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Crystallization and preliminary X-ray analyses of quaternary, ternary and binary protein–DNA complexes with involvement of AML1/Runx-1/CBFα Runt domain, CBFβ and the C/EBPβ bZip region

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Three types of protein–DNA complexes, AML1/Runx-1/CBFα(Runt)–CBFβ–C/EBPβ(bZip)–DNA (CBFα-β-C/EBPβ-DNA), AML1/Runx-1/CBFα(Runt)–C/EBPβ(bZip)–DNA (CBFα-C/EBPβ-DNA) and AML1/Runx-1/CBFα(Runt)–DNA (CBFα-DNA), were crystallized. The crystals were all orthorhombic and belonged to space groups $C222_1$, $P2_12_12$ and $P2_12_12_1$, respectively. The resolutions of CBFα-β-C/EBPβ-DNA and CBFα-C/EBPβ-DNA crystals were both 3 Å, while that of the CBFα-DNA crystal was 2.65 Å. Complete data sets were collected for all of the native crystals, along with MAD and MIR data sets for CBFα-β-C/EBPβ-DNA. The heavy-atom site was determined using MAD data for a gold derivative of CBFα-β-C/EBPβ-DNA.

1. Introduction

CBF (core-binding factor), also known as AML (acute myelogenous leukaemia-related protein) or PEBP2 (polyoma virus enhancer-binding protein 2) (for a review, see Speck & Stacy, 1995; Ito, 1999), is a critical regulator family of haematopoiesis and osteogenesis and is frequently involved in chromosomal translocations associated with acute leukaemia, familial platelet disorder and cleidocranial dysplasia (for a review, see Werner *et al.*, 1999). CBFs function as heterodimers comprised of α and β subunits (referred to in this paper as CBFα and CBFβ, respectively) (Ogawa *et al.*, 1993), of which CBFα contains a Runt domain responsible for DNA binding and dimerization with CBFβ (Kagoshima *et al.*, 1996). This heterodimerization increases the DNA-binding affinity of CBFα (Ogawa *et al.*, 1993; Golling *et al.*, 1996; Gu *et al.*, 2000) and helps CBFα to resist the action of its inhibitory domains (for a review, see Ito, 1999). Residues 1–135 of CBFβ comprise a minimum region required for both heterodimerization and modulation of CBFα–DNA binding (Ogawa *et al.*, 1993).

By interacting with CBFα, other transcriptional regulatory factors, including the Ets (Mao *et al.*, 1999; Kim *et al.*, 1999; Gu *et al.*, 2000) and CAAT/enhancer-binding protein (C/EBP) (Zhang *et al.*, 1996) families, can also increase the DNA-binding affinity of CBFα. This interaction of transcriptional regulatory factors with CBFα mediates their cooperative binding to DNA at adjacent sites on promoters. For instance, the Runt domain of CBFα cooperatively binds to DNA along with the highly conserved basic region leucine zipper (bZip) domain of C/EBPα (Petrovick *et al.*, 1998). Also, although there are so far no reports on interaction between C/EBPβ and CBFα or their cooperative binding to DNA, both C/EBPα and C/EBPβ have been shown to work synergistically with CBFα and CBFα–CBFβ to *trans*-activate the colony-stimulating factor-1 receptor (CSF-1R) promoter (Zhang *et al.*, 1996), while cooperative binding of CBFα and Ets family proteins involves regions outside the Runt domain in addition to this domain (Kim *et al.*, 1999; Gu *et al.*, 2000).

The structures of CBFα Runt domain and CBFβ are known; the regions responsible for DNA binding and heterodimerization have been determined by NMR spectroscopy (Nagata *et al.*, 1999; Goger *et al.*, 1999; Huang *et al.*, 1999; Berardi *et al.*, 1999; Perez-Alvarado *et al.*, 2000; Tang *et al.*, 2000) and by extensive mutational analysis of the Runt domain (Kagoshima *et al.*, 1996). The crystal sructures of the Runt domain (Backstrom *et al.*, 1999) and the CBFα–CBFβ heterodimer have also been reported (Warren *et al.*, 2000). However, the lack of structural information about the CBF–DNA complex means that questions still remain about the function of CBF. Namely, how does CBFα recognize the PyGPyGGTPy consensus site (Melnikova *et al.*, 1993), how does CBFβ increase the DNA-binding affinity of the CBFα Runt domain without being in contact with the DNA and how do CBFs cooperate with other transcriptional factors on the DNA molecule? To answer these questions, we initiated a structural analysis of complexes

comprised of CSF-1R promoter DNA and the CBFα Runt domain alone and coupled with the C/EBPβ bZip domain or the C/EBPβ bZip domain plus CBFβ. Here, we describe the crystallization of these complexes.

2. Experimental procedures

2.1. Protein expression and purifications

A polypeptide fragment spanning the DNA-binding and heterodimerization region of the Runt domain of mouse AML1/Runx-1 (residues 60–182) was overexpressed in *Escherichia coli* BL21(DE3) using a T7 expression system. The bacterial cells containing the overexpressed proteins were harvested, lysed with a French press and centrifuged. The resultant supernatant, containing the target protein, was purified through three column-chromatographic steps using phosphocellulose P11 (Whatman International Ltd, England), CM cellulose CM52 (Whatman International Ltd, England) and phenyl Sepharose HP (Amersham Pharmacia Biotech, USA) columns.

Polypeptide fragments containing the DNA-binding domains from mouse C/EBPβ (residues 259–345 and 259–336) and C-terminally truncated CBFβ (residues 2–141) were overexpressed in the same manner as CBFα. The supernatants containing C/EBPβ protein were purified through three column-chromatographic steps using phosphocellulose P11, CM cellulose CM52 and Superdex 75 (Amersham Pharmacia Biotech, USA) columns. The supernatant containing CBFβ protein was purified by $(NH_4)_2SO_4$ fractionation and two column-chromatographic steps using phenyl Sepharose HP and DEAE Sephacel (Amersham Pharmacia Biotech, USA) columns.

2.2. DNA syntheses and purifications

The oligonucleotide strands of the DNA sequences shown below (where I is 5-iodo-dU) were purchased from Life Technologies Asia Pacific Inc. (Yokohama), purified by reverse-phase HPLC using a Wakosil-DNA column (Wako Pure Chemical Industries Ltd, Osaka) and annealed. The double-stranded DNAs obtained were then separated from the single-stranded material using a hydroxyapatite column (Bio-Rad Laboratories, CA, USA).

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(1)      AAACTCTGTGGTTGCG
           TTGAGACACCAACGCT

(2)      GAAGATTTCCAAACTCTGTGGTTGCG
           TTCTAAAGGTTTGAGACACCAACGCC

(2-I1)   GAAGATTTCCAAACTCTGTGGTTGCG
           TICIAAAGGIITGAGACACCAACGCC

(2-I2)   GAAGATIICCAAACICIGIGGTIGCG
           TTCTAAAGGTTTGAGACACCAACGCC

(2-I3)   GAAGAITTCCAAACICTGTGGITGCG
           TICTAAAGGTTIGAGACACCAACGCC
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2.3. Protein–DNA complex preparations

Solutions, each containing one of the two native complexes, AML1/Runx-1(residues 60–182)–DNA(1), AML1/Runx-1(residues 60–182)–C/EBPβ(residues 259–345)–DNA(2) and AML1/Runx-1(residues 60–182)–CBFβ (residue 2–141)–C/EBPβ(residues 259–336)–DNA(2), hereafter referred as CBFα-DNA, CBFα-C/EBPβ-DNA and CBFα-β-C/EBPβ -DNA, respectively, or one of three iodine derivatives of CBFα-β-C/EBPβ-DNA, CBFα-β-C/EBPβ-DNA(2-I1), CBFα-β-C/EBPβ–DNA CBFα-β-C/EBPβ-DNA(2-I2) and CBFα-DNA(2-I3), were prepared by mixing equimolar amounts of each component under salt-free conditions at pH 7.4 with 10–60 m*M* dithiothreitol (DTT) and then adding 5–10% excess DNA so that the target complex was the main component of the solution. The high concentration of DTT was used to avoid oxidation of CBFα (Kagoshima *et al.*, 1996; Kurokawa *et al.*, 1996). The complex formations were monitored by electrophoresis and the concentrations of the prepared complexes ranged from 9 to 11 mg ml^{-1}. All the complex solutions were stored at a temperature of 253 K.

2.4. Crystallization

Crystallization trials were conducted at a temperature of 297 K in 24-well plates using the sitting-drop vapour-diffusion method. Initial screenings were carried out using Natrix, a crystallization reagent kit for nucleic acids supplied by Hampton Research (Scott *et al.*, 1995). In all experiments, 2–3 µl drops of protein–DNA complex solution were mixed with 2.5 µl of reservoir solution and equilibrated against 0.5 ml of reservoir solution. The best CBFα-DNA crystals, with dimensions of 0.25 × 0.25 × 0.1 mm, were produced within 1–2 d using Natrix condition number 38 (200 m*M* ammonium acetate, 150 m*M* magnesium acetate and 5%(*w*/*v*) PEG 4K in 50 m*M* Na HEPES buffer at pH 7.0) (Fig. 1*a*). On the other hand, initial screenings produced only tiny crystals of CBFα-C/EBPβ-DNA and CBFα-β-C/EBPβ-DNA. Modification of the reservoir solution and the preparation of the protein–DNA complexes slightly improved their size and shape, although crystals of CBFα-C/EBPβ-DNA still grew as aggregates. Finally, macroseeding significantly improved the size of both CBFα-C/EBPβ-DNA and CBFα-β-C/EBPβ-DNA crystals. The best reservoir solutions for these two complexes were 5 m*M* magnesium sulfate, 3%(*w*/*v*) PEG 4K and 2%(*v*/*v*) dioxane in 50 m*M* MES buffer at pH 5.6 for the former and 200 m*M* KCl, 10 m*M* $MgCl_2$, 20 m*M* DTT, 4.5%(*w*/*v*) PEG 8K, 1%(*v*/*v*) glycerol and 1%(*v*/*v*) MPD in 50 m*M* MES buffer at pH 5.6 for the latter. The drops containing the mixture of the complex sample and reservoir solution were equilibrated against the reservoir solution for 12 h, after which

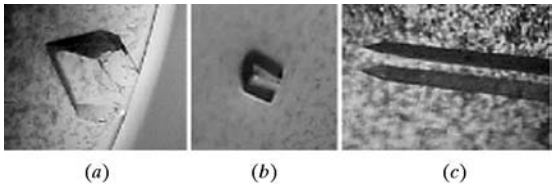

Figure 1
Photomicrographs of crystals of (*a*) CBFα-DNA, (*b*) CBFα-C/EBPβ-DNA and (*c*) CBFα-β-C/EBPβ-DNA.

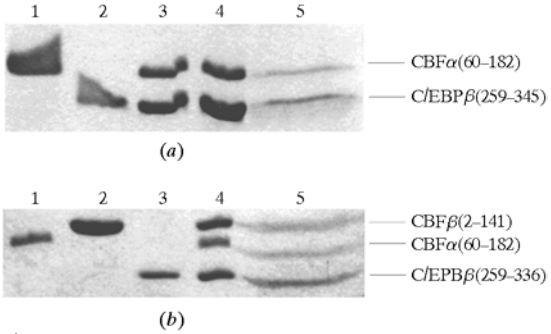

Figure 2
Presence of protein components in crystals of (*a*) CBFα-C/EBPβ-DNA and (*b*) CBFα-β-C/EBPβ-DNA analysed by 20% SDS–PAGE. (*a*) Lane 1, purified CBFα(60–182) protein; lane 2, purified C/EBPβ(259–345) protein; lane 3, freshly prepared CBFα-C/EBPβ-DNA sample; lane 4, CBFα-C/EBPβ-DNA sample after melting of the stored frozen sample at room temperature; lane 5, the sample obtained by dissolving the crystals in 0.5 *M* NaCl. (*b*) Lane 1, purified CBFα(60–182) protein; lane 2, purified CBFβ(2–141) protein; lane 3, purified C/EBPβ(259–336) protein; lane 4, CBFα-β-C/EBPβ-DNA sample after melting of the stored frozen sample at room temperature; lane 5, the sample obtained by dissolving the crystals in 0.5 *M* NaCl.

Table 1
Crystal parameters and data-collection statistics.

Crystal type 1 is CBFα-β-C/EBPβ-DNA, 2 is CBFα-DNA and 3 is CBFα-C/EBPβ-DNA.

	1								2	3
Crystal type	Native1	Native2	I1-Au	I2-Au	I3-Au	Au-λ_1	Au-λ_2	Au-λ_3	Native	Native
Unit-cell parameters (Å)										
a	119.8 (7)	117.4 (4)	121.0 (4)	121.2 (3)	121.0 (3)	121.1 (2)	121.1 (2)	121.0 (2)	51.1 (2)	102.2 (4)
b	164.4 (6)	165.0 (6)	163.6 (6)	163.6 (4)	164.4 (5)	163.6 (3)	163.6 (3)	163.6 (3)	104.1 (2)	109.2 (2)
c	109.79 (9)	110.55 (9)	109.34 (8)	109.42 (6)	109.31 (6)	109.34 (4)	109.33 (4)	109.31 (4)	116.2 (3)	127.4 (6)
Space group	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$C222_1$	$P2_12_12_1$	$P2_12_12$
Z	8	8	8	8	8	8	8	8	4	4
Solvent content (%)	69.8	69.5	69.8	69.9	70.0	69.9	69.9	69.8	61.3	64.4
Temperature (K)	100	100	100	100	100	100	100	100	100	100
Beamline	BL45XU	BL45XU	BL45XU	BL45XU	BL45XU	BL45XU	BL45XU	BL45XU	BL45XU	BL41XU
Detector	R-AXIS IV	R-AXIS IV	R-AXIS IV	R-AXIS IV	R-AXIS IV	R-AXIS IV	R-AXIS IV	R-AXIS IV	R-AXIS IV	MAR CCD
Wavelength (Å)	1.0200	1.0200	1.0100	1.0100	1.0100	1.0000	1.0398	1.0388	1.0000	0.7085
Resolution (Å)	20–3	20–3.1	20–3	20–3.04	20–3	20–3	20–3	20–3	20–2.65	30–3
Last shell	3.11–3	3.21–3.1	3.11–3	3.15–3.04	3.11–3	3.05–3	3.05–3	3.05–3	2.7–2.65	3.05–3
Observations	66643	34049	52904	60606	53564	66989	61348	65319	60234	117670
Unique reflections	20123	15398	19158	19958	19757	21113	20972	20992	17528	26407
Completeness (%)	91.8	77.7	88.3	94.4	90.5	97.1	96.5	96.7	94.4	91.3
Last shell	76.7	54.3	72.3	87.4	77.7	90.8	88.6	89.7	88.4	71.9
$R_{\rm merge}$†	0.07	0.09	0.06	0.062	0.06	0.062	0.057	0.058	0.047	0.066
Last shell	0.37	0.173	0.193	0.176	0.254	0.372	0.357	0.364	0.092	0.302
$I/\sigma(I)$	14.8	9.9	11.7	11.8	12.7	15.5	15.9	16.5	26.6	13.1
Last shell	2.0	3.2	2.6	2.3	1.6	1.7	1.6	1.6	10.9	1.4

† $R_{\rm merge} = \sum I_j - \langle I_j \rangle| / \sum \langle I_j \rangle$, where I_j is the intensity of reflection j and $\langle I_j \rangle$ is the average intensity of reflection j.

seeds of CBFα-C/EBPβ-DNA crystals were added to the mother liquid; growth of the crystals was complete in two weeks. This protocol yielded only one single CBFα-C/EBPβ-DNA crystal (0.1 × 0.06 × 0.03 mm), which was used for data collection (Fig. 1*b*). In contrast to the difficulty of reproducing CBFα-C/EBPβ-DNA crystals, the seeds of CBFα-β-C/EBPβ-DNA crystals produced readily reproducible crystals. When seeds were added to the mother liquid on the third day of equilibration, crystals grew as single elongated rods (Fig. 1*c*), reaching final dimensions of 1.5 × 0.2 × 0.1 mm in 3–4 weeks. Because of their high degree of reproducibility, CBFα-β-C/EBPβ-DNA crystals were also used for preparation of heavy-atom derivatives. The protein contents of the CBFα-C/EBPβ-DNA and CBFα-β-C/EBPβ-DNA crystals were verified by SDS–PAGE (Fig. 2).

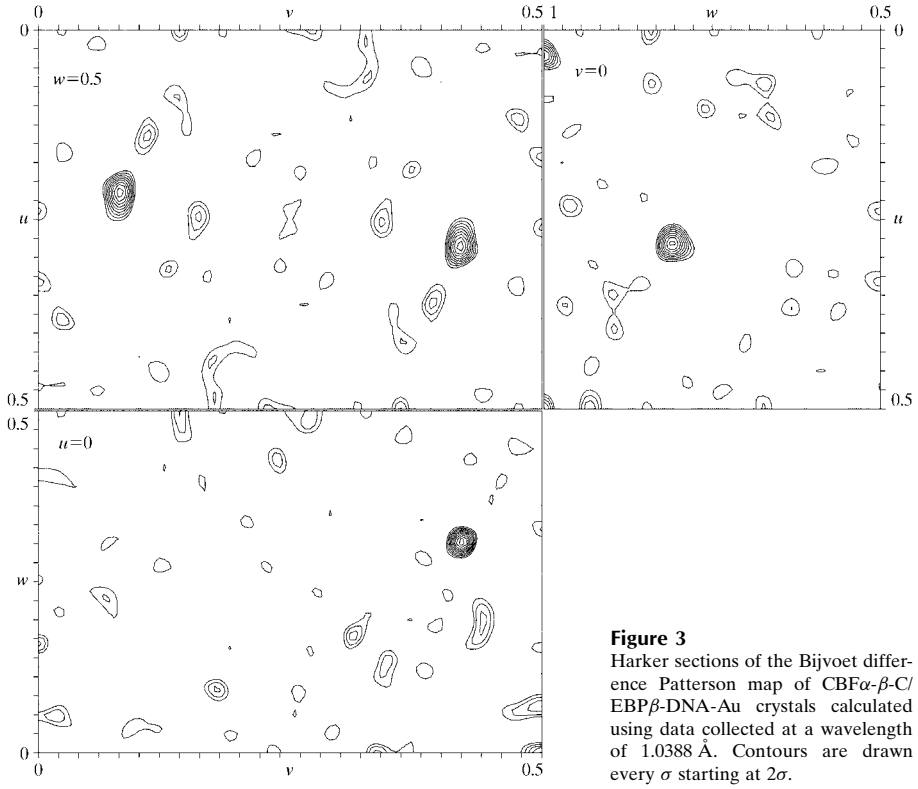

Figure 3
Harker sections of the Bijvoet difference Patterson map of CBFα-β-C/EBPβ-DNA-Au crystals calculated using data collected at a wavelength of 1.0388 Å. Contours are drawn every σ starting at 2σ.

2.5. Data collection

The characteristics of the crystals were first checked using our laboratory X-ray equipment from MacScience. X-rays were generated by a rotating Cu anode (focus size, 0.2 × 2 mm; collimator, 0.3 mm), powered by a M06XHF[22]-Fine generator operated at 50 kV and 50 mA, nickel-filtered and focused with a double mirror; the diffraction from each crystal was recorded on a DIP2030 imaging plate. For data collection at room temperature (293 K), the crystals were mounted in glass capillaries. For data collection at cryotemperature (100 K), the crystals were cryoprotected by soaking them in cryoprotectant solutions in which the concentration of cryoprotectant was gradually increased over a period of 3–10 min, after which they were mounted in nylon loops supplied by Hampton Research and flash-cooled in a stream of cold nitrogen gas. The final compositions of the best cryoprotectant solutions were 200 m*M* ammonium acetate, 150 m*M* magnesium acetate and 30%(*v*/*v*) PEG 400 in 50 m*M* Na HEPES buffer at pH 7.0 for CBFα-DNA; 3%(*w*/*v*) PEG 4K and 30% PEG 400 in 25 m*M* MES buffer at pH 5.6 for CBFα-C/EBPβ-DNA and 200 m*M* KCl, 4.5%(*w*/*v*) PEG 8K, 2%(*v*/*v*) glycerol and 25%(*v*/*v*)

MPD in 25 m*M* MES buffer at pH 5.6 for CBFα-β-C/EBPβ-DNA.

A gold derivative of CBFα-β-C/EBPβ-DNA (CBFα-β-C/EBPβ-DNA-Au) was prepared by first soaking the crystals for 1 h in solution containing 200 m*M* KCl, 4.5%(*w*/*v*) PEG 8K, 2%(*v*/*v*) glycerol, 2%(v/*v*) MPD and 25 m*M* MES at pH 5.6 and then adding $KAu(CN)_2$ to a final concentration of 1 m*M* and soaking the crystals for an additional 4 h. The unbound gold ions were then removed by back-soaking them in $KAu(CN)_2$-free solution for 30 min. Gold derivatives of crystals of iodine-containing complexes were prepared using the same protocol and all the gold derivatives were cryoprotected and flash-cooled as described for the native crystals. All data sets were collected using synchrotron radiation at SPring-8. The MAD data were collected from a single crystal using a trichromatic concept implemented on beamline BL45XU (Yamamoto *et al.*, 1998). The intensity data were indexed, integrated and scaled using the programs *DENZO* (Otwinowski, 1993) and *SCALEPACK* (Otwinowski & Minor, 1997). Details of the data collection, crystal parameters and data-processing statistics are summarized in Table 1.

3. Discussion

The crystals obtained for CBFα-β-C/EBPβ-DNA, CBFα-C/EBPβ-DNA and CBFα-DNA diffracted to resolutions of 3 Å or better. The solvent-content calculation (Matthews, 1968) (Table 1) suggested that one molecule of CBFα-β-C/EBPβ-DNA could be located in the asymmetric unit, whereas crystals of both CBFα-C/EBPβ-DNA and CBFα-DNA might have two molecules in the asymmetric unit. Crystals of native CBFα-β-C/EBPβ-DNA as well as its iodine derivative exhibited non-isomorphism (Table 1). In addition, diffraction from the native crystals was anisotropic, the highest resolutions along unit-cell axes *a*, *b* and *c* being 5, 2.8 and 3.2 Å, respectively. This anisotropy was reduced significantly in the gold derivative: the highest resolutions along the *a*, *b* and *c* axes were 3.5, 2.8 and 2.7 Å, respectively, and the mosaicity of the crystal was reduced from 0.8 to 0.4°.

The MAD data were collected using one crystal of CBFα-β-C/EBPβ-DNA-Au. The experimental values of f' and f'' at the edge and peak of the Au^+ L_{III} scattering were estimated to be −20.5 and 8.0 e, and −18.0 and 10.8 e, respectively. The Bijvoet difference Patterson map (Fig. 3), calculated with the data collected at a wavelength of 1.0388 Å (peak) using *CNS* version 0.9a (Brunger *et al.*, 1998), revealed one major site for the Au^+ atom with fractional coordinates $x = 0.141$, $y = 0.210$, $z = 0.155$. MAD phasing and subsequent density modification using *CNS* produced an electron-density map of excellent quality. Details and discussion of the structures of CBFα-β-C/EBPβ-DNA, CBFα-C/EBPβ-DNA and CBFα-DNA will be reported elsewhere.

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