5-Lipoxygenase interacts with Coactosin-Like Protein

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Running title: 5-Lipoxygenase interacts with CLP
SUMMARY

We have recently identified Coactosin-Like Protein (CLP) in a yeast two-hybrid screen using 5-lipoxygenase (5LO) as a bait. In this report, we demonstrate a direct interaction between 5LO and CLP. 5LO associated with CLP, expressed as a glutathione-S-transferase fusion protein, in a dose-dependent manner. Coimmunoprecipitation experiments using epitope-tagged 5LO and CLP proteins, transiently expressed in human embryonic kidney 293 cells, revealed the presence of CLP in 5LO immunoprecipitates. In reciprocal experiments, 5LO was detected in CLP immunoprecipitates. Non-denaturing polyacrylamide gel electrophoresis and cross-linking experiments showed that 5LO binds CLP in a 1:1 molar stoichiometry, in a Ca\(^{2+}\)-independent manner. Site-directed mutagenesis suggested an important role for lysine 131 of CLP in mediating 5LO binding. In view of the ability of CLP to bind 5LO and filamentous actin (F-actin), we determined whether CLP could physically link 5LO to actin filaments. However, no F-actin-CLP-5LO ternary complex was observed. In contrast, 5LO appeared to compete with F-actin for the binding of CLP. Moreover, 5LO was found to interfere with actin polymerization. Our results indicate that the 5LO-CLP and CLP-F-actin interactions are mutually exclusive, and suggest a modulatory role for 5LO in actin dynamics.
INTRODUCTION

5-Lipoxygenase (5LO) is of central importance in cellular leukotriene (LT) synthesis. This enzyme converts arachidonic acid, released from the membranes by the cytosolic phospholipase A₂, into 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), and subsequently into the epoxide intermediate LTA₄ (1). LTA₄ is further metabolized into LTB₄ by the LTA₄ hydrolase, or into LTC₄ through the action of the LTC₄ synthase. LTC₄ is then sequentially degraded into LTD₄ and LTE₄. Whereas LTB₄ exerts potent stimulatory effects on various leukocyte functions, including chemotaxis, adhesion, degranulation and aggregation, the cysteinyl-LTs (LTC₄, LTD₄ and LTE₄) are known to contract airway smooth muscle, increase vascular permeability, and promote mucus secretion (2). 5LO and LTs are, therefore, key components involved in inflammatory disorders, including arthritis, asthma and allergic reactions.

Recently, novel modulatory mechanisms determining cellular 5LO activity were identified. 5LO is phosphorylated by p38 kinase-activated MAPKAP kinases prepared from stimulated myeloid cells (3). In addition, Mg²⁺ increases 5LO activity in vitro (4). Furthermore, a stimulatory Ca²⁺ binding site has been localized in the N-terminal domain of 5LO, which may function as a C2 domain in the calcium regulation of 5LO catalytic activity (5). C2 domains have also been shown to mediate protein-protein interactions (6).

Additional lines of evidence indicate that cellular 5LO activity and distribution is regulated by interaction with other proteins. For example, the subcellular distribution of 5LO differs among cell types and changes in response to various stimuli. In particular, 5LO translocates to the nuclear membrane from either the cytosol (for polymorphonuclear leukocytes) (7) or from inside the nucleus (for alveolar macrophages) (8). In that perspective, an association of 5LO with cytoskeletal structures in vivo, which is a reasonable possibility after demonstration of a direct association between 5LO and actin in vitro (9), could have important implications for translocation and modulation of cellular 5LO activity.

In our attempt to determine the protein partners of 5LO using the yeast two-hybrid system, we identified coactosin-like protein (CLP) as a potential 5LO-interacting protein (10). The CLP nucleotide sequence was initially found as a sequence flanking a deletion on
chromosome 17 characterizing the Smith-Magenis syndrome (11). In a separate paper, we have characterized CLP as a human filamentous actin (F-actin)-binding protein. Here, we characterize the CLP-5LO interaction and investigate whether 5LO or the CLP-5LO tandem is recruited by F-actin.
EXPERIMENTAL PROCEDURES

Cloning, Expression and Purification of CLP and 5LO. The human CLP cDNA was obtained by screening of a human lung cDNA library using 5LO as a bait (10). The CLP cDNA was amplified by PCR, and cloned in frame into the BamHI/XhoI sites of pGEX-5X-1 vector (Amersham Pharmacia Biotech). The pGEX-5X-1-CLP construct was also subjected to site-directed mutagenesis, as described in the "Site-Directed Mutagenesis" section, to generate the CLP mutant K131A.

The pGEX-5X-1-CLP and pGEX-5X-1-CLP K131A constructs, as well as the empty pGEX-5X-1 vector, were transformed into the E. coli bacterial strain BL21 (Amersham Pharmacia Biotech) for expression and purification of the GST-CLP fusion proteins or GST only, according to the manufacturer’s instructions. In some experiments, the GST-CLP proteins were eluted from the Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) with 4 volumes of glutathione (10 mM) in 50 mM Tris-HCl pH 8.0 (elution buffer). The CLP proteins were then cleaved from the GST moiety with factor Xa (Amersham Pharmacia Biotech) and purified by anion exchange chromatography.

The E. coli bacterial strain MV1190 was transformed with the plasmid pT3-5LO, and used for expression of 5LO, as described previously (12). The 5LO protein was purified by affinity chromatography using an ATP-agarose column (12).

Protein Determination. The protein concentrations were determined by the method of Bradford (13) using the Bio-Rad dye reagent, with bovine serum albumin (BSA) as standard.

GST Binding Assays. For binding studies in vitro, 20 µg of the GST-CLP or GST-CLP K131A fusion protein, or GST alone, linked to Glutathione Sepharose 4B beads, was incubated with 0, 0.05, 0.25 and 1 µg purified 5LO and/or 0, 2, 10 and 20 µg F-actin in the presence of BSA (50 µg), in 200 µl of buffer A (2 mM Tris-Cl, 0.2 mM ATP, 0.2 mM CaCl₂ and 0.5 mM β-mercaptoethanol, pH 8.0) supplemented with 2 mM MgCl₂ and 50 mM KCl. The effect of EGTA on 5LO-CLP complex formation was examined in buffer A supplemented with 50 mM KCl. After a 30-min incubation at room temperature, the beads were washed five times in
incubation buffer, and the GST-CLP fusion proteins or GST-bound 5LO and/or F-actin complex was eluted with glutathione elution buffer (10 mM glutathione in 50 mM Tris-Cl, pH 8.0). The beads were sedimented by centrifugation and the supernatant was mixed with SDS sample buffer, and boiled. The presence of the interacting 5LO and/or actin protein in the supernatant was assayed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblot analysis.

**Mammalian Cell Culture and Transfections.** Human embryonic kidney 293 (HEK 293) cells were grown in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified incubator under 5% CO₂ at 37°C. For immunoprecipitation experiments, the 5LO cDNA, preceded by a Kozak consensus sequence (14) and a FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys), was cloned into the pcDNA3.1 mammalian expression vector (Invitrogen), whereas the CLP cDNA was cloned into the pcDNA3.1/MycHis expression vector (Invitrogen), which introduced a Myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) at the carboxy terminus. Plasmid DNAs were prepared using the QIAGEN Plasmid DNA purification kit (Qiagen), precipitated with ethanol and resuspended in water. The HEK 293 cells cultured in 100-mm tissue culture dishes were transiently transfected with 10 µg of each plasmid by the calcium phosphate method. In the case of transfection with a single plasmid, the total amount of DNA was adjusted to 20 µg with empty vector.

**Immunoprecipitation Experiments.** The HEK 293 cells were harvested 40 hours post-transfection, washed twice with ice-cold PBS, and solubilized in 1 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP-40) supplemented with Complete Protease Inhibitor Mix (Boehringer Mannheim). Total lysates were cleared by centrifugation at 15,000 g for 15 min at 4°C. One-tenth of each cell lysate was kept for protein determination and analysis by immunoblotting to verify 5LO and CLP protein expression. Nine-tenth of each cell lysate was incubated with either anti-Myc mAb (9E10) (5 µg/ml; Santa Cruz Biotechnology), anti-FLAG M2 mAb (10 µg/ml; Sigma), or normal mouse
IgG (5 or 10 µg/ml; Santa Cruz Biotechnology) under continuous rotation for 4 hours at 4°C. GammaBind Plus Sepharose (Amersham Pharmacia Biotech) was then added, and the incubation continued for an additional hour. The immune complexes were washed four times with 1 ml lysis buffer, and eluted by boiling for 7 min in Laemmli sample buffer. After centrifugation, the supernatants were analyzed by immunoblotting, as described below.

**Non-Denaturing PAGE.** Interaction between 5LO and CLP was examined by non-denaturing PAGE, as described by Safer et al. (15). 5LO and CLP were incubated, alone or in combination, at the indicated concentrations in buffer A (2 mM Tris, 0.2 mM ATP, 0.2 mM CaCl₂ and 0.5 mM β-mercaptoethanol, pH 8.0) for 15 min at room temperature. LTA₄ hydrolase was used as a non-interacting protein control. The samples were then mixed with native sample buffer, loaded immediately and run at 20 mA constant current at 4°C. Proteins were then either stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes for immunoblot analysis.

After non-denaturing PAGE of the combination of CLP or CLP K131A and 5LO, the lane was cut out and incubated in SDS sample buffer at room temperature for 20 min with gentle shaking. The lane was then inserted on top of a second dimensional SDS-PAGE gel, and the electrophoresis was performed in the presence of SDS (0.1%) in the running buffer. The gels were stained with Coomassie Blue.

**Chemical Cross-Linking Experiments.** Purified CLP (5 µM) and 5LO (1 µM) proteins were mixed and incubated for 2 h at room temperature in buffer B (10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0 containing 2 mM MgCl₂ and 50 mM KCl). The reaction mixtures were cross-linked by addition of 4 mM of the zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Pierce) and 4 mM N-hydroxysulfosuccinimide (sulfo-NHS) (Pierce). After incubation at room temperature, the reactions were quenched by addition of 20 mM Tris, and the cross-linked samples were boiled with SDS sample buffer. The presence of cross-linked CLP-5LO complexes was analyzed on SDS-PAGE gels, and either stained with Coomassie Blue or analyzed by immunoblotting.
F-Actin Binding Assay. For high-speed cosedimentation assays, actin was purified from rabbit skeletal muscle as described by Rees and Young (16). The GST-CLP fusion, CLP and 5LO proteins were purified as described above. After clearing the protein solutions at 100,000 g for 30 min at 4°C, the reaction mixtures were prepared and incubated for 2 h at room temperature in Beckman centrifuge tubes in the standard polymerization buffer B (10 mM MOPS, pH 7.0 containing 2 mM MgCl₂ and 50 mM KCl). In some experiments, the reaction mixtures were supplemented with increasing concentrations of KCl. The samples (100 µl) were then centrifuged at 100,000 g for 1 h at 4°C in a Beckman Ultracentrifuge. The pellet and supernatant of each sample were collected, and boiled with SDS sample buffer. The proteins in the pellet and supernatant fractions were resolved by SDS-PAGE, and the gels were stained with Coomassie Blue.

Actin Polymerization Assay. Actin was specifically labeled at a cysteine residue using Acrylodan (Molecular Probes), a thiol reactive adduct of Prodan (17), as described by Marriott et al. (18). G-actin-Acrylodan was used in a molar ratio of 1:7 with unlabeled G-actin for actin polymerization assays. Neither the rate nor the extent of polymerization differs significantly between the native and fluorescent actin (18). The polymerization of G-actin and G-actin-Acrylodan (2 µM) in buffer A, in the presence of 5LO, was initiated by the addition of MgCl₂ and KCl at final concentrations of 2 mM and 50 mM, respectively. In some experiments, BSA was included or salts were omitted in the assay mixtures. 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 (4-nm bandwidth), and the emission wavelength was collected through a monochromator at 465 nm (4-nm bandwidth).

Immunoblot Analysis. Protein suspensions or cell lysates were fractionated by SDS-PAGE using the Mini Protean system (Bio-Rad), transferred to nitrocellulose membranes (Amersham), and immunoblotted as described previously (10). A polyclonal anti-human CLP antiserum was raised in rabbits against amino acids 116-130 of human CLP. Monoclonal anti-
Myc antibody was from Santa Cruz Biotechnologies, anti-actin (clone AC-40) antibody was from Sigma, and rabbit polyclonal 5LO antibody 1551 was used after immunopurification. Immunoreactive proteins were visualized by using alkaline phosphatase (AP) conjugates and substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate).

**Yeast two-hybrid system.** The Gal4 DNA binding domain (BD) vector pGBT9, carrying the TRP1 gene, and the Gal4 DNA activating domain (AD) vector pACT2, carrying the LEU2 gene, were obtained from Clontech. To construct the vector pGBT9-CLP, the CLP cDNA insert generated by PCR as a BamHI/XhoI fragment and used for the pGEX-5X-1-CLP construct, as described above, was cloned in frame into the BamHI/SalI restriction sites of pGBT9. To construct the vector pACT2-5LO, a short EcoRI/KpnI fragment of the 5LO cDNA from pT3-5LO (19) was amplified by PCR, cloned into the EcoRI/KpnI sites of the construct pAS2-1-5LO, [produced by the ligation of the 5LO cDNA from pT3-5LO into the EcoRI/SalI sites of pAS2-1 (Clontech)], and the 5-lipoxygenase cDNA was cut out as a EcoRI/SalI fragment, and then cloned in frame into the EcoRI/XhoI sites of the vector pACT2 (Clontech). The constructs pACT2-ß-actin and pACT2-γ-actin were obtained by screening of a human lung cDNA library using human CLP as a bait2. The constructs pGBT9-SNF1 and pACT2-SNF4, used as controls, were described previously (10). The yeast strain PJ69-4A (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (20) was used to assay protein-protein interaction, as previously described (10).

**Site-Directed Mutagenesis.** Site-directed mutagenesis of the lysine tandem $^{130}$KK$^{131}$ of CLP, cloned in pGBT9, was performed by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer’s instructions. The desired mutations were introduced with mutagenic primers, designed with the help of The Primer Generator (21), that created a unique SstI, AflII, PstI or a new BssHII restriction site in the $^{130}$KK$^{131}$ motif. The conserved $^{71}$SKRSK$^{75}$ motif of CLP was subjected to site-directed mutagenesis by sequential PCR (22). The desired mutations were introduced with mutagenic primers that disabled a unique AvaII restriction site present in the $^{71}$SKRSK$^{75}$ motif. The presence of the mutations was
verified by restriction analysis and confirmed by sequencing the complete CLP cDNA insert using the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech) at the KISeq core facilities (Karolinska Institute, Stockholm, Sweden).

**Circular Dichroism Measurements and Analysis.** An AVIV 62DS circular dichroism spectrometer was used to measure the ellipticity of samples of purified CLP and CLP K131A at 50 µg/ml in 10 mM sodium phosphate, pH 7.0, using a 1-cm path length cell thermostated 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 interval, and averaged; the buffer background was then substrated. Measured ellipticities in millidegrees were converted into mean residue ellipticity units and expressed as degrees x cm$^2$ x dmol$^{-1}$. The fractional composition of the secondary structure of CLP and CLP K131A in terms of alpha-helix, beta-sheet and random coil was analyzed using the program K2d (available at: http://www.embl-heidelberg.de/~andrade/k2d.html) (23).
RESULTS

5LO and CLP interact directly in vitro. To study the interaction between 5LO and CLP in vitro, GST binding experiments were performed. The GST-CLP fusion protein, or GST alone, was expressed in bacteria, coupled to Glutathione Sepharose 4B beads and incubated with 0, 0.25, 1.25 and 5.0 µg/ml of purified 5LO. After a 30-min incubation, the beads were washed and the protein complexes eluted with glutathione. The presence of 5LO in the eluate was assayed by SDS-PAGE followed by immunoblot analysis using anti-5LO antibody. As shown in Fig. 1, no binding of 5LO to the beads coupled to GST alone was detected. However, a dose-dependent association of 5LO to GST-CLP was observed, indicating that CLP and 5LO interact directly with each other.

5LO associates with CLP in mammalian cells. For coimmunoprecipitation experiments, HEK 293 cells were transfected with expression plasmids producing a FLAG epitope-tagged 5LO and a Myc epitope-tagged CLP protein. No CLP (data not shown) or 5LO protein was found in cells transfected with empty vector only, and no immunoreactivity to anti-Myc or anti-5LO antibody was observed (Fig. 2A). Transfection of the HEK 293 cells induced the expression of FLAG-tagged 5LO (Fig. 2A) with enzymatic activity similar to that obtained after transfection with non-tagged 5LO (data not shown). Immunoblots also showed CLP expression in cells transfected with the Myc-tagged CLP construct; the presence of the Myc tag slightly decreasing its electrophoretic mobility as compared to wild-type CLP. A lysate fraction of transfected cells was immunoprecipitated by anti-Myc antibody and subjected to SDS-PAGE. Immunoblotting with the anti-5LO antibody revealed that 5LO was coimmunoprecipitated with CLP (Fig. 2B). In the reciprocal experiment with anti-FLAG antibody to immunoprecipitate 5LO, followed by immunoblotting with anti-Myc, CLP coimmunoprecipitated with 5LO (Fig. 2C). No 5LO or CLP was detected in immunoprecipitates prepared with normal mouse IgG (Fig. 2B and 2C). These findings indicate that 5LO forms a complex with CLP in mammalian cells.
5LO specifically interacts with CLP in a Ca\textsuperscript{2+}-independent manner. The 5LO-CLP interaction was also investigated using non-denaturing PAGE. On this gel, the rate of migration is determined by the ratio of charge to volume of a protein. Under the conditions used, purified 5LO migrated as a smeary band with a low electrophoretic mobility and remained mainly on the top of the gel (Fig. 3A). CLP had a greater electrophoretic mobility and migrated as a sharp band in the middle part of the gel. LTA\textsubscript{4} hydrolase, used as a non-binding control, migrated a little further than CLP, but as a doublet. When 5LO or CLP was incubated with LTA\textsubscript{4} hydrolase, no additional bands or changes in the migration patterns or intensity of the bands was observed. However, when equimolar amounts of CLP and 5LO were mixed, the CLP band disappeared and the intense 5LO band was slightly shifted upwards. In the presence of EGTA, the upward shift remained discernible, although the electrophoretic mobility of free 5LO was increased.

To confirm that the upward shift observed when CLP was incubated with 5LO is the result of CLP-5LO complex formation, non-denaturing PAGE was followed by either immunoblotting or SDS-PAGE as a second dimensional electrophoresis. Immunoblotting of the non-denaturing polyacrylamide gels with anti-5LO and anti-CLP antibody indicated the presence of CLP in the 5LO band (Fig. 3B). As shown in Fig. 3C, analysis of the incubation mixtures by a second dimensional SDS-PAGE confirmed the localization of CLP in the 5LO band. The effect of EGTA on 5LO-CLP complex formation was further investigated in GST binding experiments. The GST-CLP fusion protein coupled to Glutathione Sepharose 4B beads were incubated with increasing amounts of purified 5LO in the presence of calcium (0.2 mM), or, in the absence of calcium (with EGTA 1 mM). As illustrated in Fig. 3D, the presence of EGTA had no influence on 5LO binding to CLP. Taken together, these results indicate an interaction between 5LO and CLP that is Ca\textsuperscript{2+}-independent.

5LO binds CLP in a 1:1 molar stoichiometry. The stoichiometry of the 5LO-CLP complex was determined by titration binding assays on non-denaturing PAGE. In these assays, a known amount of 5LO was incubated with increasing amounts of CLP, and vice-versa, and the ratio at which unbound CLP appeared or disappeared was determined. When a fixed amount
of CLP was titrated by increasing amounts of 5LO, the intensity of the CLP band was gradually decreased and disappeared at a molar ratio of 1:1 or greater (Fig. 4A). When a fixed amount of 5LO was titrated by increasing amounts of CLP, the CLP band became apparent at a molar ratio greater than 1:1 (Fig. 4B).

In these non-denaturing PAGE experiments, the formation of multiple equimolar complexes between 5LO and CLP, such as 2:2, 3:3 or higher can not be ruled out. Therefore, the composition of the CLP-5LO interacting complex was investigated in chemical cross-linking experiments. CLP and 5LO were incubated, alone or in combination, in the absence or presence of the zero-length cross-linker EDC and sulfo-NHS, and the samples analyzed by SDS-PAGE and Coomassie Blue staining. In the absence of cross-linking agents, CLP and 5LO migrated as sharp bands at their expected molecular mass (Fig. 4C). No additional band was observed when CLP or 5LO alone were incubated with EDC/sulfo-NHS for 5 min. However, when CLP was incubated with 5LO in the presence of EDC/sulfo-NHS for 5 min, a new band of higher molecular mass appeared on the SDS-PAGE gels (Fig. 4C). A time-course experiment showed increasing amounts of this high molecular weight species with time (Fig. 4D).

To ascertain that the newly formed high molecular mass band represents a covalently linked 5LO-CLP complex, immunoblot analysis using anti-5LO and anti-CLP antibody was performed. As shown in Fig. 4E, this new band observed on Coomassie Blue stained gels was immunoreactive with both anti-5LO and anti-CLP antibodies and migrated at the expected molecular mass for an equimolar 5LO-CLP (1:1) complex. These results indicate that the 5LO-CLP protein complex is composed of one molecule of 5LO bound to one molecule of CLP.

**Role for lysine 131 of CLP in 5LO binding.** In order to identify amino acid residues of CLP involved in the binding of 5LO, the yeast two-hybrid system was used. The yeast was cotransformed with 5LO and various CLP mutant constructs. The entire CLP cDNA insert of at least one clone per mutated CLP construct was sequenced to confirm the presence of the intended mutation and the absence of unintended changes. β-Actin and γ-actin were used as positive controls, and SNF4 as a non-interacting protein control.
All the CLP mutants were able to bind 5LO, except one: the CLP mutant construct K130A/K131A. This latter CLP mutant interacted with actin, but not with 5LO (Table 1). When testing single-point mutants of CLP, we found that the mutant K130A could still interact with 5LO, whereas the alanine mutation of lysine 131 prevented 5LO binding. Similar results were obtained when lysine 131 was replaced by glutamine. Substitution of lysine 131 by arginine maintained the binding of CLP to 5LO, indicating the requirement of a basic amino acid residue at position 131.

Based on the observation that a basic amino acid residue is involved in 5LO binding, another region of CLP containing basic residues was targeted by site-directed mutagenesis: the central 71SKRSK75 motif, in which three of the five amino acids are basic residues. Individual substitution of all the residues of the 71SKRSK75 motif by alanine did not affect the 5LO-binding of CLP.

**Analysis of the CLP K131A mutant protein in vitro.** In order to confirm the two-hybrid results, the importance of lysine 131 of CLP in 5LO binding was tested in GST binding experiments and in two-dimensional gel electrophoresis. For the GST binding assays, the GST-CLP or GST-CLP K131A fusion protein was expressed in bacteria, coupled to Glutathione Sepharose 4B beads and incubated with increasing amounts of purified 5LO. The dose-dependent association of 5LO with CLP was markedly reduced by substituting lysine 131 of CLP by an alanine residue (Fig. 5A).

To exclude any contribution of the GST moiety, the interaction between purified CLP K131 mutant protein and 5LO was analyzed by non-denaturing PAGE. When CLP K131A and 5LO were mixed, no additional bands or other changes in the migration patterns relative to the individual proteins were observed (Fig. 5B, left panel). In particular, no upward shift of the 5LO band, suggestive of a protein interaction, was discernible. Subsequent analysis of the CLP K131A-5LO incubation mixtures by a second-dimensional SDS-PAGE did not show colocalization of the two proteins (Fig. 5B, right panel), thereby confirming the lack of interaction between the CLP K131A mutant and 5LO. Together, these observations support an important role for lysine 131 in 5LO binding.
The secondary structure of CLP, as well as that of the CLP K131A mutant, was evaluated by circular dichroism spectroscopy, and fractional composition in terms of alpha-helix, beta-sheet and random coil was estimated. As shown in Fig. 5C, the CD spectra of the CLP K131A mutant was slightly different from wild-type CLP. Using the program K2d (23), CLP was predicted to be composed of 9% alpha-helix, 37% beta-sheet and 54% random coil, whereas the CLP K131A mutant was found to be composed of 8% alpha-helix, 44% beta-sheet and 48% random coil. These results indicate that substitution of lysine 131 by alanine does not prevent folding of the CLP protein, but induces a subtle change in the secondary structure that might impair the binding of 5LO.

5LO interferes with the binding of CLP to F-actin. A study by Lepley and Fitzpatrick (1994) (9) reported that 5LO interacts with actin in vitro. In view of the ability of CLP to bind 5LO (this study), as well as F-actin, it was of interest to determine whether CLP provides a link between F-actin and 5LO.

This issue was first examined in GST binding experiments. The GST-CLP fusion protein was coupled to Glutathione Sepharose 4B beads and incubated with various concentrations of F-actin and/or 5LO. Whereas F-actin and 5LO individually and dose-dependently interacted with CLP, the binding of F-actin to CLP was gradually diminished by increasing concentrations of 5LO (Fig. 6A).

Since the beads could have physically interfered with the formation of an F-actin-CLP-5LO ternary complex, cosedimentation assays using purified proteins were performed. In these assays, 5LO was incubated at increasing concentrations with G-actin, in the presence or absence of CLP, and actin polymerization was induced by the addition of KCl and MgCl₂. After ultracentrifugation, the F-actin-containing pellet and the supernatant fraction containing the non-pelletable actin were analyzed for the presence of 5LO and CLP by SDS-PAGE and Coomassie Blue staining. As shown in Fig. 6B, inclusion of CLP in the incubation mixtures did not mediate 5LO binding to F-actin. As in the GST binding assays, the absence of an F-actin-CLP-5LO ternary complex in these experiments rather unveiled a mutually exclusive relationship. Binding of CLP to F-actin was observed in the absence or at low concentrations of 5LO. This
CLP-F-actin association was reduced by equimolar amounts of 5LO (Fig. 6B). In fact, under these conditions, a 5LO-CLP complex, but not a ternary F-actin-CLP-5LO complex, was detected by chemical cross-linking (data not shown).

5LO inhibits actin polymerization. In cosedimentation assays performed in the absence of CLP, no significant association of 5LO with F-actin was found (Fig. 7A). Instead, 5LO caused an increase in non-pelletable actin, with a maximal effect at 1 µM. This effect of 5LO on actin was not influenced by a 5-fold molar excess of BSA relative to 5LO, and was not mimicked by the 5LO buffer only (Fig. 7B).

The observation that 5LO increased the amount of non-pelletable actin in cosedimentation assays was further investigated in a fluorescence-based actin polymerization assay. The polymerization of Acrylodan-labeled G-actin, incubated with increasing concentrations of 5LO, was initiated by the addition of KCl and MgCl₂ and the progress of the polymerization reaction was monitored. As shown in Fig. 7C, polymerization conditions induced in the control a rapid and gradual increase in fluorescence. The addition of 5LO reduced the final extent but not the initial rate of actin polymerization; the degree of inhibition reaching about 50% at a 5LO concentration of 1 µM. 5LO buffer alone had no effect on actin polymerization (data not shown). In a control not subjected to polymerization conditions, a stable fluorescence signal was observed (dotted line in Fig. 7C). To rule out the possibility that the inhibitory effect of 5LO is due to a nonspecific interaction with other proteins, experiments were performed in the presence of 2 µM BSA. Although BSA was in an 11-fold molar excess relative to 5LO, it did not influence the degree of inhibition; 175 nM 5LO inhibited by about 40% in the presence and absence of BSA (Fig. 7D), demonstrating that this effect was specific to the interaction of 5LO with actin.
DISCUSSION

The cellular activity and localization of 5LO may depend on its physical interaction with other proteins. In order to identify interacting proteins, we have used a two-hybrid approach (10). One of the proteins discovered by this approach, CLP, shows homology to coactosin, an actin-binding protein from *Dictyostelium discoideum* (24). CLP has recently been characterized as a human F-actin-binding protein\(^2\). In the present study, we show that 5LO interacts directly with CLP in vitro and in vivo. In vitro the interaction resulted in a heterodimeric complex. The formation of this complex proved to be independent of Ca\(^{2+}\), different from the membrane association of 5LO, which is promoted by Ca\(^{2+}\). We have recently shown that binding of Ca\(^{2+}\) to the N-terminal domain of 5LO stimulates the activity of this enzyme (5). Neither Ca\(^{2+}\)-dependent activation nor the membrane association of 5LO seems to involve CLP. In fact, CLP has no direct effect on 5LO activity when purified proteins were mixed in vitro (data not shown).

In a search for amino acid residues involved in the actin binding of CLP we found one mutant, CLP K131A, with a strongly reduced capacity to interact with 5LO. The substitution of lysine 131 by arginine preserved 5LO binding, indicating the requirement of a basic, positively charged amino acid residue at position 131. The fact that the CLP K131A mutant retained its ability to bind actin indicates that its overall secondary structure was not drastically affected. In accord, the replacement of lysine 131 by alanine only slightly changed the circular dichroism spectrum. Lysine 131 thus appears to be part of the surface interacting with 5LO or to be required in particular for maintaining the conformation of CLP necessary for 5LO binding.

The regulation of actin polymerization and depolymerization, and the localized assembly of filamentous actin into a network within the cortex of polymorphonuclear leukocytes, is essential for generating the force necessary for leukocyte locomotion, shape change, phagocytosis, adhesion, and spreading (25, 26). A connection between 5LO and actin, as reported previously (9), is therefore of considerable interest. It raises the possibility that actin is involved in the intracellular translocation of 5LO, which concomitantly with LT biosynthesis follows activation of the cells (for review, see reference 27). There is evidence that the activity of 5LO is influenced by the state of actin in vivo; an upregulation of fMLP-induced leukotriene generation in polymorphonuclear leukocytes is observed when the cells were treated with...
cytochalasin B (28, 29), which interferes with actin filament formation. In turn, the catalytic products of 5LO appear to affect the actin system. For example, monohydroxy acids, including the 5LO derivate 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), are capable of binding cytosolic actin (30). LTB$_4$ induces oscillatory actin polymerization/depolymerization in polymorphonuclear leukocytes (31, 32), and LTD$_4$ triggers reorganization of the actin network in intestinal epithelial cells (33). Furthermore, leukotrienes have been implicated with the formation of stress fibers in endothelial cells subjected to mechanical stretching (34).

In our experiments, 5LO inhibited the polymerization of actin in vitro. This inhibitory effect was observed in both cosedimentation and actin polymerization assays. 5LO exerted this effect at submicromolar concentrations, in accord with the estimated concentration of monomeric actin (~ 0.1-0.2 µM) in equilibrium with polymerized actin (35, 36). These data are compatible with the possibility that 5LO inhibits actin polymerization by the sequestration of G-actin. On the other hand, the possibility remains that 5LO interferes with polymerization by the severing or capping of actin filaments, as for instance gelsolin does (37). In our actin polymerization and cosedimentation assays, which provide no evidence for the incorporation of a putative actin-5LO complex into actin, the binding of 5LO to one or the other end of actin filaments would have escaped detection. In fact, the finding that inhibition of actin polymerization by 5LO is not complete and saturates half-way (Fig. 7C), is most easily explained by assuming that 5LO caps the plus ends of actin filaments, thereby raising the critical concentration of G-actin.

An indirect relationship of 5LO to the actin system is established by the binding of 5LO to CLP. The question has been addressed whether CLP, 5LO, and actin form a ternary complex. The mutational analysis of CLP indicated that lysine 131 is important for the binding of 5LO but not for the binding of actin. On the contrary, lysine 75 is critical for actin binding but not for the interaction with 5LO$^2$. Based on these data the two binding sites on CLP appear to be distinct. However, they may be overlapping, or simultaneous binding of 5LO (78 kDa) and actin (42 kDa) to the small CLP molecule (16 kDa) may suffer from steric hindrance. Our attempts to identify a ternary complex of 5LO, CLP, and filamentous actin were unsuccessful. Data rather suggest that 5LO prevents CLP from the interaction with actin. This is relevant since
preliminary experiments suggest that CLP might antagonize the activity of gelsolin as a capper of the plus ends of actin-filaments (data not shown), a function reported for coactosin, which interfered with the actin-capping activity of fragment S1 of severin, a gelsolin-related protein in Dictyostelium (38). In the presence of CLP and gelsolin, 5LO may restore the capping activity by sequestering CLP. Thus, one could speculate that 5LO may inhibit the polymerization of actin in two ways: by maintaining the activity of capping proteins in the presence of CLP and by acting itself as an inhibitor of actin polymerization. Considering the role of 5LO in vivo, the caveat has to be taken into account that modification of the proteins involved, such as phosphorylation (3, 39), or the presence of cofactors may turn the inhibitory effect of 5LO into a supporting one.

In conclusion, our results demonstrate that 5LO directly interacts with CLP. We also show that 5LO inhibits actin polymerization and interferes with the binding of CLP to F-actin. It may be hypothesized that 5LO, in addition to its key role in leukotriene synthesis, modulates the actin dynamics in inflammatory cells, thus representing a novel regulator of actin function.
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FOOTNOTES

1 The abbreviations used are: AD, activating domain; BD, binding domain; BSA, bovine serum albumin; CLP, coactosin-like protein; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; F-actin, filamentous actin; G-actin, globular actin; GST, glutathione-S-transferase; HEK 293 cells, human embryonic kidney 293 cells; LT, leukotriene; 5LO, 5-lipoxygenase; MOPS, 3-(N-morpholino)propanesulfonic acid; NHS, N-hydroxysulfosuccinimide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

2 P. Provost, J. Doucet, A. Stock, G. Gerisch, O. Rådmark, and B. Samuelsson, submitted for publication.
FIGURE LEGENDS

Figure 1. **5LO interacts directly with CLP in vitro.** Twenty micrograms of the GST-CLP fusion protein, or GST alone, coupled to Glutathione Sepharose 4B beads were incubated with 0 to 1.0 µg of purified 5LO. After elution with 10 mM glutathione and centrifugation, the supernatant was analyzed and compared with a reference of 5LO (first lane) by SDS-PAGE and immunoblotting with anti-5LO antibody.

Figure 2. **5LO coimmunoprecipitates with CLP.** Epitope-tagged FLAG-5LO and Myc-CLP were expressed in HEK 293 cells. A, the epitope-tagged Myc-CLP (upper panel) and FLAG-5LO (lower panel) proteins in the cell lysates (10 µg proteins) were detected by immunoblot analysis with anti-Myc and anti-5LO antibodies, respectively. B, CLP was immunoprecipitated with anti-Myc antibody (IP anti-Myc) or normal mouse IgG (last lane). 5LO in the immunoprecipitates was detected by immunoblot analysis with anti-5LO (IB anti-5LO). C, 5LO was immunoprecipitated with anti-FLAG M2 antibody (IP anti-FLAG) or normal mouse IgG (last lane). Myc-CLP in the immunoprecipitates was detected by immunoblot analysis with anti-Myc antibody (IB anti-Myc).

Figure 3. **5LO interacts with CLP in a Ca\(^{2+}\)-independent manner.** A, non-denaturing PAGE followed by Coomassie Blue staining. 5LO, CLP and LTA\(_4\) hydrolase (7.5 µM each) were incubated, alone or in combination, in the presence of CaCl\(_2\) (0.5 mM), or, in absence of Ca\(^{2+}\) (with EGTA 1 mM) (last three lanes). LTA\(_4\) hydrolase was used as a non-interacting protein control. B, non-denaturing PAGE followed by Coomassie Blue staining (CB), or by immunoblotting using anti-5LO or anti-CLP antibody, as indicated. CLP, 5LO and the combination of CLP and 5LO (7.5 µM each) were incubated and subjected to the gels. C, two-dimensional gel electrophoresis. After non-denaturing PAGE of CLP, 5LO, or a mixture of CLP and 5LO (7.5 µM each), a lane run in parallel to the third lane was excised, incubated in SDS sample buffer and inserted on top of a gel for second-dimensional SDS-PAGE. Gels were stained with Coomassie Blue. D, GST binding assays. Twenty micrograms of the GST-CLP fusion protein coupled to Glutathione Sepharose 4B beads were incubated with 0 to 1.0 µg of
purified 5LO in the presence of CaCl₂ (0.2 mM), or, in absence of Ca²⁺ (with EGTA 1 mM). After elution with 10 mM glutathione and centrifugation, the supernatant was analyzed and compared with a reference of 5LO (first lane) by SDS-PAGE and immunoblotting with anti-5LO antibody.

**Figure 4.** 5LO interacts with CLP in a 1:1 molar stoichiometry. *A-B*, Analysis by non-denaturing PAGE followed by staining with Coomassie Blue. *A*, 5LO (7.5 µM), CLP (7.5 µM), or incubation mixtures of CLP (7.5 µM) with increasing concentrations of 5LO (from molar ratio 0.1 to 1.3). *B*, CLP (5 µM), 5LO (5 µM), or incubation mixtures of 5LO (5 µM) with increasing concentrations of CLP (from molar ratio 0.1 to 2). *C-D*, CLP (5 µM), 5LO (1 µM) or the combination of CLP (5 µM) and 5LO (1 µM) was incubated in the absence or presence of the zero-length cross-linking agents EDC and sulfo-NHS, as indicated. Gels were stained with Coomassie Blue. *E*, CLP (5 µM), 5LO (1 µM) or the combination of CLP (5 µM) and 5LO (1 µM) was incubated in the presence of EDC/sulfo-NHS and analyzed by SDS-PAGE followed by either Coomassie Blue (CB) staining or immunoblotting using anti-5LO or anti-CLP antibody, as indicated. An additional band of approximately 32 kDa detected by the anti-CLP antibody appears to represent a CLP dimer.

**Figure 5.** Analysis of the CLP K131A mutant protein. *A*, GST binding assays. Twenty micrograms of the GST-CLP or GST-CLP K131A fusion proteins, coupled to Glutathione Sepharose 4B beads, were incubated with 0 to 1 µg of purified 5LO. After elution with 10 mM glutathione and centrifugation, the supernatant was analyzed and compared with a reference of 5LO (first lane) by SDS-PAGE and immunoblotting with anti-5LO antibody. *B*, Two-dimensional gel electrophoresis. *Left panel*, CLP K131A (5 µM), 5LO (5 µM), or incubation mixtures of CLP K131A and 5LO (5 µM each) were analyzed by non-denaturing PAGE. *Right panel*, a lane run in parallel to the third lane was excised, incubated in SDS sample buffer and inserted on top of a second-dimensional SDS-PAGE. Gels were stained with Coomassie Blue. *C*, Circular dichroism spectra of CLP and CLP K131A mutant. Spectra were
corrected for buffer background. Measured ellipticities in millidegrees were converted into mean residue ellipticity units and expressed as degrees x cm² x dmol⁻¹.

**Figure 6.** 5LO and F-actin compete, rather than cooperate, for the binding of CLP. A, GST binding assays. Twenty micrograms of the GST-CLP fusion protein were coupled to Glutathione Sepharose 4B beads and, *left panel*, incubated with 0 to 20 µg of F-actin, or, *right panel*, incubated with 20 µg of F-actin in the presence of 0 to 20 µg of 5LO. After elution with 10 mM glutathione and centrifugation, the supernatant was analyzed and compared with a reference of actin (first lane of each panel) by SDS-PAGE and immunoblotting with anti-actin antibody. B, high-speed cosedimentation experiments. Purified actin and CLP (5 µM) were incubated without or with increasing concentrations of 5LO at room temperature for 2 h in standard polymerization buffer (10 mM MOPS, pH 7.0 containing 2 mM MgCl₂ and 50 mM KCl). After centrifugation at 100,000 g for 1 h, the pellet (P) fractions, containing F-actin and bound proteins, and supernatant (S) fractions, containing non-pelletable actin and unbound proteins were analyzed by SDS-PAGE. Gels were stained with Coomassie Blue.

**Figure 7.** 5LO inhibits actin polymerization. A-B, high-speed cosedimentation experiments. A, purified G-actin (5 µM) was incubated alone or with increasing concentrations of 5LO at room temperature for 2 h in standard polymerization buffer. B, purified actin (5 µM), 5LO (1 µM) and BSA (5 µM) were incubated in various combinations at room temperature for 2 h in standard polymerization buffer. Actin was also incubated without or with 5LO buffer only under the same conditions, as indicated. Gels were stained with Coomassie Blue. C-D, fluorescence-based actin polymerization assay. Acrylodan-labeled G-actin (2 µM) was polymerized by the addition of 2 mM MgCl₂ and 50 mM KCl. C, polymerization in the absence (5LO buffer control) or presence of increasing concentrations of 5LO. Acrylodan-labeled G-actin was also incubated without the addition of MgCl₂ and KCl, i.e. under non-polymerizing conditions. D, polymerization in the presence of BSA (2 µM) without or with 5LO (175 nM). The progress of the polymerization reaction was monitored for 2 h using a luminescence spectrometer.
Table 1. Lysine 131 of CLP is involved in 5LO binding in the yeast two-hybrid system

| Gal4 DNA-BD Constructs | 5LO | Gal4 DNA-AD Constructs | β-actin | γ-actin | SNF4 |
|------------------------|-----|------------------------|---------|---------|------|
| Wild-type CLP          | +++ | +++                    | +++     | +++     | -    |

*Mutations in the \(^{130}KK^{131}\) motif of CLP*

- CLP K130A/K131A - +++ +++ ++++ -
- CLP K130Q/K131Q - +++ +++ ++++ -
- CLP K130A ++ +++ ++++ -
- CLP K131A - +++ ++++ -
- CLP K131Q - +++ ++++ -
- CLP K131R +++ +++ ++++ -

*Mutations in the \(^{71}SKRSK^{75}\) motif of CLP*

- CLP S71A +++ +++ ++++ -
- CLP K72A +++ +++ ++++ -
- CLP R73A +++ ++ ++++ -
- CLP S74A +++ +++ ++++ -
- CLP K75A +++ - - -

The yeast strain PJ69-4A was cotransfected with the plasmids expressing the Gal4 fusion proteins, and transformants were tested for the histidine reporter gene. Three (3) to six (6) independent plasmid DNA clones were tested for each CLP mutant. Growth of the colonies was evaluated during incubation at 30°C for 7 days, and scored when visible at 1 (++++), 2 (+++), 4 (++) or 7 (+) days. -, indicates the absence of yeast growth at 7 days. In these two-hybrid experiments, actin, a CLP-interacting protein, and SNF4 served as interacting and non-interacting controls, respectively. All CLP mutants combined with actin, except for the CLP K75A mutant, indicating that the Gal4 DNA-BD moiety of the CLP mutant two-hybrid constructs was functionally preserved. In addition, all CLP mutants tested in combination with SNF4 were negative, suggesting that the mutations introduced in CLP did not induce auto-activation of the HIS3 reporter gene. Similar two-hybrid results were obtained using the Ade2 and LacZ reporter genes (unpublished data).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

(A) Beads (20 μg) GST-CLP GST-CLP
F-Actin (μg) 0.1 0 2 10 20 0.1 20 20 20 20
5LO (μg) - - - - - - - - - -

(B) Actin (5 μM) CLP (5 μM) SLO (μM)
P S P S P S P S P S
- 0.025 0.1 0.5 1.0 5.0 + + + + + +

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Figure 7
5-Lipoxygenase interacts with Coactosin-Like Protein
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