Journal of Biomolecular NMR **30:** 353–356, 2004.

Letter to the Editor: NMR structure of human coactosin-like protein

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Received and Accepted 12 August 2004

Key words: coactosin-like protein, human CLP, NMR structure

Biological context

The human Coactosin-Like Protein (CLP) gene was initially found as a sequence flanking a deletion on the human chromosome 17 characterizing the Smith-Magenis syndrome (Chen et al., 1997). The encoded CLP protein (142 amino acids, 16 kDa) is similar to *D. Discoideum* coactosin (de Hostos et al., 1993) which is a member of the ADF/Cofilin group of actin binding proteins (Lappalainen et al., 1998). Coactosin can bind to filamentous actin (F-actin) (de Hostos et al., 1993) and interfere with capping of actin filaments (Rohrig et al., 1995), thereby promoting actin polymerization.

Similarly, human CLP was characterized as an F-actin binding protein that colocalized with actin stress fibers in transfected mammalian (CHO and Cos-7) cells. Site-directed mutagenesis studies revealed the essential role of Lys75, a residue highly conserved in related proteins, in mediating F-actin binding (Provost et al., 2001b).

Most interestingly, human CLP also binds to 5-lipoxygenase (5LO; Provost et al., 1999, 2001b) which plays an important role in leukotriene biosynthesis, converting arachidonic acid to 5(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and subsequently to the epoxide leukotriene (LT) A_4 (Samuelsson 1983; Rådmark 2002). Nondenaturing PAGE and chemical cross-linking experiments showed that CLP directly binds 5LO in a 1:1 molar stoichiometry in a Ca^{2+}-independent manner. Lys131 of CLP was found to be important for 5LO binding (Provost et al., 2001a).

Although CLP is capable of interacting with F-actin and 5LO independently, no F-actin-CLP-5LO ternary complex could be observed. Instead, 5LO appears to compete with F-actin for the binding of CLP (Provost et al., 2001a). Experimental evidences obtained from studies of murine CLP also suggest that CLP exhibits different binding characteristics towards F-actin and 5LO (Doucet et al., 2002).

CLP has been linked to pancreatic cancer. CLP mRNA was found to be overexpressed in human pancreatic cell lines as compared to normal pancreatic tissue (Nakatsura et al., 2001), while CLP protein expression decreased 22-fold after treatment of the pancreatic adenocarcinoma cell line (PaCa44) with the chemotherapeutic agent 5-aza-2'-deoxycytidine (DAC) (Cecconi et al., 2003). CLP-derived peptides, which may represent human pancreatic cancer antigens, are vaccine candidates for peptide-based immunotherapy (Nakatsura et al., 2002). CLP protein levels were also found to increase during differentiation of 3T3-L1 fibroblasts into adipocytes (Welsh et al., 2004).

Here we present the NMR structure of human CLP as a prerequisite for understanding its interaction with 5LO and F-actin.

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Methods and results

Human CLP cDNA was obtained by screening of a human lung cDNA library using 5LO as a bait and cloned in frame into the *Bam*HI/*Xho*I sites of pGEX-5X-1 vector as described (Provost et al., 1999, 2001a). The GST-CLP fusion protein was expressed in *E. coli* bacterial strain BL21. Isotopically labelled CLP was produced with Silantes (Munich, Germany) media with the addition of 150 μg/ml Ampicillin. Typically, IPTG (200 μM) was added when the *E. coli* culture had reached an OD_{600} of 0.5, and the cells were harvested 9 h later (OD_{600} approx. 1.3). Cells were resuspended in lysis buffer (50 mM triethanolamine/HCl, pH 8.0, 5 mM EDTA, 2 mM DTT, 60 μg/ml soybean trypsin inhibitor, 0.5 mg/ml lysozyme), cooled for 35 min on ice and sonicated. Proteins in the $10{,}000 \times g$ supernatant were precipitated with 80% ammonium sulphate. Purification on Glutathione Sepharose 4B beads and cleavage of the fusion protein with factor Xa was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). CLP was purified by anion exchange chromatography. The factor Xa digest (typically 10 ml) was loaded onto a Mono Q HR 5/5 column, equilibrated with 20 mM Tris–HCl, pH 8.0, 0.5 mM EDTA (buffer A) at a flow rate of 0.5 ml/min. The column was washed with the same buffer, and elution was performed with a linear gradient up to 100% buffer B (1 M NaCl in buffer A). CLP usually eluted at 0.15–0.2 M salt.

For NMR measurements, pooled fractions were concentrated to 340 μM by ultrafiltration on Centricon 3 K filters and the buffer changed to 20 mM sodium phosphate, pH 7.0, 0.5 mM KCl. The protein concentration was determined according to Bradford using the Bio-Rad dye reagent with bovine serum albumin (BSA) as standard.

All NMR experiments were performed at pH 7 and 25°C. Heteronuclear NMR spectra were recorded on a Bruker DMX600 NMR spectrometer equipped with a cryoprobe operating at 600 MHz ^{1}H NMR frequency, using ^{15}N-labelled CLP at a concentration of 0.25 mM in 90% H_2O/10% D_2O. Homonuclear NMR spectra were recorded on a Varian Inova 800 MHz NMR spectrometer, using a 0.16 mM solution of CLP in 90% H_2O/10% D_2O. The homonuclear resonance assignments were based on NOESY, TOCSY (70 ms mixing time, 72 h total recording time) and DQF-COSY (recorded in D_2O in 48 h) spectra. For the collection of structural restraints, a NOESY spectrum was recorded for 144 h at 800 MHz, using 40 ms mixing time, $t_{1\max} =$ 100 ms and $t_{2\max} = 225$ ms. In addition, a NOESY spectrum was recorded in D_2O solution for 48 h at 600 MHz, using 60 ms mixing time, $t_{1\max} = 73$ ms and $t_{2\max} = 146$ ms. The ^{15}N assignments were derived from a 3D TOCSY-^{15}N-HSQC and a 3D NOESY-^{15}N-HSQC experiment, both recorded with 60 mixing time in 68 h per spectrum. A ^{15}N-HSQC spectrum with ^{1}H purge pulse (Szyperski et al., 1992) was recorded for the measurement of $^3J(H^N,H^\alpha)$ coupling constants, and a 2D version of the ^{15}N-HB experiment (total recording time 48 h, 27.8 ms coupling evolution delay) for the measurement of $^3J(N,H^\beta)$ couplings.

The linewidths observed in the NMR spectra indicated that the protein was monomeric in solution (Figure 1). Structural restraints were derived from NOEs and scalar coupling constants in the usual way (e.g., Liepinsh et al., 2003). Stereospecific assignments were obtained for 90 pairs of ^{1}H NMR resonances. The NMR assignments have been deposited in the BMRB (accession code 6283). They are in agreement with the assignments recently reported for a mutant of human CLP studied under slightly different conditions (Dai et al., 2004). The NMR structure of CLP was calculated with the program DYANA (Güntert et al., 1997), starting from 50 random conformers. The 20 best conformers were embedded in water and energy-minimized with the program OPAL (Luginbühl et al., 1996). Table 1 presents an overview of the restraints and structural statistics.

The NMR structure of human CLP is composed of a five-stranded β-sheet with two α-helices on either side (Figure 2), comprising the following residues: 7–18 (α1), 26–32 (β1), 35–42 (β2), 46–51 (α2), 57–64 (β3), 75–82 (β4), 91–104 (α3), 110–114 (β5) and 122–130 (α4). Increased mobility was indicated by narrow NMR line shapes and weak NOEs for the N-terminal residues including Ala2, residues 69–71 and residues 136–142. The coordinates have been deposited in the PDB (accession code 1WNJ). The residue numbering in the PDB file is shifted by three residues with respect to the numbering used here due to the presence of three additional N-terminal residues from the factor Xa cleavage site.

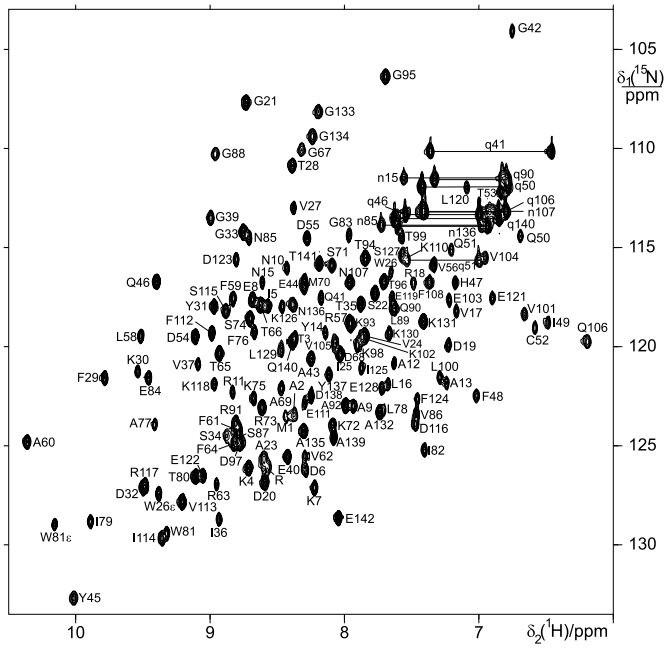

Figure 1. ^{15}N-HSQC spectrum recorded of a 0.25 mM solution of [U-^{15}N]CLP in 90% H_2O/10% D_2O, pH 7.0 at 25°C. Cross-peaks from side-chain amide protons are connected by lines and labelled with small characters. The cross-peak from the arginine of the N-terminal 3-residue cloning artifact is labelled without a sequence number.

Table 1. Structural statistics for the NMR conformers of CLP

Number of assigned NOE cross peaks	1641
Number of non-redundant NOE upper-distance limits	1300
Number of scalar coupling constants[a]	448
Number of dihedral-angle restraints	394
Intra-protein AMBER energy (kcal/mol)	−6055 ± 149
Maximum NOE-restraint violations (Å)	0.1 ± 0.01
Maximum dihedral-angle restraint violations (°)	2.5 ± 0.2
R.m.s.d. to the mean for N, C^{α} and C' (Å)[b]	0.56 ± 0.09
R.m.s.d. to the mean for all heavy atoms (Å)[b]	0.98 ± 0.09
Ramachandran plot appearance	
most favoured regions (%)	87.4
additionally allowed regions (%)	12.5
generously allowed regions (%)	0.1
disallowed regions (%)	0.0

[a]118 $^3J(H^N,H^{\alpha})$, 167 $^3J(H^{\alpha},H^{\beta})$, 163 $^3J(^{15}N,H^{\beta})$.
[b]For residues 6-136.

Discussion and conclusions

During the analysis of this structure determined of human CLP, coordinates for the murine homologue were deposited in the protein data bank (Goroncy, A., Kigawa, T., Koshiba, S., Kobayashi, N., Tochio, N., Inoue, M. & Yokoyama, S. (2004), PDB ID code 1UDM). The amino acid sequences of human and murine CLP are nearly 95% identical. Consequently, their structures are very similar, with an average pairwise r.m.s. deviation of 1.0 Å. between the backbone atoms of residues 3–130, excluding residues 66–75 of the disordered loop between strands β3 and β4. This difference is barely significant, considering that r.m.s. deviations as small as 0.24 Å were observed between individual pairs of conformers and the largest structural differences occur in the loop regions between regular secondary structure elements, where structural restraints are scarce.

The C^{α} atoms of the functionally important residues Lys75 and Lys131 are separated by about 14 Å (Figure 2). It thus appears plausible

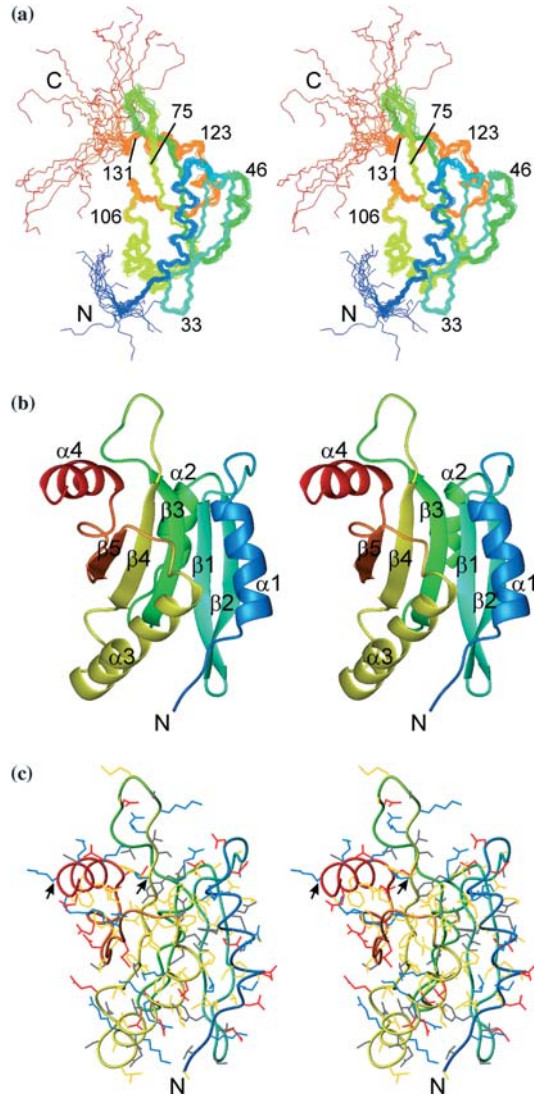

Figure 2. Stereoviews of the three-dimensional NMR structure of human CLP. (a) Superposition of the backbone atoms in the twenty NMR conformers of CLP. All residues are shown. The N- and C-termini and selected residues are labelled. (b) Ribbon drawing, comprising residues 5–136. The N-terminus and the regular secondary structural elements are labelled. The view is rotated with respect to A. (c) Representation in the orientation of B with side chains (blue, R, K, H; red, D, E; yellow, A, C, F, I, L, M, P, V, W; grey, N, Q, S, T, Y). Arrows identify the positions of Lys75 and Lys131. The figure was prepared with Molmol (Koradi et al., 1996).

that the binding sites of F-actin (involving Lys75) and 5LO (involving Lys131) overlap, explaining why these two proteins cannot bind simultaneously. The present NMR assignments and structure present the basis for an investigation of the complexes with 5LO and F-actin.

Acknowledgements

G.O. thanks the Australian Research Council for a Federation Fellowship. P.P. thanks The Arthritis Society for a New Investigator Fellowship. Financial support by The Arthritis Society, the Swedish Research Council (03X-217), EU (QLG1-CT-2001-01521), the Australian Research Council and the Karolinska Institute is gratefully acknowledged.

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