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In vitro preparation and characterization of the human CD3εε homodimer and CD3εγ and CD3εδ heterodimers

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Received May 13, 2009; Accepted July 1, 2009

DOI: 10.3892/ijmm_00000250

Abstract. The CD3 molecule is a critical component of both humoral and cellular immune responses, and yet while the structure and molecular assembly of other key mediators such as CD4 and CD8 have been reported, individual CD3 subunits have not been well characterized. Our understanding of the manner in which they interact remains limited, and the question of how many subunits are required for a functional CD3 molecular complex is yet to be addressed. It has been suggested that CD3ε pairs with CD3γ or with CD3δ, forming CD3εγ and CD3εδ heterodimers that associate with α/ß T cell receptors (TCRs) and CD3 ζ 2 dimers. In this study we investigated whether interactions between each CD3ε subunit play a role in the formation of the CD3 molecular complex. Our results revealed that the human CD3ε subunit forms a homodimer structure, which is a crucial piece of information for the elucidation of cellular signaling following TCR receptor ligation, and provide insight into our understanding of the molecular assembly of the CD3 molecular complex.

Introduction

T cell activation is the central event in the adaptive immune system, which is initiated by the interaction involving T cell receptors (TCRs), its co-receptors CD4 and CD8, the accessory molecule CD2, and the co-stimulatory receptors CD28 and CTLA-4 with antigen-MHC molecules and other ligands on the surface of an antigen-presenting cell (1,2). This interaction initiates a cascade of biochemical events in the T cell that eventually results in T cell growth, differentiation and proliferation, consequently inducing the production of different T cell subsets, such as Th1, Th2, Th17, Treg, TC1 or TC2 (3). The T cell receptor is characterized by an extremely diverse population of different surface molecules which facilitate highly specific responses to a large number of different antigens (4). The TCR is a multimeric complex comprised of two ligand-binding glycoproteins containing variable regions (α/ß TCR or γ/δ TCR heterodimer) which are expressed on the cell surface in association with CD3 molecules.

CD3 molecules consist of at least four different invariant subunits, CD3γ, δ, ε and ζ (5,6). CD3ε, CD3γ and CD3δ subunits contain an extracellular Ig-like domain, a membrane-proximal stalk region, a transmembrane (TM) helix and a cytoplasmic tail. In contrast, CD3 ζ-chains are composed of a short 9-amino acid extracytoplasmic domain, a transmembrane domain of 21 amino acids containing a cysteine involved in homodimerization as well as a negatively charged amino acid residue, and a cytoplasmic domain of 112 amino acids containing three tandem ITAM motifs (7). CD3 molecules transduce an antigen signal by forming the signaling subunits which contain ten copies of a semi-conserved cytoplasmic amino acid sequence (YxxLx6-8YxxL) termed the immunoreceptor tyrosine-based activation motif (8). This molecule serves as the nucleating point for the intracellular signal transduction machinery upon TCR engagement. Each of the CD3ε, CD3γ and CD3δ subunits contains one ITAM, and CD3ζ contains three ITAMs (9). Upon receptor ligation, two tyrosine residues within each ITAM are rapidly phosphorylated by a member of the Src-family protein tyrosine kinases (PTK), transforming them into high-affinity ligands for the Syk PTK (10). The coordinated actions of Src and Syk PTK initiate a cascade of signals that ultimately leads to T cell proliferation, cytokine secretion and effector functions (9).

The CD3 antigen is highly specific for T cells and is present in the majority of T cells. The extracellular domain of the CD3ε, CD3γ and CD3δ subunits aids in the formation of invariant dimer structure. Existing data have indicated that CD3ε can pair with CD3γ or with CD3δ, forming CD3εγ and CD3εδ heterodimers that associate with the α/ß TCRs and CD3 ζ 2 dimers. Given that CD3ε forms alternate dimers with CD3γ and CD3δ, the question is whether both types of

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Key words: human CD3, T cell receptor, homodimer, heterodimer

dimers are essential for TCR assembly or can one substitute for the other. The answer to this question remains to be addressed. Although CD3γ and CD3δ each dimerizes with CD3ε, there are differential requirements for each CD3 subunit receptor in receptor assembly and signaling transduction. It is presumably mandated by the unique structural feature of individual CD3 subunits. Furthermore, the composition of the γδ TCR/CD3 complex may differ from that of the α/ß TCR/CD3 complex. A complete understanding of the structure and assembly of each human CD3 subunit is crucial if these questions are to be addressed.

In the present study, we expressed human CD3ε, CD3γ, and CD3δ subunit molecules in *E. coli*, by *in vitro* refolding and purification. We found that, in addition to the expected CD3εγ and εδ heterodimers, the prepared CD3 subunit formed other different dimer structures. Human CD3ε may itself have the ability to form homodimer structure. Surface plasmon resonance analysis revealed that the identified CD3εε homodimers were unable to directly bind with TCR. This function correlates with the complex interaction between the different CD3 subunits and may explain the multiple regulatory activity of the TCR-CD3 molecular complex.

Materials and methods

Gene cloning and expression plasmid construction. Human CD3ε, CD3γ and CD3δ genes were amplified from the human $CD4^+$ T cell cDNA library. This cDNA library was generated by reverse transcription of the $poly(A)^+$ RNA isolated from human $CD4^+$ T cells with oligo(dT) primer (Invitrogen Life Technologies, Paisley, UK), followed by T4 DNA polymerase to obtain the double-stranded cDNA. The coding region of the human CD3 cDNA was amplified by PCR with several pairs of primers: CD3γ sense primer, 5'-GGC TCT AGA CAT ATG CAG TCT ATC AAA GGT AAC CAC TTG GTT A-3' and an antisense primer, 5'-GCA ACC ACC ACC GTC ATC CTG ACT CAT TCT GTA ATA CAC TTG GAG-3'; CD3δ sense primer, 5'-GGC TCT AGA CAT ATG TTC AAA ATT CCT ATA GAG GAA CTT GAG-3' and an antisense primer, 5'-GCA ACC ACC ACC GTC ATC CTG GCT CAC TCG ATA ATG AAC TTG CAC-3'; CD3ε sense primer, 5'-GGC TCT AGA CAT ATG CAA GAT GGT AAT GAA GAA ATG GGT GG-3', and an antisense primer, 5'-CCC AAG CTT GGA TCC TCA GTC ATC ACA CCC ACC TCC ACG GTT CTC ACT CAC TCT TGC CCT-3'. PCR was performed using standard conditions (94˚C for 1 min, 55˚C for 1 min and 72˚C for 1.5 min; 30 cycles). The PCR product was purified using PCR Purification Kit (Qiagen Ltd., Sussex, UK) according to the manufacturer's instructions and was ligated to pGEM-T Easy Vector (Promega UK Ltd., Southampton, UK). For the modification of the CD3γ and CD3δ genes, two additional PCR reactions were performed using the CD3γ and CD3δ forward primer and a common reverse primer (5'-CCC ACC AAC CTT GGA TCC TCA GTG GTG ATG GTG GTG ATG ACG TTT GCA ACC ACC ACC GTC-3') designed to generate a cysteine containing connecting peptide and His-6-Tag for protein expression identification, refolding and purification. The optimized CD3ε, CD3γ and CD3δ genes were subcloned into the T7 expression vector pGMT7. The positive clones were determined by enzymatic digestion, and three expression cassettes were constructed (Fig. 1). A further sequence was confirmed by DNA sequencing.

Protein expression and refolding. Plasmids containing the human CD3ε, CD3γ and CD3δ genes were transformed into *E. coli* Rosetta (DE3) cells (Stratagene, La Jolla, CA) and plated on LB medium supplemented with ampicillin (100 μg/ml) at 37˚C. A single colony of Rosetta (DE3) cells harboring the different human CD3 expression plasmids was chosen and inoculated in 1 liter of warm TYP medium (16 g/l tryptone, 16 g/l yeast extract, 5 g/l NaCl and 2.5 g/l K_2HPO_4) in the presence of ampicillin (100 μg/ml) with shaking at 37˚C to a cell density at OD 600 nm of 0.6. Expression of the recombinant proteins in the transformed cells was induced using 0.5 mM isopropyl b-D-thiogalactopyranoside for an additional 12 h at 30˚C. The cells were harvested by centrifugation at 6000 x g for 15 min, and the subsequent inclusion bodies were harvested by lysis in 20 ml lysis buffer (10 mM Tris, 10 mM $MgCl_2$, 150 mM NaCl and 10% glycerol), sonication and treatment with Triton wash buffer [0.5% Triton X-100, 50 mM Tris (pH 8.0), 100 mM NaCl and 10 mM EDTA]. The protein, in the form of inclusion bodies, was washed and then dissolved in guanidine buffer (6 M guanidine, 50 mM Tris, 2 mM EDTA and 100 mM NaCl). Refolding of human CD3ε, CD3γ and CD3δ was carried out as previously described (11). Briefly, the CD3ε - CD3γ and CD3ε - CD3δ heterodimers were prepared by mixing 30 mg of CD3γ or CD3δ proteins with the same amount of CD3ε at 37˚C for 15 min, while the refolding of the CD3ε homodimer was carried out using 60 mg of CD3ε in the absence of CD3γ and CD3δ using the same protocol. This was then added to 1 liter cold refold buffer [50 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0), 400 mM L-arginine, 0.74 g/l mercaptoethylamine, and 0.83 g/l cysteine]. The refold was allowed to mix at 4˚C for at least 1 h before dialysis against 10 volumes of the 10 mM of Tris (pH 8.0) dialysis buffers at 4˚C for 2 days; the dialysis buffer was changed once at 24 h after initial dialysis.

Determination of the protein concentration. The protein concentration was determined using the Bradford method with the Bradford reagent kit (Sigma-Aldrich). Bovine serum albumin was used as the standard control.

Initial purification by anion exchange chromatography. After refolding, the resulting protein was then filtered and anion exchanged using a Poros 50HQ column (Applied Biosystems) equilibrated with 10 mM Tris (pH 8.0). Purification steps were performed on an Akta Explorer FPLC system at room temperature. Different human CD3 dimers were eluted with a linear NaCl gradient of 0-1 M. The collected fractions containing protein were pooled and concentrated on an Amicon Ultra-15 centrifugal filter by centrifugation at 5000 rpm at 4˚C for 20 min. The purity of each protein was measured using an SDS-PAGE 10% Bis-Tris gel (Invitrogen).

Second purification using immobilized-metal affinity chromatography. The His-tagged human CD3ε - CD3γ and CD3ε - CD3δ heterodimer recombinant proteins were further purified using immobilized metal-ion affinity chromatography

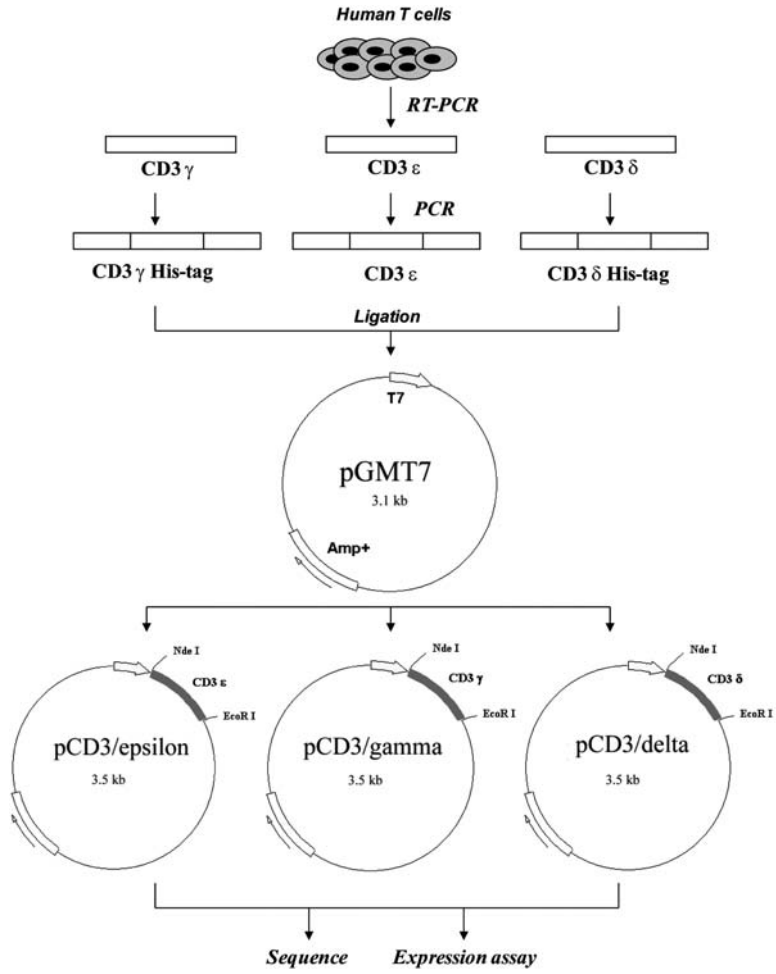

Figure 1. Schematic representation of the construction of expression vectors. Human CD3γ, ε and δ genes were amplified from PBMCs by RT-PCR. The encoding sequences of the individual protein extracellular domains were cloned into the prokaryotic expression vector pGMT7, and three expression vectors were generated. All of the constructs were confirmed by DNA sequencing.

(IMAC). The resulting solution containing different human CD3 heterodimers was dialyzed overnight at 4˚C in 10 volumes of protein binding buffer [5 mM imidazole, 300 mM NaCl and 50 mM NaH_2PO_4 (pH 8.0)] before loading onto a column containing 2 ml Ni-NTA, equilibrated with the same protein binding buffer. After washing the column with 10 CV of the washing buffer [20 mM imidazole, 300 mM NaCl and 50 mM NaH_2PO_4 (pH 8.0)], the bound protein was eluted in a step-wise manner with buffer containing 300 mM NaCl and 50 mM NaH_2PO_4 (pH 8.0) and increasing concentrations of imidazole: 50, 200 or 500 mM, respectively. The active fractions eluted from the affinity column were pooled, concentrated by Amicon-10 kDa (Millipore), and the retentate-containing concentrated protein was finally dialyzed in storage buffer [20 mM Tris-HCl and 100 mM NaCl (pH 8.0)].

Analytical gel filtrations. A Superdex 75 HR column was used for gel permeation chromatography (GPC) purification in-line on an Akta Explorer FPLC system at 4˚C. The column was equilibrated with 2 column volumes of running buffer (20 mM Tris and 100 mM NaCl) at pH 8.0. Protein (500 μl) from the IMAC column was injected onto the GPC column, and the elusion buffer [20 mM Tris, 100 mM NaCl (pH 8.0)] was used as the mobile phase. The purification diagram was recorded, and protein peaks were compared to measure the protein molecular weight. The peaks in the chromatogram corresponded to the protein of different human CD3 heterodimers or homodimers; the protein corresponding to the largest peak was stored at 4˚C.

Surface plasmon resonance. The interaction of recombinant human CD3 molecules (CD3) with TCRs was performed using surface plasmon resonance on a BIAcore™ 2000 (BIAcore, Uppsala, Sweden). TBS [20 mM Tris-HCl (pH 8.0) and 100 mM NaCl] with 1 mM cation ($CaCl_2$, $MgCl_2$ or $MnCl_2$) was used as the running buffer.

Results

Construction of expression vectors. The objective of this study was to produce and characterize the human CD3 protein for structural and functional studies. The human CD3 receptor complex consists of several different subunits, but their molecular interaction has never been previously demonstrated. Full length cDNA encoding the human CD3 molecule extracellular domain including CD3ε, CD3γ and CD3δ chains were amplified from human peripheral blood T lymphocytes using RT-PCR and sequenced. Sequences were further

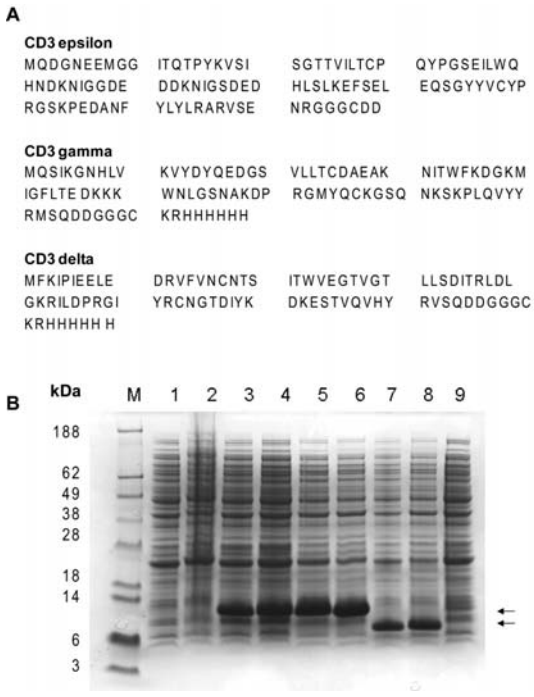

Figure 2. Expression and deduced amino acid sequences of recombinant human CD3 proteins. (A) The deduced amino acid sequences of expressed recombinant CD3γ, δ and ε molecules. (B) Coomassie-stained SDS-PAGE loaded with whole cell lysate of different samples The mobility of the protein markers (SeeBlue, Invitrogen) are shown in lane M. Lanes 1 and 2 are the sham expression control in which *E. coli* was transformed with the pGMT7 vector. Lanes 3 and 4, CD3ε expression; lanes 5 and 6, CD3γ expression and lanes 7 and 8, CD3δ expression samples. Lane 9, non-transformed *E. coli* cell lysate control. The arrow indicates the bands of recombinant proteins.

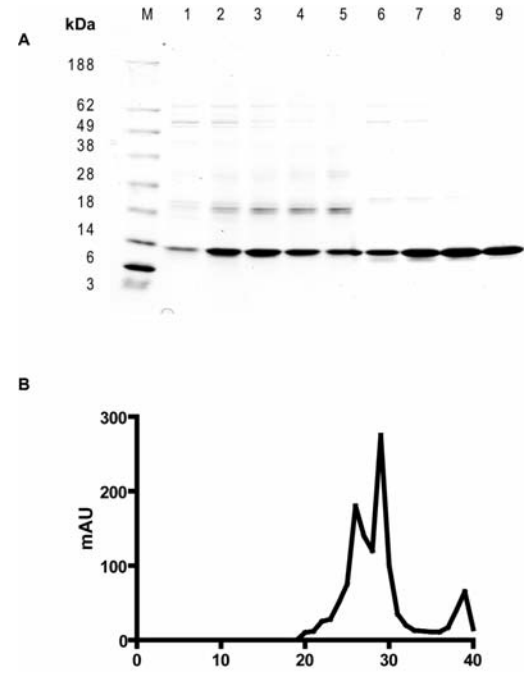

Figure 3. Refolding and purification of human CD3ε homodimers. (A) The expressed human CD3ε proteins were refolded and purified following the standard protocol described in Materials and methods. The purified products were assessed by SDS-PAGE under a no-denatured or denatured condition. Coomassie blue-stained no-denatured SDS polyacrylamide gel revealed the formation of CD3ε homodimers with molecular weight of 24 kDa (lanes 2-6); homodimers were degraded to become 12-kDa monomers when run under a denatured condition (lanes 7-10). Lane M, molecular mass marker. Following anion exchange chromatography, the eluted proteins were assessed for their molecular weights by gel filtration chromatography. (B) The purification profile of gel filtrations. UV absorbance at 280 nm and conductivity were detected real-time. Two major eluted fractions containing CD3ε homodimers and monomers are indicated by an arrow. Eluted peak 1 corresponds to SDS-PAGE lanes 3-6 (dimeric forms). Peak 2, CDε monomeric forms, SDS-PAGE lanes 7-10.

modified by a secondary PCR to introduce a carboxy-terminal flanking sequence designed to promote the formation of an interchain disulfide bridge during *in vitro* refolding (12). In addition, to facilitate the purification of the CD3γ and CD3δ protein, a fusion Histidine tag was incorporated in the C-terminal part of the protein.

The CD3 expression vectors were created to express CD3ε residues 22-118, CD3γ residues 22-103, and human CD3δ molecule residues 22-91, respectively as C-terminus fusion-proteins. The deletion of 21 residues at the N-terminus of the construct was carried out to exclude the signal peptide region. To achieve the no fusion expression in the N-terminus, a *Ned*I enzyme site was used for the vector construction. The ATC start codon contained inside of the *Nde*I enzyme was used for the start of transcription. The deduced amino sequence of the expected expressed product is shown in Fig. 2A.

Expression of human CD3ε, γ and δ subunits. Human CD3 expression vectors were transformed into *E. coli* BL21 (DE3), and the expression of different CD3 subunits was optimized by adjusting the concentration of IPTG, the time after induction (4-24 h) and the growth temperature (from 25-37˚C). Optimal expression of the CD3 subunit was obtained 16 h after induction with 0.5 mM IPTG at 30˚C. Electrophoresis of the IPTG-induced cell lysate showed the presence of a heavily stained protein band (Fig. 2B, lanes 3-8) which, by densitometry, accounted for >40% of the total cell protein while being virtually absent in the non-transformed cell lysate (Fig. 2B, lane 9) or blank vector transferred control (Fig. 2B, lanes 1 and 2). The apparent molecular mass of the overexpressed protein was estimated by SDS-PAGE at ~10-12 kDa, consistent with the value of 12,046, 11,295 and 10,159 Da calculated by the Expasy Tool Compute pI/MW for human CD3ε, CD3γ and CD3δ, respectively. The expressed products were confirmed by Western blot assay with anti-human CD3 antibody (data not shown). The cell lysate was separated into soluble and insoluble fractions. The insoluble fraction of the IPTG-induced cell lysate contained a protein band with an apparent molecular mass corresponding to that of human CD3ε, CD3γ and CD3δ, which was virtually absent in the soluble fraction of the non-induced cell lysate.

Refolding and purification of the CD3εε homodimers. The human CD3ε subunit has been reported to be able to form CD3εγ and CD3εδ heterodimers by binding with the CD3γ and CD3δ subunits. However, whether or not human CD3ε itself has the ability to form a homodimer is still unknown. To test this hypothesis, the expressed human CD3 protein was refolded and purified with ion exchange chromatography. The proper folding and purification of human CD3 was assessed by SDS-PAGE. As shown in Fig. 3A, the

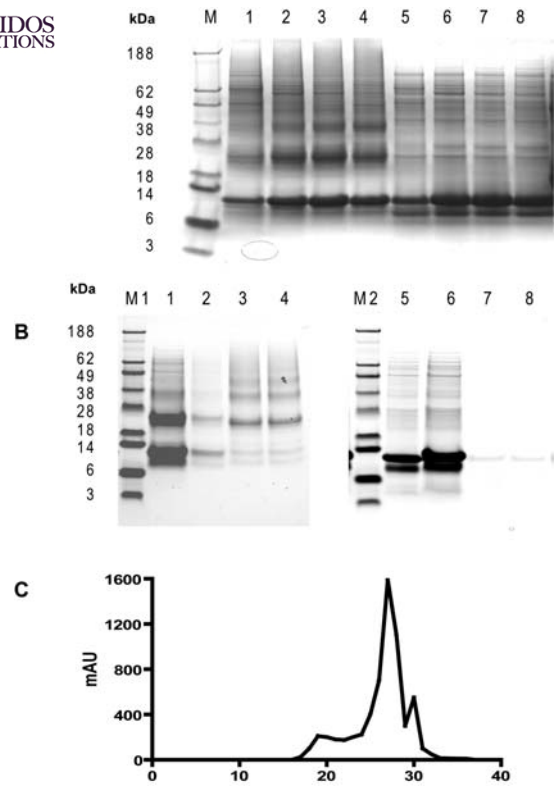

Figure 4. Refolding and purification of human CD3εγ heterodimers. (A) Human CD3εγ heterodimers were refolded and purified using the Poros 50HQ column. The initial purified products were assessed by SDS-PAGE under a no-denatured or denatured condition. Lane M, molecular mass marker; lanes 1-4, elution fractions assessed by SDS-PAGE in no-denatured condition; lanes 5-8, elution fractions assessed by SDS-PAGE in denatured condition. (B) Following initial anion exchange chromatography, the refolded proteins were further purified by immobilized-metal affinity chromatography (IMAC). SDS-PAGE illustrates secondary purification results using Ni-NTA column. Lane M, molecular mass marker; lanes 1-4, elution fractions assessed by SDS-PAGE under a no-denatured condition; lanes 5-8, elution fractions assessed by SDS-PAGE under a denatured condition. (C) Following IMAC, the eluted proteins were assessed for their molecular weights by gel filtration chromatography. The diagram shows the purification profile of gel filtrations. UV absorbance at 280 nm and conductivity were detected real-time. Two protein elution peaks were observed, representing the molecular weights of 24 and 12 kDa, which correspond to the CD3εγ heterodimer and CD3εγ monomer.

prepared target protein was already homogeneous to >80% purity. Notably, in addition to a normal band of 12 kDa CD3ε monomer, a separate protein band appeared with an estimated molecular weight of 24 kDa. This band did not exist when the refolded proteins were first treated with 5 mM DTT. The disappearance of the 24 kDa band was accompanied by increased production of 12-kDa proteins. This finding indicated the degradation of CD3ε molecules from 24-kDa homodimers into 12-kDa monomers.

To confirm this finding, a separated gel filtration chromatography was performed to directly analyze the molecular size of the refolded CD3ε molecules. As seen in Fig. 3B, human CD3ε refolded into two different types of proteins with different molecular sizes in the natural condition. In addition to the expected 12-kDa monomers, 24-kDa CD3ε homodimers also existed after refolding. This result further supported the notion that human CD3ε subunits have the ability to form εε homodimers *in vitro*.

Refolding and purification of CD3εγ heterodimers and tetramers. Having established that the human CD3ε subunit was able to form homodimers, we next examined the possibility of the formation of CD3ε-CD3γ heterodimers. Equal amounts of CD3ε and CD3γ expression products were mixed together and refolded according to the standard protocol as described in Materials and methods. After refolding and initial purification using ion-exchange column, the purified products were analyzed using SDS-PAGE with or without DTT treatment. As illustrated in Fig. 4A, purified refolded CD3ε and CD3γ proteins formed different dimer structures with quite different sizes, under non-reducing conditions; the refolded products ran as several bands of apparent molecular masses of 10-12, 22-25 and 30-35 kDa (Fig. 4A, lanes 1-4). In contrast, after a 5-mM DTT treatment, the refolded protein ran as two discrete bands with molecular masses of 10 and 12 kDa (Fig. 4A, lanes 5-8), representing CD3ε and CD3γ subunits at molecular masses of 12 and 10 kDa, respectively. The 12-kDa protein bands had stronger and broader staining which indicated the contamination of the CD3ε homodimers or the possibility that there was more CD3ε involved in the formation of the dimer structures. To answer this question, using His-tag affinity purification, we isolated the proteins of the CD3γ dimers from the contaminated CD3ε - CD3ε homodimers. The affinity purified protein was homogeneously pure and contained equal amounts of CD3ε and CD3γ in denatured Coomassie gel (Fig. 4B, lanes 5-8). This suggests that CD3ε and CD3γ would either form heterodimers or tetramers (Fig. 4B, lanes 1-4), rather than CD3ε - CD3γ - CD3ε trimers. Using gel filtration chromatography assay, we further confirmed that CD3ε and CD3γ proteins have the capacity to form tetramers along with heterodimer structures (Fig. 4C).

Refolding and purification of CD3εδ heterodimers and tetramers. Several reports have indicated that CD3ε and CD3δ molecules can form disulfide linked heterodimers. To assess whether CD3ε and CD3δ molecules expressed in these systems are able to form different dimer structures, the same refolding and purification strategy was applied to the CD3ε and CD3δ subunits. Similar to the CD3ε and CD3γ subunits, the resulting refolded CD3ε and CD3δ proteins contained a mixture of disulfide- and non-disulfide-linked CD3ε- CD3ε homodimers, CD3ε and CD3δ monomers, CD3ε - CD3δ heterodimers and CD3ε - CD3δ - CD3ε - CD3δ tetramers, which were seen in the Coomassie gel staining (Fig. 5A). Since the C-terminal extracellular segment of the CD3δ subunit contained a His-tag, using the Ni Sepharose affinity chromatography purification, the CD3δ contained proteins were further separated from the CD3ε - CD3ε homodimers. As shown in Fig. 5B, under non-reducing conditions (Fig. 5B, lanes 1-4), CD3ε and CD3δ molecules formed several different dimers, with molecular masses of 22-25 and 44-50 kDa. These results were confirmed by gel filtration chromatography assay (Fig. 5C). Under reducing conditions (Fig. 5B, lanes 5-8), the purified proteins ran as two bands with closely spaced CD3ε and CD3δ monomers at molecular masses 12 and 11 kDa, respectively. This differential molecular weight permitted us to assess the formation of different dimer structures that confirmed the identity of the individual chains

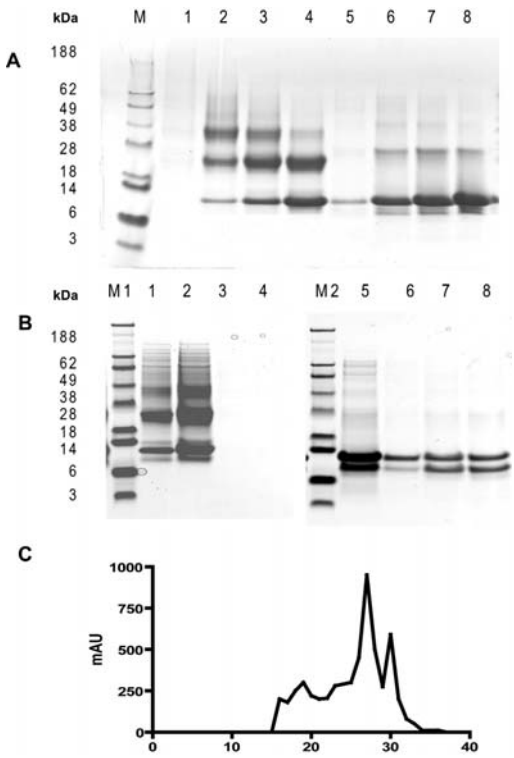

Figure 5. Refolding and purification of human CD3εδ heterodimers. (A) Human CD3εδ heterodimers were refolded and purified using the Poros 50HQ column. The initial purified products were assessed by SDS-PAGE under a no-denatured or denatured condition. Lane M, molecular mass marker; lanes 1-4, elution fractions assessed by SDS-PAGE under a no-denatured condition; lanes 5-8, elution fractions assessed by SDS-PAGE under a denatured condition. (B) Following initial anion exchange chromatography, the refolded proteins were further purified by immobilized-metal affinity chromatography. SDS-PAGE illustrates secondary purification results using Ni-NTA column. Lane M, molecular mass marker; lanes 1-4, elution fractions assessed by SDS-PAGE under a no-denatured condition; lanes 5-8, elution fractions assessed by SDS-PAGE under a denatured condition. (C) Following IMAC, the eluted proteins were assessed for their molecular weight by gel filtration chromatography. The diagram shows the purification profile of gel filtrations. UV absorbance at 280 nm and conductivity were detected real-time. Two protein elution peaks were observed, representing the molecular weights 24 and 12 kDa, which correspond to the CD3εδ heterodimer and CD3εδ monomer.

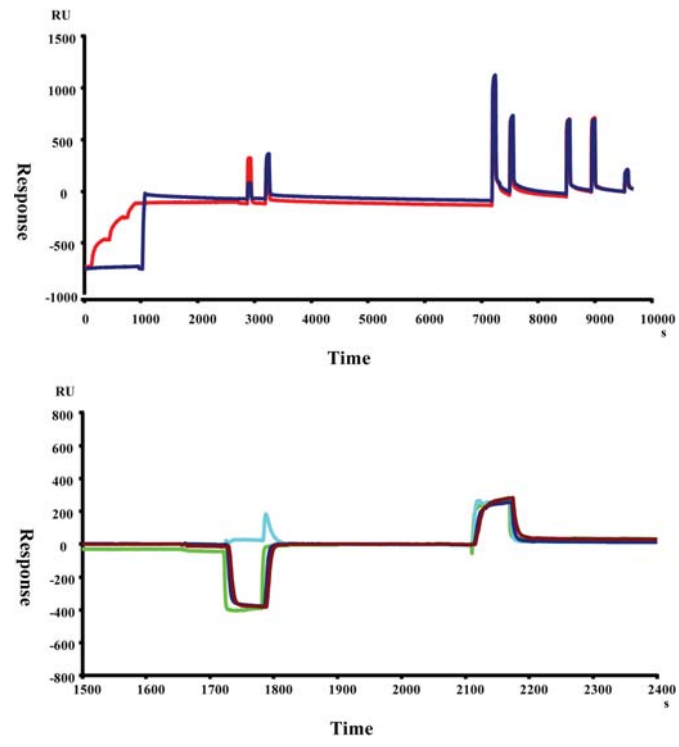

Figure 6. Assessment of TCR interactions with the CD3 homodimer or pMHC molecules. SPR kinetic measurements were performed using BIAcore 2000 with protocols as described in Materials and methods. JM22 TCR (10 μM) was immobilized and reacted with serial 2-fold-diluted CD3 homodimer or the synthetic peptide/MHC complex (A2-Flu). The figures illustrate the binding curves of the different proteins. Upper panel: binding of TCR to the CD3 homodimer. Lower panel: binding of TCR to the synthetic peptide/MHC complex (A2-Flu).

and suggested a 1:1 ratio of CD3ε and CD3δ. Therefore, we concluded, similar to the CD3ε and CD3γ subunits, that the CD3ε and CD3δ subunits also formed either heterodimer or tetramer structures. These findings were in agreement with previous studies which demonstrated that the CD3ε subunit can pair with CD3γ and CD3δ subunits to form heterodimer structures.

BIAcore affinity measurements of the interaction between CD3ε homodimers and TCRs. The interactions between the soluble CD3ε homodimer and TCRs were examined using a surface plasmon resonance (SPR) biosensor, which allows direct measurement of kinetic interactions between immobilized and solution-phase molecules (13). The affinity for TCR-CD3ε was measured using the purified refolded CD3ε homodimers. The proper folding of these soluble CD3 proteins was confirmed by BIAcore analysis using anti-CD3ε mAb (data not shown). For affinity measurements, 20 μl of the individual TCR (at 10 μM) proteins was first immobilized on the chip. Subsequently, purified CD3ε was injected at 1-64 μM concentrations. As a control, the synthetic peptide/MHC complex (HLA A2-Flu peptides) at identical concentrations of 1-64 μM was injected (14). Fig. 6 shows a typical sensorgram of the TCR binding to HLA A2 Flu peptides as comparison; no direct binding of TCR and CD3ε was detectable. This result suggests that the CD3 extracellular domain is unable to bind with the TCR without the involvement of other molecules.

Discussion

Despite many genetic, structural, and functional studies in recent years, due to its multichain structure, essential questions concerning the TCR/CD3 complex remain unanswered. Further study is required to determine the precise number of polypeptides in each TCR/CD3 complex, their interactions and spatial arrangement, the roles of each polypeptide in antigen recognition and/or in receptor signal transmission, and the relationship between the TCR/CD3 complex and other membrane or cytoplasmic molecules involved in downstream signaling (15). Different TCR/CD3 assembly models have suggested that during assembly, the CD3ε/CD3γ dimer interacts exclusively with TCRß and the CD3ε/CD3δ dimer with TCRα to form a complex with a single TCRα/ß heterodimer (16). Other reports propose different models which presume that the CD3ε/CD3γ and CD3ε/CD3δ dimers

e with both TCR chains simultaneously and ly (17). In contrast, a recent study proposed that CD3ε, γ and δ subunits can form tetramer complexes first, prior to interacting with the TCR ß-chain, and that the CD3ζζ subunits interact exclusively with the TCR α-chain (18).

In agreement with the last structural model mentioned above, we showed that different CD3 subunits can be expressed, refolded and purified *in vitro*. Furthermore, we demonstrated that, in addition to forming CD3εγ or CD3εδ heterodimers, the CD3ε subunit itself has the capacity to form homodimer structures. This finding challenges the previous hypothetical model of TCR/CD3 molecular structure which proposes that a non-covalently associated CD3δε εγ tetramer complex exists on the T cell surface. The formation of the special CD3 tetramer complex may explain the distinct functions of individual CD3 subunits in T cell activation events such as signaling, proliferation and effector functions.

CD3 molecules are highly specific for T cells and are present in the majority of T cells. Variable αß and γδ TCRS are associated with the invariant CD3γ, δ, ε and ζ proteins, thus forming the TCR/CD3 complex. Within the TCR-CD3 complex, a division of labor exists: TCRαß or γδ recognizes the antigen, and CD3γ, δ, ε and ζ control assembly and signal transduction (19). Extensive biochemical studies have shown that CD3 also plays an important role in the co-ordinated assembly of TCR/CD3 receptors in the endoplasmic reticulum for efficient transport to the cell surface (20). Furthermore, mice deficient in CD3γ, CD3δ, CD3ε or CD3ζ demonstrate impairment of thymocyte maturation (21).

Based on existing data, different CD3 subunits can form invariant dimer structures which include CD3εγ, CD3εδ and CD3ζζ, and each subunit can exert a variety of functions. The crystal structure of the human T cell receptor and CD3εγ and CD3εδ heterodimers has recently been described (21,22). However, the intact assembly pattern between each CD3 subunit has never been described. Moreover, in this study, a conjunction antibody was used to stabilize the heterodimer structure which indicated that the assembly of the CD3 molecular subunit may involve a much more complex structure. Our study demonstrated that, in addition to the formation of CD3εγ and CD3εδ heterodimers, the CD3ε subunit itself has the capacity to form homodimer structures. These results may explain the different involvement of each CD3 subunit in the regulation of T cell functions. For example, CD3γ-deficient patients show only slightly reduced numbers of peripheral T cells (22), while CD3ε or CD3δ deficiencies are present in infants with life-threatening SCID and very severe αß and γδ T lymphocytopenia (23).

In our study, to measure the interaction between the individual invariant CD3γ, δ and ε proteins, we first expressed these recombinant proteins using the prokaryotic system. For the construction of the recombinant expression vector, Histidine C-terminal tags and inter-chain disulfide bridge were employed to aid in purification and refolding. The addition of these C-terminal purification tags had no effect on the expression efficiency of the target protein, since a high level of expression was achieved for both of the CD3 subunit proteins. The observed high efficiency of expression is probably due to the special nature of the CD3 subunit molecules. All of the CD3 molecules are ~15 kDa which is optimal for *E. coli* expression (24). In addition, the absence of rare amino acids in their protein sequence also contributes to the high expression level, since no rare tRNA or chaperones were required for effective gene transcript and translation (25). The high frequency usage of C, G rather than A, T in the encoding sequence also makes the initiation of gene translation easier. All of these contributed to the successful expression of our target proteins. The expression of the inter-chain disulfide bridge cysteines allows the formation of an inter-chain disulfide bridge during *in vitro* refolding. The function of these inter-chain disulfide bridge cysteines are similar to the traditional leucine zipper structure (26).

It is worth noting that normal refolding techniques using different CD3 subunits results in the formation of more CD3εε homodimers than CD3εγ and CD3εδ heterodimers. This suggests that different kinetics are involved in the formation of CD3 homodimer and heterodimer structures. CD3εε homodimers have been proven to be very easy to form regardless of whether CD3γ and CD3δ are used for the refolding with CD3ε proteins. The homodimer forming ability of CD3ε molecules also contributes to the formation of CD3εγ - CD3εγ and CD3εδ - CD3εδ tetramers. This is why the different sizes of protein bands were detected after initial anion exchange chromatography purification. The co-expression of Histidine C-terminal tag is only restricted in CD3δ and CD3γ subunit, but not CD3ε subunit. Our result shows that the co-expression of Histidine C-terminal tag did not compromise the protein yield, since similar expression efficiencies for CD3δ, CD3γ and CD3ε were detected. The co-expression of the His-tag benefited the specific identification and purification of these two proteins. With Ni-NTA affinity purification, we easily avoided the contaminated CD3εε homodimers and analyzed the purified product using denatured gel. We found equal amounts of CD3εγ and CD3εδ, suggesting that only CD3εγ and CDεδ heterodimers and CD3εγ - CD3εγ and CD3εδ - CD3εδ tetramers, but not any trimer structures were formed.

The study of the interaction between TCRs and CD3 molecules was another focus of this study. αß TCRs and CD3 molecules determine the functional specificity of T cell responses. The transmembrane (TM) domain charge interaction is critical for the assembly of the TCR-CD3 complex, while the extracellular contacts between the TCR and CD3 subunits contribute to stability of the structure and signal transduction. Assembly of the T cell receptor (TCR) with its dimeric signaling modules CD3ε, CD3γ, CD3δ and CD3ζ is organized by TM interactions. Each of the assembly steps requires formation of a three-helix interface involving one particular basic TCR TM residue and two acidic TM residues of the respective signaling dimers. It has been reported that the CD3δε and CD3γε subunits interact with the TCR via adjacent Cα DE and Cß CC' loops, respectively (14), and TCR interaction sites are located on the membrane-proximal cysteine regions (26), but the exact function of the CD3ε subunit on the molecular assembly has not been reported. Our finding suggests that, although CD3ε molecules are crucial to the formation of the different dimer structures by binding with other CD3 subunits, the CD3ε subunit is unable to directly interact with TCR.

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