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Coactosin-like protein functions as a stabilizing chaperone for 5-lipoxygenase: role of tryptophan 102

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Abbreviations. 5-H(P)ETE, 5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid; 13-HPODE, 13(S)-hydroperoxy-9-cis-11-trans-octadecadienoic acid; 5-LO, 5-lipoxygenase; AA, arachidonic acid; CLP, coactosin-like protein, LT, leukotriene; Mono Mac 6 cells, MM6 cells; PC, phosphatidyl choline; wt, wild type

ABSTRACT

The activity of 5-lipoxygenase (5-LO), which catalyzes two initial steps in biosynthesis of proinflammatory leukotrienes (LT), is strictly regulated. One recently discovered factor, Coactosin-like protein (CLP), binds 5-LO and promotes LT formation. Here we report that CLP also stabilizes 5-LO and prevents non-turnover inactivation of the enzyme *in vitro*. Mutagenesis of Trp residues in the 5-LO β -sandwich showed that 5-LO-W102 is essential for binding to CLP, and for CLP to support 5-LO activity. In addition, also the stabilizing effect depended on binding between CLP and 5-LO. After mutations which prevent interaction (5-LO-W102A, or CLP-K131A), the protective effect of CLP was absent. A calculated 5-LO-CLP docking model indicates that CLP may bind to additional residues in both domains of 5-LO, thus possibly stabilizing the 5-LO structure. To obtain further support for binding between CLP and 5-LO in a living cell, subcellular localization of CLP and 5-LO in the monocytic cell line Mono Mac 6 was determined. In these cells, 5-LO associates with a nuclear fraction only when differentiated cells are primed with phorbol ester and stimulated with ionophore. The same pattern of redistribution was found for CLP, indicating that the two proteins associate with the nucleus in a coordinated fashion. Our data support a role for CLP as a chaperoning scaffold factor, influencing both the stability and the activity of 5-LO.

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INTRODUCTION

5-Lipoxygenase (5-LO) catalyzes two initial steps in leukotriene (LT) biosynthesis, oxygenation of arachidonic acid (AA) to 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and subsequent dehydration into the epoxide LTA₄ [1]. LTs are inflammatory lipid mediators which cause leukocyte chemotaxis and increased vascular permeability. The effects of LTs are well established in pathogenesis of asthma, and accumulating data indicate a role for LTs also in atherosclerosis [2]. In addition, 5-LO products are implicated in cancer cell survival [3], recently also for leukemia stem cells [4].

A model of the 5-LO structure, based on the crystal structure of the ferrous form of rabbit reticulocyte 15-LO [5, 6] consists of an N-terminal β -sandwich (residues 1-114) and a larger C-terminal catalytic domain containing prosthetic iron (residues 121-673). The validity of this 5-LO model structure is supported by various mutagenesis studies, as reviewed [7]. Several factors which influence 5-LO enzyme activity bind to the C2-like β -sandwich, e.g. Ca²⁺ and phosphatidylcholine, and when cells are stimulated to produce LTs, 5-LO is typically associated with the nuclear membrane [8]. However, recent observations indicate a different subcellular distribution in neutrophils from males [9].

Coactosin-like protein (CLP) is similar to coactosin [10], a member of the ADF/Cofilin group of actin binding proteins. By two-hybrid screening with 5-LO as bait, we found that CLP can bind to 5-LO [11]. Binding was also demonstrated by coimmunoprecipitation from lysates of transfected cells, and *in vitro* assays (GST pull-down assay, native PAGE, chemical cross-linking) showed binding with molar stoichiometry 1:1 [12]. Human CLP also binds F-actin, and was found to colocalize with actin stress fibres in transfected CHO and COS-7 cells [13]. Also mouse CLP was found to bind 5-LO with stoichiometry 1:1 [14]. Mutagenesis showed the involvement of CLP Lys-75 and Lys-131 in binding to F-actin and 5-LO, respectively [12]. In the CLP structure [15] K75 and K131 are close, indicating overlapping binding sites, which could explain why a ternary complex of F-actin-CLP-5-LO has not been found. We described that CLP can serve as a scaffold for Ca²⁺- induced activation of 5-LO [16]. CLP has appeared in several array/proteome analyses, connecting CLP with cancer and inflammatory disease, e.g. rheumatoid arthritis [17]. Recently, Hyperforin, an anti-inflammatory compound from St. John's wort which inhibits 5-LO activity, was found to impair the binding between CLP and 5-LO [18].

Here we present that CLP stabilizes 5-LO against non-turnover inactivation, and that one particular residue in the 5-LO β -sandwich (Trp-102) is essential for binding of CLP to 5-LO, and for the effects of CLP on 5-LO activity and stability. A model of the docking complex was calculated (DOT algorithm) taking the available mutagenesis results into consideration.

MATERIALS AND METHODS

Expression of CLP and 5-LO

Recombinant human CLP was expressed as GST fusion protein, using the plasmid pGEX-5X-1-CLP [15, 12]. The fusion partner was removed by digestion with factor Xa, and anion exchange chromatography on MonoQ. Recombinant human 5-LO was

expressed from the plasmid pT3-5-LO and purified on ATP-agarose (Sigma A2767) [19, 20]. Two *E. coli* strains were used. Expression in BL21 at 27°C using a rich medium (TB) gave 5-LO preparations with specific activities around 10 μmol 5-H(P)ETE/mg, while expression in MV1190 at 27°C using minimal medium resulted in specific activities around 20 μmol 5-H(P)ETE/mg. Mutated plasmids (W13A, W75A, W102A) were constructed from pT3-5-LO using the QuikChange kit (Stratagene).

Time and heat inactivation

Solutions of purified 5-LO (14 $\mu\text{g}/\text{ml}$, total volume 500 μl), with or without purified wt-CLP or CLP mutants (stoichiometry 1:1) were prepared in AB+ buffer (50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.1% (v/v) β -mercaptoethanol), and kept in closed Eppendorf tubes. For time dependent inactivation at RT, the tubes were kept on the lab bench, and at the indicated intervals, aliquots (10 μl) were removed for 5-LO activity assay (described in Methods). For heat inactivation, tubes were immersed in a water bath at 50°, 55°, and at 60°C. At the indicated intervals, aliquots (10 μl) were removed and assayed for 5-LO activity

HPLC assay of 5-LO enzyme activity

Incubations were performed in Eppendorf tubes at RT for 10 min. Buffer AB (50 mM Tris-Cl pH 7.5, 2 mM EDTA) was added to tubes, followed by the addition of substrate mix containing Tris-Cl pH 7.5, CaCl_2 , phosphatidyl choline (PC, Sigma P-3556), AA (Nu-Chek Prep Inc., Elysian, MN), 13-HPODE, and ATP. Tubes were immersed in a sonication bath for 1 min. The reaction was initiated by addition of aliquots of 5-LO from inactivation experiments containing about 150 ng of 5-LO. When CLP (stoichiometry 1:1) was present during inactivation experiments, it was also present in the assay. Final concentrations in the 100 μl incubation volume were: 77.4 mM Tris-Cl pH 7.5, 100 μM AA, 1.2 mM EDTA, 1.9 mM Ca^{2+} , 25 $\mu\text{g}/\text{ml}$ PC, 10 μM 13-HPODE, 4.9 mM ATP.

Incubations were terminated with 300 μl of cold stop solution (60% acetonitrile, 40% water, and 0.2% acetic acid (v/v)) containing 3.3 μM of 17(*S*)-hydroxy-(7*Z*,10*Z*,13*Z*,15*E*)-docosatetraenoic acid (internal standard at 235 nm, a kind gift from Mats Hamberg). Aliquots (100 μl) were injected onto a C_{18} HPLC column (Waters Nova Radial Pak) and AA metabolites were isocratically eluted with acetonitrile/water/acetic acid (60:40:0.2, v/v) at flow rate 1.2 ml/min. The eluate was monitored at 235 nm for 5-HPETE and 5-HETE. 5-LO enzymatic activity was calculated from the sum of peak areas, relative to internal standards.

GST Pull-Down Assay

For binding studies *in vitro*, 20 μg of the GST-CLP fusion protein linked to glutathione-Sepharose 4B beads, was incubated with purified 5-LO protein (5 μg) in presence of BSA (50 μg) in 200 μl of buffer A (2 mM Tris-Cl pH 8.0, 0.2 mM ATP, 0.2 mM CaCl_2 , 2 mM MgCl_2 , 50 mM KCl, 0.5 mM β -mercaptoethanol). After a 30-min gentle rotation at RT, beads were washed five times in buffer A (without BSA). Bound proteins were eluted with 150 μl of elution buffer (10 mM GSH in 50 mM Tris-Cl, pH 8.0) during 60-90 min rotation at RT. Beads were sedimented, and aliquots of the supernatant eluate were assayed by SDS-PAGE followed by 5-LO Western blot analysis.

Pulse proteolysis with thermolysin

A stock solution of thermolysin (Sigma T7902) was prepared in 2.5 M NaCl, 10 mM CaCl₂ and the concentration was determined by spectroscopy as previously described [21]. Purified 5-LO (4 µg) with or without CLP (0.8 µg, stoichiometry 1:1) was pre-incubated for 10 min at RT in PBS containing 10 mM CaCl₂ and 50 mM NaCl. Thermolysin (4 µg) was added, and after 20-120 sec incubations were stopped by addition of 3.5 µl EDTA (50 mM) and cooling on ice. Sample loading buffer (10 x conc) was added and 4 µl aliquots were subjected to SDS-PAGE on a Pharmacia FAST system, followed by Coomassie staining.

Subcellular fractionation after detergent lysis

Mono Mac 6 cells were grown in cell culture, and differentiated with TGFβ (5 ng/ml) and vitamin D3 (50 nM) for 96 h as described [22]. Cells (10 x 10⁶) were resuspended in 1 ml PGC buffer (PBS containing 1 mg/ml glucose and 1 mM CaCl₂). After preincubation for 10 min at 37°C, cells were primed with PMA (100 nM, 10 min) and activated with ionophore (5 µM) and arachidonic acid (40 µM) for another 10 min, as indicated. Cells were then chilled on ice for 5 min, lysed with NP-40, and subcellular fractionation was performed as described [22]. Aliquots (45 µl, derived from approximately 1 million cells) of pair-wise nuclear and nonnuclear fractions were immediately mixed with 9 µl of concentrated SDS loading buffer, heated for 5 minutes at 95°C, and analyzed for 5-LO and CLP protein by SDS-PAGE and immunoblotting. In-house antisera for 5-LO (1551) and CLP were used. Antibodies for Lamin B (nuclear membrane marker, control for the fractionation procedure) and for β-actin were from Santa Cruz Biotechnology.

Protein-protein docking of CLP and 5-LO

Protein-protein docking of CLP and 5-LO was performed using the ClusPro server [23] and applying the DOT algorithm [24, 25]. Since an experimentally determined 3D structure for 5-LO is not available, we used our previous homology model based on the crystal structure of rabbit 15-LO [26]. For CLP the averaged structure of 20 NMR determinations (PDB: 1WNJ, [15]) was used as the starting conformation for protein-protein docking. The flexible ends (residues 1-4 and 137-145) were truncated. Parameters for the docking algorithm were: radius of clustering = 9, number of electrostatic hits to be clustered = 1500, number of retained output-structures = 10. Hydrogen atoms were added to both input structures and the proteins were minimized with a constrained backbone using the CHARMM force field [27]. DOT performs a systematic, rigid-body search of one molecule translated and rotated relative to a fixed second molecule by evaluating conformations during docking with a shape complementarity function. Intermolecular energies of all configurations generated are calculated as the sum of electrostatic and van-der-Waals energies. These energy terms are evaluated as correlation functions and used to rank the docking solution [24]. The obtained docked complexes were filtered by our experimental data retaining only those complexes where CLP-K131 could interact with 5-LO and 5-LO-W102 with CLP. CLP-K131, 5-LO-W102 and nearby amino acids in the resulting complex were finally relaxed using the CHARMM27 force field to optimize the modeled interacting geometry.

Data analysis

Origin 8 was used for data analysis and Student's t-test for two independent samples was performed to determine statistical differences between the means of groups of independent experiments. P-values < 0.05 were considered statistically significant.

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RESULTS

Enzyme activity of wt-5-LO and three Trp mutants in presence of CLP and/or phosphatidylcholine

Efficient Ca^{2+} induced dioxygenase and LTA_4 synthase activities of wt-5-LO requires the presence of a scaffold factor, to which 5-LO can bind. Binding of 5-LO to PC has been shown to involve three surface-exposed Trp residues in the 5-LO β -sandwich (W13, W75, W102). When these were exchanged to Ala, mutagenesis of Trp-102 gave the most prominent effect, reducing the affinity of the isolated 5-LO C2-like domain to PC about 20-fold [28]. Because of the similar effects of PC and CLP (both support Ca^{2+} -induced 5-LO activity) we performed the same three single-point mutations in intact 5-LO, and determined the effect on enzyme activity in presence of CLP and/or PC. As shown in Fig 1A, in presence of PC (25 $\mu\text{g}/\text{ml}$) the formation of dioxygenase products (5-HPETE and 5-HETE) was rather similar for wt-5-LO and the mutants. Enzyme activity was much lower in absence of scaffold factors. In assays from which PC was excluded, addition of CLP (stoichiometry 1:1) increased dioxygenase activity of wt-5-LO and two of the mutants (5-LO-W13A, 5-LO-W75A). In contrast, for 5-LO-W102A, CLP did not support the dioxygenase activity, which was barely detectable. When both PC and CLP were added, activities were again quite similar for all four proteins. Concomitantly, for wt-5-LO, 5-LO-W13A, and 5-LO-W75A, addition of CLP resulted in an increased 5-HETE/5-HPETE ratio (data not shown).

Formation of LTA_4 was determined as the nonenzymatic hydrolysis products 6-trans- LTB_4 and 12(S)-6-trans- LTB_4 . For wt-5-LO, inclusion of both CLP and PC led to 6-fold up-regulation of LTA_4 production, compared to incubations including only PC (Fig 1B). Similar upregulation of LT production was found for 5-LO-W13A and 5-LO-W75A, but not for 5-LO-W102A. For all four 5-LO proteins, LT biosynthesis was minute when PC was absent. Thus, as observed before [16] CLP alone can not support formation of LTA_4 , but CLP together with PC leads to considerable increase of LTA_4 . Fig 1C, shows the total 5-LO product formation, with and without PC and CLP. Taken together, the activity data show that Trp-102 in 5-LO is essential for the effects of CLP on 5-LO activity.

Enzyme activity of wt-5-LO and three Trp mutants, at reduced concentrations of phosphatidylcholine and arachidonic acid

To study the role of the three Trp residues on 5-LO activation (by Ca^{2+}) in presence of only PC as scaffold factor, activity assays were performed at reduced concentrations of AA and PC (20 μM AA, 5 $\mu\text{g}/\text{ml}$ PC). Only at such relatively low concentrations, differences in activities were detected for the Trp-mutants, in comparison to wt-5-LO (Fig 2). Thus, at high PC and AA (our standard conditions: 100 μM AA, 25 $\mu\text{g}/\text{ml}$ PC) no significant reduction of oxygenase activity was observed for the mutants in relation to wt-5-LO (compare also Fig 1A). However, reduced amounts of PC and AA resulted in significantly lower formation of 5-HPETE and 5-HETE, which was observed for all three 5-LO mutants (Fig 2). Thus, all three Trp residues contribute to PC support of Ca^{2+} -induced 5-LO activation. None appears to be of particular importance. Accordingly, when all three Trp residues were mutated to Ala, there was a more pronounced reduction of 5-LO activity, at reduced concentrations of PC and AA [16].

Mutant 5-LO-W102A does not bind GST-CLP

To ascertain that the effects of CLP on 5-LO enzyme activity mirror specific binding of 5-LO proteins to CLP, pull down assays, using GST-CLP fusion protein, were performed. Wild type 5-LO, 5-LO-W13A and 5-LO-W75A all bound to GST-CLP, while 5-LO-W102A did not (Fig 3). There was no association of 5-LO to GST, used as a negative control. The enzyme activity of 5-LO-W102A was about the same as for wt-5-LO, when assayed in presence of PC (25 µg/ml) (Fig 1). This indicates that the loss of binding of 5-LO-W102A to CLP should not be due to compromised overall structure of this 5-LO mutant.

CLP prevents inactivation of 5-LO over time

In addition to supporting Ca²⁺ induced enzyme activity, we found that CLP prevents inactivation of 5-LO. Solutions of purified 5-LO were kept at RT (on the lab-bench) for up to five days in sealed Eppendorf tubes under normal atmosphere. At intervals, aliquots were removed and subjected to enzyme activity assay. 5-LO alone was gradually inactivated, with half activity remaining after 24 h, and about 20% after 120 h. However, in presence of CLP (molar stoichiometry 1:1), enzyme activity was preserved, considering that about 2/3 of the initial activity remained after 120 h (Fig. 4A). Since no substrate was present during the 5 days, thus CLP protected 5-LO against non-turnover inactivation. Exposure to oxygen is an important factor previously shown to lead to non-turnover inactivation of 5-LO, see [29] for review.

The effect of CLP was also tested regarding inactivation of 5-LO during turnover. For this purpose 5-LO, with or without CLP (1:1), was subjected to repeated addition of substrate. 5-LO was incubated under standard assay conditions (see Methods), including 100 µM AA. After 10 min, half of the assay mixture was removed, and analyzed for 5-HETE and 5-HPETE. To the remaining half, AA (100 µM) was added again, and incubated for additional 10 min. Prominent formation of 5-HETE and 5-HPETE was observed after the first addition of substrate (24 and 23.7 µmol/mg) in both cases (samples with and without CLP). There were only minor increases of products after the second substrate additions, going up to 27 and 24 µmol/mg, respectively. This result showed that CLP could not prevent turnover related inactivation of 5-LO.

CLP prevents heat-inactivation of 5-LO

Samples of 5-LO, with or without CLP (stoichiometry 1:1), were subjected to different temperatures for 10 min. Samples were then cooled on ice, and aliquots were taken to 5-LO standard assay. At 37°C there was no inactivation of 5-LO, while exposure to 80°C resulted in complete inactivation. CLP could not protect at 80°C, probably due to denaturation also of CLP itself. However, at 50°, 55°, and at 60°C, CLP had a protective effect. The protective effect was most prominent at 55°C, and this temperature was chosen for a time curve. In this experiment, samples of 5-LO, with or without presence of an equimolar (1:1) amount of CLP, were kept at 55°C for up to 90 min. Aliquots were removed at intervals, for activity assay. As shown in Fig. 4B, the presence of CLP gave considerable reduction of heat-inactivation of 5-LO, at 55°C.

Mutant 5-LO-W102A is not protected by CLP

The protective effect of CLP against heat inactivation was investigated also for the three Trp-mutants. Heat treatment (55°C) of 5-LO proteins for 80 min resulted in

reduced 5-LO product formation in subsequent oxygenase activity assays (formation of 5-HPETE and 5-HETE) compared to RT controls (Fig. 5A). These heat-induced reductions of activity were statistically significant ($p < 0.001$ for all four proteins (Fig. 5A). However, in presence of CLP, heat treatment did not have any effect on the enzyme activities of wt-5-LO, 5-LO-W13A and 5-LO-W75A. It should be observed that the formation of 5-HPETE and 5-HETE, for wt-5-LO, 5-LO-W13A and 5-LO-W75A, was generally reduced in presence of CLP, due to a shift of the 5-LO product profile in favour of LTs (compare Fig 1). In contrast, CLP failed to protect the 5-LO-W102A mutant against heat-inactivation. In addition, among the four 5-LO proteins, this 5-LO mutant was the most sensitive to heat treatment, with 90% reduction of activity. In comparison, heat exposure reduced the activity of wt-5-LO by 65%, but only by 35% for 5-LO-W75A, suggesting that mutation of Trp75 renders 5-LO less susceptible.

Inactivation at RT over time was determined for the mutant 5-LO-W102A, in comparison to wt-5-LO. As shown in Fig 5B, without CLP both 5-LO enzymes lost activity similarly over 5 days. On the other hand, in presence of CLP (stoichiometry 1:1) wt-5-LO maintained about 90 % of its starting activity after 5 days (similar to Fig 4B), while CLP did not prevent inactivation of 5-LO-W102A over time.

CLP mutant K131A does not protect 5-LO

Two CLP mutants, K131A with reduced binding to 5-LO, and K75A which binds to 5-LO (but not to F-actin), were tested regarding protection of 5-LO against non-turnover inactivation at RT. Unlike wild type CLP and CLP-K75A, which reduced 5-LO inactivation over time, the mutant CLP-K131A did not have this protective effect (Fig. 6). The two CLP mutants were also tested regarding protection of 5-LO against heat inactivation (55°C for 80 min). Again it was found that CLP-K75A prevented inactivation of 5-LO while CLP-K131A was ineffective (data not shown).

CLP prevents digestion of 5-LO by Thermolysin

The thermostable protease thermolysin (from *B. thermoproteolyticus*) can be used in so called “pulse proteolysis” to determine effects of ligands on protein stability [21]. 5-LO, which is known as an unstable enzyme was rapidly cleaved by this protease, already after 20 sec (Fig 7A, lane 1). On the other hand, CLP was quite resistant to thermolysin. A major part of CLP remained intact after 20 or 120 sec incubations with the protease (Fig 7B). 20 sec was chosen as the time for “pulse proteolysis”. 5-LO was pre-incubated with CLP (1:1 stoichiometry) for 10 min, and the sample was subjected to thermolysin for 20 sec. Subsequent SDS-PAGE of the samples showed that 5-LO was preserved in presence of CLP (Fig 7A, lanes 1 and 2). However, when the proteolysis time was increased to 90 sec, 5-LO was digested also in presence of CLP (Fig 7A, lanes 3 and 4). These results indicate that proteolytic cleavage of 5-LO can be delayed by CLP. A similar protective effect was shown for the Trp-13 and Trp-75, but not for the Trp-102 mutant of 5-LO (data not shown). Several weak bands ranging in size between approximately 15 and 70 kDa appeared when 5-LO was incubated with thermolysin. Bands larger than 34.6 kDa (size of thermolysin) can be attributed to large 5-LO fragments while smaller bands could also stem from auto-proteolysis of thermolysin. In Fig 7A, only 0.24 μ g of CLP were applied to the FAST gel (compared to 1.2 μ g of 5-LO and thermolysin), while in Fig 7B 2.4 μ g of CLP was applied. This explains the absence of visible CLP bands in Fig 7A.

Distribution of CLP and 5-LO in nuclear / non-nuclear fractions from Mono Mac 6 cells

One way to study possible association between 5-LO and CLP in a cell, is to determine if the two proteins translocate in a similar fashion upon cell stimulation. Subcellular fractions were prepared from MM6 cells which had been subjected to various treatments. First, undifferentiated MM6 cells (expressing CLP but not 5-LO) were analysed. Cells were treated in four different ways: ionophore-stimulated only; primed with PMA and stimulated with ionophore; primed with PMA and stimulated with ionophore and AA; and control cells. In all cases, most CLP was found in the non-nuclear fractions. Only weak Western blot bands were detected in nuclear fractions (Fig 8A). As a loading control, β -actin appeared with similar band intensities for all non-nuclear fractions. Weaker bands were observed for the nuclear fractions. Thus, for undifferentiated MM6 cells, lacking 5-LO, association of CLP with the nucleus was minute.

Next, differentiated MM6 cells (expressing 5-LO and CLP) were analysed. It was found before that these cells require priming with PMA, for 5-LO to associate with the nuclear membrane upon ionophore stimulation [22]. Accordingly, when differentiated MM6 cells were challenged with ionophore only, 5-LO was recovered primarily in the non-nuclear fraction, and the same pattern was found for CLP (Fig 8B). However, following priming with PMA and subsequent activation with ionophore, both 5-LO and CLP were redistributed to the nuclear fraction. The same result was seen after PMA-priming, and activation with ionophore and AA (Fig 8B). These observations indicate, that in differentiated MM6 cells, trafficking of CLP and 5-LO occur by similar patterns. CLP has not been observed to change subcellular localisation, without 5-LO doing that as well. Also with the samples from the differentiated MM6 cells, β -actin gave similar bands for the non-nuclear fractions, However, in the nuclear fractions from PMA-primed cells β -actin was increased, similar to 5-LO and CLP. Interestingly, β -actin can bind both to CLP [13] and to 5-LO (reviewed in [29]).

Protein-protein docking of CLP-5-LO interaction

The combination of computational docking results from DOT and experimental data has been established as a useful tool for understanding potential molecular interactions [30]. To better understand the interaction between CLP and 5-LO, docking was performed using our 5-LO model structure and the CLP NMR structure (PDB: 1WNJ, [15]). Assuming that the impairment of complex formation by the two mutations CLP-K131A and 5-LO-W102A points to close interaction of CLP-K131 with 5-LO, and of 5-LO-W102 with CLP, only one model complex remained (Fig 9). In particular, in this model CLP-K131 is directed towards 5-LO and 5-LO-W102 points towards CLP. This model suggests a direct interaction *via* a cation-pi-interaction between the side chains of CLP-K131 and 5-LO-F14. 5-LO-W102 could be involved in an interaction network also including 5-LO-R165, and the carbonyl oxygen of CLP-K131. In such an interaction network, the 5-LO-W102 side chain might engage in a cationic-pi-interaction with 5-LO-R165 which in turn would form a hydrogen bond with the backbone-carbonyl oxygen of CLP-K131 (Fig. 9).

Several other amino acids, located both in the 5-LO β -sandwich and in the catalytic domain, may potentially form hydrogen bonds stabilizing the complex (5-LO-E70 – CLP-R91, 5-LO-D106 – CLP-V113, 5-LO-E108 – CLP-R91, 5-LO-T137 – CLP-

K102, 5-LO-K140 – CLP-Q106). Four of these five residues are identical in other mammalian 5-LOs, one is conserved. All predicted interacting residues of CLP are identical in mouse CLP, which is also known to bind 5-LO [14].

CLP also binds F-actin [13], and F-actin binding residues were determined [31]. Many of these are located on the predicted CLP-5-LO interface, indicating overlapping binding sites. This is in line with the observation that a ternary complex of F-actin-CLP-5-LO has not been found [12].

DISCUSSION

5-LO has always been considered to be an unstable enzyme and 5-LO is inactivated both during turnover, and during storage (non-turnover inactivation). For review see [29]. Inactivation of 5-LO during turn-over can be due to reactions with the enzyme products 5-HPETE and LTA₄. Non-turnover inactivation is thought to depend on oxygen, and deliberate exposure to oxygen inactivated 5-LO, due to loss of the prosthetic iron. Also treatment with H₂O₂ inactivated purified 5-LO, and catalase or glutathione peroxidase can protect against such inactivation.

CLP was previously found to bind 5-LO by coimmunoprecipitation from lysates of transfected cells. In addition, GST pull-down assay, native PAGE, and chemical cross-linking showed binding *in vitro* with molar stoichiometry 1:1 [14, 12]. CLP serves as a scaffold for 5-LO activity [16], apparently by replacing or complementing membrane (phosphatidylcholine (PC)) in this role. When CLP was present in the *in vitro* assays (no PC added) Ca²⁺ activation led to formation of 5-HPETE plus 5-HETE, while the presence of CLP together with PC gave considerable upregulation of LTA₄ formation. Here, we confirm these effects of CLP on 5-LO activity and we show that CLP also has another effect, i.e. to prevent non-turnover inactivation of 5-LO. Thus, CLP present in molar stoichiometry 1:1, could prevent both inactivation over time and during heat treatment. This stabilizing effect of CLP was found to be specific, since it strictly depended on binding to 5-LO. The CLP mutant K131A, with considerably reduced binding ability to 5-LO, did not protect 5-LO. Also, the 5-LO mutant W102A which did not bind CLP, was neither stabilized nor activated by CLP. The 5-LO Trp-mutants studied here were as active as wt-5-LO (in presence of PC, Fig 1) indicating that these mutations did not compromise the overall structure of 5-LO.

Phosphatidylcholine is the major phospholipid constituent of the nuclear membrane. In addition to its role as a scaffold for Ca²⁺- induced 5-LO activity, PC can also stabilize 5-LO. In fact, PC has been used as a stabilizing agent during purification of 5-LO [32, 33], and membrane binding of 5-LO stabilized the structures of both 5-LO and the membrane [34]. Addition of leukocyte protein fractions improved stability and activity of purified 5-LO [32, 35]. In addition to the “macromolecular crowding” provided by mixed proteins at relatively high concentrations, it appears possible that these protein fractions contained CLP. The observation that CLP is effective already at stoichiometry 1:1 supports a specific interaction between 5-LO and CLP, different in nature from the protective effect of proteins in general. Also, the dependence on particular amino acid residues (both in 5-LO and in CLP) strongly suggests that the protective effect of CLP is due to specific interaction between these two proteins.

Binding of PC to the 5-LO β-sandwich involves three Trp residues (W13, W75, W102) [28]. Mutagenesis of these residues (to Ala) reduced the affinity of the isolated

5-LO C2-like domain to PC, substitution of Trp-102 gave the most prominent effect with 20-fold reduced affinity [28]. When all these residues were mutated to Ala in intact 5-LO, Ca^{2+} induced enzyme activity in presence of relatively low concentrations of PC and AA was reduced to about 25% of wild type 5-LO [16]. Here we show that for the three single Trp-mutants, the activity at low PC and AA was about 50% of wild type 5-LO. Thus all three residues seem to contribute, to about the same extent, for PC to support 5-LO enzyme activity. In similarity to rabbit 15-LO [36] it has been suggested that for intact 5-LO, residues also in the catalytic domain contribute to membrane binding, and it was noted that the K_D for binding of intact 5-LO to PC rich vesicles was considerably lower than for binding of the isolated β -sandwich [37]. Thus, it appears that for intact 5-LO, Trp-102 is not as influential for PC binding, as for the isolated 5-LO β -sandwich. Also, it has been proposed that 5-LO can bind to membranes in different modes, non-productive and productive [34]. Presumably, our determinations of Ca^{2+} induced enzyme activity in presence of PC reflect a productive binding mode. In view of this model, it thus appears that Trp-102 is more important for non-productive binding of 5-LO to PC, in comparison to the productive binding. Similar considerations can apply also to the role of 5-LO Trp-75 for binding to PC. In the 5-LO model structure [7] this residue is located on the very tip of a surface exposed loop of the β -sandwich, and in a model of 5-LO-phospholipid interaction, this residue inserts into the hydrophobic part of the membrane [37]. However, the present mutagenesis data (Fig 2) as well as previous findings (Trp-75 mutated also to Arg, Phe, Ser) [38] do not point to a particular role for Trp-75 regarding PC supported 5-LO activity.

On the other hand, for CLP to support 5-LO, Trp-102 in 5-LO is essential, and mutagenesis of this residue obliterated binding of CLP to 5-LO. When both PC and CLP were added to 5-LO, total 5-LO product formation was about the same as with only PC present (Fig 1C) but strikingly, the LT production was prominently increased (about 5-fold, Fig 1B). As suggested before [16] this indicates a three-partner complex, comprising 5-LO, PC, and CLP. Our findings show that Trp-102 in 5-LO is important for binding to CLP. Apparently, Trp-13 and Trp-75 are free to bind PC, possibly, Trp-102 can bind also to PC (at the same time as binding CLP).

Trp-102 is part of a stretch of conserved surface residues (FPCYRW) [39], and in the 5-LO model structure Trp-102 is partially hidden in the cleft between the two domains, as previously demonstrated for the corresponding residue (Trp-100) in 12/15-LO [40]. Thus, it appears possible that binding of CLP to 5-LO may have an allosteric effect on the association of the two domains in 5-LO, these may open up for CLP to directly access Trp-102. Alternatively, as suggested by our model of the CLP-5-LO complex, 5-LO-W102 may influence CLP binding via an interaction network involving 5-LO-R165. The model also suggests a direct pi-cation interaction of CLP-K131 with 5-LO-F14, as well as six hydrogen bonds between 5-LO and CLP, three of which involve residues in the 5-LO catalytic domain, while the other three involve residues in the β -sandwich. In either case, binding of CLP to 5-LO may influence the relative positions of the two domains in 5-LO. We like to point out, that in our modelling of the CLP-5-LO complex, Ca^{2+} was not included. Ca^{2+} is known to bind 5-LO β -sandwich and different sets of Ca^{2+} ligands have been suggested [39, 41, 19, 28]. Although CLP can bind to 5-LO in absence of Ca^{2+} [12] possibly the mode of binding will be different in presence of Ca^{2+} .

CLP could protect 5-LO against thermolysin catalysed proteolysis. Thermolysin is a Ca^{2+} -dependent endopeptidase with preference for hydrophobic target sites, which can be used to study protein stability and ligand binding by so called “pulse proteolysis” [21]. In short time incubations (20 sec), CLP protected 5-LO against thermolysin, however when the incubation time was extended to 90 sec, protection was not observed. This indicates that proteolysis of 5-LO was delayed in presence of CLP, and not entirely prevented. An attractive explanation for this effect of CLP is that by binding to 5-LO, it renders the 5-LO structure more compact, and consequently bulky hydrophobic residues (preferred cleavage sites of thermolysin [42]) may become less accessible. In soybean lipoxygenase-1, hydrophobic interactions contribute to association of the two domains [43] and hydrophobic residues are present between the two domains in 5-LO model structures [39]. The importance of 5-LO-W102 suggests that CLP binds at or near the domain interface of 5-LO. Possibly, CLP prevents access of thermolysin to a target site on hydrophobic interdomain areas of 5-LO.

When cells are activated to produce LTs, 5-LO typically migrates to the nuclear membrane. In previous studies with human neutrophils, CLP displayed the same changes in subcellular distribution as 5-LO [9, 16], i.e. following ionophore stimulation, both 5-LO and CLP were associated with a nuclear fraction. Recently, a gender difference regarding subcellular localization of 5-LO was found [9]. It was shown that for male neutrophils a substantial amount of 5-LO was associated with the nucleus already in non-stimulated cells, and the same was found for CLP. Here we analysed subcellular fractions from MM6 cells, which offer some experimental options. First, undifferentiated MM6 cells express CLP but not 5-LO. Regardless of stimulation, for these undifferentiated cells almost all CLP was recovered in the non-nuclear fractions. Second, differentiated MM6 cells (expressing 5-LO) required priming with PMA, for 5-LO to associate with the nuclear membrane in response to ionophore [22], in accordance with a role for kinases in 5-LO translocation [7]. Accordingly, when differentiated MM6 cells were stimulated with ionophore only, 5-LO was recovered primarily in the non-nuclear fraction, and the same was found for CLP (Fig 8B). However, following priming with PMA and subsequent activation with ionophore, substantial amounts of both 5-LO and CLP were found in the nuclear fraction. Thus, also in MM6 cells, it appears that migration of CLP is connected with migration of 5-LO.

In summary, our findings show that Trp-102 in the 5-LO β -sandwich is important for binding of CLP. Our data also provide further support for CLP functioning as a chaperone for 5-LO, stabilizing 5-LO in the resting cell and functioning as a scaffold during Ca^{2+} -induced 5-LO activity.

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FIGURE LEGENDS

Fig 1. Enzyme activity of wt-5-LO and three Trp mutants, in presence of CLP and/or phosphatidylcholine

A) The Ca^{2+} - activated formation of 5-HPETE plus 5-HETE was determined in 10 min incubations of purified 5-LO proteins (expressed in BL21) as described in Methods. Phosphatidylcholine (PC, 25 $\mu\text{g}/\text{ml}$ or CLP (stoichiometry 1:1) were present as indicated.

B) The formation of LTA_4 hydrolysis products was determined in the same incubations as in A.

C) Total products (5-HPETE, 5-HETE and LTA_4 hydrolysis products) in the same incubations. All data are mean \pm SEM for three independent experiments.

Fig 2. Enzyme activity of wt-5-LO and three Trp mutants, at low concentrations of phosphatidylcholine and arachidonic acid

The Ca^{2+} - activated formation of 5-HPETE plus 5-HETE was determined in 10 min incubations of purified 5-LO proteins (expressed in BL21) as described in Methods. Arachidonic acid and PC was 100 μM and 25 $\mu\text{g}/\text{ml}$, or 20 μM and 5 $\mu\text{g}/\text{ml}$, as indicated. All data are mean \pm SEM for three independent experiments.

Fig. 3. Mutant 5-LO-W102A does not bind GST-CLP

GST pull-down assay was performed as described in Methods. Aliquots of bead eluates were analyzed for 5-LO by Western blot. Similar results were obtained in four separate experiments.

Fig 4A. CLP prevents inactivation of 5-LO over time

Solutions of purified 5-LO (expressed in MV1190, 14 $\mu\text{g}/\text{ml}$, total volume 500 μl), with or without CLP (stoichiometry 1:1) were prepared in AB+ buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.1% (v/v) β -mercaptoethanol), and kept in closed Eppendorf tubes at rt. At the indicated intervals, aliquots (10 μl) were removed and assayed in triplicate for 5-LO activity (described in Methods). Data are mean \pm SEM for three independent experiments.

Fig 4B. CLP prevents heat-inactivation of 5-LO

Solutions of purified 5-LO (expressed in MV1190, 14 $\mu\text{g}/\text{ml}$, total volume 500 μl), with or without CLP (stoichiometry 1:1) were prepared in AB+ buffer, and kept in closed Eppendorf tubes immersed in a water bath at 55°C. At the indicated intervals, aliquots (10 μl) were removed and assayed in triplicate for 5-LO activity (described in Methods). Results are given as mean \pm SEM for two independent experiments.

Fig 5. Mutant 5-LO-W102A is not protected by CLP

A) Solutions of purified 5-LO proteins (wt and mutants expressed in BL21, 14 $\mu\text{g}/\text{ml}$, total volume 500 μl), with or without CLP (stoichiometry 1:1) were prepared in AB+ buffer, and kept in closed Eppendorf tubes immersed in a water bath at 55°C for 80 min. Before and after heat-treatment, aliquots (10 μl) were removed and assayed in triplicate for 5-LO activity (described in Methods). Relative activities are shown, for each 5-LO protein the activity obtained before heat-treatment, in presence of PC (no CLP added) is set as 1. When CLP was present during heat-treatment, assay was performed in presence of PC plus CLP.

B) Inactivation of wt-5-LO and 5-LO-W102A over time. Solutions of purified 5-LO (wt and 5-LO-W102A expressed in BL21, 14 $\mu\text{g}/\text{ml}$, total volume 500 μl), with or

without CLP (stoichiometry 1:1) were prepared in AB+ buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.1% (v/v) β -mercaptoethanol), and kept in closed Eppendorf tubes at rt. At the indicated intervals, aliquots (10 μ l) were removed and assayed in triplicate for 5-LO activity (described in Methods). All data are given as mean \pm SEM for three independent experiments.

Fig 6. Effects of CLP mutants on inactivation of 5-LO over time

Solutions of purified 5-LO (expressed in MV1190, 14 μ g/ml, total volume 500 μ l), with or without wt or mutated CLP proteins (stoichiometry 1:1) were prepared in AB+ buffer, and kept in closed Eppendorf tubes at rt. At the indicated intervals, aliquots (10 μ l) were removed and assayed in triplicate for 5-LO activity (described in Methods). Data are mean \pm SEM for three independent experiments.

Fig 7. Effect of CLP on thermolysin digestion of 5-LO

A) Purified 5-LO (1.2 μ g) with or without CLP (0.24 μ g, stoichiometry 1:1) was pre-incubated for 10 min at room temperature in a total volume of 9 μ l PBS containing 10 mM CaCl_2 and 50 mM NaCl. Thermolysin (1.2 μ g in 1 μ l 10 mM CaCl_2 and 50 mM NaCl) was added. After 20 or 90 sec, the incubations were stopped by addition of 3.5 μ l EDTA (50 mM) and cooling on ice. Sample loading buffer (10 x conc., 1.5 μ l) was added and 4 μ l aliquots were subjected to SDS-PAGE (Phast-gel) followed by Coomassie staining. The results are representative for four different experiments.
B) Purified CLP (2.4 μ g) was treated with thermolysin (1.2 μ g) for 20 or 120 sec. For conditions, see legend to 7A.

Fig. 8. Distribution of CLP and 5-LO in nuclear / non-nuclear fractions from Mono Mac 6 cells

Undifferentiated and differentiated (TGF β and vitD3 for 96 h) MM6 cells (10 x 10⁶ cells in 1 ml PGC buffer) were primed with PMA (100 nM) for 10 min, and activated with ionophore (5 μ M) and arachidonic acid (40 μ M) for another 10 min, as indicated. After lysis with NP-40, pair-wise nuclear and non-nuclear fractions were prepared as described in methods. Aliquots (40 μ l, corresponding to about 10⁶ cells) of pair-wise nuclear and non-nuclear fractions were analyzed for 5-LO, CLP and β -actin by Western blots. Aliquots of a 10,000 x g supernatant derived from non-stimulated cells, was analysed as a control for adequate expression of CLP and 5-LO in the cell batch used. Similar results were obtained in two other experiments.

Fig 9. Protein-protein docking of complex CLP – 5-LO

Model of the CLP-5-LO complex. Left: Side-view of the complex, right: close-up of the interface region (90° rotated about the y-axis). The side-view shows 5-LO in surface representation (light blue and dark blue) and CLP as backbone (dark purple). K131_{CLP} is shown as spheres (green, colored by element). The location of W102_{5-LO} is indicated in orange, as the other two Trp residues (W135_{5-LO} and W75_{5-LO}). The close-up shows the interaction network in the interface region. Possible hydrogen bonds formed between amino acids of the complex partners are indicated: 5-LO-E70 – CLP-R91, 5-LO-D106 – CLP-V113, 5-LO-E108 – CLP-R91, 5-LO-T137 – CLP-K102, 5-LO-K140 – CLP-Q106, 5-LO-R165 – CLP-K131. These hydrogen bonds link CLP to both domains in 5-LO. The proposed cation-pi-interactions of CLP-K131 and 5-LO-F14 and of 5-LO-W102 and 5-LO-R165 are indicated (red lines).

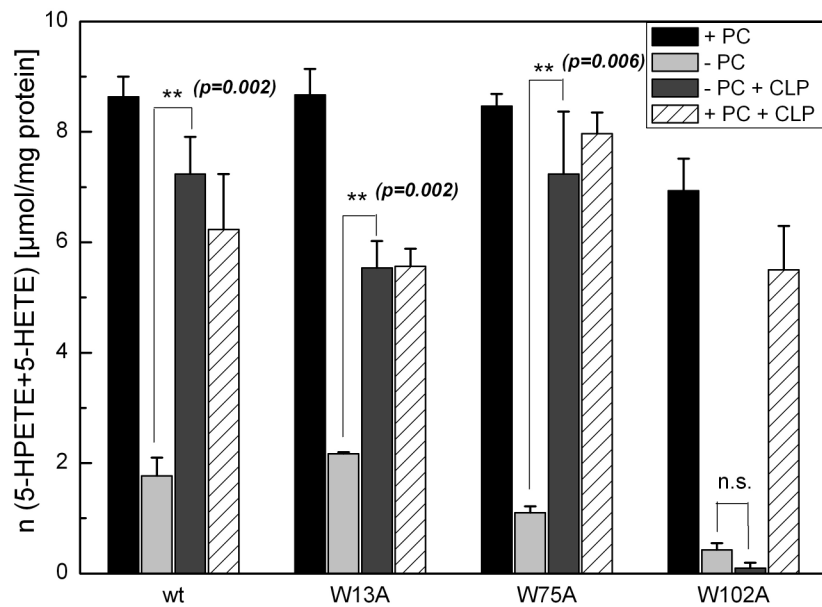


Figure 1A

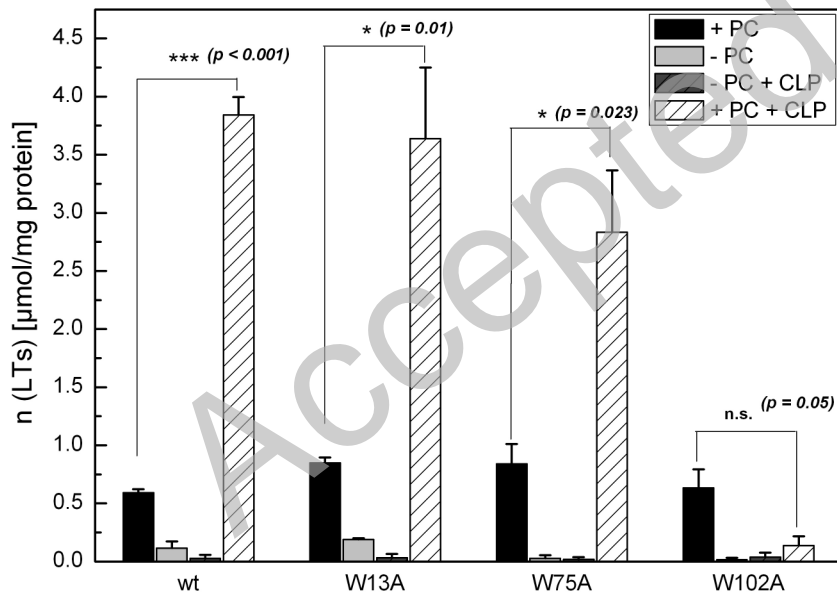


Figure 1B

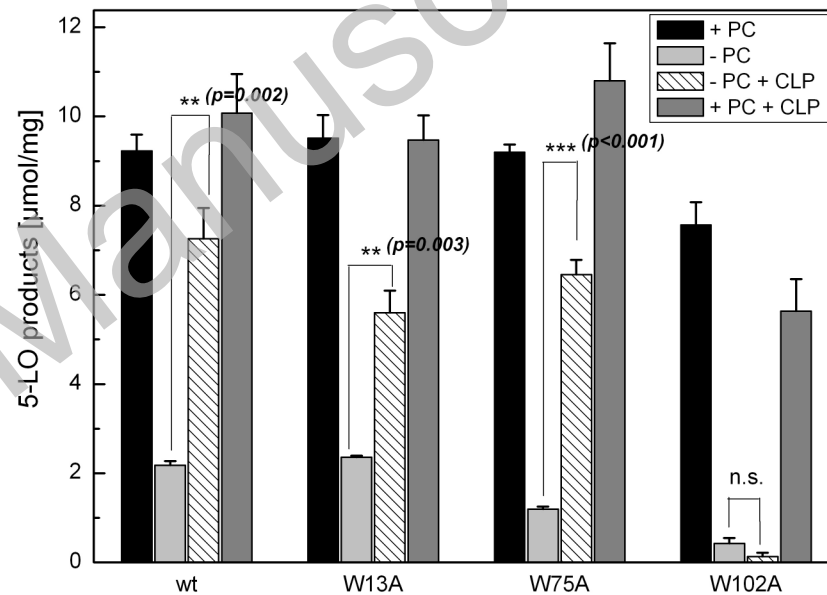


Figure 1C

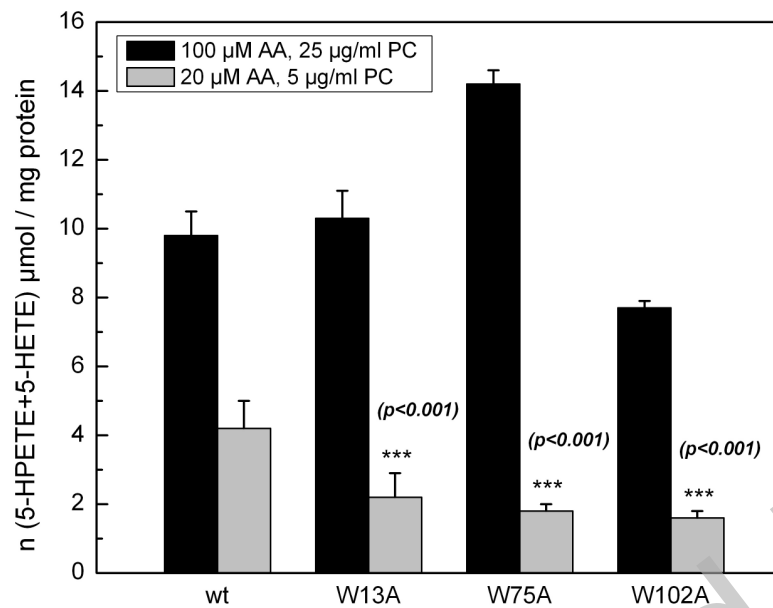


Figure 2



Figure 3

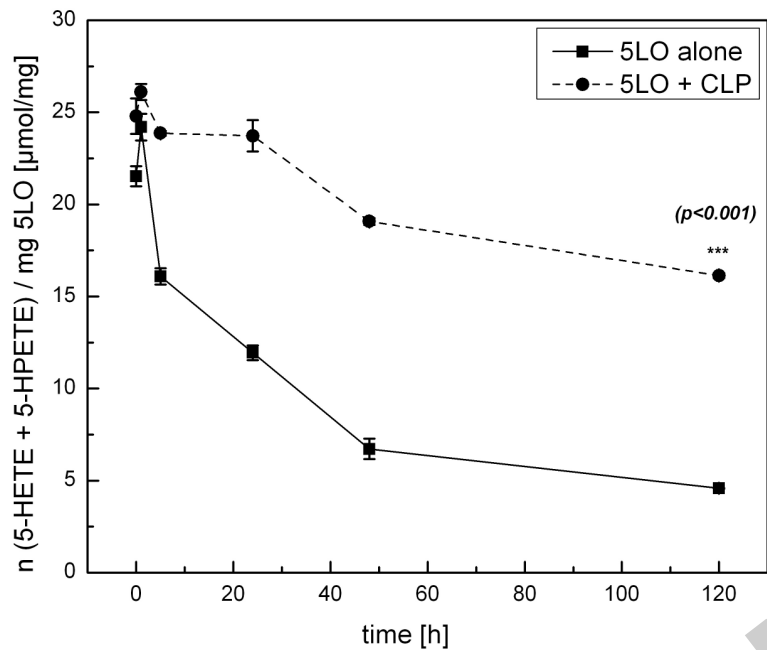


Figure 4A

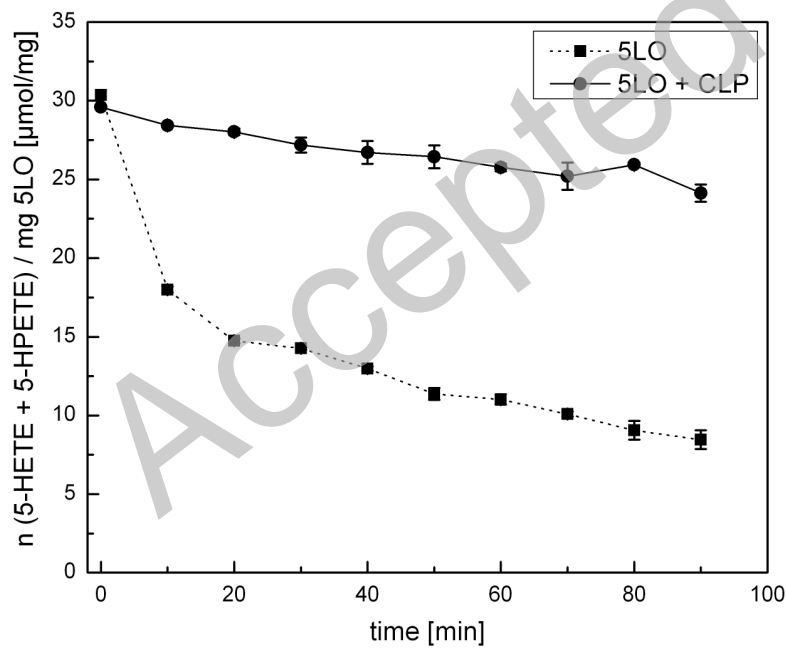


Figure 4B

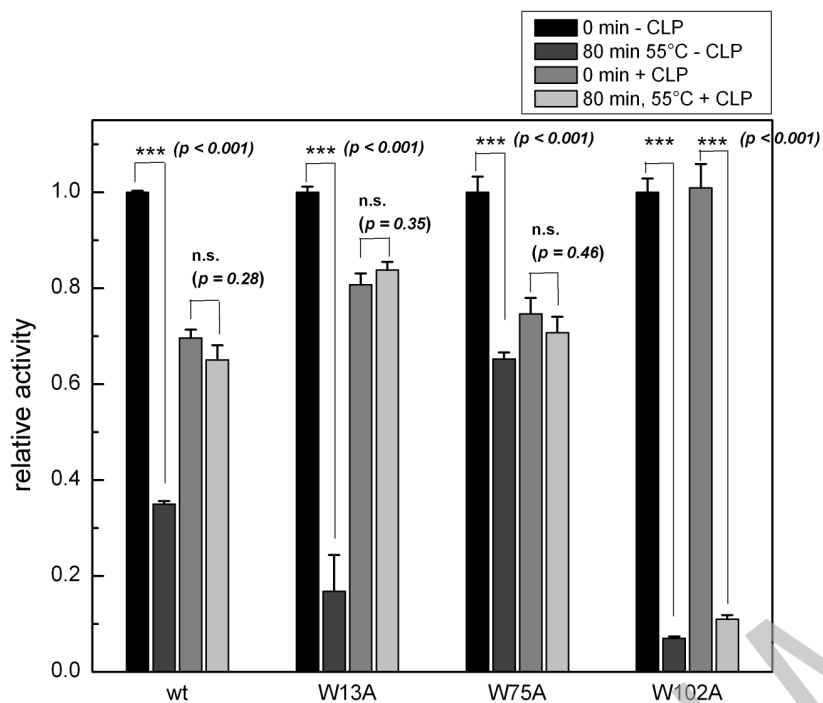


Figure 5A

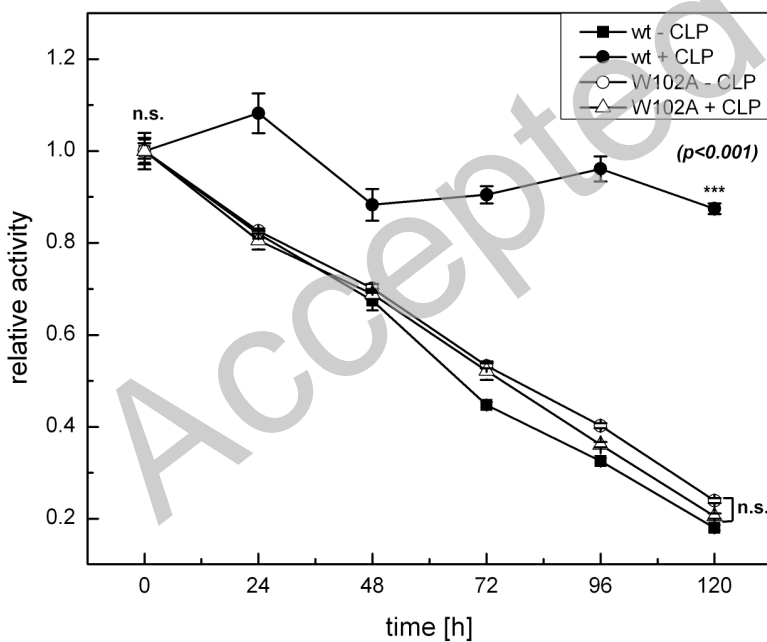


Figure 5B

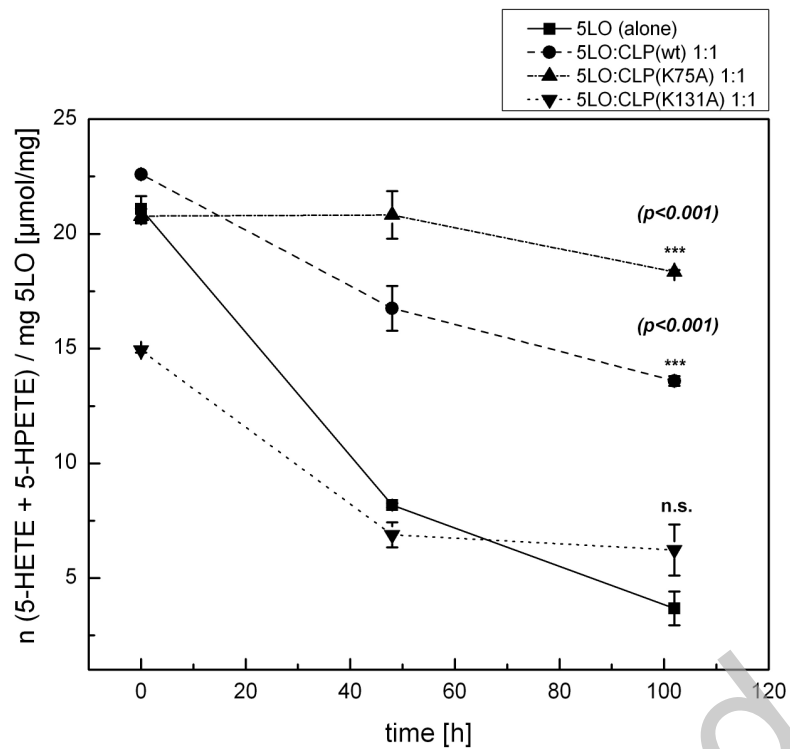


Figure 6

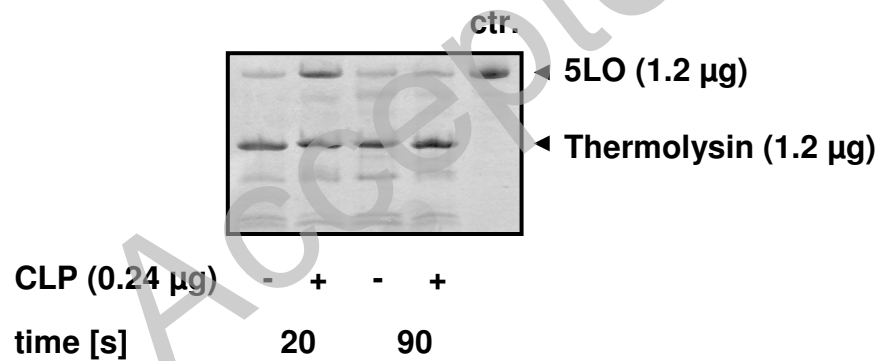


Figure 7A

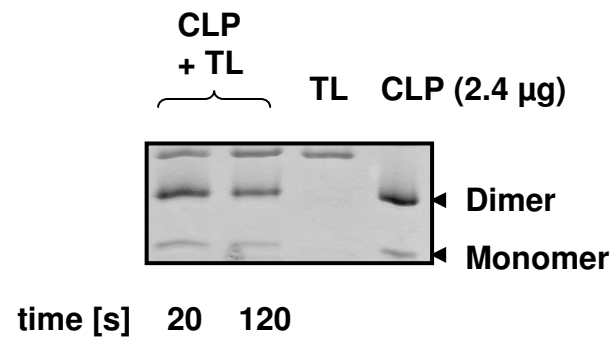


Figure 7B

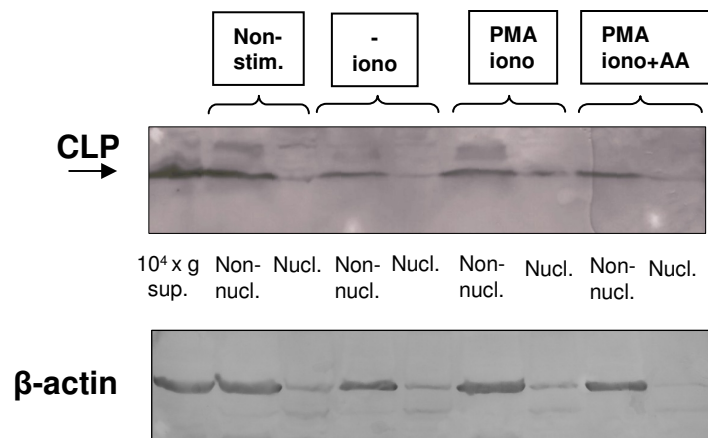


Figure 8A Non-differentiated MM6 cells

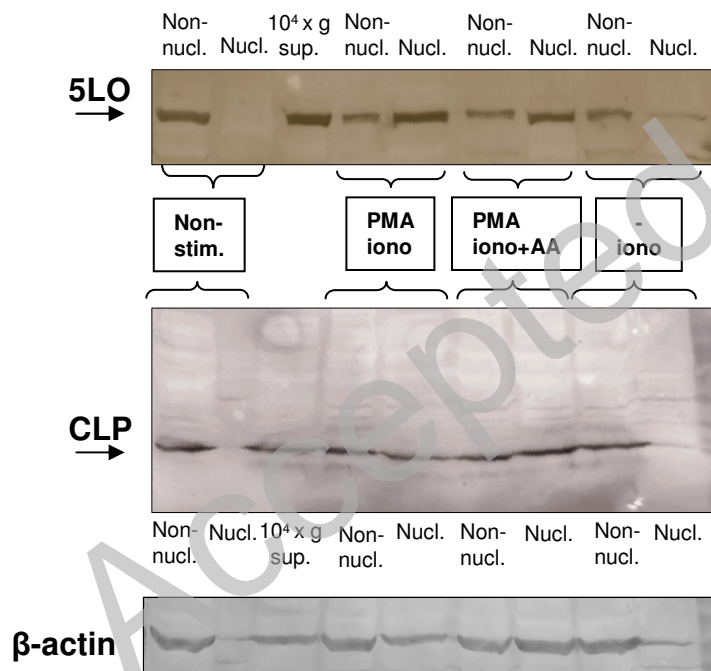


Figure 8B Differentiated MM6 cells

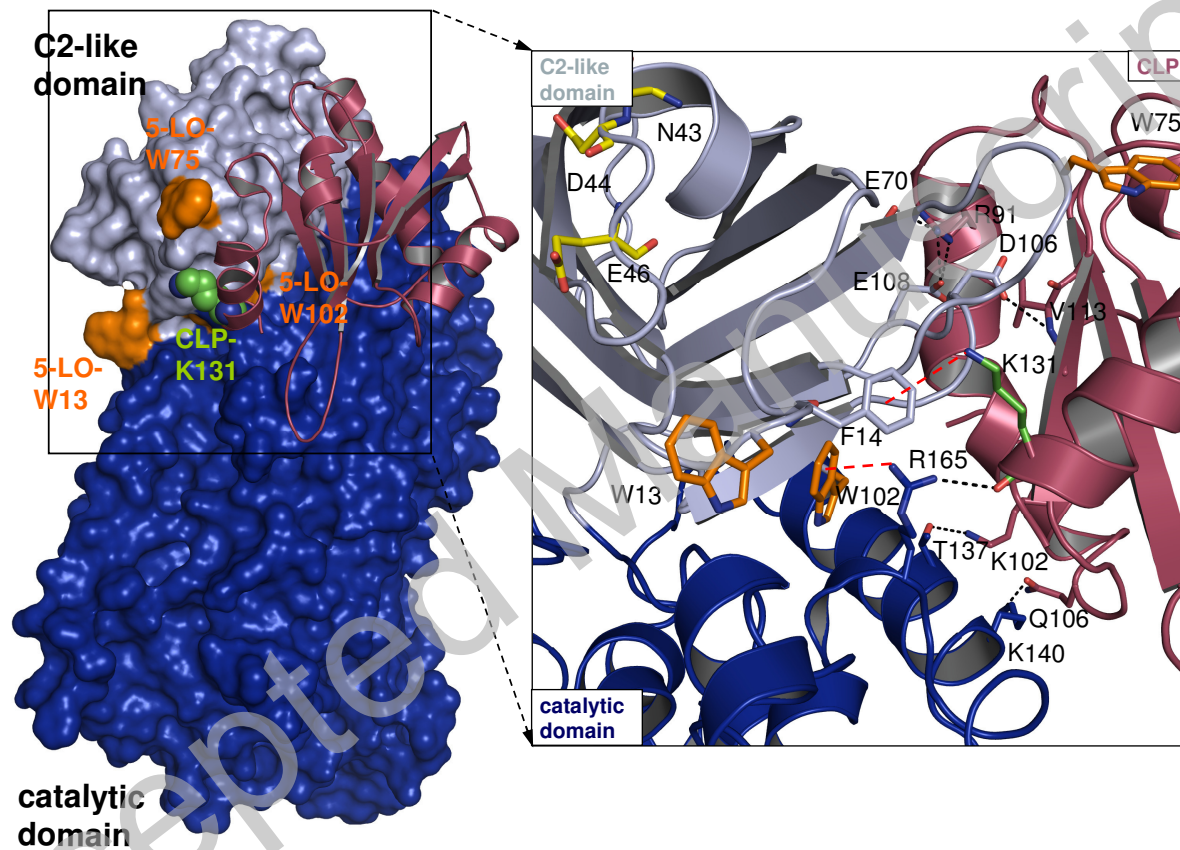


Figure 9