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Backbone Resonance Assignment of N15, N30 and D10 T cell Receptor β subunits

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Abstract

The $\alpha\beta$ T Cell receptor (TCR) governs T cell immunity through its interaction with peptide bound to major histocompatibility complex molecules (pMHC). Previously, soluble ectodomain constructs have been used to elucidate the binding mode of the TCR for the MHC. However, the full heterodimeric $\alpha\beta$ TCR has proven difficult to produce reproducibly in recombinant systems to the extent seen in the routine production of novel antibodies. Particularly, the route of production in *E. coli*, which is most convenient for isotopic labeling of proteins, is challenging for a wide range of $\alpha\beta$ TCR, including N15 $\alpha\beta$, N30 $\alpha\beta$, but not D10 $\alpha\beta$. With the aim of understanding the TCR-pMHC interaction through the use of dynamic binding measurements, we set out to produce TCR β subunits with which we could investigate binding with pMHC. The TCR β constructs are more readily produced and refolded than their $\alpha\beta$ counterparts and have proven to be an effective model of preTCR in pMHC binding studies. As a first step towards characterizing potential interactions with protein ligands, we have assigned the backbone resonances of three TCR β subunits, N15 β , N30 β and D10 β .

Keywords

T-cell Receptor; TCR; MHC; immunology; protein refolding

Biological context

The T-cell receptor (TCR) is a transmembrane cell surface protein ensemble of eight subunits including an $\alpha\beta$ heterodimer with specificity for peptide bound to major histocompatibility complex molecules (pMHC), reviewed in Wang and Reinherz (2012). While the identity of the ligand for the $\alpha\beta$ TCR has been known for some time and high-resolution structures of the complex are available, less is known about the dynamics of binding surfaces as binding occurs. It is the understanding of the details of this interaction that will facilitate better control of immune response to optimize preventative vaccination

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efficiency, stimulate therapeutic immune responses or alternately, prevent autoimmunity through targeted immunosuppressive interventions.

Protein dynamics may be crucial to understanding general aspects of $\alpha\beta$ TCR-pMHC recognition as well as details of ligand binding as it occurs under force. Specifically, $\alpha\beta$ TCR function requires physical force to yield productive binding that stimulates T cell activation (Kim et al. 2009). Furthermore, catch bonds have been shown to occur during the $\alpha\beta$ TCR-pMHC ligation at the juxtaposed cell surfaces of a T cell-antigen presenting cell conjugate, with rearrangement of specific elements within the CDR loops of the TCR and both the peptide and the MHC within pMHC (Liu et al. 2014; Das et al. 2015). Moreover, it has been recently revealed that the preTCR possesses ligand-binding functions that control development of early thymocytes to more mature $\alpha\beta$ T cells (Mallis et al. 2015). One can speculate that sparsely populated conformational states (Baldwin and Kay 2009), i.e., “hidden states”, are present in ligand free TCR which are selected during binding under force. Conformations of these hidden states may become dominant when the TCR or preTCR is presented with pMHC ligand. NMR analysis of TCR surface loops have shown potentially significant dynamic behavior in the few systems amenable to such analysis (Hare et al. 1999; Hawse et al. 2014), suggesting that study of a more diverse array of receptors will yield knowledge on TCR and preTCR ligand-binding behavior. Here we present the backbone resonance assignment of three different TCR β subunits, providing a critical step in the NMR study of the TCR and preTCR.

Methods and experiments

N15 β , N30 β , and D10 β were produced as V β -C β ectodomain constructs essentially as described (Zhou et al. 2011) with the following modifications for isotopic labeling. Uniformly ^{15}N labeled proteins were produced by growing T7ExpressI^q *E. coli* (New England Biolabs) in M9 media supplemented with ^{15}N NH_4Cl . For backbone assignment, uniformly $^2\text{H}^{13}\text{C}^{15}\text{N}$ proteins were produced in cultures grown in M9 media dissolved in >98% $^2\text{H}_2\text{O}$, supplemented with ^{15}N NH_4Cl and $\text{U-}^2\text{H}, ^{13}\text{C}$ -Glc. All stable isotopes were purchased from Cambridge Isotopes or Isotec (Sigma). While the growth rate of the bacteria was approximately 50% and 25% that of LB grown cultures for ^{15}N and $^2\text{H}^{13}\text{C}^{15}\text{N}$ protein labeling, respectively, maximal protein production was still attained by 3 hours after induction at 37C with 1mM IPTG (Sigma) in both cases. After expression, cells were lysed during two freeze/thaw cycles in the presence of 1mg/ml lysozyme, a tablet of protease inhibitor cocktail (Complete, EDTA-free Tabs, Roche) and 200U/ml Deoxyribonuclease I (from bovine pancreas, Sigma). Pellets containing inclusion bodies were washed at 4C by centrifuging ($50,000 \times g$, 30min), discarding supernatants and resuspending pellets in fresh buffer, consecutively in 30mM Tris-HCl, pH 8.0 + 150mM NaCl (TBS), TBS + 1% Triton X-100 twice, and TBS twice again. Purified inclusion bodies were then resuspended in 6M guanidine hydrochloride + 0.1M Tris-HCl, pH 8. Yields were typically greater than 100 mg protein per liter of expression as determined by UV-spectrophotometry at 280nm and quantification with the extinction coefficient from amino acid sequence determined using ProtParam (Gasteiger et al. 2003) for each protein. Proteins were then refolded by dilution to 1mg/ml into a buffer containing 5.4M guanidine hydrochloride, 1M L-Arginine, 0.1M Tris-HCl pH 8.0, 0.1mM oxidized glutathione disulfide (GSSG) and 1mM reduced glutathione

(GSH). After 1hr proteins were dialyzed against TBS for 16 hours or longer at 4C. Proteins were then centrifuged at $50,000 \times g$ for 30 min to remove particulate matter, concentrated and purified by size exclusion chromatography (SEC) using preparative sephacryl S300P and analytical superdex S200A columns (GE Healthcare Life Sciences) consecutively. The proteins were exchanged into NMR buffers (50mM NaPO₄ + 150mM NaCl, pH 7.0 for N30 and D10 β or 6.0 for N15 β , PBS) during the second SEC step. ²H₂O was added to 10% final concentration prior to NMR measurements. Typically 25% of initial protein from inclusion bodies was refolded successfully into essentially pure monomeric protein as judged by SEC, SDS-PAGE and NMR.

D10 $\alpha\beta$ TCR variable domain construct (scD10 $\alpha\beta$) was produced as described (Hare et al. 1999). Backbone assignments were obtained from the Biological Magnetic Resonance Data Bank (BMRB, <http://www.bmrb.wisc.edu>), accession number 4330 (Hare et al. 1999).

NMR spectroscopy

All NMR spectra were acquired on either a Bruker 600, 750MHz or a Varian 600MHz spectrometer equipped with a cryogenically cooled probe. Backbone experiments included TROSY versions of HNCA, HNCOC, HNCO, HNCACB and HNCACB (Salzmann et al. 1998) for each TCR β . The concentration of the protein samples were in the range of 200–400 μ M, and the samples were in PBS buffer as described above. The NMR experiments were conducted at 298K. All spectra were processed using NMRPipe (Delaglio et al. 1995). Spectra were visualized and analyzed and backbone assignments completed using CARA (Keller 2004) and AutoLink (Masse and Keller 2005).

Extent of the assignments and data deposition

Backbone amide resonances were assigned for 88% of the non-proline residues of N15 β , 94% for N30 β and 88% for D10 β (Fig. 1). Assignment of N15 β provided some challenges and it was necessary to acquire spectra at pH 6.0 in order to assign several residues. Additionally N15 β appeared to have some structural heterogeneity within the CDR2 (C'-C'' loop), C'' strand and C''-D loop, as judged by broadening of resonance peaks for residues flanking this region. These difficulties which possibly resulted from conformational exchange within the monomer may also have been due to the dimerization events which were observed in the X-ray structure of N15 β (Zhou et al. 2011). Unassigned residues in D10 β were largely clustered near the C-C' loop, which is distal to the ligand binding region. Otherwise near complete assignments of non-proline residues were possible. Encouragingly, CDR loops for all three TCR clones were well represented in the assignments, with the exception of CDR2 in N15 β and parts of CDR3 in N15 β and D10 β . Complete carbon resonance (CO, C α , C β) assignment has been achieved in most cases for each assigned amide resonance.

Spectral quality was good for all three TCR β , with wide chemical shift dispersion in the amide proton resonances characteristic of largely β -sheet secondary structures (Fig 2a-c). As would be predicted, constant domain (C β) residues show identity or similarity between all three TCR β with the exception of residues that border the variable domain (V β) (Fig 3a-b).

Similarly, D10 β overlays well with spectra from scD10 $\alpha\beta$ (Hare et al. 1999) with the exception of residues bordering Va within scD10 $\alpha\beta$ or C β within D10 β (Fig 3c-d).

The backbone resonance assignments for N15 β , N30 β , and D10 β were deposited to the BMRB with accession codes 26600, 26601 and 26602, respectively.

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Fig. 1.

Sequence alignment, secondary structure, and extent of assignment of TCRβ. Backbone resonances were assigned for black residues, unassigned residues are in gray. Sequences were aligned according to Hare et al. (1999). Secondary structure assignment was based on alignment with the N15β subunit within the N15αβ crystal structure (Wang et al. 1998). Extent of assignment was 199 of 226 non-proline residues for N15β, 211 of 225 for N30β and 196 of 224 for D10β.

Fig. 2.

TROSY-HSQC spectra of TCR β . Backbone assignments are indicated by one-letter code and residue number. a. N15 β . ^1H - ^{15}N TROSY-HSQC spectrum of U- $^2\text{H}^{13}\text{C}^{15}\text{N}$ sample acquired at 750 MHz. Sample was 400 μM in PBS, pH 6 at 298 K. b. N30 β . ^1H - ^{15}N TROSY-HSQC spectrum of U- $^2\text{H}^{13}\text{C}^{15}\text{N}$ sample acquired at 600 MHz. Sample was 200 μM in PBS, pH 7 at 298 K. c. D10 β . ^1H - ^{15}N TROSY-HSQC spectrum of U- $^2\text{H}^{13}\text{C}^{15}\text{N}$ sample acquired at 750 MHz. Sample was 400 μM in PBS, pH 7 at 298 K.

Fig. 3.

Illustration of concordance of resonances for identical sequences within different TCR β constructs. a. Select regions of overlaid TROSY-HSQC spectra for N15 β (red), N30 β (blue) and D10 β (cyan) highlighting resonance peaks corresponding to various C-domain residues proximal (D151, E214, and K216) or distal (K136 and A231) to V β . Numbering is according to N15 β (Fig. 1) b. Cartoon depiction of N15 β (Zhou et al. 2011) showing the positions of residues highlighted in a. V β is the upper domain and C β is at the bottom. V β -proximal residues are shown as blue spheres while the distal residues are white spheres. c. Select regions of overlaid TROSY-HSQC spectra for D10 β (red) and scD10 $\alpha\beta$ (blue) highlighting resonance peaks corresponding to various V-domain residues proximal (Y48, K57) or distal (T5, Q25, A52, S93) to V α . Numbering is according to D10 β (Fig. 1). d. Cartoon depiction of scD10 $\alpha\beta$ (Hare et al. 1999) showing the positions of residues highlighted in a. V α is the left domain (green) and V β is at the right (blue). V α -proximal residues are shown as blue spheres while the distal residues are white spheres. Representations in b,d depicted using pyMol (Schrödinger 2010).