at room temperature. After the filters were washed five times, they were then incubated with HRP-labeled sheep anti-M13 IgG in TBS containing 1% gelatin (1000-fold-diluted) for 1 h at room temperature. Color development was carried out by a commercial immunostaining kit (Immunostain 1000, Konica, Tokyo, Japan). With regard to western blot analysis, purified TSST-1 (1 μg) and BSA (1 μg) were subjected to electrophoresis in a 0.1% SDS/5−20% polyacrylamide gel and transferred onto a nitrocellulose filter. After transfer, the membrane was treated as described above.

Synthetic Peptide (pep3). A synthetic peptide (pep3) corresponding to the most frequently identified sequence (phage3) was prepared by Sawady Technology Co., Ltd. (Tokyo, Japan). The amino acid sequence of pep3 is

GADRSYLSFIHLYPELAGA

The glycine−alanine sequence (underlined) flanking the insert was a spacer sequence derived from the fuse 5 vector. The peptide was purified by reverse-phase high-performance liquid chromatography (HPLC). Pep3 was more than 95% pure after HPLC.

The control peptide used in a competition study was a 24-mer peptide corresponding to the N-terminal portion of the core protein of hepatitis C virus (Sato et al., 1994). The

amino acid sequence of the control peptide is

PQRKTKRNTSRRPQDVKFPGGGQI

Preparation of Human Histocompatibility Leukocyte Antigen (HLA) DR Antigens from Daudi Cells. The Daudi human B lymphoblast cell line was grown in RPMI 1640 medium containing 10% fetal bovine serum. Cells were harvested, washed in PBS, and suspended in lysis buffer [20 mM Tris-HCl, pH 8.0, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)]. Cells were then disrupted by two cycles of freezing and thawing and centrifuged at 4000*g* for 5 min. The supernatant was collected and centrifuged at 55000*g* for 1 h. The pellet was resuspended in lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, and 0.1 mM PMSF and incubated on ice for 30 min to solubilize MHC class II molecules. The detergent lysate was centrifuged at 330000*g* for 30 min. The partial purification of detergent-solubilized MHC class II molecules was a variation of the methods described by Kaufman et al. (Kaufman & Strominger, 1979). The supernatant fraction was applied to a DEAE-Toyopearl 650 M column (Tosoh Co., Tokyo, Japan) equilibrated with a solution containing 20 mM Tris-HCl, pH 8.0, 0.1 mM PMSF, and 0.1% Triton X-100. After the column was washed with a solution containing 20 mM Tris-HCl, pH 8.0, 0.1 mM PMSF, 0.1% deoxycholic acid sodium salt, and 20 mM NaCl, the column was eluted with buffer E (20 mM Tris-HCl, pH 7.0, 0.1 mM PMSF, 0.1% deoxycholic acid sodium salt, and 200 mM NaCl). Then the eluted fraction was subjected to a concanavalin A− (Con A−) Sepharose column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer E. After being washed with buffer E, the column was eluted with buffer E containing 1 M methyl α-D-mannopyranoside (Fluka Chemie AG, Buchs, Switzerland). The eluate was dialyzed against a solution containing 20 mM Tris-HCl, pH 8.0, 0.1 mM PMSF, and 0.1% deoxycholic acid sodium salt for further use. The partially purified MHC class II molecules were confirmed by immunoblot analysis probed with mouse monoclonal anti-MHC class II antibodies (TH14B, VMRD Inc.) and SDS−polyacrylamide gel electrophoresis analysis.

Inhibition of Binding of TSST-1 to MHC Class II Molecules by a Synthetic Peptide (pep3). Competition of pep3 with the immobilized MHC class II molecules for binding to TSST-1 was investigated in a nonradioactive assay (ELISA). A microtiter plate was coated overnight at 4 °C with 1 μg of partially purified MHC class II molecules/well in 100 μL of a solution containing 20 mM Tris-HCl, pH 8.0, 0.1 mM PMSF, and 0.1% deoxycholic acid sodium salt. The wells were washed with 400 μL of PBS and blocked with PBS containing 0.5% BSA for 1 h at room temperature. The wells were washed twice with wash buffer (2-fold diluted PBS containing 0.025% Tween-20), and 10 ng/mL TSST-1 in an assay buffer (PBS containing 0.25% BSA and 0.05% Tween-20) was incubated with the immobilized MHC class II molecules in the presence of pep3 at the indicated concentrations for 1 h at 25 °C. After the plates were washed three times with wash buffer, 100 μL of biotinylated mouse monoclonal anti-TSST-1 antibody (Miwa et al., 1994) in an assay buffer (0.1 μg/mL) was added to each well and incubated for 1 h at 25 °C. Then the plates were washed three times and 100 μL of avidin−HRP conjugate solution (0.625 μg/mL in an assay buffer, Zymed Laboratories, San Francisco, CA) was added and incubated for 30 min at 25 °C. After the plates were rinsed three times, color developing solution [3,3′,5,5′-tetramethylbenzidine (TMB) and peroxidase] was added and incubated for 15 min at 25 °C. The enzyme reaction was terminated by adding 100 μL of 1 N H_2SO_4. Absorbances at 450 nm were measured with a microplate reader.

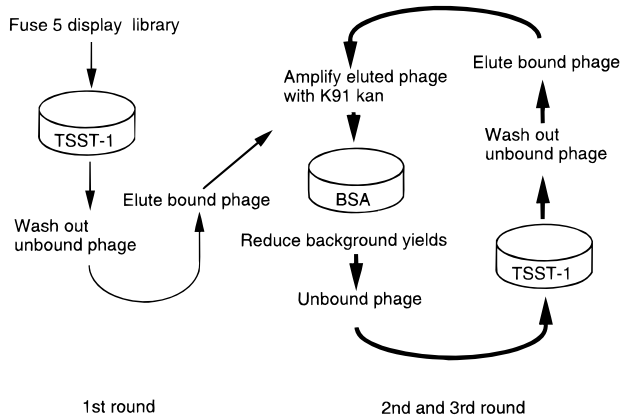

FIGURE 1: Procedure for isolating TSST-1 binding phage. Approximately 10^{12} virions of a phage display library were allowed to bind to TSST-1-coated dishes for 1 h at room temperature. After the first round of biopanning, bound phage were transferred to Petri dishes coated with BSA only to absorb background yields in the second and third rounds.

RESULTS

Screening of a 15-Amino-Acid Phage Library for Peptides Binding to TSST-1. A 15-amino-acid phage display library containing 2×10^8 independent transformants was constructed in a fuse 5 vector (Scott & Smith, 1990). This peptide library was screened with TSST-1 that had been immobilized directly on Petri dishes. Phage binding to TSST-1 coated dishes were isolated by repeated rounds of selection. Bound phage were eluted with low pH and then amplified by infection into *E. coli* K91 kan. To eliminate background yields, BSA-coated dishes were used to absorb the nonspecifically bound phage to selection with TSST-1-coated dishes in the second and third rounds (Figure 1). This step appears to be effective in preventing an enrichment of background yields, since we failed to isolate TSST-1-binding phage without this absorption step.

A total of 16 clones obtained after the third round of screening were randomly chosen and subjected to analysis by an ELISA. All but three clones (1, 6, and 10) were found to be reactive in an ELISA assay (solid bars in Figure 2). Inhibition of binding of isolated phage to the immobilized TSST-1 was observed in all reactive samples in the presence of native TSST-1, demonstrating specificity for binding to TSST-1.

Since successful screening of a cyclic hexapeptide library (CX_6C) has been reported (O'Neil et al., 1992), we undertook the screening of a conformationally constrained peptide library to identify high-affinity ligands. Unfortunately, we were not able to obtain any reactive clones from this library (data not shown).

Sequence Analysis of Isolated Phage. Twelve clones (except 1, 6, and 12 in Figure 2) showing specific reactivity in an ELISA were subjected to DNA sequence analysis. The deduced amino acid sequences of selected phage are shown in Table 1. These 12 clones comprised only four different

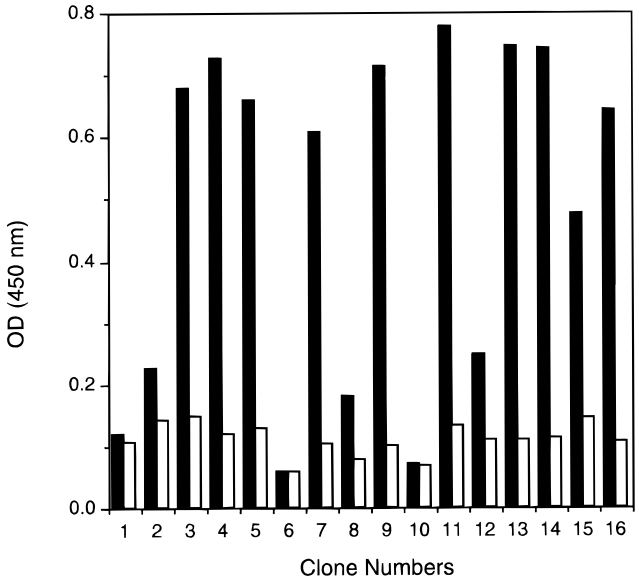

FIGURE 2: Selection of TSST-1 binding phage by an ELISA. Sixteen clones from the third-round biopanning were randomly selected to confirm the affinity and specificity by an ELISA. Absorbances at 450 nm in a noncompetitive ELISA (solid bars) and a competitive ELISA (open bars) were shown. In a competitive ELISA, 1 μg of native TSST-1 was added to each well to compare with the immobilized TSST-1 for binding to individual phage.

Table 1: TSST-1 Binding Sequences Isolated from a Phage Display Library

clone	sequence	frequency[a]	aromatic residues[b]
phage3	DRS**Y**LS**F**IHL**Y**PELA	8/12	3/15 (20%)
phage11	SARL**W**AE**Y**LPL**Y**RHM	2/12	3/15 (20%)
phage2	**F**NGGAQMG**W**D**YYWFF**	1/12	7/15 (47%)
phage8	**W**DAM**YW**N**W**RSVSE**F**H	1/12	5/15 (33%)

[a] Number of independent clones isolated from a phage display library. [b] Number of aromatic residues included in the sequence (boldface type).

sequences [samples 2 (phage2) and 8 (phage8) were independent clones in the 12 analyzed, the sequences of 3, 4, 5, 7, 9, 13, 14, and 15 were identical to each other (phage3), and 11 and 16 shared the same sequence (phage11)]. The four isolated sequences did not show obvious homology except a significant enrichment of Tyr, Phe, and Trp residues, which have aromatic side chains (20−47% included in each sequence; see Table 1).

Sequence similarity between selected clones and MHC class II receptors was investigated (Figure 3). Although no clear consensus sequence was observed in the α chain of MHC class II molecules, it was found that phage8 showed sequence similarity with two regions in the β chain of MHC class II molecules (domain A, amino acids 57−62; domain B, amino acids 188−193). Domain A was found to contain a residue that may hydrogen-bond to Gln-73 of TSST-1, as had been suggested by X-ray crystallographic analysis of the TSST-1−MHC class II complex (Kim et al., 1994), whereas domain B has not been reported as a contact region. It is attractive to speculate that phage8 mimics the three-dimensional structure of TSST-1-binding domain of MHC class II molecules, which may comprise both domains A and B.

Immunoblot Analysis of TSST-1 Binding Phage. Figure 4 shows the result of immuno-dot-blot analysis of TSST-1 binding phage (phage3) that was recovered most frequently in a screening. Phage3 showed specific reactivity for

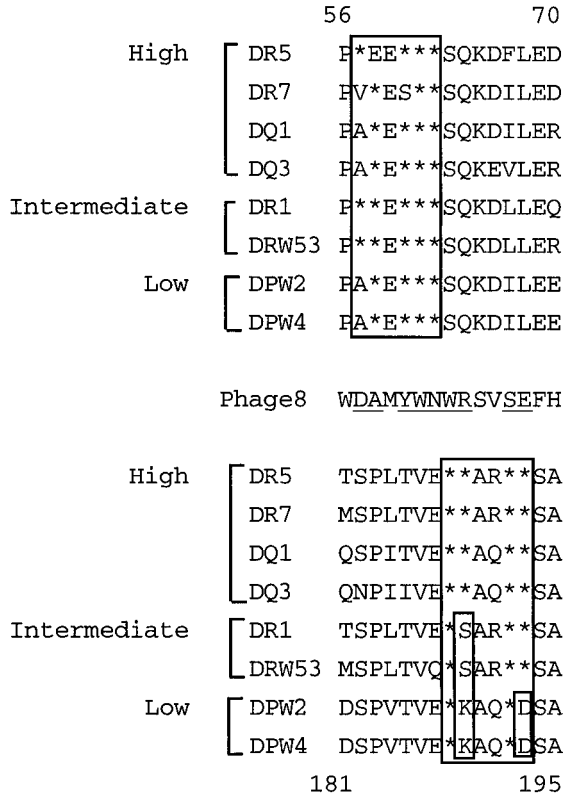

FIGURE 3: Multiple amino acid sequence alignment of phage8 and the β chain of MHC class II molecules. Sequences derived from the β chain of MHC class II molecules were arranged according to consensus sequences with phage8 and affinity for TSST-1 (Scholl et al., 1990) [high affinity, DR5 (Tieber et al., 1986), DR7 (Young et al., 1987), DQ1 (Boss & Strominger, 1984), and DQ3 (Larhammar et al., 1983); intermediate affinity, DR1 (Long et al., 1983) and DRW53 (Young et al., 1987); low affinity, DPW2 (Tonnelle et al., 1985) and DPW4 (Kelly & Trowsdale, 1985)]. Consensus sequences were boxed. Numbering indicates amino acid position in mature β chain of MHC class II molecules. Asterisks indicate identical amino acids corresponding to the underlined sequences of phage8. Boxed amino acid residues 189 and 193 might be possible amino acid substitutions associated with the affinity for TSST-1 (see the Discussion section).

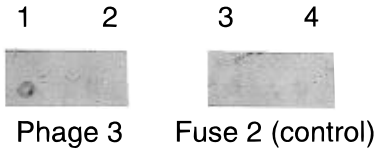

FIGURE 4: Immuno-dot-blot analysis of TSST-1 binding phage (phage3). One microgram of TSST-1 spots 1 and 3) and BSA (spots 2 and 4) as a negative control were spotted onto the nitrocellulose membrane. The membrane was treated with TSST-1 binding phage (phage3, left) and control phage (fuse2, right) as described in Materials and Methods).

immobilized TSST-1 but not for BSA, whereas the control phage (fuse 2) failed to bind to TSST-1. On the other hand, phage3 failed to recognize heat-treated (incubation at 95 °C for 5 min) or SDS (1%)-treated TSST-1 on western blots (data not shown), indicating that binding of phage3 to TSST-1 may be conformation-dependent.

Inhibition of Binding of Selected Phage to TSST-1 by a Synthetic Peptide (pep3). A representative peptide corresponding to the most frequently identified sequence (Phage3) was synthesized (designated pep3) and confirmed to inhibit binding of isolated phage to TSST-1 (Figure 5). To maximize the flexibility of the essential sequence of phage3,

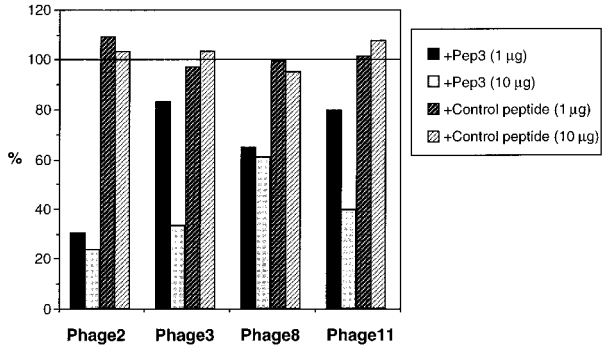

FIGURE 5: Inhibition of selected phage binding to TSST-1 by a synthetic peptide (pep3). A synthetic peptide (pep3) was used to compete with four isolated phage for binding to the immobilized TSST-1. Relative absorbance values at 450 nm were shown. One hundred percent corresponds to absorbance values at 450 nm in a noncompetitive ELISA (in the absence of pep3). The data represent means from duplicate wells.

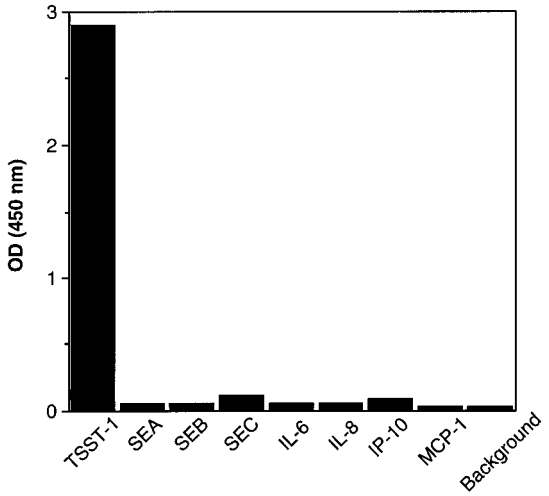

FIGURE 6: Cross-reaction of phage3 with other SEs and cytokines. Approximately 10^{10} virions of phage3 were tested to confirm the specificity in a phage ELISA as described in Materials and Methods. Absorbances at 450 nm were shown. Reactivity in a BSA-coated well was expressed as a background value. The data represent means of duplicate wells.

it was flanked by glycine−alanine spacers that were derived from the fuse 5 vector (see Materials and Methods). Despite the absence of a consensus among the four isolated sequences, binding of all four isolated phage to TSST-1 was inhibited by pep3, while no competition was observed with the control peptide. This competition experiment suggests that selected phage recognize a common or overlapping binding site on TSST-1. Moreover, the existence of a clear consensus between phage8 and MHC class II molecules allows us to consider that all four isolated phage may recognize an MHC class II binding site on TSST-1.

Cross-Reaction of Phage3 with Other SEs and Cytokines. Since little information has been reported concerning the specificity of peptide ligands isolated from a phage display library, a representative clone (phage3) was chosen to react with other purified SEs (SEA, SEB, and SEC) or cytokines (IL-6, IL-8, and IL-8 superfamily proteins) in a phage ELISA to investigate possible cross-reactivity. As shown in Figure 6, no cross-reactivity was observed with other SEs or cytokines, indicating that phage3 recognizes TSST-1 with high specificity.

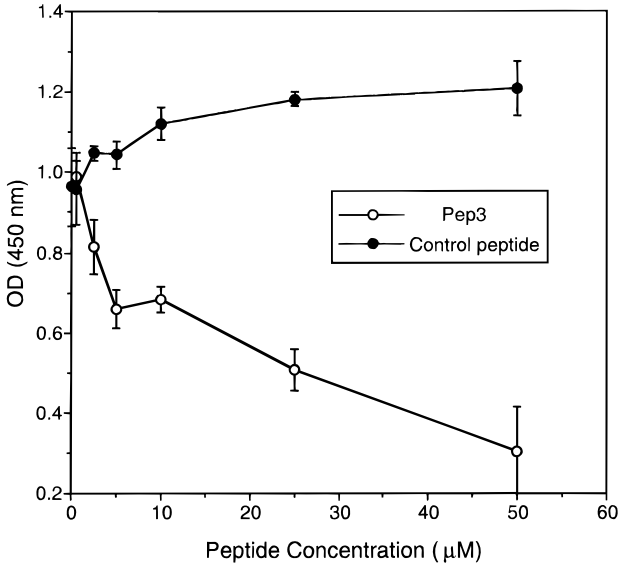

FIGURE 7: Competition between pep3 and MHC class II molecules for binding to TSST-1. MHC class II molecules, which were partially purified from Daudi cells, were coated on each well of a microtiter plate (1 μg/mL). TSST-1 (10 ng/mL) were incubated with the immobilized MHC class II in the presence of pep3 at the indicated concentrations. The biotinylated monoclonal anti-TSST-1 antibody and avidin−horseradish peroxidase conjugates were then used as described in Materials and Methods. The data represent means ± SD from triplicate wells.

Competition of a Synthetic Peptide (pep3) with MHC Class II Molecules for Binding to TSST-1. Sequence homology between phage8 and MHC class II molecules suggests that pep3 may correspond to a site of interaction between TSST-1 and MHC class II molecules. To determine whether pep3 mimics the structure of the TSST-1-binding domain of MHC class II molecules, competitive binding of TSST-1 and pep3 with the immobilized MHC class II molecules was investigated by a nonradioactive assay (ELISA). As shown in Figure 7, binding of TSST-1 to MHC class II molecules was inhibited by adding pep3 in a concentration-dependent manner, while the control peptide had no effect on TSST-1. As a control, a high concentration of pep3 (250 μM) did not show any inhibitory effects on the binding of TSST-1 to a biotinylated mouse monoclonal anti-TSST-1 antibody (data not shown). These data support the view that pep3 mimics domains of the MHC class II molecules which contact the superantigen, TSST-1.

DISCUSSION

Antibodies and receptors have frequently been used as targets for isolation of peptides from phage display libraries (Scott & Smith, 1990; Goodson et al., 1994; Renschler et al., 1994; Miceli et al., 1994; Cwirla et al., 1990; Zhong et al., 1994). Few studies, however, have used this technology to select peptides with affinity for ligand molecules. Since TSST-1, a member of the superantigen family, serves as a ligand and binds to MHC class II receptors (Uchiyama et al., 1989), we chose TSST-1 as a target to examine whether peptide ligands for TSST-1 which mimic the structure of MHC class II receptors could be identified from a phage display library. This approach should provide peptide ligands that help to elucidate the molecular basis of protein−protein interactions such as the structural features of superantigens and MHC class II receptors. From this point of view, MHC

class II receptors are also potent targets for a screening. However, since the surfaces of these receptors possess several known binding sites for superantigens or conventional protein antigens due to the multifunctional nature of MHC class II receptors, selection of peptide ligands mimicking aspects of other superantigen or conventional protein antigen recognition could potentially occur if MHC class II molecules were chosen as targets. In fact, Hammer et al. (1992) have reported the use of a phage display library in identifying sequence motifs of binding peptides for HLA-DR1. Therefore, we have used a ligand molecule (TSST-1) to screen a phage display library.

By sequencing of 12 clones which were confirmed to be reactive with TSST-1 by ELISA, four different sequences were identified as TSST-1 binding motifs. These isolated sequences shared little amino acid sequence identity except a significant enrichment of aromatic residues. The structural basis for the enrichment of aromatic residues might be due to the presence of a hydrophobic pocket in TSST-1, although there appears to be an enrichment of aromatic residues in selected phage sequences.

Recently, high-affinity peptide ligands binding to the II_b/III_a integrin have been isolated successfully from a conformationally constrained library of hexapeptides flanked by cysteine residues (O'Neil et al., 1992). However, in our hands, screening of a library of cyclic hexapeptides to obtain high-affinity ligands for TSST-1 failed to isolate any binding sequences (data not shown). None of the TSST-1 binding phage isolated from the 15-amino-acid library reported here contained cysteine residues. Moreover, the TSST-1-binding site on the β chain of MHC class II molecules corresponding to the sequence mimicked most frequently by selected phage in the present study does not contain cysteine residues (Kim et al., 1994). Thus, introduction of conformational constraints into displayed random peptides was not an effective strategy for isolation of TSST-1 binding motifs.

Comparison of phage8 with the β chain of MHC class II molecules revealed two consensus sequences, one (domain A, residues 57−62 of MHC class II) which was associated with one of three major contact regions of MHC class II with TSST-1 as had been implicated by X-ray crystallographic data (Kim et al., 1994) and another (domain B, residues 188−193) which was not reported as a functional domain for TSST-1 binding. Although phage8 may not bind TSST-1 in a similar manner as MHC class II and domain B may have no role for TSST-1 binding, the homology with domain B suggests a novel site of interaction between TSST-1 and MHC class II. Limitations in the crystallization conditions such as the proteolysis and removal of the transmembrane region of MHC class II in order to generate a soluble crystallization form (Gorga et al., 1987) or the usage of the DR1 isozyme with lower affinity for TSST-1 (Kim et al., 1994) may have prevented its identification as a potential binding site earlier. Alternatively, phage8 may mimic the complex comprising antigenic peptides and the β chain of MHC class II molecules, since TSST-1 contacts the C-terminal region of antigenic peptides which interact with domain A of the β chain of MHC class II molecules (Kim et al., 1994).

Scholl et al. (1990) have reported the effect of polymorphism on the ability of MHC class II molecules to bind to TSST-1, showing that TSST-1 binds to HLA DR and DQ but not DP molecules. Figure 3 shows a sequence alignment of HLA DR, DQ, and DP and phage8 grouped according to the affinity for TSST-1 (Scholl et al., 1990). In domain A, no apparent difference was observed among the sequences of the high-affinity HLA group (DR5, DR7, DQ1, and DQ3) and those of the low-affinity HLA group (DPW2 and DPW4). In contrast, there were two amino acid substitutions at position 189 and 193 in domain B. Residues 189 and 193 of the high-affinity HLA group (Arg and Glu, respectively) were identical with those in phage8, whereas those of the low-affinity HLA group diverged (Lys and Asp, respectively). Furthermore, DR1 and DRW53, which showed a lower affinity for TSST-1 than the high-affinity HLA DR or DQ groups as suggested by the data of Scholl et al. (1990), possess Ser at position 189; these amino acid substitutions might result in the difference of affinity for TSST-1 if domain B was associated with TSST-1 binding.

It has been reported that TSST-1 is structurally similar to SEB (Acharya et al., 1994; Prasad et al., 1993), and that SEA and TSST-1 compete for binding to MHC class II molecules (Pontzer et al., 1991), suggesting a structural conservation of MHC class II binding sites. Furthermore, our competition study (Figure 5) and the sequence similarity between phage8 and MHC class II molecules (Figure 3) suggests that selected phage recognize the MHC class II binding site on TSST-1. Taken together, these data provide the possibility of cross-reactivity of isolated phage with other SEs. However, phage3, which mimicked the binding site for TSST-1 on the β chain of MHC class II receptors, did not show cross-reactivity with other SEs. This observation is consistent with previous reports suggesting that SEB and TSST-1 bind to overlapping but not identical sites on MHC class II receptors despite structural similarity between the superantigens (Thibodeau et al., 1994). Other work has demonstrated that the binding site for SEA located on the α rather than the β chain of MHC class II molecules may overlap with that for TSST-1 (Pontzer et al., 1991).

TSST-1 was reported to have N-terminal β barrel domains that were implicated in MHC class II binding in TSST-1−HLA-DR1 complex crystal forms (Kim et al., 1994). Moreover, a peptide corresponding to residues 39−68 of TSST-1 has been reported to compete with TSST-1 for binding to MHC class II molecules (Soos et al., 1993), suggesting a role for the region in MHC class II binding. In the present study, TSST-1-binding phage isolated from a phage display library most likely recognized MHC class II binding sites on TSST-1. Thus, these phage may bind to the N-terminal β barrel domains of TSST-1. To confirm this assumption, peptide competition studies, for example, using a peptide corresponding to residues 39−68 of TSST-1, need to be performed.

Superantigens bind to MHC class II receptors outside of the antigenic peptide-binding groove and induce T-cell activation (Dellabona et al., 1990; Kappler et al., 1989). Superantigen-mediated activation of T cells results in cytokine secretion that causes illness such as toxic shock syndrome (Ikejima et al., 1984; Grossman et al., 1990). Thus, binding of superantigens to MHC class II molecules is thought to be a key step in the pathogenesis of superantigens. In the present study, pep3 inhibits binding of TSST-1 to MHC class II molecules in a concentration-dependent manner. This peptide, therefore, may present opportunities for use as a possible drug candidate for toxic shock syndrome.

In conclusion, we have identified peptide motifs binding to TSST-1 that blocked binding of TSST-1 to MHC class II receptors. Affinity selection of peptides from a random library using a receptor ligand molecule as a target represents a useful approach to understanding receptor−ligand interactions. The approach described here could be applied to other superantigens and may help to generate therapeutic agents for superantigen-related diseases.

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