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Production of a soluble γδ T-cell receptor to identify ligands for the murine intestinal intraepithelial γδ T cell population

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Abstract

Although the functions and antigen recognition requirements of αβ T cells are well characterised, the antigens recognised by γδ T cells and the consequences of this recognition are unclear. γδ T cells are enriched within epithelia, where they eradicate transformed epithelial cells and regulate inflammation. To understand how this occurs, we need to understand the cellular ligands recognised by the γδ cell through the γδ T-cell receptor (TCR). We have therefore generated a soluble TCR (sTCR) to identify ligands for the murine γδ intestinal intraepithelial lymphocyte (IEL) population. sTCR was produced in the baculovirus expression system and purified by affinity chromatography on an anti-TCRδ affinity column. sTCR was recognised by a panel of conformation-specific anti-TCRγδ antibodies. We will now use our sTCR to directly test the binding of putative ligands to the TCR using surface plasmon resonance, and to isolate the ligand biochemically.

Keywords: γδ T cells; Protein expression; Baculovirus; T-cell receptor

1. Introduction

T cells are critical effectors of the vertebrate adaptive immune system. Conventional T cells become activated through the binding of antigen-specific T-cell receptors (TCRs) on the cell surface to self-Major Histocompatibility Complex (MHC) molecules presenting antigenic peptides on the surface of target cells. The TCRs that mediate this interaction are heterodimeric members of immunoglobulin superfamily (IgSF) [1]. Each TCR chain is composed of two immunoglobulin (Ig) domains: a membrane-proximal constant (C) domain and a membrane-distal variable (V) domain. The V domain is encoded by the rearrangement of variable (V), diversity (D) and joining (J) gene segments during T cell development to generate a diverse pool of receptors capable of recognising an immense variety of antigenic peptides encountered by the organism. Each chain of the TCR heterodimer also has a transmembrane and cytoplasmic domain, which serve to associate the TCR with components of the CD3 complex to signal for effector function upon recognition of peptide-MHC.

There are four TCR gene families conserved in vertebrates: α, β, γ and δ. While the majority of T cells in the peripheral circulation express a TCR heterodimer of α and β chains, 1–10% of peripheral T cells in mice and humans express a γδ heterodimer [2]. Unlike peripheral αβ T cells, which use VDJ gene rearrangement to generate maximal diversity capable of recognising diverse antigen, intraepithelial T cells express particular V gene segments restricted to particular tissues. For example, more than 95% of

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γδ+ dendritic epidermal T cells (DETCs) in the skin of mice express Vγ5 paired with Vδ1 [3]. Similarly, γδ cells in the mouse intestine favour Vγ7 paired with Vδ4, Vδ5 or Vδ6 [4–6]. Limited V gene usage is also observed among T cells in human epithelia; the majority of T cells in the human intestine express Vγ1 paired with Vδ1 [7].

The relevance of the invariant DETC TCR was tested by targeted disruption of the Vγ5 gene segment in mice [8]. Vγ5−/− skin contained DETCs, the majority of which used a Vγ1 ''replacement'' gamma chain paired with the canonical Vδ1 chain. DETC TCRs in normal mice react with 17D1, an antibody that is specific for the Vγ5/Vδ1 conformation, and does not react with Vγ5 or Vδ1 paired with any other chain. In Vγ5−/− mice, a significant fraction of DETCs were 17D1 reactive, indicating that the Vγ1/Vδ1 ''replacement'' receptor could assume the conformation of the canonical Vγ5/Vδ1 DETC TCR. This suggests that epithelial-associated TCRs are selected to recognise important conserved ligands within the epithelia, although no ligands for these cells have yet been identified.

While αβ T cells are restricted to recognise peptide or lipid antigen presented by MHC molecules [1], γδ cells are not MHC-restricted. Diverse molecules have been proposed to activate γδ cells in cellular assays [9], although few of these have been biochemically demonstrated to bind the γδ TCR. In fact, the use of cellular assays in the search for TCR ligands can be misleading. The non-classical MHC molecule MICA was proposed as a ligand for human intestinal Vδ1+ cells [10]. MICA+ transfectants could activate human Vδ1+ cells, and this interaction could be blocked with anti-MICA and anti-TCRγδ antibodies. However, MICA was later shown to be a ligand for the NKG2D receptor expressed on γδ cells [11], while the role of the γδ TCR in this interaction remains unclear. Therefore, to avoid the problems of cellular assays, we have generated a soluble γδ TCR (sTCR) reagent that will be used to confirm or refute any proposed ligands, or to isolate novel ligands for the γδ TCR. We are specifically interested in the role of γδ cells in the mouse intestine. Here we describe the cloning, expression, purification and verification of a sTCR derived from a mouse intestinal γδ hybridoma.

2. Experimental

2.1. Cells and antibodies

The E10 hybridoma was generated by fusing C57BL/6 γδ+ IELs to BW5147 thymoma cells and was generously provided by Leo Lefrancois. Cells were maintained at 37 °C in 5% CO_2 in RPMI (Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS; Life Technologies), 2 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Sf9 and High Five insect cells were obtained from Invitrogen (Groningen, The Netherlands) and maintained at 27 °C in Ex-Cell 420 insect serum free medium (JRH Biosciences, Lenexa, KS, USA). The GL3 hybridoma, which secretes anti-TCRδ monoclonal antibody (mAb), was obtained from Robert Tigelaar and grown in DMEM supplemented with 5% Ultra-low Ig FBS (Life Technologies), 4 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. GL3 mAb was purified from hybridoma supernatants on a protein G-sepharose affinity column (Amersham Biosciences, Little Chalfont, UK). UC7 (anti-TCRδ) and H57 (anti-TCRβ) mAbs were obtained from BD Pharmingen (San Diego, CA, USA). 5F1 (anti-Vγ7) was a kind gift from Leo Lefrancois.

2.2. RNA isolation, cDNA synthesis and PCR

RNA was isolated from the E10 hybridoma using TRIzol reagent (Life Technologies) and cDNA synthesized using Superscript II RT (Life Technologies) following manufacturer's instructions. Vγ7 was amplified from E10 cDNA using the 5′ primer (CCAGATCTCCACC ATGCTGTGGGCTCTGGCCCTA) and the 3′ primer (CCGCTCAGCGATATCGCGGCCG CTTAGTAGGCAGAGGTGATCGTGAA). Vδ5 was amplified using the 5′ primer (CCGG ATCCACCATGATTGTTGCCGCGACCCTT) and the 3′ primer (GGCCCGGGTTAATCGCGCAGCgTCCATCTTCATAGCCTCGAAGATACCACCCAGGAGGGACATCATGTTTACCTT). PCR reactions were performed in 25 μl with 1× PfX Buffer, 250 μ*M* dNTPs, 1 m*M* Magnesium Sulfate, 0.25 μ*M* each primer, and 1.25 U Pfx polymerase (Invit-

rogen). PCR conditions were 95 °C for 3 min; (95 °C for 30 s, 54 °C for 45 s, 72 °C for 3 min)×35 cycles; 72 °C for 10 min. PCR reactions were carried out in a DNA Engine Tetrad (MJ Research).

2.3. Generation of recombinant baculovirus

Vγ7 and Vδ5 PCR products were digested with the appropriate restriction enzymes and cloned sequentially into the pAcAB3 transfer vector (BD Pharmingen) as described in Fig. 2. Clones were confirmed using cycle sequencing. Transfections were carried out as follows: 5 μg of transfer vector, 20 μl of INSECTIN liposomes (Invitrogen), and 5 μg of baculogold linearised baculovirus DNA (BD Pharmingen) were mixed with 1 ml of Ex-Cell 420 serum free medium for 15 min at room temperature. This mixture was then added to 2×10^6 Sf9 cells in a 60-mm dish, and rocked for 4 h at room temperature. An additional 1.5 ml of Ex-Cell 420 serum free medium was added and cells were incubated for 4 days at 27 °C. Supernatants containing budded recombinant virus were harvested and clonal viruses were isolated by plaque assay. Individual clonal viruses were then tested for protein production. A single clonal virus was chosen and amplified to produce a high titre viral stock of at least 2×10^7 pfu/ml, which was used for protein production.

2.4. Metabolic labeling of insect cells

2×10^6 Sf9 or High Five cells were infected with wild type (wt; BD Pharmingen) or sTCR virus at an MOI of 10. After 14, 22, 46 or 70 h, cells were starved in Grace's media lacking Met/Cys (Sigma–Aldrich Chemical Company, Poole, UK) supplemented with 5% dialysed FBS (Life Technologies), 2 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin for 30 min. Cells were then pulsed with 75 μCi of ^{35}S Met/Cys (Amersham Biosciences) for 1 h and chased in Ex-Cell 420 medium for 1 h. Supernatants were collected and cells were lysed in SDS–PAGE loading dye. Whole-cell lysates were analysed directly by SDS–PAGE and autoradiography.

2.5. Protein production and purification

High Five cells (1×10^6 cells/ml) grown in suspension were infected with recombinant virus at an MOI of 5 for 60 h. To reduce proteolysis of secreted TCR, FBS was added to produce a final concentration of 2%. sTCR was purified on a GL3 affinity column. Five milligrams of GL3 was coupled to CNBr sepharose (Amersham Biosciences) following manufacturer's instructions. High Five infection supernatants were centrifuged and filtered to remove cellular debris, then loaded by gravity flow onto the GL3 affinity column. The column was washed with ten column volumes each of Tris Buffered Saline (TBS; 137 m*M* sodium chloride, 20 m*M* Tris–HCl, pH 8.0) and TBS/0.5 *M* NaCl. sTCR was then eluted using Pierce Gentle Antigen–Antibody Elution Buffer (Pierce, Rockville, IL, USA) and dialysed into 10 m*M* Tris–HCl, pH 8.0. sTCR was concentrated using a Centriprep-10 (Millipore Ltd., Watford, UK) and biotinylated using the BirA enzyme (Avidity, Denver, CO, USA) following manufacturer's instructions.

2.6. Surface plasmon resonance

Experiments were carried out on a Biacore X (Biacore International AB, Uppsala, Sweden). Biotinylated sTCR (2500 RU) was coupled to one flow cell on a sensor chip-SA (Biacore AB), while an irrelevant protein was coupled to the other flow cell. Relevant antibodies were diluted in HBS-P (Biacore AB).

3. Results

sTCRs have been generated in several different expression systems, although no method has thus far proven applicable to all receptors. Several αβ TCRs have been expressed in *E. coli* and refolded in vitro [12–14]. This method was also used to generate a single human γδ TCR for crystallisation [15]. However, our previous attempts to use a similar strategy for the production of murine γδ TCRs proved unsuccessful. γ and δ chains were expressed with a histidine tag [13], or as fusions with the coiled coil

heterodimerisation domain of v-Fos or c-Jun to encourage chain pairing and increase heterodimer stability [14]. All such constructs were expressed at high levels in *E. coli* and purified from inclusion bodies to high purity, but refolding attempts were unsuccessful due to aggregation. This propensity for aggregation may have been due to the absence of glycosylation in prokaryotic cells. Hydrophobic surfaces, which are usually masked with carbohydrates in mammalian cells, are left exposed in *E. coli*-produced protein, and could have allowed aggregation of γδ chains rather than refolding.

Insect cell expression systems such as baculovirus and *Drosophila* have been used successfully to produce several αβ TCRs [16,17] and one γδ TCR [18]. Therefore, a baculovirus expression system was selected to produce sTCR. Although random γ and δ chains may be able to associate, for the study of TCR specificity it is important to isolate γ and δ cDNAs from a true TCR pair expressed by a single γδ cell which has been positively selected to recognise a ligand. We are interested in the specificity and function of γδ cells in the mouse intestine. To ensure we amplified a true γδ pair from a mouse intestinal γδ IEL, we obtained a panel of hybridomas generated by the fusion of C57BL/6 γδ+ intestinal IELs with the TCR-negative BW5147 thymoma. cDNA was made from the different hybridomas and was tested by RT-PCR for expression of Vγ7 and Vδ4, Vδ5 and Vδ6, which are the common V gene segments used by intestinal IELs. The E10 hybridoma was found to express Vγ7 paired with Vδ5 (Fig. 1). PCR products were cloned and sequenced, and the junctional sequence of the E10 Vγ7-Jγ1-Cγ1 and Vδ5-Dδ1-Dδ2-Jδ1-Cδ chains are shown in Table 1.

To produce sTCR, a strategy that allows the expression of both TCR chains from a single recombinant virus was preferable to the generation of separate Vγ7 and Vδ5 viruses; the latter strategy requires careful optimisation of co-infection conditions to produce sTCR. The tricistronic transfer vector pAcAB3 was selected, and contains three promoter sites: Vγ7 was cloned into the BglII and BlpI sites downstream of one p10 promoter, while Vδ5 was cloned into the XmaI site downstream of the other p10 promoter. The polyhedrin (polH) promoter in this vector was not utilised. Vγ7 and Vδ5 cDNAs were amplified from E10 cDNAs, using

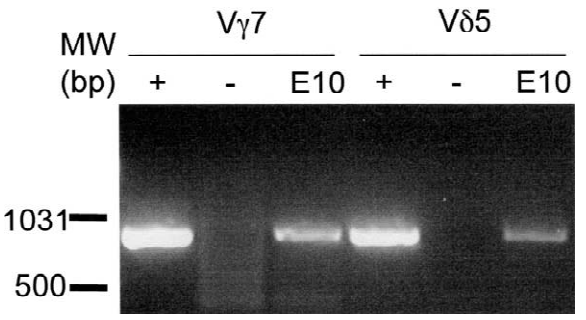

Fig. 1. The E10 hybridoma expresses Vγ7 and Vδ5. E10 cDNA was amplified using primers specific for Vγ7 and Cγ1, and Vδ5 and Cδ. Previously cloned Vγ7 and Vδ5 cDNAs were used as a positive control for the PCR.

Table 1
Vγ7 and Vδ5 cDNA junctional sequences amplified from E10 hybridoma

Vγ7-Jγ1-Cγ1

Vγ7|N|Jγ1

tgt	gcc	tcc	tgg	aat	agc	tca	ggt	ttt	cac	aag	gta
C	A	S	W	N	S	S	G	F	H	K	V

Jγ1

ttt	gca	gaa	gga	act	aag	ctc	ata	gta	att	ccc	tct
F	A	E	G	T	K	L	I	V	I	P	S

Jγ1|Cγ1

gac	aaa	agg	ctt
D	K	R	L

Vδ5-Dδ1-Dδ2-Jδ1-Cδ

Vδ5|Dδ1 |Dδ2

tgt	gcc	tcg	ggg	tat	ggc	ata	tcg	gag	gga	tac	gag
C	A	S	G	Y	G	I	S	E	G	Y	E

N|Jδ1

ctt	**gag**	**gcc**	gac	aaa	ctc	gtc	ttt	gga	caa	gga	acc
L	E	A	D	K	L	V	F	G	Q	G	T

Jδ1|Cδ

caa	gtg	act	gtg	gaa	cca	aaa	agc	cag
Q	V	T	V	E	P	K	S	Q

TCR chains were amplified from E10 cDNA using primers specific for Vγ7 and Cγ1, and Vδ5 and Cδ. PCR products were cloned, and several clones were sequenced. The junctional sequences are shown below. N-nucleotides, added by terminal deoxynucleotide transferase (TdT) are shown in bold.

primers which provided restriction sites for cloning into the pAcAB3 vector (Fig. 2). In addition, 5′ PCR primers added a consensus Kozak sequence [19] for enhanced ribosome binding to the mRNA just upstream of the ATG. The 3′ PCR primer truncated Cγ1 and Cδ just before the transmembrane region, and added an in vitro biotinylation signal sequence to the C terminus of Vδ5. The cysteines involved in the interchain disulphide bond were retained in this construct to allow proper γδ association and folding of the sTCR. This transfer vector was co-transfected into Sf9 insect cells along with linearised baculovirus DNA. After 4 days, supernatants containing recombinant baculovirus were harvested and clonal viruses were isolated by plaque assay. All clonal viruses analysed expressed sTCR at similar levels.

To optimise the production of sTCR, we analysed expression levels in two cell types and performed a time course to study expression levels at various times post-infection (pi). High Five cells are derived from ovarian cells of the cabbage looper (Trichoplusia ni), and may express as much as 25 times the level of recombinant proteins as Sf9 cells [20]. Sf9 and High Five cells were infected at an MOI of 10 for varying lengths of time with wild type (wt) virus or sTCR virus. Two hours before each time point, cells were starved in Met/Cys-deficient medium for 30 min. Cells were then pulsed with ^{35}S Met/Cys for 1 h, and chased in normal medium for 1 h to allow proteins to mature through the secretory pathway. Whole cell lysate was analysed by SDS–PAGE and autoradiography (Fig. 3). Sf9 cells infected with wt virus showed expression of p10 and polH proteins at 48 h pi, but Sf9 cells infected with sTCR virus expressed little Vγ7 or Vδ5. High Five cells expressed higher levels of p10 and polH proteins than

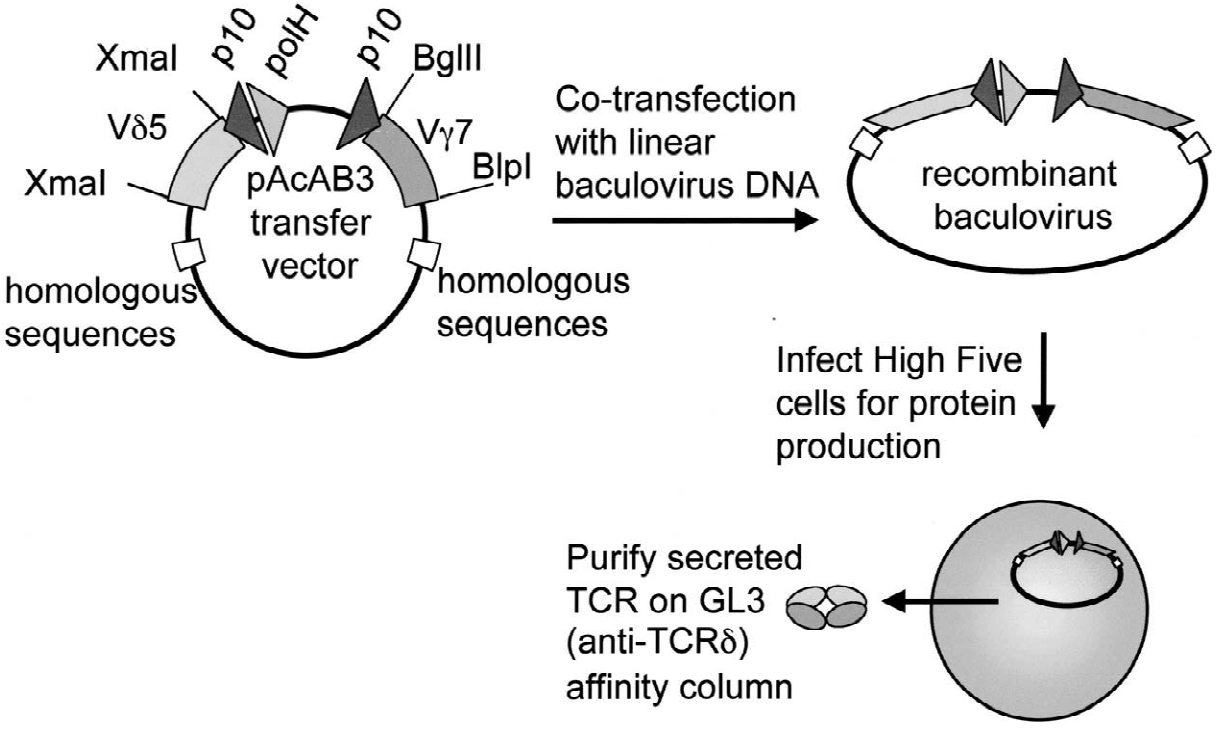

Fig. 2. sTCR expression strategy. Vγ7 and Vδ5 were amplified from E10 cDNA. Primers added restriction sites for cloning, a consensus Kozak sequence (CCACC), and introduced a stop codon just before the transmembrane domain. In addition, the 3′ Cδ primer added a biotinylation signal sequence to the C terminus. Vγ7 and Vδ5 PCR products were digested and sequentially cloned into the pAcAB3 transfer vector. Sf9 cells were co-transfected with transfer vector and linearised baculovirus DNA, and recombinant baculovirus was generated by homologous recombination. After 5 days, supernatants were harvested and clonal viruses were isolated by plaque assay. Clonal viruses were tested for protein production, and a single virus was amplified and used to infect High Five cells for protein production.

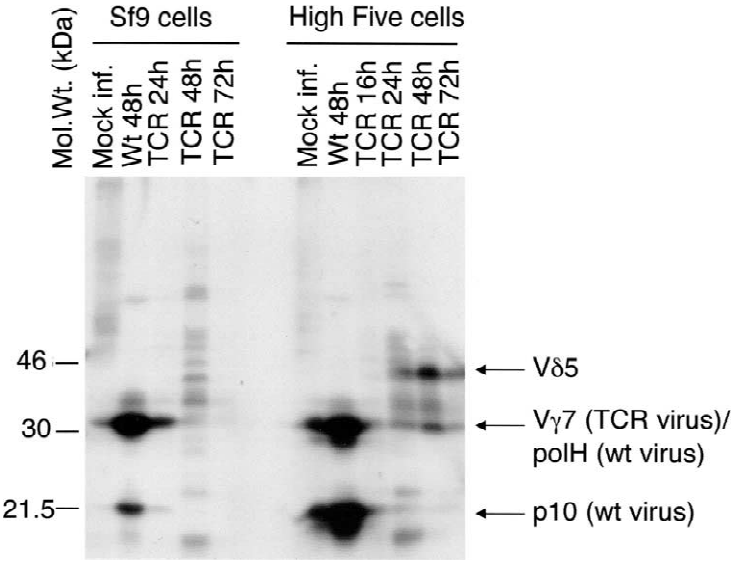

Fig. 3. Optimisation of sTCR expression. Sf9 and High Five cells were mock infected, or infected with wild type (wt) virus or TCR virus for the indicated times. Cells were then starved in Met/Cys-deficient medium for 30 min, pulsed with 75 μCi of ^{35}S Met/Cys for 1 h, then chased for 1 h in normal medium. Cells were lysed in SDS–PAGE loading dye and lysate analysed by SDS–PAGE and autoradiography.

Sf9 cells, and detectable levels of Vγ7 and Vδ5 by 24 h pi, with a peak of expression at 48 h pi, decreasing by 72 h. sTCR could be immunoprecipitated from the cell supernatant after a 1 h chase using the conformation-dependent GL3 (anti-TCRδ) mAb, although at very low levels (data not shown). Pilot experiments also indicated that expression of sTCR was equivalent at an MOI of 5 or 10 (data not shown).

For large-scale production of sTCR, 1×10^9 High Five cells were infected at an MOI of 5, at 1×10^6 cells/ml in ExCell 420 medium supplemented with 2% FBS to reduce proteolysis of sTCR. Supernatants were harvested after 60 h of infection, and sTCR was affinity purified from High Five cell supernatant on a GL3 affinity column and analysed by SDS–PAGE (Fig. 4). Under non-reducing conditions, sTCR was visible as a disulphide-linked heterodimer that migrates at approximately 62 kDa, while under reducing conditions, the Vγ7 and Vδ5 chains migrated at 30 kDa and 42 kDa, respectively. No contaminating proteins can be observed by silver staining.

In order to demonstrate that the purified sTCR was folded properly, we used surface plasmon resonance (SPR) to show binding of a panel of conformation-dependent anti-TCRγδ antibodies to the sTCR

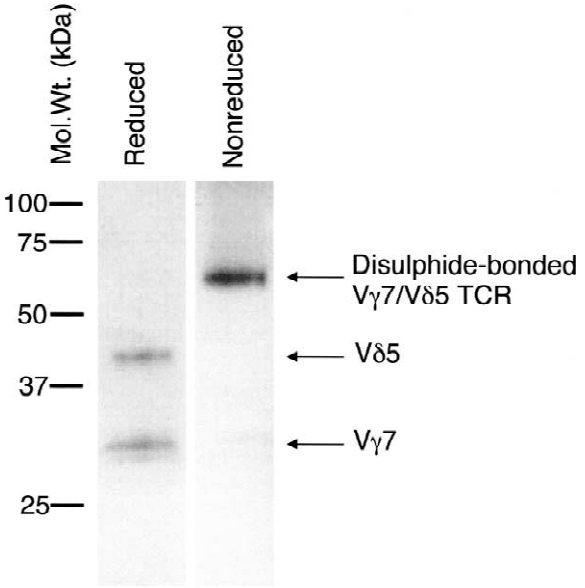

Fig. 4. Purified Vγ7/Vδ5 sTCR. sTCR was purified from High Five infection supernatants on a GL3 affinity column, subjected to SDS–PAGE under reducing and non-reducing conditions, and visualised by silver staining.

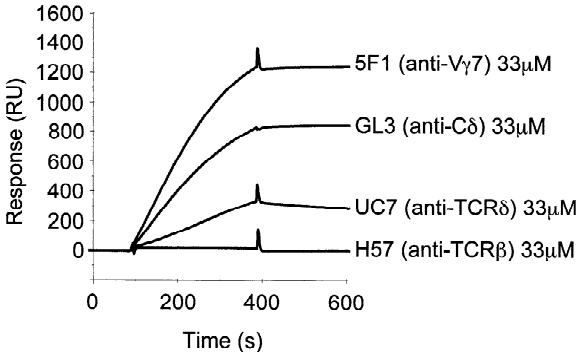

Fig. 5. Purified biotinylated Vγ7/Vδ5 TCR is recognised by anti-TCRγδ monoclonal antibodies. Conformation-dependent anti-TCRγδ mAbs 5F1 (anti-Vγ7), GL3 (anti-TCRδ), and UC7 (anti-TCRδ) bind specifically to sTCR coated on one flow cell of a streptavidin sensor chip, while H57 (anti-TCRβ) does not bind. Responses to the CD4-coated control surface have been subtracted.

coated on the chip (Fig. 5). The antibodies did not bind to an irrelevant protein, CD4, on the control flow cell. An anti-TCRβ antibody of the same isotype did not bind to the sTCR.

4. Discussion

γδ cells have been conserved in all vertebrates, and intraepithelial γδ cells express TCRs of limited variability that are selected for a particular conformation. Diverse ligands have been proposed to activate γδ cells, although little direct biochemical evidence has been provided to support these claims. Here we present a strategy for the production of soluble γδ TCRs, which will be used to biochemically confirm or refute any ligands for the γδ TCR identified using cellular assays, or to biochemically isolate the ligand.

The TCR of interest, a Vγ7/Vδ5 pair, was cloned from a C57BL/6 γδ+ IEL hybridoma. Several soluble αβ TCRs and one soluble γδ TCR have also been produced in *E. coli* and refolded in vitro, although this strategy was unsuccessful for the production of our murine γδ TCRs, as they aggregated and precipitated out of solution. Refolding efficiency may be directly dependent on the sequence of individual TCRs. Alternatively, these TCRs may require the presence of post-translational modifications such as glycosylation for folding and stability. Therefore, sTCR was produced in a baculovirus expression system, which supports glycosylation. sTCR was purified by affinity chromatography and bound to all relevant anti-γδ TCR antibodies. Purified sTCR (150 μg/l) was obtained per litre of High Five culture supernatant. We hypothesise that the low yield of sTCR is due to a failure of γδ chains to associate and fold properly. Large amounts of Vγ7 and Vδ5 protein are expressed in the cells at 48 h pi but little can be immunoprecipitated from the cell supernatant using a conformation-dependent antibody (Fig. 3 and data not shown). This method for the production of sTCRs can now be applied to other TCRs isolated from epithelial γδ cell populations, including the conserved DETC population and the human intestinal Vγ1/Vδ1 population.

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