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Structural mutations in the constant region of the T-cell antigen receptor (TCR)β chain and their effect on TCRα and β chain interaction

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SUMMARY

The region responsible for T-cell receptor (TCR)α and β chain assembly has previously been shown to reside in their extracellular domains. In an attempt to delineate further the structural requirements for TCRα and β chain assembly, chimeric TCRβ chains with increasing length of constant (C) region and mutant TCRβ chains with C-domain point mutations were constructed. Their ability to assemble with wild-type TCRα chain was evaluated in non-T (COS cells) or T cells. The results reveal that the C_β domain is the binding region to TCRα chain, whereas the intact variable (V), diversity (D) and joining (J) regions with a short C-domain of β chain are not sufficient for the TCRα and β chain assembly. The unique interchain disulphide bond between TCRα and β chains is not required for the TCR$\alpha\beta$ heterodimer formation.

INTRODUCTION

The T-cell antigen receptor (TCR) subserves both antigen and major histocompatibility complex (MHC)-restricted recognition and is composed of at least seven integral membrane proteins.[1–3] On the majority of peripheral lymphocytes, the TCR contains a clonally distributed disulphide-linked heterodimer consisting of a TCRα and a TCRβ chain. These clonotypic chains are subdivided into variable (V), joining (J) and constant (C) segments for TCRα, and a V, diversity (D), J, and C segments for TCRβ, respectively. Associated with the TCRα and β chains are three invariant chains that form the CD3 complex (γ, ε, δ), and the disulphide-linked ζ-ζ or ζ-η chains.[4,5] The TCRα and β together are sufficient for antigen recognition and MHC restriction,[6,7] while the CD3-γ, -ε, -δ and -ζ chains are believed to play an important role in signal transduction after the TCR binds its appropriate ligand.[8–10]

Recent assembly studies have focused on the structural features of the TCR complex that are important in the formation and function of this receptor. It has been reported that the surface expression of the TCR complex is not affected by the removal of V_β domain[11] or the cytoplasmic tail of the TCRβ.[12] Presumably these regions are not involved in the TCR assembly and/or its intracellular transport to the cell surface. Consistent with these findings, we have previously demonstrated that the region responsible for TCRα and β chain assembly is localized to their extracellular domains.[13] In this study, the extracellular domain of TCRβ involved in the assembly with TCRα has been further defined, and the effects of structural mutations in C_β on TCRα and β chain assembly been examined. Results show that the C-domain, but not the V_β, D_β and J_β segments, of TCRβ chain is involved in the binding to TCRα chain. The interchain disulphide bond between TCRα and β chains is not required for the TCRα and β heterodimer formation in both fibroblasts and T cells.

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Abbreviations: NEPHGE, non-equilibrium pH gel electrophoresis; PCR, polymerase chain reaction; Tac, interleukin-2 receptor α chain; TCR, T-cell antigen receptor; V, D, J and C-domains, variable, diversity, joining and constant domains.

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MATERIALS AND METHODS

Site-directed mutagenesis

The chimeric and mutant TCRβ chains used in this study are schematically represented in Fig. 1. Complementary DNA encoding these hybrid TCRβ/Tac or mutant TCRβ proteins were constructed by polymerase chain reaction (PCR) using overlap extension by splicing as described previously.[13] Briefly, to construct TCRβ1, β2, β3, β6 and β7, the wild-type TCRβ and Tac cDNA were used as templates and PCR was performed in three steps using GeneAmp reagent (Perkin Elmer Cetus, Norwalk, CT). TCRβ portions of the chimeric TCRβ1, β2, β3, β6 and β7 constructs were amplified using external 5′ primers and matched internal 3′ primers (Table 1). To amplify Tac portions of these chimeric TCRβ chains, external 3′ primers and internal 5′ primers responsible for Tac segments of TCRβ1, β2, β3, β6 and β7 were used, respectively. Enzyme sites were designed at both ends of these constructs. These include: *Not*I/*Not*I for TCRβ1 and β2, *Pst*I/*Bgl*II for β3, and *Not*I/*Bgl*II for β6 and β7.

The mutant TCRβ4 chain was constructed by a single base-pair substitution (TGT to AGT) which converted the interchain

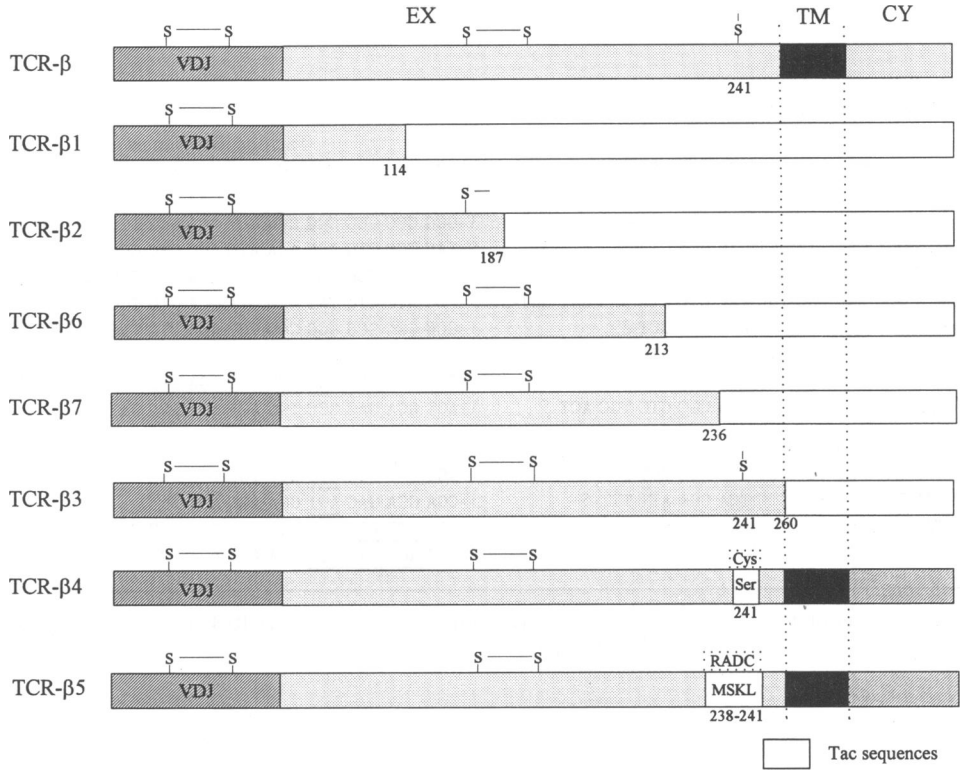

Figure 1. Diagrammatic representation of wild-type, chimeric and mutant TCRβ constructs. TCRβ1 from codon 20 including leader sequence to 425 of C-domain fused to Tac from 462 to stop codon 999; TCRβ2 from codon 20 to 644 of C-domain linked to Tac from 684 to stop codon 999; TCRβ3 contains the entire extracellular domain of wild-type TCRβ linked to transmembrane and cytoplasmic sequences of Tac; TCRβ4 from codon 20 to stop codon 846 of β chain with substitution of T with A (TGT to AGT) at position 803 converting cysteine to serine; TCRβ5 from codon 20 to stop codon 846 with nucleotide substitution (CGAGCAGACTGT to ATGAGTAAGCTT) at position 794–805. This altered the amino acid sequence from ArgAlaAspCys to MetSerLysLeu; TCRβ6 and β7 from codon 20 to 721 or 790, were fused with Tac sequences from 754 or 793 to stop codon 999, respectively. EX, extracellular domain; TM, transmembrane; CY, cytoplasmic domain. Numbers indicate the positions of amino acids which have been mutated or truncated. The length of each chimeric construct was the same as the wild-type TCRβ.

bond-related Cys^{241} to Ser^{241} in the C_β domain. The external 5′, and 3′ primers, and two mismatched internal primers were used in this approach (Table 1). Similarly, to generate the substitution of ArgAlaAspCys (RADC, 238–241) to MetSerLysLeu (MSKL) in TCRβ5 construct, the same external 5′ and 3′ primers used for TCRβ4, and two internal primers containing the mismatched codons corresponding to position 238–241 of β5 were used. The TCRβ4 and β5 contain *Pst*I/*Bgl*II sites at both ends.

The final PCR products were digested with appropriate enzymes, purified using diethylaminoethyl (DEAE) membrane (Schleicher & Schuell, Keene, NH), and cloned into the polylinker of the expression plasmids pCDM8,[14] pCDL-SRα[15] and pRSV.5.neo (gift from Dr R. Klausner, National Institutes of Health (NIH), Bethesda, MD) respectively. A truncated TCRα (α_t) in pCDM8 vector comprising almost all of the extracellular domain of TCRα, including the inter-chain bond-related cysteine,[16] was a gift from Dr J Bonifacino, NIH.

Sequence analysis

The nucleotide sequences of all the chimeric and mutant cDNA were determined using the chain termination DNA sequencing method.[17] The sequence reactions were performed by using the Sequenase Version 2 kit (US Biochemical Corporation, Cleveland, OH).

Cells and antibodies

COS-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM, ICN Biomedicals Inc. Costa Mesa, CA) supplemented with 10% fetal calf serum. A variant of the 2B4 T-cell hybridoma (21·2·2) lacking 2B4 TCRα and β chains[18] was used as host for stable transfection of 2B4 TCRα, and β genes. The mAb used in these studies were: A2B4, a mouse IgG2a specific for the TCRα chain of the T-cell hybridoma 2B4;[19] KJ25, a mAb recognizing the TCRβ chain (gift from Dr P. Marrack, Denver, and Dr A. Kruisbeek, Amsterdam); R223, an affinity-purified rabbit mAb raised against a peptide consisting of cytoplasmic residues 242–251 of the mature Tac protein (gift from Dr R. Robb, Wilmington, Delaware); 145-2C11 (2C11), a hamster mAb directed against extracellular domain of the CD3-ε chain;[20] and H57–597, a hamster anti-mouse mAb specific to the C_β domain[21] (provided by Dr A. Kruisbeek).

DNA transfection and production of chimeric proteins

cDNA encoding the mutant β constructs were cotransfected with TCRα chain (20 μg in total) into COS-7 cells using the

Table 1. Primers used in the construction of mutant and chimeric TCRβ chains

	Sequences	
	β portion primers	Tac portion primers
β1	5′-GCGGCCGCGCGGCCGCCCCACCATGGCTACAAGGCTC-3′	3′-GCG GCC GCG CGG CCG CCT AGA TTG TTC TTC TAC TCT T-5′
	3′-AGG TTG AGG TGT TAA CAC GAG GAG CCG AGT-5′	5′-CTC CTC GTG TTA ACA CCT CAA CCT GAA GAA-3′
β2	5′-GCGGCCGCGCGGCCGCCCCACCATGGCTACAAGGCTC-3′	3′-GCG GCC GCG CGG CCG CCT AGA TTG TTC TTC TAC TCT T-5′
	3′-GTG GGT CAT TTT GCT CAG GCA GTA GCT ATA-5′	5′-TAC TGC CTG AGC AAA ATG ACC CAC GCG AAG-3′
β3	5′-GCGGCCGCGCGGCCGCCCCACCATGGCTACAAGGCTC-3′	3′-GCG GCC GCG CGG CCG CCT AGA TTG TTC TTC TAC TCT T-5′
	3′-ACA GCC GGC CAC TGC TAC CTC ATA GAG GAT GGT-5′	5′-ACC ATC CTC TAT GAG GTA GCA GTG GCC GGC TGT-3′
β6	5′-CTG CAG CTG CAG CCC ACC ATG GCT-3′	3′-GCG GCC GCG CGG CCG CCT AGA TTG TTC TTC TAC TCT T-5′
	3′-TTC ACC TGG AAA CTG AAG CCC ATG GAA-5′	5′-TCC CAT GGG CTT CAG TTT CCA GGT GAA-3′
β7	5′-CTG CAG CTG CAG CCC ACC ATG GCT-3′	3′-GCG GCC GCG CGG CCG CCT AGA TTG TTC TTC TAC TCT T-5′
	3′-AAA ATC TGT TGT TGT CCA GGC CTC TGC ACT-5′	5′-AGT GCA GAG GCC TGG ACA ACA ACA GAT TTT-3′
	External primers	Mismatch primers
β4	5′-CTG CAG CTG CAG CCC ACC ATG GCT-3′	3′-AGT GAT TCC ACT GTC TGC TCG-5′
	3′-AGA TCT AGA TCT GTC TCA GGA ATT TTT-5′	5′-CGA GCA GAC AGT GGA ATC ACT-3′
β5	5′-CTG CAG CTG CAG CCC ACC ATG GCT-3′	3′-AAG CTT ACT CAT GCC CCA GGC CTC TGC ACT GAT GTT-5′
	3′-AGA TCT AGA TCT GTC TCA GGA ATT TTT-5′	5′-ATG AGT AAG CTT GGA ATC ACT TCA GCA TCC TAT-3′

* Underlined nucleotides are codons responsible for converting Cys^{241} to Ser in TCRβ4 or converting ArgAlaAspCys to MetSerLysLeu from position 238 to 241 of TCRβ5.

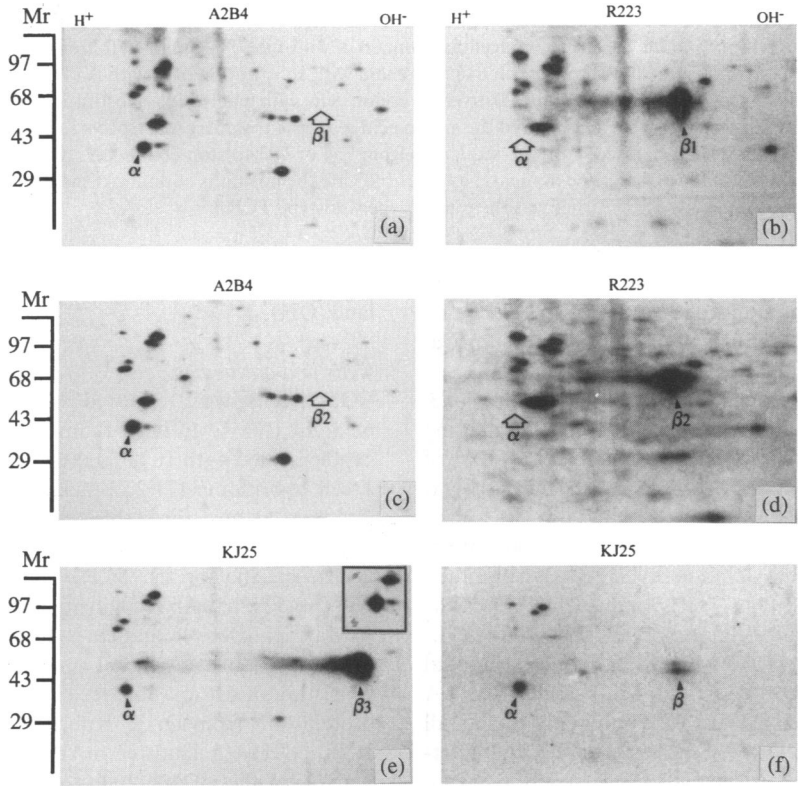

Figure 2. Two-dimensional NEPHGE/SDS–PAGE gel analysis of cotransfection of TCRα chain with TCRβ, β1, β2 and β3 in COS-7 cells, respectively. Cells were metabolically labelled, solubilized and immunoprecipitated with A2B4, R223, or KJ25 mAbs. Positions of the TCR components are indicated. TCRα and TCRβ were identified as an acidic 35 000–38 000 MW and a neutral 45 000 MW protein, respectively. Open arrows show locations of predicted chains. Inset (e) shows the total amount of TCRα precipitated by mAb A2B4. TCRα was coprecipitated with TCRβ3 (e) and β (f), but not β1 (a, b) and β2 (c, d). The molecular mass (MW) standards are indicated on the left in kDa.

calcium phosphate precipitation procedure as previously described.[22] To generate stable cell lines, 21.2.2 cells (1×10^7) were transfected with 20 μg of caesium chloride-purified plasmid DNA by electroporation, using a Bio-Rad Gene Pulser apparatus under conditions of 200 V and 250 μF. After 24 hr, the cells were transferred into 96-well plates and selected in medium containing 1 mg/ml Geneticin (Gibco BRL, Grand Island, NY). Clones of resistant cells were visible within 2–3 weeks, and assessed by flow cytometry (Coulter, Hialeah, FL) using A2B4, 2C11 and H57–597 mAb.

Metabolic labelling and immunoprecipitation

These methods have been described in detail previously.[13] In brief, cells transfected with cDNA were labelled with ^{35}S-labelled methionine (0·5 mCi/ml) and ^{35}S-labelled cysteine (0·5 mCi/ml) (ICN Biomedicals, Irvine, CA), and solubilized with lysis solution (0·5% Triton-X-100, 0·3 M NaCl, 50 mM Tris–HCl buffer, pH 7·4) containing phenylmethylsulphonyl fluoride (1 mM), iodoacetamide (10 mM) and leupeptin (10 μg/ml). The lysate was then incubated with specific antibody-coated protein A agarose beads for 1 hr at 4°. The beads were then washed, and resuspended in isoelectric focusing sample buffer containing 9·5 M urea, 0·2% Nonidet P-40 and 0·2% ampholytes at pH 3·5–10 (Serva), followed by incubation at 37° for 1 hr.

Two-dimensional gel electrophoresis

The samples were run under two-dimensional non-equilibrium pH gel electrophoresis (NEPHGE)/sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) according to O'Farrell *et al.*[23] In the first dimension, samples were electrophoresed in a 5% acrylamide tube gel containing 0·5% of pH 3·5–10 ampholytes, 8 M urea, 0·2% Nonidet P-40 for 2500 V hr. The second dimension consisted of SDS–PAGE using 13% acrylamide gels cross-linked with acrylaide attached to Gel-Bond (FMC, Rockland, USA) and were run after equilibration in pH 6·8 buffer. Gels were fixed, salicylated and oven dried prior to autoradiography.

RESULTS

Extracellular C-domain of TCRβ is the binding region between TCRα and β chains

To define the TCRβ region responsible for assembly with TCRα, full length TCRα was cotransfected with TCRβ, β1, β2 or β3, respectively, into COS-7 cells, followed by metabolic labelling and immunoprecipitation. The cotransfected TCRα chain was immunoprecipitated with TCRβ3 or TCRβ using mAb KJ25 specific to TCRβ chain (Fig. 2, e, f). Similarly, TCRβ or β3 was coprecipitated with the TCRα chain using mAb A2B4 specific to the TCRα. By contrast, the TCR$\alpha$was not coprecipitated with TCRβ1 and β2 using mAb A2B4 or R223 (Fig. 2, a–d). These results were confirmed using the TCRα_t construct which lacks the transmembrane and cytoplasmic domain of TCRα. There was no coprecipitation of TCRα_t with TCRβ2 (Fig. 3) or β1 (data not shown) when assessed by R223 mAb. These findings suggest that the C$_\beta$ domain is the binding region of TCRβ to α chain, whereas neither the intact VDJ$_\beta$ with a short portion of the C$_\beta$ (included in TCRβ1 and β2), nor the transmembrane domain and cytoplasmic tail (excluded from β3) are required for the TCRα and β chain assembly.

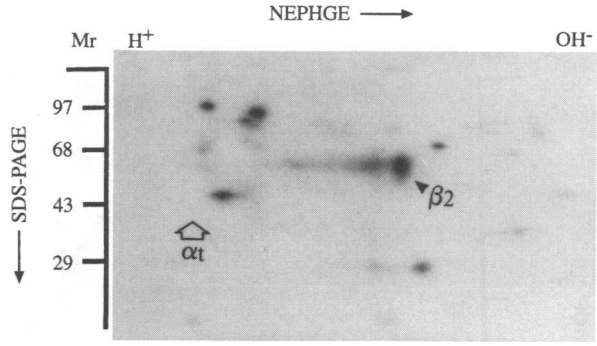

Figure 3. Two-dimensional NEPHGE/SDS–PAGE gel analysis of cotransfection of the TCRα_t and β2 in COS-7 cells. TCRβ2, but not TCRα_t, is immunoprecipitated by R223 mAb. The position of predicted TCRα_t chain is indicated by open arrow. The relative positions of the molecular size standards are indicated on the left in kDa.

To define further the rest of the C$_\beta$ domain excluded from β1 and β2 chains, another two chimeric TCRβ chains (β6 andβ7) with longer C$_\beta$ lacking the last 57 and 24 amino acids of the extracellular C$_\beta$, respectively, were constructed (Fig. 1). Cotransfection of these constructs with TCRα into COS-7 cells resulted in TCR$\alpha\beta$6 and $\alpha\beta$7 heterodimers formation as shown by A2B4 or KJ25 mAbs (Fig. 4). These findings suggest that

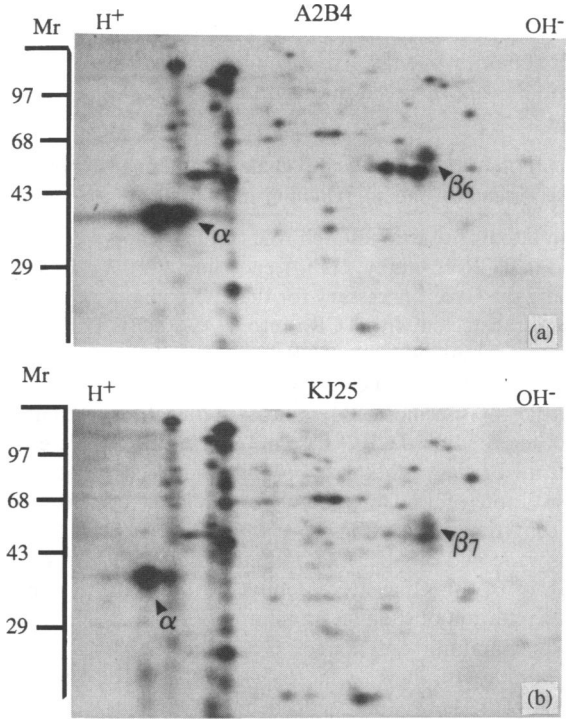

Figure 4. Two-dimensional NEPHGE/SDS–PAGE gel analysis of the interaction between TCRα chain and chimeric TCRβ6, or TCRβ7 proteins. COS-7 cells were cotransfected with wild-type TCRα and TCRβ6, or TCRβ7 genes. Heterodimer TCR$\alpha\beta$6 (a) or TCR$\alpha\beta$7 (b) was immunoprecipitated using A2B4 or KJ25 mAb. Positions of the TCR components are indicated. The molecular size standards are marked on the left in kDa.

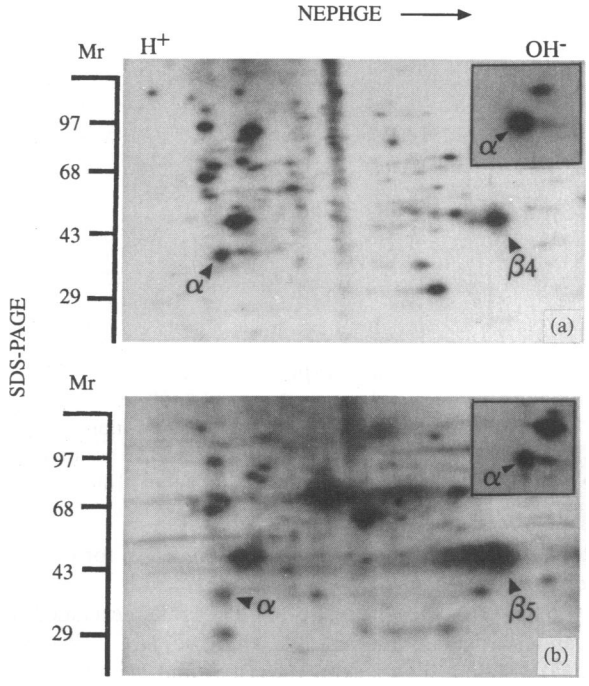

Figure 5. Two-dimensional NEPHGE/SDS–PAGE gel analysis of the interaction between TCRα and TCRβ4 or β5. Co-precipitation of TCRα with TCRβ4 (a) or β5 (b) are shown after immunoprecipitation with KJ25 mAb. Positions of TCR proteins are indicated. Insets show the total amount of TCRα as precipitated by A2B4. Molecular markers in kDa are marked on the left.

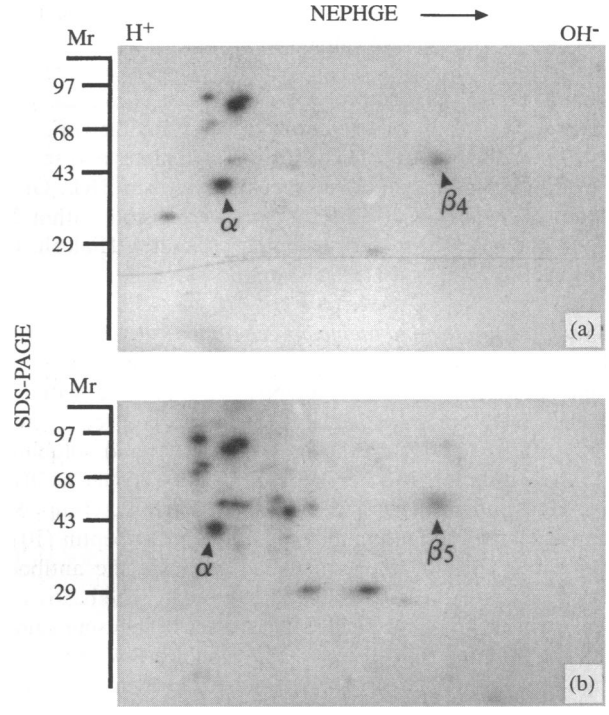

Figure 6. TCRα and β interaction in T cells in the absence of the interchain disulphide bond. TCRαβ4 or TCRαβ5 transfected cells were immunoprecipitated with KJ25 mAb, and analysed by NEPHGE/SDS–PAGE. The assembly of TCRαβ4 (a) or TCRαβ5 (b) is shown by the ability to coprecipitate TCRα using KJ25 mAb. Molecular sizes in kDa are indicated on the left.

the C_β region included in β6 is sufficient for assembly with TCRα chain, while the last 57 amino acids of the Cβ excluded from β6 is not involved in this process.

Formation of the TCRα and β chain heterodimer in non-T cells in the absence of the interchain disulphide bond

In an attempt to evaluate the role of the clonotypic interchain bond in TCR assembly, cDNA encoding a mutant TCRβ (β4) lacking the Cys^{241} necessary for the interchain bond formation was cotransfected with TCRα into COS-7 cells. TCRα protein was coprecipitated with TCRβ4 using TCRβ specific KJ25 (Fig. 5a), while the TCRβ4 was coprecipitated with α chain using TCRα-specific A2B4, indicating that the interchain bond is not required for TCRαβ assembly. These results were further confirmed using another two constructs: TCRβ5, carrying a substitution of the interchain bond-related cysteine and its adjacent three amino acids; and $TCR\alpha_t$, lacking the transmembrane and cytoplasmic domains of α chain. As shown in Fig. 5b, TCRβ5 efficiently assembled with TCRα, evidenced by coprecipitation of the two proteins by KJ25 mAb. In addition, $TCR\alpha_t$ containing only VJ and C domains displayed the ability to assemble with TCRβ4, shown by A2B4 and KJ25 mAb (data not shown). These experiments demonstrate that the interchain disulphide bond between the TCRα and β is not required for TCRαβ heterodimer formation in COS-7 cells.

TCRα and β chain assembly in T-cells without participation of the interchain disulphide bond

To study the impact of deleting the clonotypic interchain disulphide bond on TCRαβ interaction in T cells, TCRα was electroporated with TCRβ4 or TCRβ5 into 21.2.2 cells to form stable cell lines. The cell clones were selected in Geneticin, followed by limiting dilution. Coexpression of TCRα and β chains on the cell surface of each clone was confirmed by dual staining and flow cytometry (manuscript submitted). In these transfectants, TCRα chain was coprecipitated with TCRβ4 or TCRβ5 using KJ25 mAb (Fig. 6). Similarly, the mutant β chains were coprecipitated with the TCRα chain by A2B4 mAb. These results are in agreement with the findings from COS-7 cell transfections, suggesting that TCRβ4 and β5 could assemble with TCRα in T-cells in the absence of the TCRαβ interchain disulphide bond.

DISCUSSION

The crystal structure of a TCRβ chain has recently been resolved by Bentley *et al.*[24] The extracellular portion of the β chain, in general, shows structural homology to immunoglobulin, but the V_β and C_β are in closer contact with each other than those of immunoglobulin, presumably restricting the flexibility of the β chain, which may be biologically required. This finding is supported by a previous V-C shuffling study showing that the V and C domains of TCRα and β are structurally intimate.[25] We found that when a portion of the C_β domain was deleted, the TCRβ despite having an intact VDJ region and an adjacent short C_β (TCRβ1, β2), was unable to bind to the TCRα chain. However, TCRαβ assembly was observed when a longer C_β domain was included (β3, β6, β7). It

appears that the C_β domain contains a segment of amino acids which are structurally responsible for TCR$\alpha\beta$ chain assembly. The extracellular C_β domain is completely exposed to solvent,[24] and it may provide the required interface necessary for binding to the TCRα chain. Taken together with our previous study showing that alterations in the C_β region altered the antigen epitope in the V domain recognized by a V_β-specific mAb,[26] it is suggested that the extracellular C_β region is the binding site to the α chain, and also influences the overall shape of the V_β domain. The close proximity of the V_β–C_β domains is probably essential to maintain the functional structure of the TCR molecules.

The structural features of the C_β region remain to be characterized, but the presence of a large number of conserved residues and a net positive charge in this domain may be responsible for the TCRα and β chain interaction. The charged amino acids present on TCRα and TCRβ chains may facilitate the heterodimer formation by electrostatic attraction induced by the net positive charge on the C_β and the negative charge on the C_α.[24] The direct effect of these amino acids on TCRα and β chain assembly has not been assessed, but it is possible that they contribute to the conformational structure of the C_β domain, and thereby influence TCRα and β chain interaction. It has been shown that the cell surface expression of TCR can be prevented by substitution of the conserved cysteine^{191} or phenylalanine^{195} in the C_β domain.[27,28] In the present study, loss of a portion of the C_β from Ser^{188} to Leu^{213} (excluded from β2) led to failure of TCR$\alpha\beta$ assembly, whereas assembly occurred in the presence of this sequence (β6). It appears that the Ser^{188}–Leu^{213} region, containing two conserved and seven positively charged amino acids, may be responsible for the extracellular TCRα and β chain interaction. As at present, we have not made a construct that solely lacks the Ser^{188} to Leu^{213} region and transfected this construct into T cells to examine for cell surface expression. Presumably since this construct also lacks Cys^{191} and Phe^{195} residues it would not be expected to be expressed to the cell surface. It is likely that these specific residues are not only involved in TCR$\alpha\beta$/CD3 and ζ chain interaction,[29] but are also the structural requirements for TCRα and β heterodimer formation.

The exact role of the $\alpha\beta$ interchain disulphide bond in TCRα and β chain interaction and T-cell activation has not previously been evaluated. We have shown in this study that mutation of the interchain disulphide bond did not prevent the binding between TCRα and β chains in both fibroblasts and T cells. Moreover, the TCR$\alpha\beta$ interaction was not affected by removal of the interchain bond-containing region, consisting of the tetrapeptide RADC (β5), and the last 57 amino acids of extracellular C domain at the C-terminus of the TCRβ chain (β6). These findings imply that the interchain bond is not critical for TCRα and β chain interaction. The initial $\alpha\beta$ heterodimer formation may depend largely on extensive contact, such as formation of salt-bridges and van der Waals interactions between the C_α and C_β sites. A precedence exists in the $\gamma\delta$ T cells where the clonotypic dimers do not need to be disulphide-linked for TCR surface expression. This may endow the $\gamma\delta$ T cells with different stoichiometry and signal transduction features.

In conclusion, the C domain of TCRβ is responsible for binding to the TCRα chain. A number of charged and conserved residues in the C_β region, primarily between Ser^{188} and Leu^{213}, may be involved in the TCRα and β chain interaction. Substitution of the TCR$\alpha\beta$ interchain bond-related cysteine^{241} with different amino acid did not abrogate TCRα and β assembly. It appears that the unique interchain bond is not required for the initial TCRα and β chain assembly but may play an important role in stabilizing the molecular structure so that a functional TCR complex can occur.

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