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Expression, purification and preliminary crystallographic studies of human coactosin-like protein

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Coactosin-like protein (CLP) is an actin-binding protein as well as a 5-lipoxygenase binding partner. Human coactosin-like protein has been expressed in high yield and the His-tagged protein was purified by affinity chromatography. Several different crystal forms were obtained by the hanging-drop vapour-diffusion method. X-ray diffraction data to 2.0 Å resolution were collected from the best crystal. The space group was determined to be $P2_12_12_1$, with unit-cell parameters a = 38.4, b = 48.7, c = 72.6 Å.

1. Introduction

Human coactosin-like protein (CLP) shows significant homology to coactosin, a 17 kDa actin-binding protein originally isolated from *Dictyostelium* (de Hostos *et al.*, 1993), with 33% identity in amino-acid sequence (Chen *et al.*, 1997). The mRNA of CLP contains 1824 base pairs and is expressed predominantly in the placenta, lung, liver and kidney, but not in the heart, brain, skeletal muscle or pancreas (Chen *et al.*, 1997). Smith–Magenis syndrome (SMS), which causes clinical symptoms of mental retardation, neurobehavioural abnormalities, sleep disturbance, short stature, minor craniofacial and skeletal anomalies, congenital heart defects and renal anomalies, is caused by deletion of the short arm of chromosome 17 in band p11.2. The CLP gene was mapped to the SMS common deletion region (Chen *et al.*, 1997). The SMS critical region overlaps with a break-point cluster region associated with primitive neuro-ectodermal tumours, suggesting that CLP is involved in DNA rearrangements of somatic cells (Chen *et al.*, 1997). CLP is also defined as a human pancreatic cancer antigen by the SEREX method (Nakatsura *et al.*, 2002).

Human CLP was first found in a yeast two-hybrid screen by using 5-lipoxygenase (5LO) as bait (Provost, Doucet, Hammarberg *et al.*, 2001). 5LO is a 78 kDa enzyme of central importance in cellular leukotriene synthesis, catalyzing a two-step conversion of arachidonic acid to leukotrienes, which are potent lipid mediators of inflammation and of allergic disorders including arthritis, asthma and allergic reactions (Rådmark, 2000; Rohrig *et al.*, 1995). In resting cells, 5LO is localized in soluble compartments in the cytosol and/or within the nucleus. In response to stimulus, 5LO translocates to and becomes associated with the nuclear membrane. The regulation of localization and translocation of 5LO involves interactions with other proteins such as CLP.

CLP directly binds filamentous actin (F-actin), but does not form a stable complex with globular actin (G-actin; Provost, Doucet, Stock *et al.*, 2001). Under physiological conditions, G-actin is in equilibrium with F-actin, which forms the actin cytoskeleton that is responsible for maintaining and modifying cell shape in motility, phagocytosis and cytokinesis. It has been demonstrated that CLP is able to counteract the activities of capping proteins that retard actin polymerization, although CLP itself has no effect on actin polymerization (Rohrig *et al.*, 1995).

About 20 coactosins have been characterized from different organisms, but no three-dimensional structure has been reported. Thus, the successful crystallization of human CLP will provide a solid foundation for structure–function studies of coactosin that will be useful for understanding its role in binding to 5LO, in cellular leukotriene synthesis and in the physiological modulation of actin polymerization. In this paper, we report the gene cloning, expression, purification and preliminary crystallographic studies of human CLP.

2. Materials and methods

2.1. cDNA cloning and expression

The cDNA of CLP was amplified from a human brain cDNA library by polymerase chain reaction (PCR), introducing an *Nde*I restriction site in the front of the presumed ATG start codon and a *Xho*I site before the TAA stop codon, and cloned in-frame into the *Nde*I/*Xho*I sites of the pET22b(+) vector (Novagen). The pET22b(+)-CLP was transformed into *Escherichia coli* bacterial strain BL21(DE3) for expression. CLP with a C-terminal His tag was expressed in LB medium, which was incubated at 310 K until the OD_{600} reached about 0.6. The cultures were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final

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concentration of 800 µ*M* and incubated at 310 K for an additional 4 h.

2.2. Purification

Cells were harvested by centrifugation and resuspended in buffer (500 m*M* NaCl, 50 m*M* Tris–HCl pH 8.0) supplemented with 1 m*M* of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) and 1 m*M* ethylene diamine tetraacetic acid (EDTA). After sonication, cell debris was removed by centrifugation and the supernatant was applied onto a Chelating Sepharose Fast Flow (Amersham Pharmacia) column pre-equilibrated with the same buffer. After extensive washing, the His-tagged CLP was eluted in steps with 20, 50, 200 and 500 m*M* imidazole, respectively. The collected sample was concentrated to 20 mg ml^{-1}, which was determined by the method of Bradford (1976) using the Bio-Rad dye reagent with bovine serum albumin (BSA) as the standard.

2.3. Crystallization

C-terminally His-tagged CLP was crystallized using the hanging-drop vapour-diffusion method at 277 K (McPherson, 1982). A preliminary crystallization screen was carried out using Crystal Screens I and II from Hampton Research (Jancarik & Kim, 1991) by mixing 1 µl protein solution (20 mg ml^{-1} protein in 50 m*M* Tris–HCl pH 8.0, 5 m*M* NaCl, 2 m*M* imidazole) and 1 µl well solution. Needle-like crystals were obtained under several conditions which all contained PEGs (polyethylene glycol) of various molecular weights. Further optimization based on these conditions, including adjusting the pH value and the concentrations of precipitant and salt, did not result in any improvement. After analysis of these results, we systematically redesigned a series of conditions using a grid-screening method with different kinds of PEG (PEG 400, PEG 1K, PEG 2K, PEG 4K, PEG 8K, PEG 10K) combined with different pH values (from 6.0 to 9.0). This led to better crystals on mixing 1 µl of protein solution (20 mg ml^{-1}) with 1 µl well solution of 22%(*w*/*v*) PEG2K, MES (2-morpholinoethanesulfonic acid) pH 6.5, which differed from any of the conditions we originally obtained from Crystal Screens I and II.

2.4. Data collection and processing

The crystals were flash-frozen in liquid nitrogen prior to cooling in a 100 K nitrogen-gas stream for data collection. Diffraction data were collected on an R-AXIS IV^{++} imaging-plate detector with Cu $K\alpha$ X-rays (1.5418 Å) generated by a rotating-anode generator (Rigaku, Japan). The crystal-to-detector distance was set to 150 mm. The exposure time for each image was 500 s. The diffraction data were integrated and scaled using *d*TREK* as implemented in the *Crystal Clear* suite v.1.3 (MSC).

Table 1
Summary of diffraction data collections.

No.	Resolution (Å)	Space group	Unit-cell parameters (Å, °)
1	3.0	$P2_12_12_1$	a = 38.4, b = 48.8, c = 72.6
2	3.0	Tetragonal	a = b = 70.1, c = 151.3
3	2.8	$C2$	a = 128.6, b = 36.9, c = 60.3, β = 104.8

3. Results and discussion

3.1. Gene cloning and protein purification

The result of gene sequencing (TaKaRa Bio Inc), after cloning into pET22b(+), was consistent with the data from the NCBI (Genbank accession No. BC010039). The fractions eluted with 50 and 200 m*M* imidazole containing CLP were collected. SDS–PAGE results showed two bands corresponding to the molecular weights of a monomer and dimer, respectively. This unusual electrophoretic behaviour was also observed by another group (Provost, Doucet, Stock *et al.*, 2001), which suggests that part of the CLP might exist as a dimer *in vitro*. However, a MALDI–TOF mass-spectrometry experiment showed a single sharp peak corresponding to a molecular weight of 16 885.8 Da, consistent with the weight of a monomer.

3.2. Preliminary crystallographic studies

Interestingly, under the same conditions crystals appeared with different shapes, such as needle clusters, rectangular or hexagonal plates and single crystals with the shapes of a brick or a pyramid. Most of the crystals were too highly mosaic or diffracted to too low a resolution for data collection. Soaking the crystal with various cryoprotection buffers or X-ray exposure at ambient temperature did not improve the diffraction quality.

Of the more than 50 crystals tested, several diffracted to 3.0–2.8 Å resolution and belonged to a number of different space groups (Table 1). Only one CLP crystal diffracted to 2.0 Å resolution; a complete data set was collected from this crystal. Analysis of the diffraction symmetry and systematic absences indicated that the crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 38.4, b = 48.7, c = 72.6 Å (Table 2). Assuming the presence of one monomer in the crystallographic asymmetric unit, the corresponding V_M value is 2.0 Å^3 Da^{-1} (Matthews, 1968).

Table 2
Summary of diffraction data collection and processing.

Values in parentheses are for the last resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 38.4, b = 48.7, c = 72.6
Temperature (K)	100
Oscillation range per image (°)	1.0
Resolution (Å)	2.0 (2.07–2.0)
Total No. reflections	25682
No. independent reflections	9391
Completeness (%)	97.2 (93.1)
R_{merge}	0.034 (0.125)
$I/\sigma(I)$	17.7 (5.4)

Molecular replacement was performed with *AMoRe* (Navaza, 1994) with phosphorylated actophorin from *Acantamoeba polyphaga* (PDB code 1cnu) as the search model, which has only 16.9% identity with CLP in amino-acid sequence. Molecular replacement yielded a solution with relatively high values of the rotation and translation functions (RF and TF), with the highest values of RF/σ and TF/σ being approximately twice the second highest values of these functions; the solution also gave reasonable packing in the unit cell. However, further refinement with using different strategies failed, with the R factor remaining above 0.5 and the electron density being poor, which suggests that CLP and actophorin might be similar in their folding but quite different in their detailed structures. We are now in the process of trying to prepare heavy-atom derivatives and a selenomethione variant to solve the phase problem.

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