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Display of functional αβ single-chain T-cell receptor molecules on the surface of bacteriophage

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Abstract

The ability to display functional T-cell receptors (TCR) on the surface of bacteriophage could have numerous applications. For instance, TCR phage-display could be used to develop new strategies for isolating TCRs with unique specificity or it could be used to carry out mutagenesis studies on TCR molecules for analyzing their structure–function. We initially selected a TCR from the murine T-cell hybridoma, DO11.10, as our model system, and genetically engineered a three domain single-chain TCR (scTCR) linked to the gene p8 protein of the *Escherichia coli* bacteriophage fd. Immunoblotting studies revealed that (1) *E. coli* produced a soluble scTCR/p8 fusion protein and (2) the fusion protein was packaged by the phage. Cellular competition assays were performed to evaluate the functionality of the TCR and showed the DO11.10 TCR-bearing phage could significantly inhibit stimulation of DO11.10 T hybridoma cells by competing for binding to immobilized MHC/peptide IA^d/OVA(323–339). Flow cytometric analysis was carried out to evaluate direct binding of DO11.10 TCR-bearing phage onto the surface of cells displaying either IA^d containing irrelevant peptide or OVA peptide. The results revealed binding of DO11.10 TCR-bearing phage only on cells expressing IA^d loaded with OVA peptide showing TCR fine specificity for peptide. To illustrate the generality of TCR phage-display, we also cloned and displayed on phage a second TCR which recognizes a peptide fragment from human tumor suppressor protein p53 restricted by HLA-A2. These findings demonstrate functional TCR can be displayed on bacteriophage potentially leading to the development of novel applications involving TCR phage-display. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Antibodies; MHC; T-cell receptors; Immunochemistry; Phage display

Abbreviations: abs, antibodies; α, alpha chain; aa, amino acid; β, beta chain; BSA, bovine serum albumin; C, constant domain; EE-tag, glutamic acid rich peptide tag; FBS, fetal bovine serum; HLA, human leukocyte antigen; IPTG, isopropyl-β-D-thiogalactopyranoside; MCF, mean channel fluorescence; MHC, major histocompatibility complex; p8, gene VIII protein; m.w., molecular weight; mAb, monoclonal antibody; PCR, polymerase chain reaction; TCR, T-cell receptor; scTCR, single-chain T-cell receptor; cfu, colony forming unit; DPBS, Dulbecco's phosphate buffered saline; OD_{600}, optical density at 600 nm wavelength; NFDM, non-fat dry milk; HRP, horseradish peroxidase; PFU, plaque-forming units; TBS, Tris buffered saline; TBST, Tris buffered saline containing Tween-20; V, variable domain; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TM, transmembrane

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1. Introduction

T lymphocytes play a crucial role in many diseases, such as controlling the replication of infectious agents or malignant cells and mediating pathogenesis (Cohen, 1993; Houbers et al., 1993; Boon et al., 1994; Blass et al., 1997; Rickinson and Moss, 1997; Rowland-Jones et al., 1997). The antigen specificity of these lymphocytes is controlled by the TCR displayed on their surface. Therefore, much interest has been generated recently in exploring the structure and function of these molecules and their interaction with the peptide/MHC complex (Bentley and Mariuzza, 1996; Fields and Mariuzza, 1996).

Isolation of antigen-specific lymphocytes, particularly from human sources, remains a difficult task. Currently, the limiting dilution procedure continues to be the method of choice for isolating antigen-specific lymphocytes (Riddell and Greenberg, 1995). This process is time consuming, costly and not always successful. To overcome this difficulty, we propose an approach that may allow direct isolation of antigen-specific TCRs. In this approach, TCR gene libraries will be constructed via PCR and their gene products will be engineered to be displayed on the surface of bacteriophage. In vitro panning methods using peptide-linked MHC molecules or cell lines expressing MHC/peptide complexes are then implemented for their enrichment and isolation (Felici et al., 1991; Greenwood et al., 1991). In essence, this approach adapts the phage-display technology, which has revolutionized the way in which antigen-specific mAbs are presently isolated (Winter et al., 1994; Dunn, 1996), to the selection of antigen-specific TCRs.

In contrast to the success of phage-display systems for Abs, display of α/β TCR molecules on the surface of phage has not been successfully achieved; only the display of an atypical TCR Vα chain on the surface of bacteriophage has been previously reported (Onda et al., 1995). The lack of success is the result of technical problems associated with the expression of soluble TCR in *Escherichia coli* (Novotny et al., 1991; Soo Hoo et al., 1992). To address these technical difficulties with phage-display of TCR, we selected the murine TCR from T cell hybridoma DO11.10 as our model system. We chose this TCR because its ligand (a peptide from chicken ovalbumin (OVA 323–339) in the context of the class II MHC IA^d molecule) is known (Haskins et al., 1983). Additionally, the DO11.10 TCR is well characterized (a heterodimer comprised of a Vα13.1 plus a Vβ8.2 chain) and its nucleotide sequence has been determined (Kabot et al., 1991). In addition, to show the generality of TCR phage display, we expressed a different TCR (Vα2.3, Vβ11.0) specific for peptide fragment p149–157 from wild-type human tumor suppressor protein p53 in the context of HLA-A2.1 (Theobald et al., 1995).

The development of TCR phage-display technology, as described in this manuscript, would be expected to have many applications besides isolating TCRs with unique specificity from TCR phage-display libraries. Other applications would include the development of (1) a well defined system to carry out further characterization of TCR structure and function and (2) a rapid and reliable way to perform random mutagenesis to improve TCR binding affinity for cognate MHC/peptide. The outcome of these applications could be used for the generation of novel TCR-based immunotherapeutic strategies. For example, several recent reports have generated interest in a novel immunotherapeutic strategy for treating cancer patients using retargeted T cells (Cole et al., 1995). Reconstitution of T cells is carried out using gene therapy to deliver a gene encoding for a unique antigen-specific TCR that in essence redirects the antigen-specificity of the T cell. A general method, therefore, to rapidly identify TCRs with unique specificity would facilitate the development of this approach as well as other TCR based immunotherapeutics.

2. Materials and methods

2.1. Cell lines

The IA^d-restricted T hybridoma cell line, DO11.10 (Haskins et al., 1983) and gD12 (Grammer et al., 1990) cell lines, are specific for peptide derived from chicken OVA (amino acids 323–339) and HSV-1 glycoprotein D (amino acid 246–261), respectively. The B-lymphoma cell line CH12 (Arnold et al., 1983) has been shown to express surface IE^k. These cell lines were kindly provided by Susan Grammer

(University of Miami, Miami, FL) and were maintained in RPMI complete medium [(RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA), 100 U:100 ug/ml penicillin/streptomycin (Sigma, St. Louis, MO), 2 mM glutamine (Mediatech)]. The murine plasmacytoma cell line NSO (kindly provided by Baxter International, Duarte, CA) was cultured in IMDM non-selective medium [IMDM (Sigma) supplemented with 10% FBS (Irvine Scientific), penicillin/streptomycin (100 U:100 ug/ml; Sigma) and 2 mM glutamine (Mediatech)]. Membrane bound heterodimer IA^d/OVA expressing transfectants (NDO.B5) of the NSO cell line were maintained in IMDM selective medium [glutamine free IMDM (Sigma), 10% dialyzed FBS (Irvine Scientific), penicillin/streptomycin (100 U:100 ug/ml; Sigma), nucleosides, glutamate and asparagine (1 × GS supplement, JRS Biosciences)].

2.2. Bacterial and bacteriophage strains and phagemids

E. coli strain XL1-Blue (F′::Tn*10* *proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)M15/*recA*1 *endA*1 *gyrA*96 (Nal^r) *thi* *hsdR*17 ($r_k^- m_k^+$) *supE*44 *relA*1 *lac*) (Bullock et al., 1987), and helper phage VCSM13 were obtained from Stratagene (La Jolla, CA). The expression of p3 and p8 TCR fusion products was carried out using phagemid vectors pSUN18 and pSUN26, respectively, which were derived from the pBluescript phagemid vector (Stratagene). In these phagemid, the pBluescript vector was modified to contain a *pelB* leader sequence for secretion of the TCRs and a polylinker region was cloned into the vector backbone to introduce several new restriction sites, including *SfiI*, *SpeI*, *XhoI* and *XmaI* (see Section 2.3).

2.3. Construction of scTCR fusions

mRNA was prepared from 10^6 DO11.10 T hybridoma cells using oligo-dT coated magnetic beads (Dynal, Oslo, Norway). cDNA was prepared from the mRNA using Moloney murine leukemia virus reverse transcriptase (BRL/Life Technologies, Gaithersburg, MD) and the Constant α-1 and Constant β-1 primers for the alpha and beta chains, respectively (see below). Vα13.1 cDNA was amplified with Vα5′ and Vα3′ primers and the Vβ8.2Cβ cDNA was amplified with Vβ5′ and Vβ3′ primers (see below) using 20 cycles of PCR (1 min 96°C; 30 s 55°C; 1 min 72°C) in the Gene Amp System (Perkin Elmer, Norwalk, CT). The resulting DNA was digested with the appropriate restriction enzymes, electrophoresed through a 1% low melting point agarose gel (NuSieve, FMC Bioproducts, Rockland, ME), and purified using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The Vα domain was first ligated into either pSUN18 or pSUN26 at the *SfiI* and *SpeI* sites, and the VβCβ domains subsequently inserted at the *XhoI* and *XmaI* sites. The Vα and VβCβ domains were linked together by a $(G_4S)_4$ peptide linker (Huston et al., 1988) using a pair of annealed oligonucleotides L5′ *SpeI* and L3′ *XhoI* inserted at the *SpeI* and *XhoI* sites. The nucleotide insert from the resulting phagemid (pSUN18 and pSUN26) was then sequenced using an Applied Biosystems (ABI)/Perkin Elmer 373A automatic DNA sequencer (Forster City, CA).

cDNA encoding for the anti-p53 TCR specific for peptide fragment p149–157 from wild-type human tumor suppressor protein p53 was kindly provided by Dr. Linda Sherman (SCRIPPS Research Institute, La Jolla, CA). Amplification of the Vα 2.3 and the Vβ 11.0 genes was carried out as described above except that the oligonucleotides used were specific for anti-p53 TCR (see below) and the amplification of the Vα gene also included the first seven N-terminus amino acid residues of the Cα chain (APEPNQI). Cloning of the anti-p53 TCR was carried out in the same manner as the DO11.10 TCR genes for expression as a three-domain scTCR.

2.4. Oligonucleotides used in cloning

Oligonucleotides used in this study were made on an automatic DNA synthesizer (ABI) and purified with Oligonucleotide Purification Cartridges (ABI). The sequences of the oligonucleotides used to amplify the DO11.10 and the anti-p53 scTCR follow below with the restriction sites underlined: DO11.10 specific oligonucleotides are shown first. Constant-α-1: 5′-GAC TAG CCC GGG ACA GGG AAC GTG TGA ACT GGG-3′; Constant-β-1: 5′-CTT CCT

CAC TAG TAC AGT CTG CTC GGC CCC AG-3′, Vα front: 5′-CTC GCG GCC CAG CCG GCC ATG GCC GAG CAG GTG GAG CAG CTT CCT-3′, Vα back: 5′-GTG CTC ACT AGT GTT TGG CTC TAC AGT GAG TTT GGT G-3′, Vβ front: 5′-CTC GCG CTC GAG CGA GGC TGC AGT CAC CCA AAG C-3′, Cβ back: 5′-GTG GAG CCC GGG GTC TGC TCG GCC CCA GGC CTC-3′, Cβ back-EE-tag: 5′-GAG GTG GAA TTC TCA CCC GGG TTC CAT CGG CAT GTA CTC TTC CTC GTC TGC TCG GCC CCA G-3′, L-5′: 5′-CTA GTC TGG CGG TGG CAG CGG CGG TGG TGG TTC CGG TGG CGG CGG TTC TGG CGG TGG CGG TTC C-3′, L-3′: 5′-TCG AGG AAC CGC CAC CGC CAG AAC CGC CGC CAC CGG AAC CAC CAC CGC CGC TGC CAC CGC CAC CAG A-3′.

Oligonucleotides specific for amplification of anti-p53 scTCR with restriction sites underlined. Vα front: 5′-GAG GTG GAG GCC CAG CCG GCC ATG GCC CAG CAG GTG AGA CAA AG-3′, Vα-Cβ back: 5′-GAG GTG GAG ACT AGT AGC AGG TTC TGG GTT CTG-3′, Vβ front:5′-GAG GTG GAG CTC GAG CAA TGC TGG TGT CAT CCA AAC-3′, Cβ back: same as Cβ back used to amplify DO11.10.

2.5. Expression of scTCR / p8 fusion protein

For production of the scTCR/p8 fusion protein, pSUN26 was transferred into *E. coli* strain XL1-Blue cells. The transformed cells were grown overnight at 37°C in 2× Luria Broth (LB) medium containing 0.5% glucose (Sigma), 100 mg/ml ampicillin (Sigma) and 15 mg/ml tetracycline (Sigma). The overnight cell culture was diluted 1 to 50 into fresh 2× LB medium containing ampicillin and tetracycline, grown for 2 h with shaking at 37°C and then the cells were induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Alexis, Laufelfingen, Switzerland) to the medium. After 4 h of induction, cell density was measured at an OD_{600} using a spectrophotometer, and 10 ODs of cells were spun down and stored at −20°C.

2.6. Isolation of scTCR / p8 fusion protein

The cell pellet was thawed and resuspended at 10 OD_{600} per ml in cold extraction buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100) and disrupted using sonication (Branson 450, Danbury, CT) for 1 min. After lysis the sample was clarified by centrifugation at 25,000 × *g* for 20 min and the supernatant collected was defined as the soluble fraction.

2.7. Production of bacteriophage bearing TCR

To display scTCR/p3 and p8 fusion proteins on the surface of bacteriophage, XL1-Blue cells carrying either the pSUN18 and pSUN26 phagemid, respectively, were used to inoculate cultures of 2× LB medium containing 0.5% glucose, 100 mg/ml ampicillin and 15 mg/ml tetracycline. *E. coli* cells were pelleted from an overnight culture, washed twice in LB medium without glucose and then were resuspended in 50 ml of 2× LB containing 100 mg/ml ampicillin, 15 mg/ml tetracycline and 1 mM IPTG. After a 4 h induction, helper bacteriophage (VCSM13) were added to the culture at 10^{10} PFU/5 ml and incubated for 15 min before diluting the mixture 50-fold into pre-warmed 2× LB containing 100 mg/ml ampicillin, 15 mg/ml tetracycline and 1 mM IPTG. Kanamycin (5 mg/ml)(Sigma) was added to cultures 1 h later and the phage-infected cultures were grown overnight. Phage were precipitated from the supernatant with 1/5 volume of 20% polyethylene glycol (PEG) 6000 (Sigma) and 2.5 M NaCl. After a second PEG precipitation, phage were resuspended in Dulbecco's Phosphate Buffered Saline (DPBS, Sigma) and filtered through a 0.2 μm filter (Nalgene, Rochester, NY) to remove *E. coli* membrane debris. The phage preparation was buffer exchanged into DPBS containing 1% FBS using a centricon-100 microconcentrator (Amicon, Beverly, MA). Phage titers ranged from 10^{10} to 10^{14} CFU/ml. In some experiments, inductions were carried out by glucose starvation. The method is similar to the IPTG induction method described above except IPTG is not added to the medium.

2.8. Immunoblotting and immunoprecipitation

Proteins were separated by SDS-PAGE on 12% Tris–glycine gels (Novex, San Diego, CA). The proteins were blotted onto Immobilon-P transfer

membrane (Millipore, Bedford, MA) using a semi-dry immunoblotting apparatus (Integrated Separation Systems, Natick, MA). The membranes were blocked in 1 × Blotto (DPBS containing 5% non-fat dry milk (NFDM)) for 1 h at room temperature, washed three times in 0.2 × Blotto and incubated for 1 h at room temperature in 0.2 × Blotto containing 0.3 mg/ml of anti-EE tag mAb. After three additional washes in 0.2 × Blotto, the blots were incubated for another 30 min in 0.5 mg/ml of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Harbor, PA), washed three times in 0.2 × Blotto, developed with SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to X-ray film (Kodak, Rochester, NY).

Immunoprecipitation was carried out using sample derived from the soluble fraction as described above. One of the following mAbs, MR5-2, F23.1, KJ-1 or control mAb, anti-Vβ17 was added (2.5 μg of each) to 1 ml aliquots of soluble fraction material and incubated with rocking overnight at 4°C. To precipitate immune complexes formed between the scTCR/p8 and the mAb, 25 μl of magnetic beads coated with goat anti-mouse Ab (Dynal) were added to each sample and incubated with rocking for 30 min at room temperature. A magnet was used to capture beads containing the immune complexes and non-specifically bound protein was removed by washing the beads four times in DPBS. The isolated protein was suspended in 2 × cracking buffer (0.15 M Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-ME, 0.02% Bromophenol blue), boiled for 5 min and run on a 12% SDS-PAGE gel for subsequent immunoblotting.

2.9. Phage ELISA assay

A TCR-specific sandwich ELISA assay was used to detect display of TCR molecules on the surface of phage. A 96 well plate was coated with streptavidin (Pierce) at 200 ng/well in 0.1 M sodium bicarbonate buffer, pH 8.2 and incubated at 4°C overnight. The plate was blocked using 5% NFDM for 1 h followed by the addition of 50 μl per well of one of the following biotin labeled anti-DO11.10 TCR mAbs at 10 μg/ml (H57–597 hamster IgG, MR5-2 mouse IgG_{2a}, F23.1 mouse IgG_{2a}; PharMingen, San Diego, CA) or anti-idiotype specific mAb (KJ-1 mouse IgG_{2a}; a kind gift from Dr. P. Marrack, National Jewish Center for Immunology, Denver, CO), or with biotin labeled anti-p53 TCR mAbs (B20.1, anti-Vα 2.0 rat IgG_{2a}; RR3-15, anti-Vβ 11.0 rat IgG_{2b}; PharMingen) at the same concentration. After incubating mAbs for 1 h at room temperature, the plate was washed six times in Tris buffered saline (50 mM Tris–HCl, pH 8.0 and 150 mM NaCl) containing Tween-20 (0.5%) (TBST) to remove unbound Ab. Titrated samples of bacteriophage diluted in 2.5% NFDM were added to wells containing plate-bound Ab, incubated for 1 h at room temperature, washed six times in TBST and then incubated for 1 h with 100 μl per well of a 1 to 2000 dilution of HRP-conjugated sheep anti-M13 (phage) polyclonal Ab (Pharmacia). The plate was washed eight times in TBST, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate (TMB) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well and the reaction was quenched with an equal volume of 1 M sulfuric acid. The signal was read at absorbance 450 nm. Non-specific binding (background) was defined as the absorbance reading from phage bound to BSA (Sigma) and anti-Vβ17 mAb (PharMingen) coated wells.

2.10. Cellular inhibition assay

Two T hybridoma cell lines, DO11.10 and gD12, were used in the inhibition assay. The assay was carried out by immobilizing in wells 0.2 μg of single-chain IA^d (Rhode et al., 1996) containing covalently bound OVA or HSV peptide, or 0.1 μg of mAb anti-CD3ε (145-2C11, hamster IgG, PharMingen). Titration of DO11.10 TCR-bearing phage and Ab-bearing phage (control) were made in DPBS supplemented with 10% FBS. Wells were washed twice with DPBS and phage samples were incubated in wells for 15 min at 37°C in the presence of 10% CO_2. DO11.10 and gD12 T cell hybridomas were suspended at 10^6/ml in RPMI complete medium, and 100 ul (10^5 cells) of either DO11.10 or gD12 cells was added to wells containing plate-bound MHC/peptide or anti-CD3ε Ab and incubated for 4 h at 37°C in 10% CO_2. Supernatant was removed and assayed for IL-2 protein in a sandwich ELISA.

2.11. IL-2 ELISA

The IL-2 sandwich ELISA assay was carried out as described by PharMingen. Wells were coated with 50 μl of a 2 μg/ml rat anti-mouse IL-2 Ab (JES6-1A12; PharMingen) and incubated overnight at 4°C. The plate was washed twice in DPBS/0.5% Tween-20, blocked 1 h in 10% FBS and incubated for 4 h at room temperature with 100 μl of cell assay supernatant. The plate was washed six times in DPBS/Tween and 1 μg/ml of biotin-labeled rat anti-mouse IL-2 Ab (JES6-5H4; PharMingen) was used to detect captured IL-2. After an hour incubation, the plate was washed six times in DPBS/Tween and 2.5 μg/ml of strepavidin–peroxidase (Sigma) was added to wells for 30 min at room temperature. The plate was washed eight times in DPBS/Tween, 100 μl of ABTS substrate (2,2′-azino-di-3-ethyl-benthiazoline sulfonate, Kirkegaard and Perry Labs) was added and the absorbance was read at 405 nm.

2.12. IA^d/OVA plasmid construction and transfection of NSO cell line

RT-PCR was used to amplify the IA^d α chain gene and the IA^d β chain signal sequence and β1/β2/TM gene fragments from total RNA isolated from IA^d expressing A20.11 cells (Kim et al., 1979). In order to facilitate cloning, the PCR primers were designed to introduce unique restriction sites at the ends of the gene fragments. Sequence encoding a 10 amino acid peptide linker (ASGGGGSGGG) was introduced to the 5′ end of the β1/β2/TM gene fragment and the Kozak consensus sequence was introduced 5′ to both the α and β signal sequences. The β chain PCR products were cloned into pBluescript II SK^- such that a full length IA^d β chain cDNA was generated containing *AflII* and *NheI* sites and a 10 amino acid linker sequence between the signal sequence and the first amino acid of the β1 domain. The addition of the *AflII* site at the end of the signal sequence fragment resulted in a change in the last two amino acids (Glu–Gly) to residues common at the end of immunoglobulin signal sequences (Leu–Ser). Oligonucleotides encoding the OVA peptide were annealed and cloned between the *AflII* and *NheI* sites. The β chain fusion genes were subcloned in front of the cytomegalovirus (CMV) promoter of pEE6 (Celltech, Berks, UK) and the α chain gene was subcloned in front of the CMV promoter of pEE13 (Celltech). In order to make a single construct that expressed both the α and β chains, a fragment containing the CMV promoter and OVA-linked β chain was excised from the pEE6 construct and inserted into the pEE13/α chain vector. The resulting construct containing the IA^d linked OVA peptide sequence was designated as pJRS165.1.

Transfection of NSO cells was carried out as follows. Briefly, 1×10^7 NSO cells were washed twice in ice cold PBS, resuspended in 760 μl of ice cold PBS, and mixed with 40 μg (1 μg/μl) of *SalI* linearized pJRS165.1 plasmid DNA. After a 5 min incubation on ice, cells were electroporated using a Gene Pulsar (BioRad) set to deliver one pulse of 250 V, 960 μfd. Pulsed cells were placed on ice for 2–5 min and added to 30 ml of IMDM non-selective medium. Cells were plated in 96-well flat bottom tissue culture plates and 24 h later, 150 μl of IMDM selective medium was added to each well. Plates were fed with selective medium on a weekly basis by replacing 100 μl/well of spent medium with fresh selective medium, allowing the cells to gradually deplete the medium of all residual glutamine. Colonies of transfected cells became evident after 14–21 days, and were expanded and screened for expression of MHC/peptide molecules, IA^d/OVA. The NDO.B5 cell clone (NSO cell line transfected with IA^d/OVA construct) was analyzed for increased levels of surface IA^d (A. Edwards and S. Grammer, unpublished results). Cells were incubated with FITC-conjugated anti-IA^d antibody (AMS 32.1; PharMingen, 1:100 dilution) in cold staining buffer (PBS, 1% FBS) for 45 min. After washing three times in staining buffer, fluorescence was examined using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). An isotype matched FITC-conjugated anti-IA^k (10-3.6; PharMingen, 1:100 dilution) was used as a negative control.

2.13. Flow cytometry of TCR-bearing phage using membrane bound IA^d/OVA transfectants

Direct binding studies with phage displaying TCR were carried out using either 1×10^6 NSO (IA^d),

NDO.B5 (IA^d/OVA) or CH12 (IE^k) cells. To minimize potential differences in the expression and/or folding of TCR molecules, the total TCR signal in each phage preparation was normalized by assaying in a TCR-phage ELISA. After normalizing for TCR signal, DO11.10 TCR/p8 phage and DO11.10 TCR/p3 phage were incubated with cells for 2 h on ice. Samples were then washed in 1% FBS/PBS and resuspended in 0.1 ml of 1% FBS/PBS containing 2 μg of anti-M13 polyclonal sera (Pharmacia). After a 20 min incubation on ice, the cells the were washed once in 1% FBS/PBS and the pellet was resuspended in 0.1 ml of 1% FBS/PBS containing 1 μg of donkey anti-sheep IgG-FITC (The Binding Site, CA). Following a 20 min incubation on ice, the cells were washed 2 × before being suspended in 0.5 ml of 1% FBS/PBS and analyzed. The presence of bound phage was detected using a FACScan flow cytometer (Becton Dickinson). Data were analyzed with CELLQuest Software (Becton Dickinson).

2.14. Statistical analysis

A Student *t*-test was used to statistically evaluate significant differences between mean values from the cellular inhibition assay. Significance was set at a *P*-value of < 0.05 and was calculated using SigmaPlot statistical software.

3. Results

3.1. Design of the scTCR

As mentioned above, the major technical difficulty in phage-display of TCRs has been expressing soluble scTCR molecules in *E. coli* (Novotny et al., 1991; Soo Hoo et al., 1992). If the scTCR is not soluble then it cannot be transported to the periplasm or the periplasmic membrane of *E. coli* and subsequently packaged into the phage capsid. Review of recent TCR crystallography data (Fields et al., 1994, 1995; Bentley et al., 1995; Bentley and Mariuzza, 1996; Fields and Mariuzza, 1996; Garboczi et al., 1996; Garcia et al., 1996) indicates a prominent elbow region present at the N-terminus of the Cβ domain that displays extensive interaction with the Vβ domain. We presumed based on this finding that the Cβ domain may act as a scaffold that promotes folding of the Vβ domain into a stable conformation. In contrast to the Cβ domain, the less extensive interaction observed between the Cα and Vα domains suggests deletion of the Cα domain may not affect folding of the Vα domain (Fields et al., 1994; Fields et al., 1995). Furthermore, Chung et al. (1994) have observed that a three-domain scTCR design containing only the Vα, Vβ and Cβ regions fused to the CD3ζ gene is soluble and expressed as a functional molecule on the surface of transfected BW5147α/β-T thymoma cells. In light of this information, we postulated that a soluble and functional TCR could be produced in *E. coli* using the Vα–VβCβ three-domain design. Therefore, we selected to construct an equivalent three-domain scTCR, using either TCR from DO11.10 or anti-p53 T cells, to evaluate their solubility in *E. coli*. In the case of the DO11.10 scTCR, the carboxyl-terminal end of the variable region of the TCRα chain (Vα13.1) was fused via a flexible linker $(G_4S)_4$ (Huston et al., 1988) to the N-terminus of Vβ8.2 to generate the antigen-binding portion of the TCR and thus generate the DO11.10 scTCR (Fig. 1A). The Cβ domain included in our scTCR constructs was truncated at the amino acid residue just prior to the final cysteine; inclusion of the final cysteine resulted in the formation of insoluble aggregates when the scTCR was expressed in *E. coli* (data not shown).

3.2. Expression of the scTCR fusions in E. coli

To enable packaging of the scTCR for display on the surface of phage, the scTCR was genetically fused to either protein p3, the minor coat protein gene, or to the capsid protein p8, the major coat protein gene of *E. coli* bacteriophage fd. Expression of the scTCR/p3 or p8 fusion gene was under the control of the *lacZ* promoter in the pSUN18 or pSUN26 phagemid vector, respectively (Fig. 1B). Induction of scTCR expression in *E. coli* was initiated either by glucose starvation or by the addition of 1 mM IPTG into the growth medium. The induced *E. coli* in later experiments were infected with helper phage (VCSM13) to produce phage bearing the scTCR. The scTCR design used in some experiments also included an EE tag (amino acid sequence EEEEYMPME) (Grussenmeyer et al., 1985) inserted

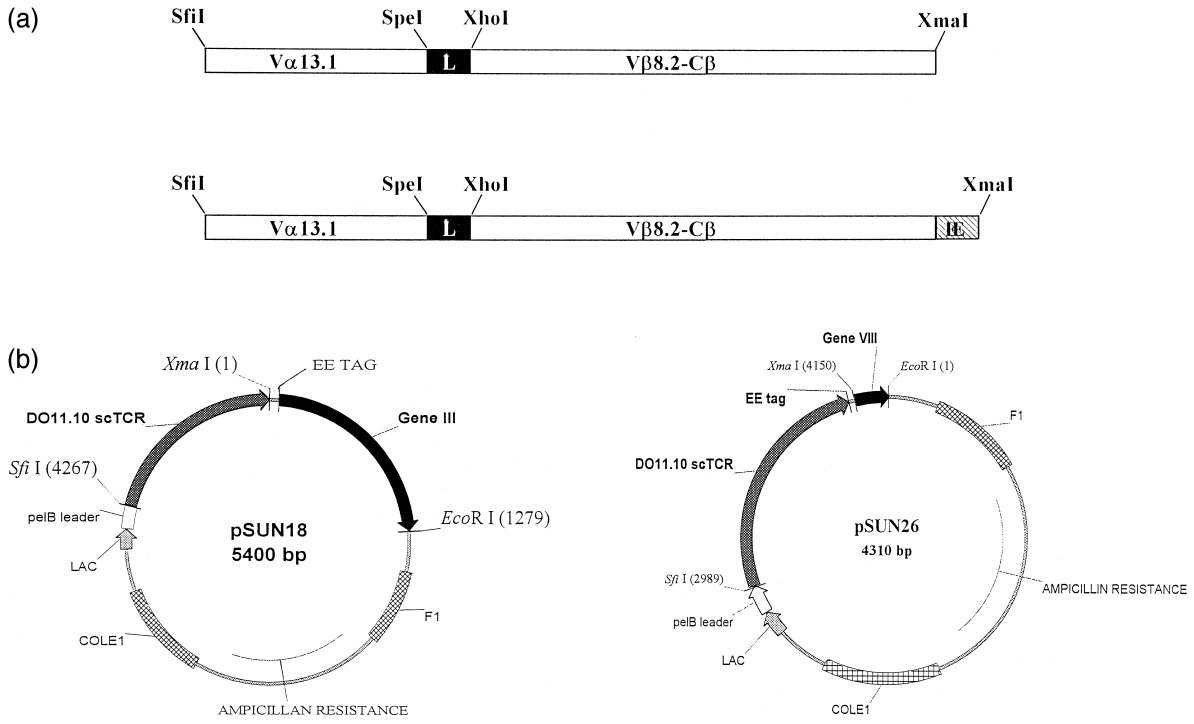


Fig. 1. Schematic representation of construct encoding the DO11.10 single-chain TCR and the phagemid vector, pSUN26. (A) The three domain scTCR construct without EE tag (top) and with EE tag (bottom); L, 20 amino acid linker $(G_4S)_4$ (Fields et al., 1995); EE, EEEEYMPME peptide tag (Bentley et al., 1995). (B) pSUN18 and pSUN26 phagemid vectors modified from pBluescript (Stratagene) contain either the p3 gene or p8 gene, respectively and an improved polylinker region to facilitate cloning of the TCR gene segments.

between the C-terminus of Cβ and the N-terminus of gene p3 or p8 (Fig. 1A) to allow for detection of the scTCR molecule by an anti-EE tag mAb (Grussenmeyer et al., 1985). To determine whether our DO11.10 scTCR/p8 fusion was soluble when expressed in *E. coli*, we performed immunoblotting analysis on extracts from induced *E. coli* and probed the blot with the anti-EE tag mAb. A protein with a m.w. of approximately 50 kilodaltons (kD) (the anticipated size of our DO11.10 scTCR/p8 molecule) was detected by the anti-EE tag mAb in the soluble fraction of *E. coli* lysate (Fig. 2A). In contrast, the 50 kD m.w. protein was not detected with lysates prepared from non-induced *E. coli*. These results suggest that the three-domain scTCR fused to p8 is soluble when expressed in *E. coli*.

To determine whether the scTCR/p8 expressed in *E. coli* was correctly folded, we immunoprecipitated the scTCR/p8 with a panel of Abs that only bind the DO11.10 TCR in the correct conformation. The selected mAbs recognize either conformational epitopes on the Vβ8.2 chain (F23.1 and MR5-2) (Staerz et al., 1985; Kanagawa, 1988) or the complementarity-determining region (CDR3) formed by correct pairing of α/β chains (KJ-1; this anti-idiotypic mAb was generated against native DO11.10 TCR) (Yague et al., 1985; Kappler et al., 1994). These three mAbs immunoprecipitated a 50 kD m.w. protein from the soluble fraction of induced *E. coli* bearing our DO11.10 scTCR/p8 fusion construct (Fig. 2B). These same mAbs did not immunoprecipitate detectable amounts of protein from non-induced *E. coli* transformed with the DO11.10 scTCR/p8 construct (data not shown). Furthermore, an irrelevant mAb, anti-Vβ17, was used as an antibody control and did not precipitate the scTCR/p8 fusion protein (data not shown). These immunoprecipitation studies further suggest that a soluble and correctly folded scTCR molecule can be produced in *E. coli* when it is fused with the p8 protein.

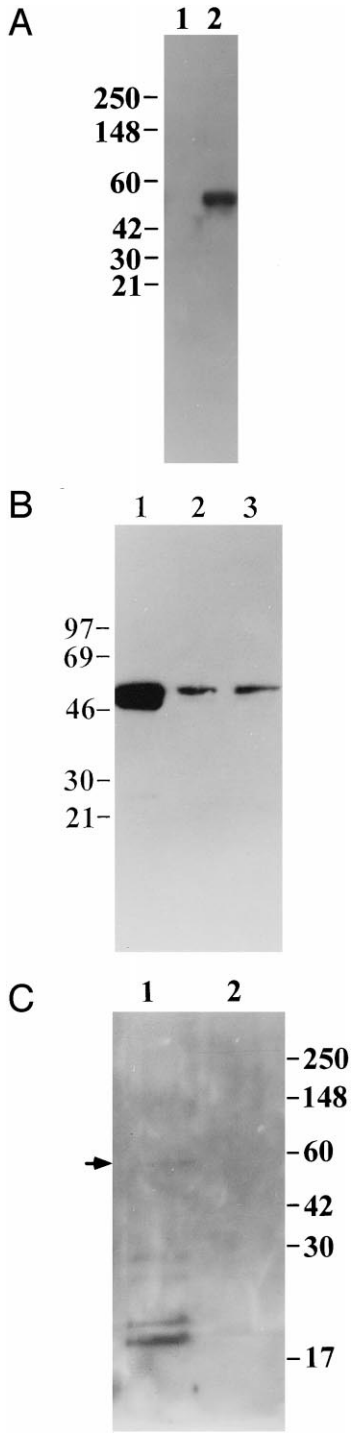


Fig. 2. Expression of soluble single-chain TCR/p8 fusion. (A) Protein samples from *E. coli* lysates transferred to membrane were probed with the anti-EE tag mAb and horseradish peroxidase (HRP)-labeled conjugate goat anti-mouse (GAM). Lane 1 represents protein sample from non-induced lysate; lane 2 represents protein sample from IPTG induced culture. (B) Lysates were immunoprecipitated with anti-TCR Abs. Immunoprecipitates were probed with anti-EE tag mAb followed by HRP-labeled conjugate GAM to detect the scTCR/p8 fusion protein. Abs used in the immunoprecipitation were as follows: lane 1-MR5-2; lane 2-F23.1; lane 3-KJ-1. (C) Immunoblot of PEG purified phage bearing DO11.10 scTCR/p8. ^{10}11 TCR-bearing phage with (lane 1) or without (control; lane 2) the EE tag were run on a 12% SDS-polyacrylamide gel under reducing conditions. The blot was probed with anti-EE tag mAb and HRP-labeled conjugate GAM to detect scTCR/p8 protein containing EE tag. The arrow indicates the position of the full-length scTCR/p8 fusion protein. Molecular size markers are indicated in kilodaltons.

3.3. Packaging of scTCR/p8 into filamentous bacteriophage fd

The soluble scTCR/p8 fusion must be transported to the periplasmic membrane of *E. coli* to allow incorporation of the fusion protein into the capsid of the phage and thus obtain preparations of TCR-bearing phage (Hunter et al., 1987). Because the phagemid contains only the DNA sequence for expression of the scTCR/p8 fusion protein, assembly of the phage capsid and packaging of the phagemid DNA is not possible without infection with helper phage. Therefore, *E. coli* containing the phagemid vector for expressing the scTCR/p8 fusion were infected with helper phage VCSM13 to produce TCR-bearing bacteriophage.

To determine whether the DO11.10 scTCR/p8 fusion was indeed incorporated into the phage capsid, we purified the phage by PEG precipitation and analyzed the phage for EE tag labeled proteins by immunoblotting analysis. A 50 kD m.w. protein with the EE tag (the expected size of our scTCR/p8 protein) was detected in phage isolated from *E. coli* transformed with the vector containing the DO11.10 scTCR/p8 gene (Fig. 2C, lane 1). Several smaller m.w. proteins presumably representing breakdown products of the scTCR fusion were also observed. Phage isolated from *E. coli* containing the control scTCR/p8 vector (DO11.10 TCR without EE tag) did not display this 50 kD m.w. protein nor any breakdown products (Fig. 2C, lane 2). This finding

suggests that the detected signal is specific for protein bearing the EE tag.

This 50 kD m.w. protein was also absent in culture supernatants recovered from *E. coli* transformed with the DO11.10 scTCR phagemid vector but not infected with VCSM13 helper phage (data not shown). Because these mock preparations did not contain detectable levels of protein bearing the EE tag, it is unlikely that the detected protein represents contamination of the phage preparations with bacterial protein. We further purified the PEG-precipitated phage on a cesium chloride (CsCl) density gradient before detecting the scTCR fusion protein in an ELISA assay. Only wells containing phage isolated from *E. coli* with the DO11.10 scTCR/p8 vector displayed significant signal (20–30-fold) above background levels. To provide further evidence that the scTCR detected in the phage preparations was not due to secretion or release of scTCR/p8 protein by the transformed bacteria into the culture medium, we selected for scTCR-bearing phage with plate-bound Abs and then infected new *E. coli* with the selected phage. The putative DO11.10 TCR-bearing phage were diluted 1:50,000 into a phage-display single-chain Ab library, then selected using Abs specific for TCR$\alpha\beta$ (H57–597) (Kubo et al., 1989) or a conformational epitope on the Vβ8 chain (MR5-2). A 2500-fold enrichment in the percentage of phage expressing DO11.10 scTCR was observed after panning against either mAb MR5-2 or H57–597; whereas no enrichment was detected by panning against a control Ab recognizing Vβ17 (data not shown). Together, the immunoblotting, ELISA and enrichment of phage analyses indicate that the DO11.10 scTCR is packaged within the phage isolated from *E. coli* containing the DO11.10 scTCR vector.

3.4. Orientation of scTCR on the surface of bacteriophage

Appropriate display of the TCR on the phage is essential for panning TCR libraries using in vitro panning techniques or for other uses such as affinity improvement and structure–function studies. Therefore, we also used ELISA assays with a panel of Abs to assess the orientation of the scTCR displayed on the surface of the phage. As shown in Fig. 3A and B,

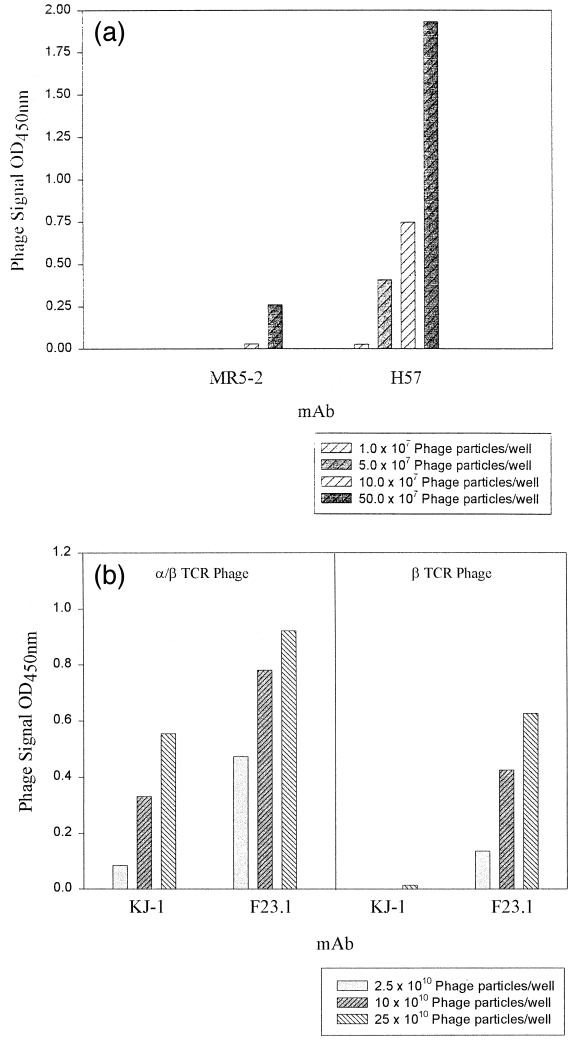


Fig. 3. Enzyme-linked immunoassay of phage bearing the DO11.10 TCR detected with a panel of mAbs. (A) Titration of TCR-bearing phage captured with either MR5-2 or H57–597 mAb. (B) Titration of phage-displaying $\alpha\beta$TCR or phage displaying the β chain alone captured with either KJ-1 or F23.1 mAb. Background signal (< 0.05 at $OD_{450\ nm}$) was subtracted from plotted signal.

phage expressing DO11.10 scTCR bound to Abs specific for conformation dependent epitopes on Vβ8.2 (F23.1 and MR5-2) and the Cβ domain (H57–597; Fig. 3A,B). In contrast, the level of binding in the ELISA assay by phage binding to wells coated with either BSA or a mAb specific for Vβ17 was low (< 0.05 OD units); this background absorbance was subtracted from the test values. This

result demonstrates that the scTCR was displayed appropriately on the surface of the phage. In addition, the KJ-1 Ab also recognized the DO11.10 scTCR displayed on the surface (Fig. 3B). Kappler et al. have shown in a previous study that the KJ-1 Ab recognizes only properly paired α and β chains which have folded correctly to form stable CDR3. The Vα and Vβ CDR3 have been shown by X-ray diffraction studies to straddle the peptide between the helices around the central position of the peptide (Garcia et al., 1996; Rickinson and Moss, 1997). Therefore, our KJ-1 Ab binding data suggests the presence of properly folded CDR3 on phage-displayed scTCR with its antigen binding domain exposed to the solvent. When we used phage expressing the β chain alone as a p8 fusion in an ELISA assay, the phage were recognized by the Ab specific for a conformational epitope on Vβ8.2 but were not recognized by the KJ-1 Ab (Fig. 3B).

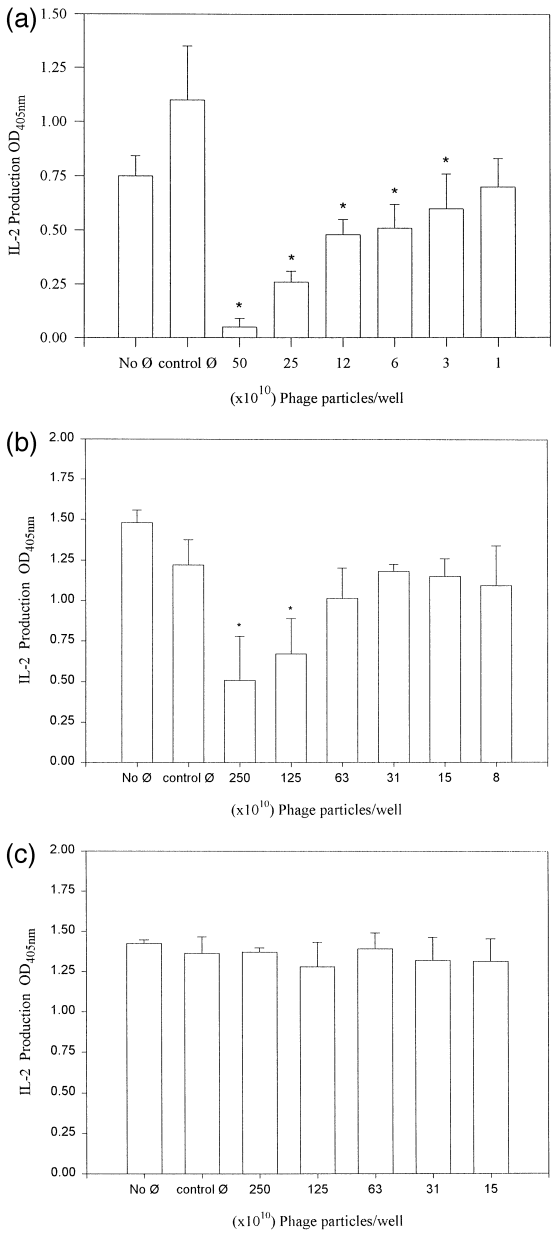


Fig. 4. Cellular inhibition assays. 10^5 T hybridoma cells were stimulated with 0.2 μg/ml of immobilized single-chain IA^d containing OVA or HSV-1 peptide, or 0.1 μg/ml anti-CD3ε mAb. T cell activation was monitored by measuring interleukin-2 (IL-2) secretion using an IL-2 specific ELISA. The relative level of IL-2 production by stimulated hybridomas in the absence or presence of phage was measured at an $OD_{405\ nm}$. (A) IL-2 inhibition of IA^d/OVA stimulated DO11.10 T hybridoma cells with DO11.10 scTCR-bearing phage. (B) IL-2 inhibition of IA^d/HSV-1 peptide stimulated gD12 T hybridoma cells with DO11.10 scTCR-bearing phage. (C) IL-2 inhibition of anti-CD3ε stimulated DO11.10 T hybridoma cells with DO11.10 scTCR-bearing phage. Data presented in figures A–C are each representative of three separate experiments using different preparations of DO11.10 scTCR/p8-bearing phage. Experimental controls included wells without any phage (no Ø) and wells containing control Ø (2.5×10^{12} phage displaying single-chain antibody molecules). Asterisk (∗) indicates significant inhibition ($P < 0.05$) calculated using the Student *t*-test.

3.5. Cellular competition assay

To assess the functionality of the DO11.10 scTCR on the surface of the phage, we next examined whether these DO11.10 TCR-bearing phage can specifically inhibit TCR-peptide/MHC interactions. DO11.10 T hybridoma cells produce significant amounts of IL-2 when stimulated by their MHC/peptide complex (plate-bound single-chain (sc) IA^d/OVA (Rhode et al., 1996)). Therefore, we expect blocking of the TCR-MHC/peptide interaction to correspond to a decrease in the amount of IL-2 secreted by the T hybridoma cells into the supernatant. Addition of phage particles bearing the DO11.10 scTCR to the T hybridoma cells stimulated by scIA^d/OVA significantly ($P < 0.05$) inhibited the amount of IL-2 protein produced by the T hybridoma

cells when compared with control wells containing either recombinant phage displaying irrelevant single-chain antibody molecules or no phage (Fig. 4A); this inhibition of IL-2 production depended on the number of scTCR-bearing phage added (Fig. 4).

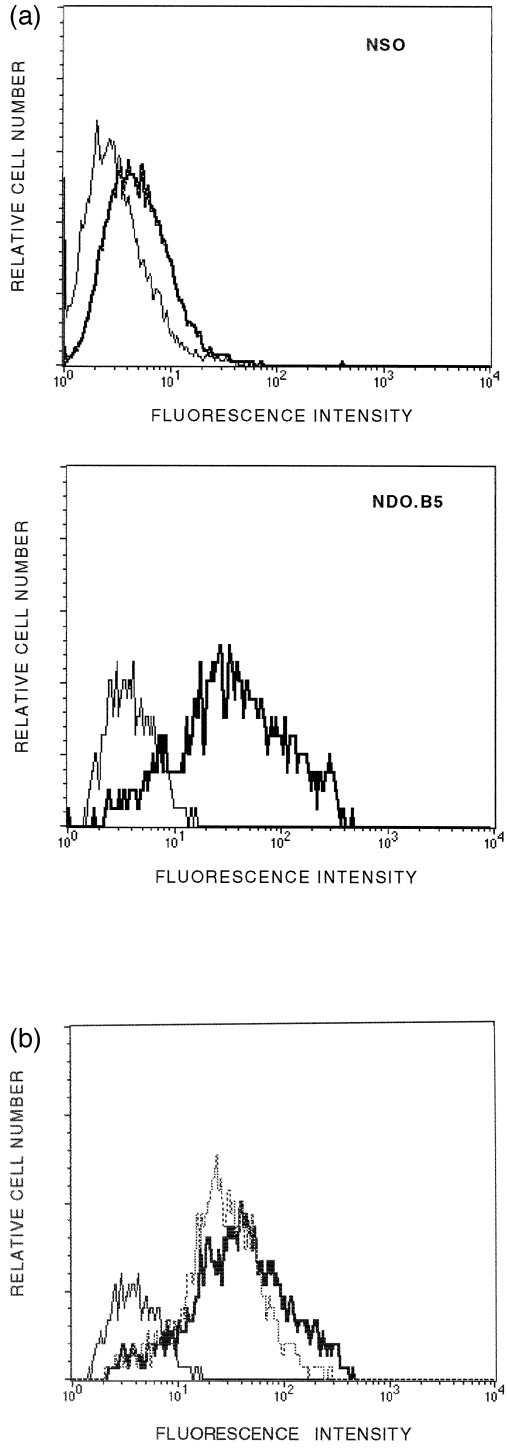
To further examine whether phage expressing the DO11.10 TCR only inhibit interaction of TCRs recognizing OVA peptide presented in the context of IA^d, we performed a similar IL-2 inhibition experiment using another T cell hybridoma. We selected the gD12 T cell hybridoma because its TCR is restricted by the same MHC class II glycoprotein (IA^d) as the DO11.10 TCR, but requires a different peptide (HSV-1 glycoprotein D peptide, aa 241–261) (Grammer et al., 1990) for stimulation and production of IL-2. Control phage expressing recombinant single-chain antibody molecules, but lacking the scTCR, did not inhibit the level of IL-2 produced by DO11.10 or gD12 T hybridoma cells after stimulation with their respective ligands (Fig. 4A and B). The DO11.10 scTCR-bearing phage inhibited IL-2 production by stimulated DO11.10 T hybridoma cells to a much greater degree than they inhibited IL-2 production by stimulated gD12 T hybridoma cells (Fig. 4A and B). At least 40-fold more DO11.10 scTCR-bearing phage were required to achieve a significant inhibition of the gD12 T hybridoma cells. The detectable level of inhibition by DO11.10 scTCR-bearing phage on IL-2 production by the gD12 T hybridoma cells may be due to the low, but significant affinity of the DO11.10 TCR for IA^d with irrelevant peptide (HSV); however, this affinity is much less compared to IA^d with its specific peptide (OVA 323–339). For example, Weber et al. (1992) reported that a soluble TCR which recognizes a haemagglutonin peptide of influenza virus in the context of MHC class II molecule IE^d can inhibit IL-2 production by a T cell hybridoma that binds the same MHC but has different peptide specificity. This may imply that TCR has an intrinsically low affinity for the MHC molecule in the absence of the specific peptide.

To demonstrate the scTCR-bearing phage inhibits the IL-2 protein production by the induced DO11.0 T hybridoma cells through blocking the TCR-MHC/peptide interaction, we directly stimulated these hybridoma cells with immobilized anti-CD3ε mAb (Leo et al., 1987). No inhibitory effect by the phage on the T hybridoma cells' ability to produce IL-2 was observed (Fig. 4C) supporting the claim that inhibition by the DO11.10 TCR bearing-phage is specific and likely occurs through blocking TCR interaction with MHC/peptide ligand. Collectively, these results indicate the DO11.10 scTCR fusion, when displayed on the surface of bacteriophage, markedly inhibits the TCR interaction with its MHC/peptide ligand.

3.6. TCR binding specificity for cognate MHC/peptide

Flow cytometry was used as a direct binding method to confirm that the phage-displayed TCRs retain their peptide recognition specificity. In these studies, the NSO, NDO.B5, and CH12 cell lines were utilized. The NDO.B5 cell line has been engineered to express and display IA^d molecules covalently linked with a peptide spanning the amino acid residues 323 to 339 of chicken ovalbumin on its cell surface. This novel peptide linked molecule on the NDO.B5 cell surface has been shown to stimulate DO11.10 T hybridoma cells to produce IL-2. Unpublished studies by A. Edwards and S. Grammer also demonstrated that the NDO.B5 cell line displays slightly higher levels of IA^d molecules on its surface than NSO cells.

When DO11.10 TCR-bearing phage were incubated with NDO.B5 cells and phage binding to the surface of these cells was measured using immunofluorescent staining and flow cytometry, we observed strong binding between the TCR-bearing phage and NDO.B5 cells (Fig. 5A). In contrast, we did not observe binding between the DO11.10 TCR-bearing phage and the NSO cells using identical methods (Fig. 5A). This suggested that the DO11.10 TCR displayed on the phage surface can distinguish the IA^d molecules based on their bound peptide supporting the notion that the DO11.10 TCR displayed on the bacteriophage surface is biologically functional. In addition, we found that the phage displaying the DO11.10 TCR exhibited strong IA^d restriction. In the flow cytometric analysis, we observed that the DO11.10 TCR-bearing phage did not bind to the B lymphoma cell line, CH12, known to express and display high levels of IE^k molecules on its cell surface (data not shown).



3.7. p3 vs. p8 TCR fusion proteins

In general, the affinity of the TCR for its MHC/peptide ligand is low (on the order of 10^5 to 10^7 M^{-1}) (Alberti, 1996; Lyons et al., 1996; Schlueter et al., 1996). In the case of the DO11.10 TCR the affinity for IA^d/OVA is 3.0×10^5 M^{-1} (Seibe et al., 1997). Such a low affinity between the receptor and its ligand could impose difficulty in using phage-display and panning methods to isolate antigen-specific TCRs from randomly created libraries. Similar challenges have been encountered by other laboratories in attempting to isolate antibody fragments from naive antibody libraries with affinity constants in the range of 10^4 M^{-1} to 1×10^7 M^{-1} (Clackson et al., 1991; Marks et al., 1991; Gram et al., 1992). In one case, Gram et al. (1992) overcame this difficulty by increasing the valency or number of TCR copies displayed on the bacteriophage surface. To examine whether a similar approach could be used to increase the level of binding of TCR-bearing phage to cognate MHC/peptide on the cell surface, we displayed the DO11.10 TCR either as a p3 or p8 fusion protein. It has previously been shown that generally one copy of a p3 fusion protein is displayed on the surface of bacteriophage particles when phagemid vectors and helper phage are used, whereas considerably more copies of the same protein are expressed when p8 fusion proteins are displayed (Marks et al., 1992). The flow cytometric study was carried out using phage preparations, normalized for TCR signal, that displayed either the DO11.10 TCR/p8 fusion protein or DO11.10 TCR/p3 fusion protein. As shown in Fig. 5B, some improvement in binding was observed with phage displaying the TCR as a p8 fusion protein (mean channel fluorescence (MCF 57)) compared to phage displaying the TCR as a p3 fusion protein (MCF 35). Therefore, the

Fig. 5. Direct binding of DO11.10 TCR-bearing phage on the surface of cells. FACS analysis of 8×10^9 DO11.10 TCR/p8-bearing phage binding to either (A) NSO or NDO.B5 cells; and (B) comparative binding of 10^{11} phage particles displaying either the DO11.10 TCR/p3 (hatched line; MCF = 35) or DO11.10 TCR/p8 (bold solid line; MCF = 57) to NDO.B5 cells. In (A) and (B) the thin solid line represents NSO and NDO.B5 cells stained with antibodies alone (anti-M13 polyclonal and anti-sheep IgG-FITC).

use of the TCR/p8 fusion protein design in phage-display may represent an important technical advantage when carrying out future panning experiments against cognate MHC/peptide.

3.8. Display of an anti-p53 TCR

To demonstrate the generality of our method to display functional TCR on bacteriophage, we expressed another TCR molecule with different specificity on the surface of bacteriophage. In this case, the scTCR (Vα2.1; Vβ11.0/Cβ) recognizes a processed peptide fragment from human wild-type p53 tumor suppressor antigen presented in the context of human HLA-A.2 (Theobald et al., 1995). Fig. 6 shows that the displayed scTCR was detectable using conformation-sensitive mAbs to Vα2.0 and Vβ11.0 in a sandwich ELISA. To detect a signal of comparable intensity to the DO11.10 TCR-bearing phage, larger numbers of phage particles carrying the anti-p53 TCR (generally two to three orders of magnitude higher) were necessary in the ELISA assay. The need for so many more anti-p53 TCR-bearing phage particles in the ELISA assay may be attributed to lower expression of anti-p53 scTCR in *E. coli* compared to that of DO11.10 scTCR. We observed a significantly lower expression level (25–50-fold less) of soluble anti-p53 TCR when compared to the expression of soluble DO11.10 TCR (data not shown). The expression differences observed between the two molecules is not unique to TCRs as numerous reports have described variability of expression of single-chain antibody fragments in *E. coli* (Makrides, 1996; Bothmann and Pluckthun, 1998). It is possible that the different intensity observed in the phage ELISA assays is also due to differences in binding affinities of the mAbs used for DO11.10 and p53 TCR detection. Regardless, our demonstration that a second scTCR molecule can be displayed on the surface of bacteriophage indicates the potential for broad application of phage-display to other TCR molecules.

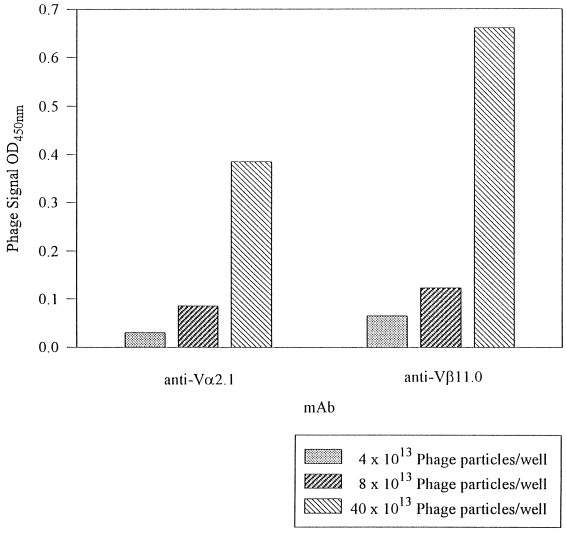


Fig. 6. Enzyme-linked immunoassay of phage displaying the anti-p53 TCR. Phage expressing the anti-p53 TCR on their surface were captured using immobilized mAb anti-Vα2.0 or anti-Vβ11.0. Control antibody F23.1, specific for Vβ8.2, was used to measure non-specific binding of the anti-p53 TCR-bearing phage and has been subtracted from the specific signal.

4. Discussion

The successful development of a TCR phage-display system is dependent on the ability to produce soluble scTCR protein in bacteria. Expression of soluble scTCR has not been an easy hurdle to overcome as demonstrated by the large number of reports describing the expression of only insoluble scTCR in *E. coli* (Novotny et al., 1991; Soo Hoo et al., 1992). Although we do not fully understand the variables which are responsible for the production of soluble TCR in *E. coli*, we believe the design of a three-domain (Vα–VβCβ) scTCR contributes significantly to the expression of soluble scTCR. For example, ongoing studies in our lab have explored the level of soluble protein expressed by variant forms of scTCR and scTCR fusion proteins, including two and three-domain (Vα–Vβ) scTCRs. We have observed the presence of soluble three-domain scTCR protein in *E. coli*; however, in contrast, the two-domain scTCR protein was expressed but only as an insoluble product suggesting the addition of the Cβ region may contribute to the production of soluble scTCR molecules. Our findings are supported by earlier studies of TCR expression in mammalian cells. In the study by Chung et al. (1994), proper assembly of variable domains of a human TCR that recognizes the HLA-DR2β/myelin basic protein (85–99) pep-

tide complex was shown to be critically dependent on the addition of the Cβ domain to the single-chain construct. In the same study, they also found that single-chain molecules with the three-domain design, but not with the two-domain design, were expressed in an eukaryotic cell as chimeric molecules linked either to glycosyl phosphatidylinositol or CD3 zeta chain. Additional evidence supporting the direct influence of the Cβ domain on Vβ folding comes from KJ25 mAb binding studies carried out using recombinantly produced β-chain variants. The KJ25 mAb is a conformation-sensitive monoclonal Ab that recognizes an epitope on the Vβ3.0 domain of the native β-chain (Li et al., 1996). Ab binding results revealed a loss of epitope recognition for the Vβ3.0 domain when expressed alone whereas the Ab strongly recognized the epitope on Vβ3.0 when the Cβ domain was included to yield a Vβ3.0/Cβ fragment. These findings indicate that close contact or interaction with the Cβ domain is a requisite for proper folding of the Vβ domain (Li et al., 1996).

The apparent requirement of the β-constant domain for cooperative folding of the two variable domains may reflect structural differences between TCR and Ab molecules. Chain shuffling experiments carried out to investigate if the variable and constant domains of the α/β TCR form independent domains have revealed a high level of domain dependence. In the study by Casorati et al. (1993), variable and constant chain shuffling was performed whereby constant domain α was shuffled to the C-terminal end of the Vβ domain and the constant domain β was shuffled to the C-terminal end of the Vα domain. The shuffling resulted in a lack of TCR dimer expression on the surface of the T cell hybridomas. This would imply that domain dependence is indispensable and eliminating the interaction probably results in the improper folding of the TCR molecule. This finding is in contrast to studies carried out with immunoglobulin domains involving variable and constant chain shuffling which showed little if any bearing on the secretion level of the Ab molecule (Simon and Rajewsky, 1990). It may be due to the fact that the TCR molecule has a more compact structure with greater interaction occurring between domains than has been reported for immunoglobulins. A likely explanation for our observations, as well as the observations of others, could be that the Cβ domain acts as a scaffold for Vβ domain folding resulting in a conformation which enhances its solubility. Structural data supporting this explanation comes from recent TCR crystallography studies (Garboczi et al., 1996; Garcia et al., 1996) that describe a strong interaction between the Vβ and Cβ domains with the greatest interaction occurring between the N-terminus or elbow region of Cβ and the C-terminus of the Vβ domain (Bentley et al., 1995). Results from both structural and molecular engineering studies suggest expression of soluble and functional TCR molecules is dependent on including the Cβ domain.

Fusion of the scTCR gene to the p8 gene may be another important factor for producing soluble scTCR protein in bacteria cells. The p8 protein of bacteriophage has been characterized (Nakashima et al., 1981) and shown to contain three distinct domains of approximately 20 amino acids in length. These domains include an N-terminal region comprised primarily of negatively charged amino acid residues, a hydrophobic central region responsible for insertion of the p8 protein into the periplasmic membrane and a C-terminal region with positively charged amino acid residues. In our scTCR design described here, the N-terminus of the p8 protein is fused to the C-terminus of the scTCR. Inclusion of the p8 protein as a fusion may serve to facilitate the transport of the scTCR to the periplasmic space where folding generally occurs under more favorable conditions (Makrides, 1996). By comparing expression levels of soluble scTCR to scTCR/p8 fusion protein produced in *E. coli*, we observed that the fusion protein was expressed at least two-fold higher than the non-fusion scTCR indicating the p8 protein may actually increase production of soluble scTCR by possibly improving the transport efficiency to the periplasmic space. Moreover, bacterial chaperons which normally promote folding and transport of the p8 protein may facilitate the TCR portion of the p8 fusion molecule to obtain its native conformation.

In this study, we have demonstrated that functional TCRs can be displayed on the surface of the *E. coli* filamentous bacteriophage fd. This may lead to future applications for TCR phage-display including development of an in vitro panning method to rapidly isolate antigen-specific TCRs from gene libraries derived from human or animal sources. The

weak interaction between the TCR and its ligand may cause difficulty implementing the phage-display and in vitro panning method for isolation of antigen-specific TCRs. To address this concern, we have attempted to increase the avidity of TCR binding by creating TCR/p8 fusions. This strategy has been successfully employed to isolate low affinity antibody fragments from naive antibody libraries (Gram et al., 1992). The enhanced fluorescence observed with the p8 fusion compared to the p3 fusion in our cell staining assays suggests that this simple maneuver might also increase the feasibility of using the phage-display/in vitro panning system for TCR isolation. Furthermore, using cell lines engineered to over-express peptide/MHC complexes on their surface or using soluble peptide/MHC molecules engineered as tetramers instead of monomers may also lead to improvement in the binding avidity between the TCR and its ligand. The supra binding affinity of tetrameric peptide/MHC molecules has been proven as the method of choice in T cell characterization using flow cytometry (Altman et al., 1996; Crawford et al., 1998). The preparation of peptide/MHC reagents coupled with our design for displaying scTCR on phage may hold the key to successful panning of TCR libraries.

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