Brief Definitive Report

Identification of HLA-DRα Chain Residues Critical for Binding of the Toxic Shock Syndrome Toxin Superantigen

By Paola Panina-Bordignon,*‡ Xin-ting Fu,§ Antonio Lanzavecchia,* and Robert W. Karr§

*From the *Basel Institute for Immunology, CH-4005 Basel, Switzerland; the ‡Institut de Chimie Biologique, Université de Strasbourg, 67000 Strasbourg, France; and the §Department of Immunology, Monsanto Company, St. Louis, Missouri 63198*

Summary

Staphylococcal toxic shock syndrome toxin 1 (TSST-1) binds to major histocompatibility complex class II molecules, and the toxin–class II complexes induce proliferation of T cells expressing Vβ2 sequences. To define the residues involved in TSST-1 binding, a set of transfectants expressing 21 HLA-DRα chain mutants were analyzed for their abilities to bind and present TSST-1 and to present an antigenic peptide. Mutations at DRα positions 36 and 39 markedly decreased the ability of the DR7 molecule to bind and present TSST-1 but did not affect the ability to present an antigenic peptide. These data indicate that DRα residues 36 and 39, predicted to be located on an outer loop, are important in the formation of the TSST-1 binding site on DR molecules.

Bacterial superantigens bind to MHC class II molecules and are recognized by specific TCRs inducing potent activation of T cells (1–3). They include *Staphylococcus aureus* enterotoxins (SE)A, SEB, SEC-1, -2, and -3, SED, and SEE, and the exotoxin toxic shock syndrome toxin (TSST-1). TSST-1, the focus of this report, is produced by most strains of *S. aureus* that cause toxic shock syndrome and appears to be important in the pathophysiology of this disorder (see reference 4 for review and references). In humans, TSST-1 binds well to HLA-DR, interacting with a not-yet-defined region of the DRα1 domain (5), but poorly or not at all to DP molecules (5–7), and it is specifically recognized by TCRs expressing Vβ2 sequences. However, despite the remarkable degree of structural, regulatory, and functional conservation between DRα and I-Eα chains (8), TSST-1 does not bind or binds only at a very low degree to murine I-E molecules (7, 9). Based on previous studies, the view has emerged that bacterial superantigens interact with class II molecules differently than antigenic peptides, perhaps by interactions with the outer surface of these molecules (5, 10, 11).

The purpose of this study was to define specific residues in the DRα chain that are involved in binding and presentation of TSST-1 as compared with presentation of an antigenic peptide. Residues from I-E, DP, or DQα chains were substituted into several analogous positions in the DRα chain, and mutant DRα chains were expressed in transfectants with the wild-type DR(β1*0701) chain. The abilities of these mutant and wild-type molecules to bind and present TSST-1 to T cells and to present an antigenic peptide to antigen-specific T cell clones were analyzed. The data indicate that the substitutions at DRα residues 36 and 39 caused a significant reduction in the ability of the DR7 molecule to bind and present TSST-1, whereas substitutions at positions 24 and 65 abrogated recognition by the tetanus toxin–specific T cell clones.

Materials and Methods

cDNA Clones and Site-directed Mutagenesis. cDNA clones encoding full-length HLA-DR(α) and DR(β1*0701) chains (12) in the pcD expression vector (13) were used in this study. Site-directed mutagenesis of the DRα chain was performed by the PCR overlap extension method (14), using Taq DNA polymerase and a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). The four 17-mer primers used in the amplifications were located on the pcD vector near the 5′ end of the cDNA, around the desired mutation positions, and codons corresponding to amino acids 145–150 of the DRα chain, respectively. After the two-step PCR amplifications, the PCR-amplified fragment containing the mutation was digested with the restriction enzymes SacI (New England Biolabs, Beverly, MA) and Csp45I (Promega Biotec, Madison, WI). The 476-bp SacI-Csp45I fragment from the 5′-untranslated region to the codon corresponding to amino acid 123 of the DRα chain was ligated into the SacI-Csp45I–digested pcD vector containing the remainder of the DRA cDNA. Dideoxy sequencing with Sequenase T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH) of the region between the SacI and Csp45I sites was used to confirm the presence of the desired mutation and absence of misincorporations.

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Transfection. The mutant or wild-type DRA cDNA was cotransfected with the wild-type DRB1*0701 cDNA into the DAP.3 subclone of class II–negative murine L fibroblasts as described (12).

Binding of TSST-1 to DR-transfected Cells. Purified TSST-1 was purchased lyophilized from Toxin Technologies (Madison, WI), dissolved in sterile water at a concentration of 1 mg/ml, and diluted in PBS just before use. 10^4 transfected L cells were washed, placed into individual microtiter wells, and incubated with various concentrations of TSST-1 for 2 h on ice. The cells were washed and incubated with a rabbit anti-TSST-1 serum (Toxin Technology) diluted 1:100 in PBS, 1% BSA, 0.01% NaN_3, and incubated on ice for 30 min. The cells were washed and incubated with FITC-labeled swine anti–rabbit Ig (Silenus Laboratories, Hawthorn, Australia) on ice for 30 min. The cells were washed and analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA). Controls were incubated with rabbit anti-TSST-1 and FITC antibody without prior addition of TSST-1. The results are presented as the relative fluorescence of the cells stained with TSST-1 normalized to the fluorescence of the same cells stained with an anti-DR mAb, as described in the legend to Fig. 1. L243 was used to assess expression on all mutants except those at positions 18 and 39, because these mutations interfere with binding of L243 to DR7 molecules (Fu, X., and R. W. Karr, manuscript in preparation). SG157 (obtained from Sanna Goyert, North Shore University Hospital, Manhasset, NY) (15) and SFR16-DR7 (obtained from Susan Radka, Oncogen, Seattle, WA) (16) were used to assess DR expression on the position 18 and 39 mutants, respectively.

T Cell Clones and Proliferation Assay. T cell clones specific for TSST-1 were derived from PBL stimulated in vitro with 50 ng/ml of TSST-1. After 7 d the cells were cloned by limiting dilution as described (17). T cell clones specific for the tetanus toxin (tt) pep-

Table 1. *Summary of the Ability of Transfectants Expressing DRα Mutants to Bind TSST-1 and Present TSST-1 and a Tetanus Toxin Peptide to T Cell Clones*

DRα position	Substitution	Source of substitution	TSST-1 binding*	TSST-1 presentation‡	tt 830–843 presentation§
7	I-S	DP, DQα	+ +	+ +	+
9	Q-G	DQ, I-Aα	+ +	+ +	+
11	E-A	DPα	+ +	+	+
15	N-L	I-Eα	+ +	+ +	+
18	Q-K	I-Eα	+ +	+ +	+
19	S-R	IEα	+	+ +	+
22	F-Y	I-E, DQ, IAα	+ +	+	−
24	F-H	DQ, I-Aα	+	+	−
31	I-E	DQα	+ +	+	+
36	M-I	I-Eα	−	−	+
37	A-E	I-Eα	+ +	+ +	+
39	K-S	I-Eα	−	−	+
42	V-I	I-E, I-Aα	+ +	+ +	+
47	E-L	DQα	+ +	+ +	+ +
49	G-A	I-E, I-Aα	+ +	+ +	+
50	R-K	DQ, I-Eα	+ +	+	+
55	E-D	DQ, I-Aα	+ +	+	+
65	V-I	DPα	+	+ +	−
66‖	D-V	I-Aα	−	+ +	−
66	D-L	DQ, DPα	+	+ +	ND
72	I-V	I-Eα	+ +	+	+

* The ability of each mutant to bind TSST-1 is categorized as follows: −, TSST-1 concentration required to induce 50% of wild-type binding is >5 μg/ml; +, TSST-1 concentration required to induce 50% of wild-type binding is between 5 and 1 μg/ml; + +, TSST-1 concentration required to induce 50% of wild-type binding is <1 μg/ml. TSST-1 binding to each mutant was analyzed at least five times.

‡ The ability of each mutant to present TSST-1 to at least three different TSST-1-specific T cell clones is categorized as follows: −, TSST-1 concentration required to induce 50% of proliferative response to the wild type is >10 ng/ml; +, TSST-1 concentration required to induce 50% of proliferative response to the wild-type is between 0.1 and 10 ng/ml; + +, TSST-1 concentration required to induce 50% of proliferative response to the wild-type is <0.1 ng/ml.

§ The ability of each mutant to present tt 830–843 to three different peptide-specific T cell clones is categorized as follows: −, peptide concentration required to induce 50% of proliferative response to the wild type is >10 μg/ml; +, peptide concentration required to induce 50% of proliferative response to the wild type is between 0.1 and 10 μg/ml; + +, peptide concentration required to induce 50% of proliferative response to the wild type is <0.1 μg/ml.

‖ In this case, the expression of DR was always low (for analysis of DR expression see Materials and Methods).

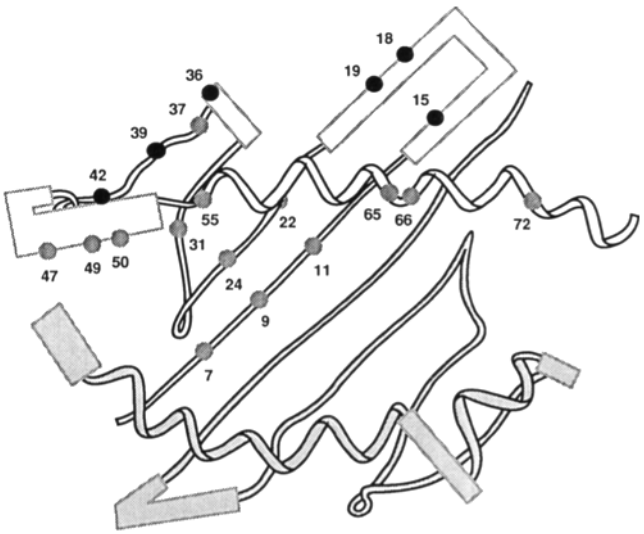

Figure 1. Locations of the substituted amino acids in the class II model of Brown et al. (18) (reprinted by permission from *Nature [Lond.]* 332:845, 1988). The DRα chain is open and the DRβ chain is shaded. DRα residues whose side chains are predicted to point into the peptide binding groove are shown as shaded circles and those on the outer loops whose side chains point away from the binding groove are shown as filled circles.

tide 830–843 were isolated and characterized as described (17). Three independent DR7-restricted, tt830–843-specific T cell clones (SP6.6, SP2.2, and SP3.12) and four TSST-1-specific T cell clones (ALp2.12.2, AL15.1, BR7.3, and GM2.11) were used. Cultures were set up in 200 μl of RPMI 1640 with 10% FCS in flat-bottomed microtiter plates. T cells (4×10^4) were cultured with 2×10^4 mitomycin C–treated L cells that had been pulsed for 2 h at 37°C with various concentrations of either TSST-1 or tt 830–843 and washed four times. After 2 d at 37°C, 5% CO_2, the cultures were pulsed with 1 μCi (37 kBq) [^{3}H]thymidine (sp act, 5 Ci/mM; Amersham Corp., Arlington Heights, IL), and the incorporated radioactivity was measured after an additional 18 h by liquid scintillation.

Results and Discussion

Production of DRα Mutants. To analyze the contribution of individual amino acids in the α chain of HLA-DR molecules in the interaction with TSST-1, 21 mutated DR7 molecules containing single amino acid substitutions in the α chain were constructed by site-directed mutagenesis and transfected in mouse L cells. Since human HLA-DP and murine I-E molecules do not bind TSST-1, or bind it poorly, and I-E molecules do not present TSST-1 (5–7, 9), most of the mutations involve replacement of the DRα residue with the corresponding residue from the α chain of HLA-DP or I-E molecules, as shown in Table 1. The predicted location of the substituted positions in the class II molecule, according to the model of Brown et al. (18), are shown in Fig. 1. The residues at positions 7, 9, 11, 22, 24, and 31 are predicted to be located on the floor of the peptide binding groove, with their side chains pointing up toward the peptide binding groove. The residues at positions 15, 18, and 19 are predicted to be located on the outer loop that connects the first and second β strands. The residues at positions 36, 37, 39, and 42 are predicted to be located on an outer loop that connects the third and fourth β strands. The residues at positions 47, 49, 50, 55, 65, 66, and 72 are predicted to be located in the α helix with their side chains pointing in toward the peptide binding groove. The level of DR expression by the various transfectants was tested before each experiment by indirect immunofluorescence utilizing three different anti-DR mAbs and flow cytometric analysis. All the transfectants utilized in this study showed good levels of DR expression with at least one of the mAbs utilized, except the 66 D-V mutant that had a low level of expression.

Binding of TSST-1 to HLA-DR7 Molecules and Stimulation of Vβ2$^+$ T Cell Clones Are Affected by Mutations at Two Residues on the Outer Loop of the α Chain. Mouse L cells expressing wild-type or mutant DR7 molecules were tested

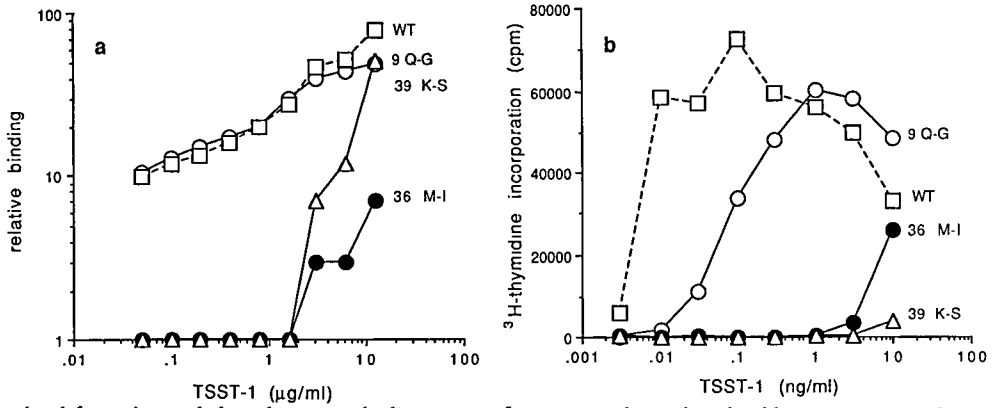

Figure 2. Binding of TSST-1 to HLA-DR7 molecules and stimulation of a Vβ2$^+$ T cell clone. (*a*) Relative binding of TSST-1 to wild-type and α chain–mutated DR(β1*0701) molecules. Mouse L cells were transfected with wild-type or α chain–mutated molecules and tested for their abilities to bind different concentrations of TSST-1. The *y*-axis is the relative binding of TSST-1 as measured by cytofluorometry. The relative binding was calculated for each transfectant in the following three steps. The net mean fluorescence with TSST-1 was calculated for each sample by subtracting the linear mean fluorescence obtained with rabbit anti-TSST-1 and FITC-swine anti-rabbit in the absence of TSST-1 from that obtained in the presence of TSST-1. The net mean fluorescence with anti-HLA-DR was calculated by subtracting the linear mean fluorescence obtained with FITC-goat anti-mouse alone from that obtained with the anti-DR antibody and FITC-goat anti-mouse. The relative fluorescence intensity for TSST-1 was normalized for variations in the level of DR expression on various transfectants by dividing the net mean fluorescence with TSST-1 by the net mean fluorescence with anti-DR. L cells expressing wild-type DR7 (WT), DR7β1-DRα9Q-G point mutant (9Q-G), DR7β1-DRα36M-I point mutant (36 M-I), or DR7β1-DRα39K-S point mutant (39 K-S) were used. (*b*) Proliferation of T cell clone ALp2.12.2 to TSST-1 presented by wild-type and α chain–mutated HLA-DR7β1 molecules. The T cell clone ALp2.12.2, expressing the Vβ2 segment of the TCR, was incubated with L cells transfected with wild-type or α chain–mutated DR7β1 molecules pulsed with the indicated concentrations of TSST-1. The induced T cell proliferation was measured by [^{3}H]thymidine incorporation. Each point represents the mean of triplicate determinations.

for their abilities to bind TSST-1. The data from one representative experiment are shown in Fig. 2 *a* and data from multiple experiments are summarized in Table 1. We found a marked difference in the level of binding of TSST-1 (corrected for DR expression) between the wild-type and the mutants at residues 36, 39, and 66 (D-V). The level of class II expression by the transfectant expressing the 66 D-V mutant was consistently lower than the other transfectants; therefore, the apparent absence of binding to the 66 D-V mutant must be interpreted cautiously. There was no significant difference in the level of binding of TSST-1 between the wild-type and the other mutants. The residues at positions 36 and 39 were replaced with the residues found at the same positions of the murine I-Eα chain, which binds TSST-1 poorly or not at all (6, 7, 9). We also calculated the estimated affinity of TSST-1 for the wild-type molecule and for the various mutant molecules using the double reciprocal plot method previously described for the calculation of the affinity of hapten-antibody interaction (19). The estimated affinity is expressed as K_0 and it is equal to the reciprocal of the free TSST-1 concentration at which half the concentration of MHC binding sites are bound to TSST-1. We found that the K_0 of TSST-1 binding for the wild-type is 1.8×10^{-7} M, while the K_0 for the point mutants at residues 36 and 39 are 10-fold higher, being 1.6 and 1.1×10^{-6} M, respectively. This indicates that the methionine at position 36 and the lysine at position 39 are critical for TSST-1 binding.

In a previous study, it was found that T cell proliferation is a more sensitive assay for TSST-1–class II interaction than the binding assay. TSST-1 binding to HLA-DP was not detected, although DP efficiently presented TSST-1 to T cells (7, 20). Therefore, the ability of the DRα mutants to present TSST-1 to three specific T cells was tested. The dose-response curves of one representative T cell clone with transfectants expressing wild-type or mutant DRα chains (Fig. 2 *b*) show that, consistent with the results obtained in the direct binding assay, mutations at positions 36 and 39 significantly reduce the recognition of TSST-1 by the T cell. In contrast, the 66 D-V mutant presented TSST-1 to the T cell clones, suggesting that the failure to detect TSST-1 binding to this mutant was due to low class II expression. All the other mutants presented TSST-1 to the T cell clones, indicating that the MHC-TSST-1 complex is not altered by the introduction of the mutation. Interestingly, the nonconservative 37 K-S substitution had no effect on TSST-1 binding or presentation, in contrast with the results with the position 36 and 39 mutations. These results are consistent with the predictions of the class II model and the view that superantigens interact with the outer surfaces of class II molecules: although residues 36, 37, and 39 are predicted to be located on an outer loop, only the side chain of residue 37 is predicted to point in toward the peptide binding groove.

Presentation of tt 830–843 by Transfectants Expressing DRα Mutants. To assess whether any of the single substitutions in the DRα chain causes gross alterations of the conformation of the DR molecules, we tested the ability of these mutants to present peptide antigen. Three independent DR7-restricted T cell clones were tested for their abilities to recognize peptide tt 830–843 presented by transfected cells. The proliferative response of one representative clone is shown in Fig. 3. We found that the substitutions of a methionine to an isoleucine at residue 36 and of a lysine to a serine at residue 39 do not affect the ability of these mutants to present peptide antigen. This indicates that the mutants bind the peptide and that the peptide-MHC complex is recognized by the specific TCR. Our results are consistent with the view that the binding site for toxin superantigens is different from the conventional antigen binding site (5, 10, 11). We also found that substitutions at positions 24 and 65 abrogated recognition by the tt-specific T cells, although they have no effect on TSST-1 binding and presentation. The substitutions at positions 22 and 66 D-V reduced the ability of the DR7 molecule to present peptide. The findings that only 2 of the 21 DRα mutations abrogated the ability of the DR7 molecule to present the tt peptide to the three T cell clones are in marked contrast to the results of our previous analysis of DRβ mutations on tt presentation in which multiple mutations abrogated T cell proliferation (21). For example, proliferation by clone SP6.6 was abrogated by mutations at DRβ positions 11, 57, 60, 67, and 70, and clone SP3.12 at positions 11, 13, 57, 67, 70, 74, and 78.

Concluding Remarks. The present study demonstrates that residues 36 and 39 on the DRα chain are critical for TSST-1 binding and presentation to specific T cells. Recently it has been demonstrated that the second hypervariable region of the mouse Aα chain (αHV2) is the single most important α chain polymorphic region that contributes to the TSST-1 binding site (22). Indeed, residues 36 and 39 are predicted

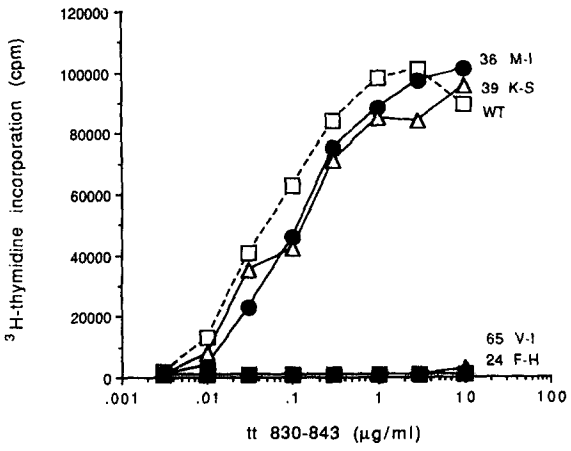

Figure 3. Proliferation of T cell clone SP6.6 to tt 830–843 presented by wild-type and α chain–mutated HLA-DR7β1 molecules. The tt 830–843-specific T cell clone SP6.6 was incubated with L cells transfected with wild-type or α chain–mutated DR7β1 molecules pulsed with the indicated concentrations of tt 830–843. The induced T cell proliferation was measured by [^{3}H]thymidine incorporation. Each point represents the mean of triplicate determinations. L cells expressing wild-type DR7 (WT), DR7β1-DRα24F-H point mutant (24 F-H), DR7β1-DRα36M-I point mutant (36 M-I), DR7β1-DRα39K-S point mutant (39 K-S), or DR7β1-DRα65V-I point mutant (65 V-I) were used.

to be located in the region analogous to αHV2 on the outer loop of the α chain that connects the third and the fourth β strands. Our data suggest the existence of a high affinity binding site on DRα chain for TSST-1, since alteration of two closely located residues, 36 and 39, can significantly reduce TSST-1 binding and presentation. However, at high TSST-1 concentrations a certain level of binding and presentation by these mutants is observed (Fig. 2), indicating that a low affinity binding site might still be available. Therefore, the substitution at one residue may decrease the affinity of TSST-1 binding to MHC, rather than abolishing the binding.

Previous studies indicate the existence of different binding sites, some of which may be overlapping, for staphylococcal toxins on class II molecules (23, 24). Two SEA binding sites have been postulated, but previous studies indicate a single TSST-1 binding site. DRβ residue 81, which is predicted to be located in a region of interchain interaction with the α chain, has been shown to be involved in the SEA and SEE binding site (11, 25). We found that a histidine to alanine substitution at position 81 in the DR(β1*0701) chain does not prevent binding and presentation of TSST-1 (data not shown). However, analysis of TSST-1 binding to additional mutants will be required to further investigate the possible contributions of DRβ chain residues to TSST-1 binding (22).

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Address correspondence to Robert Karr, Monsanto/AA4C, 700 Chesterfield Parkway North, St. Louis, MO 63198.

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