Role of the T Cell Receptor Ligand Affinity in T Cell Activation by Bacterial Superantigens*

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Similar to native peptide/MHC ligands, bacterial superantigens have been found to bind with low affinity to the T cell receptor (TCR). It has been hypothesized that low ligand affinity is required to allow optimal TCR signaling. To test this, we generated variants of Staphylococcus enterotoxin C3 (SEC3) with up to a 150-fold increase in TCR affinity. By stimulating T cells with SEC3 molecules immobilized onto plastic surfaces, we demonstrate that increasing the affinity of the SEC3/TCR interaction caused a proportional increase in the ability of SEC3 to activate T cells. Thus, the potency of the SEC3 variants correlated with enhanced binding without any optimum in the binding range covered by native TCR ligands. Comparable studies using anti-TCR antibodies of known affinity confirmed these observations. By comparing the biological potency of the two sets of ligands, we found a significant correlation between ligand affinity and ligand potency indicating that it is the density of receptor-ligand complexes in the T cell contact area that determines TCR signaling strength.

T lymphocytes are one of the central components of the acquired immune system. Each T lymphocyte carries a unique T cell receptor (TCR),1 which recognizes foreign peptides bound to the major histocompatibility complex (MHC) molecules (1, 2). The molecular mechanism by which the ligand-bound TCR transmits a signal across the plasma membrane is not known. Based on the observation that a few peptide/MHC complexes can cause down-modulation of a large number of TCRs, a model for TCR triggering proposes that low affinity (and fast off-rate) is necessary to allow “serial triggering” (3); that is, to permit one ligand to stimulate a large numbers of TCRs (up to 200) within a relatively short period of time. Thus, the model predicts that high affinity TCR ligands are suboptimal activators of T cells and that optimal dissociation kinetics exists (4). Additional models for TCR signaling (5, 6), based on kinetic proofreading principles, suggests that too brief a ligand occupancy, leading to partial assembly of the signaling complex, transmits a negative signal (antagonism) whereas contact times that enable complete assembly of the signaling complex delivers a full positive signal (agonism). Common to these considerations is that the off-rate, which defines the average time of receptor/ligand contact, is thought to determine the signaling strength of the TCR/ligand interaction.

Bacterial superantigens, which are involved in serious diseases such as toxic shock syndrome and food poisoning, elicit their biological function by cross-linking TCR and MHC class II molecules. They bind to relative invariant areas of the receptors and can thereby activate large fractions (5–20%) of the T cell population (7). Like peptide/MHC ligands, bacterial superantigens bind only weakly to TCRs with affinities in the μM range (8–11). The SAG-TCR interaction is therefore closely related to the endogenous TCR-antigenic-peptide-MHC interaction, and the molecular mechanism by which the ligand-bound TCR transmits a signal across the cell membrane is most likely the same for the two types of ligands.

To investigate if an affinity optimum for TCR ligand potency exists and to understand further the role of TCR affinity of SAGs, we generated variants of the bacterial superantigen Staphylococcus enterotoxin C3 (SEC3) with increased affinity toward TCR Vβ8.2 domains. The variants were generated by phage display and characterized by biosensor technology. Changes in binding strength shifted the affinity of the SEC3/TCR interaction from that of weak TCR agonists up to ~10-fold above the range reported for strong TCR agonists. By measuring the mitogenic potency of the SEC3 variants, we present a simple relationship between the affinity of the SEC3-TCR interaction and the functional responses; stronger binding resulted in stronger T cell responses. This observation was further supported by comparing the SEC3 experiments to similar studies using a panel of monoclonal antibodies of variable TCR Vβ8.2 affinity.

EXPERIMENTAL PROCEDURES

Lymphocytes and T Cell Lines—Lymphocytes were isolated from single transgenic mice expressing the 14.3d TCRβ chain (12). Transgenic animals were bred and maintained at the animal facility of Basel Institut for Immunology. A5 T cell hybridomas (14.3d TCRβ+) were grown in the presence of 0.5 mg/ml hygromycin (Calbiochem, La Jolla, CA). DO11.10 (Vβ8.2+) is an OVA-(323–339)-specific, Iaα-restricted T cell hybridoma (14). T cell hybridomas were cultured in RPMI 1640 medium supplemented with penicillin, 2 × 105 units/liter (Leo Pharmaceutica, Ballerup, Denmark), streptomycin, 50 μg/ml (Merck, Darmstadt, Germany), and 10% (v/v) fetal calf serum (Life Technologies, Paisley, UK) at 37 °C in 5% CO2.

Protein Expression and Purification—Soluble 14.3d TCRβ heterodimers were produced in Drosophila cells basically as previously described (15). The recombinant protein was purified from culture supernatant using affinity chromatography followed by ion exchange chromatography. Human MHC class II molecules were produced by in vitro refolding according to previously published methods (16). Briefly, the extracellular domains of the α- and β-polypeptides of HLA-DR1 was expressed as insoluble protein in Escherichia coli and subsequently isolated and solubilized under denaturing conditions. Correctly folded

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† The abbreviations used are: TCR, T cell receptor; SEC3, Staphylo-

coccus enterotoxin C3; MHC, major histocompatibility antigen; HA, hemagglutinin; GFP, green fluorescent protein; FACS, fluorescence-

activated cell sorter; IL, interleukin; WT, wild type; ITAM, immunore-

ceptor tyrosine-based activation motifs.
Protein was achieved by diluting inclusion bodies into a large volume of oxidizing buffer in the presence of molar excess of HA peptide (corresponding to influenza virus hemagglutinin position 306–318). After 2 days of gentle agitation at low temperature, soluble heterodimers were isolated using anion exchange chromatography.

Selection of SEC3 Variants of Enhanced TCR Affinity—Phage display was done essentially as previously described (8). Briefly, SEC3 disulfide loop residues were mutated by polymerase chain reaction using two complementary oligonucleotides (ACATACAGTGTGACACCTCCCGTGAGAATTCATATTATTCGCAATTCCTTTAG and CCAAGAGCTTTGAGATATGATNNNSNNNNNNNNNNNGGTTAAAACCTTGTATG; and flanking primers matching vector sequence AATGTANNSNNSNNSNNSNNSGNNNGTAAAACTTGTATG). The two gene products were assembled in a second PCR step using the flanking primers only, and the full-length product was subsequently cloned into the expression vector pCANTAB-5E (17). The SEC3 library was estimated to consist of 1.2 × 10^9 unique clones and was expressed as the gene III fusion protein on the surface of filamentous bacteriophage particles (18). Selection was done using a truncated form of the 14.3.d TCRβ-chain (19) coated on plastic surfaces. After three rounds of panning single colonies were picked and analyzed for β-chain binding by whole phage ELISA. Accordingly, 73% of the clones showed more binding to the β-chain relative to phages expressing SEC3 wild type on their surface. Soluble protein of selected SEC3 variants was isolated from bacterial periplasm and purified to homogeneity using dye affinity and ion exchange chromatography prior to any further analysis.

Biosensor Analysis—Binding studies were performed using BIAcore 1000 (Fig. 1) and BIAcore 2000 (Fig. 2) instruments essentially as described previously (8, 10). TCRβ heterodimers were immobilized onto the sensor surface using standard amine-coupling chemistry. Graded concentrations of purified SEC3 molecules were passed over immobilized TCR and allowed to bind until equilibrium was reached. Purified SEC3 molecules were highly soluble and showed no signs of aggregation. TCR-coupled surfaces could therefore be used repeatedly without any regeneration procedure needed. Binding curves were fitted using Grafit (Microsoft) software. K_d at equilibrium was determined by linear fitting of Scatchard plots. K_d was obtained by linear fits of apparent on-rates plotted as a function of ligand concentration. K_d was determined by single exponential fits of the first half of the dissociation phase under saturating conditions. Values were confirmed by independent fitting using the BIAevaluation 3.0 software. Data presented in Table I is the average of at least three independent data sets for each SEC3 variant. Standard errors were typically less than 20%. Separately calculated values of the equilibrium binding constant from Scatchard plots (K_d) or by division of rate constants (K_d) corresponded well (Table I) confirming that the fits were valid and not significantly biased.

T Cell Stimulation—T cells were stimulated using maxiSorb microtiter plates (NUNC A/S Denmark) coated with serial dilutions of the SEC3 variants. To ensure uniform coating at different concentrations SEC3 molecules were diluted into phosphate-buffered saline containing 5 μg/ml bovine serum albumin. Plates were coated overnight at 4 °C and subsequently blocked for >1 h at room temperature using 2% bovine serum albumin in phosphate-buffered saline. The efficacy of coating (Fig. 3A) was evaluated by ELISA using a sheep anti-SEC3 antisemur (a kind gift from P. Schlievert). T cells were stimulated by incubating ~10^5 cells per well at 37 °C for different periods of time. TCR down-regulation studies was done by using neubodies DO11.10 T cell hybridomas for 4 h, and TCR surface expression was subsequently determined by FACS analysis using phycocerythrin (PE)-conjugated anti-CD3 mAb 145–2C11 (BD Pharmingen, San Diego, CA). A5 T cell hybridomas, containing a nuclear factor of activated T cells (NFAT)-GFP expression cassette (13), were stimulated in SAC-coated wells for 4–5 h, and NFAT activation was determined by the presence of intracellular GFP. Fluorescent cells were detected by FACS and scored as positive in the NFAT assay. For IL-2 studies, DO11.10 cells were stimulated for 20 h; the supernatant was removed and diluted serially. HT-2 cells (21) were transferred to the diluted medium, and after 24 and 48 h the viability of the cells was evaluated by microscopy and used as a measure of IL-2 production. T cell proliferation was measured by culturing lymph node T cells for 48 h together in SAC-coated wells. [3H]Thymidine uptake was added for 12 h, and incorporation of radioactivity was then used as a measure of proliferation.

Characterization of Ligand-coated Surfaces—Maxisorb microtiter plates were coated with SACs as above or with 50 μl of protein A at 10 μg/ml followed by incubation with F23.1 variants at 10 μg/ml. Antibodies were diluted into a non-binding F23.1 variant to keep the level of protein A binding constant and thereby achieve ideal conditions for dilution. The density of TCR binding sites on F23.1-coated surfaces was determined at maximal ligand density using radioimmunoassays and subsequently used to calculate the density at lower dilutions according to the dilution factor. SEC3 variants were diluted into 5 μg/ml bovine serum albumin to avoid loss of protein at very low concentrations. The resulting coating density of each SAC dilution was determined by adding trace amounts of radiolabeled SEC3 to the coating mixture. Based on the binding of plastic-bound SEC3 1D1 to radiolabeled SAC, SEC3 was assumed to be 50% active on the plastic surface.

RESULTS

Increasing the Affinity of the SEC3-TCR Interaction—The structure of SEC3 bound to the extracellular domains of the TCR β-chain of the mouse TCR 14.3.d (Vβ8.2, Jβ2.1, Cβ1) has been determined by x-ray crystallography (22). CDR2, and to a lesser extent the hypervariable region 4 (HV4) of the Vβ8.2 domain, bind in a cleft between the two domains of SEC3. In addition, residues 102–106 of SEC3 could potentially contact residue Asn-28, Asn-30, and Gln-72 of the Vβ domain. These residues form part of a loop structure defined by two disulfide-bonded cysteines placed at either end of the loop that is featured by most bacterial SACs (except TSST-1) with variability of both sequence and length. This disulfide loop is most likely highly flexible as indicated by the high B factors and poor or missing electron density associated with the loop residues in most crystallographic analyses of SACs (22–25). Alanine-scanning mutagenesis has shown that the energetically important side chains of the SEC3-TCR interaction are located at the center of the combining site (residues are shown in area I in Fig. 1C) (10, 26). The disulfide loop was therefore chosen as target for random mutagenesis because it contributed little if any to the stability of the interface although the crystal structure suggests that it could potentially contact the Vβ domain (22, 25). Five SEC3 loop residues (amino acids 102–106; GKTG; see area II in Fig. 1C) were randomized by polymerase chain reaction, and the resulting library was expressed on the surface of filamentous phages. Phages were passed through multiple rounds of selection on immobilized TCR fragments and subsequent amplification (see “Experimental Procedures” for details). Soluble protein was produced for selected SEC3 variants and further analyzed by biosensor technology. Based on an even distribution in binding reactivity, six clones were chosen for further characterization (for representative sensorgrams see Fig. 1A). Binding to 14.3.d TCR was clearly enhanced, and no binding was observed to an empty sensor surface (Fig. 1B). Surprisingly, DNA sequencing of the SEC3 variants revealed that the most reactive clones only had three randomized residues (Table I). The loss of two codons probably originates from errors during the synthesis of the DNA oligonucleotides used in the construction of the library. However, regardless of length a consensus motif was evident in the mutated region as seen by the overrepresentation of tryptophans.

The affinity of the SEC3 variants to the αβTCR (Table I) varied between 5.9 μM and 150 nM with all mutants displaying stronger TCR binding than SEC3 wild type. The on-rates (k_on) of the SEC3 variants were generally faster than reported for peptide/MHC ligands whereas the off-rates (k_off) were in the same range as those of native interactions. Thus, the maximal TCR affinity reached by the SEC3 variants was ~10 times above TCR usually reported for peptide/MHC or superantigens affinities determined by biosensor technology (8–11, 27–32).

Mutations in the SEC3 Loop Affected the Ability to Form Triomolecular TCR-SEC3-MHC Complexes—Superimposing the structure of uncomplexed SEC2 (25) onto that of Staphylococcus enterotoxin B (SEB) bound to HLA-DR1 (33) indicated that the N-terminal part of the SEC3 disulfide loop could sterically interfere with the MHC molecule (data not shown).
Changes in both loop flexibility and volume could therefore affect MHC binding directly or affect the ability of SEC3 to bind TCR and MHC simultaneously. Previous biosensor studies showed that soluble MHC enhanced the binding of SEB and SEC3 to immobilized αβ TCR (8, 34). To determine the effect of altered loop sequences on formation of the trimolecular complex, selected SEC3 variants (WT, 1D8, and 1D3) were passed over immobilized TCR/H9251/H9252 heterodimers alone or mixed with soluble human MHC class II molecules (sHLA-DR1) (Fig. 2). SEC3 WT showed a significant increase in binding in the presence of sHLA-DR1, whereas the enhanced binding gradually disappeared as the affinity toward the TCR increased. Thus, the ability to form the trimolecular TCR/SEC3/MHC complex was affected by the mutations in the disulfide loop.

Enhanced TCR Affinity Resulted in Stronger T Cell Responses—TCR ligands attached to planar surfaces are commonly used as surrogate APC in the study of T cell biology. Even some of the ultrastructural features associated with the immunological synapse such as localized TCR and LAT accumulation (35) and actin polymerization (36) are effectively mimicked on ligand-coated surfaces. To avoid considering the reduced ability of high affinity SEC3 variants to bind simultaneously to TCR and MHC, purified SEC3 and mutant molecules were adsorbed directly onto plastic surfaces and used to stimulate T cells. Changes in loop composition of SEC3 did not affect the efficacy of the plastic coating procedure as judged by ELISA (Fig. 3A).

Table I: Characterization of SEC3/αβTCR interactions

| Clone  | Pos. 102–104 | $k_{off}$ | $k_{on}$ | $K_{bind}$ | $K_{dissoci}$ | $t_{1/2}$ |
|--------|--------------|----------|----------|-----------|--------------|-----------|
| SEC3 WT| GKVTG        | n.d.     | n.d.     | n.d.      | 22           | <0.3      |
| SEC3 1E9| XYGLA  $^b$ | 0.62     | n.d.     | n.d.      | 5.9          | 1.1       |
| SEC3 1D8| ASTWH       | 0.55     | 58 000   | 9.5       | 4.7          | 1.3       |
| SEC3 1E10| YTPYH      | 0.16     | 71 000   | 2.3       | 1.3          | 4.3       |
| SEC3 1D3| WWP         | 0.11     | 130 000  | 0.85      | 0.61         | 6.3       |
| SEC3 1D1| WWT         | 0.048    | 150 000  | 0.32      | 0.25         | 14        |
| SEC3 1A4| WWH         | 0.035    | 240 000  | 0.15      | 0.15         | 20        |

$^a$n.d. indicates that kinetics were too fast to accurately measure.

$^b$X indicates ambiguous reading of the DNA sequence.

$^c$K$_{dissoci}$ was calculated from Scatchard plots.

$^d$K$_{bind}$ was calculated from the rate constants; K$_{bind}$ = $k_{off}$$k_{on}$.

$^e$t$_{1/2}$ was calculated from the off-rate; t$_{1/2}$ = ln(2)/$k_{off}$.
Role of Ligand Affinity in T Cell Activation

Comparison of the Potency/Affinity Relationship of Anti-TCR Antibodies and Superantigens—To explore the existence of a stimulatory optimum at even higher TCR ligand affinity, the potency of the SEC3 variants were compared with the potency of a panel of 11 anti-TCR antibodies. The antibody panel consists of mutated variants of the monoclonal antibody F23.1 that, as SEC3, recognizes mouse TCR Vβ9.2 domain is relatively weak (Kd = 22 μM) with very fast dissociation kinetics at 25 °C. By randomly mutating five residues in a flexible loop structure and subsequently selecting for enhanced binding using phage display, SEC3 variants were generated with up to a 150-fold increase in TCR affinity. The on-rates of the SEC3 variants were generally faster than reported for peptide/MHC ligands, whereas the off-rates were in the same range as those of native peptide-MHC ligands (28, 30–32). The mutations in the loop affected the ability to form the trimolecular TCR-SEC3-MHC II complex. To avoid disturbances from the MHC-induced alterations in affinity, T cell stimulation experiments were done using SEC3 variants coated onto plastic surfaces. As recently discussed (36), ligand-coated surfaces can effectively cause many of the morphological responses observed during early stages of physiological contact formation. Furthermore, the simplified nature of the stimulus eliminates the complicating contributions of surface receptors other than the TCR to T cell activation. Finally, in the present study we demonstrate the ability of SEC3-coated surfaces to induce multiple stages of activation of different types of T cells. It therefore seems reasonable to use SEC3 molecules coated onto plastic surfaces as a model system in the study of the molecular mechanisms of TCR signaling.

Current models for TCR triggering (3, 6) predict that slow dissociation (and consequently high affinity) would turnover fewer receptors per unit time and thus cause less T cell stimulation. Empirically, the importance of fast kinetics is supported by the finding that Kd of TCR/peptide-MHC interactions are of low affinity (2) and, as a result, possess relatively fast dissociation kinetics. Identification of a possible optimum has been elusive because mutational studies of TCR-ligand interactions predominantly lead to complexes of similar or less stability than the native starting point. Our study demonstrates the biological consequences of a substantial increase in the affinity of a native TCR-ligand interaction. Increasing the affinity of the SEC3-TCR interaction 150-fold place the equilibrium binding constants 10-fold above reported peptide-MHC ligand affinities. In most TCR systems the affinity does not go beyond 1 μM (27–32). Thus, one could expect that we,

pressed as the number of binding sites per μm² (sites/μm²) plastic surface, needed to induce 20% of maximum NFAT activation (ED₅₀). Plotting the potency as a function of the individual binding parameters Kd, KI, and t½ (Fig. 4) allowed identification of a possible optimum in regard to biological activity, in addition to identifying whether the potency correlated to any one of the three binding parameters in particular.

All binding parameters showed an upward trend in potency in response to stronger binding and thus no potency optimum was evident. Within each panel of ligands, the increase in potency followed the increase in affinity. The relative modest difference in potency between the two F23.1 variants of highest affinity could suggest a plateau in the low nanomolar range as previously proposed (38). Comparison of the superantigens and antibodies revealed a strong correlation between affinity and potency of the two sets of ligands. In contrast, faster associating SEC3 variants were less potent than their corresponding antibodies (Fig. 4B). Although potency increased as the complexes became more stable, the correlation to half-life (Fig. 4C) appeared significantly more scattered than the correlation to affinity. We therefore conclude that the affinity was the determining factor in TCR signaling for both the bacterial superantigens and the TCR-specific antibodies.

**DISCUSSION**

We have addressed the role of the TCR ligand affinity in T cell activation by increasing SEC3 affinity to TCR. The native interaction of SEC3 to the TCR Vβ 8.2 domain is relatively weak (Kd = 22 μM) with very fast dissociation kinetics at 25 °C. By randomly mutating five residues in a flexible loop structure and subsequently selecting for enhanced binding using phage display, SEC3 variants were generated with up to a 150-fold increase in TCR affinity. The on-rates of the SEC3 variants were generally faster than reported for peptide/MHC ligands, whereas the off-rates were in the same range as those of native peptide-MHC ligands (28, 30–32). The mutations in the loop affected the ability to form the trimolecular TCR-SEC3-MHC II complex. To avoid disturbances from the MHC-induced alterations in affinity, T cell stimulation experiments were done using SEC3 variants coated onto plastic surfaces. As recently discussed (36), ligand-coated surfaces can effectively cause many of the morphological responses observed during early stages of physiological contact formation. Furthermore, the simplified nature of the stimulus eliminates the complicating contributions of surface receptors other than the TCR to T cell activation. Finally, in the present study we demonstrate the ability of SEC3-coated surfaces to induce multiple stages of activation of different types of T cells. It therefore seems reasonable to use SEC3 molecules coated onto plastic surfaces as a model system in the study of the molecular mechanisms of TCR signaling.

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**FIG. 2. The effect of enhanced TCR affinity on formation of the trimolecular TCR-SEC3-MHC complex.** Overlaid sensorgrams of SEC3 WT (5.0 μM), SEC3 1D8 (1.3 μM), or SEC3 1D3 (0.15 μM) binding to immobilized αβ TCR either alone (black lines) or in the presence of 6 μM soluble MHC class II (αHLA-DR1) (gray lines).
using the present panel of SEC3 variant, could identify a possible optimum by assuming a normal distribution of reported affinities around a point of optimal binding. However, this was not the case. Furthermore, parallel studies using a panel of anti-TCR V8.2 antibodies of even higher affinities showed that the relationship between affinity and potency was almost identical for the two sets of TCR ligands, supporting that ligand affinity is the determining factor for TCR ligand potency. The observed correlation between ligand affinity, ligand density, and biological potency for the two sets of ligands furthermore indicates that the bivalency of the antibodies and the actual ligand binding site had little influence on TCR signaling as suggested by biochemical and structural studies (22, 39 – 42). Also, because TCR signaling correlates to ligand binding at equilibrium, it follows that the density of occupied TCRs in the contact area defines the signaling strength. That T cell activation obeys the laws of mass action has been suggested previously (38) and also indicated experimentally by the observations that T cell activation at low ligand density shows stochastic behavior (43) and that TCR density, ligand affinity as well as ligand density can, in turn, compensate for one another (44, 45).

In agreement with our results, TCR signaling can be viewed as a dynamic phosphorylation/dephosphorylation equilibrium of immunoreceptor tyrosine-based activation motifs (ITAMs) where the steady-state levels of phosphorylated ITAMs is low in unstimulated T cells (46). As proposed by Davis and van der Merwe (47), TCR signaling can be initiated by changing this equilibrium in favor of ITAM phosphorylation by exclusion of the relative large sized phosphatase CD45 from the T cell contact area. However, the general efficiency by which cross-linking antibodies stimulate ITAM-containing receptors suggests that a localized increase in ITAM density can influence the dynamic equilibrium and thus also be an important factor.
inactivating the TCR signaling cascades. In addition, involvement of the CD4/CD8 co-receptors can help stabilize the receptor-ligand complex as well as recruit lck kinases and thereby further tip the balance toward ITAM phosphorylation (1). If superantigens are more potent by having higher TCR affinities, then why has evolution invariably produced weak TCR binders (8) affinities, then why has evolution invariably produced weak MHC binding site, result in self-inhibition (i.e. no cross-linking because of single occupancy of MCH and TCR molecules) at much lower toxin concentrations. Furthermore, SAgs with high TCR affinity would preferentially bind T cells in the periphery of the lymphatic system, thereby reducing their chances of coming into contact with the relatively scarce source of MHC class II on the surface of specialized antigen-presenting cells and hence reduce the likelihood of strong stimulation of T cells that seems to be the major function of the bacterial SAgs.

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