Human CCL5 trimer: expression, purification and initial crystallographic studies

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The chemokine CCL5 is considered to be a potential therapeutic target because of its ability to recruit immune cells to inflammatory sites. CCL5 aggregates under physiological conditions, and high-order oligomer formation is considered to be significant for cell migration, immune-cell activation and HIV cell entry. The structure of the high-order oligomer is unknown and the mechanism by which the oligomer is derived has yet to be established. Here, a CCL5 mutant (CCL5-E66S) which is deficient in oligomer formation was mixed with native CCL5 to prepare a protein trimer. At an optimized ratio the trimeric CCL5 crystallized, and the crystal belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = 56.6$, $b = 56.6$, $c = 154.1$ Å. The Matthews coefficient (V_M) of the crystal is 2.58 Å³ Da⁻¹ (three molecules in the asymmetric unit), with a solvent content of 52.32%. Diffraction data were collected to a resolution of 1.87 Å and the statistics indicated satisfactory data quality. The new structure will reveal the interfaces in the CCL5 oligomer, therefore assisting in understanding the mechanism of CCL5 oligomerization.

1. Introduction

CCL5 is an inflammatory factor that is released from activated T lymphocytes, monocytes and other immune cells (Gordon, 2003). The inflammatory responses induced by CCL5 include not only the activation of immune cells (chemotaxis) but also the regulation of other inflammatory cytokines, further exacerbating inflammation reactions (Soria & Ben-Baruch, 2008). As an important chemokine, CCL5 plays a critical role in several diseases, including transplant rejection, atherosclerosis, rheumatoid arthritis, atopic dermatitis, asthma, airway inflammatory disorders and cancers (Appay & Rowland-Jones, 2001). In addition, because the CCL5 receptor, CCR5, serves as one of the main co-receptors of HIV-1, binding of CCL5 to CCR5 blocks HIV-1 recognition by CCR5. As a result, CCL5 should be a potent natural inhibitor to prevent HIV-1 infection (Berger *et al.*, 1999).

On the cell surface, chemokines execute their functions through different mechanisms that are related to two types of receptors: G protein-coupled seven-transmembrane receptors (GPCRs) and glycosaminoglycans (GAGs). Most chemokines have a propensity to form oligomers. Many studies have demonstrated that chemokines bind GPCRs in a monomeric form and bind GAGs as a high-order oligomer (Mortier *et al.*, 2011; Paavola *et al.*, 1998; Kufareva *et al.*, 2015). The monomer-GPCR interaction is based on the chemokine N-terminus inserting into the helical bundle of the receptor and the core domain of the chemokine interacting with the

extracellular loops and N-terminus of the GPCR (Zheng *et al.*, 2017). Meanwhile, the chemokine oligomer integrates protein surface charges to enhance its affinity for GAGs (Wang *et al.*, 2011). The binding is strong and specific because of the multivalent nature of the effect.

The chemokine CCL5 can form monomers, dimers, tetramers and high-order oligomers (Wang *et al.*, 2011). These conformers are essential for its different physiological activities *in vivo*. Solvent conditions determine the formation of monomers and dimers in an acidic environment and oligomers in neutral conditions. Structures of the monomer (Wiktor *et al.*, 2013) and dimer (PDB entry 1eqt; Hoover *et al.*, 2000) are available. However, structural elucidation of the CCL5 oligomer is very challenging owing to severe aggregation. Therefore, the structure of the CCL5 oligomer is the missing piece of the puzzle to finalize the structure–function relationship of CCL5.

To determine the mechanism of regulation of the distinct oligomeric states, it is necessary to determine the structure of the CCL5 oligomer. Therefore, it is critical to prevent protein aggregation when preparing samples. A number of mutants have been shown to have limited oligomerization properties. A particular mutant, CCL5-E66S, in which Glu66 is replaced by a Ser residue, has a lower tendency for oligomerization and mainly forms dimers when the pH is lower than 4 (Holgate *et al.*, 1997). This mutant retains its GPCR-mediated calcium mobilization and chemotactic activities; however, it has a much lower inflammatory activity in activated T cells (Appay *et al.*, 1999). On mixing CCL5-E66S with wild-type CCL5, aggregation was significantly reduced. In this study, we developed a strategy to prepare less aggregated CCL5 complexes. Under an optimized condition, trimeric CCL5 could be obtained and crystallized. To provide a foundation

for studying the structure of a chemokine oligomer, we report the purification, crystallization and preliminary X-ray studies of the human CCL5 (hCCL5) trimer.

2. Materials and methods

2.1. Protein purification

DNA sequences for hCCL5 were amplified by PCR and ligated into pET-43.1a(+) vector. The products contained an additional Met residue at the N-terminus (Met-hCCL5) for convenient expression in *Escherichia coli*. Recombinant protein expression was induced by 1 mM IPTG for 5 h at 37°C when the cell OD₆₀₀ reached 0.6. The cells were harvested by centrifugation (6000g, 20 min at 4°C), resuspended in binding buffer (25 mM Tris–HCl pH 8.0, 100 mM NaCl) and disrupted by sonication. The cell lysate was centrifuged at 15 000g for 30 min at 4°C. The hCCL5 protein was mainly distributed in the cell pellet. The cell pellet was resuspended in denaturing buffer (100 mM Tris, 6 M guanidine–HCl pH 8.0) and stirred at 25°C overnight. Cell debris was removed by centrifugation (30 000g, 20 min at 4°C). The supernatant was refolded by dialysis into refolding buffer (0.9 M guanidine–HCl, 100 mM Tris, 5 mM cysteine, 5 mM methionine pH 8.0). The refolding process took place in 1 l refolding buffer for 8 h at 4°C. The dialysis was repeated twice. Protein debris was removed by centrifugation (30 000g, 20 min at 4°C). The supernatant containing refolded Met-hCCL5 protein was further purified by high-performance liquid chromatography on a C18 column mounted on an Agilent 1100 Series HPLC System. The purified protein was analyzed by SDS–PAGE. The purification strategy for Met-hCCL5 is depicted in Fig. 1.

2.2. Crystallization

The mixed proteins were concentrated to 7 mg ml^{−1} in 20 mM acetate pH 5.0, 150 mM NaCl using Amicon Ultra-15 concentrators (3000 Da cutoff, Millipore) and were then crystallized by the sitting-drop vapour-diffusion method. The crystallization conditions were screened in 48-well crystal-growth plates using a commercial screening kit (Index from Hampton Research). After optimization, well formed crystals of Met-hCCL5 were obtained using 0.2 M ammonium sulfate, 0.1 M bis-tris pH 5, 16% (w/v) polyethylene glycol 3350 at 283 K. Crystals were observed after 7 d.

2.3. Data collection and analysis

Single crystals were transferred to reservoir solution containing 15% (v/v) ethylene glycerol and were flash-cooled in liquid nitrogen for X-ray diffraction experiments. All X-ray diffraction data were collected at 110 K on beamline BL13B1 equipped with a CCD detector (Q315, ADSC) at the National Synchrotron Radiation Research Center, Hsinchu, Taiwan. Data sets were indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). The initial structure of the CCL5 trimer was obtained by molecular replacement with *Phaser* using the monomer component of the dimeric structure of Met-hCCL5 (PDB entry 1eqt) as the search model (McCoy

Figure 1

Flow chart for Met-hCCL5 purification. The experimental procedure of Met-hCCL5 purification includes protein expression in *E. coli* and protein purification by a series of steps.

Table 1

Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P4_12_12$
a, b, c (Å)	56.6, 56.6, 154.1
V_M (Å ³ Da ⁻¹)	2.58
Molecules in asymmetric unit	3
Solvent content (%)	52.32
Source	BL13B1, NSRRC
Wavelength (Å)	1.00000
Resolution (Å)	30.00–1.87 (1.94–1.87)
$CC_{1/2}$ (%)	95.5
R_{meas} (%)	6.1 (41.8)
R_{merge} (%)	5.7 (39.0)
$\langle I/\sigma(I) \rangle$	33.2 (4.5)
Completeness (%)	99.9 (100)
Multiplicity	8.1 (7.8)
B factor (Å ²)	29.74

et al., 2007). The correctness of the trimer is indicated by a log-likelihood gain of 766.5 and a translation-function Z -score of 24.1. Several rounds of manual model building in *Coot* (Emsley *et al.*, 2010) and refinement with *REFMAC* (Murshudov *et al.*, 2011) were performed to improve the quality and completeness of the structure. Data-collection statistics are shown in Table 1. The refinement converged to a final R_{work} of 22.3% (R_{free} = 26.8%) at a resolution of 1.82 Å in space group $P4_12_12$.

3. Results and discussion

To improve the quality and expression yield of Met-hCCL5 protein, a modified purification strategy was first developed (Fig. 1). Recombinant hCCL5 with an N-terminal Met was expressed as an 8 kDa protein (molecular weight 7974.20) in *E. coli* BL21(DE3) cells; the protein aggregated and was detected in inclusion bodies. We used 6 M guanidine-HCl to extract the protein from the inclusion bodies. The unfolded protein was subsequently dialyzed into refolding buffer

consisting of 0.9 M guanidine-HCl, 5 mM cysteine, 5 mM methionine. After dialysis, we added HCl and adjusted the pH of the protein solution to 3.0, at which Met-hCCL5 was still soluble but other heterogeneous proteins were precipitated. The precipitate was removed by centrifugation and the folded Met-hCCL5 was further purified by HPLC. The protein purity was further confirmed by 15% SDS-PAGE (Fig. 2). This procedure purified a satisfactory amount of protein with a yield of up to 8 mg per litre. Compared with the conventional method (Proudfoot *et al.*, 1996), direct refolding in a buffer without guanidine-HCl caused severe precipitation. The presence of a low concentration of guanidine-HCl in the refolding buffer significantly reduced protein precipitation, and the cysteine and methionine promoted the correct protein folding.

Wild-type hCCL5 undergoes severe aggregation at neutral pH, but the E66S mutant only forms a stable dimer. Based on these distinct properties, we developed a strategy for reducing hCCL5 oligomerization by mixing the E66S mutant into hCCL5 solution. We could evaluate the size distribution using size-exclusion chromatography (SEC). Upon an increase in the concentration of the E66S mutant, the SEC data revealed a decrease in the oligomer size. At a 1:1–1:3 molar ratio of wild type to mutant, the molecular size of the mixed CCL5 was primarily a trimeric form (Fig. 3). We prepared a trimeric sample and crystallized it. The trimeric sample was concentrated to ~ 7 mg ml⁻¹ in 150 mM NaCl, 20 mM acetate for initial crystallization screening. Triangular plate-like crystals (Fig. 4) successfully appeared in the Index screen. The best single-crystal diffraction (Fig. 5) was obtained to 1.87 Å resolution on the BL13B1 beamline at NSRRC. The crystals had a tetragonal lattice and belonged to space group $P4_32_12$, with unit-cell parameters $a = 56.6$, $b = 56.6$, $c = 154.1$ Å. Matthews coefficient calculations indicated that the solvent content was 52.32%, with three molecules per asymmetric unit (Table 1), matching the observation from the SEC experiment. The structure was solved by molecular replacement using the structure of the Met-hCCL5 monomer (PDB entry 1eqt)

Figure 2
SDS-PAGE of Met-hCCL5 purification. The SDS-PAGE recorded the individual steps of protein expression and purification: lane 1, before induction; lane 2, after induction; lane 3, supernatant from cell debris; lane 4, pellet from cell debris; lane 5, supernatant after refolding; lane 6, pellet after refolding; lane 7, target protein after HPLC purification.

Figure 3
Size-exclusion chromatography data for Met-hCCL5 mixed with the E66S mutant in different molar ratios. The chromatographic data, which were recorded at 280 nm using a Bio SEC-3 300 Å column (Agilent) with a flow rate of 0.35 ml min⁻¹, show elution times of 9.9 min for the CCL5/E66S complex trimer and 10.2 min for the E66S dimer.

Figure 4
Crystals of the Met-hCCL5 trimer. Crystals were grown under an optimal condition consisting of 0.2 M ammonium sulfate, 0.1 M bis-tris pH 5, 16% (w/v) polyethylene glycol 3350.

Figure 5
X-ray diffraction image of a Met-hCCL5 trimer crystal.

determined under acidic conditions (pH 4.6). The molecular replacement indicated that the initial space group was not correct and that the crystal belonged to the enantiomorphic space group $P4_12_12$. This discrepancy could only be detected by solving the structure. Structural refinement is ongoing to accurately define the protein interface and its flexible loops and residues.

This trimer structure will help to define the protein interface that is responsible for oligomerization. Based on this, we might speculate on the formation of the oligomer structure. This study will shed light on the implications of CCL5 oligomerization for clinical investigations.

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