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Cloning, expression and interaction of human T-cell receptors with the bacterial superantigen SSA

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Superantigens (SAgs) are a class of disease-causing and immunostimulatory proteins of bacterial or viral origin that activate a large number of T-cells through interaction with the Vβ domain of T-cell receptors (TCRs). In this study, recombinant TCR β chains were constructed with human variable domains Vβ5.2, Vβ1 and Vβ2.1, expressed as inclusion bodies, refolded and purified. The *Streptococcus pyogenes* SAg SSA-1 was cloned and expressed as a soluble periplasmic protein. SSA-1 was obtained both as a monomer and a dimer that has an intermolecular disulfide bond. We analyzed the biological activity of the recombinant SAgs by proliferation assays. The results suggest that SSA dimerization occludes the TCR interaction site. Naturally occurring SSA dimerization was also observed in supernatants of *S. pyogenes* isolates. An SSA mutant [SSA(C26S)] was produced to eliminate the Cys responsible for dimerization. Affinity assays using a resonant biosensor showed that both the mutant and monomeric wild type SSA have affinity for human Vβ5.2 and Vβ1 with K_d of 9–11 μM with a fast k_{ass} and a moderately fast k_{diss}. In spite of the reported stimulation of Vβ2.1 bearing T-cells by SSA, we observed no measurable interaction.

Keywords: affinity constant; biosensor; SSA; *Streptococcus pyogenes*; T-cell receptor.

T-lymphocytes recognize a wide variety of antigens through highly diverse cell-surface glycoproteins known as T-cell receptors (TCRs). These disulfide-linked heterodimers are comprised of α and β (or γ and δ) chains that have variable (V) and constant (C) regions homologous to those of antibodies. Unlike antibodies, which recognize antigen alone, αβ TCRs recognize antigen only in the form of peptides bound to major histocompatibility complex (MHC) molecules. In addition TCRs interact with a class of viral and bacterial proteins known as superantigens (SAgs).

SAgs are microbial toxins with potent immunostimulatory properties. They circumvent the normal mechanism for T-cell activation by binding as unprocessed molecules to MHC class II and TCR. The resulting trimolecular complex activates a large fraction of the T-cell population (5–20% of all T-cells), compared with conventional peptide antigen specific activation (0.01–0.001%). The activated T-cells release massive amounts of inflammatory cytokines such as IL-2, TNF-α and IFN-γ, contributing to the symptoms caused by SAgs, which can lead to lethal toxic shock [1]. SAgs have been implicated in a number of autoimmune diseases such as diabetes mellitus, rheumatoid arthritis and multiple sclerosis, by activating T-cells specific for self-antigens [2,3]; however, the best characterized diseases caused by SAgs are food poisoning and toxic shock syndrome (TSS) [4,5].

SAgs produced by several strains of *Staphylococcus aureus* and *Streptococcus pyogenes* are structurally and immunologically the best characterized to date [6], although the crystal structures of SAgs from *Mycoplasma arthritidis* and *Yersinia pseudotuberculosis* have been solved recently [7,8]. Bacterial SAgs are 22–29 kDa molecules that are resistant to proteases and heat denaturalization. They can be absorbed by epithelial cells as immunologically intact proteins [1,9]. Most SAgs share a common three-dimensional structure, although their amino acid sequences are highly variable. The structure of bacterial SAgs shows two globular domains: a small N-terminal domain with an OB fold ('oligosaccharide/oligonucleotide-binding'), and a large C-terminal domain with a β-grasp motif [10,11]. The TCR binding site on the SAg is situated in a cleft between the two domains [12,13]. In addition, SAgs have one or two binding sites for MHC class II: a low affinity site, and a higher

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Abbreviations: C, constant region; DTT, dithiothreitol; MHC, major histocompatibility complex; NTA, nitrilotriacetic acid; PBMC, peripheral blood mononuclear cell; SAg, superantigen; SEC3, Staphylococcal exterotoxin C3; SSA, Streptococcal superantigen; SSAm, SSA monomer; SSAd, SSA dimer; SSAia, SSA–iodoacetamide; TCR, T-cell receptor; TSS, toxic shock syndrome; V, variable region; wt, wild type.

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affinity site on the opposite face of the molecule that is Zn^{2+} dependent [14,15].

The proinflamatory and procytotoxic properties of SAgs are responsible for the increased interest in these molecules in the treatment of several pathologies and because of the potential use of the toxins as biological weapons. Alteration of their MHC and TCR binding capacity by site directed mutagenesis could be useful in the development of vaccines and in cancer therapy. SAgs with mutated TCR and/or MHC binding sites could be employed as vaccines against TSS and food poisoning to generate protective antibodies without systemic effects [16,17].

Streptococcal superantigen (SSA) is a 260 residue protein produced by *S. pyogenes* that can generate symptoms similar to TSS [18]. SSA shares more molecular properties with the staphylococcal enterotoxins SEB and SEC than with other streptococcal SAgs [19]. Cellular proliferation studies show disagreement about which TCR bearing T-cell subsets are expanded by interaction with SSA. Some authors indicated clonal expansion of T-cells bearing human Vβ3, Vβ12, Vβ17 and Vβ19 [20]. Others showed proliferation of human T-cells bearing Vβ1, Vβ2, Vβ3, Vβ5.2, Vβ12, Vβ15 and Vβ17 domains and found differences in the stimulation pattern between native and recombinant SSA, or even between variants (SSA-1 or SSA-2) [19,21].

To investigate SSA–TCR binding affinity, we expressed these molecules in high yield prokaryotic systems that allow us to obtain enough recombinant protein to conduct binding studies. In order to avoid dimerization, SSA Cys26 was mutated to Ser [SSA(C26S)]. Different SSA preparations were used to study T-cell proliferation capacity with human peripheral blood mononuclear cells (PBMCs). The binding of SSA and mutant C26S to soluble TCR β-chain molecules Vβ1, Vβ2.1 and Vβ5.2 was measured in real time by a 'resonant mirror' optical biosensor method.

Materials and methods

Reagents

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO). Restriction enzymes, *Taq* DNA polymerase, T4 ligase and buffers for cloning were purchased from New England Biolabs, Inc. (Beverly, MA). Ultra pure agarose was purchased from Gibco BRL-Life Technologies (Rockville, MD).

Recombinant TCR β chains

Human Vβ5.2 (hVβ5.2) was fused to a mouse constant β chain domain (mCβ15) to facilitate purification and increase yield [22,23]. Chimeric hVβ5.2mCβ15 was cloned into the kanamycin resistant expression vector pET26b and expressed as inclusion bodies [12] in *Escherichia coli* BL21(DE3) (Stratagene, La Jolla, CA). Two other β chains, hVβ2.1hCβ2 and hVβ1hCβ2 (genes kindly provided by U. Utz and R. P. Sekaly, University of Montreal, Canada), were cloned between the *Nde*I and *Eco*RI restriction sites of the pET17b expression vector and expressed in *E. coli* BL21(DE3) as inclusion bodies. Glycerol stocks of these clones were maintained at −70 °C.

TCR production and purification

Luria–Bertani broth (LB) agar plates containing 50 $\mu g \cdot mL^{-1}$ of kanamycin or 100 $\mu g \cdot mL^{-1}$ of ampicillin were incubated overnight at 37 °C from transforming BL21(DE3) glycerol stocks. One litre of LB medium was inoculated with 10 mL overnight culture and grown with shaking at 37 °C to an attenuance of 0.8 at 600 nm. TCR expression was induced with 1 mM isopropyl thio-β-D-galactoside for 3–5 h. Cells were harvested by centrifugation at 2100 ***g*** for 20 min. The bacterial pellet of hVβ5.2mCβ15 was resuspended in lysis buffer [50 mM Tris/HCl, pH 7.5, 1 mM EDTA, pH 8, and 1 mM dithiothreitol (DTT)] and passed through a French press twice at 1300 psi. The lysate was centrifuged at 7700 ***g*** for 15 min and the pelleted inclusion bodies were washed four times with 0.5% (v/v) Triton X-100 and 100 mM NaCl in lysis buffer. The inclusion bodies were then washed with 2 M urea in 2 M NaCl, 50 mM Tris/HCl, pH 7.5, 1 mM DTT, with 4 M urea in the same buffer, and finally with 100 mM Tris/HCl, pH 7.5, 1 mM EDTA and 1 mM DTT. Inclusion bodies were then solubilized in 8 M urea, 100 mM Tris/HCl, pH 7.5, 10 mM EDTA and 1 mM DTT. Concentration of solubilized inclusion bodies was estimated in a Coommassie Blue stained SDS/PAGE, using different concentrations of BSA and then diluted 1 : 5 in 6 M guanidine, 10 mM acetate buffer, pH 4.2, and 10 mM EDTA. Denatured β chain was added dropwise to the renaturation buffer (1 M arginine/HCl, pH 7.5, 2 mM EDTA, 100 mM Tris/HCl, pH 7.5, 6.3 mM cysteamine, 3.7 mM cystamine) under vigorous stirring to a final concentration of 20–50 $\mu g \cdot mL^{-1}$ during 48 h at 4 °C.

Refolded hVβ5.2mCβ15 was concentrated and dialyzed extensively against NaCl/P_i and affinity purified using the anti-mouse Cβ mAb (H57-597) [24,25]. Alternatively, hVβ5.2mCβ15 was run on a Superdex 200 FPLC column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) to eliminate aggregated material that could interfere with affinity measurements [26]. hVβ5.2mCβ15 was dialyzed against 50 mM Mes, pH 6, and further purified on a Mono-S cation-exchange FPLC column (Amersham Pharmacia Biotech AB) equilibrated with 50 mM MES, pH 6, and developed with a linear NaCl gradient. The purified protein was dialyzed against NaCl/P_i and concentrated to 2 $mg \cdot mL^{-1}$.

hVβ2.1hCβ2 and hVβ1hCβ2 were also produced as inclusion bodies and refolded at pH 8.5. Purification steps included gel filtration on a Superdex 200 FPLC column and further purification on a Mono Q anion-exchange FPLC column (Amersham Pharmacia Biotech AB) equilibrated with 50 mM Tris, pH 8.5, and developed with a linear NaCl gradient.

Streptococcus pyogenes superantigen (SSA)

The *ssa-1* gene was PCR amplified from *Streptococcus pyogenes* DNA (ATCC 51500 strain) or clinical isolates of *S. pyogenes* with 5′ and 3′ terminal oligonucleotides specific for the region encoding the mature protein (5′ primer, 5′-CATGCCATGGCCAGTAGTCAGCCTGACCCTACT CCAG-3′; 3′ primer, 5′-CGCGCGGGATCCTTAGTG ATGGTGATGGTGATGGGTGACCGGTTTTTTGG

TAAGGTGAAC-3′) that had *Nco*I and *Bam*HI restriction sites, respectively. The amplified DNA was purified by agarose gel and ligated without previous digestion in the pGEM T Easy vector (Promega, Madison, WI). Ligation products were transformed into *E. coli* DH5α (Stratagene). pGEM T-ssa was digested with *Bam*HI and *Nco*I and the agarose gel purified product was cloned into the *Nco*I/*Bam*HI site of the bacterial expression vector pET 26b (Novagen, Madison, WI). *E. coli* DH5α cells were transformed with ligation products for amplification. Expression was carried out in *E. coli* BL21(DE3) as periplasmic protein. All transformed clones were selected in 50 $\mu g \cdot mL^{-1}$ kanamycin plates.

An SSA mutant where the Cys26 residue was replaced for a Ser [SSA(C26S)] was obtained by site-directed mutagenesis in order to avoid dimerization through intermolecular disulfide bond formation. Primers used for first PCR were: 5′ primer, 5′-CATGCCATGGCCAGTAGTCAGCCTGA CCCTACTCCAG-3′ and 3′ primer, 5′-GGTTATCATA TAAAGATCTCAAATTACCC-3′, for the second PCR the primers were: 5′ primer, 5′-GGGTAATTTGAGATC TTTATATGATAACC-3′ and 3′ primer, 5′-CGCGCGG GATCCTTAGTGATGGTGATGGTGATGGGTGACC GGTTTTTTGGTAGGTGAAC-3′. The third PCR was carried out using PCR products, the first PCR 5′ primer and the second 3′ primer. The final amplified DNA was ligated into pET26b and expressed in *E. coli* BL21(DE3) cells. Glycerol stocks were maintained at −70 °C.

SSA and SSA(C26S) DNA sequence analysis

Both wild type SSA (wtSSA) and mutant SSA(C26S) DNA were sequenced with a Thermo Sequenase Cy5.0 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech AB) as indicated by the manufacturer.

Superantigen expression and purification

LB agar plate cultures with 50 $\mu g \cdot mL^{-1}$ of kanamycin were grown overnight at 37 °C from SSA or SSA(C26S) transformed BL21(DE3) glycerol stock. One litre of LB was inoculated with 10 mL overnight culture and incubated at 30 °C with shaking to an attenuance of 1.0 at 600 nm (3–6 h). SAg expression was induced with 0.2–0.4 mM isopropyl thio-β-D-galactoside for 5 h. Cells were harvested from induced cultures by centrifugation at 7300 ***g*** for 10 min. The periplasmic fraction, which contained most of the SAg, was obtained by osmotic shock as described previously [27]. Briefly, the bacterial pellet was resuspended in 50 mL of Tes buffer (200 mM Tris/HCl, pH 8, 500 mM sucrose and 0.5 mM EDTA) on ice for 30 min and centrifuged for 10 min at 12 000 ***g***. The supernatant was saved on ice and the pellet was resuspended in 50 mL of a 1 : 5 dilution of Tes and centrifuged as before. Both supernatants were mixed and dialyzed against NaCl/P_i. His_6-tagged protein was further purified by Ni^{2+}–nitrilotriacetic acid (NTA) affinity chromatography as described by the manufacturer (Qiagen), washed with 20 mM imidazole 0.5 M NaCl, 0.1 M Tris/HCl, pH 8.5, and 20 mM imidazole, 0.5 M NaCl, 0.1 M Tris/HCl, pH 8.0. The protein was eluted with 0.3 M imidazole, pH 7.5, 10 mM EDTA [27]. Further purification of SAgs was performed using a size exclusion Superdex 75 column (Amersham Pharmacia Biotech AB) equilibrated with 50 mM Tris, pH 7.5, 150 mM NaCl and finally with a Mono-S cation exchange column (Amersham Pharmacia Biotech AB) equilibrated with 50 mM MES, pH 6.0, and developed using a linear NaCl gradient. About 15 mg of purified protein per litre of culture medium was obtained.

Reduction and alkylation of SSA

SSA after Ni–NTA purification was reduced with 10 mM DTT for 2 h at 25 °C. Solid iodoacetamide was then added and alkylation was allowed to proceed in the dark at 25 °C for 30 min. The reduced and alkylated protein was dialyzed into NaCl/P_i and analyzed by SDS/PAGE and immunoblot. SSA–iodoacetamide (SSAia), with the free Cys blocked, was purified as a monomer by S-75 column (Amersham Pharmacia Biotech AB) with 50 mM MES, pH 6, 150 mM NaCl.

SDS/PAGE and immunoblotting

Proteins were analyzed by SDS/PAGE on a 12.5% gel. Previously all the proteins were denatured in SDS buffer with or without DTT and boiled for 3 min before electrophoresis. Proteins bands were visualized using Coommassie Brilliant Blue. SAgs were also analyzed by immunoblotting using an anti-His mAb (Sigma) or a rabbit anti-SSA. Rabbit polyclonal antisera were obtained by immunization with 1 $mg \cdot mL^{-1}$ of SSAia mixed with a volume of complete Freund's adjuvant. Boosts were administered on day 7, 14 and 28. Sera obtained on day 35 were diluted 10-fold and tested by ELISA and immunoblot. Experiments using animals were carried out following rules from the National Council of Research (CONICET).

T-cell proliferation assay

Heparinized blood was obtained from healthy blood donors, previously tested for antibodies against SSA by ELISA with negative results, and diluted with RPMI 1640 or NaCl/P_i (1 : 1, v/v). Blood samples were taken with the understanding and written consent of each subject. Twenty millilitres of the diluted blood was slowly added to 10 mL of Ficoll-Paque™ (Amersham Pharmacia Biotech AB) in a 50 mL tube and centrifuged at 400 ***g*** for 20 min. The PBMCs contained in the interphase were washed with 15–20 mL of RPMI and centrifuged for 10 min at 200 ***g***; the pellet was resuspended in 5 mL of RPMI with 10% of human serum with 2 mM glutamine, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 1 mM pyruvate. The PBMC population was counted with Trypan Blue in a Newbauer camera.

Purified cells (10^6 per well) were cultured in flat-bottom 96-well plates in the presence of varying dilutions of staphylococcal exterotoxin C3 (SEC3), SSA monomer (SSAm), SSA dimer (SSAd), SSAia or SSA(C26S), in 100 μL of complete culture medium. Phytohaemagglutinin (1 $\mu g \cdot mL^{-1}$) was used as positive control. After 48 h incubation at 37 °C in 5% (v/v) CO_2, 1 mCi per well of

[^{3}H]thymidine was added for the next 18 h and then harvested onto glass fibre filters. Incorporation of radioactivity was then measured using a Liquid Scintillation Analyzer 1600 TR (Packard, Canberra, Australia). All measurements were made in triplicate.

Binding analysis

The interaction of soluble β chains with SAgs was monitored in a resonance mirror with an IAsys instrument (Labsystem, Cambridge, UK) biosensor, which allows determination of real time interactions between two molecules [28].

β Chains or SAgs (≈ 100 μg·mL^{-1}) were dialyzed against 10 mM sodium acetate, pH 5.5, and coupled to the carboxymethyl-dextran cuvettes (Labsystems) using the Amine Coupling Kit as described by the manufacturer [29]. The activation and immobilization periods were set between 5 and 7 min to couple the desired amount of proteins yielding between 400 and 600 arc seconds.

Micromolar concentrations of SAgs [SSA and SSA(C26S)] or β chains (Vβ1, Vβ2.1 and Vβ5.2) were dialyzed against NaCl/P$_i$, pH 7.5, containing 0.05% (v/v) Tween 20. Twofold dilutions were made in the same buffer (160, 80, 40, 20, 10, 5, 2.5 and 1.25 μM). All binding experiments were performed at 25 °C. Dissociation was carried out in (NaCl/P$_i$)/Tween 20. Pulses of 10 mM HCl were used to regenerate the surface. All the experiments were repeated at least three times.

Dissociation constants (K_d) were determined under equilibrium binding conditions using Scatchard plots after correction for nonspecific binding, in which the proteins were passed over blocked, empty cuvettes, as described previously [26,30]. The off rate (k_{diss}) was determined using the software FASTPLOT and the on rate (k_{ass}) was obtained as $k_{ass} = k_{diss}/K_d$.

Results

TCR β chains

Our TCR β chain expression systems yielded 35–50 mg·L^{-1} of inclusion bodies. After refolding and concentration, a first purification step was carried out for chimeric hVβ5.2mCβ15 with a H57-597 mAb affinity column or with an S-200 column, followed by ion exchange chromatography. Typically, a final yield of 1–2 mg·L^{-1} of culture for the refolded β chain TCR constructs were obtained.

Superantigen

Our expression system produced 15 mg·L^{-1} of folded wild type SSA-1. After Ni–NTA purification, two bands of protein were observed in SDS/PAGE (Fig. 1A). The weaker band has an apparent molecular mass in agreement with the theoretical value calculated from the amino acid sequence; the stronger band has about twice the expected molecular mass. Both bands were reactive in immunoblotting with anti-His mAbs and an anti-SSA serum prepared in rabbits (Fig. 1C), indicating the presence of two recombinant species. The dimer/monomer mixture could not be efficiently resolved using an S75 column, yielding a fraction with 80% monomer (SSAm) and another containing 90% dimer (SSAd) (Fig. 1B). To determine whether an intermolecular disulfide bond mediated dimerization, Ni–NTA purified SSA was gently treated with DTT and the resulting free Cys residues were alkylated with iodoacetamide. As shown in Fig. 1B, reduction and alkylation produced only one band in SDS/PAGE with the expected molecular mass of the monomer.

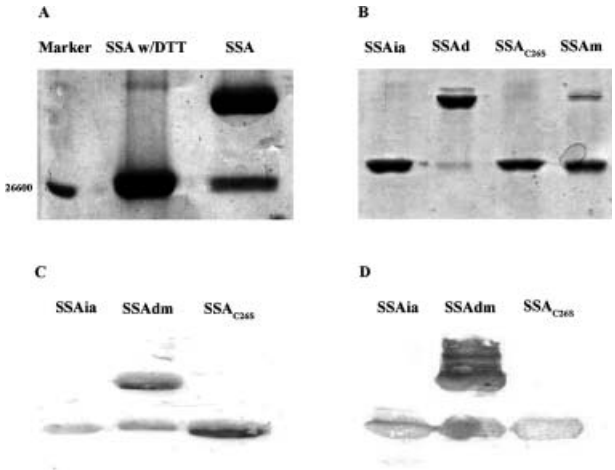

Fig. 1. SDS/PAGE and immunoblotting analysis of SSA. (A) 12.5% SDS/PAGE of SSA after Ni–NTA purification (Lane 3) and the same sample treated with DTT (Lane 2). A TCR β chain with a similar molecular mass is shown as a marker (Lane 1). (B) 12.5% SDS/PAGE of different SSA preparations. Lane 1: SSA reduced and alkylated with iodoacetamide (SSAia); Lane 2: SSA preparation enriched in dimer after purification on S75 FPLC (SSAd); Lane 3: C26S mutant (SSA$_{C26S}$) and Lane 4: SSA preparation enriched in monomer (SSAm). (C and D) Immunoblots of SSAia, wtSSA and SSA(C26S) using a commercial anti-His mAb or rabbit anti-SSA sera, respectively.

Considering that dimerization could occlude the TCR binding site, we also constructed a mutant SSA by site-directed mutagenesis. Analysis of the three-dimensional structure of SSA [31] showed that: (a) SSA has five Cys, of which two (Cys93 and Cys108) form a disulfide bond, which is present in most of the known SAgs; (b) the position of Cys101 was not determined in the crystal structure of SSA because it forms part of a loop that could not be modeled; and (c) Cys158 would not be exposed at the SSA surface. The putative TCR binding site of SSA is not known yet but an analysis based on homology with the TCR binding site of SEB and SEC3 [12,13,19,21], showed that Cys26 is not only exposed in the protein surface (Fig. 2), but would be in the putative TCR binding site. Consequently, a point mutation was introduced to replace Cys26 by Ser, which was confirmed by DNA sequencing. As can be seen in Fig. 1B–D, expression of the mutant yields only monomeric SSA, free of dimer.

T-cell proliferation assay

We next analyzed the ability of recombinant SSA to stimulate human T-cells. All SSA preparations yielded

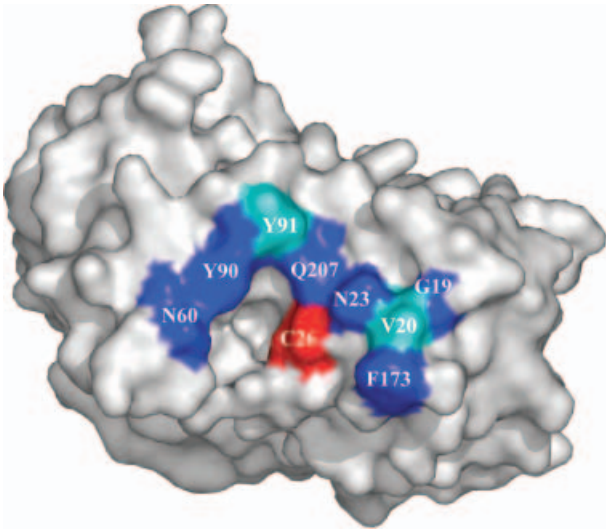

Fig. 2. SSA three-dimensional structure. Residue Cys26 of SSA is contiguous with its putative binding interface with the T-cell receptor. The common residues of SEB and SEC3 that form their respective molecular interfaces with mVβ8.2 are largely conserved in SSA. These include residues that are strictly conserved between SEB, SEC3 and SSA (shown in blue on the SSA molecular surface), as well as residues that vary between the three superantigens (shown in cyan). Residue Cys26 is shown in red.

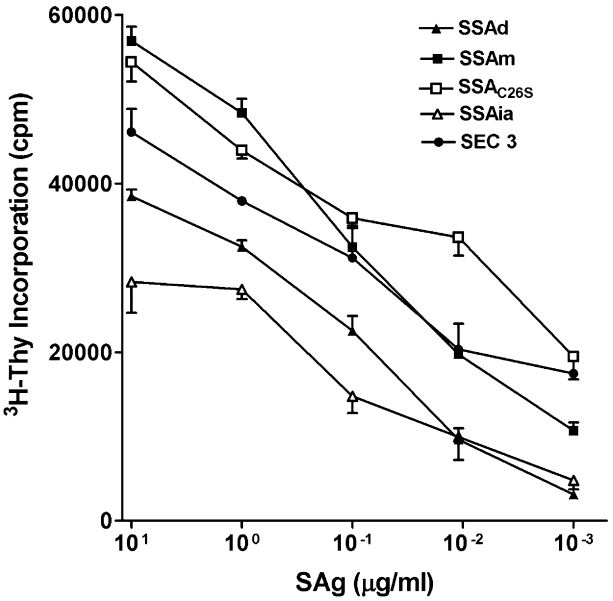

Fig. 3. Dose-dependent T-cell proliferation by the different SAg preparations. As indicated in Materials and methods, [^{3}H]thymidine incorporation was measured in a liquid scintillation analyzer. Both SSAm and SSA(C26S) produce more than 100-fold higher T-cell proliferation than SSAd and SSAia. SEC3 was included as a positive control.

dose-dependent T-cell proliferation, analyzed by [^{3}H]thymidine incorporation (Fig. 3). SSAm and the mutant SSA(C26S) caused greater proliferation than the positive control SAg, SEC3. Both SSAd and SSAia produced T-cell proliferation > 100-fold lower than SSAm or SSA(C26S) (Fig. 3).

Affinity assays

Equilibrium and kinetic parameters for SSA binding to different TCR β chains were determined in a resonance mirror using an IAsys instrument biosensor. SSA affinity for the immobilized TCR Vβ5.2 was first measured and data were evaluated by Scatchard plot analysis. Dissociation constants were estimated from the negative reciprocal of the slope of the fitted line yielding a K_d value of 153 μM (result not shown). Considering that the immobilization process could alter the molecule, rendering inaccurate results, and the fact that a high proportion of SSA was a dimer that may have the TCR binding site blocked, the purified SSAm form and the mutant SSA(C26S) were immobilized in a dextran matrix. As shown in Fig. 4, TCR Vβ5.2 concentration-dependent binding to both SSA species was observed. The association rate constant (k_{ass}) was too fast to be accurately measured. On the contrary, the dissociation rate constant (k_{diss}) could be determined using higher concentrations of SSA. Therefore, affinities (K_d) were determined under equilibrium binding conditions, in which we took report points for Scatchard analysis 5 min after injection. The k_{ass} were further calculated using equation $K_d = k_{diss}/k_{ass}$ (Table 1). Immobilization of the SAgs instead of TCRs yielded a higher binding constant of the former, which was similar for both complexes, wtSSA–Vβ5.2 and SSA(C26S)–Vβ5.2.

Different concentrations of human Vβ2.1 and Vβ1 TCRs were also used to measure the binding to the immobilized SSAm and SSA(C26S). Vβ1 showed a pattern of association and dissociation rates similar to the one obtained with Vβ5.2 (Fig. 5), yielding K_ds of the same order of magnitude (Table 1). On the contrary, no binding of Vβ2.1 TCR to SSA was detected even using a 160 μM concentration of β chain. Trials using higher concentrations were unsuccessful due to nonspecific aggregation of the Vβ2.1 TCR.

Discussion

The expression and purification of TCRs using either prokaryotic or eukaryotic systems had been troublesome for several years, delaying structural and other studies. The TCR β chain constructions we engineered allowed us to obtain large amounts of recombinant protein as inclusion bodies that could be refolded properly and used for SAg binding experiments.

SAg constructs generated large amounts of properly folded protein, but monomer and dimer forms were obtained in the wtSSA preparation. Dimerization as a prerequisite for T-cell activation has been suggested for other SAgs, such as SED [32], SPEC [33] and more recently SPEA [34]. In SED and SPEA the presence of Zn^{2+} plays an important role in dimerization; Zn^{2+} was found in the crystal structure of SPEC after soaking the crystal in a zinc solution, but dimerization of this SAg also occurred in absence of Zn^{2+}. On the other hand, SSA, which has not been reported to have a zinc-binding site, dimerized through Cys. Among the known SAgs, most have two Cys residues forming an intramolecular bridge. There are four SAgs with

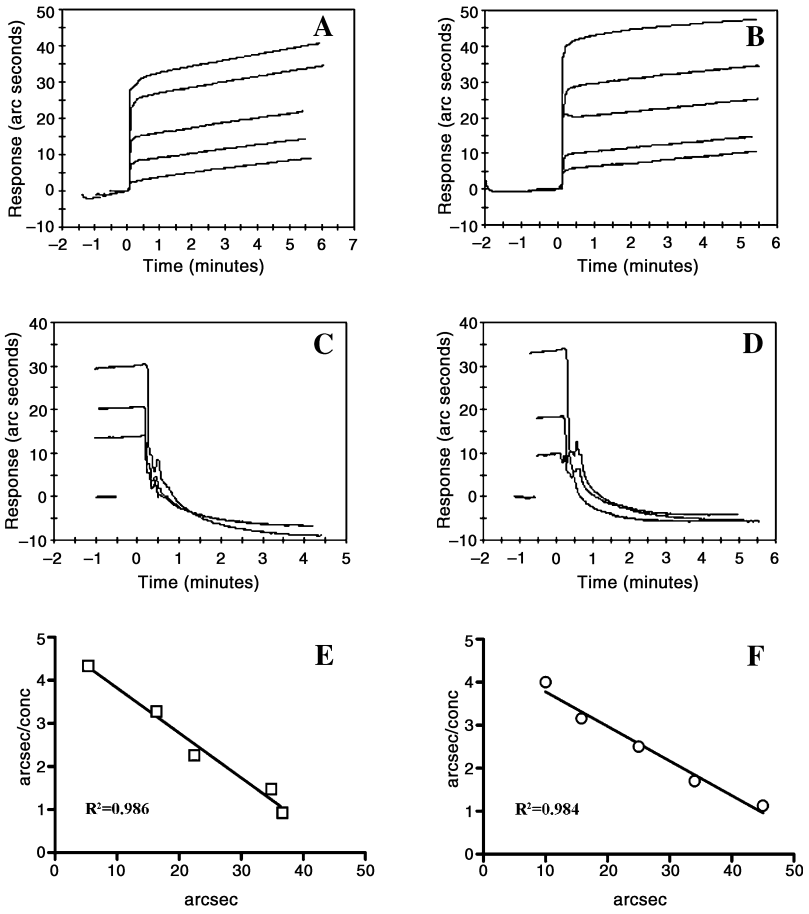

Fig. 4. TCR Vβ5.2–SSA interaction analysis. Association curves between Vβ5.2 (2.5, 5, 10, 20 and 40 μM) and immobilized SSA (A) or SSA(C26S) (B). Data sets were measured five minutes after injection. Dissociation curves between Vβ5.2 (10, 20, 40 μM) and SSA (C) and SSA(C26S). (D). Scatchard analysis for the binding of Vβ5.2–SSA with a K_d = 10.04 μM (E) and Vβ5.2–SSA(C26S) with a K_d = 10.72 μM (F).

Table 1. Binding parameters for SAg–TCR interactions. Dissociation rate constants (k_{diss}) were obtained with FASTPLOT; K_d were obtained by Scatchard analysis and association rate constants were calculated as $K_{ass} = K_{diss}/K_d$. –, No binding detected.

SAg–TCR	K_{ass} ($M^{-1}\cdot s^{-1}$) × 10^2	K_{diss} (s^{-1}) × 10^{-3}	K_d (M) × 10^{-6}
wtSSA–Vβ5.2	34.4	34.5 ± 0.6	10.0
wtSSA–Vβ2.1	–	–	–
wtSSA–Vβ1	5.1	5.5 ± 0.2	10.8
SSA(C26S)–Vβ5.2	29.9	32.0 ± 0.5	10.7
SSA(C26S)–Vβ2.1	–	–	–
SSA(C26S)–Vβ1	5.6	5.1 ± 0.1	9.1

no Cys in the mature protein sequence (TSST-1, SPEB, SMEZ1 and 2), three have one Cys (SEI, SEK and SPEC), two have three Cys (SEG and SPEA) and only SSA has more than that, five Cys. As discussed later, the fact that SSA has two Cys residues (Cys26 and Cys101) exposed to solvent would facilitate formation of an intermolecular disulfide bond, as observed in recombinant wtSSA.

In order to address whether dimerization is an artefact of overexpression in *E. coli* we analyzed the supernatant of several *S. pyogenes* isolates by immunoblotting using a rabbit SSA antiserum. As shown in Fig. 6 there is high degree of naturally occurring dimerization in eight out of ten supernatants studied, which reverted upon DTT treatment of the samples. Further studies are necessary to understand the biological significance of the natural dimerization.

The presence of a dimer in wtSSA, which could not be completely separated from the monomer, prompted us to follow two strategies to obtain a single species; reduction and alkylation, and mutation of the Cys implicated in the intermolecular disulfide bridge. Analysis of the three-dimensional structure of SSA [31] to determine which of the remaining three Cys residues could be implicated in the intermolecular disulfide bridge, showed that Cys158 is located in the core of the protein and is therefore not exposed to solvent. Cys101 could not be identified in the SSA structure because it forms part of the flexible disulfide loop of positions 93–110 with high intrinsic mobility [31]. Cys26 is exposed to solvent in the cleft between the small and large domains, which has been shown to be the TCR binding site in other SAgs. Point mutation of Cys26 to Ser prevented dimer formation and allowed TCR interaction studies.

Initial binding experiments using a biosensor gave an apparent dissociation constant for the immobilized TCR Vβ5.2 of wtSSA of 153 μM, which is near the lower limits of the known SAg–TCR interactions [26]. To avoid any altered K_d determination due to the immobilization process of the TCR, we immobilized wtSSA to analyze binding to soluble Vβ5.2 obtaining an approximately 10 times lower K_d. The K_d calculation is independent of the amount of

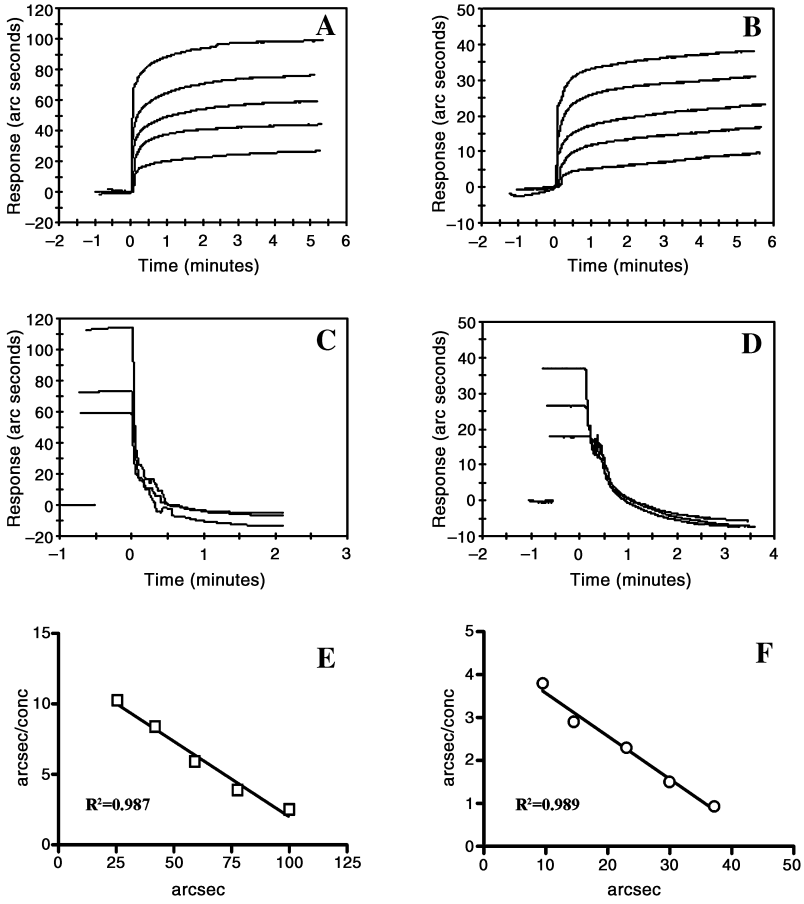

Fig. 5. Vβ1–SSA interaction analysis. Association curves between Vβ1 (2.5, 5, 10, 20 and 40 μM) and immobilized SSA (A) or SSA(C26S) (B). Data sets were measured five minutes after injection. Dissociation curves between Vβ1 (10, 20, 40 μM) and SSA (C) or SSA(C26S) (D). Scatchard analysis for the binding of Vβ1–SSA with $K_d = 10.82$ μM (E) and Vβ1–SSA(C26S) with a $K_d = 9.14$ μM (F).

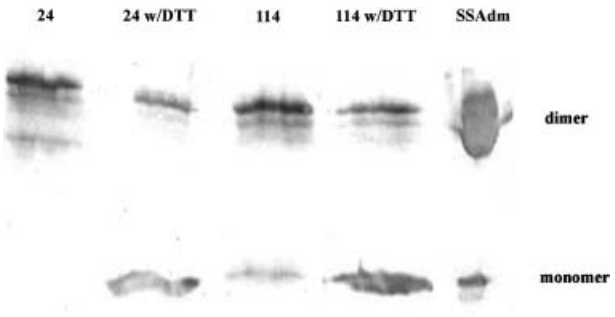

Fig. 6. SSA dimer in supernatant of *S. pyogenes*. Supernatants of isolates from patients infected with *S. pyogenes* were analyzed by SDS/PAGE and immunoblotting using a rabbit anti-SSA serum. Lanes 1 and 3: supernatants of 2 isolates; Lanes 2 and 4: supernatants treated with DTT prior SDS/PAGE showing an increase of the monomer; Lane 5: recombinant SSA produced in *E. coli*.

immobilized ligand [29]; consequently, the 5–10% of biologically active monomer contained in the wtSSA allowed an accurate affinity determination when immobilized but gave a 10–20 times higher K_d when passed over Vβ5.2, as observed. To verify Vβ5.2 availability when immobilized, soluble SSAm and SSA(C26S) were assayed yielding a K_d similar to wtSSA (150 μM). This demonstrates that the immobilization process affects the binding ability of the Vβ5.2 in a similar manner as reported for TCR β chain, Vβ8.2 [26]. On the contrary, immobilization of monomer wtSSA and SSA(C26S) yielded a $K_d = 10$ μM with soluble Vβ5.2, thus confirming the dissociation constant value for the couple SAg–TCR. In addition, these experiments showed that the C26S mutation was nondisruptive for binding to Vβ5.2.

The superantigen activity of SSA and the likelihood that dimerization occluded its TCR binding site were confirmed in human T-cell stimulation assays where SSAm and SSA(C26S) produced a higher proliferation than the positive control SEC3, which was two orders of magnitude greater than by SSAd. The residual biological activity of this sample could be explained by the SSAm contaminant (Fig. 1B).

Previous studies have shown differences in the Vβ repertoire of native and recombinant SSA. Thus, proliferation assays carried out by Reda *et al.* [21] showed that native SSA-1 but not the recombinant form, was able to stimulate T-lymphocytes bearing Vβ5.2 and Vβ1 TCRs. Similarly, native SSA-2 but not recombinant SSA-2 stimulated T-cells bearing Vβ2 [21]. Differences in the stimulation properties between SSA-1 and SSA-2 cannot be attributed to amino acid sequences because they only differ at residue 2 (Ser and Arg, respectively), which is not expected to be implicated in TCR binding. Here we found that both recombinant wtSSA and SSA(C26S) were able to bind these β chains with detectable affinity using biosensor technology.

Table 2. Residues of SEB and SEC3 that are implicated in the Vβ8.2 interaction. Numbering corresponds to SSA residues. Identical residues are shown as dashes.

SAg	19	20	23	26	60	90	91	173	207
SEB	G	L	N	V	N	Y	Y	F	Q
SEC3	–	T	–	Y	–	–	V	–	–
SSA	–	V	–	C	–	–	–	–	–
SPEA	K	N	–	F	–	–	H	N	–

On the contrary, we did not detect any significant binding between these SAgs and Vβ2.1, which is in accordance with previous cellular proliferation assays [19–21]. A nonproper folding of Vβ2.1 can be ruled out because it binds toxic shock syndrome toxin-1 [35] and its three-dimensional structure was determined as a complex with the SAg SPEC [36]. The possibility that Vβ specificity may be determined not only by SAg sequence variation within conserved regions, but also by the orientation that a SAg adopts after binding to a class II molecule, or by a particular subset of the presenting class II molecules, cannot be discarded [22]. On the other hand, it has been proposed that a small increase in the affinity of a SAg for MHC can overcome a large decrease in the SAgs affinity for the TCR [30,37].

The kinetic interaction studies of SSA with Vβ1 and Vβ5.2 showed a very fast dissociation rate, as observed in TCR–peptide–MHC interactions. In the latter case a single peptide–MHC complex is thought to sequentially bind and trigger a large number of TCRs (up to 200), as proposed in the so-called 'serial engagement' model of Lanzavecchia *et al.* [38]. A similar mechanism could be employed by the SAgs, which are able to cause TSS in human when 1–2 μg is injected [39].

SSA shares with the staphylococcal superantigens, SEB and SEC3, specificity for several mouse and human TCRs, including mouse Vβ8.2 (M. C. De Marzi & E. L. Malchiodi, unpublished results). The three-dimensional structures of SEB and SEC3 bound to mouse Vβ8.2 have already been determined [12,13], allowing identification of the most important residues in the TCR binding site. Residues N23, Y90 and Q207, which make the greatest energetic contribution (> 2.5 kcal·mol^{-1}) [29] to stabilizing the Vβ8.2–SEC3 complex, are strictly conserved in SEB, SSA and SPEA (Table 2), providing a basis for understanding why these SAgs have similar specificity for this TCR β chain. Moreover, residues N23, N60 and Y90 are conserved among bacterial SAgs reactive with mouse Vβ8.2, including SEC1–3, SPEA and SSA. The differences in residues C26 and Y91 in SSA compared with Y26 and V91 in SEC3, which make a slightly lower energetic contribution (1.5–2.0 kcal·mol^{-1}), can account for the different specificities among SSA (human 1, 2, 19; mouse 14), SEB and SEC3. As shown in Table 2, SSA residues most likely to bind Vβ chains are more similar to those presented in the staphylococcal SEC3 and SEB than in the streptococcal SPEA, indicating that SSA behaves more like a staphylococcal than a streptococcal SAg. The presence of a Cys at position 26 in SSA instead of Tyr, as in SEB, could explain why dimerization mediated by this residue occludes the TCR interaction site.

SAgs mutated in the TCR or MHC II binding site could be used to generate protective responses without systemic effects such as TSS and food poisoning. Such recombinant proteins could also be used against tumors or to treat autoimmune diseases [40]. Consequently, the molecular studies of the interaction of SAgs with their specifics ligands will not only advance understanding of the physiological mechanisms of these molecules, but may lead to the development of therapeutic agents.

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