Coactosin-like protein, a human F-actin-binding protein: critical role of lysine-75

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Coactosin-like protein (CLP) was recently identified in a yeast two-hybrid screen using 5-lipoxygenase as bait. In the present study, we report the functional characterization of CLP as a human filamentous actin (F-actin)-binding protein. CLP mRNA shows a wide tissue distribution and is predominantly expressed in placenta, lung, kidney and peripheral-blood leucocytes. Endogenous CLP is localized in the cytosol of myeloid cells. Using a two-hybrid approach, actin was identified as a CLP-interacting protein. Binding experiments indicated that CLP associates with F-actin, but does not form a stable complex with globular actin. In transfected mammalian cells, CLP co-localized

with actin stress fibres. CLP bound to actin filaments with a stoichiometry of 1:2 (CLP: actin subunits), but could be cross-linked to only one subunit of actin. Site-directed mutagenesis revealed the involvement of Lys⁷⁵ of CLP in actin binding, a residue highly conserved in related proteins and supposed to be exposed on the surface of the CLP protein. Our results identify CLP as a new human protein that binds F-actin *in vitro* and *in vivo*, and indicate that Lys⁷⁵ is essential for this interaction.

Key words: cytoskeleton, leukotriene, 5-lipoxygenase, protein binding, yeast two-hybrid system.

INTRODUCTION

Monomeric globular actin (G-actin) is, under physiological conditions, in equilibrium with filamentous actin (F-actin) that forms the actin cytoskeleton, which is responsible for maintaining and modifying cell shape in motility, phagocytosis and cytokinesis. Mammalian actin isoforms can be separated into three isoelectric variants, referred to as α -, β - and γ -actin [1]. The activities of these actin isoforms are regulated by numerous actin-binding proteins [2].

Several actin-binding proteins have been demonstrated to associate with actin through electrostatic interactions involving basic and acidic amino acid residues [3]. Actin-binding sites have been gradually narrowed from large actin-binding domains, composed of approx. 250 residues, to short hexamers. One example of such a short actin-binding consensus sequence is the hexamer LKKAET (where single-letter amino-acid notation has been used). The lysine residues in this motif are involved in the binding of actobindin [4,5], villin [6], thymosin [7] and verprolin [8] to actin.

In the present paper, we identify coactosin-like protein (CLP) as a human actin-binding protein. CLP was named in view of its similarity to coactosin, an actin-binding protein from *Dictyostelium discoideum* [9]. Coactosin has been isolated from actin–myosin complexes and has been found to bind F-actin *in vitro* [9]. In addition, while having no effect on actin polymerization, coactosin was shown to counteract the activity of capping proteins [10].

Initially found as a sequence flanking a deletion on the human chromosome 17 characterizing the Smith–Magenis syndrome [11], the CLP gene encodes a protein composed of 142 amino

acid residues with a molecular mass of 16 kDa. We previously demonstrated that CLP is capable of forming a complex with 5-lipoxygenase (5LO) [12], an enzyme involved in leukotriene biosynthesis [13], and that a LKKAET-like motif is involved in this interaction [14]. In the present study, we characterize the actin-binding activity of CLP and describe the importance of another basic residue, Lys⁷⁵, in mediating the binding of CLP to actin.

EXPERIMENTAL

Northern-blot analysis

Multiple Tissue Northern Blots (ClonTech Laboratories) containing 2 μ g of polyadenylated RNA per lane were hybridized with a 980 bp fragment (nt 239–1218) of the CLP cDNA, labelled using the T7-QuickPrime kit (Pharmacia Biotech) and [α -³²P]dCTP (Amersham). The blots were washed and exposed to X-ray film (Kodak) at -70°C . A 2 kb human β -actin cDNA probe (ClonTech Laboratories) was used as a control.

Myeloid cells

Human neutrophils were isolated from leucocyte concentrates obtained from healthy donors at the Karolinska Hospital (Stockholm, Sweden). Human promyelocytic leukaemia 60 (HL60) cells (A.T.C.C.) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified 5% CO_2 incubator.

For preparation of lysates, cells were resuspended at 2×10^7 /ml in Nonidet P40 (NP40) lysis buffer [10 mM Tris/HCl (pH 7.4),

Abbreviations used: ABP1, actin-binding protein 1; CLP, coactosin-like protein; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride; F-actin, filamentous actin; G-actin, globular actin; GST, glutathione S-transferase; HA, haemagglutinin; HL60, human promyelocytic leukaemia 60; K75A, Lys⁷⁵ $\rightarrow$ Ala; 5LO, 5-lipoxygenase; sulpho-NHS, *N*-hydroxysulphosuccinimide; NP40, Nonidet P40.

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10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 0.1 % NP40, Complete Protease Inhibitor Mix (Boehringer Mannheim) and 1 mM PMSF] and sonicated. The lysates were clarified by centrifugation at 15000 *g* for 10 min at 4 °C, and boiled in SDS sample buffer for immunoblot analysis.

Subcellular fractionation

Neutrophils were subjected to subcellular fractionation following lysis induced by NP40, as described by Pouliot et al. [15]. Human neutrophils (2×10^7) were pelleted, briefly vortex-mixed and resuspended in 600 μ l of ice-cold NP40 lysis buffer. The cells were vortex-mixed for 15 s, kept on ice for 5 min and centrifuged at 500 *g* for 10 min at 4 °C. The resulting supernatant (non-nuclear fraction) and pellet (nuclear fraction) were processed for immunoblot analysis. In parallel, another aliquot of the pellet (nuclear fraction) was washed, resuspended in 100 μ l of ice-cold relaxation buffer [10 mM Pipes (pH 7.3), 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1 mM ATP and 1.25 mM EGTA containing Complete Protease Inhibitor Mix and 1 mM PMSF], sonicated on ice and centrifuged at 180000 *g* for 20 min at 4 °C using a SW 50.1 rotor in a Beckman ultracentrifuge. The resulting supernatant and pellet fractions were referred to as nuclear soluble and membrane fractions respectively. The fractions were boiled in SDS sample buffer, and analysed by SDS/PAGE and immunoblotting.

Immunoblot analysis

Samples were fractionated by SDS/PAGE, transferred on to nitrocellulose membranes (Amersham), and immunoblotted as described previously [12]. A polyclonal anti-(human CLP) serum was raised in rabbits against amino acids 116–130 of human CLP (Innovagen, Lund, Sweden). Anti-(leukotriene A₄ hydrolase) antibody was described previously [16], and anti-actin (clone AC-40) antibody was obtained from Sigma. Immunoreactive proteins were visualized using alkaline phosphatase conjugates and substrates (Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate).

Expression and purification of recombinant human CLP

The CLP cDNA was amplified by PCR and cloned in frame into the *Bam*HI/*Xho*I sites of a pGEX-5X-1 vector (Amersham Pharmacia Biotech). This pGEX-5X-1-CLP construct was also subjected to site-directed mutagenesis to generate the CLP mutant Lys⁷⁵ → Ala (K75A).

The pGEX-5X-1-CLP and K75A mutant constructs, as well as the empty pGEX-5X-1 vector, were used to transform the *Escherichia coli* bacterial strain BL21 (Amersham Pharmacia Biotech) for expression and purification of the glutathione S-transferase (GST)–CLP fusion proteins or GST only, according to the manufacturer's instructions. In some experiments, the GST–CLP and GST proteins were eluted from the GSH–Sepharose 4B beads (Amersham Pharmacia Biotech) with 10 mM GSH [in 50 mM Tris/HCl (pH 8.0)]. The CLP proteins were then cleaved from the GST moiety with Factor Xa (Amersham Pharmacia Biotech) and purified by anion-exchange chromatography.

Protein determination

The protein concentrations were determined by the method of Bradford [17] using the Bio-Rad dye reagent, with BSA as a standard. Actin concentration was determined by measuring the absorbance at 290 nm.

GST binding assay

For binding studies *in vitro*, 20 μ g of the GST–CLP or GST–CLP(K75A) fusion protein, coupled to GSH–Sepharose 4B beads, was incubated with F-actin in the presence of BSA (50 μ g) in 200 μ l of G buffer [2 mM Tris/HCl (pH 8.0), 0.2 mM CaCl₂, 0.2 mM ATP and 0.5 mM 2-mercaptoethanol] supplemented with 2 mM MgCl₂ and 50 mM KCl. F-actin was prepared by allowing actin to polymerize for 2 h at 22 °C in G buffer supplemented with 2 mM MgCl₂ and 50 mM KCl prior to the binding assays. After incubation for 30 min, the beads were extensively washed in incubation buffer, and the GST–CLP-bound protein complexes were eluted with 10 mM GSH. The beads were sedimented by centrifugation, and the presence of the interacting actin in the supernatant was analysed by SDS/PAGE and immunoblot analysis.

Non-denaturing PAGE

Non-denaturing PAGE was used to test whether CLP can interact with G-actin, as described by Safer [18]. Purified proteins (7.5 μ M each) were incubated in G buffer for 15 min at 22 °C. The samples were then mixed with native sample buffer, loaded immediately and run at 20 mA constant current at 4 °C. Proteins were then stained with Coomassie Brilliant Blue.

After non-denaturing PAGE of the G-actin and CLP combination, the lane was cut out, incubated in SDS sample buffer and then inserted on top of an SDS/polyacrylamide gel. The electrophoresis was performed in the presence of SDS (0.1 %) in the running buffer. The gels were stained with Coomassie Brilliant Blue.

Mammalian cell culture, transfections and immunofluorescence labelling

CHO and COS-7 cells were grown on sterile glass coverslips in Dulbecco's minimal essential medium supplemented with 10 % (v/v) fetal bovine serum, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified incubator under 5 % CO₂ at 37 °C. To allow expression of an epitope-tagged CLP, a haemagglutinin (HA) epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala), preceded by a Kozak recognition sequence [19], was introduced by PCR, and cloned as a *Bam*HI–*Xho*I fragment into the mammalian expression vector pcDNA3.1 (Invitrogen). Cells were transfected with plasmid DNAs using Lipofectamine Plus (Life Technologies).

For immunofluorescence labelling, cells were washed in PBS, fixed in 4 % (w/v) paraformaldehyde, permeabilized with 0.1 % Triton X-100 and incubated in blocking buffer (PBS containing 0.1 % BSA). To visualize the epitope-tagged CLP, the cells were incubated with 2 μ g/ml anti-HA mouse monoclonal antibody (clone 12CA5; Boehringer Mannheim). After extensive washing in PBS, the cells were incubated with FITC-conjugated goat anti-mouse IgG antibodies (1:50 dilution; Sigma). Rhodamine–phalloidin (1:500 dilution; Molecular Probes, Eugene, OR, U.S.A.) was used to stain F-actin. After extensive washing in PBS, the coverslips were mounted on slides with mounting solution, consisting of 1 mg/ml phenylenediamine (Sigma), 50 % (v/v) glycerol and 50 mM Tris/HCl, pH 8.0. The dual-labelled cells were viewed on an Axiophot microscope (Zeiss), and the images were analysed using the program QED CCD Camera StandAlone (QED Imaging).

High-speed co-sedimentation assay

For high-speed co-sedimentation assays, actin was purified from rabbit skeletal muscle as described by Rees and Young [20]. G-

actin, GST, GST-CLP, CLP and CLP(K75A) solutions were centrifuged at 100 000 *g* for 30 min at 4 °C, and the supernatants were used to prepare the reaction mixtures (5 μ M of each protein), which were incubated for 2 h at 22 °C in standard polymerization buffer [10 mM Mops (pH 7.0), 2 mM MgCl₂ and 50 mM KCl] or in imidazole buffer [10 mM imidazole (pH 7.0), 3 mM MgCl₂, 1 mM ATP and either 0.2 mM CaCl₂ or 1 mM EGTA] [21]. Some experiments were performed in 10 mM Mops (pH 7.0) (in the absence of Mg²⁺ and K⁺), in standard polymerization buffer at various pH values, or in the presence of BSA.

For chemical cross-linking, G-actin was dialysed overnight against phosphate buffer [5 mM potassium phosphate (pH 8.0), 0.2 mM CaCl₂, 0.2 mM ATP and 0.2 mM dithiothreitol]. After incubation of the reaction mixtures (5 μ M of each protein) for 2 h at 22 °C in standard polymerization buffer, the zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC) (Pierce) and *N*-hydroxysulphosuccinimide (sulpho-NHS) (Pierce) were added to a final concentration of 4 mM each. After incubation for 5 min at 22 °C, the reactions were quenched by the addition of 20 mM Tris.

The samples (100 μ l) were then centrifuged at 100 000 *g* for 1 h at 4 °C. Proteins in the pellet and supernatant of each sample were boiled with SDS sample buffer, resolved by SDS/PAGE, and the gels were either stained with Coomassie Brilliant Blue or processed for immunoblotting.

Actin polymerization and depolymerization assays

Rabbit skeletal-muscle actin was specifically labelled at cysteine residues using Acrylodan (Molecular Probes), a thiol-reactive adduct of Prodan [22], as described by Marriott et al. [23]. G-actin-Acrylodan was used in a molar ratio of 1:7 with unlabelled G-actin for actin polymerization assays. Neither the rate nor the extent of polymerization differs significantly between the native and fluorescent actin [23]. The polymerization of Acrylodan-labelled G-actin (2 μ M) in G buffer was initiated by the addition of 2 mM MgCl₂ and 50 mM KCl, in the absence or presence of CLP. The progress of the polymerization reaction was monitored for 2 h using a luminescence spectrometer (Aminco-Bowman Series 2). The excitation wavelength was 400 nm (bandwidth of 4 nm), and the emission wavelength was collected through a monochromator at 465 nm (bandwidth of 4 nm).

For actin depolymerization assays, Acrylodan-labelled G-actin (35 μ M) was polymerized in a polymerization buffer [10 mM Mops (pH 7.0), 2 mM MgCl₂, 100 mM KCl, 0.2 mM CaCl₂, 0.2 mM ATP and 0.5 mM 2-mercaptoethanol] for 1 h at 22 °C. The F-actin solution was then diluted to a final concentration of 50 nM actin in the same polymerization buffer in the absence or presence of equimolar amounts of CLP. The fluorescence was measured for 1 h at an excitation wavelength of 400 nm (bandwidth of 2.5 nm) and an emission wavelength of 465 nm (bandwidth of 2.5 nm) (PerkinElmer LS50B fluorimeter).

CD measurements and analysis

An AVIV 62DS CD spectrometer was used to measure the ellipticity of samples of purified CLP and CLP(K75A) at 50 μ g/ml in 10 mM sodium phosphate (pH 7.0), using a 1 cm path length cell that was thermostatically maintained at 25 °C. For each measurement, four CD spectra were acquired in the far-UV region from 260 to 200 nm, at 0.5 nm intervals, and averaged; the buffer background was then subtracted. Measured ellipticities in millidegrees were converted into mean residue ellipticity units and expressed as degrees $\times$ cm² $\times$ dmol⁻¹. The fractional com-

position of the secondary structure of CLP and CLP(K75A) in terms of alpha-helix, beta-sheet and random coil was analysed using the K2d program (available at: <http://www.embl-heidelberg.de/~andrade/k2d.html>) [24].

RESULTS

CLP mRNA tissue distribution

The expression of CLP mRNA was investigated in various human tissues by Northern-blot analysis. Using a 980 bp fragment of the CLP cDNA as a probe, a single band of approximately 2 kb was identified. The size of this transcript is in agreement with that reported for CLP cDNA (1824 bp) (GenBank® accession number L54057). The CLP mRNA transcript is expressed predominantly in placenta, lung and kidney, with lower expression in the brain, liver and pancreas (Figure 1, left-hand panel). In the heart, an additional band of approx. 1.4 kb was found, suggesting alternative splicing. Examination of the CLP mRNA distribution in human immune tissues revealed relatively high expression of CLP in peripheral-blood leucocytes, compared with that in the spleen and lymph node (Figure 1, right-hand panel). CLP mRNA expression in the skeletal muscle, thymus, bone marrow and fetal liver was low. Nevertheless, the Northern-blot analysis indicated that CLP shows a wider tissue distribution than its interacting partner, 5LO, which is expressed primarily in leucocytes [25].

CLP is expressed in myeloid cells and is localized in the cytosolic compartment

The results from Northern-blot analysis prompted us to examine the expression and localization of CLP in myeloid cells. For that purpose, a peptide antibody was raised against human CLP, and was used to examine CLP expression in human neutrophils and in undifferentiated or DMSO-differentiated HL60 cells. Cell lysates were analysed by SDS/PAGE and immunoblotting using the CLP antiserum. A band of approximately 16 kDa was detected by the immune serum, but not by the preimmune serum (Figure 2A), in agreement with the calculated molecular mass of CLP (15945 Da). CLP expression was found to be unrelated to the differentiation status of HL60 cells, in contrast with 5LO

Figure 1 CLP mRNA distribution in human tissues

Northern blots of polyadenylated RNA from human tissues were hybridized with a 980 bp fragment of the CLP cDNA. The blots were also hybridized with a 2 kb human β -actin cDNA control probe. RNA size markers are shown in kilobases (kb). Sk., skeletal; PBL, peripheral-blood leucocyte.

Figure 2 Expression of CLP in myeloid cells and subcellular localization

(A and B) Lysates (1.5×10^6 cells) of human neutrophils [polymorphonuclear leucocytes (PMNL)] and HL60 cells, undifferentiated (u) or differentiated with 1.3% (v/v) DMSO for 4–7 days (d), were analysed by SDS/PAGE and immunoblotting (IB) with anti-human CLP serum (I) or preimmune serum (PI) (A), or with anti-5LO serum (B). (C) Human neutrophils were lysed using NP40, and subjected to cellular fractionation. Fractions representing 1×10^6 cells were analysed by SDS/PAGE and immunoblotting using the anti-CLP antibody (top panel) or the anti-leukotriene A_4 hydrolase (LTA_4 Hydr.) antibody (bottom panel). NN, non-nuclear; N, nuclear; NS, nuclear soluble; NP, nuclear pellet. Molecular-mass markers (in kDa) are shown on the left.

Figure 3 CLP does not form a stable complex with G-actin

(A) G-actin and DNase I, G-actin and leukotriene A_4 hydrolase (LTA_4 Hydr.), CLP and leukotriene A_4 hydrolase or G-actin and CLP ($7.5 \mu\text{M}$ each) were incubated alone or in combination, and analysed by non-denaturing PAGE. (B) Two-dimensional gel electrophoresis. After non-denaturing gel electrophoresis of the G-actin and CLP mixture, a lane was excised and inserted on top of a denaturing SDS/polyacrylamide gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue.

expression, which becomes apparent only after cellular differentiation (Figure 2B). The differential regulation of CLP and 5LO expression suggests that CLP has additional cellular function(s) independent of 5LO in myeloid cells.

To determine the intracellular distribution of CLP in myeloid cells, we fractionated lysates of human neutrophils, followed by immunoblot analysis. After lysis with NP40, CLP was detected in the non-nuclear fraction, like leukotriene A_4 hydrolase, a

cytosolic enzyme involved in leukotriene B_4 synthesis [26]. (Figure 2C). The nuclear fractions (nuclear soluble and nuclear pellet) showed no immunoreactivity with CLP or leukotriene A_4 hydrolase antisera (Figure 2C).

CLP directly binds F-actin and does not form a stable complex with G-actin

A yeast two-hybrid screen of a human lung cDNA library, using CLP cDNA as bait, allowed the identification of β - and γ -actin as CLP-interacting proteins [14]. GST binding experiments showed that the GST–CLP fusion protein associated with F-actin in a dose-dependent manner [14]. However, no binding of actin to GST–CLP beads was detected under non-polymerization conditions (P. Provost, unpublished work). To exclude the possibility that the GST moiety or coupling of the fusion protein to the beads may have interfered with G-actin binding, we re-examined by non-denaturing PAGE whether CLP associates with G-actin. Purified CLP and G-actin were incubated in a low-ionic-strength environment at 22 °C for 15 min, and loaded immediately on to a 10% (w/v) non-denaturing polyacrylamide gel. Proteins migrate in these gels according to the ratio of charge to volume. After electrophoresis, the gels were stained with Coomassie Brilliant Blue.

G-actin, known to interact with DNase I in non-denaturing PAGE [18], was used as an interacting protein control, and leukotriene A_4 hydrolase was used as a non-interacting protein control. G-actin and CLP migrated as single bands on non-denaturing gels, with G-actin having a greater electrophoretic mobility than CLP (Figure 3A). DNase I and leukotriene A_4 hydrolase migrated as two major bands respectively, with DNase I having a lower electrophoretic mobility. In contrast, single bands were formed on SDS gels, indicating the presence of charge heterogeneity in these purified proteins. The combination of equimolar amounts of G-actin and DNase I gave a major band of intermediate mobility, with little or no unbound G-actin or DNase I, demonstrating the formation of a binary G-actin–DNase I complex. When G-actin was mixed with CLP, no additional band was observed, and the respective band intensities of the unbound protein species remained unchanged. Again, no interaction between CLP and monomeric actin was observed in two-dimensional gel electrophoresis (Figure 3B). These observations suggest that CLP does not interact with monomeric actin at low ionic strength.

Figure 4 Intracellular localization of epitope-tagged CLP in transfected mammalian cells by immunofluorescence microscopy

CHO (A–C) or COS-7 (D–F) cells were transfected with plasmid constructs encoding HA-tagged CLP. Cells were fixed and labelled with anti-HA mouse monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG antibody (A and D). Rhodamine–phalloidin was used to stain F-actin (B and E). The yellow colour of merged images demonstrates the co-localization of CLP with F-actin (C and F).

CLP co-localizes with actin stress fibres *in vivo*

To determine if CLP interacts with F-actin *in vivo*, mammalian cells (CHO and COS-7 cells) were transfected with constructs expressing HA- or Myc-tagged CLP. At 16–24 h after transfection, the cells were fixed, dual-labelled for CLP and F-actin, and examined under a fluorescence microscope. F-actin staining with phalloidin showed rod-like structures typical of actin stress fibres (Figure 4). Labelling of CLP with anti-HA antibody showed concentration of CLP along these structures. The same observation was made with a Myc-tagged CLP (P. Provost, unpublished work). Merging of the CLP and F-actin labelling confirmed co-localization of CLP with the stress fibres. Other structures, such as the ruffles at the leading edge, were not labelled. This does not necessarily mean that these structures are devoid of CLP. For instance, since CLP is a small protein, it may be extracted from the loose actin network of the ruffles after the mild fixation necessary to preserve immunoreactivity.

CLP interaction with F-actin is pH-insensitive and Ca^{2+} -independent

The F-actin-binding properties of CLP were characterized in high-speed co-sedimentation assays based on the rapid pelleting of polymerized actin. The ability of CLP to interact with F-actin was first assayed using GST–CLP fusion protein. When incubated alone, most of the GST or GST–CLP fusion protein was found in the supernatant fraction (Figure 5A). However, when mixed with equimolar amounts of actin, GST–CLP, but not GST alone, co-sedimented with F-actin. In controls lacking MgCl_2 and KCl, most of the actin was found unpolymerized in the supernatant fraction after high-speed centrifugation (Figure 5B).

To exclude any influences of the GST moiety on F-actin binding, the same experiments were performed with purified CLP. As for the GST–CLP fusion protein, most of the purified CLP was found in the supernatant fraction in the absence of actin (Figure 5B). When mixed in equimolar amounts with actin,

approx. half of the CLP co-sedimented with F-actin. This CLP–F-actin binding was not affected by the addition of an equimolar concentration of BSA, which remained in the supernatant fraction. The CLP–F-actin association was not substantially altered by varying the pH from 6.5 to 8.0 (results not shown). The absence or presence of Ca^{2+} had no significant effect on the interaction of CLP with F-actin (results not shown). These data indicate that CLP is a Ca^{2+} -independent F-actin-binding protein.

Stoichiometry of CLP binding and chemical cross-linkage to actin filaments

In order to determine the stoichiometry of CLP binding to F-actin, we combined high-speed co-sedimentation with chemical cross-linking using the zero-length cross-linker EDC and sulphonyl-NHS. When incubated alone, actin was mainly found as F-actin in the pellet fraction and CLP in the supernatant fraction, in both in the absence and presence of the cross-linking agents (Figure 6A). The absence of covalently linked actin–actin complexes in the pellet indicated that the chemical cross-linking conditions were suitable.

When the actin–CLP mixture was incubated in the presence of cross-linkers, an additional, slower moving band was observed in the pellet, but not in the supernatant fraction. The appearance of this band coincided with a decrease in the actin band and the disappearance of the CLP band in the pellet fraction, and was not observed when actin or CLP were incubated alone, suggesting that the additional band contained a covalently linked actin–CLP complex. This was supported by the estimated molecular mass of the protein in the new band of approx. 60 kDa, and was confirmed by immunoblotting with anti-actin and anti-CLP antibodies. The additional band was immunoreactive with both anti-actin and anti-CLP antibodies (Figure 6B), indicating that one CLP molecule is cross-linked to one actin molecule. Bands of higher molecular mass were detected in the CLP–actin cross-linked sample analysed by immunoblotting using the anti-CLP

Figure 5 Characterization of the F-actin-binding properties of CLP in high-speed co-sedimentation assays

(A) GST (left-hand panel) or the GST-CLP fusion protein (right-hand panel) was incubated in the absence or presence of actin ($5 \mu\text{M}$ of each) in standard polymerization buffer [10 mM Mops (pH 7.0), 2 mM MgCl_2 , and 50 mM KCl]. (B) Left-hand panel: G-actin was incubated in 10 mM Mops (pH 7.0) with no additions (— Salts) or in standard polymerization buffer (+ Salts). Right-hand panel: Actin, CLP and BSA ($5 \mu\text{M}$ of each) were incubated in the combinations indicated in standard polymerization buffer. Samples were incubated for 2 h at 22°C and centrifuged at $100\,000 g$ for 1 h. Equivalent amounts of the pellet (P) and supernatant (S) fractions were analysed by SDS/PAGE, and the gels were stained with Coomassie Brilliant Blue.

antibody. These bands may represent higher-order CLP-actin complexes, which might also have been visible on actin blots after a more lengthy exposure. In addition, a band of approximately 32 kDa, which might represent a CLP dimer, became visible by immunoblot analysis using the anti-CLP antibody.

To determine the stoichiometry at which CLP binds to F-actin, we incubated increasing amounts of CLP with a fixed amount of actin, and determined the ratio at which binding became saturated. As shown in Figure 6(C), binding of CLP to F-actin increased with CLP concentration up to a ratio of CLP:actin subunits of approx. 1:2. A dissociation constant (K_d) of approx. $1.6 \mu\text{M}$ was calculated from a Scatchard analysis (Figure 6D). Together, these results indicate that CLP binds along the actin filaments in a 1:2 stoichiometry, but can be cross-linked to only one subunit of actin.

CLP has no effect on actin polymerization or depolymerization

To investigate the effects of CLP on actin polymerization, Acrylodan-labelled G-actin was incubated in the absence or presence of CLP in G buffer, and polymerization was initiated by the addition of MgCl_2 and KCl, as reflected by a gradual increase in fluorescence. The presence of increasing concentrations of CLP did not influence the polymerization of actin (results not shown). With no additions, i.e. under non-polymerizing conditions, the fluorescence was low and remained stable for the entire monitoring period of 120 min (results not shown).

Figure 6 Estimation of the stoichiometry and K_d of the CLP-F-actin interaction

G-actin and CLP ($5 \mu\text{M}$ of each) were incubated alone or in combination in standard polymerization buffer for 2 h at 22°C . (A) The incubation was continued for an additional 5 min in the absence (left-hand panel) or in the presence (right-hand panel) of the zero-length cross-linker EDC (4 mM) and sulpho-NHS (4 mM). After centrifugation at $100\,000 g$ for 1 h, the pellet (P) and supernatant (S) fractions of each sample were analysed by SDS/PAGE, and the gels were stained with Coomassie Brilliant Blue. (B) The presence of cross-linked F-actin-CLP complexes in the pellet fractions, or CLP alone in the supernatant fraction (last lane), was analysed by SDS/PAGE followed by either Coomassie Brilliant Blue (CB) staining or immunoblotting (IB) using anti-actin or anti-CLP antibodies. Molecular-mass markers (in kDa) are shown on the left. (C and D) Actin ($5 \mu\text{M}$) was incubated with increasing concentrations of CLP (from 0.25 to $20 \mu\text{M}$) in standard polymerization buffer for 2 h at 22°C . After centrifugation at $100\,000 g$ for 1 h, the pellet and supernatant fractions were analysed by SDS/PAGE, and the gels were stained with Coomassie Brilliant Blue. The protein bands were analysed by quantitative densitometry and the results expressed as (C) bound CLP/total actin versus unbound CLP, or as (D) bound CLP/unbound CLP versus bound CLP. Scatchard analysis was performed to obtain the K_d .

Figure 7 Characterization of the CLP(K75A) mutant protein

(A) Actin binding to GST fusion proteins. GST fusion proteins of CLP or CLP(K75A) (20 µg) were coupled to GSH–Sepharose 4B beads and incubated with 0, 2, 10 or 20 µg of F-actin. After elution with 10 mM GSH and centrifugation, the supernatant was analysed and compared with an actin control (first lane) by SDS/PAGE and immunoblotting (IB) with anti-actin antibody. Molecular-mass markers (in kDa) are shown on the left. (B) High-speed co-sedimentation assays. CLP (left-hand panel) or CLP(K75A) (right-hand panel) was incubated in the absence or presence of actin (5 µM of each) in standard polymerization buffer for 2 h at 22 °C. After centrifugation at 100 000 *g* for 1 h, equivalent amounts of the pellet (P) and supernatant (S) fractions were analysed by SDS/PAGE. Gels were stained with Coomassie Brilliant Blue. (C) CD spectra of wild-type and mutant CLP, acquired from 200 to 260 nm and corrected for buffer background. Mean residue ellipticity is in degrees × cm² × dmol⁻¹. (D) CLP and CLP(K75A) analysed by non-denaturing PAGE. The gel was stained with Coomassie Brilliant Blue.

We then tested whether CLP influences depolymerization of Acrylodan-labelled F-actin by rapid dilution. Under these conditions, actin depolymerizes and the decrease in fluorescence represents the depolymerization rate. No substantial differences could be detected between samples with or without equimolar amounts of CLP (results not shown). This indicates that, under the conditions used, CLP has no direct effect on actin depolymerization.

Involvement of Lys⁷⁵ in actin binding

Several studies implicated positively charged amino acids in the ability of a protein to bind acidic residues of actin [3]. Based on the similarity of CLP to other actin-binding proteins, two regions of CLP were previously targeted by site-directed mutagenesis: Lys¹³⁰–Lys¹³¹ in the C-terminal LKKAGG motif, and the central ⁷¹SKRSK⁷⁵ motif. When tested in the yeast two-hybrid

system, the CLP mutant K130A/K131A could still bind actin, whereas the K75A mutation abolished the actin-binding properties of CLP [13].

The importance of Lys⁷⁵ for the binding of CLP to F-actin was confirmed by GST binding assays and high-speed co-sedimentation experiments *in vitro*. As shown in Figure 7(A), a dose-dependent association of F-actin with purified GST–CLP was observed, but no binding of F-actin to GST–CLP(K75A) was detected. In high-speed co-sedimentation experiments, CLP was found together with F-actin in the pellet fraction (Figure 7B). Substitution of Lys⁷⁵ with alanine almost completely abolished the co-sedimentation, thereby confirming the crucial role of Lys⁷⁵ in the actin-binding of CLP.

Lys⁷⁵ contributes to the surface charge of the CLP protein

To ascertain that the lysine-to-alanine substitution at position 75 of CLP did not induce gross structural changes, the secondary structures of CLP and the CLP(K75A) mutant were evaluated by CD spectroscopy. As shown in Figure 7(C), the CD spectra of the CLP(K75A) mutant showed no significant changes compared with wild-type CLP. Using the K2d program [24], wild-type and mutant CLP were predicted to be composed of 9% α-helix, 37% β-sheet and 54% random coil. These results indicate that substitution of Lys⁷⁵ with alanine did not induce a gross perturbation of protein folding.

The migration of the CLP(K75A) mutant on non-denaturing PAGE was significantly changed. Wild-type CLP migrated as a sharp band towards the anode, indicating an overall negatively charged surface at pH 8.0 (Figure 7D). Substitution of the lysine residue with alanine significantly increased the mobility of the protein towards the anode, indicating a loss of positive surface charge. Together, these findings suggest that the positively charged lysine residue at position 75 is exposed on the surface of the native CLP protein.

DISCUSSION

The relative abundance of CLP mRNA and protein in myeloid cells is interesting in view of its ability to bind 5LO [14], which is found in the cytosol and nucleus of inflammatory cells [25]. However, CLP was only found in the cytoplasmic fraction. Furthermore, the broader distribution of CLP mRNA in various tissues suggests that CLP plays a more basic cellular role distinct from its interaction with 5LO. In fact, CLP interacted with β- and γ-actin in the two-hybrid system, and with skeletal-muscle actin *in vitro*, which is of the α type. CLP bound to stress fibres *in vivo*, in accord with a function as a new F-actin-binding protein in humans.

Given the sequence similarity between CLP and coactosin from *D. discoideum* [9] (33% identity and 75% similarity) (Figure 8), CLP probably represents the human homologue of coactosin, which belongs to the family of domain PD009482 proteins (ProDom release 2000.1) [27]. In fact, coactosin and CLP show similarities to the N-terminal domains of two other members of this family, actin-binding protein 1 (ABP1) and drebrin (Figure 8). These proteins bind to the sides of actin filaments, but not to G-actin [28,29]. ABP1 and drebrin differ from CLP and coactosin in that they possess a C-terminal regulatory Src homology 3 domain and coiled-coil repeat respectively [29,30]. EMBL (European Molecular Biology Laboratory) and EST (expressed sequence tag) databases revealed sequences encoding several other CLP-related proteins of unknown function, including *Drosophila melanogaster* CG6891 protein (Figure 8), as well as cDNA clones from mouse, chicken,

Figure 8 Sequence relationships of CLP

Alignment with the CG6891 gene product from *D. melanogaster*, coactosin from *Dictyostelium*, human drebrin F (residues 1–147) and ABP1 from *Saccharomyces cerevisiae* (residues 1–150). The conserved Lys⁷⁵ residue is marked by the black triangle (▼). The sequences were aligned with the Clustal W program (European Bioinformatics Institute) and shaded with the BOXSHADE program. Black shading indicates identity and grey shading indicates similarity. Amino acid identities (*) and conservative differences [: (amino acids share common structural features) or . (amino acids share less common structural features)] are indicated. A blank space means that the structural features of the aligned residues are different.

Mastigamoeba balamuthi and the plant *Oryza sativa*. CLP showed more than 41% identity and 77% similarity to the *Drosophila* gene product, which has 19 extra amino acids at the N-terminus. Thus CLPs appear to be widely expressed among different species, consistent with an important role as actin-binding proteins.

The hexapeptide sequence LKKAGG near the C-terminus of CLP is similar to the previously described LKKAET motif, which contains a lysine tandem directly implicated in actin binding of actobindin [4,5], villin [6], thymosin [7] and verprolin [8]. However, double alanine substitutions of the lysine residues of the LKKAGG motif in CLP had no effect on actin binding [14]. In addition, this motif appears not to be conserved in CLP-related sequences (Figure 8). The motif ¹²⁹LKKAGG¹³⁴ in CLP is involved instead in the binding to SLO [14].

In the present study, CLP was found to bind F-actin with a stoichiometry of approx. one CLP monomer to two actin subunits with a K_d in the micromolar range. The stoichiometry implies that CLP binds along the length of actin filaments. Almost all known F-actin-binding proteins interact with at least two subunits of an actin filament [31]. However, comitin has been reported to bind a single actin subunit in F-actin [32]. There is no evidence for CLP to interact with two subunits, as by zero-length chemical cross-linking a covalently linked 1:1 complex of actin and CLP was predominantly formed. The dissociation rate of F-actin was not significantly altered by the binding of CLP, which also argues against the possibility of CLP bridging two subunits in the actin filament. These data suggest that CLP binds to a surface of actin that is not involved in subunit contacts within the filament.

Our cross-linking data suggest that CLP molecules, free or bound to F-actin, do not effectively interact with each other, rendering it unlikely that CLP binds to F-actin as a dimer and acts as a cross-linker. In fact, no actin-filament bundling activity of CLP was detected in low-speed centrifugation assays (P. Provost and A. Stock, unpublished work). It appears that CLP does not interact with G-actin. Electrophoresis in non-denaturing

gels has provided no evidence for the formation of a complex, but in light of an estimated K_d of 1.6 μ M for the interaction of CLP with F-actin we cannot rule out a rapidly dissociating CLP-actin monomer complex.

The resistance of the CLP-actin interaction to pH 8.0 argues against an involvement of histidine residues with a pK_a below 7.0. In fact, histactophilin, a protein composed of almost 27% histidine residues, co-sediments with F-actin only at a pH below 7.2 [33]. The fact that Lys⁷⁵ is conserved in all of the CLP-related sequences shown in Figure 8 prompted us to investigate the central ⁷¹SKRSK⁷⁵ motif of CLP. In the two-hybrid system, alanine substitutions of all residues in the ⁷¹SKRSK⁷⁵ motif of CLP identified only Lys⁷⁵ as a potentially important residue [14]. Experiments *in vitro* confirmed these two-hybrid results. The suitability of our two-hybrid approach in combination with mutagenesis suggests that it provides an accelerated method for investigating interacting sites in actin-binding proteins, even if the interaction is only transient.

The observed increase in electrophoretic mobility of the CLP(K75A) protein towards the anode suggests that Lys⁷⁵ resides on the surface of CLP, creating a positively charged micro-environment that favours an electrostatic bond with an acidic site on the surface of actin. Together, these data indicate that Lys⁷⁵ plays a crucial role in the binding of actin by CLP.

The strong reduction or even loss of CLP function by substitution of one amino acid suggests that CLP has a single actin-binding site. In that respect, actin subdomain 1, in particular the acidic N-terminus of actin, comprising residues 1–12, is of interest. Actin subdomains 1 and 2 are favourite targets for F-actin-binding proteins [31]. Comitin binds a single actin subunit of F-actin via subdomain 1 [32]. Several proteins could be cross-linked to the extreme N-terminus of actin [4]. The complementary interfaces in these proteins mapped to regions rich in basic amino acid residues [3]. These data suggest that the N-terminus of actin, which protrudes from F-actin as a highly mobile unit [34], acts as a 'fishing-rod' in attracting positively charged surfaces of actin-binding proteins [3].

In summary, we have characterized CLP as a new human F-actin-binding protein and have shown that Lys⁷⁵ of CLP is essential for actin binding. This binding site of CLP differs from its site of interaction with 5LO [14], an enzyme involved in leukotriene biosynthesis [13]. Nevertheless, the 5LO-CLP and CLP-F-actin interactions appear to be mutually exclusive [14], opening the possibility that CLP binding to F-actin is regulated by 5LO in inflammatory cells.

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