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New simplified molecular design for functional T cell receptor

We have produced a chimeric single-chain T cell receptor (TcR) that combines the specific antibody recognition function and TcR/CD3 signaling properties within the same polypeptide chain. This hybrid molecule consisted of a single-chain antibody combining site that was connected over a short spacer to the transmembrane and cytoplasmic region of CD3ζ. When expressed on TcR^- or TcR^+ T cell hybridomas it could mediate recognition of relevent target cells and subsequent production of lymphokines; *i.e.* it could functionally replace the TcR/CD3 complex. Therefore, the single-chain TcR model presented here represents an interesting and useful means for the creation of T cells with new specificities.

1 Introduction

The T cell antigen receptor (TcR) is a multiprotein complex composed of at least six different transmembrane proteins. The TcR α and β chains are expressed as covalently linked αβ heterodimers which provide the antigen specificity for T cells. While these proteins have large extracellular domains, their cytoplasmic portions contain only a few amino acids. The α and β chains are noncovalently associated with the invariant subunits CD3γ, -δ, -ε, -ζ and/or -η to form TcR/CD3 complexes [1]. These associated proteins have large intracellular domains and are thought to couple the process of antigen recognition to the signal transduction pathways of the T cell [2, 3]. We wanted to bypass this complexity by creating a chimeric molecule that would retain both signaling and recognition functions within one polypeptide chain to generate an easily manipulatable system, which would allow us to change the specificity of a T cell at will.

Following several experiments which showed, that the intracellular portion of the CD3ζ chain would be sufficient to induce activating signals after cross-linking of their artificial extracellular domains [4–6] we constructed a hybrid molecule, which unified the signaling properties of the CD3ζ chain and the antigen-binding ability of an antibody. For this we used a single-chain (Fv) mAb form [7] derived from TR66, an mAb specific for human CD3ε [8], and grafted it onto a short polypeptide spacer (hinge) from the CD8α chain. This chimeric extracellular domain was then finally fused to the transmembrane and cytoplasmic region of CD3ζ to form the single-chain TCR "FvCD3ζ". When the construct coding for the single-chain TcR was transfected into TcR^- or TcR^+ T cell hybridomas, the expressed chimeric molecule was able to mediate recognition of relevant target cells resulting in the production of IL-2 and IL-3 by the transfectants.

Since the antigen specificity of our model chimera can easily be changed by replacing its Fv domain by other Fv derived from any antibody, this system could be useful in creating T cells of any desired specificity, bypassing the necessity of MHC restriction.

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Abbreviation: **Fv:** Variable region combining site

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2 Materials and methods

2.1 Construction of the chimeric FvCD3ζ

The vector pHβAPr-1-neo-FvCD3ζ was designed to express the chimeric FvCD3ζ construct under the control of the human β-actin promoter and bears a selectable neomycin marker. For its construction we removed the unique EcoRI restriction site in the plasmid pHβAPr-1-neo [9] by digesting it with EcoRI, blunting its cohesive ends and religating it. The polymerase chain reaction (PCR) was used to amplify a cDNA segment encoding for the cytoplasmic und transmembrane region of the murine CD3ζ chain and 42 amino acids of the hinge region of murine CD8α (oligonucleotide primers 5451, 5452). As a template for this reaction we used the plasmid CD8ζ [6]. The primers of this reaction contained a SalI and a HindIII restriction site so that the amplified PCR product could be cloned after digestion with SalI and HindIII into the SalI-HindIII opened pHβAPr-1-neo(RI). The resulting plasmid was opened by SalI, blunted and recut with EcoRI to receive the EcoRI-digested PCR-amplified cDNA fragment coding for the VDJ heavy and VJ light chain region of the mAb TR66. As a template for this PCR amplification we used the plasmid FvCD3 Hγ3 [10] and the oligonucleotide primers 5971 and 5979 with the latter containing an EcoRI cloning site.

The sequences of the used oligonucleotide primers are: 5′TCCTGTCGACAGTGGAATTCACTACTACCAAGCCAGTGCTGC3′ (5451); 5′GCATAAGCTTGCCAGAAGACCCAAGAGCAGGG3′ (5452); 5′TATGAGAATTCTTTCAGCTCCAGCTTGGTCCCAGC3′ (5971); and 5′ACTGCATGAGCTCGAGACAACACTGACTCTAACCATGGGA3′ (5972).

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2.2 Cell lines and monoclonal antibodies

BW5147$\alpha^-\beta^-$ is a derivative of the AKR/J lymphoma BW5147 that fails to express the TcR α, β, γ, δ and ζ/η genes [6, 11]. 14.3d is a mouse T helper hybridoma (derived from the T cell clone V_{2-15}) that recognizes a hemagglutinin peptide of influenza virus A/PR/8/34 (H1N1) in the context of MHC class II I-E^d [12]. The B lymphoma A20 [13] was used as antigen presenting cell for this peptide and Jurkat, a human acute T cell leukemia line (ATCC CRL 8162) and GC13, a human T cell clone (kindly provided by Dr. A. Lanzavecchia) were used as stimulators in the functional assays. All cells were cultured in IMDM containing 10% fetal calf serum.

For immunofluorescence and immunoprecipitations we used the following mAb: anti-FvCD3 (rat anti-FvCD3; Traunecker, unpublished data); 2C11 (hamster anti-mouse CD3ε, [14]), F23.1 (anti-mouse TcR Vβ8; [15]).

2.3 Immunofluorescence

Expression of cell surface proteins was assayed by indirect immunofluorescence. Cell suspensions of 1×10^5 viable cells were stained with 20 μg/ml of first-step mAb. After 30 min of incubation the cells were washed and incubated with the second-step FITC-conjugated goat anti-rat Ig (Southern Biotechnologies) at 10 μg/ml. Stained cells were washed and fixed in 1% formaldehyde prior to cytofluorometric analysis using a FACScan (Becton Dickinson).

2.4 Biochemical characterization of cell surface proteins

Viable cells (3×10^7) were washed five times in washing buffer (PBS, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and then biotinylated for 30 min at 4°C in Sulfo-NHS biotin (Pierce, 0.5 mg/ml in washing buffer). After another five washes the biotinylated cells were lysed in lysis buffer containing 150 mM NaCl, 50 mM Tris, 1 mM PMSF, 10 mM iodoacetamide and 0.5% NP40 or 1% digitonin. The lysate was precleared overnight with protein G-Sepharose (Pharmacia) and the immunoprecipitation was performed for 2 h with 10 μg specific mAb followed by a 1-h incubation with protein G-Sepharose. The precipitate was washed four times in lysis buffer and then boiled in 40 μl of 1.5 × Laemmli sample buffer prior to SDS-PAGE using 12.5% or gradient gels. The proteins were transferred from the SDS-PAGE to nitrocellulose which was then blocked in PBS, 0.4% Tween-20 (PBS-T) containing 5% nonfat dry milk. It was subsequently incubated for 1 h in PBS-T containing 0.08 μg/ml streptavidin-horseradish peroxidase conjugate (Southern Biotechnology). The blot was washed three times for 5 min in PBS-T before addition of the ECL chemoluminescence reagent (Amersham) and the nitrocellulose was exposed to an X-ray film (Kodak) for 30 s to 3 min.

2.5 Stimulation of T cell transfectants

In each assay 5×10^4 responder cells were stimulated with different amounts of human T cells, which were fixed before the experiment by a 30-s treatment with 0.05% glutaraldehyde. When the APC A20 was used to present influenza virus hemagglutinin peptide, the A20 cells were irradiated (8000 rad) before being used at 5×10^4 per assay with various amounts of peptide.

In each instance the supernatants of the stimulation assays were collected 24 h after the start of the experiment and tested for their level of IL-2 and IL-3 [16, 17] on the IL-2-dependent cell line CTL.L [18] and the IL-3-dependent cell line DA-1 [19].

3 Results and discussion

3.1 General strategy

To investigate if the TCR/CD3 multiprotein complex could be replaced functionally by a single-chain chimeric polypeptide, we designed a chimeric gene, which could direct the synthesis of a protein consisting of the cytoplasmic and transmembrane region of mouse CD3ζ, a 42-amino acid segment of mouse CD8α as extracellular hinge region and the antigen combining site of the mAb TR66 (Fig. 1B); TR66 is specific for the human CD3ε chain [8], which was already successfully modified to bispecific single-chain Fv molecules by Traunecker et al. [10]. The final construction (Fig. 1A) was then transfected by protoplast fusion [20] into the T hybridomas BWα$^-\beta^-$ (TcR$^-$) and 14.3d (TcR$^+$) and G418-resistant stable transfectants were isolated and analyzed for expression and function of the chimeric single-chain TcR.

3.2 Surface expression and protein characterization

The immunofluorescence analysis (Fig. 2) shows that the parental hybridomas BWα$^-\beta^-$ (Fig. 2A) and 14.3d (Fig. 2C) did not stain with the mAb anti-FvCD3, while the

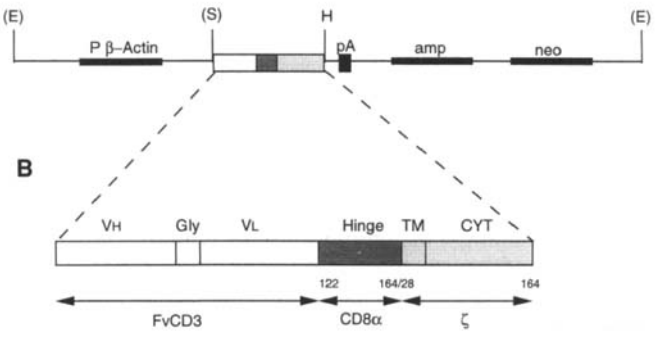

Figure 1. (A) A schematic representation of the chimeric FvCD3ζ construct. The FvCD3ζ cDNA is expressed under the control of the human β-actin promoter (P β-Actin). The plasmid further carries selectable neomycin and ampicillin resistance genes (neo, amp) and a polyadenylation site (pA) at the 3'end of the cDNA. The indicated restriction sites are EcoRI (E), SalI (S) and HindIII (H). Restriction sites in parentheses have been removed by blunt end cloning. (B) The predicted structure of the FvCD3ζ polypeptide. The FvCD3 portion consists of the heavy and light chain variable regions (VH, VL) of TR66 connected by a glycine linker (Gly) [10]. This Fv domain is followed by amino acid residues 122 to 164 of the hinge region of CD8α (numbering according to [22]) and the transmembrane (TM) and cytoplasmic portion (CY) of the ζ chain from residue 28 to 164 (numbering according to [23]).

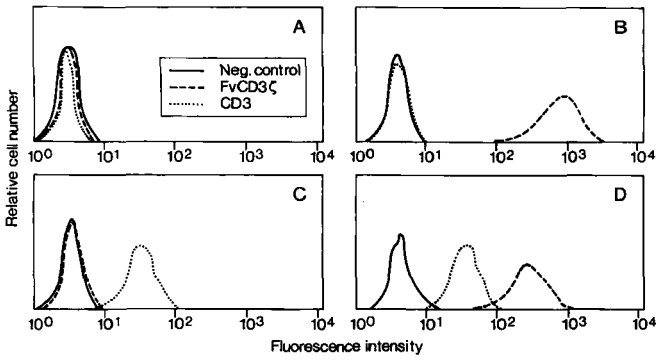

Figure 2. Flow cytometry of cells stained with antibodies specific for mouse CD3 (mAb 2C11, dotted lines), FvCD3ζ (dashed lines) and second-step fluorescein-conjugated antibody only FITC-goat anti-rat, solid lines). (A) shows the untransfected parental hybridoma BWα⁻β⁻, while (B) shows the staining of the BWα⁻β⁻ transfectant BW TF1. The cytofluometric analysis of the TcR⁺ hybridoma 14.3d and its transfectant 14.3d TF1 are shown in (C) and (D), respectively.

transfectants of each hybridoma (Fig. 2B,C) showed a surface staining indicating that the FvCD3 determinant was expressed on their surface. In contrast to 14.3d and its transfectants (Fig. 2C,D), the BWα⁻β⁻ transfectants (Fig. 2B) as well as the parental BWα⁻β⁻ (Fig. 2A) did not express endogenous TcR/CD3 complexes, as monitored with the mouse CD3ε-specific mAb 2C11 [14].

Theoretically, several possible forms of chimeric single-chain TcR could be expressed on the surface of the transfectants: while the BWα⁻β⁻ transfectants should express FvCD3ζ independently of endogenous TcR/CD3 complexes, an association could appear in the 14.3 transfectants. FvCD3ζ could then associate with endogenous TcR/CD3 complexes on the cell surface by charge pairing of its CD3ζ transmembrane region [21]. Furthermore, this CD3ζ transmembrane region could potentially permit covalent disulfide linkage between the single-chain FvCD3ζ and endogenous wild-type CD3ζ chains, resulting in the formation of CD3ζ-FvCD3ζ heterodimers. To investigate which of these was expressed on the surface of our transfectants, we biotinylated the cell surface of these cells, lysed them either in 0.5 % NP40 or 1 % digitonin and used the lysates for immunoprecipitations.

The SDS-PAGE analysis of the immunoprecipitations under reducing conditions, followed by a Western blot analysis (Fig. 3, lanes 1, 2), showed that the FvCD3ζ protein had an apparent molecular weight of 60 000 on the BW transfectant when the FvCD3-specific mAb was used for precipitation. When analyzed under nonreducing conditions the FvCD3ζ protein seemed to have a dimeric and to a lesser degree tetrameric form on the BWα⁻β⁻ transfectant (Fig. 3, lane 4).

Surprisingly we could only detect homodimers on the surface of the 14.3d transfectant (Fig. 3, lane 7) and no CD3ζ-FvCD3ζ heterodimers; these findings suggest that those kinds of heterodimers are either not transported to the cell surface or they are not formed at all.

To detect the eventual associations between FvCD3ζ and endogenous TcR/CD3 components we lysed the biotin surface-labeled 14.3d transfectant with 1 % digitonin-containing lysis buffer, which preserves the TcR/CD3 association. The digitonin lysate of the 14.3 transfectant was either used for precipitation with the anti FvCD3-mAb (Fig. 3, lanes 5, 6) or with F23.1 [15], and antibody specific for the Vβ8 segment of the TcRβ chain (Fig. 3, lanes 7, 8). The SDS-PAGE-analysis under nonreducing conditions revealed, that the F23.1 precipitation pattern of the untransfected 14.3d and its transfectant were identical: the major protein was the TcRαβ heterodimer while an additional coprecipitated FvCD3ζ homodimer on the transfectant could not be detected (Fig. 3, lanes 5, 6). *Vice versa* the immunoprecipitation with anti-FvCD3 did not coprecipitate TcR polypeptides from the 14.3d-transfectant (Fig. 3, lane 7).

This analysis of the 14.3d transfectant indicated, that the chimeric FvCD3ζ protein was expressed independently from endogenous TcR/CD3 proteins and that its major form was a homodimer. Therefore, the presence of the endogeneous TcR/CD3 complexes of the 14.3d transfectants should not sterically hinder the function of the FvCD3ζ polypeptide and *vice versa*.

3.3 Functional tests of the FvCD3ζ chimera

According to the experiments of Irving and Weiss [4] and Romeo and Seed [5] the presence of the intracellular portion of CD3ζ was sufficient to activate their transfectants when the extracellular domains of their chimeric proteins CD8ζ or CD4ζ were cross-linked by either CD8-specific mAb or gp120. We wanted to extend these findings and test if our transfectants would be able to recognize antigen (human CD3ε) through their FvCD3 domain and if this was sufficient to activate the T cell transfectants.

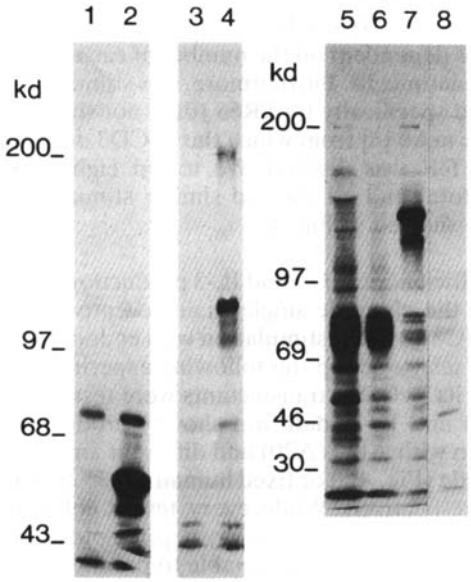

Figure 3. Biochemical analysis of cell surface proteins: BWα⁻β⁻ (lanes 1, 3) and one of its transfectants BWTF1 (lanes 2, 4) as well as 14.3d (lanes 6, 8) and its transfectant 14.3dTF1 (lanes 5, 7) were cell surface biotinylated, lysed in either 0.5 % NP40- (lanes 1–4) or 1 % digitonin- (lanes 5–8) containing lysis buffer. The lysates were used for immunoprecipitations with either FvCD3-specific mAb (lanes 1–4, 7, 8) or TCR-specific mAb F23.1 (lanes 5, 6).

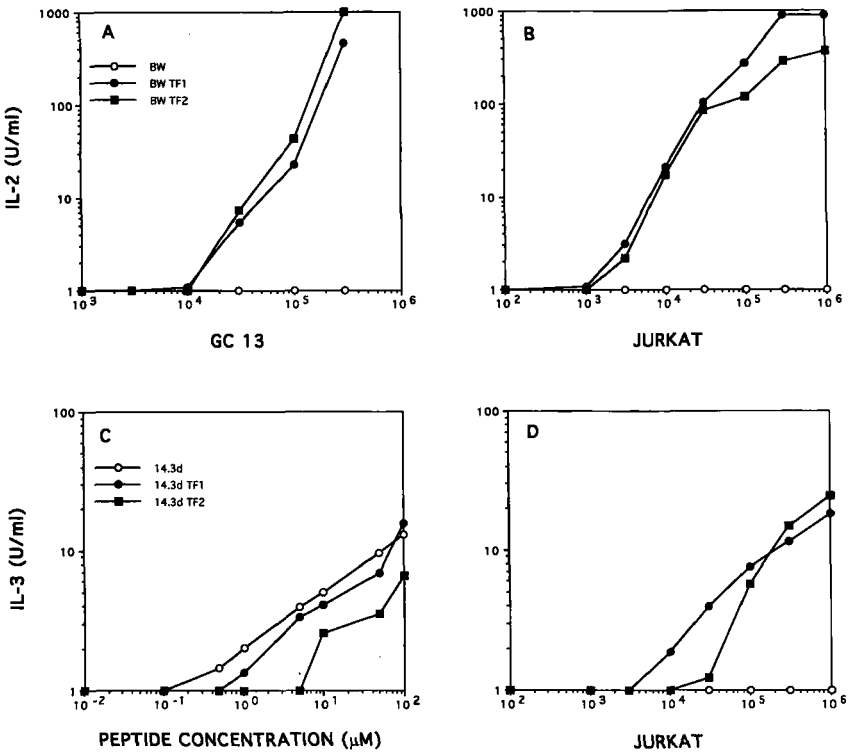

Figure 4. IL-2 (A, B) and IL-3 (C, D) production of BW and 14.3d cells transfected with the chimeric FvCD3ζ gene constructs after stimulation with the fixed human T cells GC 13 (A), Jurkat (B, D) or peptide antigen presented by A20 (C). The parental cell BWα⁻β⁻ (open circles) and two transfected subclones BW TF1 (closed circles) and BW TF2 (closed squares) were incubated with the indicated numbers of GC 13 (A) and Jurkat (B); 14.3d (open circles) and two of its transfectants [14.3d TF1 (closed circles), 14.3d TF2 (closed squares)] were stimulated either by antigen-presenting cell and different peptide concentrations (C) or the indicated numbers of fixed Jurkat cells (D). After 22 h the supernatants were assayed for their content of IL-2 or IL-3 using IL-2-dependent CTL.L or IL-3-dependent DA-1 cells.

As stimulators we used Jurkat (Fig. 4B, D), a human CD3⁺ T cell lymphoma and GC13 (Fig. 4A), a human T cell clone (A. Lanzavecchia, unpublished data). Both stimulators were fixed before the functional assays in order to make sure, that they could not be a source of lymphokine production (data not shown). Fig. 4A and B shows the results of the stimulation of BWα⁻β⁻ and two of the BW transfectants. While the untransfected parental hybridoma was apparently neither activated by the stimulator cells GC13 (Fig. 4A), nor Jurkat (Fig. 4B), the two transfectants produced IL-2 (Fig. 4A, B) and IL-3 (data not shown) in a manner which was dependent on the number of each of the two types of stimulator cells. Furthermore, this stimulation could be inhibited specifically by TR66 (data not shown), the CD3ε-specific mAb [8] from which the FvCD3 domain of our chimeric TcR was derived. We tested eight BW transfectants in total and all showed similar stimulation patterns to those showed in Fig. 4.

To compare the efficiency of IL-2 and IL-3 production after stimulation over the chimeric single-chain receptor with conventional MHC-restricted stimulation with endogenous TcR/CD3 complexes we set up the following experiments: 14.3d and two of its FvCD3ζ transfectants were tested for IL-3 (Fig. 4C, D) and IL-2 (data not shown) production during stimulation with APC (A20) and different amounts of antigenic peptide (Fig. 4C) or fixed human CD3⁺ Jurkat cells (Fig. 4D), respectively. While every tested cell was stimulated in a similarly efficient way by peptide plus A20 (Fig. 4C), only the transfectants were able to produce IL-3 (Fig. 4D) and IL-2 (data not shown) during stimulation by fixed Jurkat cells. Both kinds of assay showed that the production of lymphokines was either dependent on peptide concentration or on the number of stimulator cells, and indicated that the additional anti-human CD3 specificity on the 14.3d transfectants is caused by the expression of the single-chain TcR. A comparison of the stimulation efficiencies through endogeneous TcR (Fig. 4C) and FvCD3ζ single-chain TcR (Fig. 4D) shows that both signaling machineries seem to be similarly efficient in inducing lymphokine production in the same cell. While the peptide antigen presented by A20 is recognized in an MHC-restricted way, the CD3 recognition through FvCD3ζ resembles an antibody-antigen interaction where no restricting MHC is needed.

4 Concluding remarks

Here we have demonstrated that the biological activities of helper T cell hybridomas can be directed by a single polypeptide chain that is able to recognize antigen and to transduce activating signals into the cell, which result in the production of biologically active lymphokines, in manners indistinguishable from normal antigen-specific stimulation.

The anti-human CD3 specificity used in our experiments can be easily exchanged with Fv, of other desired specificities derived from any existing mAb. Therefore, the single-chain TcR model presented here represents an interesting and useful means for the creation of T cells with every desired specificity at will.

While our manuscript was under preparation Eshhar et al. published a similar study [24] with comparable results. Their findings and those of Moritz et al. (personal communication) combined with ours suggest that the single-chain TcR technology is generally applicable.

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