Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray diffraction analysis of the ternary human GM-CSF receptor complex

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Received 22 May 2008
Accepted 26 June 2008

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a haemopoietic growth factor that acts though a ternary receptor signalling complex containing specific α (GMRα) and common β (βc) receptor subunits. Human GM-CSF is encoded by the gene *csf2*, while the genes for GMRα and βc are *csf2ra* and *csf2rb*, respectively. Crystals of the ternary ectodomain complex comprising GM-CSF and the soluble extracellular regions of both the GMRα subunit and either βc or its glutamine-substitution mutant N346Q were obtained using the hanging-drop vapour-diffusion method. The best diffracting crystals of the ternary complex were obtained using the N346Q mutation of the βc subunit. These crystals grew using polyethylene glycol 3350 with a high concentration of proline, belonged to space group $P6_322$ and diffracted to 3.3 Å resolution.

1. Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) belongs to a family of haematopoietic cytokines that includes interleukin-3 (IL-3) and IL-5. They are produced by activated T cells to stimulate the proliferation, differentiation, survival and functional activation of haemopoietic cells (Metcalf, 1993). GM-CSF is also produced by macrophages, endothelial cells and fibroblasts to control dendritic cell and T-cell function. Conversely, these cytokines have all been implicated in multiple pathologies resulting from their excessive or aberrant expression or that of their receptors in conditions such as arthritis, asthma, chronic obstructive pulmonary disease, autoimmunity and leukaemia. In addition, recombinant human GM-CSF is used clinically to reduce the severity of chemotherapy-induced neutropenia and accelerate haematopoietic recovery after bone-marrow transplantation (Cebon & Lieschke, 1994; Armitage, 1998).

GM-CSF, IL-3 and IL-5 exert their effects on target cells by binding to heterodimeric cell-surface receptors composed of a cytokine-specific α subunit (Gearing *et al.*, 1989; Kitamura *et al.*, 1991; Tavernier *et al.*, 1991; Murata *et al.*, 1992; Takaki *et al.*, 1993) and a βc subunit (Hayashida *et al.*, 1990) that is common to all three receptors. The α subunit binds a cytokine with low affinity, forming a complex that then binds with high affinity to the βc subunit. This process of receptor dimerization results in the activation of intracellular signal transduction pathways, although the mechanism remains poorly understood.

Both the GMRα and βc receptor subunits are type I transmembrane glycoproteins. The GMRα subunit has an apparent molecular weight of 84 kDa with 11 potential N-linked glycosylation sites within the extracellular ligand-binding domain (Gearing *et al.*, 1989), while the βc subunit has a molecular weight of 130 kDa and contains three N-linked glycosylation sites that are located in the extracellular region (Hayashida *et al.*, 1990). Structurally, both of these receptor subunits are characterized by the presence of cytokine-receptor homology modules, each consisting of two fibronectin type III domains. The N-terminal fibronectin type III domain contains four strictly conserved cysteine residues, while a highly conserved WS*X*WS motif is present near the C-terminus of the second domain.

The βc subunit comprises two of these cytokine-receptor homology modules, while the GMRα has a single module together with an N-terminally located 'knob' domain of unknown function.

The stoichiometry of the fully assembled GM-CSF receptor complex has been controversial. We have shown that a soluble form of the ternary GM-CSF receptor complex contained a single molecule of GM-CSF, a single molecule of GMRα and two molecules of βc (McClure *et al.*, 2003). However, a more recent analysis revealed that at high cytokine concentrations a 2:2:2 complex can form (unpublished results).

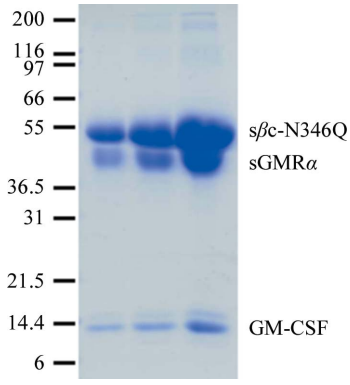

Figure 1
SDS–PAGE of the ternary GM-CSF complex containing GM-CSF, soluble GMRα and the N346Q soluble βc mutant. The concentrated ternary complex was separated by electrophoresis on a 10–20% polyacrylamide gradient gel under reducing conditions. GM-CSF migrated with an apparent molecular weight of 14 kDa, sGMRα had a molecular weight of 42 kDa and sβc-N346Q had a molecular weight of 52 kDa. Both receptor subunits migrated as broad bands owing to glycosylation.

The crystals reported here will form the basis for determining the molecular structure of the ternary GM-CSF receptor complex and will clarify the nature of the interactions involved in cytokine binding, affinity conversion, receptor activation and signal transduction.

2. Experimental procedures and results

2.1. Protein expression, purification and complex formation

Soluble recombinant human GM-CSF was expressed in *Escherichia coli* and purified by anion-exchange chromatography and reversed-phase HPLC (Hercus *et al.*, 1994). The soluble extracellular regions of human GMRα (sGMRα; residues 1–320) or human βc (sβc; residues 1–438) were cloned into the pIBV5-His expression vector (Invitrogen, Australia) and transfected into Sf21 insect cells using Cellfectin (Invitrogen, Australia). Stable transfected Sf21 cell lines were selected for by incubation with blasticidin at 50 µg ml^{-1} (Invitrogen, Australia). Once cells lines had been established, blasticidin treatment was discontinued. Sf21 cells and transfected cell lines were maintained in serum-free ExCell 420 medium (JRH Biosciences, Australia) at 300 K. Large-scale expression generated up to 30 l of conditioned medium, which was concentrated to less than 1 l prior to affinity chromatography as previously described (McClure *et al.*, 2003).

Human βc contains three N-linked glycosylation sites. Mutation of one site, Asn346, to Gln has been shown by others to improve the diffraction properties of crystallized sβc (Carr *et al.*, 2001). Full-length βc-N346Q retains wild-type function when co-transfected with full-length GMRα in GM-CSF receptor-binding assays using transiently transfected COS cells (results not shown). Site-directed mutagenesis using PCR was used to generate sβc-N346Q, which was

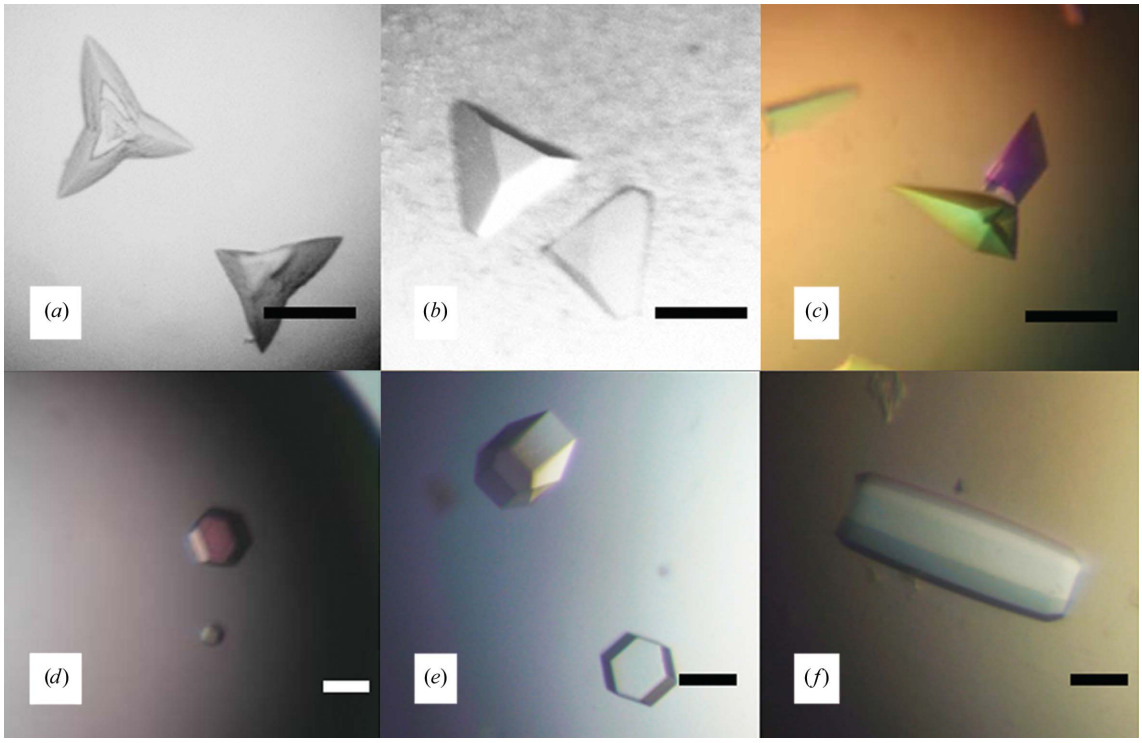

Figure 2
Crystallization montage of wild-type and sβc-N346Q complex crystals. (*a*) Wild-type crystals grown in 100 m*M* HEPES pH 7.0, 8%(*w*/*v*) PEG 4000 and 5%(*v*/*v*) MPD at 277 K. (*b*) Wild-type crystals grown in 100 m*M* MES pH 6.2, 8%(*w*/*v*) PEG 4000, 5%(*v*/*v*) MPD at 277 K. (*c*) sβc-N346Q complex crystals grown in 100 m*M* HEPES pH 7.5, 10%(*w*/*v*) PEG 4000, 5%(*v*/*v*) MPD at 277 K. (*d*) sβc-N346Q complex crystals grown in 100 m*M* HEPES pH 7.5, 10%(*v*/*v*) PEG 550 monomethyl ether, 5%(*v*/*v*) MPD at 277 K. (*e*), (*f*) sβc-N346Q crystals grown in 100 m*M* HEPES pH 7.0, 6%(*w*/*v*) PEG 3350, 200 m*M* proline at 293 K. The scale bars represent 200 µm.

Table 1
Crystal data and X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution bin.

Crystal	GM-CSF ternary complex
Crystallization conditions	100 m*M* HEPES pH 7.0, 6%(*w*/*v*) PEG 3350, 200 m*M* proline, 293 K
Cryoprotectant	100 m*M* HEPES pH 7.0, 8%(*w*/*v*) PEG 3350, 200 m*M* proline, 20%(*v*/*v*) MPD
X-ray source	Beamline 17-ID, IMCA-CAT, APS, Chicago, USA
Detector	ADSC Quantum-210 CCD
X-ray wavelength (Å)	1.0
Temperature (K)	100
Space group	$P6_322$
Unit-cell parameters (Å)	a = 166.6, b = 166.6, c = 213.1
Resolution (Å)	3.3 (3.48–3.3)
Total observations	533343
Total No. of unique reflections	26931
Redundancy	4.7 (4.8)
Data completeness (%)	99.7 (99.7)
R_{sym}† (%)	7.7 (62.1)
$\langle I/\sigma(I)\rangle$	17.7 (2.3)

† $R_{sym} = \sum_h \sum_l |I_{hl} - \langle I_h\rangle| / \sum_h \sum_l \langle I_h\rangle|$, where I_l is the lth observation of reflection h and $\langle I_h\rangle$ is the weighted average intensity for all observations of l of reflection h.

subsequently cloned into the pIBV5-His expression vector, expressed and purified as outlined for wild-type sβc. Purified sβc-N346Q showed a slight reduction in molecular weight compared with the wild-type sβc but no difference in its ability to form a ternary complex with sGMRα and GM-CSF (results not shown).

Ternary complexes consisting of GM-CSF, sGMRα and either sβc or the glutamine-substituted mutant sβc-N346Q were isolated by gel-filtration chromatography using a Superdex 200 column (26 × 600 mm; GE Healthcare, Australia) equilibrated with 150 m*M* NaCl, 50 m*M* sodium phosphate pH 7.0 at 277 K. The peak containing all three components as identified by SDS–PAGE was pooled, buffer-exchanged into 10 m*M* HEPES pH 7.0 and concentrated to between 4 and 6 mg ml^{-1} (Amicon Ultra-10 centrifugal concentrator, Millipore, Australia; Fig. 1).

2.2. Protein crystallization

Crystallization trials were performed at both 277 and 293 K using the hanging-drop vapour-diffusion method by mixing 1–2 µl protein solution with an equal volume of reservoir solution and equilibrating against 1 ml reservoir solution. The initial crystallization conditions were established using the screens described by Jancarik & Kim (1991) and Cudney *et al.* (1994).

Initial screening of the wild-type complex resulted in numerous small crystals appearing within 30 min at 293 K in conditions buffered between pH 6.0 and 8.0 using various molecular-weight polyethylene glycols (PEGs; 3350, 4000, 6000, 8000) as precipitants. Crystal size was optimized by a combination of streak-seeding, lower concentrations [7–12%(*w*/*v*)] of the high-molecular-weight PEGs, addition of 5%(*v*/*v*) 2,4-methylpentanediol (MPD) to the reservoir solution and incubation of the trays at 277 K. A number of different crystal forms were obtained (Figs. 2*a* and 2*b*). These crystals were very sensitive to handling.

Screening of the ternary GM-CSF receptor complex comprising the sβc-N346Q mutation around the conditions that produced wild-type crystals resulted in large crystals that grew within a week at 277 K (Fig. 2*c*). These crystals were once again sensitive to handling. Further screening with PEGs as precipitants resulted in the identification of a morphologically distinct hexagonal form that grew slowly over six weeks at 277 K using PEG 550 monomethyl ether as the precipitant (Fig. 2*d*). Optimization of this hexagonal crystal form resulted in large single hexagonal crystals that grew at 293 K using 100 m*M* HEPES pH 7.0, 6%(*w*/*v*) PEG 3350 and 200 m*M* proline as an additive. Crystals appeared after one week and grew to full size (800 × 800 × 400 µm) within two weeks (Figs. 2*e* and 2*f*).

2.3. Data collection and preliminary X-ray analysis

Initial X-ray diffraction studies were performed on the wild-type ternary GM-CSF receptor complex crystals using an in-house MAR345 image-plate detector with Cu $K\alpha$ X-rays generated by a Rigaku RU-200 rotating-anode X-ray generator. These crystals diffracted very weakly to 23 Å resolution following a 60 min exposure at 293 K. The crystals were extremely fragile and sensitive to cryo-protection. Eventually, conditions were determined under which the crystals could be frozen, involving stepwise addition of glycerol at 5%(*v*/*v*) intervals to a final concentration of 20–25%(*v*/*v*). The best wild-type crystals failed to diffract beyond 8 Å resolution using synchrotron radiation. Numerous methods were tried to optimize the quality of these crystals, including controlled dehydration, crystal annealing and growing the crystals in a magnetic field; however, none proved successful.

Crystals of the ternary complex containing the sβc-N346Q mutation grown under similar conditions to the wild-type crystals also failed to diffract beyond 8 Å resolution. Hexagonal crystals grown at 277 K in 100 m*M* HEPES pH 7.5, 10%(*v*/*v*) PEG 550 monomethyl ether and 5%(*v*/*v*) MPD were flash-frozen after stepwise transfer into the final cryo-buffer. The glycerol cryoprotectant was added in steps of 5%(*v*/*v*) up to 20%(*v*/*v*) in stabilizing buffer containing 100 m*M* HEPES pH 7.5, 15%(*v*/*v*) PEG 550 monomethyl ether and 5%(*v*/*v*) MPD. As the crystals were relatively large (200–300 mm in each dimension), the soaking time for each 5% increment was 20 min to ensure that diffraction quality was preserved. A complete X-ray diffraction data set was measured at 100 K using an ADSC Quantum-

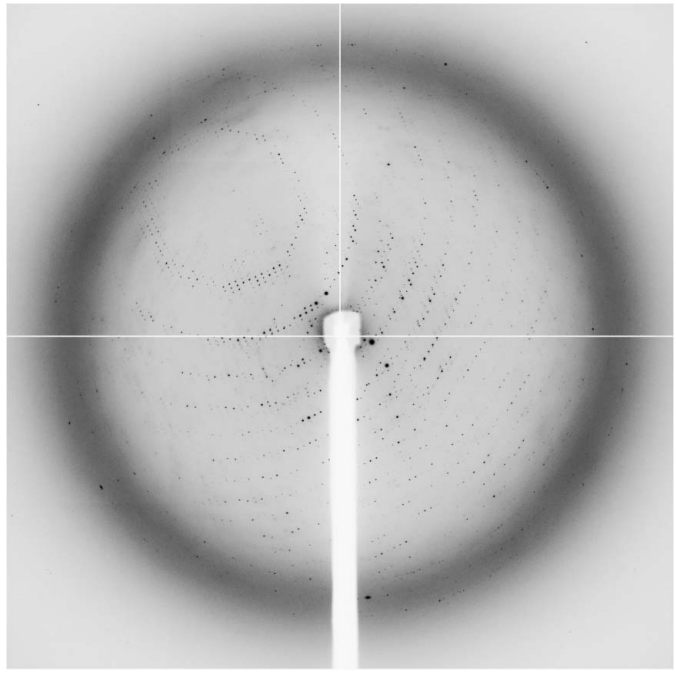

Figure 3
A representative 0.25° oscillation image of the data collected from a crystal of the ternary GM-CSF receptor complex using the sβc-N346Q construct, showing Bragg reflections extending beyond 3.6 Å resolution. This image was collected using an ADSC Quantum-210 CCD detector from a crystal frozen at 100 K on IMCA-CAT beamline 17-ID at the Advanced Photon Source, Chicago, USA.

315 CCD detector on the BioCARS beamline 14-BM-C at the Advanced Photon Source, Argonne, Illinois, USA. These crystals belonged to space group $P6_322$, with unit-cell parameters $a = b = 166.7$, $c = 213.6$ Å, with the largest crystals diffracting to 3.6 Å resolution. A second hexagonal crystal form was grown in 100 m*M* HEPES pH 7.0 containing 6%(*w*/*v*) PEG 3350 and 200 m*M* proline at 293 K. These crystals were cryoprotected by soaking in 100 m*M* HEPES pH 7.0 containing 8%(*w*/*v*) PEG 3350, 200 m*M* proline and 20%(*v*/*v*) MPD for 5 min before being flash-frozen in liquid nitrogen. A complete data set was measured at 100 K using an ADSC Quantum-210 detector on the IMCA-CAT beamline 17-ID at the Advanced Photon Source. This crystal belonged to space group $P6_322$ and had near-identical unit-cell parameters, $a = b = 166.6$, $c = 213.1$ Å, to those of the first hexagonal crystal form. The data were processed using *MOSFLM* and *SCALA* (Collaborative Computational Project, Number 4, 1994) and the statistics are shown in Table 1. The best crystal diffracted to 3.3 Å resolution (Fig. 3). Calculated Matthews coefficients (V_M) of 4.42 or 2.21 Å^3 Da^{-1} suggested the presence of one or two ternary complexes in the asymmetric unit with a solvent content of about 72% or 44%, respectively (Matthews, 1968).

The structure of the GM-CSF ternary complex has been determined by molecular replacement using the data reported here (unpublished results). Details will be published elsewhere.

This work was supported by grants from the National Health and Medical Research Council of Australia (NHMRC), the National Institutes of Health and the Australian Cancer Research Foundation. This work, including the use of the BioCARS sector, was also supported by the Australian Synchrotron Research Program, which is funded by the Commonwealth of Australia under the Major National Facilities Program. Use of the Advanced Photon Source was supported by the US Department of Energy, Basic Sciences, Office of Energy Research. We thank Dr Harry Tong, Dr Kevin Battaile and other staff at BioCARS and IMCA-CAT for their help with data collection during our visit to the Advanced Photon Source. MWP is a Australian Research Council Federation Fellow and NHMRC Honorary Fellow, and WJM was a NHMRC Industry Fellow.

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