Structural characterization of the TCR complex by electron microscopy

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Abstract

Structural information on how the TCR transmits signals upon binding of its antigen peptide MHC molecule ligand is still lacking. The ectodomains of the TCRα/β, CD3γ and CD3δ dimers, as well as the transmembrane domain of CD3ζ, have been characterized by X-ray crystallography and nuclear magnetic resonance (NMR). However, no structural data have been obtained for the entire TCR complex. In this study, we have purified the TCR from T cells under native conditions and used electron microscopy to derive a three-dimensional structure. The TCR complex appears as a pear-shaped structure of \(180 \times 120 \times 65 \AA\). Furthermore, the use of mAbs has allowed to determine the orientation of the TCRα/β and CD3 subunits and to suggest a model of interactions. Interestingly, the reconstructed TCR is larger than expected for a complex with a \(\alpha\beta\gamma\delta\epsilon\zeta\) stoichiometry. The accommodation of a second TCRαβ to fill in the extra volume is discussed.

Keywords: CD3, purification, structure, TCR

Introduction

The majority of T cells recognize antigens in the form of peptides associated to MHC molecules (pMHC) via a membrane protein complex known as the TCR (1–3). The TCR of αβ T cells is composed of the ligand-binding subunits TCRα and TCRβ, which form the disulfide-linked TCRαβ heterodimer, non-covalently bound to the signal transducing CD3 subunits \([CD3γ, CD3δ, CD3ζ, and CD3ζ’ (CD247)]. The structure of the TCRαβ heterodimer ectodomain has been characterized by X-ray crystallography (3–5). Likewise, the structures of the CD3γ and CD3ζ dimer ectodomains have been solved by X-ray crystallography and NMR (6–9). However, the structural characterization of the entire TCR complex has not yet been achieved.

Assembly of TCRαβ with CD3 is thought to rely mostly on transmembrane domain interactions (10–15). On the other hand, interactions between the TCR and CD3 ectodomains have been suggested to play an important role in early signalling events (5–9, 16). However, these interactions have not been directly confirmed on intact complexes. In this study, we have purified the native TCR complex from murine T cells in non-ionic detergents and have generated a low-resolution three-dimensional (3D) reconstruction of the complete complex by electron microscopy (EM) of negatively stained particles. This has allowed us to define the dimensions and general shape of the TCR unit. Furthermore, the analysis of TCR complexes bound to either anti-TCRβ or anti-CD3ζ has provided valuable information on how the TCRαβ and CD3 subunits are oriented in the complex. This is important to delineate how the TCRαβ heterodimer interacts with the CD3 subunits and for understanding the molecular basis for transmembrane signalling.

Methods

Expression vectors and cells

To purify the TCR complex, the MA5.8 CD3ζ-negative derivative of the murine T hybridoma 2B4 was transfected with CD3ζ tagged at the C-terminus with a hexa-histidine sequence. The His \((6\times)\) CD3ζ construct was obtained by PCR and cloned into the expression vector pSRα. A G418-resistant clone of MA5.8, clone 1D8, expressing high levels...
of the TCR on the cell surface was selected. The digitonin-solubilized TCR was purified from an MA5.8-derived cell line expressing a streptavidin-binding peptide-tagged ζ protein (MA5.8·SBP).

Antibodies and reagents

The rabbit antiserum 448 (anti-CD3ε) has been previously described (17). mAbs H57-597 (anti-TCRβ) and 145-2C11 (anti-CD3ε) were generously provided by Ralph Kubo and Jeffrey Bluestone. Goat anti-CD3ε antiserum M20 antibody was purchased from Santa Cruz Biotechnology. Concavalin A-sepharose, D-biotin and α-methyl-mannoside were purchased from Sigma and the Ni-NTA resin from Qiagen. Ultra-Link Immobilized Streptavidin Plus was purchased from Perbio.

Protein purification

The TCR complex was extracted from 1D8 cells (12 × 10⁹) and suspended in Brij96 lysis buffer [20 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 μg ml⁻¹ aprotinin, 1 μg ml⁻¹ leupeptin, 0.01% phenylmethylsulphonyl-fluoride and 1% (w/v) Brij-96]. Cell lysates were centrifuged at 3000 × g and applied onto a Concavalin A-sepharose column pre-equilibrated with lysis buffer containing 0.1% Brij-96. Proteins were eluted with 500 mM α-methyl-mannoside and fractions were analyzed by western blotting using anti-CD3ε antiserum and M20 (anti-CD3ε). Fractions were selected, diluted in Brij96 buffer plus 10 mM imidazole and applied onto a Ni-NTA column. The TCR complex was eluted in the same buffer plus 100 mM imidazole. Aliquots of purified TCR were directly used in EM analysis. For the purification of SBP-tagged TCRs, 10 mM imidazole. Aliquots of purified TCR were directly used

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Antibody labelling

mAbs anti-TCRβ H57-597 and anti-CD3ε (145-2C11) were added (15 μg) to TCRs eluted from the Ni-NTA column and the suspension was incubated for 2 h at 4°C. Beads were packed into empty columns (Bio-Rad) and washed with the same buffer containing 25 mM imidazole and 0.1% Brij-96. After extensive washing, labelled TCR was eluted from the column in 200 mM imidazole and analyzed by EM.

EM and image analysis

Aliquots (5 μl, 0.05 mg ml⁻¹) of purified TCR complex were applied to glow-discharged carbon coated grids and stained with 2% (w/v) uranyl acetate. Images were recorded at 60 000x nominal magnification on Kodak SO163 films under low-dose conditions and 1μm defocus using a JEOL 1200EX-II electron microscope operated at 100 KV. Micrographs were digitized in a Zeiss SCAI scanner with a final sampling rate of 3.5 Å per pixel. Particles were selected and normalized using XMIPP image processing software (18). 3D reconstructions were obtained with EMAN software (19). Volumes were low-pass filtered and refined until convergence was attained. 3D EM maps were aligned using BSOFT software (20) and visualized using UCSF Chimera (21).

Molecular modelling

The atomic co-ordinates of TCRβε heterodimer bound to a Fab·H57 (1nf5.pdb) (5), CD3εδ bound to UCHT1 (1xiw.pdb) (6) and CD3γε bound to OKT3 (1sy6.pdb) (7) were used to model the TCRβε·CD3εδ and CD3εγ interactions. Atomic models of TCRβε·CD3εδ human complex, generated with the programme Z-Dock (22), were filtered using the following restraints: (i) deviation from the membrane perpendicular axis <30°, (ii) interference between bound antibodies and (iii) collision with glycosylation sites. The mureine structural homologue to the best human model was constructed with MAMMOTH structural alignment programme (23). Energy minimization was performed with AMBER8 (24). Asn/Gln/His flips were corrected with molprobity web server (25). Protonation was done with the programme H++ (26). Buried surface area was calculated with NACCESS (27). The final model was fitted into the EM volumes using Situs software (28).

Results

Purification of the TCR complex

The TCR complex was cloned with two different types of tags in the C-terminal end of the CD3ε subunit: either a His (6×) or a streptavidin-binding peptide. Because CD3ε is the last subunit to be added to the TCR complex during assembly (29, 30), the use of anti-CD3ε antiserum in immunoblotting permits the detection of completely assembled complexes (Supplementary Figure 1A is available at International Immunology Online). Cells were solubilized with two different non-ionic detergents, Brij-96 and digitonin. Both detergents were used previously in the study of the oligomeric state of TCR (31). The purified murine TCR was analyzed by BN-PAGE followed by a second dimension SDS-PAGE under non-reducing conditions. Irrespective of the method used, the TCR complex appeared pure after silver staining (Supplementary Figure 1B is available at International Immunology Online). The αβγδεζζζ TCR, of an apparent molecular size of 400–420 kDa in the first dimension (BN-PAGE), is the most abundant protein complex in the sample, with a purity of >90%. More important, the second dimension (SDS-PAGE) reveals that the 400–420 kDa band is composed of the TCRγ, TCRβ, CD3γ, CD3ε, CD3ζ and CD3ζ subunits of the TCR and that no other proteins were associated with the TCR at stoichiometric amounts. Therefore, the TCR complex preparations consist exclusively of the TCR complex itself.

3D reconstruction of the TCR complex

Purified TCR was negatively stained and analyzed by EM (Fig. 1A). The low-resolution (35 Å) 3D reconstruction generated had a pear-shaped structure of 180 × 120 × 65 Å dimensions and an apparent 2-fold symmetry along the long...
The alignment of both structures suggests that binding of the TCR complex, suggesting that their epitopes in the TCR and CD3e subunits are very close to each other. Indeed, the extra volume as the membrane domain of TCR is only 20% its mass. (iv) There is a second TCRab heterodimer. In agreement with previous mAb binding analysis (32).

**Modelling of the TCRab and CD3ec interactions**

Immunolabelling of the TCR complexes with different antibodies indicated that the binding epitopes should be very proximal (Fig. 2D). The anti-TCRβ epitope is located at a loop connecting its constant (Cβ) and variable regions (Vβ). Likewise, the epitope for the CD3ec subunit is located at a loop connecting the C and Fβ-strands. Here, we have taken advantage of the crystal structures of murine TCRαβ solved in the presence of the H57-597 Fab fragment (5) and the NMR structure of murine CD3ecγ (6) to build an atomic model of the TCRαβ-CD3ecγ complex (Fig. 3). As there are no atomic structures available for the murine CD3ecγ or CD3ecδ in complex with antibodies, the human structures of CD3ecγ, complexed to the Fab fragment of anti-CD3 mAb OKT3 (7), and CD3ecδ, complexed to the Fab fragment of anti-CD3 mAb UCHT1 (6), were used to model the 145-2C11 antibody position in mouse CD3ec. Several constraints were used to create the model: the orientation of the complementarity-determining regions in TCRαβ away from the transmembrane region, the position of the epitopes in Cβ and in CD3ec, the location of the glycosylation sites and the orientation of the subunits with respect to the plasma membrane. The final model (Fig. 3) is the best fit, with the largest buried surface area (3615Å²), the lowest energy and with no steric impediments. This atomic model was docked into the 3D reconstructions obtained by EM (Fig. 4). The extra mass in the lobe region not filled with any atomic structure is due to the fact that in our EM preparations, the TCR complex was labelled with full-sized mAbs, whereas the atomic model was derived from crystal structures obtained with Fab fragments. According to this EM-derived model, the interactions between the ectodomains of TCRαβ and CD3ecγ occur primarily between Cβ and CD3ec.

**Docking the atomic model into the EM volumes**

After docking the atomic structures of TCRαβ-CD3ecγ in the EM volumes, it became evident that there was an extra volume that could not be filled by the CD3ec dimer alone (Fig. 4). We considered several possibilities: (i) the TCR complex could be associated to other proteins that fill in the extra volume. This seems unlikely, as no other proteins were detected after SDS-PAGE and silver staining of the purified TCR (Supplementary Figure S1 is available at International Immunology Online). (ii) Excessive N-linked glycosylation could contribute to the extra volume. Indeed, the reduction of the TCR size in native gels after treatment with N-glycosidase F is large (Swamy M. S. and Schamel W. S., unpublished results). Therefore, this possibility is not excluded. (iii) The extra volume is contributed by the detergent. However, it is unlikely that the detergent would fill all the extra volume as the membrane domain of TCR is only 20% its mass. (iv) There is a second TCRαβ heterodimer. In a corresponding model (Fig. 5), the two TCRαβ heterodimers are placed in a parallel slided orientation, with CD3ecδ and CD3ecγ interactions.
CD3ε located at the same face of the TCR complex. In this orientation, the Cβ domain of the second TCRβ and the second CD3ε subunit would not be accessible to mAbs H57-597 and 145-2C11, thus supporting the experimental evidence that only one anti-TCRβ and one anti-CD3ε antibodies are bound to the TCR complex (Fig. 1). The extra volume remaining after docking the second TCRβ into the EM model can be filled by N-linked sugars and all transmembrane and cytoplasmic domains.
Discussion

The mechanism by which antigen binding to the TCR results in a signal being transmitted inside the cell remains little understood. Several models have been proposed in which interactions between TCR\(\alpha\) and CD3 ectodomains participate in early signalling events (5–8, 11, 16). However, these interactions have not been proved on intact complexes. In this study, we present a protocol to produce entire complexes purified in non-ionic detergents that enabled us to generate 3D reconstructions by EM. Labelling with mAbs against TCR\(\beta\) or CD3\(e\) has allowed us to identify the orientation of TCR\(\alpha\) towards one CD3 dimer. Based on this topological analysis and biological constraints, such as the location of the antigen-binding region, the location of the glycosylation sites or the distance to the membrane plane, we suggest a tentative model of the TCR\(\alpha\)/CD3\(e\) interactions.

Previous models have been suggested where the overhanging FG loop of C\(\beta\) interacts with the BC loop at the top of CD3\(e\) (8). Such arrangement, perpendicular to the membrane plane, was proposed to permit a downward ‘piston displacement’ of the CD3 dimers after pMHC binding to TCR\(\alpha\beta\) (Fig. 6A) as a mechanism to initiate TCR signalling (8, 17, 33). However, other arrangements have been suggested in which CD3\(\gamma\) interacts with TCR\(\alpha\beta\) in an oblique orientation (7), with CD3\(e\) placed ‘side-by-side’ with C\(\beta\) (Fig. 6B). Although due to the resolution level of our 3D reconstructions we cannot fully discard any of these models, it seems more likely the ‘side-on’ than the ‘piston-like’ model.

We have generated >1000 atomic models of TCR\(\alpha\beta\)-CD3\(\alpha\) complex and using the constraints specified in Methods, the model that fits better into the 3D reconstructions was more in agreement with the side-on nature of the TCR\(\beta\)/CD3\(e\) interactions. The side-on nature of these interactions would also facilitate the communication between adjacent TCR complexes in preformed nanoclusters in non-stimulated T cells (31, 34).

Another important aspect of the present study refers to the stoichiometry of the minimal TCR complex. Although our own gel shift assays using anti-CD3\(e\) and anti-TCR\(\beta\) antibodies indicated that the stoichiometry of the TCR complex in digi-tonin was \(\alpha\beta\gamma\delta\epsilon\zeta\zeta\) (35), the volume of the EM-derived model cannot be explained by this stoichiometry, unless we assume that 50% of the extracellular mass is contributed by glycosylation. Our own BN-PAGE data show a large increase in TCR mobility after N-glycosidase treatment (Swamy and Schamel, unpublished results). However, previous data generated after N-glycosidase treatment and SDS-PAGE does not show such large contribution of glycosylation to the size of the TCR (36). Therefore, although glycosylation cannot be excluded, we favour the existence of a second TCR\(\alpha\beta\) in the minimal TCR complex. Whether the minimal TCR complex contains either one or two TCR\(\alpha\beta\) heterodimers is
a recurring issue. Coimmunoprecipitation, comodulation, sucrose density fractionation and antibody gel shift assays in BN-PAGE and FRET have been used to determine the stoichiometry of the TCR complex with discrepant results (12, 35, 37–40). Many of the discrepancies could be based on the type of detergent used and the sensitivity of the method. However, most data confirming the presence of two TCRαβ heterodimers per complex could have been equally produced if a monovalent (i.e. with one TCRαβ) is repeatedly oligomerized forming the so-called TCR nanoclusters (31, 34). Therefore, the large volume of the EM-derived TCR model brings the issue of TCR stoichiometry again to stage. The presence of two different subcomplexes (αβγδε and αβδε), perhaps glued by the CD3ε homodimer, in a parallel slided orientation (Fig. 5), could explain the inaccessibility of the second TCRβ and second CD3ε epitopes to antibodies. Furthermore, this arrangement implies that both CD3δε and CD3γε are located at the same face of the bivalent complex and that, in spite of the presence of two CD3ε-CD3δε(γε) dimers, anti-CD3 antibody-mediated cross-linking cannot occur within a single complex. Finally, the parallel but slightly ‘slided’ orientation of the two TCRαβ heterodimers bears consequences on how the TCR complex binds MHCp either as single complex, as dimers of dimers, or as higher order complexes (41, 42).

In summary, here, we present the first direct confirmation of the TCRβ—CD3ε interactions. Based on the volume, shape and position of two antibody epitopes, we propose a model for organization of the entire TCR complex in which the pMHC-binding TCRαβ subunits establish side-on interactions with the signalling CD3 dimers. Furthermore, the large volume of the EM-derived model suggests the presence of two TCRαβ heterodimers per complex. However, further structural and mutagenesis work is needed to confirm the validity of this model.

Supplementary data
Supplementary Figures 1A and B and Figure S1 are available at International Immunology Online.

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