Structure of the chicken CD3εδ/γ heterodimer and its assembly with the αβ T cell receptor

Richard Berry*1, Stephen J. Heady*2, Melissa J. Call*3, James McCluskey4, Clive A. Tregaskes5, Jim Kaufman5, Ruide Koh1, Martin J. Scanlon2, Matthew E. Call3# & Jamie Rossjohn1,6#

1Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Clayton, Victoria 3800, Australia

2Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia

3Structural Biology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia, University of Melbourne, Parkville, VIC 3010, Australia

4Department of Microbiology & Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, Victoria 3010, Australia

5Dept. of Pathology, University of Cambridge, Tennis Court Rd., Cambridge CB2 1QP, United Kingdom.

6Institute of Infection and Immunity, Cardiff University, School of Medicine, Heath Park, Cardiff CF14 4XN, UK.

* RB, SJH and MJC are joint 1st authors
# MEC and JR are joint senior and corresponding authors.

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To whom correspondence should be addressed: Matthew E Call, Tel.: +61-3-9345-2306; Fax: +61-3-9345-2686; E-mail: mecall@wehi.edu.au; or Jamie Rossjohn, Tel.: +61-3-9902-9236; Fax: 99054699; E-mail: Jamie.rossjohn@monash.edu

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Background: Chickens possess a CD3δ/γ chain that assembles with T-cell receptor to mediate immune signaling.

Results: Chicken CD3 ε δ / γ has an atypical heterodimer interface and surface but associates with TCR α β.

Conclusion: chicken CD3 δ / γ represents a hybrid chain possessing features in common with human CD3 δ and CD3 γ.

Significance: Understanding the ancestral TCR signaling complex provides insights into the evolution of this signaling apparatus.

ABSTRACT

In mammals, the αβ T cell receptor (TCR) signaling complex is composed of a TCRαβ heterodimer that is non-covalently coupled to three dimeric signaling molecules, CD3ζ, CD3ζγ and CD3ζζ. The nature of the TCR signaling complex and subunit arrangement in different species remains unclear however. Here we present a structural and biochemical analysis of the more primitive ancestral form of the TCR signaling complex found in chickens. In contrast to mammals, chickens do not express separate CD3δ and CD3γ chains but instead encode a single hybrid chain, termed CD3δ/γ that is capable of pairing with CD3ε. The NMR structure of the chicken CD3εδ/γ heterodimer revealed a unique dimer...
interface that results in a heterodimer with considerable deviation from the distinct side-by-side architecture found in human and murine CD3εδ and CD3εγ. The chicken CD3εδ/γ heterodimer also contains a unique molecular surface, with the vast majority of surface-exposed, non-conserved residues being clustered to a single face of the heterodimer. Using an in vitro biochemical assay, we demonstrate that CD3εδ/γ can assemble with both chicken TCRα and TCRβ via conserved polar transmembrane sites. Moreover, analogous to the human TCR signaling complex, the presence of two copies of CD3εδ/γ is required for ζζ assembly. These data provide insight into the evolution of this critical receptor signaling apparatus.

The αβ T cell receptor (TCR)-CD3 complex represents an extensively studied transmembrane (TM) receptor system. In humans and other mammals, the TCR signaling complex comprises eight type-I membrane-spanning polypeptides that include the TCR αβ heterodimer, the CD3εγ and CD3εδ heterodimers and the CD3ζζ homodimer (1), although higher order molecular assemblies are considered to exist (2,3). The αβ TCR functions to recognize peptide, lipid and vitamin B-precursor based antigens that are presented by Major histocompatibility complex (MHC) or MHC-like molecules (4-7). This ligand-sensing heterodimer has no intrinsic ability to transmit signals to the cell interior due to the short cytoplasmic tails of the α and β chains of the TCR. Instead, signaling is mediated by the CD3εγ, CD3εδ and CD3ζζ subunits that associate with the TCR to produce a complex with a 1TCRαβ:1CD3εδ:1CD3εγ:1ζζ stoichiometry (1). The signaling subunits contain immunoreceptor tyrosine-based activation motifs (ITAMs) within their intracellular regions that become targets of the Src-family kinase Lck upon receptor triggering; each CD3 heterodimer contributes two ITAMS to the signaling capacity of the TCR, while the ζζ homodimer contributes six (8-12). The precise mechanism by which signals are transmitted from the extracellular portion of TCR to the intracellular regions of CD3 is a major unresolved issue in T cell biology, with kinetic segregation, conformational change, mechanotransduction and other models being proposed (13-18). The uncertainty surrounding TCR triggering mechanisms is in large part due to the lack of structural information on the intact TCR signaling complex.

The structures of mammalian CD3 heterodimer ectodomain fragments have been determined by X-ray crystallography and solution NMR (19-22). Analysis of their surface features, including the sites of activating monoclonal antibody binding and carbohydrate modifications, have suggested particular regions that are likely to be closely juxtaposed with the TCR ectodomains and may therefore play a role in transmission of ligand sensing from TCR to CD3 modules (17,23-25). However, a detailed structural analysis of the extracellular interactions between TCR and CD3 subunits is complicated by the lack of any apparent affinity among these dimeric components in solution. A major energetic contribution to assembly of TCR and CD3 subunits appears to come from a network of polar interactions within the transmembrane (TM) domains (26,27). Both the TCRα and TCRβ TM domains have a central lysine residue that recruits one CD3 heterodimer via a pair of acidic TM residues (28). Within the mature complex, CD3εδ associates exclusively via the TCRα TM domain and CD3εγ via the TCRβ TM domain to create an “asymmetric” hexamer. TCRα also contains an arginine residue in the upper half of the TM domain that mediates its interaction with the ζζ homodimer (28). The ζζ module will only join a pre-assembled CD3εδ-TCRαβ-CD3εγ hexamer, signalling the completion of assembly by masking an ER retention signal in CD3εδ (29) and allowing export to the cell surface.

The evolutionary basis for the inclusion of two globally similar CD3 modules in this receptor complex is unclear. There is evidence to suggest that the mammalian CD3δ and CD3γ subunits have evolved novel, non-redundant functions. Both CD3δ and CD3γ knockout mice exhibit profound defects in the production of mature αβ T cells, confirming that both CD3εδ and CD3εγ modules are absolutely required (30,31). Furthermore, substitution of the extracellular immunoglobulin (Ig) domain of CD3γ with that of CD3δ failed to rescue surface TCR expression in CD3γ-deficient human Jurkat T cells (32) even
though exchange of TM and cytoplasmic domains was tolerated; thus the folded ectodomains, at least, are non-interchangeable. Nonetheless, the precise nature of their unique functional roles and the significance of their strictly asymmetric assembly with TCR remain unknown.

Chickens (ch) and the amphibian Xenopus laevis also express a CD3ε-like protein but do not express separate CD3δ and CD3γ chains. Instead, they encode a protein that shares equal homology with both mammalian CD3δ and CD3γ, and has thus been designated CD3δ/γ (33). At the amino acid sequence level, chicken and human CD3ε, δ and γ chains have low extracellular (32-34 %) and high TM (44-52 %) and intracellular (49-55 %) sequence identity. Analysis of the CD3 locus with both mammalian CD3εγδζζ and CD3εδγ, and has thus been designated CD3δ/γ (33). At the amino acid sequence level, chicken and human CD3ε, δ and γ chains have low extracellular (32-34 %) and high TM (44-52 %) and intracellular (49-55 %) sequence identity. Analysis of the CD3 locus suggests that mammalian CD3δ and CD3γ arose from a gene duplication event that occurred 230 million years ago (34). Accordingly, it is likely that the ch-CD3 represents a primordial form that has not diversified in a manner analogous to its mammalian counterpart. To provide further insight into the relationship between the mammalian TCR signaling complex and its evolutionary precursors, we have undertaken a structural and biochemical analysis of the chicken CD3 proteins and their assembly into the chicken TCR signaling complex. The solution NMR structure of the ch-CD3εδγ ectodomain dimer reveals significant differences from the mouse and human CD3 heterodimers in both domain orientation and surface chemistry. Furthermore, the ch-TCR signaling complex assembly demonstrates that, despite the lack of CD3 asymmetry in the chicken receptor system, two CD3εδγ dimers are required to form a fully assembled complex that is capped by ζζ association.

**EXPERIMENTAL PROCEDURES**

Cloning, expression, refolding and purification of chicken CD3- Gene fragments encoding the extracellular domains of mature ch-CD3ε (residues 24-91) and CD3δ/γ (residues 18-97) excluding the cysteine-rich stalks were synthesized de novo (Genscript). To generate a single chain construct, the carboxy terminus of ch-CD3ε was covalently linked to the amino terminus of ch-CD3δ/γ via a 26-amino acid flexible peptide using splice-by-overlap PCR. Ch-CD3εδγ was cloned into a pET28b expression vector downstream of the thrombin-cleavable histidine tag and expressed as inclusion bodies in *E. coli* BL21(DE3) cells. Inclusion bodies were solubilized in 0.2 M Tris-HCl (pH 9.5), 6M Gdn- HCl, 0.1M DTT, 10 mM EDTA and refolded essentially as described (35). Refolded protein was buffer exchanged into 10 mM tris (pH 8) containing 0.5 M NaCl using tangential flow filtration prior to loading on a His Trap HP nickel column (GE Healthcare) and eluted with 0.5 M imidazole. Histidine tag cleavage was performed using agarose-linked thrombin beads (Sigma) according to the manufacturers instructions. The final purification step involved gel filtration chromatography using a Superdex75 16/60 column (GE Healthcare) pre-equilibrated in 25 mM HEPES (pH 7.6) containing 50 mM NaCl and 0.5 mM EDTA.

NMR- Suitable NMR buffer conditions were identified as 0.5 mM CD3, 50 mM HEPES pH 7.6, 125 mM arginine, 125 mM glutamate, 0.01% azide, 0.01% Roche protease inhibitor, 0.5 mM EDTA using crystallography dialysis buttons. All NMR samples contained 10% 2H2O and the spectra were recorded at 293 K. The following NMR spectra were recorded on a Bruker AVANCE™ 600 MHz spectrometer with cryoprobe using a 13C,15N-labelled CD3 sample: HNCA, HNCO, HBHA(CO)NH, (H)CH-TOCSY, H(C)CH-TOCSY, 15N-NOESY-HSQC (τm110 ms), HD(CDCG)CB and HE(CECDCG)CB. A 2H,13C,15N-CD3 sample was used to acquire TROSY versions of a HNCA,CB, HN(CO)CAB, HN(CA), and HN(CO)CA on an 800 MHz Bruker AVANCE fitted with a cryoprobe and 13C-NOESY-HSQC (aliphatic) and 13C-NOESY-HSQC (aromatic) (τm110 ms) spectra were acquired on the same spectrometer using the 13C, 15N labeled sample. Spectra were processed using Topspin version 3.0. Backbone amide, CA, CB, HA and HB resonances were assigned manually using XEasy (36). Automated side-chain assignments were made using the ASCAN algorithms of UNIO and verified and supplemented by manual assignments using the HCCH-TOCSY spectra.

Structures were calculated using the AtmosCandid automated NOE peak picking and assignment algorithms with CNS torsion angle dynamics starting from an extended chain. The resulting structures were refined in CNS using simulated annealing with Cartesian dynamics. During refinement dihedral angle restraints...
predicted from TALOS were incorporated along with hydrogen bond restraints in regions of canonical secondary structure where unique donor-acceptor pairs could be identified by convergence. The 10 lowest energy conformers with no NOE violations >0.3 Å, no bond violations >0.05 Å, and no improper or dihedral angle violations >5° were chosen to represent the solution structure of CD3. The Ch-CD3 structures have been deposited with the Protein Data Bank (PDB code: 2MIM) and the chemical shifts with the BMRB (code 19687).

**Epitope tags and affinity reagents**-
Epitope tags were used to facilitate immunoprecipitation of chicken TCR components. In all cases, epitope tags were installed at the C-terminus of protein with a Gly-Ser linker, which was encoded by the BamHI site used for cloning. Anti-HA agarose beads (Sigma, #A2095) were used to immunoprecipitate chains carrying the HA epitope tag (GYPYDVPDYA). SA conjugated beads (Sigma #S1638) were used to precipitate chains carrying the SBP tag (DEKTTGWRGGHVEGLAGELEQLRARLEH HPQGQREPSSSGGSKLG). Anti-PC agarose beads (Roche, #11815024001) were used to immunoprecipitate chains carrying the PC tag (EDQVDPRLIDGK).

**cDNA constructs and in vitro transcription**- cDNA encoding CD3ε, CD3δγ and ζ were synthesized as G-blocks (IDT) and cloned into pSP64 (modified by M. Kozak). The chicken cell line MSB1 (37) was the source of cDNA to amplify by standard PCR conditions the coding sequence for TCR alpha chain (primers: forward CMGTGSGASRAAATGRATTTTS and reverse GCACCCAATGCTCCAGTAAT) which was cloned into the pJET3.1 vector (CloneJet, Fermentas), and for TCR beta chain (primers: forward TTCSTGCTGGTTTCTTACG and reverse TCCCTTTCAAGGCAAAAGCAT) which was cloned into the filled Bam HI site of pGEM3zf (Promega). In all cases the natural signal sequence was replaced with that of mouse H2-Kb (MVPCTLLLLALALPTQTRA) ensuring robust and equal targeting of all polypeptide chains to ER microsomes (38). Sequences were cloned into pSP64 constructs modified to append an HA, PC, SBP or no tag (Gly-Ser) to the C-terminus as appropriate and a poly A tail to the mRNA. mRNA was transcribed in vitro using the Ribomax Large Scale RNA T7 Production System (Promega #P1300) according to manufacturers instructions. m7G Cap analogue (Promega #P1712) was supplied in 5-fold excess with respect to rGTP to ensure mRNA was correctly capped. After mRNA transcription, plasmid DNA was cleaved with RQ1 DNAse (Promega #M6101) and mRNA purified with an RNeasy Mini Kit (Qiagen #74104). 25 µl transcription reactions typically yielded 60 µl of mRNA at a concentration of 250 µg/ml.

**In vitro transcription and assembly**- Each mRNA was translated alone to ensure targeting to ER microsomes, signal peptide cleavage and glycosylation occurred as expected (data not shown). mRNA concentration was adjusted to ensure equivalent translation of each polypeptide. Adjustment factors were determined by densitometry (see below) of de-glycosylated (EndoH treated) samples. 25 µl in vitro translation reactions contained 17.5 µl of rabbit reticulocyte lysate (Promega #L4960), 0.5 µl of amino acids without Cys or Met (Promega #L5511), 0.5 µl of SUPERase-In (Promega #N2511), 2.0 µl of Express Protein Labeling Mix (PerkinElmer #NEG072002MC) containing 35S Met and 35S Cys, and 2 µl of ER microsomes (prepared as previously described (28)). Total mRNA concentrations were generally kept below 250 ng per reaction but varied depending on efficiency of translation as determined by test translation experiments. mRNA was denatured at 65°C for 3 minutes prior to addition to lysate mixtures. mRNA was translated for 15-30 minutes at 30°C and then oxidized glutathione was added to a final concentration of 4 mM. Assembly proceeded in oxidizing conditions for 2 hours (three chain experiments) or 4 hours (five chain experiments). Reactions were stopped by addition of 900 µl of ice-cold tris-buffered saline containing 10 mM iodoacetamide. ER microsomes were pelleted at 20,000g for 10 minutes. Pellets were washed with an additional 500 µl of tris-buffered saline containing 10 mM iodoacetamide. After discarding the wash, ER microsome pellets were extracted in 0.5% digitonin, 200 µg/ml BSA, 10 mM iodoacetamide for 30 minutes at 4°C. For experiments involving anti-PC pull-down, 2 mM CaCl2 was included in the extraction buffer. Mixing controls were performed by translating and oxidizing certain mRNAs (detailed in figure...
Chicken CD3 complex

Immunoprecipitation and analysis - Digitonin extracts were passed through Spin-X filters (Corning #8160) to remove insoluble material and 10-15 µl of the appropriate affinity bead slurry added to immunoprecipitate polypeptides of interest. Immunoprecipitations were incubated between 2 and 24 hours before being washed. For non-denaturing elution, SBP pull-downs were eluted with 100 µM biotin in 0.5% digitonin containing 200 µg/ml BSA in tris-buffered saline before being transferred to anti-HA agarose for sequential immunoprecipitation. When non-denaturing elution was not required, samples were eluted by heating at 95°C for 3 minutes in 10 µl of 0.5% SDS in 50 mM citrate pH 5.6. Eluted complexes were incubated at 37°C after the addition of 0.5 µl of EndoH (NEB #P0702) to remove sugar moieties. Deglycosylated chains were separated by 12% NuPAGE (Invitrogen, #NP0341 or #NP0343) in MES Buffer (Invitrogen, #NP0002) and blotted onto PVDF (Millipore, #ISQ00010). To measure incorporated 35S methionine and 35S cysteine, PVDF membranes were placed on phosphorstorage screens (BAS IP TR2040) and scanned by a Typhoon (Molecular Dynamics, FLA-9410). The gel files were converted to tiff format and densitometry performed using Image Gauge V4 software (Fujifilm). Data were plotted using Prism V5 (GraphPad Software Inc).

RESULTS

For NMR structural studies we expressed, refolded and purified a single-chain construct comprising the extracellular Ig domains of ch-CD3e and ch-CD3δ/γ joined via a 26 amino acid flexible linker. Of the 178 residues present, backbone amide assignments could be made for all CD3e residues with the exception of Gly13, and all CD3δ/γ residues except Ser27-Asn33 and His75-His77 (excluding the linker sequence). Approximately 85% of other non-labile side chain resonances were assigned (Table 1). A second set of resonances could be observed for CD3e residues Ile11, Ser12, Thr17, Ile18, Thr19, Ser22, Trp29, Ile42, Asn43, His46, Asp47, Ser49, Ser54, Cys55 and His62, suggesting the presence of two conformations of CD3ε in slow exchange on the NMR timescale. Only the resonances of the major conformer (which represented 71% of total protein) were used for structure determination.

Chicken CD3 domain structure. The ch-CD3ε monomer forms an Ig domain comprising seven β-strands making up two antiparallel β-sheets (sheet 1: ABE and sheet 2: CFG) that pack via a hydrophobic core and are bridged by a disulfide bond originating from two conserved cysteine residues (Cys20 and Cys55) (Figures 1A and B). Accordingly, ch-CD3ε belongs to the C1-set Ig fold but is atypical in that, like the C11 antibody domains, it lacks the short C’ strand in sheet 2 (39). This is in contrast to both mouse and human CD3ε, which contain seven and eight β-strands and fall into the C2 and I-set Ig folds respectively (20, 21).

The ch-CD3ε Ig domain has low sequence identity with human (22%) and mouse (24%) CD3ε, but overall is structurally very similar with an r.m.s.d. of 1.65-1.75Å over the entire domain (61 Ca atoms). The primary deviations from the structure of human CD3ε are related to the configuration of the Ig domain and the length and conformation of loops connecting certain β-strands (Figure 1C). Two loop regions in ch-CD3ε are significantly different to the human CD3ε structure: the C-D loop, which in ch-CD3ε adopts a compact 310 helical turn, and the FG loop present at the membrane distal face, which forms a tight hairpin loop. Due to these differences, ch-CD3ε has a considerably diminished accessible surface area (4430 Å2) relative the human CD3ε (5640 Å2).

Ch-CD3δ/γ comprises two antiparallel β-sheets (sheet 1: ABE and sheet 2: CC’FG) linked by a single disulfide bond between Cys24 and Cys64 and supported by a hydrophobic core comprising residues Leu22, Trp36, Leu51, Pro58, Tyr62 and Leu74 (Figure 1A and B). Accordingly, ch-CD3δ/γ adopts a C2-set Ig fold. The single N-linked glycosylation site at Asn71 is present within the FG loop (Figure 1B) in a location that would likely protrude away from the cell membrane.

The ch-CD3δ/γ fold is similar to that observed for human CD3γ (20) but differs from the C1-set Ig fold found in human CD3δ due to a translocation of the D strand from the ABED face to the CFG face, where it becomes a C’ strand (Figure 1C and D). Despite this difference, the remainder of the ch-CD3δ/γ domain is reasonably
structurally conserved compared to human CD3δ, with 47 Cα atoms having an r.m.s.d of 2.15 Å. However, ch-CD3δ/γ aligns relatively poorly to human CD3γ (r.m.s.d 2.5 Å over 50 Cα atoms) primarily due to a number of structural alterations distributed throughout the molecule. Apart from the DE loop (C’-E loop in human CD3γ), the major structural difference lies in the FG loop, which adopts a flattened conformation in ch-CD3δ/γ. In both human CD3γ and sheep CD3δ (this loop is not visible in the human CD3δ structure), the F-G loop extends away from the core of the CD3 module (20,22).

The chicken CD3 heterodimer. The ch-CD3 subunits associate in a side-by-side manner, resulting in a buried surface area of 1,470 Å². The heterodimer interface is dominated by interactions between the parallel G-strands, each of which contains a continuous string of residues (Asp59-Tyr65 in CD3ε and Asn71-His75 in CD3δ/γ) that come together to form an interlocking ladder (Figure 2A). The CD3δ/γ interface is further supported by contacts between the F-strand of CD3ε and a triplet of residues (Met11-Val13) at the base of the CD3δ/γ A-strand that make extensive Van der Waals interactions with Thr52 and Tyr65 of CD3ε.

Despite playing a central role at the heart of the ε:δ/γ interface, the conformation of the ch-CD3ε G-strand is considerably different to that found in human and murine CD3s (Figure 2B). Due to a tight turn in the FG loop, the ch-CD3ε G-strand, which is extended at its base, packs tightly against the C-terminal portion of the F-strand, presenting an unusual surface to ch-CD3δ/γ (Figure 2B). Accordingly, the nature of the CD3ε:CD3δ/γ interactions are distinct from those observed in human CD3s. For example, of the 8 CD3ε residues whose side chains contribute to the dimer interface, only Gln4 and Tyr65 are present in human CD3ε; and of the 10 interacting residues in CD3δγ, 3 are conserved with human CD3εγ and 4 with CD3εδ, most of which (Leu74, His77, Tyr78 and Arg79) lie at the base of the G-strand.

The unusual conformation adopted by the base of the CD3ε G-strand has a considerable impact on the overall domain organization, giving the ch-CD3 molecule an unusual appearance relative to all mammalian CD3 structures studied to date. Whereas human and mouse CD3 heterodimers have a distinctive upright arrangement, the ch-CD3 heterodimer has a more skewed/asymmetric configuration, as evidenced by the center of mass of the δ/γ subunit that is rotated by up to 45° when viewed from side-on and 35° viewed from the top relative to human CD3δ (Figure 2C). As a consequence, the overall structural similarity between ch-CD3εδ/γ and human CD3εδ is low, with an r.m.s.d of 3.6 Å over 106 Cα atoms.

Comparison of molecular surfaces in chicken and mammalian CD3. Human, and to a lesser extent mouse CD3ε contain a prominent negatively charged surface derived from the C’-D ((37)DED(40)) and D-E loops ((46)DED(48)) (20,21) (Figure 3A). In ch-CD3ε the bulky C’-D loop is notably absent and in its place is a more compact and neutral 310 helix derived from Pro33-Leu35 (Figure 1C I and D). This, coupled with a change in nature of the D-E loop, which contains a single basic residue (Lys 40), contributes to the relatively flat, featureless appearance of ch-CD3ε (Figure 3A). Although many of the amino acid residues conserved between chicken and human CD3 lie within the core and maintain the Ig fold, several conserved residues are surface exposed. When mapped onto the structure of ch-CD3, these residues appear to cluster to a single face (the ε:ABE, δγ: C’CFG) and are particularly prominent in the membrane proximal region adjacent to where the cysteine-rich stalks (not present in our construct) would protrude (Figure 3B). In contrast, the opposing face (ε:CFG, δγ:ABE) is almost devoid of any conserved, surface-exposed residues.

Incorporation of chicken CD3εδ/γ into a complete chicken TCR signaling complex. In order to relate our structural insights on the isolated CD3 heterodimer to the broader picture of subunit organization within the complex, we performed a series of biochemical experiments interrogating the basis of chicken TCR complex assembly. Analysis of the TM domains of chicken TCRα and TCRβ chains reveals that basic residues are present at all the same positions to those in mammalian sequences (Figure 4), and each CD3 chain contains the conserved acidic and hydroxyl-bearing TM residues that are crucial for human TCR signaling complex assembly. Interestingly, the first basic residue in the TCRα chain is a lysine rather than the typical arginine found in other vertebrates (including fish). We mutated each TCR TM lysine to alanine and assessed each
mutant’s ability to assemble with ch-CD3εδ/γ heterodimers in isolated ER microsomes using an established in vitro translation-based assay (28). To facilitate immunoprecipitation (IP) and SDS-PAGE analyses, hemagglutinin (HA) and streptavidin-binding peptide (SBP) tags were installed at the C-termini of TCR and CD3ε polypeptides, respectively. Incorporation of ^35S isotope-labeled methionine and cysteine during translation provided a quantitative method to measure the relative levels of each subunit recovered from digitonin extracts of the ER microsomes. Mutation of each central lysine residue in TCRα and TCRβ resulted in loss of CD3εδ/γ association with the individual TCR chains, while the lysine in the upper half of the TCRα TM had no effect (Figure 5). Interestingly, the ability of TCRβ to support CD3εδ/γ assembly was approximately 50% compared to TCRα. This property is similar when human TCRβ is compared with TCRα for its ability to associate with CD3 heterodimers: TCRα-CD3εδ assembly is several-fold more efficient that TCRβ-CD3εγ assembly.

We next tested the ability of ch-CD3εδ/γ to associate with a chicken TCRαβ heterodimer and ζζ homodimer in in vitro assembly reactions. To facilitate isolation of TCRαβ heterodimers for analysis, we installed a SBP tag on the TCRα chain and a HA tag on the TCRβ chain. This allowed the products of a first-step capture with SA-coupled agarose beads to be gently eluted with biotin and re-probed with anti-HA agarose beads to specifically isolate folded TCRαβ heterodimers. Densitometry measurements were used to determine the ratios of different CD3 subunits that co-precipitated with TCR. For these experiments, a protein C (PC) epitope tag was installed at the C-terminus of the CD3ε chain to improve electrophoretic separation from CD3δγ. Again, mutation of each central lysine residue of TCRα and TCRβ resulted in reduced CD3 association, but only when both chains lacked the central lysine residue was CD3εδ/γ recruitment to the complex completely abolished (Figure 6). Surprisingly, mutation of a single lysine residue did not result in a 50% drop in CD3 association, implying that in the context of the whole receptor system, additional contacts may be made that partially compensate for loss of each lysine.

In contrast to previous results in the human system, a clear product at the expected molecular weight of disulfide-linked chicken ζζ homodimer was not evident in the full assembly reactions. Because chicken ζζ migrates very close to free TCR chains when separated by SDS-PAGE, we used a ζε-chain IP strategy (via C-terminal PC tag) to determine what percentage of ζζ interacted with TCRαβ. We co-translated quantities of mRNA that produced TCRα, TCRβ, CD3ε, CD3δγ, and ζ proteins at a molar ratio of 5:5:10:10:1 and probed the digitonin extracts with anti-PC-coupled agarose beads. CD3ε was again tagged with SBP to facilitate separation from CD3ζζ by SDS-PAGE. Anti-PC IP yielded substantial amounts of ζζ homodimer and monomeric ζ chain (both signal-peptide-cleaved and uncleaved) in addition to TCRαβ, CD3ε and CD3δγ chains, but only when all lysines in the TCR TM domains were present (Figure 7). Densitometric ratios in the all-wild-type assembly reaction (lane 1) indicated that only ~25% of ζζ was associated with TCRαβ, explaining why a prominent ζζ homodimer was not seen when TCRαβ was the IP target. Despite the lack of effect on CD3 heterodimer recruitment, mutation of the lysine in the upper half of the TCRα TM reduced TCRαβ recovery in the ζζ IP to near background levels. Mutation of the central lysines of TCRα and TCRβ also interrupted ζζ assembly. These results are consistent with the observation that in human TCR, ζζ is the final species to join the complex and prevention of CD3 association at either the TCRα or TCRβ TM domain is sufficient to prevent ζζ recruitment (28).

**DISCUSSION**

All jawed vertebrates have an adaptive immune system that includes the genes to make antibodies, MHC and TCR (40). Furthermore, all mature TCR genes cloned to date (excluding pre-Tα (41,42)) encode proteins with very short cytoplasmic domains that are unlikely to contain signaling motifs and are therefore thought to depend on CD3 or CD3-like signaling modules to transmit information to the intracellular signaling machinery (43). Analysis of CD3 loci across jawed vertebrate species suggests that the three genes present in mammals arose from two separate gene duplication events, the latter of which facilitated the divergence of CD3γ and CD3δ genes to produce two distinct CD3εγ and CD3εδ
signaling modules. However, in non-mammalian species, the CD3 locus encodes only CD3ε and a second protein that has features similar to both CD3δ and CD3γ (34) and is likely to be their evolutionary precursor. Avian and mammalian CD3 proteins have diverged significantly enough that chicken and human TCR and CD3 are unable to form hybrid complexes (44), although the more highly conserved chicken ζ chain can replace its mammalian counterpart in ζ-deficient T cells (45). In an effort to gain further insights into the evolutionary relationship among mammalian and non-mammalian CD3 components, we have determined the structure of the chicken CD3εδ/γ heterodimer extracellular domains and examined the biochemical requirements for incorporation of the CD3εδ/γ module into a complete chicken TCR signaling complex.

Herein we provide evidence that ch-CD3δ/γ is a genuine hybrid chain. Based on amino acid sequence, ch-CD3δ/γ is CD3δ-like in its extracellular domain and CD3γ-like in its intracellular region (34). However, our structure reveals that the extracellular region of ch-CD3δ/γ has a fold more reminiscent of that found in human CD3γ while retaining significant structural homology to CD3δ. The most striking feature of ch-CD3 is the unexpected domain juxtapositioning, which deviates significantly from the rigid, side-by-side, almost pseudosymmetric arrangement observed in murine and human CD3 dimers (20,21). Surprisingly, this difference appears to stem from the more conserved CD3ε chain rather than from the hybrid CD3δ/γ chain.

Precisely how the unusual subunit arrangement in ch-CD3 impacts on the overall TCR signaling complex structure is unclear, but based on our structure the ch-CD3 heterodimer is likely to present a unique molecular surface to the associated TCR. For example, an acidic patch on the surface of human CD3ε that has previously been suggested to participate in interactions with TCRβ is notably absent in the chicken structure. It is noteworthy that the appearance of separate CD3δ and CD3γ chains in mammals correlates with the appearance of a surface-exposed loop (the FG loop) in the mammalian TCRβ constant domain (46), a region that has long been implicated in CD3εγ association (23,25,47-49). Given that one face of ch-CD3 (ε:CFG, δγ:ABE) contains several unique surface-exposed residues and the opposing face (ε:ABE, δγ: C'CFG) is decorated with residues conserved in human CD3, it is tempting to speculate that the latter may be involved in CD3εδ-TCRα interactions that have been conserved during evolution whilst the former has diverged to accommodate the relatively newly evolved TCRβ surface. This hypothesis is supported by the observations that many of the conserved residues in the putative CD3εδ-TCRα interface are located in the membrane proximal region, adjacent to the cysteine-rich stalks that have been previously implicated in association of the TCR and CD3 subunits (48,50,51).

What unique functional features may have accompanied the CD3 diversification in mammals remains an open question. One possibility is that development of separate CD3δ and CD3γ proteins was concomitant with incorporation of two CD3 dimers in the TCR signaling complex where the evolutionary precursor utilized only one. However, our analysis of chicken TCR and CD3 subunit assembly demonstrates that, as suggested by the conservation of basic residues in the TM domains of chicken TCRαβ, the chicken TCR associates with two copies of the CD3εδ/γ heterodimer in a manner that is analogous to the incorporation of both CD3εδ and CD3εγ modules in mammals (Figure 8). Thus the octameric arrangement of one TCR with three dimeric signaling modules is an intrinsic feature of the TCR signaling complex even in organisms without separate CD3δ and CD3γ chains. This theme is echoed in the structure of the mouse γδTCR complex, which does not incorporate the CD3εδ module but instead contains two copies of CD3εγ (30,52). Whether this symmetric CD3 arrangement confers any unique signaling properties on the mouse γδTCR is unclear, but it does not appear to be a general feature common to mammals since primary human γδ T cells contain both CD3εγ and CD3εδ (53). Determination of a chicken αβTCR structure would allow comparison of putative CD3-interacting surfaces among all of these receptor types to reveal whether particular features have co-evolved with asymmetric versus symmetric CD3 assemblies.

A notable feature is that, like human TCRβ, the chicken TCRβ is less efficient than its α-chain counterpart in associating with CD3 in three-chain assembly experiments (28). This property in human TCR is expected to ensure that
the requisite asymmetric arrangement of CD3 heterodimers is achieved during biosynthesis. Observation of a similar phenomenon in the chicken TCR, where no such asymmetry exists, suggests that this feature is intrinsic to the TCRα and TCRβ proteins and may reflect a fundamental requirement for cooperative assembly with the two CD3 modules. Accordingly, we consistently recovered more ch-TCRαβ heterodimer in samples where only one of the central lysine residues was mutated to alanine, compared to samples where both were mutated (Figures 5 and 6), and poorest TCRαβ heterodimer formation was seen in control reactions where α and β chains are folded in the absence of CD3. TCRαβ formation and assembly with two CD3 modules are thus cooperatively linked. As in human TCR, joining of the ζζ homodimer appears to be the final step in chicken TCR assembly. It is unusual that the interaction between ζζ and TCR is mediated by a lysine instead of an arginine in the TM domain of TCRα, since in the human TCR mutation of this arginine to lysine results in a significant drop in affinity for ζζ (28). Indeed, the chicken TCR is comparatively poor at recruiting the ζζ homodimer based on our biochemical analysis. Whether this has functional consequences for chicken TCR signaling or is simply an artifact introduced by detergent extraction is unclear.

The unique structural features of ch-CD3 described here may reflect the distinctive demands of a “symmetric” CD3 assembly with the inherently asymmetric TCR: whereas human CD3εδ and CD3γε each appear to have evolved unique surface features that optimize extracellular complementarity with TCRα and TCRβ, respectively, the ch-CD3εδ/γ surface must complement both TCR constant domains simultaneously. Due to its reduced subunit complexity compared to the mammalian receptor, the chicken TCR signaling complex may represent a more tractable experimental system for studying ectodomain interactions between the TCR and CD3 subunits. Further structural studies on the chicken proteins may therefore provide access to some of the more conserved features of the TCR signaling complex molecular organization that will impact on our models of receptor function.

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Acknowledgements -

FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Overview of the ch-CD3 NMR structure. (A) Ensemble of 10 final NMR structures of ch-CD3. Regions with secondary structure (as defined by STRIDE (54)) are colored red. (B) Ribbon diagram of ch-CD3 with the secondary structure of CD3ε shown in pink and CD3δγ shown in cyan. The cysteine residues that form intra-domain disulfide bonds are represented as red sticks. The single predicted N-linked glycosylation site in CD3δγ (Asn 71) is shown as an orange sphere. The structures shown span from Glu-6ε to Val70ε and from Leu-9δ/γ to Met-79δ/γ and exclude the 26 amino acid linker. (C) Secondary structure-matched superimposition of human and chicken CD3ε chains (I). In (II) and (III) the structure of ch-CD3δ/γ was aligned to that of human CD3δ and CD3γ respectively. Human CD3 structures are colored grey, ch-CD3 structures are in pink (CD3ε) or cyan (CD3δγ). Only residues that were assigned in the ch-CD3 structure, and the corresponding residues in human CD3 are shown. Dashed lines highlight regions of significant structural deviation. (D) Amino acid sequence alignment of the immunoglobulin domains of human, mouse and chicken CD3ε, δ and γ chains. The secondary structure for human and chicken variants is displayed above and below the sequences respectively. Potential N-linked glycosylation sites are highlighted in bold. * Indicates absolutely conserved residues.

FIGURE 2. The ch-CD3 heterodimer. (A) Interactions at the ch-CD3εδ/γ interface. The interlocking ladder running along the parallel G-strands comprises residues Asp59ε-Tyr65ε (pink) and Asn71δ/γ-His75δ/γ (cyan). The interface is supported by contacts between the F-strand of CD3ε (residues Thr52ε and Ser54ε) and the A-strand of CD3δ/γ (Met11δγ-Val13δγ). (B) The ch-CD3 heterodimer is shown in ribbon representation with the FG loop and G-strand of human CD3ε overlaid in red. The bulkier human CD3ε loop would clash extensively with the ch-CD3εδ/γ subunit. (C) Surface representation showing side view (top) and top view (bottom) of ch-CD3. For comparison the structures of human CD3εδ (1XIW) and CD3εγ (1SY6) are shown. All structures were aligned via the CD3ε chain (pink). The black line connects the center of mass of the CD3 subunits. Center of mass calculations included only those residues for which NMR spectra was assigned (for ch-CD3) and the corresponding residues in the human CD3 variants. Subunits are colored as in Figure 1.
The contribution of individual TCR α and CD3 chains was performed and the results calculated as a ratio of CD3 heterodimer/TCR heterodimer. TCR α β select TCR with anti-HA agarose. Shown is a representative gel of the species bound to the beads in each on the graph represents three repeats of the same experiment. Two tailed T-tests indicated that TCR immunoprecipitation and a gel showing 5% of the input before immunoprecipitation. The data presented could pull down more CD3 than TCR chains and 5-fold lower than TCR chains.

FIGURE 4. Alignments of human and chicken TCR and CD3 stalk, transmembrane and intracellular juxtmembrane sequences. Residues that are identical between human and chicken sequences are marked with asterisks. In the comparison of chicken CD3δ/γ with human CD3δ and CD3γ, residues identical to human CD3δ are marked above the alignment and those identical to human CD3γ are marked below the alignment. The predicted membrane spanning regions based on analysis of TMHMM 2.0 scores (55) are shaded with grey rectangles. The polar TM residues that are known to be essential for assembly of the human TCR and CD3 subunits and the corresponding positions in the chicken sequences are marked in blue (basic), red (acidic) and orange (hydroxyl-bearing).

FIGURE 5. Both TCRα and TCRβ can bind CD3 εδγ and the interaction is dependent on the central TM lysines. HA-tagged TCR chains were translated with CD3ε and CD3δ/γ in IVT and immunoprecipitated with anti-HA agarose. Shown is a representative gel of the species bound to the beads in each immunoprecipitation and a gel showing 5% of the input before immunoprecipitation. The data presented on the graph represents three repeats of the same experiment. Two tailed T-tests indicated that TCR could pull down more CD3εδγ than TCRβ, but that the amount pulled down by TCRβ was still more than the mixing control where TCRβ and the CD3 mRNAs were translated in separate IVT reactions and mixed prior to IP. ** indicates 0.001 < P < 0.01, * indicates 0.01 < P < 0.05, ns indicated P > 0.05.

FIGURE 6. The central TM lysines of both TCRα and TCRβ are important for CD3εδ/γ recruitment in full assembly of the TCR signaling complex. SBP-tagged TCRα and HA-tagged TCRβ were translated with CD3ε, CD3δ/γ and ε and a sequential immunoprecipitation for TCRα followed by TCRβ was used to select TCRβ heterodimers. In the mixing control, TCRα and TCRβ were translated in a separate reaction from the CD3 and ε components and mixed after stopping the assembly reaction. A 5% sample before immunoprecipitation was taken to ensure input was similar. Densitometry of TCRβ heterodimers and CD3 chains was performed and the results calculated as a ratio of CD3 heterodimer/TCR heterodimer. The contribution of individual TCRα and TCRβ chains to the CD3 signal was included in the ratio calculations. ** indicates 0.001 < P < 0.01, * indicates 0.01 < P < 0.05, ns indicated P > 0.05.

FIGURE 7. ζ incorporation requires the upper TM lysine of TCRα and all CD3 chains. TCRα and TCRβ were translated with CD3ε-SBP, CD3δ/γ and ζ-PC with ζ at 10-fold lower concentration than CD3 chains and 5-fold lower than TCR chains. ζ was targeted for immunoprecipitation and the amount of co-precipitated TCR heterodimer was measured by densitometry. In mixing controls, TCRα and TCRβ mRNA were translated separately from CD3 and ζ chains and processed as described in Figure 6. A 5% sample of the digitonin extraction was loaded on a gel as an input control. The ratio of TCR heterodimer to ζζ homodimer is graphed. * indicates 0.01 < P < 0.05.

FIGURE 8. Cartoon schematic of the chicken TCR signaling complex. The grey disc represents the plasma membrane. Residues that are important in guiding TCR assembly are color-coded: light blue – lysine, red – aspartic acid. The extracellular domains are color-coded to identify each polypeptide: light blue – TCRα, green – TCRβ, orange – CD3ε, red – CD3δγ. ITAMs in the cytoplasmic tails of each species are represented as grey rectangles. While this cartoon implies both CD3ε TM domains would fall closest to ζζ in the assembled complex, this information is not known and this uncertainty is indicated with question marks.
TABLES

**TABLE I.** NMR and refinement statistics for the 10 Ch-CD3εδ/γ model structures

| Non-redundant NOE distance restraints |   |   |
|--------------------------------------|---|---|
| Total                                | 1784 |   |
| Intra (i = j)                        | 315  |   |
| Sequential ([i-j] = 1)               | 547  |   |
| Short (1 < |i – j| ≤ 5)             | 291  |   |
| Long (|i – j| > 5)            | 631  |   |
| Dihedral Angle Restraints           | 158  |   |
| Hydrogen Bond Restraints (2 per bond)| 68  |   |

| Deviations from Experimental Data   |   |   |
|-------------------------------------|---|---|
| NOEs (Å)                            | 0.016 ± 0.001 |   |
| Dihedrals (°)                       | 0.692 ± 0.071 |   |

| Deviations from Ideal Geometry      |   |   |
|-------------------------------------|---|---|
| Bonds (Å)                           | 0.0016 ± 0.0001 |   |
| Angles (°)                          | 0.327 ± 0.006 |   |
| Impropers (°)                       | 0.211 ± 0.012 |   |

| Pairwise root mean standard deviation (Å) (residues 9-73,108-123,132-174) |   |   |
|--------------------------------------------------------------------------|---|---|
| Backbone atoms                                                           | 0.69 ± 0.10  |   |
| Heavy atoms                                                              | 1.29 ± 0.10  |   |

| Ramachandran Statistics                                                   |   |   |
|--------------------------------------------------------------------------|---|---|
| Residues in most favoured regions                                        | 68.7% |   |
| Residues in additional allowed regions                                   | 25.4% |   |
| Residues in generously allowed regions                                   | 5.0% |   |
| Residues in disallowed regions                                           | 0.9% |   |
Figure 3

A

Ch-CD3ε

h-CD3ε

B

ε: ABE, δ/γ: C'CFG

ε: CFG, δ/γ: ABE
Fig 5

Table of data:

| Sample          | TCRα 1st K→A | TCRα 2nd K→A | TCRβ K→A | Mixing Control |
|-----------------|--------------|--------------|----------|----------------|
| WT TCRα         |              |              |          |                |
| TCRα 1st K→A    |              |              |          |                |
| TCRα 2nd K→A    |              |              |          |                |
| TCRβ K→A        |              |              |          |                |
| Mixing Control  |              |              |          |                |

Graphical representation:

- (CD3 dimer)/(TCR chain)
- WT TCRα
- TCRα 1st K→A
- TCRα 2nd K→A
- TCRβ K→A
- Mixing Control

**Notes:**
- NS
- *p < 0.05
- **p < 0.01

Immunoprecipitation:
- anti-HA
- 5% of input before IP
Fig 6

TCRα-SBP:TCRβ-HA
TCRα-SBP
TCRβ-HA
CD3ε-PC
CD3δ/γ

SBP => HA
Immunoprecipitation
5% of input before IP

(CD3 dimer)/(TCR heterodimer)

TCRa wt:TCRβ wt
TCRa 1st K => A: TCRβ wt
TCRa 2nd K => A: TCRβ wt
TCRa 2nd K => A: TCRβ K => A
TCRa wt:TCRβ wt:Mixing Control

TCRa 1st K => A: TCRβ wt
TCRa 2nd K => A: TCRβ wt
TCRa 2nd K => A: TCRβ K => A
TCRa wt:TCRβ wt:Mixing Control

**
*
ns

TCRa wt:TCRβ wt
TCRa 1st K => A: TCRβ wt
TCRa 2nd K => A: TCRβ wt
TCRa 2nd K => A: TCRβ K => A
TCRa wt:TCRβ wt:Mixing Control
Structure of the chicken CD3εδ/γ heterodimer and its assembly with the αβT cell receptor
Richard Berry, Stephen J. Headey, Melissa J. Call, James McCluskey, Clive A. Tregaskes, Jim Kaufman, Ruide Koh, Martin J. Scanlon, Matthew E. Call and Jamie Rossjohn

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