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# Crystallization and preliminary X-ray analysis of the complex between human CTLA-4 and B7-2

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CTLA-4 is a dimeric T-cell surface receptor responsible for transducing signals that down-regulate activated T cells upon binding B7 ligands. The disulfide-linked homodimer of the extracellular segment of human CTLA-4 and the receptor-binding domain of human B7-2 were purified and cocrystallized. Diffraction from these crystals is consistent with the monoclinic space group $P2_1$ (unit-cell parameters $a$ = 47.85, $b$ = 54.56, $c$ = 103.09 Å, $\beta$ = 91.63); native data have been collected to 3.2 Å resolution.

## 1. Introduction

The specificity of the T-cell mediated immune response is determined by the atomic interactions between the TCR and peptide–MHC complex (Garcia *et al.*, 1999). While the signal initiated by this interaction is necessary, a distinct costimulatory signal, most efficiently delivered by the binding of CD28 on the surface of T cells to its B7-1/B7-2 ligands on the surface of antigen-presenting cells (APCs) (Chambers & Allison, 1999), is also necessary for a fully functional T-cell response (Schwartz, 1990). In contrast, the negative signal transduced by the binding of CTLA-4, a related T-cell surface receptor (~30% identity with CD28), to the B7 ligands results in down-regulation of the immune response and is required for the maintenance of self-tolerance.

Both CD28 and CTLA-4 are disulfide-linked homodimeric type I membrane glycoproteins that belong to the variable (v-type) immunoglobulin (Ig) family. While the binding interfaces of CD28 and CTLA-4 with their B7 ligands are predicted to be very similar based on sequence similarity and mutagenesis studies, CTLA-4 exhibits a 10–100-fold higher affinity for the B7 ligands than does CD28 (van der Merwe *et al.*, 1997). The expression patterns of CD28 and CTLA-4 also differ markedly, as CD28 is constitutively expressed on resting T cells, while CTLA-4 can only be detected on the surfaces of activated T cells (Chambers & Allison, 1999).

B7-1 and B7-2 (~25% identity) are both type I membrane glycoproteins expressed on the surfaces of APCs and each isoform is composed of a membrane-distal V-type Ig domain and a membrane-proximal constant-like (C-type) Ig domain. While the overall structures of these molecules are predicted to be similar, an important difference between the two isoforms has been observed. The V-type Ig domain of B7-2 binds both CD28 and CTLA-4 receptors with the same affinity exhibited by the full-length extracellular region of B7-2 (Ellis *et al.*, 1996; Rennert *et al.*, 1997), while both extracellular domains of B7-1 are required for the maintenance of wild-type binding affinity (Peach *et al.*, 1995).

The recent structure determinations of both murine CTLA-4 and human B7-1 homodimers revealed unusual modes of dimerization which placed their respective ligand-binding sites distal to the dimerization interface in each molecule (Ikemizu *et al.*, 2000; Ostrov *et al.*, 2000). These observations led to the proposal that each B7 dimer could bind two independent CTLA-4 dimers, which would support the formation of a periodic assembly of these molecules that might mimic the aggregation of these molecules at the T-cell/APC interface. To test this model, the complex between the disulfide-linked homodimer of human CTLA-4 and the receptor-binding domain of human B7-2 was crystallized. Here, we describe the preparation, crystallization and preliminary X-ray analysis of this receptor–ligand complex.

## 2. Material and methods

The disulfide-linked homodimeric form of human CTLA-4 was purified as described by Cox *et al.* (1999) with minor modifications. Briefly, the extracellular region of human CTLA-4 (residues 1–126, including Cys122 which forms the intermolecular disulfide) was expressed in *Escherichia coli* and purified from inclusion bodies. These insoluble aggregates were solubilized in 8 *M* urea, 10 m*M* Tris and 10 m*M* EDTA pH 8.0 at 50 mg ml$^{-1}$ CTLA-4 and reduced for ~2 h with 50 m*M* DTT. 100 mg of these solubilized reduced inclusion

bodies were diluted into 100 ml 3 *M* guanidine hydrochloride (Gu–HCl), 10 m*M* NaOAc, 5 m*M* EDTA pH 4.2. These diluted inclusion bodies were then slowly added dropwise into 1 l of refolding buffer (0.3 *M* Gu–HCl, 100 m*M* glycine pH 9.5) at 277 K using a peristaltic pump with constant stirring over ∼40 h. Refolded disulfide-linked homodimeric CTLA-4 was purified by anion-exchange and subsequent gel-filtration chromatography. The presence of the intermolecular disulfide was confirmed by mass spectrometry, gel filtration and reducing and non-reducing SDS–PAGE (data not shown).

The purification of the human B7-2 receptor-binding domain will be described elsewhere (Zhang & Schwartz, unpublished data). In brief, the B7-2 variable region was expressed in *E. coli* and purified from inclusion bodies. 25 mg of this material was solubilized in 1 ml 8 *M* Gu–HCl, 10 m*M* Tris, 5 m*M* EDTA pH 8.0 and then diluted into 14 ml 3 *M* Gu–HCl, 10 m*M* NaOAc, 5 m*M* EDTA pH 4.2. These 15 ml were rapidly diluted over a few seconds into 1 l of refolding buffer composed of 0.4 *M* arginine–HCl, 100 m*M* Tris–HCl, 1 m*M* EDTA, 5 m*M* cysteamine, 0.5 m*M* cystamine pH 8.0 at 277 K with vigorous stirring. Two additional aliquots of B7 (25 mg each) were added with ∼12 h between additions. Refolded B7-2 was purified by anion-exchange chromatography. The monomeric state of B7-2 was confirmed by gel filtration and SDS–PAGE.

Purified human CTLA-4 and B7-2 were each exchanged into buffer composed of 10 m*M* Tris pH 8.0, 20 m*M* NaCl, concentrated to ∼10 mg ml$^{-1}$ and then mixed using a 1:1 mass ratio (monomeric CTLA-4 and the receptor-binding domain of B7-2 have approximately equivalent molecular weights of 13.6 and 12.8 kDa, respectively). Crystallization screens at room temperature were carried out by hanging-drop vapor diffusion by mixing 2 µl of the CTLA-4/B7-2 complex at 3 mg ml$^{-1}$ and 2 µl of crystallization buffer. Diffraction-quality crystals were prepared by hanging-drop vapor diffusion in buffer composed of 15–20% PEG 20 000, 0.1 *M* HEPES pH 7.0. Several crystals were washed, dissolved and subjected to reducing and non-reducing SDS–PAGE to confirm that B7-2 and the disulfide-linked dimer of CTLA-4 had been cocrystallized (Fig. 1).

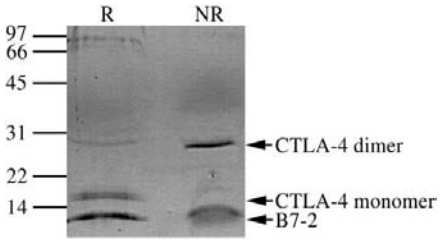


**Figure 1**
SDS–PAGE analysis of complex crystals. Electrophoresis of washed dissolved crystals was performed under reducing (R) and non-reducing (NR) conditions on a 15% SDS–polyacrylamide gel. CTLA-4 migrated as a monomer of ∼14 kDa and a dimer of ∼28 kDa in the reducing and non-reducing lanes, respectively, which confirmed that CTLA-4 exists as a disulfide-linked dimer in the complex crystals. B7-2 migrated as a monomer of ∼13 kDa under both conditions.

## 3. Results and discussion

The crystals were stabilized for flash-freezing by the direct addition of 2 µl of mother liquor containing 30% glycerol to a drop containing CTLA4–B7-2 complex crystals. Crystals were flash-frozen at 100 K and diffraction data collected at beamline X9B (National Synchrotron Light Source, Brookhaven National Laboratory) using a 2×2 ADSC CCD detector. While the overwhelming majority of crystals exhibited high mosaic spread (>2.0°), a single crystal was obtained with a mosaicity of 1.2° which diffracted to 3.2 Å resolution. Data were reduced with the *HKL* suite (Otwinowski & Minor, 1997) and diffraction from this crystal was consistent with the monoclinic space group $P2_1$, with unit-cell parameters $a$ = 47.85, $b$ = 54.56, $c$ = 103.09, $\beta$ = 91.63°.

**Table 1**
Statistics of diffraction data.

Values in parentheses refer to the last shell.

| | |
|---|---|
| Resolution (Å) | 25–3.2 (3.3–3.2) |
| No. of measured reflections | 16672 (1570) |
| No. of unique reflections | 7080 (695) |
| Completeness (%) | 78.8 (79.4) |
| $R_{sym}$ | 12.1 (32.1) |
| $I/\sigma(I)$ | 6.5 (2.2) |

Assuming four V-type Ig domains (*i.e.* two CTLA-4 monomers and two B7-2 monomers) in the asymmetric unit, the Matthews volume (Matthews, 1968) $V_M$ and solvent content were calculated to be 2.59 Å$^3$ Da$^{-1}$ and 52%, respectively. Data-collection statistics are summarized in Table 1. Structure solution by molecular replacement is in progress.

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