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Immunology

Characterization of a single-chain T-cell receptor expressed in *Escherichia coli*

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ABSTRACT **Despite progress in defining the nature of major histocompatibility complex products that are recognized by the T-cell antigen receptor, the binding properties and structure of the receptor have not been solved. The primary problem has been the difficulty in obtaining sufficient quantities of active receptor. In this report we show that a single-chain T-cell receptor gene can be expressed in *Escherichia coli*. The protein consists of the variable (V) regions of the α and β chains (V_α and V_β) encoded by the cytotoxic T-lymphocyte clone 2C (a H-2^b anti-H-2^d alloreactive cell line) linked by a 25-amino acid flexible peptide. Solubilized extracts that contain the 27-kDa $V_\alpha 3V_\beta 8$ protein are positive in solid-phase immunoassays with the anti-$V_\beta 8$ antibody KJ16 and the anti-clonotypic antibody 1B2. Approximately 1% of the protein can be specifically purified on a 1B2-conjugated column. These results indicate that a fraction of the protein is able to fold into a native conformation and that single-chain proteins should be useful not only as immunogens for eliciting anti-T-cell receptor antibodies but in the study of T-cell receptor structure and function.**

The $\alpha\beta$ T-cell antigen receptor (TCR) binds to a complex of peptide and major histocompatibility complex (MHC) product that is expressed on the surface of a presenting cell. Although unproven by direct means, it is thought that the receptor probably contacts both peptide and MHC determinants (1–3). Thus, there is an inherent difference in the functional properties of immunoglobulin and TCR despite the apparent similarities between the primary structures of these molecules (3, 4).

The tertiary structure of the TCR is unknown, but several models have been proposed over the years. Most recently, Davis and Bjorkman (2) and Chothia *et al.* (3) proposed that complementarity-determining regions 1 and 2 (CDR1 and CDR2) of the TCR binds to the two MHC helices, while CDR3 is primarily involved in peptide binding. In support of this model, several investigators have demonstrated the critical role that the CDR3 plays in antigen binding and fine specificity (5–7). The roles of CDR1 and CDR2 have yet to be studied in a similar fashion, but there are now examples of α-chain and β-chain variable (V)-region (V_α and V_β) expression correlating with the MHC product that is recognized (8–13). This may indicate that CDR1 and CDR2, which are encoded by the *V* gene, do in fact contact the MHC product.

A completely different approach has shown indirectly that the TCR interacts with self MHC molecules during positive selection in the thymus. Thus, T cells from animals that express V_α and V_β transgenes require binding to the appropriate MHC product to be exported to the periphery from the thymus (14–16). These MHC products have invariably been of the haplotype from which the donor T-cell clone was originally derived. Similarly, if a negatively selecting MHC product is present during thymic development, transgenic T cells are absent from the periphery (15, 17). Using anti-receptor antibodies to follow the fate of particular T-cell subsets, both positive and negative selection have also been shown to operate in nontransgenic animals (11, 18).

Although significant progress has been made toward understanding the general process of thymic selection, the molecular details concerning the interaction of TCR and MHC products are largely unknown. These studies will require expression of sufficient TCR and MHC product with bound peptide to perform binding studies and to attempt structural analyses. Several laboratories have now used mammalian expression systems to overproduce TCR domains (19–22). Chimeric genes that encode the TCR V and constant (C) regions together with a glycosyl phosphatidylinositol-linked membrane anchor have been expressed in mammalian cell culture (19, 21). The cell-surface molecules could be cleaved by a phospholipase and purified with appropriate anti-TCR antibodies. Recently, a chimeric gene composed of TCR *V* and *C* genes and the gene encoding the κ light chain C region (C_κ) has been expressed in secreted form (22). This secreted heterodimeric molecule could also be purified with anti-TCR antibodies.

Given the recent success in the expression of single-chain antibody (scAb; also called SCA) domains in *Escherichia coli* (23, 24) and the structural similarities between immunoglobulin and TCR, it seemed reasonable to pursue the expression of TCR domains in *E. coli*. For this purpose we chose to use the TCR expressed by the cytotoxic T-lymphocyte (CTL) clone 2C (25). Loh and his colleagues (15, 26) have shown that the 2C TCR recognizes the H-2K^b class I product during the process of positive selection and the H-2L^d class I product during the process of negative selection. In addition, there are both clonotypic (1B2) and β-chain V-region (V_β)-specific (KJ16) antibodies that recognize the native cell-surface TCR (27, 28).

We show here that a single-chain protein of the expected size (27 kDa) can be expressed in large quantities in *E. coli*. The refolded protein appears to be in a native conformation because it is both KJ16 and 1B2 reactive. This expression system has the potential to provide both the amounts and the size of soluble receptor that makes them amenable to analysis by NMR. In contrast to the mammalian systems, an *E. coli* genetic system will also provide a more convenient approach to site-directed mutagenesis and other structure–function studies.

MATERIALS AND METHODS

Cell Lines and Antibodies. Cytotoxic T lymphocyte (CTL) clone 2C, specific for the H-2L^d MHC product, was derived

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Abbreviations: TCR, T-cell antigen receptor; scTCR, single-chain TCR; scAb, single-chain antibody (also called SCA); V, variable; V_L, V light chain; V_H, V heavy chain; CDR, complementarity-determining region; C, constant; MHC, major histocompatibility complex; mAb, monoclonal antibody; CTL, cytotoxic T lymphocyte(s); HRP, horseradish peroxidase.

from a BALB.B mouse injected with the DBA/2 mastocytoma P815 ($H\text{-}2^d$) (25). Cell lines were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 5 mM Hepes, 1.3 mM L-glutamine, and 50 μM 2-mercaptoethanol. Clone 2C was maintained with the addition of 10% supernatants from concanavalin A-stimulated rat spleen cells. 2C cells were stimulated with irradiated (1500 rads) BALB/c spleen cells every 2 weeks.

1B2 is a mouse monoclonal antibody (mAb; IgG1) that is specific for the T-cell receptor of clone 2C (27). KJ16 is a rat mAb that is specific for the mouse $V_\beta 8$ region (28). 9-40 (IgG1) is a mouse mAb specific for the hapten fluorescein (29). 1B2 and 9-40 were purified from BALB/c ascites fluid as described (27, 29). KJ16 was used as culture supernatant or purified on a protein G-sepharose column.

Construction of Single-Chain TCR (scTCR) Gene. The construction of the scAb genes from the anti-fluorescein hybridoma 4-4-20 (30) and the characterization of scAb 4-4-20 have been described (23, 31, 32). The scTCR gene was constructed in several steps by using the PCR to introduce convenient restriction sites. With the scAb gene cloned into pUC19 as a template, a PCR fragment containing the light-chain V-region gene (V_L) and the 205C linker gene (31, 32) was produced by using a 101-base "linker" primer (5'-CTACGGATCCGCTAGCATGCATCTTTCTTAGCATCATCTTTCTTAGCATCATCCTTCTTAGCAGCATCCTTCTTAGCATCATCCGCGGAGGACTTAATTTC-3') and an upstream pUC primer. The linker primer contained an *Sst* II site at the 5' end and *Nsi* I and *Bam*HI sites on the 3' end. The PCR product was digested with *Bgl* II and *Bam*HI and ligated to the *Bgl* II/*Bam*HI-digested scAb plasmid (*Bgl* II supplies a unique site within the V_L gene). The α-chain *V*-region (V_α) gene from CTL 2C ($V_\alpha 3J_\alpha 58$; ref. 33; *J* = joining region gene) was amplified with a 5' primer (5'-ACCCGTGCACAGTCAGTGACACAGCC-3') containing a *Bsp* I site and a 3' primer (5'-GGTTCTGGATCCTAGATGT-3') containing a stop codon followed by a *Bam*HI site. The V_α PCR product was digested with *Bsp* I and *Bam*HI and ligated to the *Nsi* I/*Bam*HI-digested V_L-linker construct. The V_β gene from CTL 2C ($V_\beta 8 D_\beta J_\beta$; ref. 34; *D* = diversity region gene) was amplified with a 96-base 5' primer (5'-TTGTATCGATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCCGGCTTCGCTACCGTTGCGCAGGCCGACGTCGCAGTCACCCAAAGCCCAA-3') that contained a *Cla* I site followed by the *E. coli ompA* signal sequence and a 3' primer (5'-TCATCCGCGGAGGATAGGACCGATAGTCG-3') that contained an *Sst* II site. The PCR product was digested with *Cla* I and *Sst* I and ligated to the *Sst* II/*Cla* I-digested V_L-linker-V_α construct (partial *Sst* II-digested because the V_α contained an *Sst* II site). Transformations were performed by electroporation, and constructions were confirmed at each step by restriction mapping or sequencing.

Expression of scTCR. Single-chain constructions were expressed by using a hybrid O_L/P_R λ phage operator/promoter in *E. coli* strain GX6712 as described (23, 31, 32). Briefly, cells were grown to an A_{600} of 0.6–1.0 at 30°C in 2× YT medium [16 g of Bacto tryptone, 10 g of yeast extract (Difco), and 5 g of NaCl per liter of distilled water] containing 50 μg of ampicillin per ml, and protein was expressed by a temperature shift to 42°C for 1 hr. Cells were pelleted and then disrupted by a French press at a cell pressure of 20,000 psi (1 psi = 6.89 kPa) in 50 mM Tris·HCl, pH 8.0/1 mM EDTA/0.1 mM phenylmethanesulfonyl fluoride (buffer A). Unlysed cells were removed by centrifugation, and the supernatant was centrifuged at 20,000 × *g* to pellet inclusion bodies and insoluble material. Pellets were washed twice with buffer A, once with buffer A containing 0.5% Triton X-100, and once with buffer A. For SDS/PAGE, pellets derived from 50-ml cultures were solubilized in 8 M urea/1% SDS/10 mM dithiothreitol, and the equivalent of 170 μl of culture was electrophoresed through a SDS/15% polyacrylamide gel. For refolding and subsequent experiments, a portion of the insoluble material was solubilized in 6 M guanidine hydrochloride in buffer B (50 mM Tris·HCl, pH 8.0/10 mM $CaCl_2$/100 mM KCl/1 mM EDTA/0.1 mM phenylmethane-sulfonyl fluoride) to an A_{280} of 40 and was diluted 1:100 into buffer B. For some experiments, buffer B included 3 mM dithiothreitol and 4 mM oxidized glutathione. Dilution into this buffer yielded ≈3- to 5-fold more activity than did buffer lacking DTT and glutathione (data not shown). Diluted samples were allowed to refold at 4°C overnight. Samples were used at various dilutions in solid-phase antibody binding assays or they were dialyzed into phosphate-buffered saline (PBS; pH 7.3) for cell assays.

ELISA. For direct binding of KJ16, single-chain preparations were adsorbed to wells (Immulon 2, 96-well plates) at 4°C overnight. After masking with PBS (pH 7.3) containing 0.05% Tween-20 and 0.1% bovine serum albumin for 1 hr, antibodies were added and incubated for 30 min at room temperature. Binding was detected with horseradish peroxidase (HRP)-conjugated goat anti-rat immunoglobulin antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) followed by the addition of substrate (tetramethylbenzidine; Kirkegaard and Perry Laboratories). Absorbances at 450 nm were determined with a 96-well plate reader. A "sandwich" ELISA was used to detect binding by the clonotypic antibody 1B2. Wells were incubated with 1B2 at 20 μg/ml in PBS (pH 7.3) overnight at 4°C. After masking, single-chain preparations were added at various concentrations. In some assays, scTCR preparations were added in the presence of soluble 1B2 or control antibody 9-40 (each at 200 μg/ml). To detect scTCR binding, KJ16 (20 μg/ml) was added for 30 min. After the sample was washed, HRP-labeled goat anti-rat antibodies were added for 30 min followed by the addition of substrate.

Purification of TCR Protein on 1B2-Conjugated Columns. Refolded scTCR preparations were applied to columns containing immobilized 1B2 or 9-40 (≈2 mg of IgG1 per ml of Affi-Gel-10 resin; Bio-Rad). After washing with binding buffer (50 mM Tris·HCl, pH 8.0/10 mM $CaCl_2$/100 mM KCl), columns were eluted with 0.2 M glycine·HCl (pH 2.6) into tubes containing 0.1 volume of 1 M Tris (pH 8.0). Fractions were monitored for absorbance at 280 nm and for activity in the 1B2 ELISA. Various fractions were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis through 15% gels, visualized by staining with Coomassie Blue.

Cytotoxicity Assays. P815 cells were labeled with ^{51}Cr, and 4×10^4 cells were incubated with 2C in a 96-well plate at various effector-to-target cell ratios for 4 hr at 37°C. In some experiments, scTCRs dialyzed against PBS (pH 7.3) were preincubated with P815 target cells for 30 min at room temperature prior to incubation at 37°C. After incubation, supernatants were removed and monitored for ^{51}Cr. Specific release of ^{51}Cr was calculated by the following equation: % specific ^{51}Cr released = (experimental counts − spontaneous counts)/(maximal counts − spontaneous counts) × 100.

Flow Cytometry. Approximately 2×10^6 P815 cells were incubated with 75 μl of scTCR that had been dialyzed against PBS. After 30 min on ice, the cells were washed twice with PBS and incubated for 30 min with 10 μg of KJ16 (protein G-purified) per ml. After washing twice, a 1:50 dilution of fluorescein-conjugated goat anti-rat immunoglobulin (Kirkegaard and Perry Laboratories) was added, and cells were incubated for 30 min. Flow cytometric analysis was performed with a Coulter Electronics EPICS 752.

RESULTS

Construction of the scTCR Gene. The scAb and scTCR genes were designed to contain the *ompA* signal sequence to facilitate export to the periplasmic space (Fig. 1). However, all of

the scAb genes that have been examined with this signal sequence and expression system have yielded insoluble single-chain proteins with cleaved signal sequences (refs. 23, 31, and 32; also unpublished observations). The construction of the scTCR from CTL 2C was also designed to provide restriction sites at the 5′ end of the V_β gene (*Aat* II), the 5′ end of the linker gene (*Sst* II), the 3′ end of the linker gene (*Nsi* I), and the 3′ end of the V_α gene (*Bam*HI) for convenient cloning of other scTCR genes. The first two residues of the V_β protein have been changed from Glu-Ala to Asp-Val to accommodate the *Aat* II site, but these are conservative changes, and many of the V_H genes encode Asp-Val in these positions. The 205C linker sequence was chosen because our previous studies with scAbs indicated that proteins with this linker had binding affinities most similar to that of the intact antibody (32).

Expression and Folding of $V_\alpha V_\beta$ Protein. Small-scale cultures (50 ml) of cells containing the scAb and scTCR genes were subjected to lysis by a French press. The insoluble pellets were washed and solubilized in urea and SDS/PAGE sample buffer and were electrophoresed through a 15% gel. Both samples contained a major protein component of ≈27 kDa that was absent in the control host cell (Fig. 2). This insoluble component represented as much as 50% of the protein as judged by Coomassie staining of the SDS/PAGE gel. The total amount of the scTCR varied to some degree among independent small-scale preparations but was usually present as one of the most predominant bands.

Rapid dilution of guanidine hydrochloride-solubilized single-chain antibody preparations resulted in the refolding of a fraction of the protein. The SCA preparation used in this study as a control yielded ≈10% antigen-binding protein after dilution and concentration (data not shown). The identical refolding procedure was followed with single-chain $V_\alpha V_\beta$ material. A similar SDS/PAGE profile was obtained with the soluble, diluted material (scTCR′, Fig. 2). The scTCR is the most prominent component in the diluted sample.

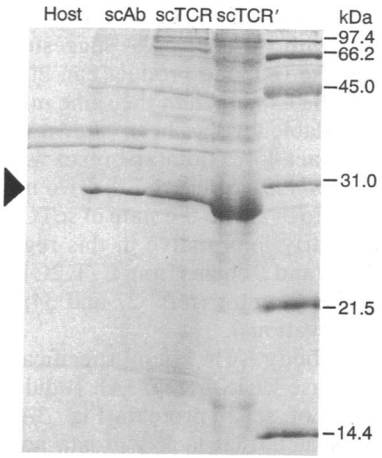

FIG. 2. SDS/polyacrylamide gel electrophoretic analysis of single-chain constructions expressed in *E. coli*. Cells without plasmid expression vector (host), with scAb (also called SCA) 4-4-20 expression vector, or with scTCR expression vector were cultured as described in text. After lysis with a French press, insoluble pellets were washed and solubilized in urea and SDS sample buffer containing dithiothreitol. Samples equivalent to 170 μl of original culture were electrophoresed through a 15% gel, which was then stained with Coomassie blue. scTCR is material that was diluted for refolding and precipitated with trichloroacetic acid. The arrow indicates the position of single-chain proteins (≈27 kDa).

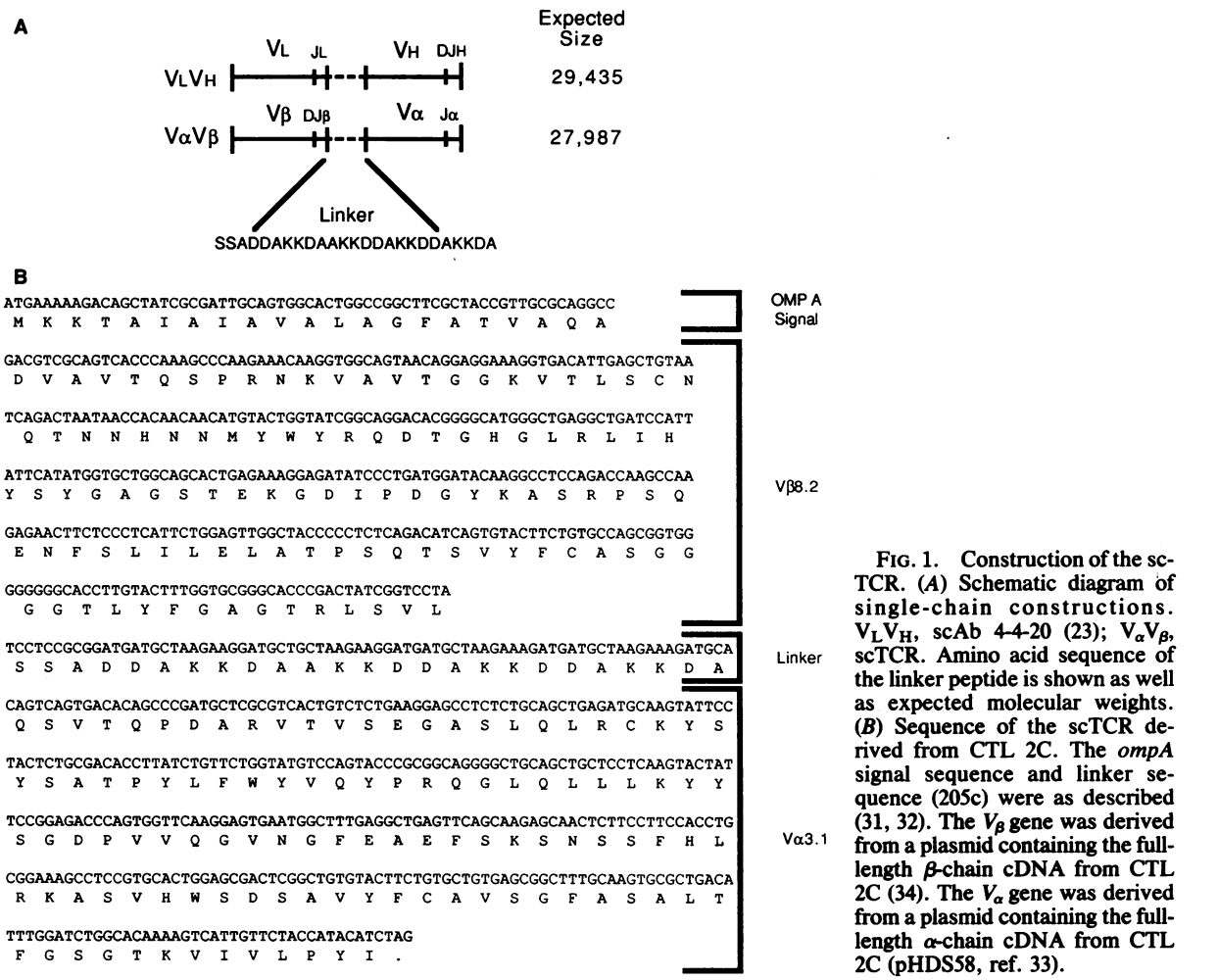

FIG. 1. Construction of the scTCR. (*A*) Schematic diagram of single-chain constructions. V_LV_H, scAb 4-4-20 (23); $V_\alpha V_\beta$, scTCR. Amino acid sequence of the linker peptide is shown as well as expected molecular weights. (*B*) Sequence of the scTCR derived from CTL 2C. The *ompA* signal sequence and linker sequence (205c) were as described (31, 32). The V_β gene was derived from a plasmid containing the full-length β-chain cDNA from CTL 2C (34). The V_α gene was derived from a plasmid containing the full-length α-chain cDNA from CTL 2C (pHDS58, ref. 33).

Reactivity of Single-Chain $V_\alpha V_\beta$ with Anti-TCR Antibodies. Although results with various scAbs suggested that a significant fraction of the refolded product can bind antigen, the scTCR could not be examined in the same manner since the ligand is not available. It has been shown that antibodies specific for the intact 4-4-20 idiotype react with scAb 4-4-20 derived from *E. coli* (35). Similarly, the two mAbs KJ16 and 1B2 could be used to evaluate the state of scTCR protein. 1B2 would be particularly informative in this regard because it requires both the α and β chains from CTL 2C in order to bind to the cell surface receptor (refs. 27 and 34; also D.M.K., unpublished observations).

The anti-V_β antibody KJ16 bound specifically to refolded $V_\alpha V_\beta$ in a solid-phase ELISA (Fig. 3*A*). It did not react with similar quantities of scAb protein (Fig. 3*A*) or to other single-chain constructs that lack V_β (data not shown). Because a direct binding assay with 1B2 gave a minimal signal above background, a sandwich assay with 1B2 was performed. In this assay, 1B2 was absorbed to a solid-phase well, followed by incubation with the scTCR preparation. Bound scTCR was detected with KJ16 followed by HRP-labeled anti-rat immunoglobulin. This method specifically detected the scTCR protein (Fig. 3*B*) but not scAb or other single-chain constructions (data not shown). Binding was detected at total protein concentrations as low as 0.3 μg/ml (1:100) (Fig. 3*B*), and the binding by 1B2 in the solid phase could be inhibited with soluble 1B2 but not with an isotype-matched control antibody (9-40, IgG1, κ).

To purify and concentrate the refolded scTCR, diluted material was run through a column containing immobilized 1B2 antibody. Eluted protein was examined by SDS/PAGE and the 1B2 ELISA (Fig. 4*A*). Eluted fractions contained the 27-kDa protein, and they were enriched at least 10-fold in 1B2 binding activity. Passage of the scTCR material through a control column that contained an equivalent amount of the 9-40 IgG1 did not yield eluted material that was 1B2-reactive (Fig. 4*B*). Furthermore, the 1B2 column but not the 9-40 column was effective in reducing the amount of ELISA-positive material as judged by activity of the pass-through material. From experiments in which covalently linked 1B2 antibody was in molar excess of the refolded scTCR, we estimate that ≈1% of the 27-kDa component is 1B2 reactive. This result, together with the fact that single-chain antibody preparations yielded no more than 10% ligand binding material (data not shown), further suggests that the 1B2 binding form of the scTCR is in native conformation.

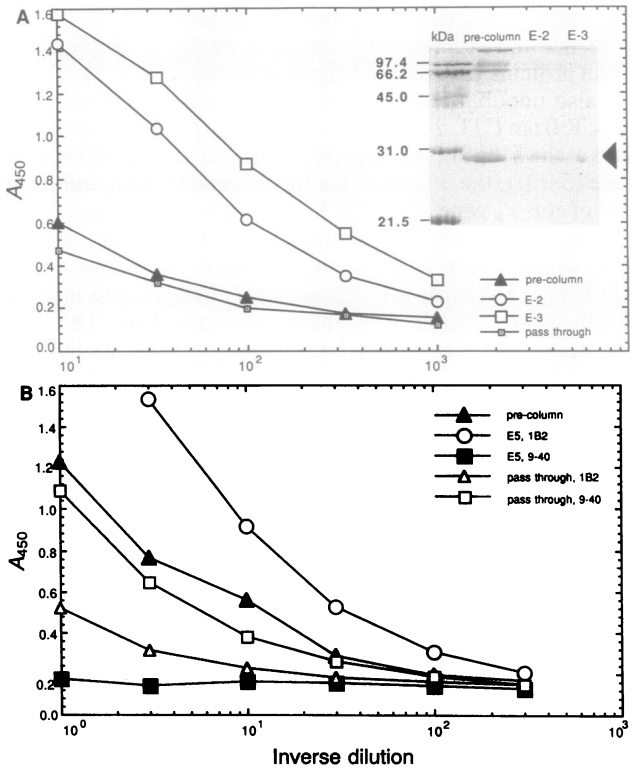

FIG. 4. Absorption of scTCR protein to 1B2 columns. (*A*) 1B2 ELISA and SDS/PAGE of fractions eluted from a 1B2-Affi-Gel column. Approximately 100 ml of scTCR diluted material was run over a 1B2 column (≈500 μl of resin). After the column was washed, fractions were eluted with 500-μl aliquots of 0.2 M glycine·HCl (pH 2.6). Fractions 2 and 3 (E-2 and E-3) were electrophoresed through a 15% gel and tested at various dilutions in the 1B2 ELISA. (*B*) 1B2 ELISA of scTCR from columns prepared with 1B2 or 9-40 IgG1. Approximately 100 ml of diluted scTCR material was applied to either a 1B2 or 9-40 column (≈1 ml of resin each), and 500-μl fractions were eluted. Fractions 1–10 were examined in the 1B2 ELISA. Fraction 5 is shown for both 1B2 and 9-40 eluates (E5). All 9-40 fractions were negative (data not shown).

Effect of Single-Chain $V_\alpha V_\beta$ in Functional Assays. It was of obvious interest to determine if the scTCR could bind to its ligand (H-2L^d) on the surface of target cells. Two types of experiments were performed to examine such binding. The first involved direct binding to P815 (H-2^d) target cells. In

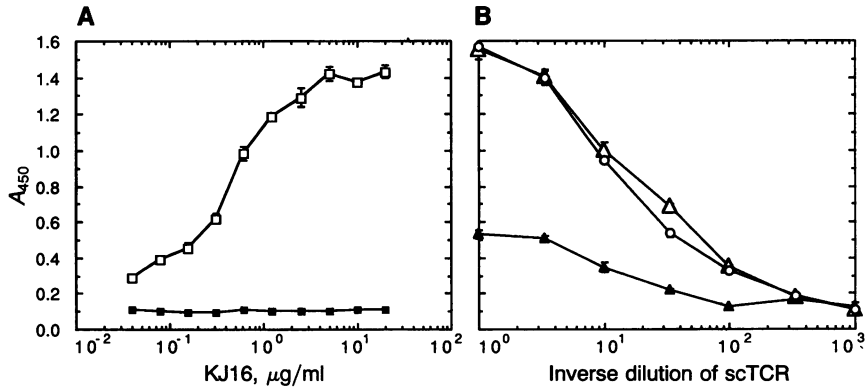

FIG. 3. Specific binding of anti-TCR mAbs to single-chain $V_\alpha V_\beta$ (scTCR) in a solid-phase ELISA. (*A*) Binding of the anti-$V_\beta 8$ antibody KJ16 to scTCR. scTCR (□) and scAb 4-4-20 (■) (at 30 μg of protein per ml) were absorbed to wells of a 96-well plate. KJ16 mAb at the indicated concentrations was added for 30 min; after the wells were washed, HRP-labeled goat anti-rat immunoglobulin (Ig) was added; after another wash, substrate was added and the absorbance was monitored at 450 nm. (*B*) Binding of scTCR to the anti-clonotypic antibody 1B2. 1B2 mAb (20 μg/ml) was absorbed to wells overnight. Various dilutions of scTCR were added in the presence of soluble 1B2 (▲) or 9-40 mAb (○) or without antibody (no inhibitor) (△). KJ16 mAb, followed by HRP-labeled goat anti-rat Ig (mouse Ig absorbed) were used to detect bound scTCR. Host cell extracts, scAb 4-4-20, and another single-chain antibody construction did not give a signal above background (data not shown).

these experiments, P815 was incubated with diluted scTCR material (Fig. 3) or 1B2-column-eluted material (Fig. 4). After incubation with KJ16, cells were stained with fluorescein isothiocyanate-labeled anti-rat immunoglobulin. Flow cytometric analyses of P815 using several different scTCR preparations were all negative (data not shown).

In addition, a cytoxicity assay with 2C as effector cells and P815 as target cells was performed in the presence and absence of soluble receptor. No inhibition was observed at any of the effector-to-target ratios tested or at any concentration of scTCR tested (up to 40 μg of protein was eluted from the 1B2-columns per ml; data not shown). Several possible explanations for the lack of detectable binding or inhibition, or both, by the soluble TCR are discussed below.

DISCUSSION

We show here that a simple *E. coli* expression system can be used to produce scTCR domains that refold into a conformation that is similar to that found on the T-cell surface. The observation that this protein, even without rigorous purification, will react with the KJ16 and 1B2 antibodies suggests that this approach will be useful in generating antibodies to various TCR determinants. Furthermore, the proteins may be useful as modulators of the immune response, as has been shown recently with specific TCR peptides in experimental allergic encephalomyelitis (36, 37).

The 27-kDa protein expressed in *E. coli* differs from the TCR expressed on the surface of 2C in at least two significant ways. First, the 2C-expressed receptor is associated with other accessory molecules such as CD3 and perhaps CD8. Second, the molecule on 2C is glycosylated (38). Thus, the fact that *E. coli*-derived material reacts with KJ16 and 1B2 was not necessarily an expected finding; TCR proteins that have been examined with antibodies in the past presumably have been glycosylated. In fact, glycosylation has been shown to have an effect on the binding by one clonotypic anti-TCR antibody (39). Whether either of these properties will affect future studies involving binding of the single-chain protein to their natural ligands remains to be seen. However, studies with scAb 4-4-20 show that it exhibits a binding affinity that is similar to that of the intact antibody (23). Four other scAbs that we have produced recently also had binding affinities that are nearly identical to the intact antibodies (unpublished observations, E.W.V. and L.K.D.).

Our preliminary binding studies with P815 (H-2L^d) and the scTCR have yielded negative results. Among the possible explanations are: (*i*) the affinity of the receptor for the target-cell ligand is low, and the concentration of soluble scTCR is below that required to detect binding; (*ii*) in flow cytometry, the number of H-2L^d molecules that contain the appropriate peptide may be below the level of sensitivity; (*iii*) in the cytotoxicity assay, even with 90% saturation of the ligand, target cells may still bear sufficient unbound ligand for recognition by 2C.

During the course of these studies, expression of a different scTCR was reported (40). This scTCR was derived from a T cell that is specific for the fluorescein hapten. In those studies (40), the solubility of the receptor was improved by substitution of several exposed hydrophobic residues. Although solubility of the scTCR from 2C might also be improved by such an approach, we show here that it may be unnecessary for many purposes, including antibody studies.

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