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Immunology

A soluble, single-chain T-cell receptor fragment endowed with antigen-combining properties

(protein engineering/immunoglobulin domains/variable regions/*Escherichia coli* expression)

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ABSTRACT **A strategy for the production of small, soluble, single-chain T-cell receptor (scTCR) fragments that carry an intact TCR antigen-combining site is presented. The rationale is based on structural similarity between TCR and antibody molecules and use of computer modeling methods to derive a model structure of a human scTCR variable (V)-domain dimer. A gene encoding the RFL3.8 TCR protein, specific for the hapten fluorescein in the context of major histocompatibility complex class II and composed of one V_α and one V_β domain joined via a flexible peptide linker, was assembled in an *Escherichia coli* plasmid. Subsequently, the protein was produced in a bacterial expression system, purified, refolded, and found to be poorly soluble at neutral pH in aqueous buffers. An inspection of the computer-generated V_α–V_β domain model showed several surface exposed hydrophobic residues. When these were replaced by water-soluble side chains via site-directed mutagenesis of the corresponding gene, a soluble protein resulted and was shown to have antigen-binding properties equivalent to those of the intact TCR of the RFL3.8 T-cell clone. These results demonstrate the feasibility of obtaining TCR fragments endowed with antigen-combining properties by protein engineering in *E. coli*.**

The T-cell receptor (TCR) is a polypeptide multi-chain complex that mediates functional recognition of antigen in the context of a particular major histocompatibility complex (MHC) protein (1, 2). The clonally unique antigen/MHC-binding moiety, termed Ti, is a disulfide-linked heterodimer consisting of one α and one β subunit. Both subunits have each been thought to be immunoglobulin-like, folding into variable (V) and constant domains (3). As with immunoglobulin, V_α- and V_β-domain polypeptide chains contain hypervariable regions analogous to those found in antibody complementary-determining regions (4, 5). Structural analyses have elaborated multiple points of similarity between antibodies and TCRs (6–8). In immunoglobulins, the antigen-combining site arises by noncovalent association of the V domains of the heavy (V_H) and the light (V_L) chains. The V_H–V_L interface forms a close-packed, twisted β-barrel, the geometry of which is preserved via invariance of some 15 side chains (9). Most of these side chains are conserved in TCR sequences as well, indicating that the antigen-combining sites of the TCRs and of antibodies are similar in their overall dimensions and geometry. Sequence profiles of hydrophobicity, secondary structure propensity, and charge distribution also supported the notion that the antiparallel β-sandwich topology of the antibody V_H and V_L domains is present in the TCR V_α and V_β domains (6).

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Because of its tight association with the cell membrane, molecular attributes of the TCR antigenic function (e.g., antigenic specificity, affinity, size, and nature of the antigen combining site, etc.) could only be studied indirectly. Clearly, our understanding of cellular immunity would be greatly enhanced if TCR–antigen interactions could be studied in solution, akin to the methodology used to study antigen–antibody reactions. We report here a method for producing soluble TCRs with antigen-binding properties based on a single-chain construct.

MATERIALS AND METHODS

Methods of Computation. All calculations were done on a Silicon Graphics IRIS workstation 4D/80 running the CONGEN (10) and the Biosym Technologies INSIGHT programs under the UNIX operating system. Cartesian atomic coordinates of the McPC 603 V_H and V_L domains were obtained from Brookhaven Protein Data Bank (11). An explicit hydrogen representation of protein structures was used, with all polar hydrogen atoms explicitly constructed. The CONGEN model of the single-chain TCR (scTCR) was constructed in five stages with the McPC 603 atomic coordinates serving as the point of departure. (*i*) The β-sheeted framework of the RFL3.8 $V_\beta V_\alpha$ domain model was obtained by copying directly atomic coordinates of the backbone and the conserved side chains from the corresponding parts of the McPC 603 V_H and V_L domains, respectively. (*ii*) Three short framework segments, residues 6–9, 70–73, and 199–203 (Fig. 1), showing insertions/deletions between the RFL3.8 and McPC 603 structures, were generated by using the uniform conformational search. (*iii*) All the remaining framework side chains were constructed using an iterative search procedure on all the side-chain torsional degrees of freedom (12). (*iv*) The backbones of the six hypervariable loops comprising the RFL3.8 antigen-combining site (loops defined as shown in Fig. 1) were successively constructed one after another with computer modeling essentially following the construction protocol developed by Bruccoleri *et al.* (13). In a loop, all the torsional degrees of freedom were sampled in the N to C direction on an angular grid of 30°, and both cis and trans proline peptide bonds were considered. All conformations that satisfied the fixed endpoint condition (the modified Go and Scheraga algorithm) (14) and had acceptable potential energies were retained. The "best conformation" was incor-

Abbreviations: TCR, T-cell receptor; scTCR, single-chain TCR; V, variable; V_H and V_L, V region heavy and light chains; MHC, major histocompatibility complex; Fl, fluorescein; IPTG, isopropyl β-D-thiogalactopyranoside; NP-40, Nonidet P-40; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; AP-Fl, 5-[(5-aminopentyl) thioureidyl]fluorescein; FITC, fluorescein 5-isothiocyanate; RITC, rhodamine B isothiocyanate; EITC, eosin 5-isothiocyanate; CNF, 5-(and 6-)carboxynaphthofluorescein, succinimidyl ester.

porated into the final model as described (13). (*v*) Due to their excessive length, the β3 hypervariable loop and the linker (residues 112–134) could not be searched completely, and results of partial searches were incorporated into the model. In the β3 run, 19,842 conformations were generated in ≈7 days of central processing unit time; the lowest energy conformation of this incomplete collection was included in the model. In the linker run, only narrow ranges of the helical and extended, β-like regions of the conformational space were explored. In this run, 6464 conformations were generated in ≈17 h of central processing unit time, and the lowest energy conformation was used.

Cloning of Fluorescein (Fl)-Specific TCR α- and β-Chain cDNAs and Construction of scTCR Expression Vector. Ti α and Ti β cDNAs were isolated from a λgt10 library prepared from RFL3.8 as described (15). Standard PCR and cloning techniques were used to excise the V domains and a synthetic oligonucleotide "linker" was cloned between the C terminus of V_β and the N terminus of V_α (Fig. 1). The resulting scTCR was subcloned into the pSS1 secretion vector. pSS1 was created by excising the polylinker of pBluescript II SK⁻ (*Sac* I/*Kpn* I) and replacing this region with the pelB leader sequence (with its ribosome binding site) and a new polylinker. The pelB leader was obtained by PCR using pSW1-VHpolyTagQ (16) as template. The new polylinker was constructed from two overlapping synthetic oligonucleotides (Fig. 1). The resultant expression vector (pSS1/Fl-scTCR) encodes the pelB leader followed immediately in-frame by V_β, the linker, and then V_α segments. The expression of this construct is under the control of the lacZ promoter and is therefore isopropyl β-D-thiogalactopyranoside (IPTG) inducible. pSS1 based plasmids were maintained in a derivative of strain XL1-Blue (Stratagene), which contains the plasmid pRG1 (Robert Garcea, Dana–Farber Cancer Institute). The latter constitutively expresses lacIQ, a repressor of the lac promoter. pSS1/Fl-scTCR2 was created via site-directed mutagenesis using the Amersham kit as directed by the manufacturer.

Induction, Purification, and Refolding of scTCR. To induce the expression of scTCRs, a 165-ml overnight culture of XL1-Blue/pRG1 containing the pSS1/Fl-scTCR vector grown at 37°C in Luria broth containing kanamycin (30 μg/ml), ampicillin (50 μg/ml), and tetracycline (12.5 μg/ml) was diluted to 1000 ml with the same medium containing 5 mM IPTG. After 7 h, the cell pellet was resuspended in 50 mM Tris·HCl, pH 8.5/5 mM EDTA/lysozyme (0.3 mg/ml)/1 mM phenylmethylsulfonyl fluoride (PMSF), and incubated on ice for 2 h. Nonidet P-40 (NP-40) was then added to 0.75% and NaCl was added to 0.35 M. The suspension was sonicated (Branson 250 sonifier) and centrifuged at 10,000 × *g* for 20 min. The pellet was resuspended in high salt wash buffer (1.0 M NaCl/10 mM Tris·HCl, pH 8.5/0.5% NP-40/1 mM PMSF/1 mM EDTA) and repelleted. The process was repeated followed by two washes with low salt wash buffer (10 mM Tris·HCl, pH 8.5/0.5% NP-40/1 mM PMSF/1 mM EDTA). Finally, the pellet was resuspended in 5 ml of solubilization buffer [20 mM Tris·HCl, pH 8.0/50 mM dithiothreitol (DTT)/1 mM PMSF/8 M urea], centrifuged for 15 min at 10,000 × *g*, and the supernate was collected. The urea solubilized Fl-scTCR was subjected to further purification by HPLC on a Vydac C4 column (0.1% trifluoroacetic acid/acetonitrile at a flow rate of 1 ml/min). Fractions containing the scTCR were combined and resuspended in 8 M urea (≈3 mg/ml), 10 mM Tris·HCl (pH 8.0), and 20 mM DTT. The scTCR was then refolded by rapidly diluting the sample 1:100 into 20 mM sodium acetate (pH 5.6) containing 5 mM reduced and 0.5 mM oxidized glutathione. After 3 h, the solution was dialyzed against 10 mM sodium acetate (pH 5.6). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (0.1%; Pierce) was added and the protein was concentrated to ≈1 mg/ml in Centricon 10 microconcentrators (Amicon). For solubility studies, aliquots containing purified scTCR in 8 M urea (3 mg/ml) were diluted to 30 μg/ml in 20 mM sodium acetate (pH 5.0), 20 mM sodium phosphate (pH 7.0), PBS (10 mM sodium phosphate, pH 7.4/150 mM NaCl), or 20 mM Tris·HCl (pH 8.0). Samples were incubated at room temperature for 2 h and microcentrifuged for 10 min, and the supernatants were analyzed by reducing SDS/PAGE.

Preparation of Dye-Conjugated Sepharose Affinity Resins and Dye-Binding Studies. CNBr-activated Sepharose 4B (Pharmacia) was coupled to fluorescein 5-isothiocyanate (FITC) (Molecular Probes), rhodamine B isothiocyanate (RITC) (Aldrich), eosin 5-isothiocyanate (EITC) (Molecular Probes), or 5-(and 6-)carboxynaphthofluorescein, succinimidyl ester (CNF) (Molecular Probes) as described (17). Dye-conjugated Sepharose beads (100 μl) were washed with PBS in microcentrifuge tubes. PBS (1 ml) containing 10% fetal calf serum was added, and the tubes were wrapped in aluminum foil and rotated at 4°C overnight. After resuspending in 200 μl of the same buffer containing 20 μl of ≈20 μg of refolded, concentrated Fl-scTCR2, the samples were wrapped in aluminum foil and rotated for 4 h at room temperature. The beads were then rapidly washed and the Fl-scTCR2 was eluted by resuspending the beads in 50 μl of PBS containing various concentrations of 5-[(5-aminopentyl)thioureidyl]fluorescein (AP-Fl) (Molecular Probes). The eluates were analyzed by SDS/PAGE and Western blotting using affinity-purified anti-Fl-scTCR. Polyclonal antisera against the scTCR were raised by immunizing rabbits with purified Fl-scTCR, and specific antibodies were purified by affinity chromatography using Affi-Gel 10 beads (Pierce) coupled with purified Fl-scTCR.

Large Scale Purification and Refolding. The Fl-scTCR2 was purified by S-Sepharose chromatography in 6 M urea followed by reverse-phase HPLC using a Vydac C4 column in 0.1% trifluoroacetic acid/acetonitrile. Pure Fl-scTCR2 was dialyzed versus 2 M urea/50 mM Mes/5 mM EDTA/0.05% Tween-80/0.1 mM DTT, pH 6.5, and refolded in the presence of 5 μM thioredoxin for 24 h at 4°C. The refolded Fl-scTCR2 was then dialyzed in step fashion to remove urea in the same buffer without DTT. Epoxy-activated Sepharose 6B was coupled to AP-Fl according to the manufacturer's directions. AP-Fl-conjugated Sepharose was equilibrated in 50 mM Mes/5 mM EDTA/0.2 mM PMSF/0.05% Tween 80, pH 6.5. Fl-scTCR (10 mg) was applied to the column and recirculated for 48 h. The column was washed with equilibration buffer, followed by AP-rhodamine (100 μM) in equilibration buffer. The Fl-scTCR2 was specifically eluted with 200 μM AP-Fl.

RESULTS AND DISCUSSION

Homology Modeling of TCR $V_\beta V_\alpha$ Fragment Based on the Structure of Antibody V-Region Fragments ($V_H V_L$ Dimer). Amino acid sequences of the Fl-specific, MHC class II-restricted human TCR α and β subunits from the RFL3.8 $CD4^+$ T-cell clone (15, 17) were aligned with sequences of immunoglobulin chains of known three-dimensional structure (Fig. 1*A*). Structural similarity of the TCR V domains with the aligned immunoglobulin domains was 29 identities between V_β and V_H and 38 identities between V_α and V_L. When occurrences of similar residues were considered as identities, the alignments displayed a homology of close to 40%. As expected, the key residues known to be important for the stability and conservation of the immunoglobulin fold (i.e., Cys-23, -91, -157, and -224; Gln-6 and -140; Trp-34 and -169; Arg-68 and -195; Asp-218; Leu-21; Tyr-222; Phe/Trp-102 and -236; Gly-103, -105, -237, and -239; see ref. 9) were present in the RFL3.8 V_β and V_α sequences as well. Similarly, the side chains known to be important for mediating the tight noncovalent interaction between the two V domains

A

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                         5                   10                  15                  20                  25
RFL3.8 b asn ala gly val thr gln thr pro lys[phe]arg val leu lys thr GLY gln SER met thr LEU leu CYS ALA gln asp met asn  -   -
M603 H   glu val lys leu val glu  -  ser gly gly gly leu val gln pro GLY gly SER leu arg LEU ser CYS ALA thr ser gly phe thr phe

             ******************* A *********************                         ***************** B *****************
                                                                                                            ++++++ b1 +++++

                 30                  35                  40                  45                  50
RFL3.8 b  -  his glu TYR MET tyr TRP tyr ARG GLN asp PRO GLY[met]gly LEU arg leu ILE his tyr SER val gly glu GLY  -   -   -
M603 H   ser asp phe TYR MET glu TRP val ARG GLN pro PRO GLY lys arg LEU glu trp ILE ala ala SER arg asn lys GLY asn lys tyr

                     ********** C **************                ********************* C' *********************
             ++ b1 +                                                                                  ++++++ b2 +++++

             55                      60                  65                  70                      75
RFL3.8 b  -  THR thr ala lys gly glu VAL pro asp gly tyr asn VAL SER ARG leu lys lys  -  gln asn phe leu LEU gly leu glu
M603 H   thr THR glu tyr ser ala ser VAL lys gly arg phe ile VAL SER ARG asp thr ser gln ser ile leu tyr LEU gln met asn

                                                     ************ D ****************                  *********** E *********
             ++ b2 +

         80                  85                  90                  95                  100
RFL3.8 b ser ala ala pro ser gln THR ser val TYR phe CYS ALA ser arg thr ala thr gln pro gln his  -   -   -  phe GLY asp
M603 H   ala leu arg ala glu asp THR ala ile TYR tyr CYS ALA arg asn tyr tyr gly ser thr trp tyr phe asp val trp GLY ala

         ** E **                     ************ F ****************                                  ******** G ********
                                                                 +++++++++++ b3 ++++++++++++++

         105                 110                 115                 120                 125                 130
RFL3.8 b GLY THR arg leu ser ile[leu]pro gly gly gly gly ser gly gly gly gly ser gly gly gly gly ser gly gly gly gly ser gly ala
M603 H   GLY THR thr val thr val ser glu asn gly

         ** G **
                                     ++++++++++++++++++++++++++++++++++++++++ Linker ++++++++++++++++++++++++++++++++++++++++

         135                 140                 145                 150                 155                 160
RFL3.8 a gln gln gln val lys GLN SER PRO gln SER LEU[ile]VAL gln lys GLY gly[ile]ser ile ile asn CYS ala tyr glu asn thr ala phe
M603 L   asp ile val met thr GLN SER PRO ser SER LEU ser VAL ser ala GLY glu arg val thr met ser CYS lys ser ser gln ser leu leu

             ******************* A *********************                             ********* B ***********
                                                                                                         ++++++++ a1 ++++++

                                 165                 170                 175                 180                 185
RFL3.8 a  -   -   -   -   -   -  asp tyr phe pro TRP TYR GLN GLN phe PRO GLY lys gly PRO ala LEU LEU ILE ala ile arg pro asp
M603 L   asn ser gly asn gln lys asn phe leu ala TRP TYR GLN GLN lys PRO GLY gln pro PRO lys LEU LEU ILE tyr gly ala ser thr

                                             ****** C **********                     ************* C' **************
                                 ++ a1 +                                                                 +++++ a2 ++++++

                 190                 195                 200                 205                 210                 215
RFL3.8 a val ser glu lys lys glu gly ARG PHE THR ile SER phe asn lys SER ala lys gln PHE ser LEU his ILE met asp ser GLN pro gly
M603 L   arg glu ser gly val pro asp ARG PHE THR gly SER gly  -   -  SER gly thr asp PHE thr LEU thr ILE ser ser val gln ala glu

                                         ******* D *********                             ******** E ********

                 220                 225                 230                 235                 240
RFL3.8 a ASP ser ALA thr TYR phe CYS ala ala ser phe SER gly asn thr pro LEU val PHE GLY lys GLY THR arg LEU ser val ile
M603 L   ASP leu ALA val TYR tyr CYS gln asn asp his SER tyr pro  -   -  LEU thr PHE GLY ala GLY THR lys LEU glu ile lys
                     ******* F ********                                              ************** G **************
                                             +++++++++++ a3 ++++++++++
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B

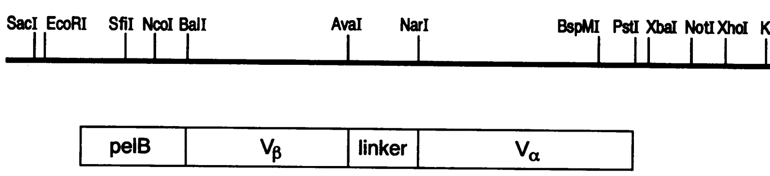

FIG. 1. Fl-scTCR. (A) Alignment of Fl-scTCR and McPC 603. Amino acid sequence of the human anti-Fl clone RFL3.8 is aligned with that of the mouse immunoglobulin McPC 603. The RFL3.8 α- and β-chain V domains are labeled a and b, respectively, and the residues conserved between TCR and immunoglobulin sequences are capitalized. Residue numbering is that of the single-chain RFL3.8 $V_\alpha V_\beta$ fragment. Asterisks denote positions of β strands in the McPC 603 three-dimensional structure. In each domain, the strands are labeled A–G. The hypervariable loops (b1 through a3) of the RFL3.8 sequence, constructed by CONGEN conformational searches (cf. Fig. 2), are marked by + signs under the sequence. The hydrophobic residues on the surface of the RFL3.8 that were mutated in order to increase solubility of the single-chain RFL3.8 $V_\alpha V_\beta$ (Fig. 3) are boxed. (B) Restriction map of scTCR cDNA insert in pSS1 polylinker and schematic of scTCR protein.

(Gln-37 and -171; Phe/Tyr-90, -170, and -222) have been conserved in the RFL3.8 sequences. These sets of conserved residues served as anchor points in delimiting the extent of the β-sheet framework as depicted in Fig. 1A.

The basic premise of our computer modeling was the essential conservation of the immunoglobulin fold in the TCR V domains, which are expected to represent self-standing, independent folding units made of seven or eight antiparallel β-strands stabilized by disulfide bonds formed between a pair of invariant cysteine residues. It was previously established that the average length between the C terminus of immunoglobulin V_H and the N terminus of immunoglobulin V_L is $\approx$3 nm and that this distance could be comfortably spanned by a 15- to 20-residue-long polypeptide chain (18). Furthermore, it was found that linkers connecting the C terminus of the V_H domain to the N terminus of the V_L domain (18) or vice versa (19) were equally capable of giving rise to antigen-binding single-chain V region fragments. In the present work, an eicosapeptide linker consisting of four repeats of the motif Gly-Gly-Gly-Gly-Ser has been introduced in between the C terminus of the V_β domain and the N terminus of the V_α domain. Three more residues—namely, Pro at the N terminus of the linker and Gly-Ala at the C terminus of the linker—were included to facilitate gene cloning. The CONGEN model of this TCR fragment was constructed as described in *Materials and Methods*. Fig. 2 demonstrates the approximate

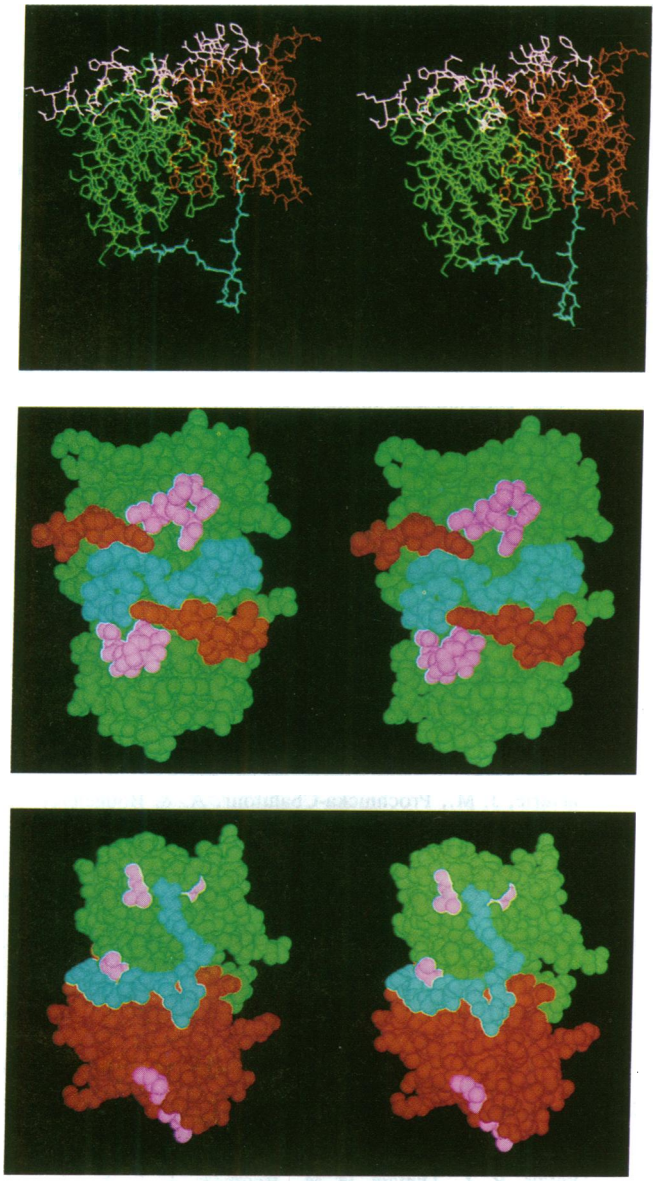

FIG. 2. scTCR model. (*A*) Stereoscopic picture of the Fl-scTCR2, stick representation. Color coding is as follows: green, V_β domain; blue, linker; red, V_α domain; magenta, hypervariable loops. (*B*) Stereoscopic, space-filling picture of Fl-scTCR2 antigen-combining site. V_β domain is on the bottom; V_α domain is on the top. Amino acid residues color coded in red constitute the β1 and α1 hypervariable loops, respectively (cf. Fig. 1), those in magenta correspond to the β2 and α2 loops, and those in light blue correspond to the β3 and α3 loops, respectively. (*C*) Stereoscopic, space-filling picture of the distal, "bottom" part of Fl-scTCR2, showing the linker and the hydrophobic solvent-exposed residues that were replaced by site-directed mutagenesis with polar side chains. In this orientation of the molecule, the antigen-combining site is not visible, being on the back part of the molecule. V_β domain (green) is at the top; V_α domain (red) is at the bottom. Linker is color-coded blue, and surface-exposed hydrophobic side chains are magenta.

twofold symmetry of our model and the fact that the linker is distal to the complementarity-determining regions.

Expression, Purification, and Refolding of scTCR. To produce a Fl-binding scTCR protein in *E. coli*, the Fl-scTCR1 cDNA sequence was constructed and subcloned into the pSS1 expression vector at *Bal* I and *Pst* I sites (Fig. 1*B*). In this vector, the pectate lyase B (pelB) leader of *Erwinia carotovora* is fused in-frame upstream of the Fl-scTCR1 cDNA and should direct scTCR accumulation to the periplasm. A specific band of ≈30 kDa was detected in total cell lysates of several *E. coli* strains such as XL1-blue/pRG1 cells upon IPTG induction. Although the apparent mobility of the Fl-scTCR1 was slightly larger than that predicted (M_r, 25,896), its identity was confirmed by amino acid sequence analysis from a poly(vinylidene difluoride) blotted band (data not shown). The latter analysis showed that the pelB leader sequence had been cleaved from the Fl-scTCR1 and that cleavage occurred at the predicted N terminus of the scTCR. However, cell fractionation experiments failed to detect scTCR in the periplasm. After cell lysis, Fl-scTCR1 fractionated with the insoluble pellet but could be solubilized using strong denaturants and purified by reverse-phase HPLC (Fig. 3). The protein was purified to >95% as judged by staining with Coomassie blue and N-terminal sequence analysis. Subsequent refolding of this material was as described in *Materials and Methods*. Fig. 3 shows that in addition to the Fl-scTCR1 species at 30 kDa, there are slower-migrating oligomers as well as faster-migrating monomer bands. The mobility of such monomers could result from formation of intrachain disulfide bonds between or within domains. Approximately 5–10 mg of Fl-scTCR is produced per liter of *E. coli* (1–2 mg of protein per g wet weight).

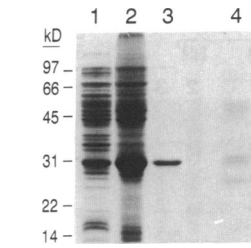

FIG. 3. Purification and SDS/PAGE analysis of *E. coli*-produced Fl-scTCR. Coomassie staining of total cell extract (lane 1), 8 M urea extract (lane 2), reverse-phase HPLC purified and renatured scTCR (lanes 3 and 4). Lanes 1–3 were run under reducing conditions; lane 4 was run under nonreducing conditions.

Modification of scTCR Solubility by Site-Directed Mutagenesis. The Fl-scTCR1 was poorly soluble in physiologic buffers (Fig. 4*A*). A detailed examination of amino acid alignments, atomic solvent accessibilities, and molecular surfaces of the CONGEN-constructed model disclosed 10 solvent-exposed positions distal from the antigen-combining site occupied by large hydrophobic residues: Phe-10, Leu-22, Met-41, Phe-74, Leu-111, Ile-146, Ile-152, Phe-173, Met-212, and Ile-245 (Fig. 1). In immunoglobulins, including McPC 603, analogous positions generally contain water-soluble residues. To improve on solubility of the Fl-scTCR, five of the above-mentioned residues were mutated to charged or polar residues—namely, Phe-10 to Ser, Met-41 to Lys, Leu-111 to Thr, Ile-146 to Ser, and Ile-152 to Arg (Fig. 2*C*). While the

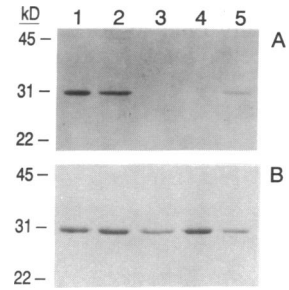

FIG. 4. Improved solubility of Fl-scTCR2 vs. Fl-scTCR1. Lane 1, total input; lanes 2–5, soluble material after 2 h of incubation in the following buffers: 20 mM sodium acetate (pH 5) (lane 2), PBS (pH 7.4) (lane 3), 20 mM Tris·HCl (pH 7) (lane 4), 20 mM Tris·HCl (pH 8) (lane 5). Staining was as in Fig. 3. (*A*) Fl-scTCR1. (*B*) Fl-scTCR2.

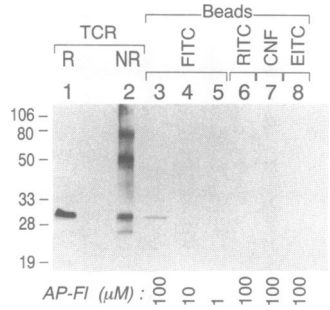

FIG. 5. Specific binding and elution of scTCR2 by AP-Fl from dye-conjugated Sepharose beads. SDS/PAGE analysis of eluates. The input, refolded Fl-scTCR2 is shown under reducing (R) (lane 1) and nonreducing (NR) (lane 2) conditions. Lanes 2–8, nonreducing analyses of AP-Fl eluates from beads conjugated with FITC (lanes 3–5), RITC (lane 6), CNF (lane 7), and EITC (lane 8).

expression in *E. coli* and fractionation characteristics of the mutated scTCR, termed Fl-scTCR2, appeared virtually identical to Fl-scTCR1 (data not shown), the resulting surface-modified protein was substantially more soluble than Fl-scTCR1 (Fig. 4*B*).

Fl-scTCR2 Specifically Binds to FITC-Sepharose Beads and Is Eluted by AP-Fl. The antigen-specific binding properties of Fl-scTCR2 were then tested at neutral pH in physiologic buffer. FITC, RITC, CNF, and EITC were conjugated to Sepharose as described. After 4 h of incubation with purified, refolded TCR protein (Fig. 5, lanes 1 and 2), specifically bound Fl-scTCR2 was eluted with AP-Fl. Subsequently, eluates were analyzed by nonreducing SDS/PAGE (Fig. 5). A single band at ≈29 kDa was eluted by 100 μM AP-Fl from FITC Sepharose but no band was eluted from the structurally related dyes RITC-, CNF-, or EITC-conjugated Sepharose beads. Although not shown, the 29-kDa species could not be eluted from FITC Sepharose by RITC, further supporting the specificity of the Fl-scTCR2 for FITC. Binding inhibition studies with free AP-Fl suggest that the K_d of Fl-scTCR2 for hapten is ≈50 μM (data not shown). When the specific binding experiment was repeated on a larger scale with 10 mg of refolded Fl-scTCR2 using an AP-Fl affinity column, ≈1.0 mg of the 29-kDa protein was eluted from the column with AP-Fl. These data indicate that 10% of the renatured receptor in this preparation binds antigen specifically and can be recovered from the affinity column by competitive elution with the free ligand.

The present studies demonstrate the feasibility of expressing a soluble, biologically active scTCR analogous to an immunoglobulin V-domain fragment in *E. coli*. Although the RFL3.8 human T-cell clone from which the TCR Ti α and β cDNAs were derived was shown to bind Fl in the context of class II MHC, the apparent MHC restriction could be overcome at high concentrations of Fl (17). Moreover, Fl polymers could specifically bind to RFL3.8 in the absence of antigen-presenting cells. Such binding presumably results from the structural rigidity of the hapten, which may not require the MHC binding pocket to lock into a configuration necessary for TCR recognition. The specificity and affinity of the scTCR binding to dyes is virtually identical to that of the transmembrane TCR of RFL3.8 expressed in Ti αβ cDNA transfectants of Jurkat (15, 17).

In the course of this work, we introduced nonconservative mutations into the TCR V-region framework at solvent-exposed positions predicted to be distal to the antigen-binding site without affecting antigenic specificity. These findings provide additional support for the predicted similarity of TCR and immunoglobulin V domains. Furthermore, the methodology of protein "redesign" described here may be exploited to solubilize other receptor molecules.

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