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Crystallization of a 2:2 complex of granulocyte-colony stimulating factor (GCSF) with the ligand-binding region of the GCSF receptor

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The granulocyte-colony stimulating factor (GCSF) receptor receives signals for regulating the maturation, proliferation and differentiation of the precursor cells of neutrophilic granulocytes. The signalling complex composed of two GCSFs (GCSF, 19 kDa) and two GCSF receptors (GCSFR, 34 kDa) consisting of an Ig-like domain and a cytokine-receptor homologous (CRH) domain was crystallized. A crystal of the complex was grown in 1.0 *M* sodium formate and 0.1 *M* sodium acetate pH 4.6 and belongs to space group $P4_12_12$ (or its enantiomorph $P4_32_12$), with unit-cell parameters $a = b = 110.1$, $c = 331.8$ Å. Unfortunately, this crystal form did not diffract beyond 5 Å resolution. Since the heterogeneity of GCSF receptor appeared to prevent the growth of good-quality crystals, the GCSF receptor was fractionated by anion-exchange chromatography. Crystals of the GCSF–fractionated GCSF receptor complex were grown as a new crystal form in 0.2 *M* ammonium phosphate. This new crystal form diffracted to beyond 3.0 Å resolution and belonged to space group $P3_121$ (or its enantiomorph $P3_221$), with unit-cell parameters $a = b = 134.8$, $c = 105.7$ Å.

1. Introduction

Granulocyte-colony stimulating factor (GCSF) is a member of a cytokine family, having a four α-helical bundle structure, and regulates the proliferation and differentiation of neutrophilic progenitor cells (Metcalf, 1989). These responses are initiated by interaction with a specific receptor (GCSFR), which is expressed on the surface of target cells (Nagata & Fukunaga, 1991). GCSFR has a composite structure consisting of an immunoglobulin-like (Ig) domain, a cytokine-receptor homologous (CRH) domain and three fibronectin type III (FNIII) domains in the extracellular region. It also has a series of conserved cysteine residues and a 'WS*X*WS' motif commonly seen in type I receptors (Bazan, 1990). The CRH region of GCSFR is an essential domain for ligand binding and mediating the signal (Fukunaga *et al.*, 1991) and GCSFR dimerization induced by GCSF binding has been demonstrated to be a common signal transduction mechanism. Previous studies also show that not only the CRH (through site II) but also the Ig domain (through site III) is important for GCSF binding and receptor dimerization (Layton *et al.*, 1999) and it has also been shown that even a soluble Ig domain of GCSFR can bind GCSF (Ishibashi *et al.*, 2001). The crystal structure of the chimeric complex of human GCSF and murine CRH domain has been reported (Aritomi *et al.*, 1999); however, it does not provide details of site III interactions owing to the absence of the Ig domain. Recently, not only site II but also site III interactions were elucidated by the structural analysis of a complex of the Ig-CRH domains of human gp130 with viral IL-6 (vIL-6; Chow *et al.*, 2001) and with human IL-6 (Boulanger *et al.*, 2003). The extracellular region of GCSFR shares 46% sequence similarity with that of gp130 (Hammacher *et al.*, 1998); additionally, the Ig-CRH domain of GCSFR forms a 2:2 stoichiometric complex with GCSF (Mine *et al.*, 2004), suggesting a receptor-activation mechanism similar to that of the vIL-6–gp130 complex (Layton *et al.*, 2001). A model of GCSF–Ig-CRH based on the structure of the vIL6–gp130 complex is plausible since it would explain previous data obtained using a chimeric receptor (Layton *et al.*, 1999) and neutralizing mAbs (Layton *et al.*, 1997); however, there has been no structural evidence for the ligand-recognition scheme in GCSF. Here, we report the purification, crys-

tallization and preliminary X-ray data analysis of a 2:2 GCSF–Ig-CRH complex. Elucidation of the structure of this complex reveals the true structural details of the binding between the receptor and the ligand.

2. Materials and methods

2.1. Materials

Recombinant human GCSF expressed in *Escherichia coli* was obtained from the pharmaceutical division of Kirin Brewery Co. Ltd (Tokyo, Japan). The receptor-binding region (308 amino acids) of human GCSF receptor (GCSFR) comprised of an Ig-like domain (Ig) and a cytokine-receptor homologous region (CRH) was prepared as a fusion protein with the Fc region of immunoglobulin (Mine *et al.*, 2004). Three cysteine mutations (Cys79→Ser, Cys164→Ser and Cys229→Ser) were incorporated into Ig-CRH to reduce cysteine-mediated aggregation (Mine *et al.*, 2004). The Ig-CRH-Fc expressed in cell culture was recovered using GCSF-immobilized Sepharose, which was prepared by coupling 1 mg recombinant human GCSF with 1 ml NHS-activated Sepharose Fast Flow (Amersham Biosciences). Ig-CRH-Fc (0.6 mg) was mixed with a twofold molar excess of GCSF and then subjected to thrombin digestion (298 K, overnight) using a Thrombin CleanCleave Kit (Sigma) to remove the Fc region. The GCSF–Ig-CRH complex was applied onto a Sephacryl S-200 column (2.6 × 60 cm, Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl buffer pH 7.0 containing 0.2 *M* NaCl and excess G-CSF was eliminated. The complex was concentrated to 1.5 mg ml^{-1} and used for crystallization screening (crystallization sample 1). The average molecular weight of this complex was confirmed to be approximately 112 000 Da by light-scattering analysis (Mine *et al.*, 2004), indicating that sample 1 formed a 2:2 stoichiometric complex.

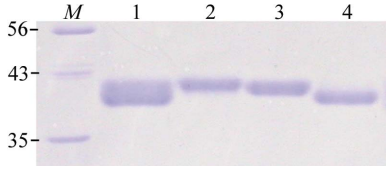


Figure 1
SDS–PAGE analysis of fractions I–III from ion-exchange chromatography. Lane 1, crystallization sample 1, lane 2, fraction I; lane 3, fraction II; lane 4, fraction III (crystallization sample 2).

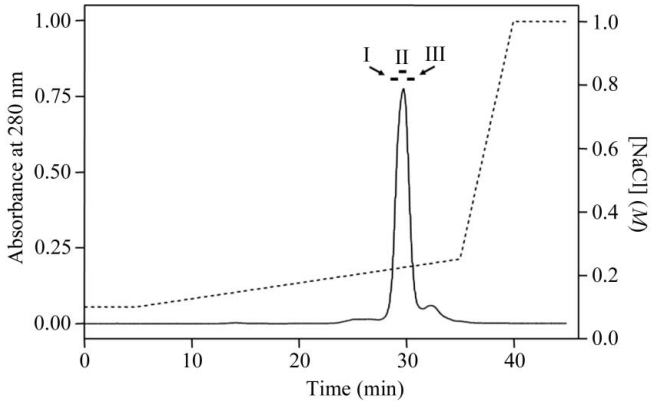


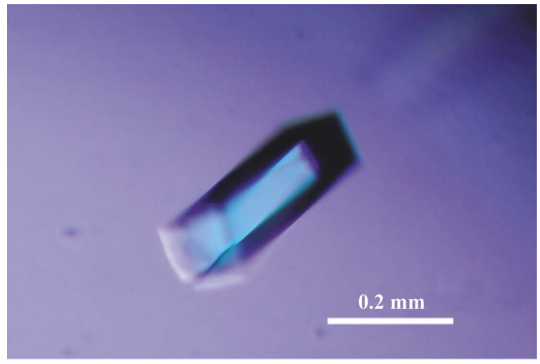
Figure 2
Second ion-exchange chromatography of Ig-CRH on an ES502N column. The Ig-CRH peak was divided into three fractions (I–III).

2.2. Purification of Ig-CRH

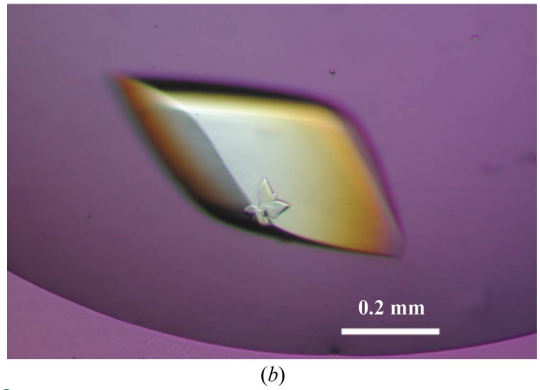
Since the SDS–PAGE analysis showed that sample 1 still contained two bands (a major band with lower molecular weight and a minor band with higher molecular weight; Fig. 1, lane 1), further purification of Ig-CRH was attempted in order to obtain better diffracting crystals. 0.6 mg Ig-CRH-Fc was digested by thrombin in the absence of GCSF (298 K, overnight) and was then applied onto an ES502N column (Shodex, Japan) equilibrated with 20 m*M* Tris–HCl pH 8.0 and eluted with a linear gradient of NaCl from 0 to 0.5 *M*. The Ig-CRH was eluted with a small shoulder peak and separated from the Fc fragment (data not shown). The Ig-CRH was further purified using the same column with a linear gradient of NaCl from 0.1 to 0.26 *M* (Fig. 2). The symmetrically shaped peak of the Ig-CRH fraction was tentatively divided into three fractions (fractions I–III, labelled according to the order of elution). The molecular size of each fraction was found to be slightly different on SDS–PAGE (Fig. 1, lanes 2–4), suggesting a difference in the sugar-chain content. Since fraction III appeared to be the most uniform (Fig. 1, lane 4), it was purified by gel filtration after adding the GCSF and was used for crystallization (sample 2).

2.3. Crystallization

Crystallization conditions for sample 1 were screened by the hanging-drop vapour-diffusion method using Crystal Screen kits (Hampton Research). 3 µl drops consisting of 1.5 µl protein solution (1.5 mg ml^{-1}) and 1.5 µl mother liquor were equilibrated against 0.7 ml reservoir solution at 293 K. Microcrystals were found using Crystal Screen Lite (well Nos. 9, 34, 35 and 46) and a few crystals grew from 0.1 *M* sodium acetate pH 4.6 containing 1.0 *M* sodium formate (well No. 34) to dimensions of approximately 0.15 × 0.15 × 0.7 mm (form I; Fig. 3*a*).



(*a*)



(*b*)

Figure 3
Crystals of GCSF–Ig-CRH complex: (*a*) crystal from sample 1, (*b*) crystal from sample 2.

Table 1
Comparison of diffraction data from crystals of GCSF–Ig-CRH complex.

Values in parentheses refer to the high-resolution shell: 5.7–5.5 Å resolution for form I and 2.9–2.8 Å resolution for form II.

	Tetragonal (form I from sample 1)	Trigonal (form II from sample 2)
Space group	$P4_12_12$ or $P4_32_12$	$P3_121$ or $P3_221$
X-ray source	BL41XU, SPring-8	BL38B1, SPring-8
Wavelength (Å)	1.0000	1.0000
Resolution (Å)	5.5	2.8
Unique reflections	6965	25214
Measured reflections	66413	153280
$I/\sigma(I)$	10.6 (1.3)	20.2 (1.9)
Completeness (%)	96.7 (77.6)	91.0 (78.0)
R_{sym}†	0.102 (0.253)	0.058 (0.355)

† $R_{sym} = \sum|I(h) - \langle I(h)\rangle|/\sum I(h)$, where $\langle I(h)\rangle$ is the mean intensity value of the reflection h for all measurements of $I(h)$.

Crystallization conditions for sample 2 (2.3 mg ml^{-1}) were also screened using Crystal Screen kits. Sample 2 was crystallized using the same conditions as were used for sample 1 and also using three additional conditions: 0.2 *M* ammonium phosphate (Crystal Screen Lite, No. 3), 0.05 *M* potassium phosphate containing 10%(*w*/*v*) PEG 8000 (Crystal Screen Lite, No. 42) and 0.1 *M* sodium acetate pH 4.6 containing 2.0 *M* sodium chloride (Crystal Screen II, No. 9). After optimization of these conditions, crystals were grown at 293 K using 0.1 *M* sodium acetate pH 4.6 containing 1.4 *M* NaCl and brittle and thin long plate-shaped crystals were grown using 1 m*M* potassium phosphate containing 2%(*w*/*v*) PEG 8000. Although both crystals grew to about 0.1 × 0.1 × 0.6 mm in 3–5 d, these crystals did not diffract beyond 5.0 Å resolution and the space group was the same as that obtained from sample 1. On the other hand, a crystal with a different shape (form II; Fig. 3*b*) was grown from 0.2 *M* ammonium phosphate in 2–3 weeks. The reproducibility of crystallization was very low, but the crystals of this form grew to dimensions of 0.3 × 0.3 × 0.15 mm.

2.4. Data collection

Diffraction data from form I crystals were collected under a nitrogen-gas stream at 100 K and recorded on a CCD camera, with a total oscillation range of 180°, at BL41XU (SPring8). The diffraction data from form II crystals were collected at BL38B1 (SPring8) in the same manner.

3. Results and discussion

The Ig-CRH region of the GCSF receptor contains three free cysteines and four potential N-glycosylation sites. The cysteine mutations (Cys79→Ser, Cys164→Ser and Cys229→Ser) appeared to be important to obtain a useful form of Ig-CRH for crystallization. There is little published information regarding the effect of glycosylation on protein crystallization and it may prove useful to compare the form I crystal from sample 1 and form II crystal from sample 2 owing to their different extent of glycosylation.

Form I crystals belong to the tetragonal space group $P4_12_12$ (or its enantiomorph $P4_32_12$), with unit-cell parameters $a = b = 110.1$, $c = 331.8$ Å. The Matthews coefficient (V_M) was 2.4 Å^3 Da^{-1} assuming the presence of two 2:2 complexes in the asymmetric unit. The intensity data were processed with the *HKL* programs *DENZO* and *SCALEPACK* (Otwinoski & Minor, 1997). Data-collection and processing statistics are shown in Table 1. However, no crystals diffracted beyond 5.0 Å resolution even using synchrotron radiation.

On the other hand, the form II crystals diffracted to 2.8 Å resolution with sufficient quality for further structure analysis (Table 1). This crystal form belongs to the trigonal space group $P3_121$ (or its enantiomorph $P3_221$), with unit-cell parameters $a = b = 134.8$, $c = 105.7$ Å. The Matthews coefficient (V_M) was 3.2 Å^3 Da^{-1} assuming the presence of one 2:2 complex in the asymmetric unit.

Although the Ig-CRH region of the GCSF receptor is highly glycosylated (containing four N-glycosylation sites), we succeeded in obtaining a diffraction-quality crystal of the signalling complex of GCSF and the Ig-CRH region of GCSFR. Removal of the heterogeneity caused by N-glycosylation by further purification of the sample may be an effective way to obtain high-quality diffraction data from crystals of glycosylated proteins.

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